12-2017

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Recommended Citation

Roy, N.M., Zambrzycka, E., Santangelo, J. (2017). Butyl Benzyl Phthalate (BBP) induces caudal defects during embryonic development. *Environmental Toxicology and Pharmacology, 56*, 129-135. doi:10.1016/j.etap.2017.09.009

Accepted Manuscript

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PII: S1382-6689(17)30267-3
DOI: http://dx.doi.org/10.1016/j.etap.2017.09.009
Reference: ENVTOX 2882

To appear in: *Environmental Toxicology and Pharmacology*

Received date: 21-6-2017
Revised date: 11-9-2017
Accepted date: 14-9-2017

Please cite this article as: Roy, Nicole M., Zambrzycka, Ewelina, Santangelo, Jenna, Butyl Benzyl Phthalate (BBP) Induces Caudal Defects During Embryonic Development.Environmental Toxicology and Pharmacology http://dx.doi.org/10.1016/j.etap.2017.09.009

Butyl Benzyl Phthalate (BBP) Induces Caudal Defects During Embryonic Development

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Highlights

- BBP causes concentration dependent defects in caudal tail development
- BBP alters expression of *ntl* in the notochord and *myoD* in the muscle
- BBP causes defects in myofibrils and alteration to the vasculature

Abstract

Butyl benzyl phthalate (BBP) is commonly added during the manufacturing of plastics to increase flexibility and elasticity. However, BBP leaches off of plastic and environment presence has been

detected in soil, groundwater and sediment potentially effecting organisms in the environment. Given the widespread uses of BBP in household, consumer goods and the presence of BBP in the environment, studies on developmental toxicity are needed. Here, we use a zebrafish model to investigate the early developmental toxicity of BBP. We treated gastrula staged embryos with increasing concentrations of BBP and noted concentration-dependent defects in caudal tail development, but the effect was caudal specific with no other developmental defects noted. *In situ* hybridization studies using muscle and notochord markers show alterations in muscle development and non-linear, kinked notochord staining. A more detailed antibody staining using a myosin specific marker shows disorganized myofibrils and a loss of chevron shaped somites. Furthermore, vascular development in the tail was also disrupted in a concentration dependent manner. We conclude that BBP is toxic to caudal development in zebrafish. The sensitivity of zebrafish during development to environmental toxins and chemicals has been useful in assessing the health of the aquatic environment. The results presented here are a useful early warning system for contamination that could affect human health.

Keywords: Zebrafish; Toxicity; Butyl Benzyl Phthalate, Caudal Development

1. Introduction

Esters of phthalic acids or phthalates are chemicals routinely used in the manufacture of plastics to increase the flexibility and durability of plastic polymers. Each year, over three million metric tons of phthalates are produced globally and used primarily in polyvinyl chloride (PVC) (Schettler, 2006), but are also found in vinyl flooring, building materials, toys and medical devices. Additionally, as an inert component, phthalates can be found in cosmetics, pesticides, pharmaceuticals, detergents and wood finishes (Chatterjee and Karlovsky, 2010; Schettler, 2006). Phthalate contamination occurs readily as phthalates are not chemically bound to plastics and leach easily into the environment (Adams et al., 1995; Chatterjee and Karlovsky, 2010; Schettler, 2006).

With massive annual production and continuous release into the environment, phthalates can be found in measurable concentrations in aquatic ecosystems worldwide (Oehlmann et al., 2008) including river waters and sediments (Yuwatini et al., 2006) and municipal waste waters (Wibe et al., 2002). Studies have documented the aquatic toxicity of numerous phthalate esters on

fresh and saltwater microorganisms, algae, invertebrates and fish (Adams et al., 1995; Staples et al., 1997). However, phthalate detection is not isolated to aquatic environments. Phthalates have been found in food and food packing materials (Cao, 2010; Page and Lacroix, 1995; Schecter et al., 2013), human blood (Wan et al., 2013), breast milk (Main et al., 2006; Zimmermann et al., 2012), urine (Lin et al., 2011) and home dust (Abb et al., 2009; Bornehag et al., 2004; Rudel et al., 2003). Phthalates easily pass the placenta from maternal blood to the developing fetus (Latini, 2005; Planello et al., 2011).

Amongst the phthalate esters family, butyl benzyl phthalate (BBP) is the environmentally present phthalate with the most documented health effects in humans and animals (Bornehag et al., 2004; Chatterjee and Karlovsky, 2010; Swan, 2008). Most of the effects of BBP in fish and rats have been documented on adults or on the developing reproductive system and demonstrated estrogen-mimicking properties (Jobling et al., 1995; Sharpe et al., 1995). For example, in pikeperch exposed to BBP, disrupted gonadal differentiation, delayed testicular development and feminization were observed (Jarmolowicz et al., 2014). In zebrafish, BBP exposure disrupted sex hormone balances by altering steroidogenic genes (Sohn et al., 2016) and decreased sperm quality in the males exposed to BBP (Oehlmann et al., 2009). Teratogenic effects in rats have also been reported including testicular toxicity, decreased testosterone, decreased sperm production and altered sexual development (Ema et al., 1991; Ema and Miyawaki, 2002; Gray et al., 2000; Lyche et al., 2009; Parks et al., 2000; Piersma et al., 2000). Behavioral effects of BBP have been noted in the threespine stickleback and *Fundulus heteroclitis* each having demonstrated alterations in shoaling and bottom-dwelling behaviors (Kaplan et al., 2013; Wibe et al., 2002). BBP also alters social interactions and fear conditioning in rats (Betz et al., 2013) as well as alters feeding behavior in threespine stickleback (Wibe et al., 2004). There are limited investigations into the embryotoxicity of BBP, but previous reports using zebrafish embryos have documented tail curvature and a lack of touch response in a 72 hour acute toxicity test as well as the estrogenic activity of BBP (Chen et al., 2014). Mice also display a concentration-dependent embryolethality and developmental malformations (Saillenfait et al., 2003).

Besides BBP's anti-androgenic effects on sex development, mechanistically, not much is known about how BBP induces toxic effects. However, a recent study in zebrafish has shown 7-day BBP exposed fish demonstrated decreased acetylcholinesterase (AChE) activity as well as

alterations in superoxide dismutase (SOD) activity (Zhang et al., 2014). BBP has also been shown to suppress calcium signaling coupled to nicotinic acetylcholine receptors in cell lines (Liu et al., 2009).

The massive production volume and widespread usage of phthalates has made the presence of phthalates ubiquitous in the environment. Fish become exposed in the water column, by sediment or food and phthalates can work their way up the food webs to affect higher order vertebrates and humans. Thus, understanding the developmental effects of BBP exposure is an important consideration. Here, we investigated the embryotoxicity of BBP using zebrafish as a model. Zebrafish are a commonly used vertebrate model in developmental toxicology to monitor environmental contaminants given their high fecundity, rapid external development, transparent embryos, ability to absorb chemicals and their genetic similarity to higher order vertebrates (Hill et al., 2005).

2. Materials and Methods

2.1 Zebrafish Husbandry and Breeding

Adult zebrafish were housed in a zebrafish module (Aquatic Habitats, Inc) on a 14:10 hour light:dark cycle. Water quality (pH, ammonia, nitrate, nitrite, salinity) was monitored daily and fish were fed brine shrimp supplemented with TetraMin® flake food twice a day. For breeding, two male and two female adult fish were placed in a standard breeding box at night (Westerfield, 1993). The following morning, embryos were collected and placed in 30% Danieau Buffer (Westerfield, 1993) prior to treatment.

2.2 Embryo Treatments

Butyl benzyl phthalate was obtained from Sigma-Aldrich (308501). Treatment concentrations of 10-30μM were diluted in 30% Danieau Buffer. Methanol was used as a vehicle (Chen et al., 2014) to increase solubility with a final concentration in diluted samples not greater than 0.1%. All treatments were performed in glass petri dishes. Treatments began at the onset of gastrulation (6 hpf (hours post-fertilization)) and embryos were continuously treated until 24hpf when they were either live imaged, fixed or processed. This developmental time window was chosen as it covers

gastrulation through segmentation, somitogenesis and neurulation, which establishes the larval and adult body. Embryos were staged according to the Kimmel staging series (Kimmel et al., 1995). Treatment protocols were approved by the Sacred Heart IACUC (Institutional Animal Care and Use Committee) committee as meeting ethical standards. Treatments were repeated three times on separate occasions, minimum number of embryos per treatment concentration (0, 10, 20 and 30μM) was 20.

2.3 Imaging and Microscopy

Live images were attained using a Leica dissection microscope with a Nikon Digital Sight DS-2Mv digital camera with Q-Capture software. Live embryos were sedated for imaging with tricaine methanesulfonate (MS-222) (Westerfield, 1993). Fluorescent images of live transgenic embryos (*fli1-gfp*) (green fluorescent protein) (Lawson and Weinstein, 2002), live acridine orange staining or fixed fluorescent antibody staining were imaged with a Nikon Eclipse E400 fluorescent microscope attached to an Andor Zyla sCMOS cooled CCD (charge-coupled device) camera using Q-Capture software. For all live images, transgenic or fixed, embryos were placed in a depression slide in a pool of 3% methylcellulose thickening medium to aid its positioning.

2.4 Whole Mount in situ Hybridization and Immunohistochemistry

In situ hybridization protocols were previously described (Roy et al., 2015; Sagerstrom et al., 1996). The *ntl* (*no tail*) probe, a common notochord marker and the *myoD* (*myogenic differentiation*) probe, a common muscle marker, were generous gifts from the Sagerstrom Lab at the University of Massachusetts Medical Center. The antisense *ntl* and *myoD* probes were digoxigenin (DIG) labeled (Roche Life Sciences, 11209256910) and transcribed using a T7 and SP6 in vitro transcription kit respectively (Promega, P1450). Probes were visualized in whole mount using an anti-DIG antibody (Roche Life Sciences, 11093274910) conjugated to nitroblue tetrazolium and bromo-4-chloro-indolyl phosphate (NBT/BCIP, Promega, S3771).

Whole mount immunohistochemistries were performed as previously described (Barresi et al., 2001; Devoto et al., 1996; Roy et al., 2015) using a myosin heavy chain specific antibody (F59, Developmental Studies Hybridoma Bank, University of Iowa). A 1:5 dilution of F59 antibody was used along with a 1:200 dilution of a FITC-labeled goat anti-mouse secondary antibody (Santa Cruz Biotechnology).

2.5 Cell Death

Acridine Orange was obtained from Sigma-Aldrich (A6014) and prepared to a stock solution of 1mg/ml in distilled water. Embryos were treated in 1µg/ml dilution in 30% Danieau Buffer for 1 hour and 30 minutes in the dark, washed extensively and imaged as described above.

3. Results

3.1 Live Gross Morphology

To investigate general body morphology after BBP treatment, live embryos were imaged at 24hpf. All treatment levels demonstrated normal neural morphology. Fore, mid and hindbrains were normal, otic vesicles were present and lens and retinas were developed in relation to controls (Fig. 1, A-D). However, caudal tail development was altered. Control tails demonstrated straight extension, with a linear notochord and classic, chevron shaped somites (Fig. 1A'). BBP treatments demonstrated a concentration-dependent increase in tail defects. The low concetration (10µM) demonstrated a slight loss of linear extension with a slightly curvy notochord. Somites were still present and boundaries clearly defined (Fig. 1B'). At the 20µM concentration, the tails started to curve and extension was limited, the notochord was wavy and the somites were irregularly shaped and spaced and difficult to define. Necrosis at the tail tip was also seen (Fig. 1C'). At the high concentration (30µM), there was no tail extension, the notochord demonstrated an undulating pattern, no somites were detected and necrosis was present (Fig. 1D').

3.2 Notochord Development

The *ntl* gene is a commonly used marker for the notochord, (Schulte-Merker et al., 1994; Yamada et al., 1991) which serves as a signaling source to pattern tissue including the paraxial (somatic) mesoderm (Stemple, 2005). Control embryos demonstrated smooth *ntl* staining along the length of the notochord up to the tailbud by 24hpf (Fig. 2A, A'). Increasing concentrations of BBP induced alterations in *ntl* staining in a concentration-dependent manner (Fig. 1 B-D') in correlation with the wavy and undulating patterns seen in live imaging (Fig. 1) by 24hpf. Examination of *ntl* staining at an earlier developmental timepoint, the bud stage, marking the

beginning of the segmentation period when somites form, showed no change in axial mesoderm staining in any treatment level (Fig. 2, E-H).

3.3 Muscle Development

As changes were seen in somite boundaries and patterning in live imagery, we sought to more specifically define the somatic changes via in situ hybridization with *myoD*, a basic helix-loop-helix (bHLH) transcription factor with a major role in myogenesis (Weinberg et al., 1996). At 24hpf, control embryos demonstrated *myoD* staining within each somite block with the strongest staining at the tail (Fig.3 A). Increasing concentrations of BBP induced alterations in *myoD* staining in a concentration dependent manner (Fig. 3, B-D). At 10µM BBP it was difficult to note major changes as *myoD* staining appeared normal, but by 20µM, loss of patterning was seen, with somites becoming disorganized. By 30µM, no somite patterning with *myoD* staining was detected.

To further characterize muscle changes, an F49 immunohistochemistry specifically marking myosin heavy chain was performed. In control embryos, myosin stained muscle fibers were apparent in a linear pattern within each chevron shape somite (Fig. 4, A). At the 10µM BBP treatment level, the somite boundaries were noted, but individual muscle fibers lost their linear shape demonstrating wavy muscle fibrils (Fig.4, B). At the 20µM concentration, myofibrils started to lose any pattern and somite boundaries were becoming unclear (Fig. 4, C). This phenotype became more severe at 30µM (Fig. 4, D), no muscle pattern or fibers were detected.

3.4 Vasculature

To investigate if the alterations detected in musculature affected vascularization of the trunk and tail, we utilized *fli-1 gfp* transgenic embryos where the *fli-1* promoter drives expression of GFP in developing endothelial blood vessels (Lawson and Weinstein, 2002). By 24hpf in control embryos, GFP was expressed in the intersegmental vessels (ISVs) which extend up through the somites from the dorsal aorta (DA) to the dorsal longitudinal anastomotic vessel (DLAV) (Fig 5. A,B). Increasing concentrations of BBP induced alterations in the developing vasculature in a concentration-dependent manner. These defects included truncation of the vessels and a lack of migration through the somite to the DLAV (Fig. 5C) or branching of the vessels (Fig. 5D, E) for

10 and 20μM concentrations of BBP. By 30μM BBP, little to no vascularization was seen in the tail region.

3.5 Cell Death

Lastly, we tested if BBP caused an increase in cell death. At 24hpf in control embryos, cell death was not noted in the trunk somites or tail (Fig. 6A). At 10μM, few spots were seen throughout the somite region, but clusters could be seen in tail region. At the 20μM concentration, the tails started to curve (Fig. 1C') and cell death was noted within the somite bodies and the tail (Fig. 6C). At the high concentration (30μM), where there was no tail extension (Fig. 1D'), cell death was also noted throughout the curved tail with clustering at the tail tip (Fig. 6D).

4. Discussion

Here we find that treatment with BBP results in loss of proper somite patterning and ventral bending of the tail. It has recently been reported that BBP exposure in zebrafish causes decreased acetylcholinesterase (AChE) activity (Zhang et al., 2014). Acetylcholine (ACh) is normally released from motoneuron terminals and migrates through the neuromuscular junction (NMJ) to bind to acetylcholine receptors (AChRs) clustered on the muscle membrane (Buss and Drapeau, 2001). Once bound, the muscle is depolarized causing calcium to be released from the sarcoplasmic reticulum into the cytosol. Increasing cytosolic calcium levels activate troponin, which initiates sliding actions of actin and myosin inducing muscle contraction. Excess ACh within the NMJ is rapidly degraded by the enzyme acetylcholinesterase (AChE). The rapid removal of excess ACh within the NMJ along with decreasing cytosolic calcium levels performed by Ca^{+2}-ATPase pumps is critical for normal muscle relaxation (Hirata et al., 2004). Thus, acetylcholinesterase is a crucial enzyme for muscle functionality. In zebrafish AChE mutants that lack ACh hydrolysis, embryos demonstrate abnormal myofibril organization and develop paralysis (Behra et al., 2002). Exposure to the AChE inhibitor diisopropylfluorophosphate (DFP) induced abnormal somites and a ventral bend of the tail (Hanneman, 1992). Here we find that treatment with BBP results in loss of proper somite patterning similar to what is seen in AChE mutants and the ventral bending seen with DFP treatment phenocopies what we see in our higher treatment concentrations (Fig. 1C,D). Thus, it might suggest that the defects we see are a result of BBP

blocking acetylcholinesterase causing overstimulation of muscle activity. However, we did not specifically test this and the exact mechanism by which BBP blocks AChE is not yet known (Zhang et al., 2014). We also find that myofibril organization is lost and results in a thinner disorganized pattern in the lower concentration of BBP (Fig. 4B). This agrees with what is noted by others investigating myofibrils in *ache* and *twister* mutants (Behra et al., 2002; Hirata et al., 2004; Lefebvre et al., 2004) who note altered arrangement and/or integrity of myofibrils, myofibrils that were splayed apart or absent and fibers that were less fasciculated.

Here, we find that increasing concentrations of BBP induce alterations in *myoD* staining and by the highest concentration, *myoD* somatic staining is absent (Fig. 3). This is not surprising given the defects noted in somites in live images. *myoD* expression occurs during the period when somites are forming (Weinberg et al., 1996) and if somatic development is affected, we would expect to see correlative defects in *myoD* staining.

In zebrafish muscle, superoxide dismutase (SOD), the enzyme responsible for eliminated dangerous superoxide radicals, has been shown to be induced in BBP treatments. As the dose of BBP increased and more radicals developed, SOD synthesis was likely induced (Zhang et al., 2014). Oxidative stress induced necrosis is a common phenomenon when SOD cannot keep up with the level of radicals being produced. We find in higher concentrations of BBP, necrosis was noted (Fig. 1C,D). The necrosis seen is likely the reason for the lack of myofibrils noted in F59 staining (Fig. 4C,D). Acridine orange staining also noted cell death. Increasing concentrations of BBP induce higher levels of cell death (Fig. 6). These results indicate that higher concentrations of BBP are toxic to the developing embryo possibly due to increases in SOD although that was not specifically tested here.

Lastly, we examined the effect BBP has on the vasculature. Specifically, we investigated intersegmental vessels (ISVs) which extend up through the somites from the dorsal aorta (DA) to the dorsal longitudinal anastomotic vessel (DLAV). We found that increasing concentrations of BBP induced truncation and migratory defects and/or branching of the vessels (Fig. 5). This is not a surprising result given the ISVs run in parallel with the somite borders and somite changes were detected. By the highest concentration, little to no vascular pattern in the tail was seen suggesting that the higher concentration of BBP is toxic to the developing embryo.

Alternatively, one could argue BBP alters notochord formation (seen in Fig. 2) and the effect seen on muscle is secondary to the notochord defects. The notochord is necessary for somite patterning and is required to induce muscle cell pioneers, which develop into the adaxial mesoderm (Felsenfeld et al., 1991; Halpern et al., 1993). Specificaly, *ntl* is necessary to induce adaxial muscle development and *myoD* expression in the paraxial mesoderm (Odenthal et al., 1996; Weinberg et al., 1996). However, in contrast to our results, by 24hpf in *ntl* mutant embryos, somites do not become lost or disorganized as we see, but are reported to become block-like and columnar shaped (Halpern et al., 1993) or described as U-shaped (Odenthal et al., 1996). Additionally, a decreased number of muscle fibers have been noted in *ntl* mutants (Stickney et al., 2000) whereas we did not detect a decrease or loss in muscle fibers until the higher toxic concentration (Fig. 4D), but rather noted disorganized myofibrils (Fig. 4 B,C).

Zebrafish are a common toxicological model. Zebrafish have high fecundity allowing for large sample sizes and high throughput screens for toxicity (Hill et al., 2005). Their rapid *ex utero* and transparent development allows one to test toxicological endpoints at various stages of development, asses behavior in response to chemical exposure as well as address transgenerational outcomes (Sipes et al., 2011; Yang et al., 2009). Most importantly, zebrafish can be used in monitoring the aquatic environment (Dai et al., 2014). Zebrafish have been used to monitor common environmental pollutants like heavy metals, endocrine disruptors and organics (Bambino and Chu, 2017; Dai et al., 2014) . Use of zebrafish embryos have been well documented as sentinels in aquatic ecosystems (Carvan et al., 2000; Mizell and Romig, 1997; Scholz et al., 2008). Determining if a chemical effects embryonic development is essential to determining if the chemical is a risk to the environment and ultimately to human health (Mizell and Romig, 1997). Here, our endpoints on BBP toxicity in zebrafish during development will help environmental toxicologists in monitoring water contamination and provide key endpoints that provide an early warning signal for waters that could be dangerous for human health.

5. Conclusion

Here, we have shown that BBP is toxic to caudal development in zebrafish embryos. We demonstrate that somite patterning, myofibril arrangement and a muscle specific marker, *myoD* expression are altered in a concentration-dependent manner in which higher concentrations become toxic. Vasculature is also altered as a result of changes in somite development. Increases

in cell death are noted in a concentration-dependent fashion. Further studies will investigate the effect BBP treatments have on movement and behavior as well as innervation of primary and secondary motorneurons, but that was beyond the scope of this preliminary study.

Acknowledgements

The authors wish to thank the Lawson Lab (University of Massachusetts Medical Center) for the *fli-1* transgenic fish and Dr. Charles Sagerstrom for the *ntl* and *myoD* DNA plasmid.

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Figure Legends

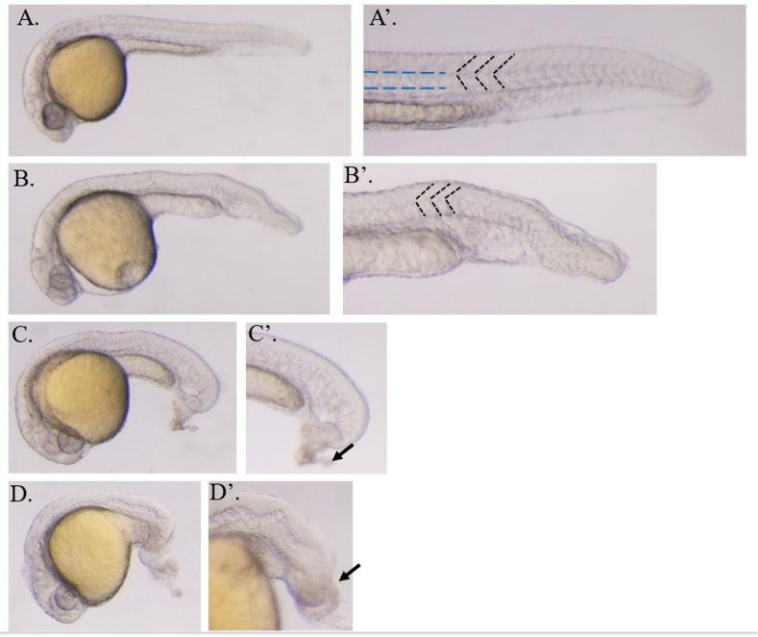

Fig. 1. Live Images of control and BBP treated embryos at 24hpf in lateral views. Zoomed images of tails (A'-D'). Control (A, A') embryos demonstrate linear extension of the tail marked by a straight notochord (large dashes) and classic chevron shaped somites (small dashes). Increasing concentrations of BBP (B-D') cause concentration-dependent malformations in tail extension and development including wavy notochords, loss of somite patterning or somites, curved tail and necrosis (arrows). (A, A') Control, (B, B') 10μM BBP, (C, C') 20μM BBP and (D, D') 30μM BBP.

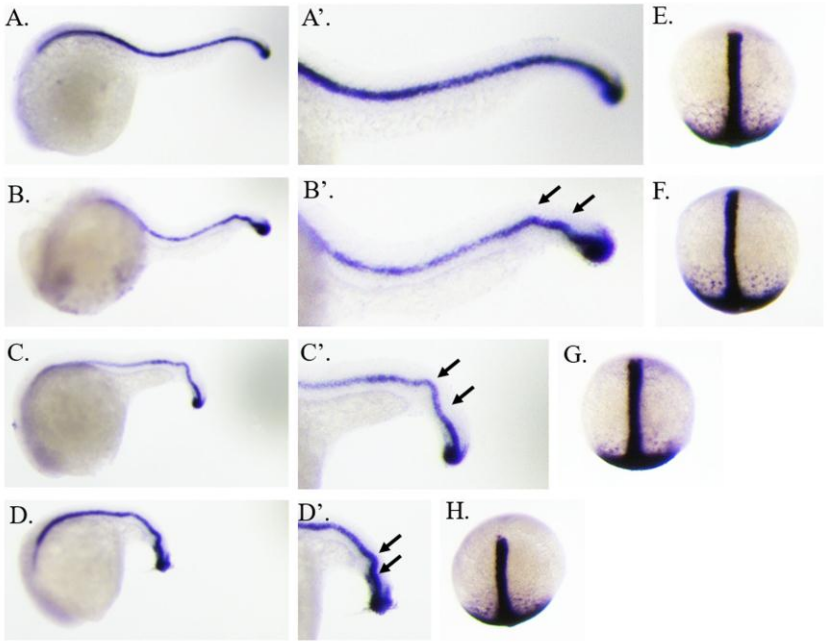

Fig. 2. *ntl* in situ hybridization of control and BBP treated embryos. (A-D) Whole body lateral views and (A'-D') zoomed images of the tails at 24hpf in lateral view. (E-H) Dorsal views at 12hpf. (A, A', E) Control, (B, B', F) 10μM BBP, (C, C', G) 20μM BBP and (D, D', H) 30μM BBP. Arrows note wavy notochords.

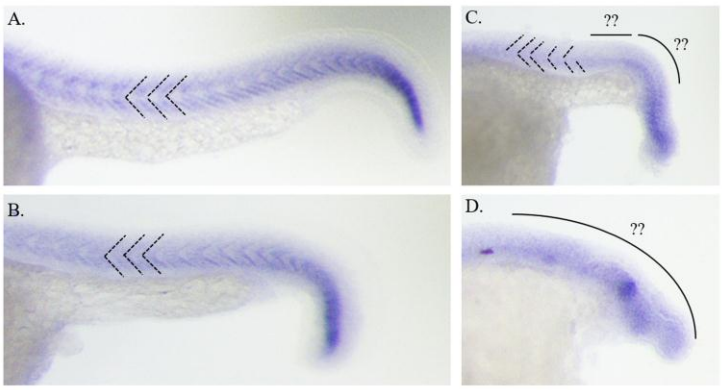

Fig.3. *myoD* in situ hybridization of control and BBP treated embryos. (A-D) Zoomed images of the tails at 24hpf in lateral view. (A) Control, (B) 10µM BBP, (C) 20µM BBP and (D) 30µM BBP demonstrating concentration-dependent alterations to *myoD* staining. Dashes represent normal chevron shaped somites (A,B) or show disorganized somite boundaries (C). Lines represent areas where *myoD* expression is not defined.

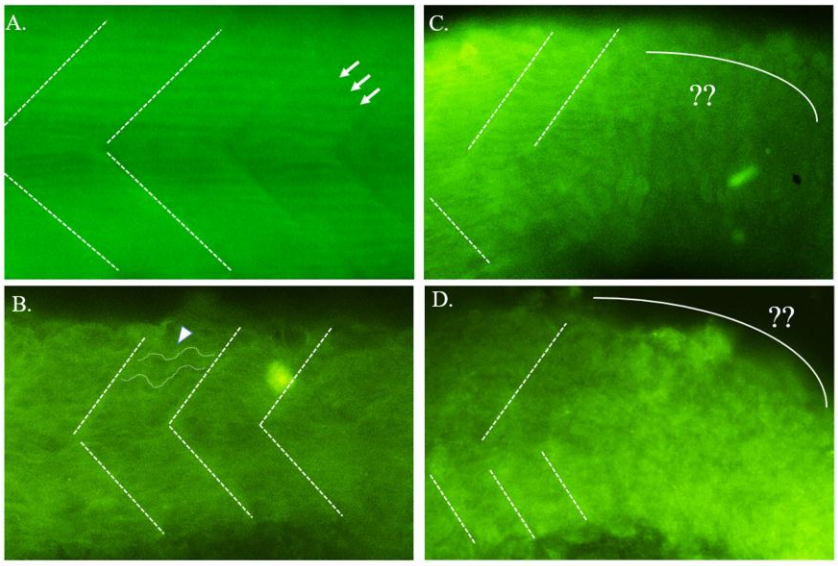

Fig.4. Whole mount fluorescent immunohistochemistry using F59 myosin specific antibody at 24hpf. (A-D) Zoomed images of the tails at 24hpf in lateral view. (A) Control, (B) 10µM BBP, (C) 20µM BBP and (D) 30µM BBP demonstrating concentration-dependent alterations to myofibril arrangement within somites. Dashed lines represent normal chevron shaped somites (A,B) or show disorganized somite boundaries (C,D). Arrows in A show linear myofibril arrangements within somites. Dotted lines demarcate wavy myofibril arrangement shown by arrowhead (B). Solid arches represent areas where F59 staining is not defined and loss of myofibrils is detected.

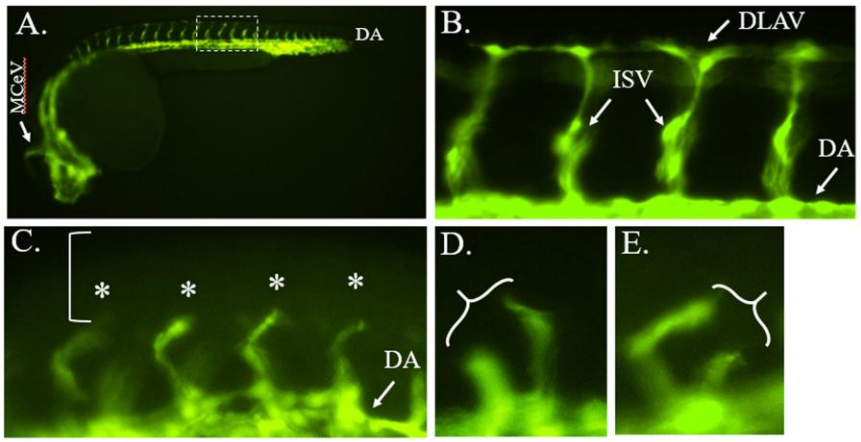

Fig.5. *fli-1* transgenics and vasculature. Live lateral images of vasculature as seen by green fluorescent protein. Whole image of control embryo at 24hpf (A) with dashed box denoting area of imaging in zoomed view (B). Increasing concentrations of BBP induced vascular defects including truncation of vessel migration (C, asterisks) and branching of ISVs (D,E, brackets) in both 10µM and 20µM concentrations. Vascular staining is absent at the highest BBP concentration. ISV: intersegmental vessels, DA: dorsal aorta, DLAV: dorsal longitudinal anastomotic vessel, MCeV: mid-cerebral vein

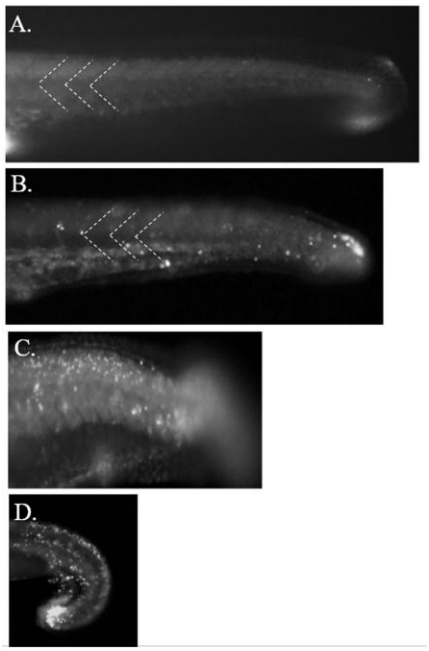

Fig. 6. Cell death in control and BBP treated embryos. Live images of zoomed tails in lateral view at 24hpf (A-D). Control (A) embryos demonstrate no cell death as marked by acridine orange staining. Dashed lines represent chevron shaped somites. Increasing concentrations of BBP (B) 10µM BBP, (C) 20µM BBP and (D) 30µM BBP induce cell death in a concentration-dependent manner as noted by fluorescent dots.