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# A screening assay for thyroid hormone signaling disruption based on thyroid hormone-response gene expression analysis in the frog *Pelophylax nigromaculatus*

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ABSTRACT

Amphibian metamorphosis provides a wonderful model to study the thyroid hormone (TH) signaling disrupting activity of environmental chemicals, with *Xenopus laevis* as the most commonly used species. This study aimed to establish a rapid and sensitive screening assay based on TH-response gene expression analysis using *Pelophylax nigromaculatus*, a native frog species distributed widely in East Asia, especially in China. To achieve this, five candidate TH-response genes that were sensitive to T3 induction were chosen as molecular markers, and T3 induction was determined as 0.2 nmol/L T3 exposure for 48 hr. The developed assay can detect the agonistic activity of T3 with a lowest observed effective concentration of 0.001 nmol/L and EC50 at around 0.118–1.229 nmol/L, exhibiting comparable or higher sensitivity than previously reported assays. We further validated the efficiency of the developed assay by detecting the TH signaling disrupting activity of tetrabromobisphenol A (TBBPA), a known TH signaling disruptor. In accordance with previous reports, we found a weak TH agonistic activity for TBBPA in the absence of T3, whereas a TH antagonistic activity was found for TBBPA at higher concentrations in the presence of T3, showing that the *P. nigromaculatus* assay is effective for detecting TH signaling disrupting activity. Importantly, we observed non-monotonic dose-dependent disrupting activity of TBBPA in the presence of T3, which is difficult to detect with *in vitro* reporter gene assays. Overall, the developed *P. nigromaculatus* assay can be used to screen TH signaling disrupting activity of environmental chemicals with high sensitivity.

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## Introduction

Thyroid hormones (THs) are critical for vertebrate growth, development, metabolic balance, and particularly brain organization and functions throughout life (Mullur et al., 2014; Remaud et al., 2014; Yen, 2001). TH (notably T3) actions are predominantly mediated by TH signaling, which involves TH-response gene transcriptional regulation by TH receptors (TRs, TRα and TRβ) (Zhang and Lazar, 2000). Additionally, the

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transcriptional activation requires the release of corepressors and recruitment of coactivators to the complex of TR associating with retinoid X receptor (RXR) (Astapova and Hollenberg, 2013).

With similar chemical structures to THs, some environmental chemicals, such as hydroxyl polychlorinated biphenyls (OH-PCBs), hydroxyl polybrominated diphenylethers (OH-PBDEs), bisphenol A and its derivatives, and perfluorinated chemicals, have been shown to disrupt TH signaling by binding to TR (Boas et al., 2012; Ren et al., 2013, 2015). To detect the TH signaling disrupting activity of chemicals, several research groups developed *in vitro* bioassays, which were based on transcription analysis of reporter enzymes as a consequence of TR activation in mammalian cells or yeasts (Freitas et al., 2011; Zoeller et al., 2007). For example, Matsubara et al. (2012) developed a reporter gene assay using a pituitary cell line to detect the TH-signaling disruption activities of environmental chemicals (Matsubara et al., 2012), whereas Shiizaki et al. (2010) established a reporter yeast for screening TRα and TRβ ligands to examine their response to endogenous THs and chemicals. Due to the lack of complex interaction of cofactors with TRs, however, these *in vitro* bioassays are not potent enough to detect the TH signaling disrupting effects of chemicals *in vivo* (Zoeller et al., 2007). For example, Ishihara et al. (2011) reported that 5–500 nmol/L OH-PCBs caused an alteration of TH-response gene expression as well as a delay of T3-induced vertebrate development, demonstrating TH signaling disrupting activity, but no TH signaling disrupting activity was observed in a T3-dependent reporter gene assay *in vitro*. Therefore, it is necessary to develop simple and rapid *in vivo* screening methods that can sufficiently detect TH signaling disrupting activity (Coady et al., 2010; Shi, 2009).

Given that amphibian metamorphosis serves as an ideal model for studying TH actions (Laudet, 2011; Opitz et al., 2005; Tata, 1993), this model has begun to be used to investigate the TH signaling disruption of chemicals (Veldhoen et al., 2006, 2014b; Zhang et al., 2014). In this model, pre-metamorphic tadpoles, which can be induced by TH to metamorphose precociously, are treated with test chemicals in the presence or absence of TH for several days. At the end of treatment, if a chemical inhibits TH-induced metamorphosis at morphological and transcriptional levels, this chemical will be determined to be a TH signaling antagonist. In contrast, if a chemical promotes metamorphosis at morphological and transcriptional levels in the presence or absence of TH, this chemical will be determined as TH signaling agonist (Heimeier et al., 2009; Zhang et al., 2014). So far, the model organism *Xenopus laevis* is the most commonly used species to study TH signaling disruption (Crump et al., 2002; Fini et al., 2007; Searcy et al., 2012; Zhang et al., 2014). Also, certain native amphibian species are promising as test species for TH signaling disruption research (Hammond et al., 2013; Helbing et al., 2006; Veldhoen et al., 2014a). The black-spotted frog (*Pelophylax nigromaculatus*, formerly *Rana nigromaculata*) is a native frog species widespread in East Asia. This species has been used in toxicological studies as well as biological studies for decades (Freytag et al., 1953; Jia et al., 2014; Huang et al., 2014). Furthermore, our research group has recently presented the utilizability of *P. nigromaculatus* as a test species for assaying the TH signaling disrupting activity of chemicals (Ge et al., 2014; Lou et al., 2014a,b).

In this study, we aimed to develop a simple and rapid screening assay for TH signaling disruption based on the expression analysis of TH-response genes in *P. nigromaculatus* intestines, including the selection of TH-response genes sensitive to TH induction as well as the selection of an appropriate time and concentration for TH induction. Furthermore, we applied this assay to detect the TH signaling disrupting action of the flame retardant tetrabromobisphenol A (TBBPA), a known TH signaling disruptor, to validate the screening assay established (Jagnytsch et al., 2006; Terasaki et al., 2011; Zhang et al., 2014).

## 1. Materials and methods

### 1.1. Chemicals

T3 (purchased from Geel Belgium, New Jersey, USA) was dissolved in ultrapure water with NaOH to prepare a stock solution (6.60 g/L). Dimethyl sulfoxide (DMSO) and 3-aminobenzoic acid ethyl ester (MS-222) were from Sigma-Aldrich (St. Louis, MO, USA). A stock solution of TBBPA (54.4 g/L, obtained from Geel Belgium) was prepared in DMSO. Luteinizing hormone releasing hormone (LHRH) was from Ningbo Second Hormone factory (Zhejiang, China). Human chorionic gonadotropin (HCG) was purchased from Yantai north pharmaceutical Co. Ltd. (Shandong, China). TRIzol, Fast Quant RT Kit, Real Master Mix (SYBR Green) Kit, RNase-free water, and ethidium bromide (EB) were purchased from Tiangen (Beijing, China). PCR primers were synthesized by Sangon Biotech (Beijing, China).

### 1.2. Animals

*P. nigromaculatus* frogs (from our amphibian house) were maintained in glass tanks containing charcoal-filtered tap water and fed with *Terzebrio molitor* daily. Housing and breeding conditions were reported in our previous study (Lou et al., 2014a). The water quality was as follows: without chlorine, iodine concentration 2–4 μg/L, pH 6.5–7.0, dissolved oxygen concentration > 5 mg/L, and water hardness ($CaCO_3$) approximately 150 mg/L. Fluorescent lighting provided a photoperiod of 12 hr light/12 hr dark. Water temperature was maintained at 24°C ± 1°C. Adult frogs were induced to breed by injecting 25 μg LHRH and HCG (male 200 IU, female 300 IU) accompanied by simulating rainfall. Fertilized eggs were incubated in the dechlorinated tap water at 24°C ± 1°C. On the fifth day post-fertilization, the tadpoles were fed with commercial diets (Totoro Supplies, Hong Kong, China) twice daily. The tadpoles were staged according to the Gosner system (1960).

### 1.3. Expression analysis for candidate TH-response genes in the intestine during metamorphosis

TRα, TRβ, basic transcription element binding protein (BTEB), TH-responsive basic leucine zipper transcription factor (TH/bZIP), matrix metalloproteinase 2 (MMP2), and sonic hedgehog

(SHH) have been demonstrated to be TH-response genes in *X. laevis* (Fini et al., 2012a; Hasebe et al., 2011; Heimeier et al., 2009). Their expression levels in the intestine parallel endogenous TH levels (Heimeier et al., 2010; Hoopfer et al., 2002; Wang et al., 2008). Changes in TH-response gene expression levels during metamorphosis can reflect to some degree the responsiveness of TH-response genes to TH. Here, we chose these genes as candidate TH-response genes in *P. nigromaculatus* intestines, and investigated the developmental expression profiles during metamorphosis, in order to understand the relationship between their expression and endogenous TH levels. Four genes (TH/bZIP, MMP2, SHH and BTEB) were analyzed except for TRα and TRβ, which were reported in our previous study (Lou et al., 2014a).

The tadpoles ($n$ = 8–10) at stages 26, 30, 34, 38, 40, 41, 42, 44, and 46 were collected from the stock populations from two different sets of adults, respectively. The tadpoles were anesthetized and killed in 100 mg/L MS-222 buffered with 200 mg/L of sodium bicarbonate. Then their abdomens were opened, and intestine tissues were sampled and immediately immersed in TRIzol reagent for RNA extraction.

All animal procedures were conducted according to Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission of the People's Republic of China, 1988).

### 1.4. Choice for T3 induction time

According to our previous reports (Lou et al., 2014a), TR expression was regarded as one of the molecular biomarkers for assaying TH signaling disruption in *P. nigromaculatus*, with the intestines of stage 27 tadpoles as test tissues. Here, we chose an appropriate time for T3 induction in the screening assay by comparing the response of TR expression for 24 hr-, 48 hr-, and 72 hr-T3 induction in the intestines of stage 27 tadpoles.

Tadpoles at stage 27 from a clutch were exposed to 1 nmol/L T3 for 24–72 hr, and three replicate test beakers (12 tadpoles per beaker) were employed for each treatment group. Exposure was conducted at 24°C ± 1°C under a 12-hr light/12-hr dark cycle. Following water changes, chemical replacements were performed every 24 hr. Before changing water, four tadpoles from each test beaker (12 tadpoles) were randomly selected and anesthetized in MS-222, and then two intestine tissues were pooled into one mixed sample and immersed in TRIzol reagent for RNA extraction. TR expression was analyzed by RT-qPCR. The experiment was repeated three times using tadpoles from different sets of adults.

### 1.5. Choice for T3 induction concentration

To choose an appropriate T3 concentration to induce TH-gene expression, we investigated the dose–response relationship between T3 and TH-response gene expression. Tadpoles at stage 27 from a clutch were exposed to a series of concentrations of T3 (0, 0.001, 0.01, 0.1, 1, 10, and 100 nmol/L) for the chosen time for T3 induction. Expression of six TH-response genes (TRα, TRβ, BTEB, TH/bZIP, SHH, and MMP2) was analyzed by RT-qPCR.

Three replicate test beakers (four tadpoles per beaker) were employed for each treatment group. Exposure condition and sample collection were as described above. The experiment was repeated three times using tadpoles from different sets of adults.

### 1.6. Thyroid hormone signaling disrupting activity of TBBPA in P. nigromaculatus

To validate the *P. nigromaculatus* screening assay established in this study, we used this assay to detect the TH signaling disrupting action of TBBPA, a known TH signaling disruptor. Tadpoles at stage 27 were exposed to TBBPA (1, 10, 100, and 1000 nmol/L) in the presence or absence of the chosen concentration of T3 for the chosen time for T3 induction. The DMSO concentration was 0.001% ($v/v$) in all beakers. Three replicate test beakers (four tadpoles per beaker) were employed for each treatment group. Exposure conditions and sample collection were as described above. Expression of TH-response genes (TRβ, BTEB, TH/bZIP, MMP2, and SHH) was analyzed by RT-qPCR. Additionally, expression of type 3 iodothyronine deiodinase (DIO3), which was used as a molecular biomarker to assay exogenous TR agonists and antagonists in previous studies using *X. laevis* (Fini et al., 2012a; Nakajima et al., 2012), was also detected. The experiment was repeated three times using tadpoles from different sets of adults.

### 1.7. RNA extraction and RT-qPCR

Total RNA was isolated from intestines using TRIzol reagent following the manufacturer's instructions. RNA quality was verified by electrophoresis on ethidium bromide-stained 1% agarose gels and by A260 nm/A280 nm ratio (Nanodrop ND-1000, Nano-Drop, Wilmington, USA) in the range of 1.8–2.0. Total RNA concentration was calculated from absorbance at 260 nm. Then first-strand cDNA was synthesized from 1 μg total RNA using the Fast Quant RT Kit following the manufacturer's instructions.

To analyze TH-response gene expression levels, we conducted qPCR using SYBR Green I with the MX Real-time Polymerase Chain Reaction system (Light Cycler 480, Roche, Switzerland). Ribosomal protein L8 (rpl8) was used as a reference gene to normalize mRNA expression (Lou et al., 2014b). Specific primers are 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 (listed in Table 1) 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. Additionally, we determined the specificity of these primers by sequencing PCR products.

### 1.8. Data analysis

The fold change of gene expression compared to the reference was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Quantitative data are shown as mean ± standard error of the mean (SEM). To identify developmental expression of candidate TH-response genes, fold-changes were calculated relative to stage 26. Statistical differences in mRNA expression between T3 treatments and controls were assessed by one-way analysis of variance followed by Student–Newman–Keuls (SNK) post-hoc analyses (ANOVA, SPSS 16.0, USA) ($p < 0.01$). Statistical differences among TBBPA treatments and controls in the absence or presence of T3 were analyzed by two-way ANOVA

**Table 1 – Primers used for RT-qPCR and related information.**

| Gene | Primer sequences (5′–3′) | GenBank ID | Amplify length | Annealing temperature | PCR efficiency |
|---|---|---|---|---|---|
| rpl8 | F: GCTGTCGACTTCGCAGAAAGGCA<br>R: ACCTGTAAGGGTCACGGAAGGCA | KF585213 | 102 bp | 60°C | 100% |
| TRα | F: TCTCCGGGCTGCTGTGCGTTA<br>R: GCATCTGAGACCACCCCAAGTCCA | KC139354.1 | 114 bp | 60°C | 108% |
| TRβ | F: GGAGGACTAGGAGTGGTATCTG<br>R: CAACAAGGCGACTTCGGTAT | KC139355.1 | 90 bp | 63°C | 107% |
| BTEB | F: ACACAGGCGAGAAGCAGTT<br>R: CAGATGAAGGTTGAGCTGGATC | KF724718 | 145 bp | 62°C | 97% |
| TH/bZIP | F: GACGCAAGAACAATGAGGCT<br>R: AACGGAGAGCAAGAAGTTCAG | KF724721 | 132 bp | 62°C | 104% |
| SHH | F: TTACTAGGAACTCGGAGAGGTT<br>R: TGTCGGCTGCTGTGTTCT | KF724720 | 90 bp | 62°C | 98% |
| MMP2 | F: ACCACAGAAGACTATGACAAGG<br>R: GAAGACACAAGGAGAGCCATC | KF724719 | 99 bp | 60°C | 106% |
| DIO3 | F: ACGGACAGAAGCTCGACTT<br>R: TGCCGAAGTTGACCACTAGG | SRP041632 | 144 bp | 64°C | 106% |

F: forward; R: reverse; rpl8, ribosomal protein L8; TRα, thyroid hormone receptor alpha; TRβ, thyroid hormone receptor beta; BTEB, basic transcription element binding protein; TH/bZIP, TH-responsive basic leucine zipper transcription factor; SHH, sonic hedgehog; MMP2, matrix metalloproteinase 2; DIO3, type 3 iodothyronine deiodinase.

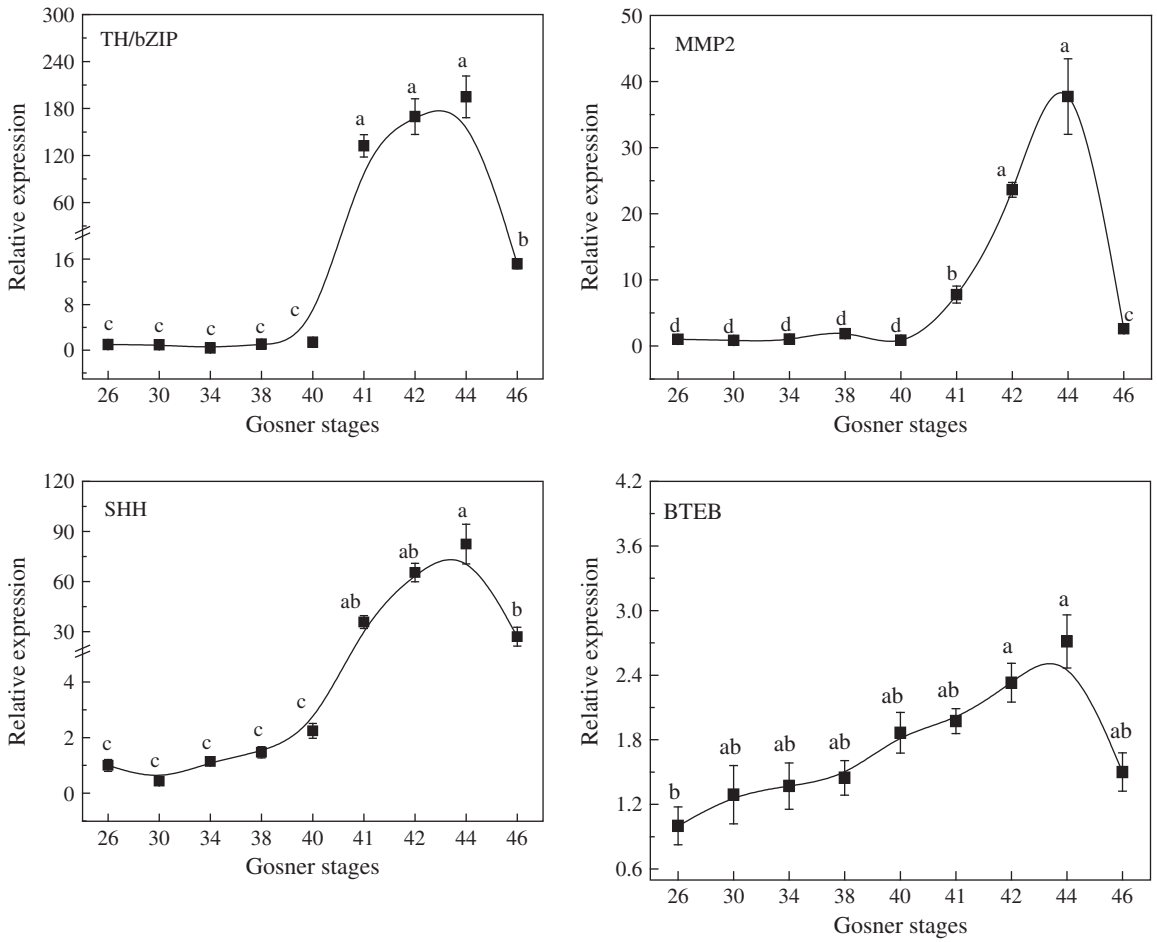


**Fig. 1 – Developmental expression of candidate TH-response genes in the intestine during *Pelophylax nigromaculatus* metamorphosis. Data are shown as means ± SEM ($p < 0.01$), and the tadpoles are from two different sets of adults ($n$: 8–10). TH/bZIP: TH-responsive basic leucine zipper transcription factor; SHH: sonic hedgehog; MMP2: matrix metalloproteinase 2; BTEB: basic transcription element binding protein.**

followed by Duncan post-hoc analyses ($p < 0.05$). The developmental expression of candidate TH-response genes in Fig. 1 was fitted by B-spline curves (Origin 8.5, Origin Lab., Northampton, USA). The dose–response curves of TH-response genes to exogenous T3 in Fig. 3 were fitted with a sigmoidal model (Origin Lab) to derive the value of EC50.

## 2. Results

### 2.1. Expression changes of candidate TH-response genes in the intestine parallel changes in endogenous TH levels during metamorphosis

The expression levels of TH/bZIP were relatively low and remained stable before stage 41, but increased sharply after stage 41 and peaked at stages 41–44 (Fig. 1). Then, TH/bZIP expression decreased towards the end of metamorphosis (stage 46), but was still significantly higher than the levels observed before stage 40 ($p < 0.01$). Similarly, SHH mRNA expression during metamorphosis exhibited a parallel profile with TH/bZIP, although the fold change of SHH expression at metamorphic climax relative to stage 26 was smaller than that of TH/bZIP. Like TH/bZIP and SHH, MMP2 peaked around stages 42–44 and declined towards stage 46. However, the decline of MMP2 mRNA levels towards stage 46 was so dramatic that it returned to the level at stage 26. Different from the other three genes, changes in BTEB mRNA levels were not dramatic during the whole metamorphosis. Compared with the mRNA level at stage 26, BTEB expression displayed a slowly increasing trend with development, peaked at stage 42–44, and decreased at stage 46.

Overall, TH/bZIP, SHH and MMP2 as well as TRs in the intestine displayed roughly similar expression profiles during *P. nigromaculatus* metamorphosis, with a similar trend of BTEB expression (Lou et al., 2014a). The expression profiles of these genes parallel changes in endogenous TH levels, which remain low at premetamorphic and most prometamorphic stages, increase beginning in late prometamorphic stages, peak at the metamorphic climax, and decline towards the end of metamorphosis (Ge et al., 2014; Lou et al., 2014a). It is suggested that changes in TH-response gene expression levels reflect to some degree the responsiveness of TH-response genes to endogenous TH during *P. nigromaculatus* metamorphosis. The peak levels of TH/bZIP, SHH, MMP2, and BTEB expression were about 195-, 82-, 38-, and 3-fold the levels at stage 26, respectively. The data demonstrate that TH/bZIP expression has higher responsiveness to endogenous T3, with lower responsiveness for BTEB expression.

### 2.2. Determination of T3 induction time (48 hr)

To determine an appropriate time for T3 induction in the screening assay, we compared the effects of 24 hr-, 48 hr-, and 72 hr-T3 induction on expression of TRβ and TRα, as the representatives of TH-response genes, in intestines from stage 27 tadpoles. It was found that T3 significantly up-regulated TRβ transcriptional levels in intestines, with no significant difference among 24 hr-, 48 hr-, and 72 hr-T3 induction times (Fig. 2). T3 exposure for 24 hr and 48 hr also induced TRα expression, whereas 72 hr-T3 induction was noneffective (Fig. 2). Based on these observations and a consideration of the time for test chemicals to concentrate in tadpoles, we chose 48 hr-T3 treatment to induce TH-response gene expression in the screening assay.

### 2.3. Dose–response curves between exogenous T3 and TH-response gene expression and determination of T3 induction concentration (0.2 nmol/L)

To determine an appropriate concentration for T3 induction in the screening assay, we investigated the responsiveness of the

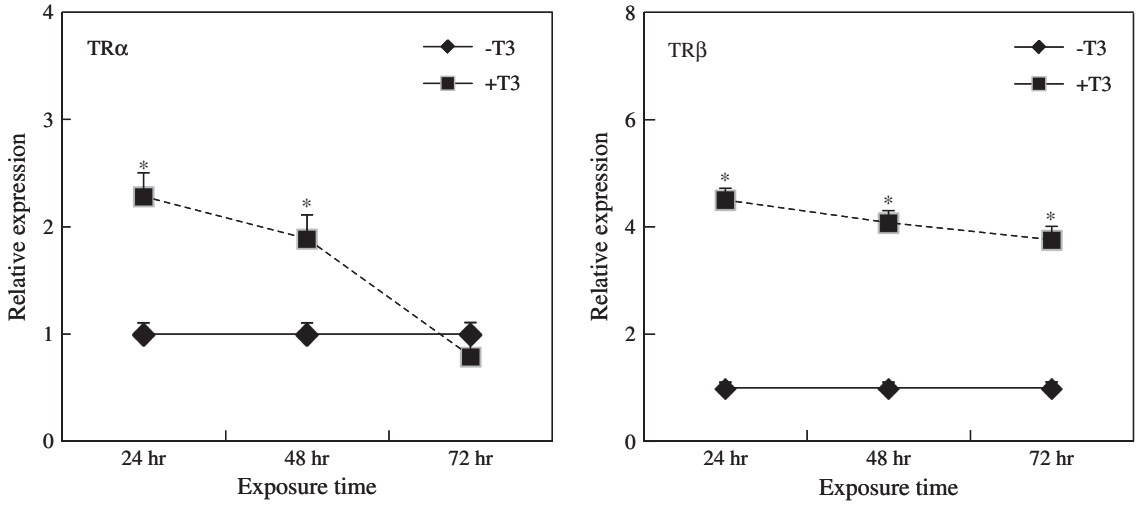


**Fig. 2 – Thyroid hormone receptor (TRα and TRβ) expression in the intestines of Gosner stage 27 *Pelophylax nigromaculatus* tadpoles following T3 exposure for 24–72 hr. Data are shown as means ± SEM ($n$ = 12, 24 tadpoles). Significant differences between T3 treatment groups and the control were indicated by $^*p < 0.01$. All experiments were repeated three times with similar results. Here we show the combined data from two experiments because the tadpoles displayed higher sensitiveness to T3 in another experiment.**

six candidate TH-response genes to 48 hr-T3 treatment (0.001–100 nmol/L) in the intestines from stage 27 *P. nigromaculatus* tadpoles. Fig. 3 shows the dose–response curves between T3 concentrations and the expression levels of TRα, TRβ, BTEB, TH/bZIP, SHH, and MMP2. T3 up-regulated expression levels of these genes compared with the control, with 0.01 nmol/L as the lowest observed effective concentration (LOEC) for TRα, TRβ, BTEB, and MMP2 and 0.001 nmol/L for TH/bZIP and SHH (Table 2). With increasing T3 concentrations, the agonistic actions of T3 on gene expression increased. TRβ and BTEB

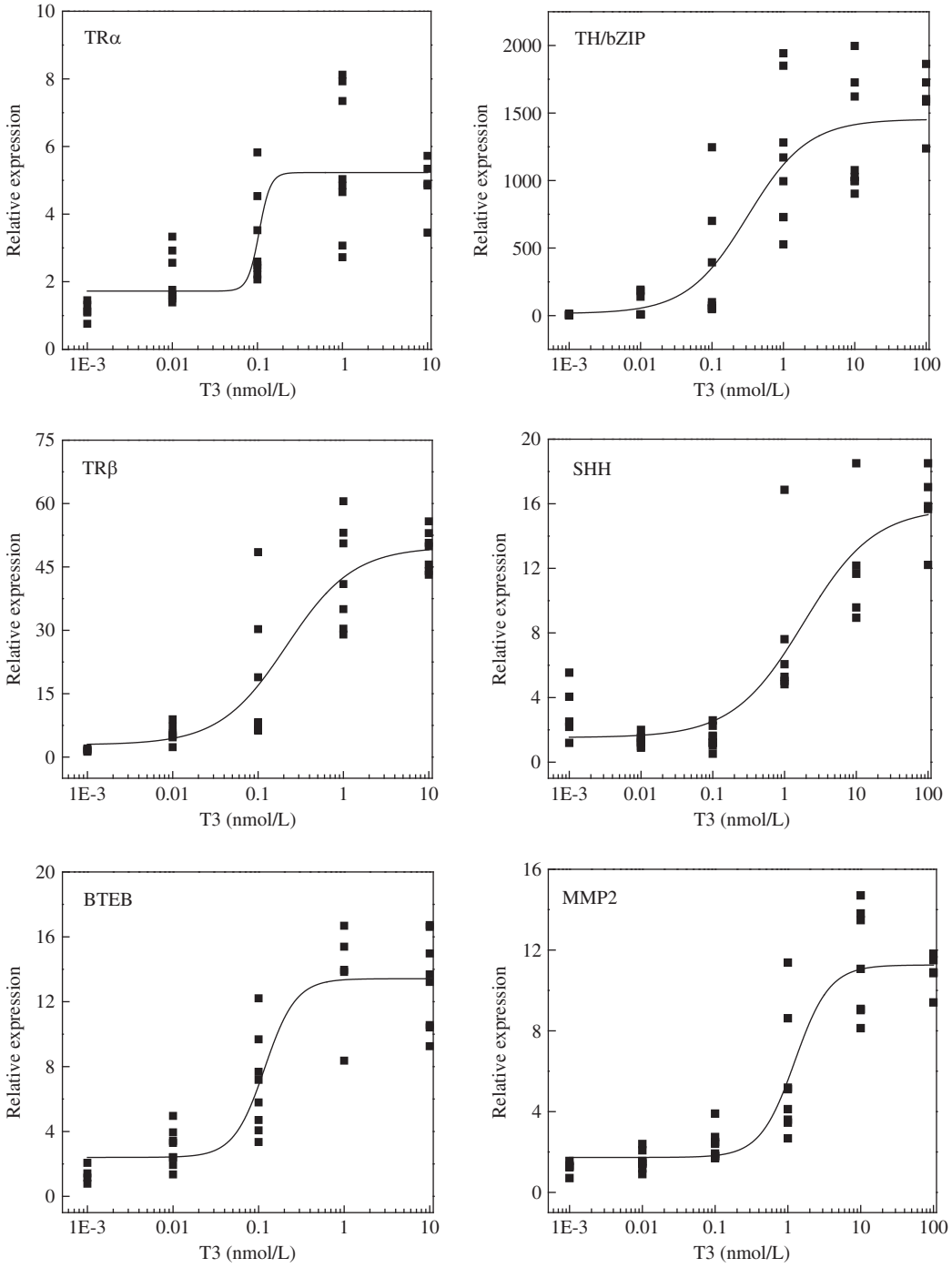


**Fig. 3 – Dose–response curves between T3 concentrations and TH-response gene expression in the intestines of *Pelophylax nigromaculatus* tadpoles at Gosner stage 27. All experiments were repeated three times using tadpoles from different sets of adults with similar results. Here we show the combined data from two experiments ($n$ = 12) because the tadpoles displayed higher sensitiveness to T3 in another experiment. TRα: thyroid hormone receptor alpha; TRβ: thyroid hormone receptor beta; BTEB: basic transcription element binding protein; TH/bZIP: TH-responsive basic leucine zipper transcription factor; SHH: sonic hedgehog; MMP2: matrix metalloproteinase 2.**

**Table 2 – T3 induction on TH-response gene expression in the intestines of Gosner stage 27 *Pelophylax nigromaculatus* tadpoles.**

| | TRα | TRβ | BTEB | TH/bZIP | SHH | MMP2 |
|---|---|---|---|---|---|---|
| LOEC (nmol/L) | 0.01 | 0.01 | 0.01 | 0.001 | 0.001 | 0.01 |
| HIE (fold) | 3.952 ± 0.558 | 48.87 ± 1.802 | 13.18 ± 1.806 | 1608 ± 189.8 | 15.91 ± 1.901 | 10.91 ± 1.312 |
| EC50 (nmol/L) | – | 0.215 ± 0.087 | 0.118 ± 0.068 | 0.291 ± 0.147 | 1.075 ± 0.426 | 1.299 ± 0.427 |

LOEC: the lowest observed effective concentration; EC50: the median effective concentration (obtained from the sigmoidal model); HIE: the highest inductive effect (data are shown as means ± SEM).
TRα, BTEB, TH/bZIP, SHH, and MMP2 refer to Table 1.

expression appeared to reach plateaus at 1-10 nmol/L, whereas the plateaus of TH/bZIP, SHH, MMP2 expression occurred at concentrations higher than 10 nmol/L. The highest inductive effects (HIE) of T3 on TRα, TRβ, BTEB, TH/bZIP, SHH, and MMP2 were about 4-, 49-, 13-, 1600-, 16-, and 11-fold of the control, respectively. The dose–response curves between T3 and the expression levels of these genes (except TRα) were well fitted with a sigmoidal model. The effective concentrations (EC50) of T3 for expression of the other five genes ranged from 0.118 to 1.229 nmol/L. Given that T3 induction on TRα expression was very weak, we chose TRβ, BTEB, TH/bZIP, SHH, and MMP2 as molecular endpoints for the *P. nigromaculatus* screening assay. Based on the LOECs and EC50 values for these TH-response genes, we chose 0.2 nmol/L T3 to induce gene expression in the *P. nigromaculatus* screening assay.

Taken together, we have established a *P. nigromaculatus* screening assay for TH signaling disruption, in which tadpoles at stage 27 are treated with a test chemical in the presence or absence of 0.2 nmol/L T3 for 48 hr. By investigating the effects of the test chemical on TRβ, BTEB, TH/bZIP, SHH, and MMP2 expression in tadpole intestines in the presence or absence of T3, the chemical is determined as a TH signaling agonist or antagonist or noneffective factor.

### 2.4. TBBPA exhibits TH signaling disrupting activity in the *P. nigromaculatus* screening assay

Using the *P. nigromaculatus* screening assay, we detected the TH signaling disrupting activity of TBBPA in order to validate the screening assay. In the absence of T3, 1000 nmol/L TBBPA promoted TH/bZIP and MMP2 expression, with no obvious effect on the other genes (Fig. 4). The data suggest that TBBPA alone had a weak agonistic action on TH signaling. In the presence of 0.2 nmol/L T3, 1 nmol/L TBBPA significantly promoted T3-induced expression of all the TH-response genes compared with T3 treatment ($p < 0.05$). With increasing TBBPA concentrations, however, the expression levels of these genes decreased. TBBPA at 10 nmol/L inhibited or had no effect on T3-induced gene expression. Higher concentrations (100–1000 nmol/L) of TBBPA inhibited the T3-induced expression of all genes tested (Fig. 4).

In the *P. nigromaculatus* screening assay, in the presence of T3, lower concentrations of TBBPA had agonistic effects on TH-response gene expression, whereas higher concentrations had antagonistic effects, exhibiting a non-monotonic dose–response manner. In contrast to TBBPA plus T3 treatment, TBBPA alone may have a weak agonistic action on TH signaling.

## 3. Discussion

*X. laevis* has been most commonly used to study TH signaling disruption of chemicals (Crump et al., 2002; Fini et al., 2007; Zhang et al., 2014). However, it is necessary to develop some other assays using native species, considering species differences and the accessibility of test species. Recently, we presented the utilizability of *P. nigromaculatus* as a test species for assaying the TH signaling disrupting activity of chemicals (Lou et al., 2014a). In particular, the TR expression in *P. nigromaculatus* intestine was demonstrated to be sensitive to TH induction, and can be used as a molecular endpoint for screening TH signaling disrupting effects (Lou et al., 2014a). This study aimed to develop a *P. nigromaculatus* screening assay for TH signaling disruption using additional TH-response genes as molecular markers. We chose four candidate TH-response genes, including TH/bZIP, SHH, MMP2 and BTEB, which have been used as molecular markers to study TH signaling disruption in *X. laevis* (Fini et al., 2012a; Heimeier et al., 2009; Zhang et al., 2014). The expression changes of the four candidate genes in *P. nigromaculatus* intestines were found to parallel endogenous TH levels during metamorphosis, which is consistent with previous observations in *X. laevis* (Heimeier et al., 2010). The peak levels of TH/bZIP, SHH, MMP2 and BTEB expression at metamorphic climax were about 195-, 82-, 38- and 3-fold the levels at stage 26, respectively (Fig. 1). The data demonstrate that these genes are responsive to endogenous T3 in *P. nigromaculatus*, and their expression is suitable for use as molecular endpoints for assaying TH signaling disruption.

In accordance with the response of these genes to endogenous TH, the dose–response curves between exogenous T3 and TH-response gene expression further demonstrate the high responsiveness of TRβ, BTEB, MMP2, SHH and TH/bZIP to exogenous T3 in *P. nigromaculatus* intestines, with low responsiveness for TRα (Fig. 3). Similarly, higher responsiveness of TRβ, BTEB, MMP2, SHH and TH/bZIP than TRα to TH was reported previously in premetamorphic tadpoles in *X. laevis* and *Rana catesbeiana* (Heimeier et al., 2009; Stolow and Shi, 1995; Veldhoen et al., 2014b; Zhang et al., 2014). To ensure high sensitivity for the *nigromaculatus* screening assay, thus, we chose TRβ, BTEB, TH/bZIP, SHH, and MMP2 but not TRα as molecular endpoints. In the literature, several screening assays for TH signaling disruption using TH-response gene expression or TH-regulated reporter gene expression as endpoints were reported previously (Table 3). We compared the sensitivity of molecular endpoints for the *P. nigromaculatus* screening assay

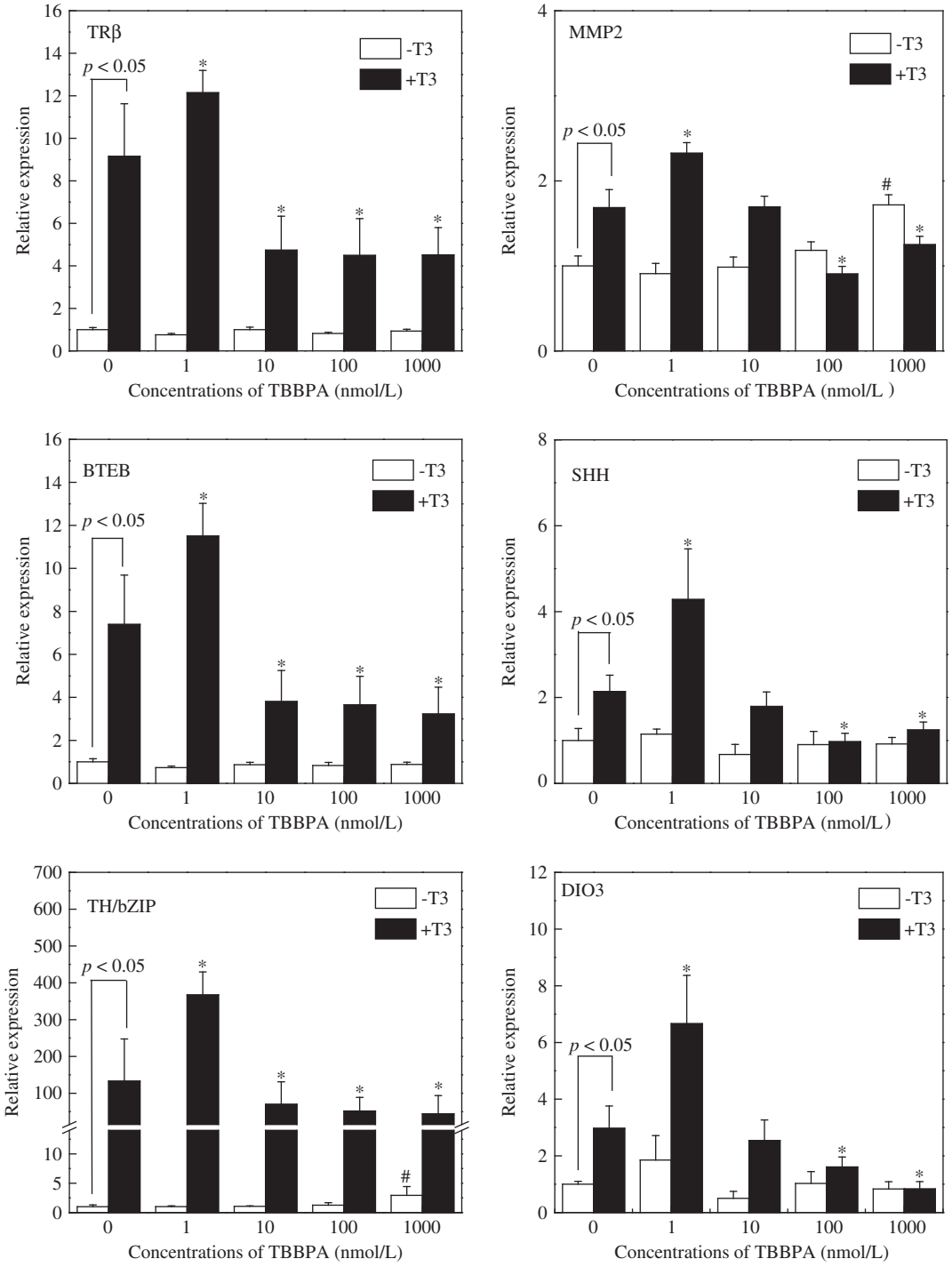


**Fig. 4 – Effects of TBBPA on TH-response gene expression in the intestines of Gosner stage 27 *Pelophylax nigromaculatus* tadpoles following 48 hr-exposure to tetrabromobisphenol A (TBBPA) in the absence or presence of 0.2 nmol/L T3. All experiments were repeated three times using tadpoles from different sets of adults with similar results. Here we show the combined data from two experiments (n = 12) because the tadpoles displayed higher sensitiveness to T3 in another experiment. Data are shown as mean ± SEM. # and * indicate significant differences between TBBPA treatment and the control, and between TBBPA + T3 treatment and T3 treatment, respectively ($p < 0.05$). TRβ: thyroid hormone receptor beta; BTEB: basic transcription element binding protein; TH/bZIP: TH-responsive basic leucine zipper transcription factor; MMP2: matrix metalloproteinase 2; SHH: sonic hedgehog; DIO3: type 3 iodothyronine deiodinase.**

with previously reported assays according to LOEC and EC50 values of T3 for gene expression. Turque et al. (2005) constructed a TH/bZIP-GFP reporter system in *X. laevis* tadpoles, which can detect significant transcription up-regulated by 0.001 nmol/L T3. In *P. nigromaculatus* intestines, TH/bZIP and SHH expression induced by 0.001 nmol/L T3 was also found. That is, molecular

**Table 3 – Comparison of sensitivity to T3 between our study and other assays in the literature.**

| Assay organism | | T3 (nmol/L) | | | Reference |
|---|---|---|---|---|---|
| | | CR | LOEC | EC50 | |
| *In vivo* | *P. nigromaculatus* intestine | 0.001–100 | 0.001–0.01 | 0.118–1.299 | This study |
| | *X. laevis* caudal muscle | ND | 0.001 | ND | Turque et al. (2005) |
| | *R. pipiens* brain | 0.05–50 | 50 | ND | Hogan et al. (2007) |
| *In vitro* | MtT/E-2-rat TRα or β | 0.001–10 | 0.01 | ND | Matsubara et al. (2012) |
| | Rat GH3.TRE-Luc | 0.001–100 | 0.01 | 0.1 | Freitas et al. (2011) |
| | HEK293-vertebrate TRs | 0.001–100 | 0.01 | 0.243 | Oka et al. (2012) |
| | Rat PC-DR-LUC | 0.03–10 | 0.03 | 0.18 | Jugan et al. (2007) |
| | CV-pGal4-LTRβ | 0.1–10000 | 0.1 | ND | Hu et al. (2013) |
| | HepG2-human TRs | 0.01–10000 | 0.1 | 1.2 | Hofmann et al. (2009) |
| | Yeast-human TRβ-SRC-1 | 0.1–10000 | 0.2 | 1.5 | Shiizaki et al. (2010) |
| | GH3 cells-DIO1 | 0.5–100 | 0.5 | ND | Davey et al. (2008) |
| | GH3.TRE-Luc high-throughput | 0.0003–4600 | ND | 0.33 | Freitas et al. (2014) |
| | *X. laevis* cell-*X. laevis* TR | 0.1–1000 | ND | 10 | Furlow et al. (2004) |
| | CV-1 cell-TRβ | 0.01–1000 | ND | 11.3 | Sun et al. (2009) |
| | Yeast-human TR-GRIP1 | 0.05–1000 | 3 | 26 | Li et al. (2014) |
| | Yeast-human TRα | 0.1–1000 | ND | 55 | Terasaki et al. (2011) |
| | Yeast-human TR-GRIP1 | 0.01–5000 | ND | 110 | Li et al. (2008) |
| | CHO-human TRα | 0.01–10000 | 10 | ND | Kitamura et al. (2005) |

CR: Concentration range; LOEC: the lowest observed effective concentration; EC50: the median effective concentration; ND: not determined.

endpoints in *P. nigromaculatus* display a comparable sensitivity to the assay of Turque et al. However, the LOEC value of T3 in *Rana pipiens* reached up to 50 nmol/L (Hogan et al., 2007). In TH-regulated transcription assays *in vitro*, the LOEC and EC50 values of T3 varied in the range of 0.01–10 nmol/L and 0.01–110 nmol/L, respectively. In our study, the LOEC and EC50 values of T3 for the molecular endpoints in *P. nigromaculatus* were 0.001–0.01 nmol/L and 0.118–1.229 nmol/L, respectively. These data show that the molecular endpoints in *P. nigromaculatus* intestines in our study are comparable to or more sensitive than previously reported assays for detecting TH signaling disrupting activity.

Following determination of molecular markers and T3 induction time and concentration, we validated the *P. nigromaculatus* screening assay using TBBPA as a model TH signaling disruptor. In previous studies, TBBPA alone generally exhibited a weak TH stimulatory activity in the absence of TH, with a TH antagonistic activity in the presence of TH (Fini et al., 2012b; Jagnytsch et al., 2006; Zhang et al., 2014), as shown in Table 4. In accordance with previous reports, we found that 1000 nmol/L TBBPA alone weakly activated intestinal TH-response gene expression (Fig. 4), whereas 10–1000 nmol/L TBBPA exhibited a TH antagonistic action in the presence of T3, showing that the *P. nigromaculatus* screening assay we established is effective for detecting the TH signaling disrupting activity of TBBPA. Additionally, we found that in contrast to the results for higher concentrations, 1 nmol/L TBBPA promoted TH-induced gene expression in the presence of T3, showing a non-monotonic dose–response manner (Fig. 4). Our recent study concerning *X. laevis* also reported a non-monotonic dose–response manner of TBBPA in affecting TH-response gene expression, with 10–100 nmol/L TBBPA as a TH agonist and 500–1000 nmol/L as a TH antagonist in the presence of T3 (Zhang et al., 2014). In accordance with the agonistic effect on T3 induction of 1 nmol/L TBBPA in our study, Veldhoen et al. (2006) also found 10 nmol/L TBBPA increased gel B transcript in the presence of T3 in the tail from *Pseudacris regilla* tadpole, and 100 nmol/L TBBPA promoted T3-induced TRα expression in the brain. In addition, one *in vitro* study using GH3.TRE-Luc cell lines also revealed a non-monotonic dose-dependent TH signaling disrupting action of TBBPA ranging from 100 nmol/L–10 μmol/L in the presence of T3 (Freitas et al., 2011). However, other *in vitro* reporter gene assays could not detect non-monotonic dose-dependent effects of TBBPA on TH signaling, even when a wide concentration range of TBBPA was tested (Hofmann et al., 2009; Kudo et al., 2006; Oka et al., 2012). It is known that non-monotonic dose–response for endocrine disrupting chemicals is observed frequently (Beausoleil et al., 2013; Vandenberg, 2014). Our study strongly demonstrates a non-monotonic dose–response of TBBPA in terms of TH signaling disrupting activity. Comparing our assay and other *in vivo* amphibian assays in the literature (Table 4), the LOECs of TBBPA for TH signaling disrupting effects *in vivo* amphibian assays were close to or higher than those in our study, demonstrating that our assay has more or comparable sensitivity for detecting TH signaling disruption compared with other *in vivo* amphibian assays (Fini et al., 2012a; Veldhoen et al., 2006; Zhang et al., 2014). Compared with *in vitro* assays (Table 4), in which the LOECs of TBBPA for TH signaling disrupting effects are higher by 2–3 orders than those in our assay, the sensitivity of our assay is superior. Possibly, the fact that the biphasic effects of TBBPA on TH signaling occurring in *in vivo* assays were not detected in *in vitro* assays is due to their low sensitivity. In particular, the LOEC of TBBPA (1 nmol/L, 0.54 μg/L) in our study is in the range of environmentally relevant concentrations, which generally range from pg/L to several μg/L levels in surface water (He et al., 2013; Labadie et al., 2010; Yang et al., 2012). Taken together, the *P. nigromaculatus* screening assay we established is capable of detecting TH signaling disrupting activity of environmental chemicals with high sensitivity. Given that the *P. nigromaculatus* population is declining in East

**Table 4 – Comparison of TH signaling disrupting activity of TBBPA between in our study and other assays in the literature.**

| Assay organism | | TBBPA (μmol/L) | | | | Reference |
|---|---|---|---|---|---|---|
| | | LOEC | CR (Toxicity) | −T3 | +T3 | |
| *In vivo* | *P. nigromaculatus* intestine | 0.001 | 0.001–1 (ND) | 1 ↑ | 0.001 ↑<br>0.01–1↓ | This study |
| | *X. laevis* intestine | 0.01 | 0.01–1 (ND) | 0.01–1 ↑ | 0.01–0.1 ↑ ↓<br>0.1–1 ↓ | Zhang et al. (2014) |
| | *P. regilla* tail and brain | 0.01 | 0.01–0.1 (ND) | 0.01–0.1 ↑ | 0.01 ↑<br>0.1 ↑ ↓ | Veldhoen et al. (2006) |
| | *X. laevis* head | 0.184 | 0.184–0.920 (ND) | 0.920 ↑ | 0.184–0.920↓ | Jagnytsch et al. (2006) |
| | *X. laevis* brain | 1 | 1 (ND) | 1 ↑ | ND | Fini et al. (2012a) |
| *In vitro* | Rat GH3.TRE-Luc | 0.1 | 0.1–100 (>10) | ND | 0.1–5 ↑<br>5–10 ↓ | Freitas et al. (2011) |
| | *X. laevis*-TRE-Luc | 0.1 | 0.01–16 (>4) | 0.1–1 ↑ | 0.1–1 ↓ | Kudo et al. (2006) |
| | Yeast-human TRα | 1 | 0.001–100 (>20) | 1 ↑ | 4 ↓ | Terasaki et al. (2011) |
| | HepG2-human TRs | 1 | 0.001–100 (>10) | 1–10 ↑ | 1–10 ↓ | Hofmann et al. (2009) |
| | MtT/E-2-rat TRα or β | 1 | 1–10 (ND) | ND | 1–10 ↓ | Matsubara et al. (2012) |
| | Yeast-human TRβ-SRC-1 | 1 | 0.001–100 (≥5) | 1–5 ↑ | ND | Shiizaki et al. (2010) |
| | HeLa cell-TRα LBD | 3 | 1–10 (ND) | 3–10 ↑ | 3–10 ↓ | Fini et al. (2012b) |
| | CHO-human TRα | 3 | 0.01–100 (>50) | 10 ↑ | 3–50↓ | Kitamura et al. (2005) |
| | Rat PC-DR-LUC | 10 | 10–100 (>100) | 10–60 ↑<br>60–100 ↓ | 20–100 ↓ | Jugan et al. (2007) |
| | CV-1 cell-TRβ | 1 < | 1–100 (>100) | ND | 30 < EC50 ↓ | Sun et al. (2009) |
| | HEK293-vertebrat-e TRs | 10 | 0.01–100 (>10) | ND | 10 ↓ | Oka et al. (2012) |
| | Yeast-human TR-GRIP1 | ND | ND | ND | 0.7 (EC20) ↓ | Li et al. (2008) |
| | Cultured *X. laevis* tail | 1< | 0.010–1 (ND) | – | ND | Hinther et al. (2010) |

↑: induction; ↓: repression; –: no statistically significant effect; ND: not determined.

Asia (Dai et al., 2011; IUCN, 2014), the use of *P. nigromaculatus* as an alternate test species for *X. laevis* is very helpful for identifying the TH signaling disrupting effects of chemicals on native frogs.

## 4. Conclusions

We established a 48 hr-assay for TH signaling disruption using TH-response gene expression as endpoints in *P. nigromaculatus*, which can detect the agonistic activity of 0.001 nmol/L T3. In further validation of the assay, we found TH agonistic activity of TBBPA in the absence of TH and TH antagonistic activity in the presence of TH, which was reported in previous studies. Moreover, this assay detected a non-monotonic dose–response of TBBPA in disrupting TH signaling over a wide concentration range, including low concentrations. These results show that the *P. nigromaculatus* screening assay we established is suitable for detecting TH signaling disrupting activity of environmental chemicals with high sensitivity.

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