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353-NONYLPHENOL INDUCES EXPRESSION OF THE T-BOX6 GENE IN ZEBRAFISH EMBRYOS – LINKING TRANSCRIPTIONAL INFORMATION WITH DEFORMITIES

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Abstract: Nonylphenols (NP) can be detected in the environment worldwide, the major component of the technical NP mixture, *p353*-NP, produce embryonic misdevelopment in zebrafish (*Danio rerio*). Phenotypes with comparable appearance were generated with knock out or other mutants in zebrafish embryos. The genes involved in these studies were: *no tail (ntl) gene*, *spade tail (spt) gene* and *tbox 6 (tbx6)gene*. Aim of this study was to correlate the observed teratogenic effect of *p353*-NP in zebrafish with expression analysis of genes of the T-Box family involved in normal development of the tail and therefore increase the understanding of the mechanisms by which NP-induced disease. Zebrafish embryos were treated with sub lethal concentrations of different NP isomers. A unique phenotype with massively swollen tail tip was only seen using *p353*-NP. Expression of *ntl gene* and *spt gene* measured by real time PCR was unaltered while expression of *tbx6* massively increased after *p353*-NP administration.

Keywords: Danio rerio, Nonylphenol, T-Box, no tail, Spade tail, Quantitative gene expression, Embryonic development

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Özet: **353-Nonylfenolün Zebra Balığı Embriyolarında T-BOX6 Geninin Ekspresyonuna Neden Olması ve Bunun, Deformitelerle Tanımsal Bilgiyle İlişkilendirilmesi**

Nonylpehoneller (NP) dünya genelinde çevremizde bulunurlar. Teknik olarak NP karışımını oluşturan ana bileşen p353-NP, zebra balıklarında (*Danio rerio*) embriyonik gelişme bozukluğuna neden olmaktadır. Zebra balığı embriyolarında dış görünüş olarak ayırt edilebilen fenotipler knock-out veya diğer mutantlar sayesinde elde edilir. Bu çalışmalarda kullanılan genler: kuyruksuz (ntl) geni, mahmuz kuyruk (spt) ve t box 6 *(tbx6)* genidir. Çalışmanın amacı *p353*-NP'nin normal kuyruk gelişiminde T-box ailesine mensup genlere olan teratogenik etkisini inceleyerek NP kaynaklı hastalıkların mekanizmalarının daha iyi anlaşılmasıdır. Zebra embriyoları sub-lethal konsantrasyonda NP izomerleri ile muamele edilmiştir. Özel bir fenotip olan kuyruk ucunda aşırı şişme sadece p353-NP kullanıldığında görülmüştür. Ntl geni ve spt geninin PCR ile sayımı sonucu değişmemiş olmakla birlikte tbx6 geninde p353-NP sonrası aşırı artış görülmüştür.

Anahtar Kelimeler: *Danio rerio*, Nonylphenol, T-Box, kuyruksuz, Mahmuz kuyruk, Kantitativ gen ekspresiyonu, Embriyonik gelişim

Introduction

Nonylphenols (NP) have been found in almost every environmental compartment such as rain and surface water, snow, sewage sludge and sediments (Lin et al, 2004; Kawahata et al. 2004; , 2006; Stachel et al. 2005; Isobe et al. 2004; Kannan et al. 2003). NP are microbiological metabolites of alkylphenol polyethoxylates. Alkylphenol polyethoxylates have exceptional surface properties, are used widely in industrial processes, and account for 5% of the tenside market (Sharma et al. 2009).

NP directly interact with the estrogen receptor in vertebrates (Owens et al. 2003; Folmar et al. 2002; Bevan et al. 2003) and exert effects on the reproduction of fish especially in vitellogenin synthesis (Harries et al 1997, Christiansen et al. 1998, Giesy et al. 2000) – a precursor of lipoproteins and phosphoproteins in egg yolk-production. Additionally, NP disturb early differentiation in fish inducing development of intersex gonads and circulatory and other systemic abnormalities (Gray et al. 1997). NP bind to the 17-β-estradiol receptor, induce morphologic deformations, increase apoptosis, and alter the deposition and differentiation of neural crest-derived melanocytes in the tailbud stage of *X.laevis* embryos (Bevan et al 2003). Their tendency to partly mimic estrogen and disrupt the natural hormonal balance supports the inclusion of NP to the group of endocrine disrupters. A detailed analysis of the risk posed by NP was conducted by the European Union and led to restricted use within the EU (ECB 2002).

Due to the synthetic methods used, technical NP (*t*-NP*)* is a mixture of approximately 20 para-substituted isomers with differently branched alkyl chains (Russ et al. 2005; Wheeler et al. 1997) whereas isomeric composition varies between mixtures *p*353-NP is the most abundant isomer. The absolute concentration of *p*353-NP in technical mixtures depends on the production process (Russ et al. 2005). It was shown previously that NP isomers varied in their estrogenic potency in the Yeast Estrogen Screen (YES) assay (Gabriel et al. 2008), in the E-screen (Preuss et al. 2010) as well as the MVLN reporter gene cell assay (Preuss et al. 2006, Preuss et al. 2010) demonstrated that *t*-NP and *p*353-NP, as well as *p*363-NP, *p*33-NP and *p*252-NP can be classified as partial agonists in the MVLN cell assay, whereas *p*262-NP, *p*22-NP and 4*n*-NP can be classified as antagonists in the MVLN cell assay.

Embryos of zebrafish (*Danio rerio*) have often been used in toxicity studies of environmentally relevant substances (Hollert et al. 2003; Kosmehl et al. 2006; Scholz et al. 2008). An advantage of zebrafish as test organism in toxicological research is the transparency of the eggs. Spinal malformation observed after exposure of zebrafish embryos to *p*353-NP (Kammann et al. 2009) is comparable to those reported form several groups working on developmental processes: Zebrafish expressing a mutant (non-functional) of the *no tail* (*ntl*) gene or a mutant of the *spade tail gene* (*spt, tbx16*) lack the notochord and tail (Halpern et al. 1993, Amacher et al. 2001;

Schulte-Merker et al. 1994). Studies with knock out mutants showed that homozygous *spt*¯(double knock out embryos) lack trunk somites and trunk muscle later in development (Griffin et al. 1998; Ho et al. 1990). Ntl and spt proteins belong to the T-box6/16 protein subfamily of T-box transcription factors which contain a DNA binding and protein dimerization domain: The T-box (Naiche et al. 2005). The T-box6/16 subfamily has important functions in the development of the mesoderm in all mammals investigated (Wardle et al. 2008). The damages of *ntl* gene knock out and *spt* gene knock out phenotypes in zebrafish are less severe than mutations in or depletion of the homologous genes in other vertebrates indicating a functional redundancy among zebrafish T-box genes.

Understanding the molecular mechanisms involved in misdevelopment produced by NP is a prerequisite for further investigations in limiting the effects of NP release to the environment. Aim of the present study was to correlate the observed tail deformation produced by *p*353-NP with expression data of genes of the T-box6/16 subfamily. Zebrafish embryos were exposed to sublethal concentrations of different isomers of NP and real time PCR analysis for genes belonging to the T-box6/16 family was performed.

Materials and Methods

Chemicals

The synthesized NP used in this study (Table 1) were synthesized and purified as described in Preuss et al (2006). All isomers had a purity >99%. Technical nonylphenol (*t*-NP) and dimethyl sulfoxide (DMSO 99.9%) was purchased from Sigma-Aldrich/Fluka (Deisenhofen, Germany). Substances used for RNA extraction were purchased from AppliChem (Darmstadt, Germany), enzymes used for cDNA synthesis and real time PCR were supplied by MBI Fermentas (St. LeonRot, Germany).

Breeding of zebrafish

250 zebrafishes were held in breeding groups of 20 females and 30 males. Fish were kept at 26°C and a light/dark period of 14h/10h in tap water. For collection of eggs translucent plastic spawning boxes (12 x 20 x 12 cm) with a stainless steel mesh insert (3 - 4 mm mesh size) were placed in the aquaria. On top of the mesh some green plastic net material and green glass marbles animated the fish to spawn into the box. Although not all fish spawned into the boxes, up to 3000 eggs/day could be collected from the stock using 2 boxes for each breeding group. Eggs are 1.0 - 1.2 mm in diameter and have a transparent chorion. Fertilized and well developed eggs in the 4 to 8 cell stage were selected for the test and placed in 24-well dishes (Nunc GmbH, Wiesbaden, Germany). The mean spontaneous lethality rate was 2%. Fertilization rates were > 80%.

Treatment of zebrafish

(1) For morphological effects observation zebrafish embryo test was carried out according to [31]: In each well of a 24well dish 5 zebrafish embryos (4-8-cell stage) were exposed for 48 hr at 26°C to 1 ml test solution containing one NP isomer (2.5-50 µmol/L) and 1% DMSO resulting in a total number of 60 embryos for each test, either exposure or control. Since all experiments were repeated twice a total of 120 zebrafish embryos were used for each concentration of each chemical. Negative controls were exposed to the 1% DMSO solvent only. EC_{50} was calculated from concentration-effect curves for the spinal malformations. Measurement was the amount of embryo showing any spinal deformation without respecting a certain degree of the misdevelopment. Four-parameter logistic curves were used to describe the concentration-effect relation. From these, EC_{50} values were calculated.

(2) For RT-PCR 3-4 hr old zebrafish embryos were placed into 24-well dishes (5 embryos per well). For each experiment, either exposure or control, 30 embryos were used. The embryos were treated with the indicated NP at a final concentration of 10 µmol/L for 1 hr in water containing DMSO at 26 °C. These parameters were chosen because in preliminary experiments expression effects were observable after 1 hr and the concentration of NP were selected on the basis of our previous study (Kammann et al. 2009). After incubation, the embryos were washed in fresh water and subsequently shock frozen in liquid nitrogen. All experiments - covering 6 NP isomers, the technical NP mixture (Table 1) and a negative control with DMSO only - were repeated 5 times at different days. The concentration of the test substances was 10 µmol/L, i.e., in the range of EC_{10} - EC_{50} of acute morphological effects of NP isomers in zebrafish after 48h exposure (Kammann et al. 2009).

RNA Extraction

RNA was extracted using a modification of the method of Chomczynski P, Sacchi (1987). In brief, 30 frozen embryos were carefully homogenized in 1 ml of GuaSCN-buffer (3 M guanidinthiocyante, 50 mM TRIS, 10 mM EDTA, 8% sarcosyl, 1% β-mercapethanol pH 7.0). After addition of 0.1 ml of 2 M sodium acetate (pH 4), 1 ml water saturated phenol and 0.2 ml chloroform the samples were centrifuged at 15.000g for 15 min. The supernatant was collected and RNA was precipitated by adding of 1 ml of isopropanol followed by two ethanol (70%) washes. Concentration and purity of RNA was determined by absorption measurement at 230, 260 and 280 nm.

Table 1. Characterization of the nonyphenol isomers including induced spinal malformations with effective concentration (EC_{50})

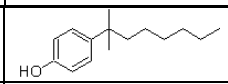
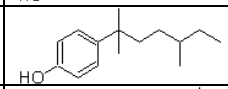
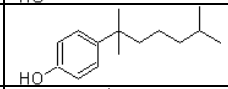
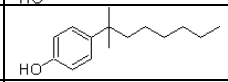
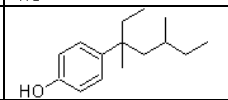
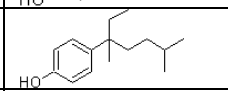
Abbreviation	Name	Structure	Spinal malformation	EC_{50} ± SEM spinal malformation [µmol/L]
*p*22 NP	4-(2'methyl-2'-octyl)phenol		very short tail	n.d.
*p*252 NP	4-(2',5'-dimetyl-2'heptyl)phenol		bended tail	7.9 ± 16.2
*p*262 NP	4-(2',5'-dimetyl-2'hexyl)phenol		bended tail	7.4 ± 0.8
*p*33 NP	4-(3'methyl-3'-octyl)phenol		bended tail	n.d.
*p*353 NP	4-(3',5'-dimetyl-3'heptyl)phenol		broadened tail tip	14.5 ± 0.45
*p*363 NP	4-(3',5'-dimetyl-3'hexyl)phenol		swollen tail tip	10.0 ± 0.25
t- NP	technical mixture		bended tail	9.5 ± 0.58

Table 2. Primers used for Real time PCR

	Forward Primer 5'-3'	Reverse Primer 5'-3'	Amplicon size (bp)	System efficiency (E)	Reference
β-actin	CGAGCTGTCTTCCCATCCA	TCACCAACGTAGCTGTCTTTCTG	86	2.00	Lin et al. 2009
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	148	1.91	Lin et al. 2009
ef1α	AGACGCACAATCTTGAGAGCAG	ATCAAGAAGAGTAGTACCGCTAGCATTAC	87	1.95	Lin et al. 2009
ntl	ACGAATGTTTCCCGTGCTCA	CGTTCACGTATTTCCACCGAT	112	1.91	this paper
spt	CTGGTGCCGTATGCAAAGTACA	AGCTTTACCTGCAACCTCCCA	103	2.00	this paper
tbx6	CACAGCTCTCGATCTTGCAGTG	TATGGATTAGATTGCCGGACT	93	1.93	this paper

cDNA Synthesis

Two hundred ng total RNA was digested with DNase for 30 min at 37°C, terminated by adding EDTA to final a concentration of 25µmol/l) and then transcribed. Reverse transcription was carried out in a total volume of 20 µl containing 10 mmol/l Tris-HCl, (pH 8.8), 50 mmol/l KCl, 5 mmol/L $MgCl_2$, 1 mmol/l each dNTPs, 0.5 µg oligo (dT) primers, 0.5 µg random primers, 25 U RNase inhibitor, and 200 U M-MuLV reverse transcriptase for 5 min at 25°C followed 1 hr at 42°min. The reaction was stopped by incubation for 5 min at 70°C

Real time PCR

Real time PCR was performed in an Applied Biosystems 7500 Real-Time PCR System using 1 µl of the synthesized cDNA in a final volume of 15 µl. Details on the primer combinations are shown in Table 3. Each assay included a no template control and all measurements were performed in duplicate. The efficiency of the system was measured by using serial 2-fold dilutions of a mixture of different cDNAs. The efficiency indicates the amplicon doubling rate of a primer pair. The Ct value is defined as the number of cycles needed for the fluorescence signal to rise above a threshold level of detection. Obtained Ct values of the dilution series were plotted and the resulting slope of the linear graph was used for calculation of the system efficiency using the equation: $E=1/10^{1/slope}$ (Scheffe et al. 2006) The system efficiency ranged between 1.91 and 2.11 (Table 2). The dissociation plots (melting curve analysis) indicated a single peak for all primer pairs further supporting specificity. To evaluate the different housekeeping genes used in the study the original concentration of the transcript was calculated using the formula $Conc_{trans} = E^{-Ct}$. The concentration of the transcripts *ntl*, *spt* and *tbx6* related to the housekeeping genes *ef1α* and *rpl13a* was calculated using the formula:

$$C_{transcript} = \frac{2 \bullet E_{transcript}^{-Ct_{transcript}}}{(E_{ef1\alpha}^{-Ct_{ef1\alpha}} + E_{rpl13\alpha}^{-Ct_{rpl13\alpha}})}$$

The concentrations of the transcript (arbitrary units) were multiplied by a transcript-specific factor to gain a scale from 0 to 100. These concentrations are comparable within one transcript but not between transcripts.

Statistics

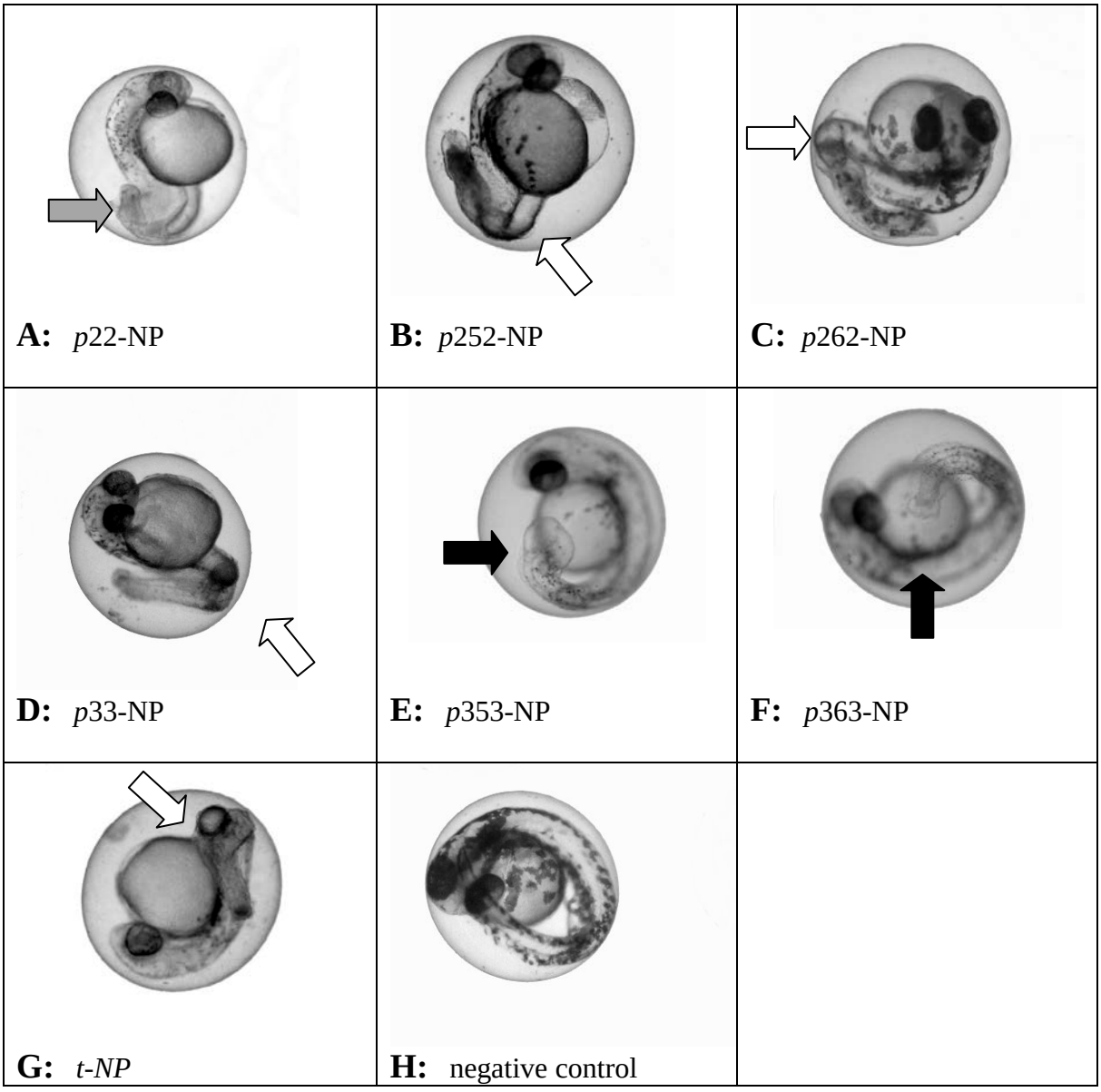
Statistical significances of relative mRNA expression between different NP isomers were determined using Prism®4.03 (GraphPad Software, La Jolla, CA USA). The calculated concentrations related to the expression of *rpl13a* and *ef1α* were log transformed and univariate analyses of variance (ANOVA) were performed to detect statistically significant differences between any groups. Data are expressed as mean ±standard error of the mean (SEM), arbitrary units, values related to the expression of the gene of interest in the DMSO control and related to the mean of the expression observed with *rpl13a gene* and *ef1α gene.* As post test to determine the actual differences between individual groups the Tukey-Kramer test was employed. The p-value of $p<0.05$ was considered to be significant.

Results and Discussion

The development of zebrafish embryos after incubation with various isomers of NP is demonstrated in Figure 1. Normal development after 48 hr of incubation results in a long extended tail without any bending or tip broadening (Figure 1H). The malformations in development of the tail produced by treatment with different isomers of NP are shown in Figure 1A-G. In some cases the malformation were accompanied by necrosis of the tail tip (results not shown). All NP isomers produced spinal malformations: Zebrafish embryos treated with *p*22-NP showed short tails which appeared to be incomplete developed (Figure 1A). Exposure of zebrafish embryos to *p*252-NP, *p*262-NP, *p*33-NP and *t*-NP results in bended tails. These tails showed a sharp kink in the middle of the tail length combined with a turn in the tail orientation (Figure 1B, C, D and G). Since the concentration of *p*22-NP and *p*33-NP required to induce the malformation described was 50µmol/l, calculation of EC50 was not possible. *p*353-NP produced a tail tip broadening (Figure 1E) which was described previously (Kammann et al 2009). At a concentration of 16 µmol/l 50% of the embryos showed that deformation (Table1). Slight swelling of the tail tip was observed after exposure of embryos to *p*363-NP (Figure 1F). The phenotype of tail tip broadening is unique for *p*353-NP and was never seen with other isomers of NP. After 1 hr of treatment of 3-4 hr old embryos no marked differences in

phenotypes could be detected. The first time when tail tip shape was visible was around 24 hr after fertilization – independent of the treatment. No general alterations in the tail were observed from then until 48 hr after fertilization, when the tail tip was visible best. This time frame was selected because in preliminary experiments one could demonstrate that a change in expression of the selected genes is observable after 1 hr exposure, demonstrating that gene activation of transcription factors is rapid.

Figure 1. 48h old zebrafish embryos after exposition with different isomers of nonylphenols (A-F), technical mixture of nonylphenol (G) and a control embryo without any treatment (H). The arrows indicate the tail malformations: White: bended tail; black: broadened tail tip; grey: very short tail.



To combine the effects of NP with expression data, several possible housekeeping genes used for quantification were investigated. The measured concentrations of the housekeeping genes *rpl13* (mitochondrial), *ef1α* and *β-actin* based on the amount of RNA used for reverse transcription is shown in Figure 2. No significant differences were found. However, the expression of *β-actin gene* appeared smaller in the *p*262-NP/ *p*22-NP/ *p*252-NP group compared to the other groups (*p*33-NP/ *p*353-NP/ *p*363-NP, *t*-NP and DMSO alone). Zebrafish embryos treated with NP methylated on the 2' position (*p*22-NP, *p*252-NP, *p*262-NP) compared with zebrafish embryos treated with NP methylated on the 3' position (*p*33-NP, *p*353-NP, *p*363-NP) showed a mean difference of 0.47 (relative expression). This difference failed to be statistical significant. Assigning the data of the NP known for antiestrogenic effects (*p*22-NP and *p*262-NP,) to one group and data for tNP known to exert estrogenic effects (*p*33-NP, *p*252-NP, *p*353-NP and *p*363-NP) to the other group reveals that the expression of *β-actin gene* in the antiestrogenic group is significantly different from the DMSO control while the comparison between the estrogenic group and DMSO control shows no significant differences (0,07±0.04 and 0,54±0.17 respectively). However, since no marked differences were observed using the other housekeeping genes all further data were related to the mean of the expression observed with *rpl13a gene* and *ef1α gene*. This combination shows a homogeneous distribution over all groups (Figure 2d) and was used to normalize the expression data.

Expression of *ntl gene, spt gene* and *tbx16 gene* is shown in Figure 3. Although the zebrafish were harvested within a short period of time the variation within the single groups is remarkably high. Therefore statistical significant differences are seldom observed. Highest expression of *ntl gene* (38.8±30.6) was seen after exposition the embryos to *p*252-NP, followed by the expression after treatment with *p*33-NP (16.8±11.7). The expression of *ntl gene* in the other zebra fish embryos varied between 7.7±4.4 in *p*22-NP and 0.8±0.6 in the *p*353-NP group. Expression of *spt gene* was highest in zebrafish embryos treated with *t*-NP embryos (33.3±15.6). The *spt gene* expression in zebra fish embryos under treatment of the different isomers of nonyphenol used varied between 0.72±0.63 (*p*353-NP) and 20.9±16.1 (*p*252-NP). Performing linear regression using the expression of *spt gene* and *ntl gene* based on the response of the individual embryo revealed a significant correlation with a coefficient of determination R^2= 0.9172.

The expression of *tbx6 gene* measured for zebrafish embryos exposed to all isomers but *p*353-NP and *t*-NP is comparable to the expression observed in zebrafish embryos treated with DMSO (values between 0.3±0.1 for *p*252-NP and 2±2 for *p33*-NP). Zebrafish embryos treated with *t*-NP showed an expression of 9.8±3.7. A marked increase in *tbx6 gene* expression was observed in zebra fish embryos treated with *p*353-NP (83.5±14.7). This induction is statistically significant different from the expression of *tbx6 gene* in zebrafish exposed to DMSO alone.

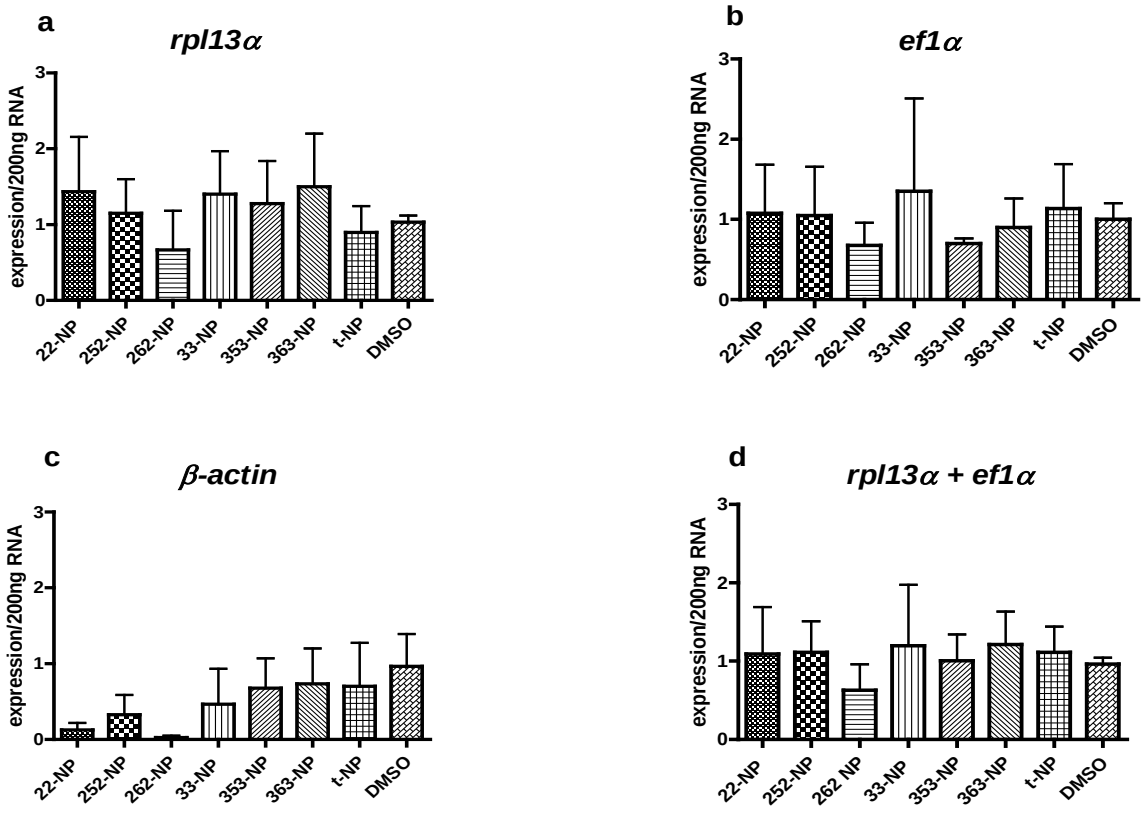
In addition to the often described action of NP as estrogen like substances it was possible to demonstrate that some NP have the potential to induce tail-malformation in fish embryos. The observed tail malformation seen in our experiments may be due to miss-migration of precursor cells of the mesoderm in early development. The correct migration is a prerequisite of normal development (Christ et al. 2002). The various spinal malformations induced by NP isomers underline the different effects and possible modes of action known for NP isomers. *p*353-NP showed a unique morphological alteration: A broadened tail tip. This deformity might be related to the *tbx6* expression as discussed below.

To investigate gene expression pattern of the tbx6/16 family in zebrafish embryos it is necessary to study the role of the housekeeping genes which might be regulated depending on developmental stage and/or treatment of the organism (Guenin et al. 2009). Data showed found that the often used expression of housekeeping gene *ß-actin gene* is not suitable in experiments using NP. HSU et al. (1987) previously demonstrated that the *ß-actin gene* is upregulated by estrogens, interestingly a down regulation was found in the two antiestrogenic NP isomers (*p*22-NP and *p*262-NP;Figure 2a). The partial estrogenic isomers show quantitative down regulation. This gene expression pattern, in line with the known mode of action for the isomers, indicates that at the selected time point gene expression react already to NP exposure. It was decided to use the average expression of two genes, i.e. *ef1α gene* and *rpl13a gene*, to normalize our data. An effect of NP on two other housekeeping genes (*ef1α, rpl13a*) used in our study was not observed, although chemical regulation of *ef1α gene* was re-

ported when incubation was prolonged over 96 hr (Mc Curley et al 2008). The short incubation time of 1 hr in our study is probably the reason for our different observations.

Although the variation of the measure expression within the groups is not small, the correlation between the measured values for *spt gene* and *ntl gene* is remarkable high. This indicates that the observed variation is not due to experimental conditions but to different stages in development of the used embryos. However, the effects of different isomers of NP on the expression of transcription factors of the T-Box family in zebrafish embryos presented here shows that the increased expression of *tbx6 gene* was associated with the malformation of the tail produced by *p*353-NP (Kammann et al. 2009). A numerical increase in *tbx6 gene* expression was also observable by using *t*-NP. This rise is only 1/10 of the increase observed with *p*353-NP (Fig 3c). The main components of *t*-NP are two diasteromeric forms of *p353*-NP with combined concentrations between 12.2 and 20 % (Russ et al. 2005; Katase et al. 2008; Eganhouse et al. 2009). Given that still several of the components of *t*-NP are not identified so far, and that some isomers of NP act estrogenically while other act antiestrogenically (Preuss et al. 2010) the expression of *tbx6 gene* as reaction of incubation the zebrafish embryos with *t*-NP is probably due to the *p*353-NP present in *t*-NP. The other genes belonging to the T-box6/16 subfamily (*spt, ntl*) are not fundamentally regulated by *p*353-NP within the incubation time of 1hr (Figure 3a,b). However, it needs to be emphasized that these observations are not a proof for a cause effect relationship, which further experiments may provide.

Figure 2

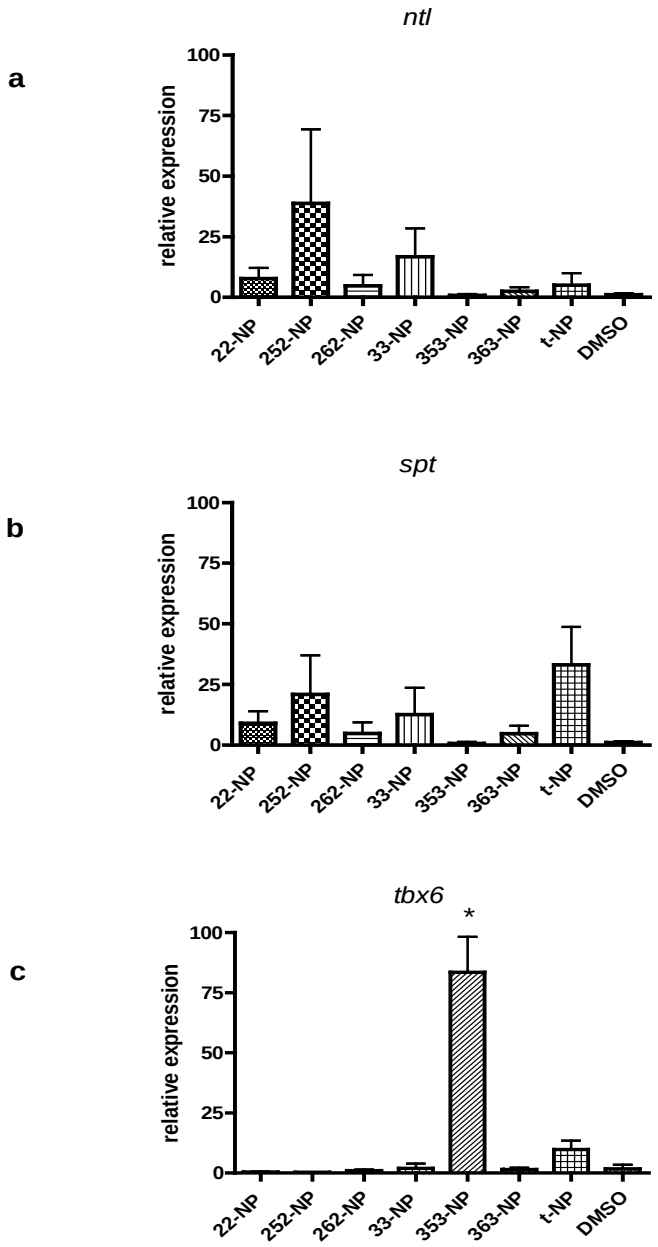


Expression levels of the housekeeping genes *rpl13α* (a), *ef1α* (b), *β-actin* (c) of zebra fish embryos treated with different synthetic isomers of nonylphenols (NPs) with a concentration of 10 nmol/L for 1 hr. Technical NP (t-NP) is a mixture of more than 20 NP isomers. Dimethyl sulfoxide (DMSO) was used as vehicle control. Fig 1d represents the mean of *pr113α* and *ef1α* mRNA levels. Values are means of 5 independent experiments with SEM.

The family of T-Box genes have been investigated using knock out zebrafish with single knock out and multiple knock outs. Characterization of *spt knock out*, *ntl knock out* and *ntl double knockout* embryos shows that spt protein and ntl protein function in a partially redundant manner (Amacher et al. 2002). The regulation of the expression of these genes is apparently similar, as a high correlation in the expression values of *spt* gene and *ntl gene* was found, Spt protein, ntl protein and tbx6 protein might interact in three ways: (a) two factors activate target genes that neither factor can activate on its own (combinatory interaction), (b) two factors contribute to the activation of a gene in an additive manner and (c) one factor prevents activation of a gene by another factor (competitive antagonism). Summarizing the studies on gene expression in zebrafish regarding the T-box 6/16 subfamily and the demonstrated increase of *tbx6* mRNA due to *p*353-NP exposure; it is most likely that tbx6 protein is abundant when zebrafish embryos are treated with *p*353-NP. It is presumed that the tbx6 protein competes with spt protein and antagonized the spt protein effect, resulting in similar phenotype (deformed tail) as *spt knock out* zebrafishes. This postulation is supported by the fact that tbx6 protein compete effectively with ntl protein promoted expression of some T-site dependent gene transcription (Goering et al. 2003).

It is not known which molecular mechanisms regulate the expression of *tbx6 gene*. Thus far only regulation via spt/ntl proteins is described: *Tbx6 gene* contains six spt/ntl protein binding sites within 500 bp of the transcription site, and expression-driven by a 500 bp *tbx6 gene* promotor is mainly regulated by spt protein and ntl protein, spt protein exerting more influence (Garnet et al. 2009). In our experiments an over expression of *spt gene* or *ntl gene* was not observed in embryos treated with *p*353-NP compared to the other NP isomers or the controls. The regulation of *tbx6 gene in vivo* is probably more complex with more regulatory elements in greater distance to the transcription site and not yet analyzed by reporter gene experiments.

Figure 3



Gene expression levels of *ntl* (a), *spt* (b) and *tbx6* (c) relating to the housekeeping genes *rpl13α* and *ef1α* of zebra fish embryos treated with different synthetic isomers of nonylphenols (NP) with a concentration of 10 Nmol/L for 1 hr. Technical NP (*t*-NP) is a mixture of more than 20 NP isomers. Dimethyl sulfoxide (DMSO) was used as vehicle control. Values are means of 5 independent experiments with SEM. Asterisks indicate significant difference ($p<0.05$) as compared to DMSO control (Tukey-Kramer test).

Conclusions

It was necessary to investigate gene expression of tail formation in an early stage of embryonic development, whereas the fully developed tail was visible considerably later. The developmental stage of 48 hr was selected to visually investigate the embryos, because the visibility of the tail tip was best at this time. It is in general not possible to investigate gene expression during migration of precursor cells of the mesoderm and phenotype of tail tip formation at the same time. It was therefore necessary to combine two experiments conducted a different time points. One could detect reasonable results for *ß-actin* gene regulation at the time point select for investigating the gene expression. This indicates that changes in gene expression due to NP might be investigated at the selected time point. As discussed above, the observed relation of *p*353 NP and *tbx6 gene* expression is plausible as cause and effect, but this can not be proven with the shown experiments alone. With this investigation a short snapshot in embryonic gene expression was covered. Other genes related to NP toxicity might be expressed in different time frames. The combination of gene knock out experiments to analyze phenotypes induced by administration of NP may reveal new insights into the biological mechanisms of these xenobiotics. With our investigation the starting point for such future experiments is provided. Only this hypothesis driven approach shed some light on the transcriptions factors initial responsible for gene activation or gene inactivation and in conclusion for the mode of action of NP in fish embryos.

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