Effects of Di-butyl Phthalate (DBP) on Developing Medaka Embryos

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ABSTRACT

Plasticizers are chemical additives that enhance plastic flexibility. They are ubiquitous environmental contaminants and are commonly found in river and lake waters (Fromme et al 2002). The present study aimed to investigate the effects of a water-soluble plasticizer, dibutyl phthalate (DBP) on developing Medaka (Oryzias latipes) embryos. Three concentrations of DBP (5μg/L, 25μg/L, and 45μg/L) were used to treat groups of 10 eggs, and death, developmental stages, and any morphological abnormalities were observed for 5 consecutive days. Embryos with asymmetrical eyes and missing eyes were observed in the DBP groups. In addition, dose-dependent mortality and developmental delays were also observed. 45μg/L and 25μg/L were lethal by the 5th day. The results indicate that DBP poses an environmental hazard to developing fish.

INTRODUCTION

Plasticizers are chemical additives that enhance the flexibility of plastic products. The most common plasticizers are phthalates, which are used in polyvinyl chloride (PVC) plastic products. Plasticizers are found in nearly all plastic products, including medical devices, toys, tools, wall insulation, and paint (Shin et al. 2002). Plasticizers are reported to be ubiquitous environmental contaminants because they leak out over time into the environment (Marcilla et al, 2003; Singletary et al. 1997). Consequently, plasticizers can be found in waste dumps, river water, and purified drinking water (Liu et al, 2008; Barnabé et al. 2007). Plasticizers have received wide attention because they have been reported to mimic the natural estrogen 17-β-oestradiol and induce developmental irregularities, decreases in sperm counts, and feminization of male characteristics (Ohlson and Hardell, 2000; Wong and Gill, 2002).

In addition, there are direct correlations between occupations with high PVC exposure and occurence of testicular cancer (Ohlson and Hardell, 2000). Many such studies focus on exposing the study animals by feeding diet dosed with plasticizers. However, more and more evidence points to the fact that plasticizers are contaminants often present in water sources. With the wide spread plasticizer contamination, it is clear that studies on the embryonic development of aquatic organisms are necessary in order to gain further insights into the effects of plasticizers. This study aims to investigate the effects of a water-soluble plasticizer, dibutyl phthalate (DBP), on developing embryos of Oryzias latipes.

MATERIALS AND METHODS

Study Organism

Japanese Medaka (Oryzias latipes) embryos were used in this experiment to assay the effects the plasticizer DBP may have on development of fish embryos. Medaka embryos are widely used in such studies because they have a clear chorion that allows easy observation and their fairly long period of development allows observations of many stages during development, even with a large gap between observations. In addition, studies on Medaka embryos have been standardized according to a summary by Iwamatsu (Iwamatsu, 2004), thus description of the developmental stages can be done consistently, and any abnormalities can be described in reference to Iwamatsu's description of normal devel-

opment. Embryos were obtained from Dr. David E. Hinton's laboratory in Duke. The Embryo Rearing Medium (ERM), a water-based multi-salt solution, was made for these embryos according to the recipe obtained also from Dr. Hinton's laboratory.

Experimental Design

Healthy embryos were treated in different solutions all made from ERM. For a negative control, only ERM was used. From literature and preliminary data, it has been shown that ethanol can cause a variety of adverse effects on developing Medaka embryos (Oxendine et al, 2006), so ethanol was used as a positive control to reference possible abnormalities that could be expected. A concentration of 2.5% ethanol was chosen based on preliminary data. Three concentrations of DBP were used. The highest concentration was selected from literature describing the river water concentrations of DBP (Fromme et al, 2002). A lower 25μg/L and 5μg/L dose were chosen. During the first trial of the experiment, 5 glass jars containing 10 eggs each were used in each group. During the second trial, 10 such jars were used for the DBP groups. The eggs were observed for 5 consecutive days, once per day, in order to see any dead embryos, the developmental stages, and any observable morphological abnormalities. In order to avoid repeated disturbances, only one jar was observed each day and solution changes were performed on the other jars.

Source	DF	Sum of Squares	Mean Square	F Ratio	Probability > F
Treatment	4	3202.390	800.597	38.2343	<0.0001
Error	338	7077.465	20.939		
C. Total	342	10279.854			

Figure 1. Analysis of variance

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Methods

Healthy eggs were screened and only eggs from stage 10 ± 1 were used. The eggs were placed into the 20ml glass jars with a glass micropipette. All treatment solutions were made from ERM solution: the 2.5% ethanol by diluting 95% ethanol (Sigma-Aldrich) and the DBP solutions by adding calculated amounts of 99% DBP (Sigma-Aldrich) into ERM. Aliquots of 5ml of each solution were then added to each glass jar, and the jars were sealed and placed into a sealed plastic container for isolation and incubation at around 22 ± 1oC. Embryos from each group were observed under a dissection microscope in a small glass petri dish that facilitated observations. For careful observation, the eggs were rolled around with micropipettes.

JMP 6.0.3 was used for statistical analysis of data collected from the experiment. The average developmental stages from each group were compared using ANOVAs and means comparisons using student's t test and Tukey-Kramer's test with an alpha level of 0.05. The same was done on the observed morphological abnormalities.

RESULTS

For both the first and the second trial, no embryos died in the control group. The mortality rate was highest for the 45μg/L treatment group, followed by the 25μg/L group and then the 5μg/L group (see Figure 1). The 2.5% ethanol group was higher when compared to the control group. The developmental stages reached by all of the DBP groups on the fifth day were significantly lower than the control group ($F_{4,342}=38.23$, $p<0.0001$ from ANOVA; Dunnett's with control, $p<0.05$ for means comparisons). The means comparisons reported significant differences between the control and the DBP groups for the second trial ($p<0.05$). The trend in development seen in both trials was consistent, so data from the two trials were combined for statistical analysis as summarized in Figure 1, Figure 2, and Figure 3 on the previous page.

Figure 4 shows the average devel-

	c	e	5	25	45
c	0.000	2.446	4.355	7.719	11.001
e	-2.466	0.000	1.910	5.273	8.555
5	-4.355	-1.910	0.000	3.363	6.645
25	-7.719	-5.273	-3.363	0.000	3.282
45	-11.001	-8.555	-6.645	-3.282	0.000

Figure 2. Difference matrix; c is the control group

Level	Abs(Dif)-LSD	p-value
c	-1.61	1.0000
e	0.796	0.0011
5	2.715	<0.0001
25	5.631	0.0000
45	8.159	0.0000

Figure 3. Positive values show pairs of means that are significantly different

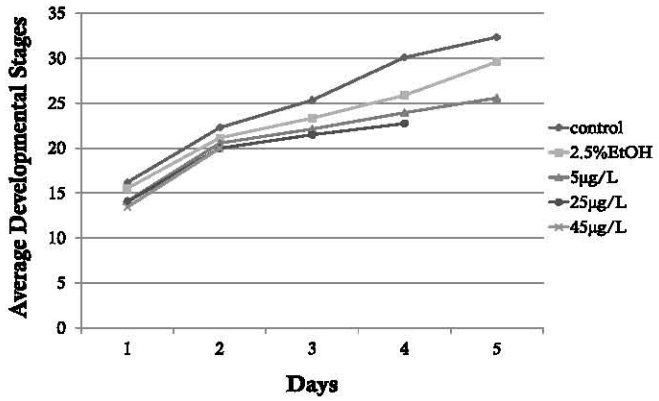

Figure 4. Average developmental stages of embryos during trial 1. All embryos were at stage 11±1 during the initial observation (day 0).

opmental stage of the embryos during trial 1. No data is shown for the fifth observation for the 25µg/L group because all embryos were dead by then. Similarly, no data is shown for the 45µg/L group past the second observation because all embryos had died. No data points were taken if no embryos were alive by that observation (Figure 4). The same graphical technique was used for the second trial. The embryos in the second jar of the 45µg/L group were all dead on the second observation, and such was the case for the third, fourth and fifth jars, so no data value was available after the first observation for the 45µg/L group.

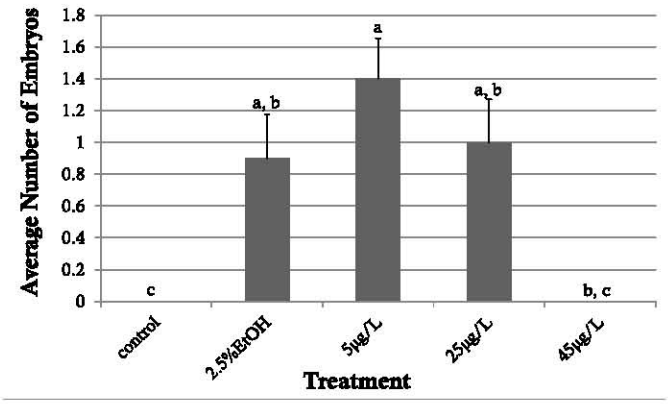

Figure 5. The average number of embryos that showed asymmetrical eyes during development with both trials combined. .

Morphological abnormalities observed were also recorded. The data obtained from the two trials were consistent, so the two sets of data were combined. The average number of eggs with a specific deformity was calculated from 2 jars for the control groups and 3 jars for the DBP groups. In both trials, some embryos with asymmetrical eyes (one eye lower than the other) and some embryos with no visible eyes were observed. As Figure 5 shows, the 2.5% ethanol group had a higher average number of embryos with asymmetrical eyes when compared to the control. The 5µg/L group showed a higher average number when compared to the 2.5% ethanol group in all observations. All observed embryos from the 45µg/L were dead by the second observation, so no data was collected from that point on. The average number of embryos no visible eyes was also calculated and is displayed in Figure 6. In both figures, the bars that are labled with different letters are statistically different according to a Tukey-Kramer test with $p<0.05$. For example, bars labeled "b" are statistically different from those labeled "a", but ones labeled "a,b" are not statistically different from "a" or "b"

Only the DBP groups showed such morphological abnormalities for both trials. The 5µg/L group showed a significant difference in the average number of abnormal embryos

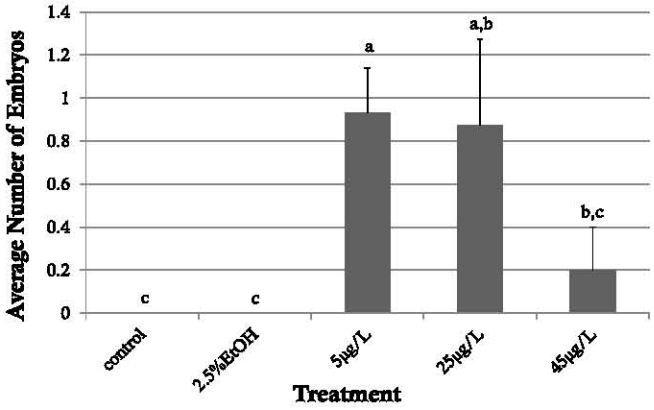

Figure 6. The average number of embryos that had no visible eyes during development per jar with both trials combined. The value from day 3 for the 25µg/L group is lower because the mortality was too high.

when compared to both of the control groups. Embryos in the 45μg/L group were all dead by the third observation, so no data was collected from the 3rd day.

DISCUSSIONS AND CONCLUSIONS

The data suggest that DBP slows the development when compared to the control group. The differences were especially visible during the fourth and fifth days after treatment. The 45μg/L and 25μg/L doses were both lethal; all embryos were dead either by the second day or the fifth day. It was apparent that DBP seems to lead to asymmetrical and missing eyes. Interestingly, the asymmetrical eyes also showed in the 2.5% ethanol group in addition to in the 5μg/L and 25μg/L groups, which suggest that some morphological characteristics or organs maybe especially susceptible to environmental contaminants.

The embryos dosed with DBP showed two types of morphological abnormalities. While some embryos developed asymmetrical eyes, where one eye was placed higher than the other on the head, other embryos had no visible eye structure even though they were able to continue growth. This experiment also showed that doses of 45μg/L and 25μg/L were lethal to the embryos. Although the two trials showed consistent morphological abnormalities in the DBP groups and sometimes also the 2.5% ethanol groups, further studies must be done in order to draw more conclusions. In addition to employing a larger sample size, further studies can be done to link the morphological abnormalities observed to possible genetic mutations or gene regulation changes. DNA taken from the embryos with deformities can be screened for differences compared to the DNA taken from the control groups. In addition, studies employing knock-out strains of Medaka embryos can be used to test if the deformities are from genetic mutations, gene regulation changes or gene expression changes. Such studies would help pin point the possible source of the visible deformities and help us better understand the effect DBP has on developing Medaka embryos.

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