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Thyroid hormone-dependent gene expression as a biomarker of short-term 1,1-dichloro-2,2-*bis* (*p*-chlorophenyl)ethylene (DDE) exposure in European common frog (*Rana temporaria*) tadpoles

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Abstract
The effects on thyroid hormone-dependent gene biomarker responses of the persistent organochlorine pesticide metabolite 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE) were investigated after exposure of 4-week-old European common frog (*Rana temporaria*) (stage 36) tadpoles to two (0.001 and 0.01 ppm) DDE concentrations. Total body weight, total length, and tail length and width increased after 3-day exposure to DDE. Expression patterns of genes encoding for growth hormone, thyroid-stimulating hormone (TSHβ) and thyroid hormone receptor (TRα and TRβ) isoforms were evaluated in the head, body and tail regions using a validated real-time polymerase chain reaction (PCR) method. The mRNA expression of growth hormone in the body, and TSHβ in the head showed significant DDE concentration-dependent decreases. While DDE caused variable effects on TRα mRNA steady-state, the expression of TRβ was significantly decreased in the tail by DDE in a concentration-specific manner. The effect of DDE exposure on TRβ mRNA expression showed a negative correlation with tail length and width during the exposure period. The unique pattern of a DDE-induced decrease of tail TRβ expression probably reflects the significant role of this thyroid hormone receptor isoform in tail re-absorption and overall metamorphosis in anuran species. Therefore, the present study shows that the evaluation of thyroid hormone-dependent genes may represent quantitative biomarkers of acute exposure to organochlorine pesticides in anuran species during critical developmental periods such as metamorphosis. Given the widespread environmental levels of DDT and its metabolites, these pollutants will remain a subject of concern and their effects on anuran species should be studied in more detail.

Keywords: Rana temporaria, *1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDE), thyroid hormone pathway, gene expression, endocrine disruption*

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Introduction

A large number of chemicals released into the environment disrupt endocrine homeostasis in humans and animals by interfering with developmental processes and endocrine systems (Wade et al. 2002, Brown et al. 2004). Organochlorine

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pesticides such as 1,1,1-trichloro-2,2-*bis* ethane (DDT) have been progressively banned in much of North America and Europe owing to their long-term persistence and multiple chronic toxicities. Presently, exposure to such pesticides is through accumulated compounds in the sediments, where they were deposited from past usage (Turusov et al. 2002). Aerial dispersal from other areas of the world in which organochlorines are still in use are also potential source of exposure (Turusov et al. 2002). Laboratory and field data have implicated organochlorines in impaired reproductive success and abnormal sexual development in wildlife species. For example, DDT and its metabolites altered population structure by causing eggshell thinning and endocrine and reproductive toxicity in wild birds (Forsyth et al. 1994). 1,1-Dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE) is a persistent metabolite of DDT (Wade et al. 2002). The sexual abnormalities reported in alligators in Florida, USA, are assumed to be a result of the demasculinizing effects of DDT metabolites, including DDE (Guillette & Gunderson 2001). DDT and its metabolites have been shown to have variable effects on steroid hormone receptors (Kelce et al. 1995, Sohoni & Sumpter 1998, Arukwe & Jenssen 2005).

Declines in amphibian populations have been reported in many parts of the world, but the causes for the decline are still subject to ongoing discussions (Alford et al. 2001, Reeder et al. 2005). Suggested causes include parasitic infection, ultraviolet light radiation and xenobiotic chemicals (Carey 1995). There have been reports that the presence of DDE in lakes and wetlands harms the development of several amphibian species (Fellers et al. 2004). For example, two DDT metabolites (*p*,*p*-DDE and *o*,*p*-DDT) were observed in surface water at Sierra Nevada Mountain sites in California at 0.31 and 0.46 ng l^{-1}, respectively (Fellers et al. 2004). *p*,*p*-DDE was found at significantly greater concentrations in frog tissue at some sites (Fellers et al. 2004). The metamorphosis of anuran species is a complex process where the gills and tails are replaced with lungs and limbs, respectively. These structural and functional changes are dependent on endogenous thyroid hormones (Crump et al. 2002). The dependence on endogenous hormones suggests that metamorphosis will be vulnerable to endocrine disruptors at this developmental stage. Several research groups have investigated the effects of organochlorine pesticides on frogs. The herbicide acetochlor has been shown to accelerate triiodothyronine (T_3)-dependent metamorphosis in ranid species (Cheek et al. 1999a, Veldhoen & Helbing 2001) and in *Xenopus laevis* (Crump et al. 2002).

Thyroid hormones (T_3 and thyroxine; T_4) are required for their roles in various aspects of vertebrate development, metabolism, homeostasis, cellular proliferation and differentiation (Oppenheimer & Schwartz 1997). The pleiotropic effects of T_3 and T_4 are mediated through two thyroid hormone receptor proteins: TRα and TRβ (Gauthier et al. 1999). TRs function as hormone-inducible transcription factors that regulate the expression of target genes and are members of the hormone and orphan nuclear receptor superfamily (Gauthier et al. 1999). The homeostasis of the thyroid hormone system is maintained by thyroid-stimulating hormone (TSH). TSH positively regulates the thyroid hormone synthesis in anuran species (DeVito et al. 1999). Thyroid-disrupting chemicals such as organochlorines may target any of the multiple pathways within the thyroid hormone system in a chemical-dependent manner (Finnson & Eales 1997, O'Connor et al. 1999, Findlay et al. 2000, Kaptein et al. 2000, Chen et al. 2003, Ulrich 2003). Growth hormone is a pluripotent hormone produced by the anterior pituitary. In amphibians growth hormone

functions mainly in the stimulation of growth and metabolism, whereas its specific role in metamorphosis is still unclear. The expression pattern of growth hormone in tadpoles during metamorphosis shows that the level of growth hormone drops right before climax and increases post metamorphosis (Buckbinder & Brown 1993).

The present study was designed to investigate: (1) the acute effects of DDE on the expression of genes involved in growth, development and metamorphosis in different body regions (head, body and tail) of European common frog (*Rana temporaria*) tadpoles; and (2) to determine the utility of these genes as biomarkers of acute DDE exposure. It is hypothesized that exposure of tadpoles to different concentrations of DDE will cause differential body region-specific expression patterns for thyroid hormone-dependent genes, whose functional products may modulate growth and metamorphosis. These responses will indicate potential adverse metamorphic, health and physiological effects and represent quantitative biomarker of effect and exposure responses.

Materials and methods

Chemicals and reagents

1,1-Dichloro-2,2-*bis*(4-chlorophnyl)ethane (*p*,*p*-DDE or DDE) was purchased from Sigma Aldrich Co. (St Louis, MO, USA). The trizol reagent for RNA purification and TA Cloning kit were purchased from Invitrogen Corporation (Carlsbad, CA, USA). IScript cDNA Synthesis Kit and iTAQ™SYBR® Green Supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and GeneRuler™ 100-bp DNA Ladder and dNTPs from Fermentas GmbH (St. Leon-Rot, Germany).

Tadpoles and exposure

Stage-36 tadpoles (Gosner 1960) were divided into three groups ($n = 15$ per group) and kept in separate 10-litre aquaria at $14 \pm 0.5^{\circ}$C with a 12:12-h photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facility in Trondheim. During the experimental period, the tadpoles were fed TetraMin fish feed. The tadpoles were exposed once to waterborne concentrations of DDE (0.001 and 0.01 parts per million (ppm)) using dimethyl sulfoxide (DMSO; 7.5 ppb or 0.00075%) as carrier vehicle (control). The water was not changed during the treatment, and after day-3 post-exposure, five individual tadpoles from DDE and DMSO (solvent control) groups were anaesthetized with MS222 (0.5 mg ml^{-1}) and head, body, and tail tissues were removed and homogenized in Trizol (Invitrogen). The head region was defined as the distance from the tip of the snout to an imaginary line directly behind the eyes. The junction of the posterior body wall with the tail axis was defined as the body terminus. By using this convention for the body region, the tail was defined as the distance from the body terminus to the absolute tail tip. Moreover, tail width was measured as an imaginary line at the point of maximum tail width (McDiarmid & Altig 1999).

RNA purification and cDNA synthesis

Total RNA was purified from tissues homogenized in Trizol reagent according to manufacturer's protocol. Total cDNA for the real-time PCR reactions were generated

from 1 μg of total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

Primer optimization, cloning and sequencing

PCR primers were designed to amplify 108–280-bp products from conserved regions of the genes of interest. The primer sequences, their amplicon size and the optimal annealing temperatures are shown in Table I. Before PCR reactions, all primer pairs were used in titration reactions in order to determine optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCRII vector in INVαF′ *Escherichia coli* (Invitrogen). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Department of Biology, NTNU. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

Quantitative (real-time) polymerase chain reaction (PCR)

Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analysed using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25-μl DNA amplification reaction contained 12.5-μl of iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 1 μl of cDNA, and 400 nM of each forward and reverse primers. The three-step real-time PCR programme included an enzyme-activation step at 95°C (5 min) and 40 cycles of 95°C (30 s), 50–62°C (30 s, depending on the primers used) (Table I), and 72°C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification. The absence of genomic DNA in the real-time PCR reactions was also verified by using cDNA synthesized without the reverse transcriptase enzyme (RT control). Cycle threshold (C_t) values obtained were converted into nanogram (ng) of DNA using standard plots of C_t versus log ng DNA standards. Standard plots for each target sequence were generated using known amounts of plasmid containing the amplicon of interest. This method for quantifying gene expression patterns is well validated in the authors' laboratory and is routinely used (Arukwe 2005, Arukwe & Jenssen 2005, Mortensen & Arukwe 2006). Target cDNA amplification was averaged and expressed as ng μg^{-1} total RNA used in the RT reaction. The use of the standard curves was based on equal amplification efficiency (usually >90%) between target gene and plasmid-containing gene of interest.

Statistical analyses

Statistical analysis was performed with GraphPad Prism, version 2.1 (GraphPad Software, Inc., 1996). One-way analysis of variance (ANOVA) was used to detect significant differences between control and exposure groups. The level of significance was set at $p = 0.05$ unless otherwise stated.

Table I. Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time polymerase chain reaction (PCR).

	Primer sequence*				
Target gene	Forward	Reverse	Amplicon size (bp)	Annealing temperature (°C)	Accession number
TSHβ	TGCATGACAAAGGATCCAAA	AGCCAGGAATGGTCACTGTC	108	57	DQ358696
TRα	TCATCGACAAAATCACCC	TTCTTCAATCAGCTTCCG	128	53	DQ372976
TRβ	AGTGCCAAGAAGGTTTCC	CCAAGAATCCTGAAGCAC	232	55	DQ323118
GH	CAACAACCAGGTGTTTGGAA	GTGCAGTTGCTCTCCACAAA	280	55	DQ372977

*Sequences are given in the 5′ to 3′ order.

Results

Morphometric parameters

Morphometric parameters, including total weight (Figure 1A), total body length (Figure 1B), tail length (Figure 1C) and tail width (Figure 1D), were all increased in DDE exposure groups compared with the control group after day 3.

Effects on TSHβ and growth hormone gene expressions

Tadpoles exposed to 0.001 and 0.01 ppm DDE showed significantly decreased levels of TSHβ in the head region (Figure 2A). Waterborne exposure of tadpoles to DDE concentrations showed an apparent concentration-dependent decrease of growth hormone mRNA expression in the head region of *R. temporaria* tadpoles (Figure 2B). Particularly, exposure to the highest concentration (0.01 ppm DDE) caused significant decrease in growth hormone mRNA expression in head compared with control, while exposure to 0.001 ppm DDE caused non-significant decrease in growth hormone mRNA steady-state levels (Figure 2B). In the body, DDE exposure caused a significant concentration-dependent decrease of growth hormone mRNA levels (Figure 2C).

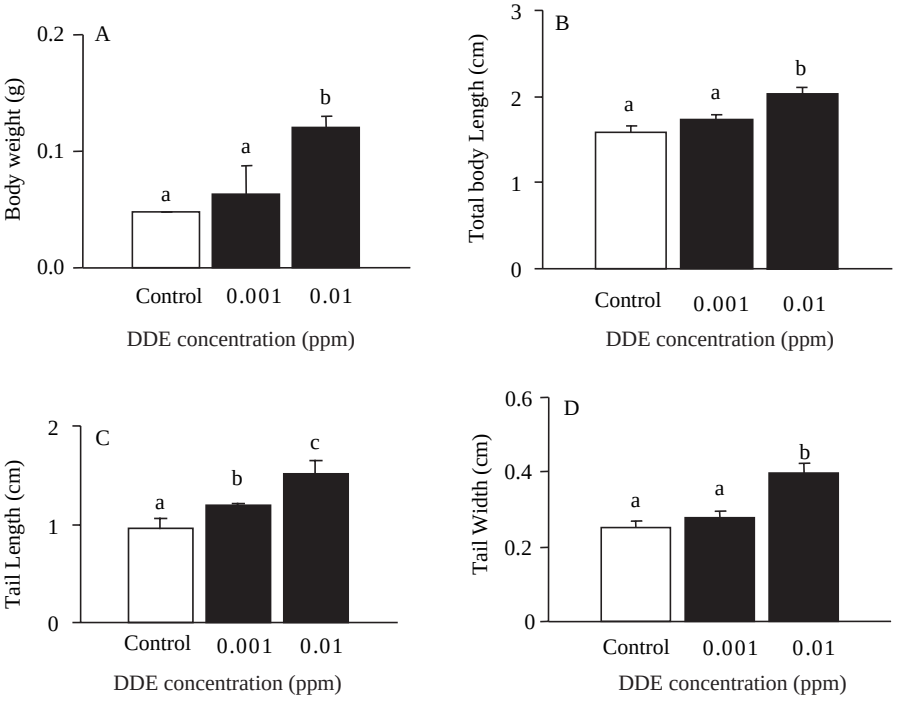

Figure 1. Morphometric parameters of control and treated (0.001 and 0.01 ppm 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE)) *Rana temporaria* tadpoles and sampled at day 3 post-exposure. Total bodyweight (A), total body length (B), tail length (C) and tail width (D). Data are given as means ± standard error of the mean (SEM) ($n = 5$). Different letters denote exposure group means that are significantly different, using the analysis of variance (ANOVA) followed by a Student's *t*-test ($p < 0.05$).

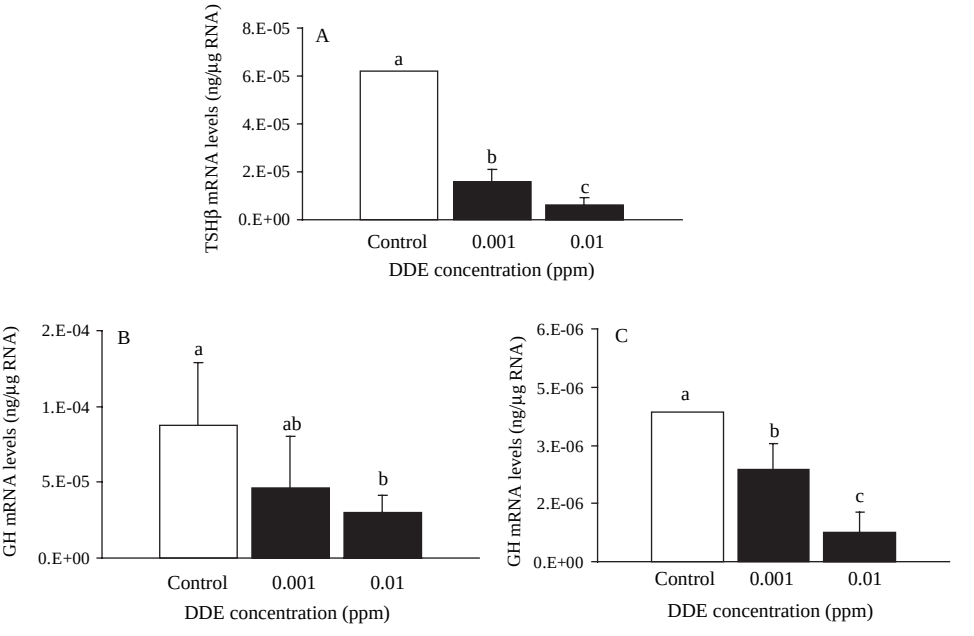

Figure 2. Expression of the thyroid-stimulating hormone β-subunit (TSHβ) and growth hormone (GH) mRNA in control and treated (0.001 and 0.01 ppm 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE)) *Rana temporaria* tadpoles and sampled at day 3 post-exposure. Head region TSHβ (A), head region GH (B) and body region GH (C) mRNA expression levels were quantified using real-time polymerase chain reaction (PCR) with specific primer pairs. Data are the means ± standard error of the mean (SEM) ($n = 5$). Different letters denote exposure group means that are significantly different, using the analysis of variance (ANOVA) ($p < 0.05$).

Effects on thyroid hormone receptor gene isoforms

Waterborne exposure of tadpoles to DDE (0.001 and 0.01 ppm) showed no significant alterations in TRα gene expression compared with the control group (Figure 3). In the head region, a non-significant decrease of mRNA levels was observed in the DDE exposed groups (Figure 3A) and no change in TRα mRNA steady-state levels were observed in the body (Figure 3B) and tail (Figure 3C) regions. Transcription of TRβ in head region was decreased (albeit not significant) after exposure to DDE concentrations (Figure 4A). In the body, TRβ mRNA was significantly induced and reduced after 3-day exposure to 0.001 and 0.01 ppm DDE, respectively (Figure 4B). Tail TRβ mRNA expression showed an apparent DDE concentration-dependent decreases compared with the control group (Figure 4C). TRβ mRNA levels were more abundant in the tail compared with head and body regions (Figure 4). It should also be noted that the low DDE concentration, 0.001 ppm, resulted in increased and decreased TRβ transcript levels in body and tail (Figure 4B and C, respectively).

Discussion

Metamorphosis is an essential commitment to adulthood. Thyroid hormones have a permissive influence on the regulation of anabolic metabolism, cellular differentia-

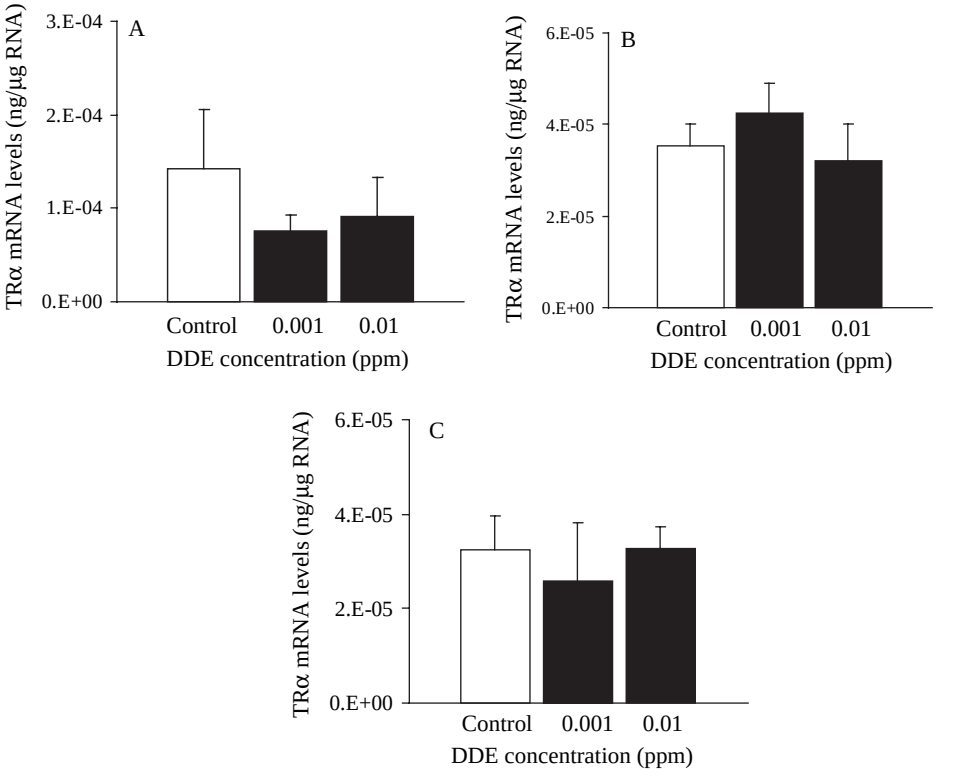

Figure 3. Expression of thyroid receptor-α (TRα) mRNA in the head (A), body (B) and tail (C) of control and treated (0.001 and 0.01 ppm 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE)) *Rana temporaria* tadpoles and sampled at day 3 post-exposure. TRα mRNA expression levels were quantified using real-time polymerase chain reaction (PCR) with specific primer pairs. Data are given as the means ± standard error of the mean (SEM) ($n = 5$). Different letters denote exposure group means that are significantly different, using the analysis of variance (ANOVA) followed by a Student's *t*-test ($p < 0.05$).

tion, growth and development, including metamorphosis, of organ systems. The overt toxicity of DDE to the hypothalamus–pituitary–thyroid (HPT) axis would have the greatest consequences for foetal or juvenile animals where cellular differentiation is occurring in many tissues through the body (Sachs et al. 2000, Power et al. 2001). We studied the effects of DDE on thyroid hormone-dependent gene expression patterns in relation to morphological changes. Changes in the transcriptome occur before morphological changes, but are indicative of molecular and ultimately cellular events. Because molecular and cellular events are causal and occur in a defined temporal sequence, and these molecular events branch out into different directions. Therefore, up- and down-regulation of genes (i.e. molecular profiles of gene expression patterns) are molecular approaches with a potential to serve as predictors (biomarkers) of xenobiotic effects on development. Molecular events also provide crucial and reliable information for specific mechanisms of thyroid hormone action on the transformation to adulthood in anuran species. The present data demonstrate that DDE concentrations decreased mRNA expression of TSHβ and TRβ gene expression in the head and tail regions, and growth hormone in the body region. Interestingly, DDE exposure caused a

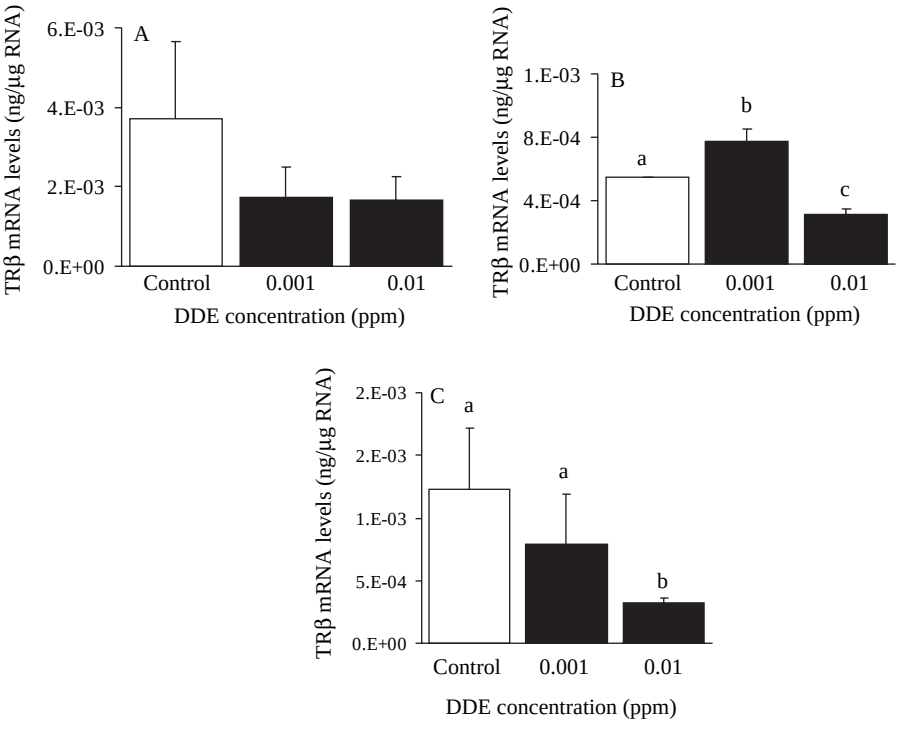

Figure 4. Expression of thyroid receptor-β (TRβ) mRNA in the head (A), body (B) and tail (C) of control and treated (0.001 and 0.01 ppm 1,1-dichloro-2,2-*bis*(*p*-chlorophenyl)ethylene (DDE)) *Rana temporaria* tadpoles and sampled at day 3 post-exposure. TRβ mRNA expression levels were quantified using real-time polymerase chain reaction (PCR) with specific primer pairs. Data are given as the means ± standard error of the mean (SEM) ($n = 5$). Different letters denote exposure group means that are significantly different, using the analysis of variance (ANOVA) ($p < 0.05$).

concentration-dependent increase in the average weight, total length, tail length and width. The effects of DDE on morphometric parameters, especially the increased tail length and width showed a negative relationship with decreased expression of TRβ mRNA. These responses therefore serve as potential biomarkers in the respective anatomical regions, for exposure to and effect of DDE and related environmental chemicals.

Effects on TSHβ, TRα and TRβ gene expressions

TSH plays an important role in the maintenance of the thyroid hormone system homeostasis. A rise in TSH synthesis will occur as a result of decreased thyroid hormone levels and vica versa. It was observed that TSHβ gene expression in the head region decreased in response to DDE exposure. The brain is the main TSH-producing organ in frog; the decreases observed in this experiment are in accordance with previous observations demonstrating decreased expression of TSHβ mRNA in salmon presmolt exposed to DDE and thyroxine singly and in combination (Mortensen & Arukwe 2006). In rats, it was reported that DDE significantly decreased serum T4, but not T3 or TSH (Yamada et al. 2004). Decreased TSHβ expression in the head region may indicate that DDE interferes with the molecular control of the HPT axis,

specifically targeting the TSH receptors rather than TRs. This aspect of DDE as an endocrine disruptor needs further investigation. Exposure of tadpoles to DDE inhibited transcription of tail TRβ gene in apparent concentration-dependent manner. It should be noted that TRβ mRNA showed a negative relationship with tail length and width after DDE exposure. It is well known that TRβ is the most abundant of the receptor isoforms at the time of tail re-absorption. In this regard, the decreases in TRβ levels suggest a potential for DDE-induced inhibition of metamorphic re-absorption of tail tissues through a thyroid hormone-dependent mechanism. Additional experiments should be conducted during the tail re-absorption stage to determine if these impacts on TRβ in the tail would actually inhibit the process. Given that DDE does not bind to TR isoforms (Cheek et al. 1999a), other factors involved in the HPT axis could be considered as possible targets for DDE.

The DDE-induced decrease of TRβ reported in the present study may cause alterations in hormone sensitivity (loss of function) or hypersensitivity (gain of function) (Yamada et al. 2004). It may also result to compensatory response to reduced T_3 levels, as reduced plasma T_3 levels have been observed after organochlorine exposure (Braathen et al. 2004). It might also result in other epigenetic effects that might change the nature of TR-containing complexes. The general significance of induction or reduction of TRβ in different body regions after exposure to DDE is still a subject of speculation. An abnormal expression pattern of TRβ might cause a failure of thyroid hormones to bind and activate the appropriate post-receptor response cascades (Oppenheimer 1979, Wu & Koenig 2000). Both of these effects might also affect circulating thyroid hormone levels through the de-iodinase enzyme activity (Sambroni et al. 2001). However, all these effects will have significant consequences to the long-term health of embryo and pre-metamorphic juveniles. No significant DDE-related alteration of TRα mRNA steady-state levels was observed in the present study. The non-significant effect of DDE on TRα observed in this study probably reflects the metabolic demands of tadpoles during metamorphosis. The TRα isoform mediates the actions of thyroid hormone-dependent metabolism and homeostasis and therefore less susceptible to toxicant exposure at this developmental stage (and thus not a good biomarker).

Due to very low blood levels in the experimental animals, we did not investigate plasma hormone levels, and as a result of this the scope of the study was limited to the evaluation of DDE-induced transcriptional changes in thyroid hormone-dependent gene expression in different body regions. Nevertheless, there are several aspects of the present study that can be related to other findings on the effects of persistent pollutants on the thyroid pathways of wildlife species. The major mechanism of thyroid hormone action involves hormone binding to nuclear thyroid hormone receptors (TRα and TRβ), resulting in tissue-specific activation/repression of gene transcription (Helbing & Atkinson 1994, Sachs et al. 2002). In a competitive inhibition experiment using hydroxylated polychlorinated biphenyls (OH-PCBs), DDT and its metabolites, and several organochlorine herbicides, Cheek et al. (1999b) reported that only the OH-PCBs competed for binding to TR sites. The present study shows a possible effect of DDE on the induction of other signal transduction pathways that results in TSHβ, TRα and TRβ gene transcription that might affect survival and growth.

Recently, we showed that exposure of adult male *R. temporaria* to subcutaneous DDE doses caused differential organ-specific expression patterns of TRα and TRβ

mRNA (Arukwe & Jenssen 2005). The mechanisms of chemical disruption of the thyroid hormone system still remain speculative. An extrapolation of the present data from controlled laboratory gene expression data to other vertebrate species is necessary to suggest potential mechanisms of disruption of the thyroid axis (Arukwe & Jenssen 2005). For example, the differential effects of DDE on TSHβ, TRα and TRβ mRNA expressions in the different body regions suggests a possible direct effect of DDE on the thyroid hormone feedback system or the pituitary (Wu et al. 2001). Another mechanism of chemical disruption of the thyroid endocrine system involves the interference of contaminants with thyroid hormone plasma carrier proteins (Brouwer 1989, Cheek et al. 1999b).

Organochlorine compounds were shown to alter thyroid-mediated homeostasis in many species, including humans. Several reports show that organochlorines including OH-PCBs affect plasma levels of thyroid hormones and/or their transport proteins (transthyretin; TTR) in humans (Hagmar et al. 2001), rats (Zoeller et al. 2000, Schmutzler et al. 2004), fish (Brown et al. 2002), birds (Bishop et al. 1998, Verreault et al. 2004), minks (Restum et al. 1998), polar bears (Braathen et al. 2004) and other wildlife species (Colborn 2002). Elsewhere, the herbicide acetochlor was shown to accelerate thyroid hormone-induced metamorphosis in *X. laevis* (Veldhoen & Helbing 2001, Crump et al. 2002).

Effect on growth hormone

Growth hormone plays an essential role in the regulation of growth and development by promoting cell division, differentiation, enlargement and metamorphosis (Buckbinder & Brown 1993). The present study demonstrates that exposure of tadpoles to DDE causes concentration-dependent decreases in growth hormone gene expression in the head and body region. The data for the body actually show a more pronounced and significantly different pattern of expression at both DDE concentrations. The specific role of growth hormone in amphibian metamorphosis is not well established. It has previously been reported that amphibian pituitary glands contain a growth-promoting hormone(s) (Mosconi et al. 2002). The expression pattern of growth hormone in tadpoles during metamorphosis shows that the level of growth hormone might decrease before and increase after metamorphosis (Buckbinder & Brown 1993). In an experiment with transgenic frog tadpoles over-expressing growth hormone, the overall growth was stimulated in various parts of the tadpole including limbs and tail (Huang & Brown 2000). Growth hormone has also been reported to promote limb growth and development during pre-metamorphosis, but no effect on spontaneous or induced metamorphosis thereafter was observed (Wright et al. 1994). Therefore, the effect of DDE on growth hormone mRNA steady-state levels warrants attention.

Conclusion

The data indicate that DDE affected the transcriptional levels of TSHβ, growth hormone and TRβ expression in different anatomical regions of *R. temporaria* tadpoles. Before phenotypic manifestation, the first interaction between contaminants and organisms occur at the molecular levels. Therefore, changes in gene expression as a result of environmental stressors and the subsequent molecular processes leading to

adverse health and developmental effects may be used as quantitative biomarkers for cellular, organismal and population effects. Therefore, the present data suggest that these responses can potentially lead to higher levels of effects and the usefulness of these gene responses could be validated and considered as biomarkers for acute DDE exposure in anuran species. These responses may also be applied to non-invasive approaches with tissue biopsy, as previously demonstrated by Veldhoen & Helbing (2001). Given the high persistency in the environment and continued use in developing countries coupled with the tendency for global atmospheric transport, DDT and its metabolites such as DDE will remain a focus of concern for both scientific and societal reasons.

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