

Effects of Bis(2-ethylhexyl) Phthalate on Cortisol Production and Development in *Xenopus laevis*.

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By

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April, 2016

A Senior Comprehensive Project in Partial Fulfillment of the Requirements
for a Bachelor of Science Degree from Allegheny College

***I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code
and to maintain the integrity of both myself and the College community as a whole.***

Pledge

Approval by the Senior Thesis Committee:

Dr. 1st Reader

Dr. 2nd Reader

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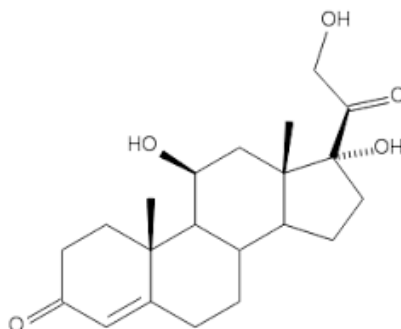
Abstract

Bis (2-ethylhexyl) phthalate (DEHP) is a suspected endocrine disruptor (ED). Phthalate levels in the environment are rising and so are the concerns for wildlife and human health, because exposure to phthalates can be detrimental. Amphibian populations are declining rapidly, and they are at the highest risk for exposure to EDs. However, DEHP exposure did not affect the amount of cortisol excreted in urine as assessed by enzyme-linked immunoassay. *X. laevis* tadpoles exposed to 14 µg/ml DEHP from NF-stage 39-40 to NF-stage 51-53 had increased body mass and decreased limb development in post-hatching developing tadpoles compared to controls. These results give us further insight into potential explanations for the amphibian population decline and increase in the non-communicable diseases in human health.

Introduction

The endocrine system plays a vital role in the balance and integration of all the organ systems in an organism. Hormones produced by the endocrine system regulate metabolism, physiology, cell differentiation, development, and embryonic morphogenesis. These hormones can be steroids, peptides, or amino acid derivatives that can directly affect a target cell or synthesis of secondary hormones or molecules resulting in a signaling cascade. Disruptions in hormones lead to chronic diseases and disorders, such as metabolic syndrome, diabetes, and obesity. Glucocorticoid adrenal hormones cortisol and aldosterone are synthesized early in

Image 1. Chemical structure of glucosteroid, Cortisol. (Dartmouth J. Science 2011)



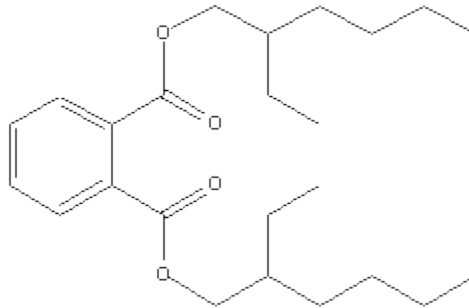
embryogenesis and play an important role in cell differentiation during organogenesis (Ishimoto & Jaffe 2011; Nesan and Vijayan 2013). Cortisol is a hydrophobic steroid hormone that can freely diffuse through the plasma membrane (Image 1). Once bound to its receptor within the cytoplasm of a cell, the receptor-cortisol complex can move to the nucleus and bind to a complementary regulatory region on the DNA. The target gene is then activated and transcription is initiated. A cell responds to cortisol more slowly than it does to a hormone like epinephrine that binds to a surface receptor for immediate activation or inhibition; however, nuclear receptor hormones result in longer, more prolonged changes to the organism (Fox 2013). Steroid metabolism has been associated with insulin resistance, diabetes, metabolic syndrome, and vascular disease mainly as a result of increased tissue-specific cortisol metabolism (Nesan and Vijayan 2012; Traish et al 2014). Glucocorticoids, including cortisol, inhibit insulin sensitivity of tissues and promote lipolysis, gluconeogenesis, and glycogenolysis in the liver and adipose tissues. An increase in cortisol titer elevates levels of free fatty acids, triglycerides, and glucose in the blood, increasing the risk of physiological complications and non-communicable diseases (Morton, 2010). Cortisol released under stress also inhibits the inflammatory response of the immune system. This is supposed to increase healing by preventing further epithelium damage caused by inflammation. If cortisol levels should increase, then the resulting suppression of the immune system would be disadvantageous in the presence of bacteria or other foreign material (Fox 2013). In fact, any disruption can cause major complications and malfunctions of organ systems that can be fatal (Hung et al 2011; Rafacho et al 2014).

The incidence of chronic disease and disorders have risen worldwide, especially those related to the endocrine system. Diabetes, cardiovascular disease, obesity, cancer, genital malformations, and low semen quality are all on the rise (WHO 2012). Such increases suggest

that environmental factors increase vulnerability to non-communicable diseases, possibly by endocrine disrupting chemicals (EDCs) that disrupt the synthesis and signaling pathways of hormones (WHO 2012).

Endocrine disrupting chemicals (EDC) are emerging pollutants (EPs) in environmental shallow water, streams, surface water, and effluent wastewater from water treatment plants. These exogenous chemicals can disrupt by either inhibition or enhancement of synthesis, secretion, transport, binding, and metabolism of natural hormones deregulating the homeostatic mechanisms, reproduction, and development (Annamalai and Namasivayam 2015; Chen et al., 2014; Colborn et al 1993). Much of the environmental and organismal effects of the toxicants are unknown, but the global production of anthropogenic chemicals increased from 1 million to 4 million tons per each year between 1930 and 2000 (Gavrilescu et al 2015). Although little is known about the toxicity and environmental impact, environmental concerns are emerging. For example, amphibian populations have declined as early as the 1970s in western United States, which is within the range that the US Environmental Protection Agency saw the increase in synthetic human-made materials of everyday house hold supplies (Stuart et al 2004). Exposure to synthetically made materials has been increasing. Phthalates are one of the most common EDCs and are one of the many materials that cause concern (Thomaidi et al 2015). Phthalates are toxicants that affect hormones that in turn, disrupt reproduction and development. Specifically how these phthalates disrupt hormone biosynthesis and development remains unknown. It is unlikely that wastewater treatment plants are able to remove the components due to the hydrophobicity and chemical stability of the toxins. For instance, phthalates are manufactured to increase flexibility, durability, and longevity of plastic products, making them too stable to react with most eliminating reagents. Phthalates have been found to be released into the environment

Image 2. Chemical structure of Bis-2-ethylhexyl-phthalate (DEHP) (ec.europa.eu Glossary)



from treatment plants and have been identified in the urine output of aquatic organisms.

Phthalates are synthetic di-esters of phthalic acids used in the manufacture of plastics, personal care products such as perfumes, hairsprays, lubricants, floor and wall coverings, toys, food packaging, and medical devices (Gavrilescu et al 2015). However, these plasticizers are not physically bound to the polymers allowing them to be easily released into the environment. The stability and low water solubility of phthalates allows the possibility for them to bypass water treatment removal. The phthalate bis (di-ethylhexyl) phthalate (DEHP) was found in 74% of common U.S foods and is the most abundant phthalate in agricultural soil (Image 2) (Schechter et al 2013; Annamalai and Namasivayam 2015; Thomsen et al 1999). Phthalates decrease fertility, alter sex ratios, testosterone levels, and testicular differentiation in zebrafish and African clawed frogs as well as increase DNA damage in sperm (Lee and Veeramachaneni 2005; Schechter et al 2013; Zhai et al 2014). Low concentrations of phthalates also enhanced estrogenic activity in zebrafish while decreasing thyroid-T3 hormone in African clawed frogs (Chen et al 2014; Sugiyama et al 2005). These disruptions affect a species' ability to reproduce and could lead to a decline in population size.

Aquatic species are susceptible to hormone disruptions and developmental malformations, which could explain the severe decline in amphibian populations. The amphibian population decline, especially in stream-associated species, has been seen worldwide. Population sizes of frogs have declined 48%. Disruption of sex organ morphogenesis due to phthalates has

contributed to the continuing population decline (Stuart 2004). African clawed frogs, *Xenopus laevis*, exposed to exogenous phthalates have reduced reproductive success.. Hermaphroditism among populations of *X. laevis* have increased. *X. laevis* exposed to low concentrations of DBP, di-butyl phthalate, have decreased levels of testosterone and impaired spermatogenesis (Lee and Veeramachaneni 2005; Chen et al., 2014). Malformed gonads or hermaphroditism in frogs would lead to a female-biased sex ratio and an eventual decrease in population size due to limited reproduction (Annamalai and Namasivayam 2015). Amphibians are highly exposed to endocrine disrupting chemicals because their skin is highly permeable, providing a large surface area for aquatic environmental toxins to be absorbed into the body (Stuart 2004). Furthermore, human communities surrounding water treatment plants are also at risk for developing negative effects from phthalate exposure through drinking water and locally grown food (Gavrilescu et al., 2015; Schechter et al., 2013; Wang et al., 2015). Concentrations of DEHP found in the effluent of water-treatment plants were as high as 14.2 µg/L in the UK (Gavrilescu et al., 2015)

The ability of phthalates to disrupt development of reproductive organs has been examined. The gonads are derived from the mesoderm during embryogenesis (Chen et al., 2014). Proper organ development in amphibians depends on the hypothalamus-pituitary-interrenal hormone axis (HPI) which is homologous to the hypothalamus-pituitary-adrenal hormone axis (HPA) in humans and other mammals. Specifically, glucocorticoid hormones secreted from steroidogenic cells are responsible for cell differentiation and migration during organ development (Ishimoto & Jaffe, 2011). The physiology of the adrenal gland in mammals and interrenal tissue of amphibians are the same, but they are anatomically different. In all vertebrates, the adrenal gland is located near the top of the kidneys. In mammals, the steroidogenic cells and catecholamine-secreting cells are located in distinct zones called the

cortex and medulla, respectively. Amphibians have no distinct zonation. The interrenal tissue (steroidogenic cells) and the chromaffin cells (catecholamine-secreting cells) are intermingled (Magath 1915). In amphibians, cortisol is a glucocorticoid that is secreted from interrenal tissue in response to adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. These hormones raise the glucose level in the blood by increasing gluconeogenesis in the liver, kidneys, and intestines, regulating pancreatic hormone secretion (Rafacho et al 2014, Rajesh and Balasubramania 2013). Aldosterone is a mineralocorticoid secreted in response to low blood volume. Aldosterone works with the kidneys to maintain salt and water homeostasis by increasing sodium reabsorption in the distal convoluted tubule of the nephron (Fox 2013). These hormones are crucial for proper blood pressure, heart rate, and glucose and insulin levels as well as proper organ development (Ishimoto & Jaffe, 2011).

In zebrafish, the cortical sex hormones, androgens and estrogens, decreased following exposure to phthalates; however, the specific origin of the developmental level of disruption is still unclear (Chen et al 2014). Zebrafish embryos were exposed to bis (2-ethyl-hexyl) phthalate during embryonic stages in which the gonads differentiate. Such treatment disrupted testicular weight, sex differentiation, gonad production, and oogenesis and spermatogenesis (Chen et al., 2014; Lee and Veeramachaneni 2005). In *X. laevis*, gonad malformation corresponded with the decreased androgen levels; a disruption of interrenal tissue and tissue differentiation from the mesoderm may have contributed to decreased androgen levels (Lee and Veeramachaneni 2005). Furthermore, since increased adrenal hormones in humans are ultimately seen in insulin-resistant Type II diabetes patients, a disruption in these glucocorticoid hormone levels could result in additional chronic health issues (Rafacho et al., 2014). The kidney tubules, cardiac muscle, blood vessels, and the interrenal tissue in amphibians (analogous to adrenal cortex in mammals) are

also derived from the mesoderm during organogenesis. Organogenesis is regulated by the hypothalamic-pituitary-interrenal (HPI) axis in amphibians (homologous to the hypothalamic-pituitary-adrenal (HPA) hormone axis in birds and mammals). The HPA axis in humans develops prior to organogenesis in the late gestational stages of embryogenesis, regulating organ development and post-development organ function (Ishimoto & Jaffe, 2011). A disruption of glucocorticoid hormone levels as the origin of phthalate disruption could result in organ malformation and amphibian population decline.

The specific aim of this study was to determine whether phthalate DEHP (bis-2ethylhexyl) exposure altered cortisol produced in *X. laevis* tadpoles during development. Cortisol can be measured in the blood, feces, urine, and saliva. Serum cortisol concentrations range from about 25-800 nM, but 90-95% of the cortisol is bound to proteins. Urinary cortisol is not bound to proteins making it a good measurement to determine if there is an excess amount in the body (Fox, 2013). Another aim was to observe the potential developmental effects of DEHP exposure by monitoring mass, length, and stage of tadpole development. We hypothesized that exposure to DEHP would result in abnormal cortisol production in the interrenal tissue. A decrease in cortisol would result in organ abnormalities leading to a higher mortality. In contrast, an enhancement of production would lead to increased body mass and increase developmental rate increasing risk of malformations and excess tissue growth (Rafacho et al 2014; Thomas et al 2000).

The completion of this research should help in the explanation of how environmental exposure to synthetic chemicals affects development and hormone regulation in wildlife and humans by identifying the effect of the phthalate, bis-2-ethylhexyl (DEHP), on cortisol hormone production in the interrenal gland of developing tadpoles. My central hypothesis is that long term

exposure of developing tadpoles to DEHP would disrupt regulation and hormone production in the interrenal gland, and that an increase in hormone production will lead to increase of adipose tissue and growth abnormalities present in the tadpole body (Rafacho et al, 2014). These results are important additional information about the impact of synthetic material exposure on developing organ systems and assist in the search for the cause of the increased prevalence of chronic diseases such as cardiovascular disease and diabetes in humans and the decrease in amphibian populations.

Materials and Methods

Experimental set up: Eight tanks, two per treatment group, were set up with aeration tubing and air stones. Each tank contained 3 liters of treated water marked by a line. Any water that was lost due to evaporation was replaced to keep a constant water volume. The incubation room was 24 degrees Celsius with a 10 hr dark:14 hr light photoperiod.

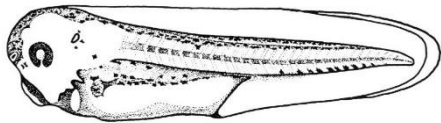
Stock solutions of DEHP: The stock solution of DEHP was made November 2 of 2015.

Solubility of DEHP is approximately 40 µg/L (ppb) in water. The 14 ppb high and 8 ppb low DEHP concentrations are based on the average and maximal concentrations found within the environment (Gavrilescu et al., 2015). DEHP (purity >98%) was purchased from ChemService in West Chester, Pa. A stock of 8µg/ 100µl EtOH (8ppm) was made from 2µg of DEHP dissolved in 25ml of EtOH. At the start of the experiment, “Low treatment” DEHP tanks (8ppb) received 300µl per tank and “high treatment” tanks (14 ppb) received 525 µl per tank. EtOH control tanks received 525 µl of 100 % EtOH. Additionally, for every 10 ml of water replaced due to evaporation, 1.75 µl of DEHP stock was added for “High treatment” tanks and 1 µl of DEHP stock was added to “Low treatment” tanks.

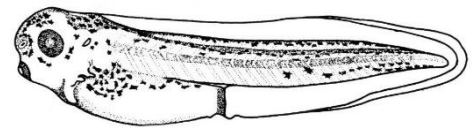
***Xenopus laevis*:** Adult male and female *X. laevis* were injected with 500 μ l hCG on November 2, 2015, but spawning was unsuccessful. Another pair of adults produced fertilized eggs when injected November 11, 2015. Eggs hatched on Nov. 13. Approximately 50 tadpoles were placed into each of the 8 tanks and treatments were started. Thus, I began with 100 tadpoles per treatment group and 400 tadpoles in the experiment. Preliminary mass (mg), lengths (mm), and NF developmental stage were taken with 4 tadpoles immediately before treatments commenced (Table 1). Daily 300 μ l of prepared food was added to each tank and any dead tadpoles were removed.

Table 1.
Preliminary data collection
11/13/15 Treatment started
Average stage of development:
NF 39.5 (right) (Nieuwkoop and
Fabor 1994)

tadpole	mass(mg)	length (mm)	NF-Stage
1	2.7	5.7	37-38
2	2.4	6	40-41
3	3.2	5.2	39-40
4	2.7	5.7	41-42



NF Stage 39



NF Stage 40

Data Collection: Mortality was recorded daily for each group. Urine samples for ELISA analysis were collected from 4 tadpoles from each treatment group every Monday for four weeks starting November 23, 2015 i.e., after 10 days of exposure. A control sample of water from each treatment group was also collected. Mass (mg), length (mm), and developmental stage were also recorded. Each tadpole was placed individually in a 5 ml tube with approximately 3-3.5 ml water for 60 minutes. Afterwards, tadpoles were transferred to 1.5 ml centrifuge tubes containing 1 ml of MS-222, ethyl 3-aminobenzoate methane sulfonate, for approximately 20 seconds to euthanize them prior to further data collection. Tubes containing urine samples were stored at -80°C until further analysis.

Preparation prior to ELISA.

Buffer and Reagent Preparation. *EIA Buffer* Concentrate (10X) (Item No. 400060) was diluted with 90 ml of pure water. The *wash buffer* Concentrate (400X) (Item No. 400062) was diluted to a volume of 1 L with pure water and 500 µl of Polysorbate 20 (Item No. 400035) was added. The cortisol *AChE Tracer* (100dtn) (Item No.10005272) was prepared by adding 6ml of already prepared EIA Buffer solution. Cortisol *EIA monoclonal antibody* (100dtn) (Item No. 400362) was prepared adding 6 ml EIA buffer. All buffers and reagents were stored at 4°C.

Cortisol Preparation from water samples. The cortisol in the tadpole urine sample had to be isolated and purified before quantifying. After thawing samples, we used Sep Pak C18 filtration (Waters Corporation Milford, MA) first washing with 4 ml of methanol and 4 ml of water before applying samples. Each treatment group had one urine sample plus one water control per week, giving a total of 8 samples /week; 30 samples per 4 weeks. [Ethanol treated for week 4 were used in the pre-trials]. Pre-trials consisted of an ELISA analysis of a urine sample from 1 tadpole, and another from 3 tadpoles. Quantifiable cortisol was found in the 3-tadpole sample but not in the 1-

tadpole sample. Therefore, all four tadpole urine samples from each treatment group were combined to obtain quantifiable levels of cortisol. The cortisol sample was then eluted into glass tube using 4ml methanol. The samples were then dried at 37°C (Biological 98.6 °F) for at least 6 hours or until methanol was gone. All samples were re-suspended in 20µl 100% ethanol. After 1 minute vortex, 380µl EIA buffer was added.

Assay Protocol

EIA Standard Preparation. Bulk standard (40 ng/ml) was prepared with dilution of 100µl of the cortisol *EIA Standard* (Item No. 400364) with 900µl of pure water into 1.5ml centrifuge tube. A new centrifuge tube (S1) was aliquoted with 900 µl EIA buffer. Tubes S2-S8 contained 600µl EIA buffer. Dilution of bulk standard began with a transfer of 100µl bulk standard to tube S1 resulting in a concentration of 4 ng/ml. Serial dilution of 400µl was then completed giving a final S8 concentration of 6.6 pg/ml.

Performing Assay. The 96-well Cortisol EIA kit assay (ELISA) was bought from Cayman Chemical Company in Ann Arbor, MI (Item No. 500360) and stored at -20°C. The assay was completed to measure amounts of cortisol from previously collected urine samples. This competitive assay between cortisol and cortisol-acetylcholinesterase (AChE) had an IC₅₀ (50% B/B₀) of approximately 180 pg/ml and a detection limit (80% B/B₀) of approximately 35 pg/ml. Addition of reagents started with 100µl EIA buffer to Non-specific binding (NSB) well and 50µl EIA buffer to Maximum Binding (B₀) well. For cortisol EIA standards, 50µl from each standard tube, starting with S8, were added to an individual well. For each treatment group and each week, 50µl of sample was added to individual well on plate. Every well except Blank (Blk) and Total activity (TA) received an additional 50µl of cortisol AChE Tracer. Every well except Blk,

TA, and NSB received an additional 50µl of cortisol EIA monoclonal Antibody. The entire plate was incubated overnight at 4 °C.

After incubation, the plate's wells were emptied and rinsed with Wash buffer five times.

Ellman's Reagent (100dtn) (Item No. 400050) was reconstituted with 20 ml pure water immediately before adding 200 µl to each well. An additional 5 µl of AChE Tracer was added to TA well. The plate was covered and placed on orbital shaker in dark for 120 minutes.

Reading the Assay. Absorbances were read at wavelength 405nm after 120 minutes.

$$\text{Absorbance} \propto [\text{Bound AChE Tracer}] \propto 1/[\text{Cortisol}]$$

This is a competitive assay between cortisol and cortisol-acetylcholinesterase (AChE tracer). The plate is pre-coated with goat anti-mouse IgG. There are a limited number of cortisol specific mouse monoclonal antibody binding sites per well. Tracer and free cortisol compete for the antibodies. The concentration of the tracer is held constant while the cortisol per sample varies. Therefore the amount of tracer that binds to the antibody is inversely proportional to the concentration of cortisol in the well. Ellman's Reagent contains highly reactive acetylthiocholine and 5, 5'-dithio-bis-(2Nitrobenzoic Acid). The addition of the Ellman's reagent to the wells causes enzymatic AChE to hydrolyze acetylthiocholine which then reacts with 5,5'-dithio-bis-(2Nitrobenzoic Acid). The product 5-thio-2-nitrobenzoic acid has a strong absorbance at 412nm.

Absorbances were corrected by subtracting the Blk absorbance (0.2nm) from all wells on the plate. The corrected maximal binding B_0 was calculated by $B_0 - \text{NSB}$. To calculate B/B_0 ratio (Sample or standard bound/maximum bound), NSB was subtracted from all Standard and samples and divided by corrected B_0 . To get % B/B_0 all values were multiplied by 100. The spectrophotometric absorbances are proportional to the amount of cortisol tracer bound to well,

which is inversely proportional to the amount of free cortisol present (Cayman Chemical Company, 2015).

$$[\text{Cortisol}] \text{ in well} = 1/\text{Absorbance}$$

Statistical Analysis. After testing for the assumptions of the analysis of variances (ANOVA), a one-way ANOVA was used to analyze the collected data. Adjusted cortisol concentrations were used during the comparisons (water control [cortisol] ng/ml – Urine [cortisol] ng/ml).

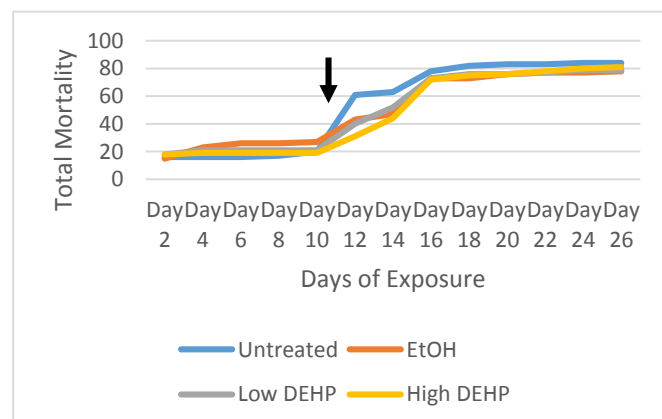
Differences in variances among treatments, weeks, mass, length, and developmental stage were all considered. A Nonparametric test was used for multivariable comparisons due to failure of normal distribution of the small sample size. A Chi-Squared test was used to compare mortality frequencies among treatment groups.

Results

Due to the difficulty of getting the adult *X.laevis* to spawn and produce viable, fertilized eggs, the data collection was delayed and limited the range of data collection to four weeks.

Mortality. Treatment did not affect frequency of deaths of tadpoles. ($X^2=16.034$; $df=12$; $X^2_{\text{critical}}=21.026$) (Figure 1). Mortality among all groups was high on Day 10-16 of exposure (Figure 1).

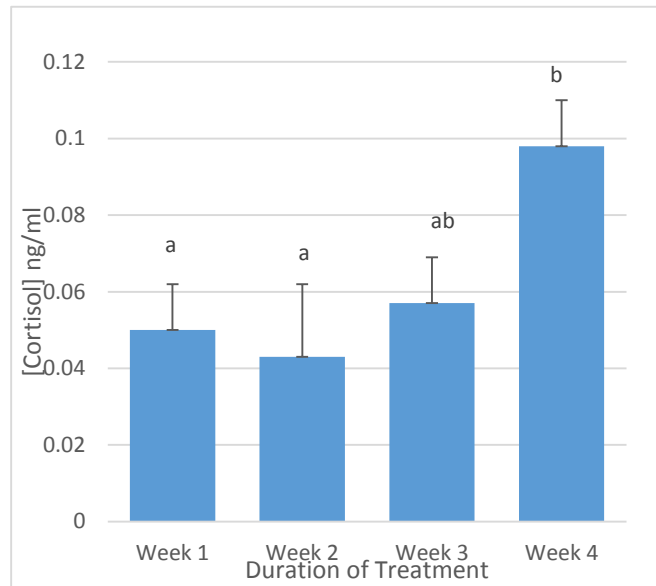
Figure 1. Tadpole deaths over the 26 days of exposure per treatment group. Mortality was high in all groups during week 2 (arrow). n=100 per group at the start.



Cortisol Production. Treatment did not affect the amount of cortisol excreted ($p=0.38$).

However, when all tadpole cortisol production was compared by week, cortisol excretion was significantly enhanced by week 4 (NF-stage 50-53) (Figure 2).

Figure 2. Cortisol levels increased significantly ($p=0.03$) during the 4 weeks ($N=16$). Weeks with different data labels (a) or (b) are significantly different. $n=4$ for each histogram. Vertical bars represent standard errors of the means.



Mass, length, and stage comparisons. Mass (mg)/Length (mm) ratios were averaged weekly.

This determined whether the body mass per length of tadpole differed among groups. For weeks 1 through 3, the DEHP-treated tadpoles weighed more per body length than did those of the other groups. By week 4, there were no significant differences between groups (Figure 3). All animals grew over the 4 weeks of exposure ($p < 0.001$) (Figure 4). During weeks 1 and 2 the fastest growth was among High-DEHP treated tadpoles ($p < 0.01$)

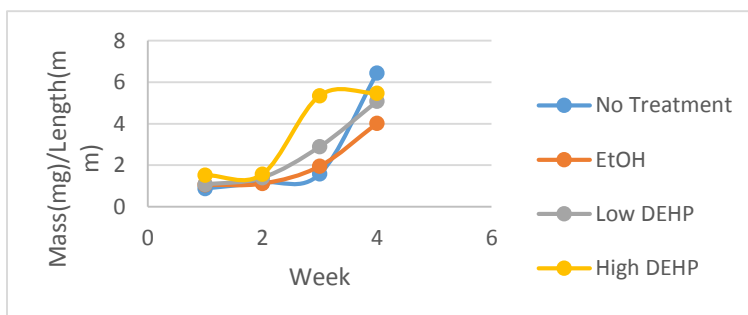


Figure 3. DEHP animals weighed more per body length for weeks 1-3 (arrow). $N=64$; $n=16$ per group.

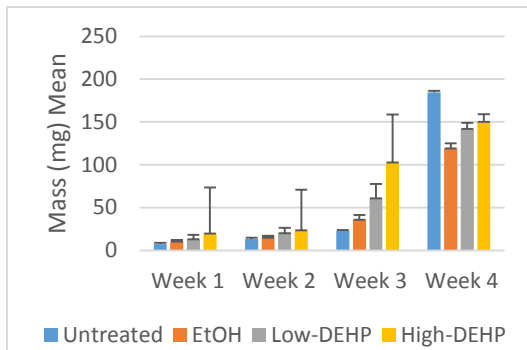


Figure 4. Comparison of Mass (mg) among groups showed growth among all animals over the 4 weeks ($p < 0.001$). $N=64$; $n=4$ per group per week

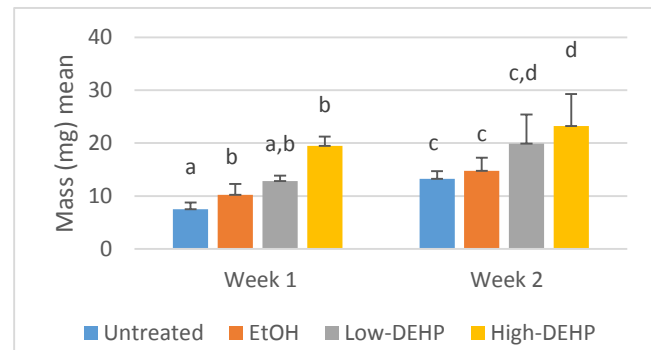


Figure 5. All tadpoles grew from week 1 to 2. ($N=32$; $n=8$ per group) High DEHP grew faster than other groups ($p < .01$)

(Figure 5). All tadpoles continued to developed throughout the 4 weeks ($p < .001$). High DEHP-treated tadpoles developed at a faster rate than did Ethanol-treated tadpoles per week ($p=0.006$), but overall there were no differences between stages (Figure 6). Body length (mm) increased in all groups; treatment did not affect this growth ($p < .001$) (Figure 7).

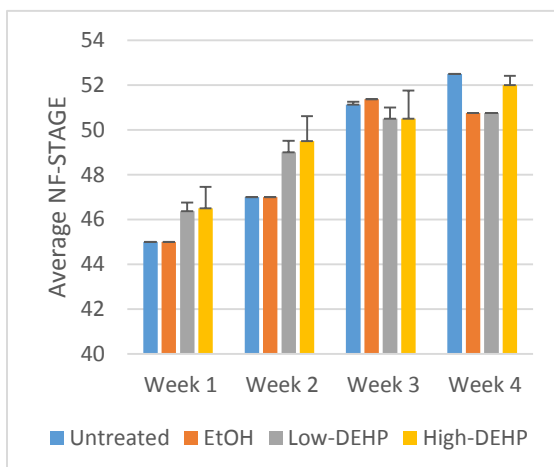


Figure 6. Developmental NF-stage increased with weeks ($p < .001$) starting at NF-Stage 39-40. High DEHP significant faster development than Ethanol tadpoles ($p=0.0056$) but no difference overall

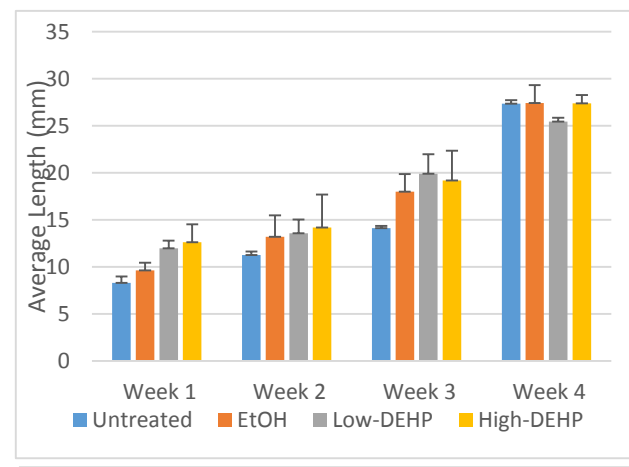


Figure 7. All groups got longer in length over the 4 weeks ($N=64$) ($p < .001$). However, there are essentially no differences in length between groups. ($n=16$ per group)

Further Observations. Tadpole behavior and physical characteristics were observed throughout the experiment. The ethanol-treated animals and the DEHP-treated animals differed in color from the untreated tadpoles. The untreated tadpoles were clear in color, facilitating identification of the development of the heart, peripheral cardiovascular system, and other characteristics used to determine developmental stage. The ethanol and DEHP-treated groups were cloudy and yellowish, hindering identification of cardiovascular development. The tadpole behavior was also observed. Tadpoles in the untreated and ethanol groups roamed more among the middle and surface of the tanks while the DEHP-treated animals stayed near the bottom and the walls of the tanks. When responding to sound, the DEHP-treated animals had faster, spastic movements for a longer period of time than the non-treated and ethanol groups. Early in the study, the DEHP-exposed tadpoles had a higher rate of development than the controls. However, during the third week of observations, developmental characteristics to determine NF-stages were inconsistent. The tail lengths, body shape, and size of the DEHP-treated groups were consistent with a higher developmental staging than the non-treatment tadpole (Figure 8). Based on the hind-limb development and growth, the DEHP-treated tadpoles were less developed than those of the two controls (Figure 9). Another developmental observation between exposure of DEHP and unexposed, was the formation of what looks to be the peritoneal membrane (membrane that surrounds abdominal cavity) extending upwards and surrounding the heart (Figure 10).



Figure 8. Untreated (left) and High DEHP-treated tadpole (right). Comparison of tadpole's size, shape, and development. DEHP-treated tadpoles visually larger than untreated

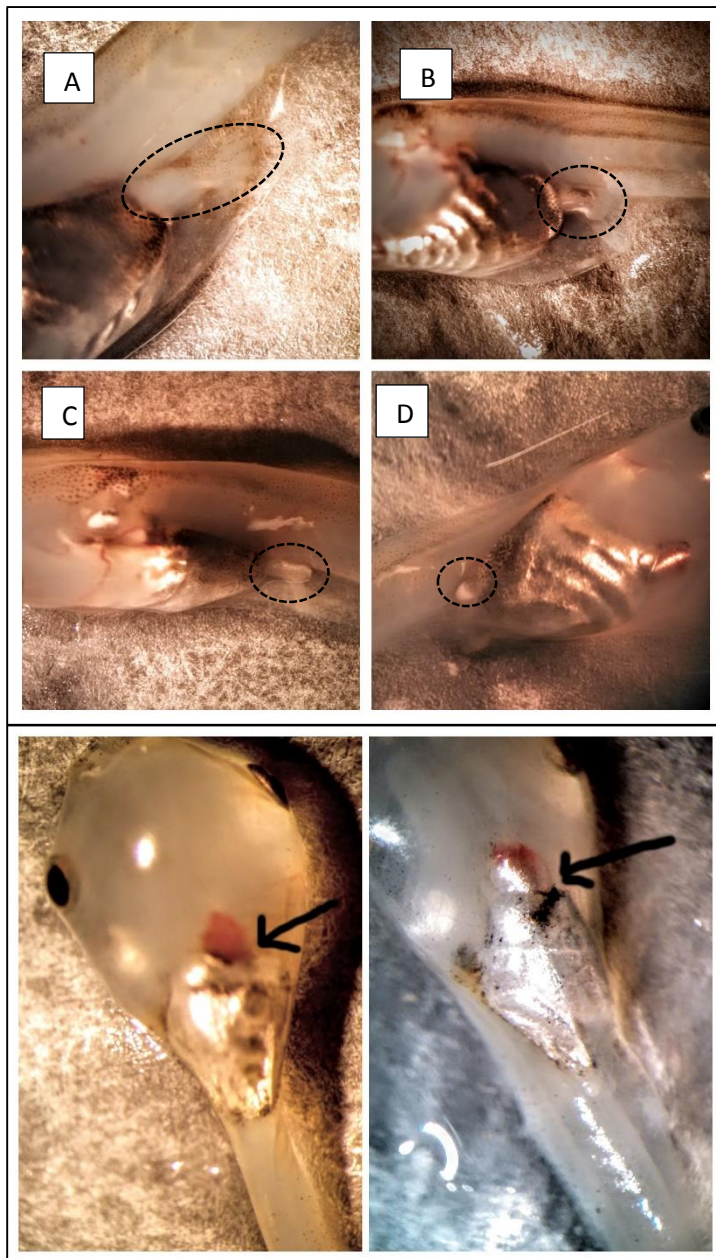


Figure 9. Hind limb development (marked by circles) of DEHP-treated tadpoles (C,D) was reduced compared to controls (A,B).

Week 3 DEHP-treated Stage 50 (D) compared to Week 3 control Stage 53 (B).

Week 4 DEHP-treated Stage 52 (C) compared to Week 4 control Stage 54 (A)

Figure 10. Week 3 NF-stage 50-51 tadpoles Untreated (left) compared to High DEHP (right). The silver peritoneal membrane that surrounds the gut of DEHP tadpole has ascended further upward, encompassing the heart. This was not seen in any of the controls.

Discussion

Exogenous exposure to DEHP had no significant effect on excreted overall cortisol level in *X.laevis*. Tadpoles exposed to high amounts of DEHP had slightly (but not significantly) elevated levels throughout weeks of exposure. This was to be expected since exposure to a toxin

such as a phthalate would create a stressful environment. Physiological mechanisms would increase cortisol release when under stress (Pijanowski et al 2015). DEHP also had no effect on survival, which is consistent with other recent studies (Li et al 2015). The increase in mortality among all groups on day 10-13 of exposure occurred during the time period of a flood in the building that housed the experiment. Heavy maintenance and repair were being done above and around ventilation system. Hence, *X.laevis* tadpole sensitivity to changes in environment and sound likely account for increased mortality at that time.

Levels of cortisol in the urine account for the concentration of unbound (free) cortisol (Gabor et al 2013). An increase in cortisol may not be detected in urine samples. Zebrafish exposed to exogenous cortisol quickly responded with an increase in *hsd11b2* mRNA transcripts within 24 hours (Berdusco et al 2013). This transcript is homologous to the mammalian one from the gene encoding 11 β -hydroxysteroid dehydrogenase type 2 (11 β HSD2) that converts bioactive cortisol into inactive cortisone metabolites. This supports the notion that during an increase of cortisol, homeostasis is maintained through physiological mechanisms and a significant increase would not be found for long term exposure (Berdusco et al 2013). About 90% of cortisol in blood plasma is bound to the transport protein Corticosteroid-Binding Globulin (CBG). These proteins cannot pass through renal filtration, so that only excess free cortisol is excreted. CBG regulates the plasma cortisol levels and bioavailability (Lin et al 2010). CBG is encoded by *SERPINA6* gene which is known to sustain naturally occurring mutations that affect gene expression (Simard et al 2015). This leads to disruptions in CBG binding affinity and binding capacity if such substitutions occur in the steroid-binding site. Amino acid substitution at two locations inhibited steroid-binding completely (Lin et al 2009). Non-functional CBG increases concentration of free cortisol (Lin et al 2010). Since our results show that cortisol levels are

unaffected, we suspect that the interrenals glands and CBG transport proteins are not the target of the phthalate DEHP disruption. However, DEHP could still be a ligand competitor post-hormone production for the glucocorticoid receptor (GC) or other proteins that helps regulate gene expression (Rose et al 2010; Hsieh et al 2012; Zhang et al 2015; Prokkola et al 2016).

We did see differences among treated tadpoles and controls. DEHP exposure lead to an increase in body mass and decrease in limb development. This supports the hypothesis that long-term DEHP exposure could lead to obesity, growth abnormalities, and other health related problems. The cause of the weight gain is still unknown. The largest gain of body mass occurred in the first two weeks post fertilization when exposed to DEHP. An epidemiological study in Iceland showed that faster early childhood weight gain was associated with risk factors for cardiovascular morbidity and mortality (Imai et al 2014). Two DEHP-treated tadpoles presented with yellow growths around the heart, presumably adipose cells (Figure 11). Increase in adipocytes is common among those with metabolic syndrome and liver disease. The disruption in

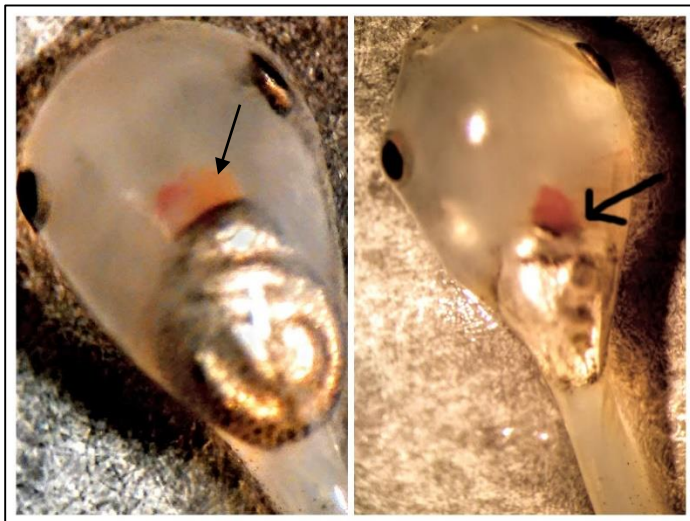


Figure 11. Excess yellow tissue growth (arrow) around DEHP-treated tadpole's heart (left). Possibly accumulation of adipose cells. Untreated tadpole heart (right, arrow) for comparison.

metabolic function in hepatocytes increases plasma triglyceride concentration and lipid accumulation (Woods et al 2015; Masuzaki et al 2001; Klöting et al 2015). All DEHP-treated tadpoles had a yellowish tint to their epidermal membrane, which is seen when bilirubin blood

levels are elevated. Bilirubin is a yellow product of HEME catabolism when the body rids itself of old red blood cells. It is broken down by the liver in order to be expelled from the body (Woods et al 2015). This suggests that the target of DEHP disruption might not be the production of hormones but the regulation of metabolic function. Disruption of liver metabolism, seen in fatty liver disease, results in disruptions of lipolysis, gluconeogenesis, and glycolysis (very similar to disruptions in cortisol production). Long term health issues include heart disease, diabetes, and obesity (Woods et al 2015)

Regulation of metabolic function starts with the regulation of gene expression. Phthalates disrupt gene expression and molecular docking of ligands (Annamalai and Namasivayam 2015; Chen et al 2014). Some hypothesize that this is due to epigenetic mechanisms, such as methylation, where the environment plays a critical role in gene expression and molecular functioning (Xiang et al 2016). Liver cells exposed to phthalate BBP had decreased gene expression for Sir-1 and Sir-3 regulator proteins involved in mitochondrial metabolism (Zhang et al 2015). Down regulation of sir-3 expression is seen in those with obesity, insulin resistance, and lipid abnormalities. DEHP, BBP, and DBP had the strongest affinity for Sir-1 binding sites with up to 5 hydrogen bonds (Zhang et al 2015) (Figure 12). DEHP and other phthalates disrupt spermatogenesis and sex determination and in rats suppression of DNA replication in testicular cells may be the root of the cause (Lee and Veeramachaneni 2005; Chen et al 2014; Li and Fang et al 2014). Epithelium and hind limb growth also originates from gene expression. Inhibition or disruption of regulators in the Hedgehog signaling pathway results in abnormalities and reduced limb growth in *X.laevis* (Stopper et al 2016). DEHP-treated tadpoles seemed to be larger and more developed than the other groups had decreased hind-limb growth compared to controls (Results Figure 10). Our DEHP-treated tadpoles also had excess membrane growth ascending

around the heart (Results Figure 9). Pregnant mice exposed to DEHP had increased cell proliferation and altered cell proliferated-related gene expression in mammary glands (Li et al

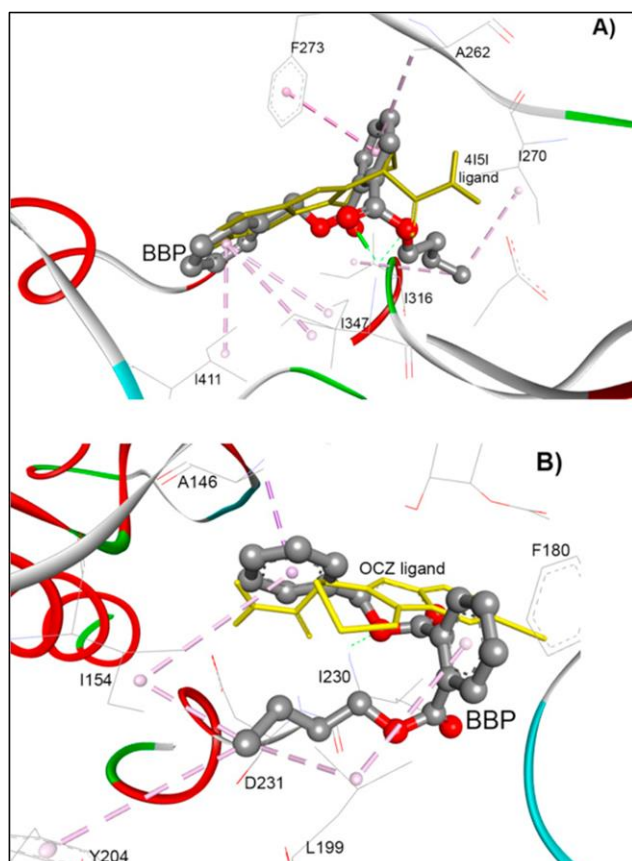


Figure 12. (Fig. 1. From Results of Zhang et al. 2015)

Binding affinity of BBP into Sirt1 and Sirt3. (A) BBP (ball and stick, colored by element) closely aligned with Sirt1 co-crystallized ligand 4I5 (yellow sticks), and exhibited two hydrogen bonds (shown as green dashed lines) between its C=O and COO moieties and NH of I347. It interacts hydrophobically with A262, I270, I316, I347, F273, and I411 amino acids. (B) BBP exhibited one hydrogen bond (green dashed lines) between its PhCH₂O moiety and HN of I230 of Sirt-3; and it interacts via hydrophobic interactions with A146, I154, L199, and Y204.

2015). Phthalates were also found to stimulate excess cell growth and metastasis in human breast cells, along with the increase stimulation of estrogen receptors (Hsieh et al 2012).

After cortisol production, the steroid hormone freely diffuses through the membrane to bind to the glucocorticoid receptor (GR). This receptor binds to DNA to regulate gene expression. The regulation of Na⁺ uptake to changing environmental water pH in zebrafish is through the gene regulation of the cortisol and GR complex (Kumai et al 2012). DEHP as a ligand competitor to cortisol could alter GR binding to DNA and overall gene expression. DEHP disruption of gene regulators of gene expression should be studied further as the target of DEHP. Up-regulation of cell growth can be seen in the excess epidermal membrane growth around the

hearts of DEHP-treated tadpoles, along with possible down-regulation or inhibition of signaling pathways during limb growth and development.

If repeated, the immunoassay plate should be kept on the orbital shaker for at least 120 minutes. The longer the development time the better results we received. However, potential experimental errors that were encountered by immunoassays like anti-reagent antibody interference and cross-reactivity with structurally similar compounds, may be avoided by liquid chromatography coupled to mass spectrometry (LC-MS/MS). This lab technique has shown a high quality of measurement with enhanced analytical specificity and higher sensitivity (Persichilli et al 2010; Monaghan et al 2013; Miller et al 2013).

More research is needed on the physiological effects of DEHP and other environmental chemicals. Phthalates are an increasing potential risk to humans and are being produced in increasing amounts (Li et al 2015). In an analysis of household dust, DEHP was the most abundant phthalic acid ester (phthalate) at 562 mg/kg. The next closest was DBP, at only 170 mg/kg. Household dust also increased gene expression of pro-inflammatory mediators and tumor necrosis factor α (TNF- α) while decreasing growth factor- β (TGF- β) expression in human corneal epithelial cells (Xiang et al 2016). Future studies should focus on metabolic regulations and functions post-steroid hormone production that could affect gene expression. This includes the expression of protein regulators and DNA binding receptors such as glucocorticoid receptor (GC), considering the proper molecular functioning of biochemical properties such as binding affinity and binding site conformations of receptors when exposed to DEHP. This will continue to give us insight on the effects that environmental toxins like phthalates have on the health of organisms.

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