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Benzo(a)pyrene reduces osteoclast and osteoblast activity in ex-vivo scales of zebrafish (*Danio rerio* [Hamilton-Buchanan, 1822]) and goldfish (*Carassius auratus* [Linnaeus, 1758])

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Summary

Environmental contaminants have previously been demonstrated to cause bone deformities mediated through the aryl hydrocarbon receptor (AhR) in fish and mammals. Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment, many of them capable of activating AhR. In the present study, fish scales were utilized as a model system to examine possible AhR-mediated effects of PAHs on bone forming osteoblasts and bone resorptive osteoclasts, using the AhR-ligand benzo(a)pyrene (BaP) as a model compound. Elasmoid scales from goldfish and zebrafish were exposed to 0.005–50 μM BaP for up to 48 hr, and the activity of osteoblastic and osteoclastic markers were measured, as well as mRNA levels of bone related genes and *cyp1a* and *cyp3a*. Using the sp7:luciferase zebrafish assay, a decrease in *sp7* promoter activation was observed at the two highest concentrations (5 and 50 μM). Gelatin zymography revealed significantly reduced activity of the osteoclastic protease matrix metalloproteinase 9 (Mmp9) at the highest concentration. Furthermore, transcriptional analysis showed a dose-dependent increase in *cyp1a*, however, no significant differential expression was observed for the bone related genes. The findings indicate that BaP might decrease differentiation and activation of osteoblasts, and reduce osteoclastic activity, and thus ultimately cause decreased bone formation. Further investigation is necessary in order to confirm the role of AhR in mediating these effects.

1 | INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants originating from natural as well as anthropogenic sources, mainly through incomplete combustion of organic compounds (Collier et al., 2014). Thus, PAHs are released into the environment from a wide range of sources such as vehicular traffic, industry and petroleum operations (e.g., drilling, oil spills and combustion). Increased use of crude vegetable oils has also caused increased concentrations of PAHs in aquafeeds (Berntssen, Ornsrud, Hamre, & Lie, 2015). Thus far, hundreds of PAHs have been identified. Some of these, such as benzo(a)pyrene (BaP), are listed as possible mutagenic/genotoxic or carcinogenic compounds due to their ability to bind to the aryl hydrocarbon receptor (AhR) and induce cytochrome P450 enzymes, which in turn could induce the formation of reactive toxicant intermediates (EFSA, 2008; Hahn & Hestermann, 2008).

Developmental abnormalities, such as bone deformities, have previously been observed following exposure to environmental contaminants, including PAHs (Kingsford & Gray, 1996). For this reason, bone abnormalities have also commonly been used as biomarkers in environmental field studies (Kingsford & Gray, 1996). Effects on bone development observed in the field are for obvious reasons most often sublethal. However, studies on fish larvae following large oil spills suggest that craniofacial deformities induced during the larval stage can result in reduced food intake and ultimately increased

mortality in the population (Carls, Rice, & Hose, 1999). Although much of the knowledge on the effects of PAHs on bone and bone metabolic processes are derived from studies on the effect of cigarette smoke on osteoporosis (Lee, Lee, Waldman, Casper, & Grynpas, 2002), studies also show that BaP exposure can have detrimental effects on bone development in fish (Corrales, Thornton, White, & Willett, 2014; He et al., 2011; Seemann et al., 2015).

In early life stages of fish, increased prevalence of developmental deformities such as craniofacial and spinal abnormalities are typical observations following oil exposure (Carls et al., 1999; Di Toro, McGrath, & Stubblefield, 2007; Incardona, Collier, & Scholz, 2004; Incardona et al., 2005, 2013; Rice, Short, Carls, Moles, & Spies, 2007). Although the mechanisms inducing the observed abnormalities following crude oil exposure are poorly understood, PAHs are believed to be possible culprits (Brown & Carls, 1998; Carls et al., 1999; Incardona, Day, Collier, & Scholz, 2006; Incardona et al., 2004, 2013). Incardona et al. (2004) previously demonstrated that individual PAHs might cause skeletal deformities in zebrafish (*Danio rerio*) in vivo in a similar fashion as crude oil (Incardona et al., 2005, 2006). However, environmental contaminants do not only induce severe abnormalities but can also affect the mineralization process and cause a "weaker" skeleton (Corrales et al., 2014; Herlin et al., 2010; Hodgson et al., 2008; Korkalainen et al., 2009; Naruse, Ishihara, Miyagawa-Tomita, Koyama, & Hagiwara, 2002; Seemann et al., 2015). Although effects on the bone metabolic process might not lead to deformities directly, a weaker skeleton could make the subject more prone to mechanical damage.

The skeleton is metabolically active and subjected to constant remodeling (renewal), mediated by bone resorptive osteoclasts and bone forming osteoblasts. Balanced activity between these cells is tightly coordinated, ensuring a healthy and functional skeleton (Hadjidakis & Androulakis, 2006; Witten & Huysseune, 2009). Hence, any disturbance in the equilibrium between osteoblasts and osteoclasts could have a major impact on the net effect of the remodeling process (Feng & McDonald, 2011). Several in vitro studies have shown that PAHs are capable of interfering with these bone metabolic processes (Naruse et al., 2002, 2004; Tsai, Sen Yang, & Liu, 2004; Voronov, Li, Tenenbaum, & Manolson, 2008). Transgenerational effects of BaP exposure on bone metabolism was recently observed in the offspring of BaP exposed medaka (*Oryzias latipes*) through three generations (F1–F3) (Seemann et al., 2015). Similar transgenerational effects on developmental abnormalities have been observed in zebrafish larvae following BaP exposure (Corrales et al., 2014). In a previous study on Atlantic cod (*Gadus morhua*) larvae, dispersed oil and water-soluble fractions (WSF) of crude oil caused a decrease in osteoblast-related genes and an increase in osteoclast-associated genes (Olsvik et al., 2011). These effects were correlated to the cytochrome p450 1a (*cyp1a*) and aryl hydrocarbon receptor (*ahr2*), suggesting AhR-mediated transcriptional effects on bone metabolism.

Elasmoid scales have previously been used as a model to study the effect of toxicants on skeletal metabolism (Suzuki & Hattori, 2003; Suzuki et al., 2009; Suzuki, Yachiguchi et al., 2011; Yachiguchi et al., 2014). These scales are small independent bone-like units covered with a monolayer of osteoblasts on the inner layer and osteoclasts on the mineralized outer layer. Elasmoid scales have been regarded as dentin-derived tissue (Sire & Huysseune, 2003), but recent studies show that scales develop from the mesoderm (Mongera & Nusslein-Volhard, 2013; Shimada et al., 2013). Although distinct in evolutionary origin and ossification mode, scale cells are remarkably similar to bone cells. Compared with bone cells, many of the same genes and mechanisms are involved in the mineralization process (De Vrieze, Metz, Von den Hoff, & Flik, 2010; Thamamongood et al., 2012). Furthermore, the response of elasmoid scale cells to hormonal treatment can be related to the predicted response of mammalian bone cells (Hamazaki et al., 2009; Omori et al., 2012; Rotllant et al., 2005; Suzuki, Danks et al., 2011; Yoshikubo et al., 2005). This further implies that the same metabolic pathways and processes are involved in the regulation of matrix formation, mineralization and resorption of bone-like tissue in elasmoid scales compared with bone (De Vrieze, Moren, Metz, Flik, & Lie, 2014). Osteoblastic markers such as sp7, alkaline phosphatase (ALP) and bone gamma-carboxyglutamate (gla) protein (BGLAP), as well as osteoclastic markers such as matrix metalloproteinase and -9 (Mmp2 and Mmp9) and cathepsin k (CTSK), are all expressed in the scales (De Vrieze, Sharif, Metz, Flik, & Richardson, 2011; Nishimoto, Araki, Robinson, & Waite, 1992; Thamamongood et al., 2012). Sp7 (osterix) is a transcription factor essential for osteoblast differentiation and activation (DeLaurier et al., 2010). The main advantage of using scales as a model system rather than cell culture-based assays, is the preservation of the pivotal cell-cell and cell-matrix interactions (De Vrieze, Zethof, Schulte-Merker, Flik, & Metz, 2015). The close interaction between these cells is, among other factors, regulated by the binding of receptor activator of nuclear factor κB ligand (RANKL) on osteoblasts to receptor activator of NF-κB (RANK) on osteoclasts. This binding triggers osteoclast differentiation and activation (Hadjidakis & Androulakis, 2006; Witten & Huysseune, 2009).

This study aimed at examining the effects of benzo(a)pyrene (as a well-known AhR agonist) on bone forming osteoblasts and bone resorptive osteoclasts using elasmoid scales of two cyprinids (*Carassius auratus* and *Danio rerio*) as model systems.

2 | MATERIALS AND METHODS

2.1 | Exposure setup and scale collection

A 50 mM (96%) (Sigma-Aldrich, St. Louis, MO) stock solution was prepared by dissolving benzo(a)pyrene (BaP) in dimethyl sulfoxide (DMSO) in a glass bottle. From this, 10-fold serial dilutions were prepared. Stock solutions were further diluted 1:1,000 in osteogenic-DMEM (o-DMEM) (Pombinho, Laize, Molha, Marques, & Cancela, 2004), yielding exposure medium with BaP concentrations ranging from 0.005 to 50 μM and DMSO concentration of 0.1%.

Handling of zebrafish was approved by the animal ethics committee of Radboud University (Permit number RU-DEC2014-259). Zebrafish were anesthetized in 0.05% (v/v) 2-phenoxyethanol

(Sigma Aldrich, St. Louis, MO, USA), or euthanized in 0.1% (v/v) 2-phenoxyethanol. Goldfish were sacrificed by pithing the brain using a sharp scalpel. All efforts were made to minimize suffering. Elasmoid scales of Tg(Ola.sp7:luciferase) zebrafish (De Vrieze et al., 2015) or goldfish were removed from the area between the dorsal fin and the operculum, and distributed over 96-well plates (for cell viability, Sp7:luciferase zebrafish scale assay and gelatin zymography) or 6-well plates (for RT-qPCR) containing osteogenic-Dulbecco's Modified Eagle Medium (o-DMEM). The o-DMEM has been shown to be the most suitable medium for sustaining scale-cultures (De Vrieze et al., 2015; Pombinho et al., 2004). Two controls were included in the setup: a dimethyl sulfoxide (DMSO) vehicle control with a DMSO concentration equal to the exposures (0.1%), and a no-treatment control (only scale-culture medium).

2.2 | Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) based on the In Vitro Toxicology Assay Kit (Sigma-Aldrich, St. Louis, MO) was used to measure viability of scale cells after BaP treatment. Scales from six adult goldfish (11.8 ± 2.2 g) were collected and washed as described above, and further treated with 0.005–50 μM BaP. After 48 hr, the exposure medium was carefully replaced with o-DMEM comprising MTT. After 4 hr incubation at 28°C, MTT medium was replaced with 100 μl MTT solubilization solution. The resulting purple formazan crystals in living cells were quantified spectrophotometrically in an iEMS Reader MF (Labsystems, Helsinki, Finland) by measuring the absorbance at 570 and 690 nm. Finally, the background values at 690 nm were subtracted from the absorption values at 570 nm, and the latter expressed relative to the DMSO vehicle control. Initially, this assay was used to measure viability of exposed zebrafish scales. However, this resulted in detection signals that were too low (zebrafish scales are smaller than goldfish scales), thus goldfish scales were used.

2.3 | Sp7:luciferase zebrafish scale assay

An sp7 luciferase assay using transgenic zebrafish (Ola.sp7:luciferase) was used to assess activity of osteoblasts. Ontogenetic scales from six male fish (four technical replicates per fish) were individually distributed over white luminometer plates, and washed as described above. Baseline luciferase activity was measured as described by De Vrieze et al. (2015). Scales were washed in 200 μl o-DMEM and further treated with 100 μl of 0.005–50 μM BaP for 48 hr at 28°C. Following treatment, endpoint luciferase activity measurement and calculation of relative luciferase activity was performed as described by De Vrieze et al. (2015), and expressed relative to the DMSO vehicle control. Four technical replicates were used for each fish. The mean of the four technical replicates from each of the six fish were used for statistical analysis.

2.4 | Gelatin zymography

Matrix metalloproteinases (MMPs) enzymatic activities were used as markers for bone resorption (osteoclasts) activity. Scales from six adult goldfish were collected and washed as described previously, and further treated with 0.05–50 μM BaP for 24 hr at 28°C. Medium from each well was diluted 1:1 in sample buffer and 10 μl was loaded on a polyacrylamide gel (10%) gel containing 1 mg/ml gelatin. 10 μl Novex® Sharp Pre-stained Protein Standard (Invitrogen, UK) was used as molecular weight reference. A 0.25 ng human recombinant pro MMP9 (Sigma-Aldrich, Dorset, UK) was used as reference sample, allowing comparison of bands on different gels (Bildt, Bloemen, Kuijpers-Jagtman, & Von den Hoff, 2009). Gels were electrophoresed through stacking gel at 60 V for 20 min, and through running gel for 2.25 hr at 110 V. Subsequent steps were performed according to Bildt et al. (2009). Finally, the gels were scanned and relative MMP activity (band intensity) was analyzed using ImageJ, version 1.48 (National Institute of Health [NIH], Washington, DC, USA). Relative MMP activity was further normalized to human recombinant MMP9 on the corresponding gel, and expressed relative to the DMSO vehicle control. Relative activity values were generated by dividing the individual values for each sample with the mean control (C1) value.

2.5 | cDNA synthesis and qPCR analysis

Scales from six adult goldfish were collected in 6-well plates (30–40 scales per well), washed as described above, and further treated with 0.5–50 μM BaP for 13–15 hr at 28°C. After treatment they were washed once in phosphate buffered saline (PBS) water. Scales were further homogenized in Qiazol Lysis Reagent (Qiagen, Hilden, Germany), and The EZ1 RNA Universal Tissue Kit (Qiagen) was used to extract total RNA from scale cells, according to the manufacturer's guidelines. Total RNA concentration and purity was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). All samples had A260/A280 ratios ≥1.8 and A260/A230 ratios >1.8. The RNA integrity numbers (RIN) of 12 samples were measured using the Agilent RNA 6000 Nano Kit together with the Agilent 2100 Bioanalyzer instrument (Agilent Technologies Inc., Santa Clara, CA, USA), according to the manufacturers' protocol. The range value for RIN was between 7.80 and 8.20 for eight randomly-selected analyzed samples.

The cDNA was synthesized using the TaqMan Reverse Transcription Reagents cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA) in 50 μl reaction volume, according to the manufacturer's instruction. The reverse transcription PCR (RT-PCR) reaction was performed as described by Lie and Moren (2012). Two-fold serial dilutions for downstream efficiency calculations (1,000–31.25 ng) were prepared in triplicate using pooled total RNA from all samples for each group). All other samples (n = 6 fish for each group) were prepared using technical duplicates. Each RNA sample consisted of a pool of all scales from each well, representing one fish. In addition, two negative controls were included: a no-amplification control, and a no-template control.

TABLE 1 Sequences of forward and reverse (5′–3′) primers used for qPCR of goldfish target genes

Gene name	Accession number	Primer sequence (5′–3′)
Alkaline phosphatase (*alp*)	AB459538	FW: TGGACACAGCGGTGAGGAAA
		RV: GTGGGCATATGCTGCACTCG
Bone gamma-carboxyglutamate (*gla*) protein (*bglap*)	AB685220	FW: ATGCCTGAGCGCAGGTCTTC
		RV: CACAGGCCAGGTTTGCTTCA
sp7 (*osterix*)	AB274888	FW: GACTGCCTGACCAGCGTCAA
		RV: GAGGCACCAAGCCTCTCCAA
Receptor activator of nuclear factor-κB ligand (*rankl*)	AB459540	FW: GCGCTTACCTGCGGAATCATATC
		RV: AAGTGCAACAGAATCGCCACAC
Tartrate-resistant acid phosphatase (*trap/acp5*)	JX477207	FW: TGCTGGACACTGTGCTGCTG
		RV: GGAACCTGGTTTCGGTGTCG
Cathepsin K (*ctsk*)	AB236969	FW: TGGGAGGGCTGGAAACTCAC
		RV: CATGAGCCGCATGAACCTTG
Cytochrome P450 1A (*cyp1a*)	DQ517445	FW: ACCGGAAACTGGACGAGAAC
		RV: GACGACACCCCAAGACAGAG
Cytochrome P450 3A (*cyp3a*)	JN555609	FW: CAGCGGCAGGTTAAAGGAGA
		RV: GGGTCTCTGGGGTTGTTGAG
β-actin	AB039726	FW: CGAGCGTGGCTACAGCTTCA
		RV: GCCCGTCAGGGAGCTCATAG
Elongation factor-1 α (*ef1α*)	AB056104	FW: ATGGGCTGGTTCAAGGGATG
		RV: CGGGCACTGTTCCAATACCT
Ribosomal protein L4 (*rpl4*)	NM_213107	FW: CGTTATGCCATGTGCTCTGC
		RV: CACGGCTTCCTTGGTCTTCT

β-actin, elongation factor-1 α (*ef1α*) and ribosomal protein L4 (*rpl4*) were used as reference genes.

Primer sequences for target genes and reference genes are listed in Table 1. Primer3web, version 4.0.0 was used to design gene specific primers for *cyp1a*, cytochrome p450 3a (*cyp3a*), transcription elongation factor 1a (*ef1α*) and ribosomal protein L4 (*rpl4*), and a primer analysis was further conducted using NetPrimer (Premier Biosoft). The other primer sequences were obtained from Thamamongood et al. (2012). The qPCR analysis was performed as described by Lie and Moren (2012) using the SYBR Green Master Mix (Roche Applied Sciences, Basel, Switzerland) and a Light Cycler 480® real time qPCR system (Roche Applied Sciences). The geNorm (version 3.5) was used to determine a normalization factor based on the three reference genes β-*actin*, *ef1α* and *rpl4*, from which the mean normalized expression (MNE) of target genes was calculated. Normalization of gene expression was further conducted in accordance with Vandesompele et al. (2002).

2.6 | Statistical analysis

STATISTICA version 12 (StatSoft Inc., Tulsa, OK, USA) and Graphpad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) were used for statistical analyses. Levene's test was used to check for homogenity of variance ($p < .05$). Data that violated Levene's test were log-transformed. One-way ANOVA followed by Dunnett's post hoc test was further used to test for significant differences in results. $p < .05$ was used as significance level. Graphs were made in GraphPad, and all data are presented as mean ± standard deviation (*SD*).

3 | RESULTS

3.1 | Goldfish scale cell viability

Using the MTT based In Vitro Toxicology Assay, no significant cytotoxicity was detected in goldfish scales treated with 0.005–50 μM BaP when compared with the DMSO vehicle control (C1) (Figure 1).

3.2 | Down-regulation of sp7 in zebrafish scale osteoblasts following benzo(a)pyrene treatment

The *sp7* promoter-driven expression of the luciferase in the tg (Ola.sp7:luciferase) zebrafish allows for measurement of sp7 before and after exposure. Relative to the DMSO vehicle control (C1), the activity of Sp7 in scale osteoblasts was significantly down-regulated at 5 μM ($p < .05$) and 50 μM ($p < .001$) BaP after 48 hr treatment (Figure 2). No significant change was found in the sp7 promoter activity following treatment with 0.005–0.5 μM (Figure 2).

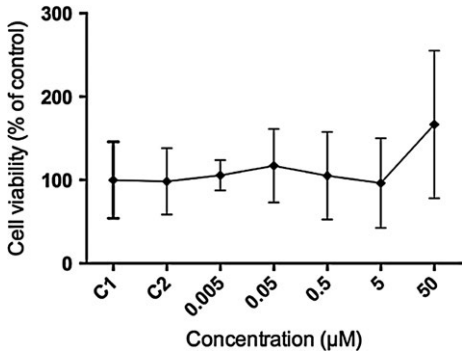

FIGURE 1 Cell viability of goldfish scale cells following BaP treatment for 48 hr. The MTT cytotoxicity assay was used to measure living scale cells after treatment with DMSO vehicle control (C1), no-treatment control BaP (C2), 0.005–50 μM BaP 0.05. *n* = 6 for C1, C2, and 0.005 μM BaP. *n* = 5 for the other groups due to contamination in wells. Viability of treated scale cells are expressed relative to the in %C1 (mean% ± standard deviation)

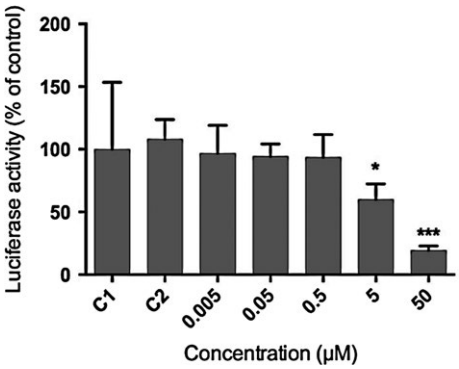

FIGURE 2 Luciferase activity (expression of *sp7*) in scale osteoblasts of transgenic zebrafish was significantly down-regulated following BaP treatment for 48 hr. Each bar represents mean normalized signal intensities ± standard deviation from six fish (*n* = 6). Relative luciferase activity was calculated based on scale luciferase measurements before and after treatment, and further expressed relative to the DMSO vehicle control (C1) (scales treated with DMSO concentration equal to exposures). *p < .05, ***<.001. C2 = no-treatment control

3.3 | Benzo(a)pyrene caused reduced Mmp9 activity in goldfish scales

A representative gel from zymographic detection of pro (incative) and active forms of Mmp2 and Mmp9 in medium from treated scales is shown in Figure 3a. Mmp9 and pro Mmp9 are localized around 75 and 130 kDa, respectively, and Mmp2 and pro Mmp2 at approximately 65 and 70 kDa, respectively. Compared with the DMSO vehicle control (C1), BaP treatment at 50 and 5 μM significantly decreased the activity of Mmp9 (p < .05) (Figure 3b). The activity of pro Mmp9 was not significantly affected (Figure 3b). No significant changes were detected in the activity of pro Mmp2 and Mmp2 (Figure 3c).

3.4 | Benzo(a)pyrene induced expression of *cyp1a* in goldfish scales

Goldfish scales treated with BaP showed a clear dose-dependent increase in the expression of *cyp1a*, while scales in the DMSO vehicle control and the no-treatment control showed very low expression of *cyp1a*. Compared with the DMSO vehicle control, all tested concentrations of 0.5, 5 and 50 μM (p < .001), resulted in a 89, 226 and 413 fold up-regulation of *cyp1a* in the scale cells, respectively (Figure 4). No significant differential expression was observed for any of the other examined genes (Figure 4b–d). Due to low mRNA levels and low amplification efficiency, the expression of *alp*, *sp7*, *trap* and *ctsk* could not be quantified.

4 | DISCUSSION

The present study shows that BaP at 5 μM concentration interferes significantly with bone metabolic processes in vitro. This is also the first study to demonstrate that BaP exposure induced the transcription of *cyp1a* in scales, up to 400-fold. This corresponds with observations in mammalian cells where the ability of both osteoclasts and osteoblast to induce an AhR mediated response has been demonstrated (Herlin et al., 2010; Korkalainen et al., 2009; Ryan et al., 2007). Ryan et al. (2007) found that osteogenesis in cultured cells from AhR deficient mice was reduced, demonstrating the importance of AhR mediated signaling in bone formation. The magnitude of the observed *cyp1a* induction (89-fold at 0.5 μM) in the present study suggests that there is a potential for induction of *cyp1a* at much lower doses. It also shows that the *cyp1a* induction in the zebrafish scale is surprisingly comparable to previous observations in whole Atlantic cod larval homogenates (Olsvik, Lie, Nordtug, & Hansen, 2012; Olsvik et al., 2011) and in hepatocytes from common carp (*Cyprinus carpio*) (Smeets, van Holsteijn, Giesy, & van den Berg, 1999). Several investigations studying the effects of environmental toxicants clearly demonstrate the importance of AhR-ligand interaction as a modulator of bone metabolism through which bone toxicity is mediated (Herlin et al., 2010; Incardona et al., 2006; Jamsa, Viluksela, Tuomisto, Tuomisto, & Tuukkanen, 2001; Ryan et al., 2007; Singh et al., 2000). Most of the knowledge on bone AhR interactions are derived from 2,3,7,8-tetrachlorodiben zo-p-dioxin (TCDD) exposure studies. Results of a previous study conducted by Olsvik et al. (2011) suggested AhR mediated transcriptional effects on bone related genes in Atlantic cod larvae exposed to dispersed oil. In that study both increased expression of genes related to bone resorption (osteoclast activity) as well as decreased expression of genes related to bone formation were observed. The authors speculated that such a shift in the balance between resorption and formation of bone could in turn lead to a demineralized skeleton, making the skeleton of Atlantic cod larvae more susceptible to mechanical damage. These studies and others (Yu, Pang, & Yang, 2015) clearly suggest that AhR has a role in bone homeostasis.

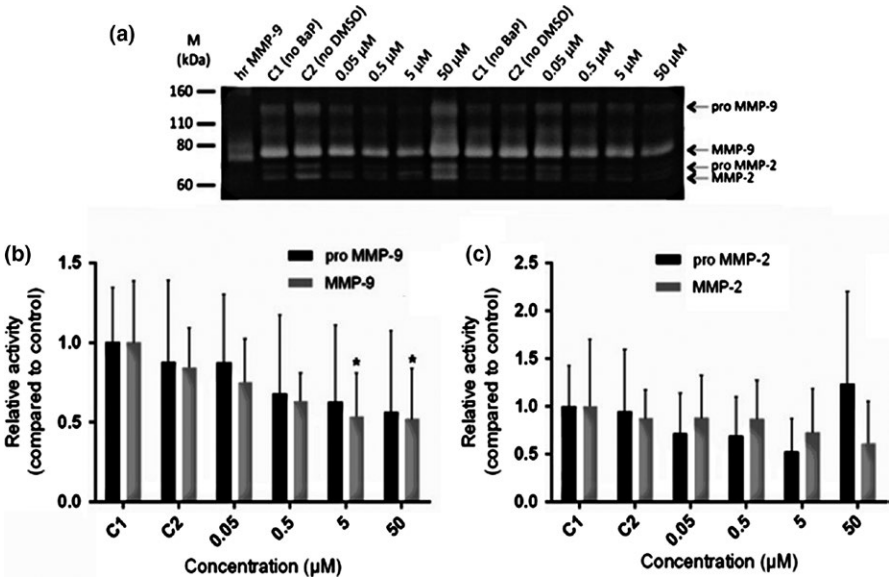

FIGURE 3 Zymographic detection of Mmp9 and Mmp2 in culture medium of goldfish scales treated with BaP for 24 hr. (a): Representative gel showing Mmp9 and Mmp2 activity in two of totally six fish. (b and c): Relative activity (mean ± standard deviation) of pro and active forms of Mmp9 (b) and Mmp2 (c) was analyzed using ImageJ, normalized to human recombinant Mmp9 (hr Mmp9), and expressed relative to the DMSO-vehicle control (C1). *$p < .05$. C2 = no-treatment control, M = molecular weight standard in kilodaltons (kDa). Relative activity values were generated by dividing the individual value for each sample with the mean control (C1) value

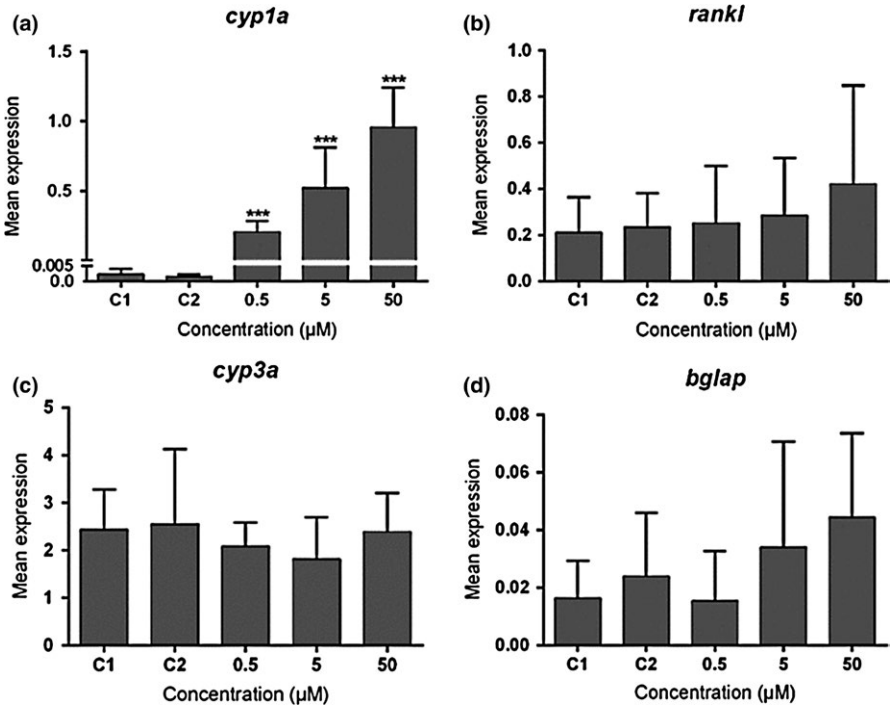

FIGURE 4 Mean normalized expression (mean ± standard deviation, $n = 6$) of a) *cyp1a*, b) *rankl*, c) *cyp3a* and d) *bglap* in goldfish scale cells treated with 0.5, 5 and 50 µM BaP for approx. 13–14 hr. (a): Mean expression of *cyp1a* presented with scale break due to low expression in control groups. Statistical significance denoted with ***$p < .001$. C2 = no-treatment control

In the present study, using the sp7:luciferase zebrafish scale assay, BaP inhibited activation of the *sp7* promoter at the two highest concentrations (5 and 50 µM), at which no difference in cell death was observed compared to the control. The transcription factor Sp7 has been previously demonstrated to be a key regulator of osteoblast differentiation and bone formation in zebrafish (Spoorendonk et al., 2008) and medaka (Renn & Winkler, 2014). Thus, the inhibition of *sp7* promoter activity in zebrafish scales indicates that BaP might decrease the formation of osteoblasts as well as the mineralization process. The effect on *sp7* is consistent with the findings of Olsvik et al. (2011), who observed decreased expression of *sp7* in Atlantic cod larvae. Interestingly, Seemann et al. (2015) observed a decrease in *sp7* gene expression in the third and fourth generation after ancestral BaP exposure. Inhibition of osteoblast differentiation has also been demonstrated in rat calvarial osteoblast-like cells (ROB cells) following 3-methylcholanthrene (3MC) exposure (Naruse et al., 2002), and in osteoblast differentiation models in vitro following TCDD exposure (Carpi et al., 2009; Ryan et al., 2007; Singh et al., 2000). Monohydroxylated PAHs have also been shown to inhibit osteoblast activity in goldfish scales (Suzuki et al., 2009;

Suzuki, Yachiguchi et al., 2011). In contrast to the decreased osteoblast differentiation observed in the present study, Tsai et al. (2004) demonstrated that BaP stimulates differentiation of cultured rat osteoblasts through estrogen receptor-related (ER) mechanisms (Tsai et al., 2004). This further suggests that the effect on *sp7* in the present study was not mediated through ER.

The BaP also decreased the activity of Mmp9 in goldfish scales in the present study, indicating decreased bone resorption activity. A link between the enzyme activity and changes in the mineralized scale matrix was demonstrated in zebrafish (De Vrieze et al., 2014). Voronov et al. (2008) showed that BaP was able to inhibit osteoclast differentiation of a cultured mouse macrophage cell line. This was suggested to be a consequence of an AhR-RANK "crosstalk" inhibiting RANKL activation of osteoclasts (Voronov et al., 2008). A similar observation was made for another AhR agonistic PAH, 3MC (Naruse et al., 2004). Furthermore, TCDD has also been shown to cause AhR mediated inhibition of osteoclast differentiation (Korkalainen et al., 2009).

Despite a strong induction of *cyp1a* (89-fold) in the goldfish scales following exposure to 0.5 μM BaP, *sp7* and Mmp9 were only induced at the two highest concentrations (5 and 50 μM). If the inhibitory mechanisms of *sp7* and Mmp9 were mediated through the AhR receptor pathway, one would expect to observe an effect at 0.5 μM, a concentration resulting in a 89-fold induction of *cyp1a*. In addition, no differential transcriptional effects were observed for any of the bone-related genes, including *bglap* and *rankl*. However, we cannot exclude an effect on the genes that were below the quantification threshold (*alp*, *sp7*, *trap* and *ctsk*). Furthermore, as exposures in the present study were conducted in short-term cultures, we thus cannot exclude any effects on the protein- or gene-level after prolonged exposure. In contrast to the present study, TCDD decreased the mRNA expression of *runx2*, *alp* and *bglap* in mammalian bone marrow stem cells (Korkalainen et al., 2009). Despite most of the literature pointing towards an AhR-mediated inhibition of osteogenesis and osteoclastogenesis, examples of the opposite also exist. Ilvesaro, Pohjanvirta, Tuomisto, Viluksela, and Tuukkanen (2005) demonstrated that TCDD did not mediate osteoclast inhibition in vitro despite strong AhR activation. In addition, another AhR agonist, the polychlorinated biphenyl (118) (PCB 118), induced osteoclast and osteoblast activity in scales from PCB-exposed goldfish (Yachiguchi et al., 2014). Altogether, these studies show rather contradictory effects of AhR-ligands on bone metabolism, which further indicates that pathways additional to the AhR pathway might be involved in mediating bone toxicity. Incardosna et al. (2006) showed that the developmental abnormalities in zebrafish induced by exposure to two different 4-ring PAHs were differentially dependent on tissue-specific activation of AhR isoforms and CYP1A metabolism.

The present study shows that BaP, a model compound for AhR-mediated effects following PAH exposure, negatively influences osteoblast differentiation and activity, as well as osteoclast activity. Although BaP caused a dose-dependent *cyp1a* induction, no alterations in bone related gene expression were observed. Furthermore, although *cyp1a* was strongly induced at the lowest concentration (0.5 μM), Sp7 nor Mmp9 nor Mmp2 activity were significantly affected at this level. However, we cannot exclude an effect on these markers at lower concentrations following prolonged exposure. The present results suggest that AhR activation might not be as central for the osteotoxic effects following BaP exposure as previously demonstrated for TCDD. However, further investigation is necessary in order to confirm which role AhR plays in modulating bone metabolic processes following BaP exposure. Furthermore, as any disruption in the balanced activity of osteoblasts and osteoclasts could have major effects on the bone remodeling process, this notion warrants investigation into the long-term effects on skeletal development following PAH exposure.

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