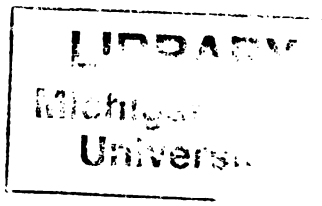

This is to certify that the

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MORPHOLOGICAL EFFECTS OF BISPHENOL A ON THE EARLY LIFE STAGES OF MEDAKA (Oryzias latipes)

presented by

STEPHANIE D. PASTVA

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John P. Giesy

Major professor

John P. Giesy

Date May 2000

MORPHOLOGICAL EFFECTS OF BISPHENOL-A ON THE EARLY LIFE STAGES OF MEDAKA (*ORYZIAS LATIPES*)

By

Stephanie D. Pastva

A THESIS

Submitted to

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ABSTRACT

MORPHOLOGICAL EFFECTS OF BISPHENOL-A ON THE EARLY LIFE STAGES OF MEDAKA (*ORYZIAS LATIPES*)

By

Stephanie D. Pastva

Bisphenol-A (BPA), a widely used polycarbonate plasticizer, has been of concern because it has been shown to leach out of plastics and other epoxy products. Primary sources of environmental releases are expected to be from BPA and epoxy manufacturing facilities. Although environmental concentrations may be limited, little is known about the effects of this compound on fish, particularly during their most sensitive early life stages. A 96-hr, 200μg BPA/L, lethality exposure was conducted with newly hatched larvae, but no differences in mortality between treatments were observed. In addition, medaka embryos were exposed beginning 5-hr post-fertilization, for 9-d at 25°C, to concentrations of 20 μg BPA/L or 200 μg BPA/L (24-h static renewal). Embryos were monitored daily for stage of development and gross abnormalities. Embryos exposed to 200 μg BPA/L did not exhibit abnormalities until after day 4, between days 4-8 the severity index score of embryos was significantly greater than that for embryos exposed to lesser concentrations. By day 9, severity index scores were no longer statistically different among treatments. BPA caused transient embryonic deformities in medaka embryos at environmentally relevant concentrations, but these deformities healed prior to hatching.

DEDICATION

This thesis is dedicated to the Pastva family…

I would like to thank my parents, Dawn and Robert who have always encouraged me to do my best and also to figure things out for myself. They even made me look things up in the dictionary when I asked how to spell a word! I know I didn't appreciate it then, but I'm thankful for the way it made me ask questions and pursue the answers.

I would also like to thank my grandparents, Betty and Elmer Pastva. They have done so many things that have helped me get where I am today that I couldn't possibly mention them all. They have taught me what the word family really means.

ACKNOWLEDGMENTS

A big thanks to Dr. Giesy for taking a chance and taking me on as a grad student- even though I didn't have a toxicology background. The opportunities I have been presented with are enormous. I hope I have met and continue to both meet and exceed his expectations.

During this time, K. Kannan was instrumental in teaching me about the chemistry lab. Some of the lessons were hard and quite frustrating, however, I now have a much better understanding of and appreciation for chemistry.

Although his name is listed last, it is by no means an indication of how I feel about him. Alex Villalobos was there for me the entire time during my M.S. research. Although his job was only as a post-doc, he was much more. He was my mentor, someone that I looked to for help and advice (at work and with personal problems). He taught me all I know about medaka and their development. Alex was (and still is) also a good friend, he's always been there for the good and bad in my life cheering me on and with good advice.

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INTRODUCTION

The embryonic and/or larval periods have consistently been shown to be the most sensitive life stage of a variety of fish species to chemical stressors (McKim, 1985). These early life stages are thought to be especially sensitive due to the numerous critical events that occur in a short time period (McKim, 1985). *In vivo* assays, instead of *in vitro* assays, maintain biological complexity and observations about effects can be directly observed instead of inferred. Thus, partial exposures are relevant for determining the potential effects of chemicals on fish.

The medaka (*Oryzias latipes*) serves as an excellent fish model for early life-stage tests for the following reasons: 1.) adults are small (3-4 cm long); 2.) It is easily cultured in the laboratory; 3.) under proper conditions females produce eggs daily (Hyodo-Taguchi and Egami, 1989), and 4.) adults do not need be killed to obtain eggs. In addition, the chorion, is clear allowing observation, without damage, of the developing embryo under a light microscope. Finally, the embryological and larval stages of medaka development have already been well-documented (Iwamatsu, 1994). Observing the developing embryo provides an abundance of information including survival, growth, and developmental abnormalities.

Bisphenol-A (4,4' isopropylidenediphenol or 2,2'-Bis(p-hydroxyphenyl propane)), BPA, is an industrially important compound, widely used as a monomer in the production of polycarbonates, epoxy resins, and coatings. BPA

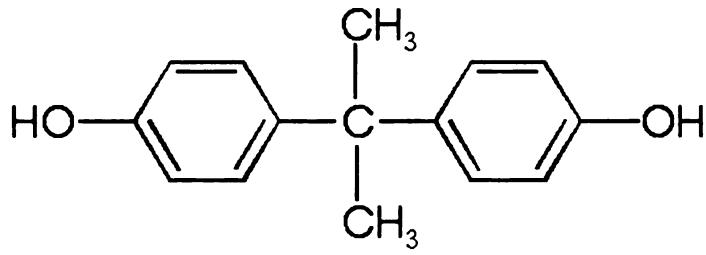
is very soluble in water and has a relatively short environmental half-life (Table 1). In 1993, 109 tons of BPA were estimated to have been released into the air, surface water, or wastewater treatment plants from the United States alone (Staples, et al., 1998). BPA has been described as "slightly to moderately toxic" to select aquatic organisms (Staples, et al., 1998). BPA has also been confirmed to be an endocrine disrupter by interacting with the estrogen receptor (Brotons, et al., 1995, Krishnan, et al., 1993, Perez, et al., 1998, Steinmetz, et al., 1997). It is for this reason that BPA has attracted so much attention.

BPA occurs at relatively small concentrations in streams and rivers. In the 1970's, BPA from various rivers in the Tokyo area were reported to range from 0.06 µg/L - 1.9 µg/L (Matsumoto, 1982). According to a 1996 study, in five U.S. receiving streams of facilities that manufacture or use BPA in the manufacturing process, BPA was < 1.0 µg/L (Markham, et al., 1998). In 1997, BPA concentrations in these same 5 streams ranged from < 1.0 µg/L - 8 µg/L (Staples, et al., 2000).

Previous studies have examined acute toxicity of BPA to adults of various fish (Alexander, et al., 1988, Staples, et al., 1998). Although BPA is classified as slightly to moderately toxic (Staples, et al., 1998), information about the sublethal effects of BPA to fish, especially during their early life stages is lacking. The overall objective of this research was to determine if waterborne BPA concentrations affected medaka early life stages. To accomplish this objective, medaka embryos were exposed to BPA to quantify morphological effects on embryological stages and acute lethality to newly hatched larvae. This study

only looked at embryological developmental deformities as an endpoint. Further research should be designed to determine if BPA at environmentally relevant concentrations causes abnormalities related to the endocrine system in fish species.

Table 1. Physical and chemical properties of bisphenol-A (Staples, et al., 1998).



CAS No.	80-05-7
Formula	$C_{15}H_{16}O_2$
Molecular Weight	228 g/mol
pKa	9.6 - 11.3
Water Solubility	120 mg/l - 300 mg/l
Log Kow	2.2 - 3.82
Half-life	2.5 - 4 days

METHODOLOGY

Culture of Broodstock and Egg Collection:

Adults were housed in glass aquaria at a constant temperature of 25°C (± 2°C), with a photoperiod of 16:8 (light:dark) hours. Adults were fed brine shrimp and 1:1 TetraMin® (Tetra; Blacksburg, VA) flake food:ground trout chow pellets were fed *ad lib* daily.

Eggs were collected less than 5-hr after fertilization, from individually netted females. Clusters of eggs were carefully removed from the female's abdomen using fingertips. Filaments attaching adjacent eggs together were removed by gently rolling the clusters between moistened fingertips, and using a sharp pair of forceps to pull filaments from the egg. The cleaned eggs were then observed using a dissecting microscope. Eggs that were greater than stage 10 (Iwamatsu, 1994), unfertilized, or abnormal in appearance were discarded. Eggs were then disinfected by placing them in a 0.9% solution of hydrogen peroxide for 10 min, after which eggs were placed in embryo rearing medium (ERM) until initiation of exposure. ERM (Table 2) is osmotically balanced with the medaka embryo.

Embryo Exposure:

Embryos were exposed in 10 ml scintillation vials (Research Products International; Mount Prospect, IL). Vials were washed beforehand using Luqui-nox detergent (VWR Scientific Products; South Plainfield, NJ) followed by

Table 2. Salts needed to make Embryo Rearing Medium

Salt	Amount needed (grams/liter)
NaCl	1.0
KCl	0.03
$CaCl_2 2H_20$ -OR-	0.04 -OR-
$CaCl_2$ anhydrous	0.03
$MgSO_4$	0.08
aerate and adjust pH (7+/- 0.2) with	
0.14M $NaHCO_3$ (0.25g/20ml H_2O)	

acetone (Burdick and Jackson; Muskegon, MI) and finally hexane (Burdick and Jackson). Powder BPA was obtained from Sigma Chemical Company (St. Louis, MO). Dilutions were made from a 20 mg/L BPA standard dissolved in nanopure water, no organic carrier solvent was used. ERM was used for the controls and to make the 200 μg/L and 20 μg/L dilutions.

Individual eggs were randomly placed in vials until there were 5 embryos in 5 ml of test solution. There were 5 replicates per concentration tested, for a total N=25 embryos per concentration. Individual vials were labeled and capped with a double layer of teflon tape. The vials were placed in a water bath, and maintained at 25° C. The exposure was a 24-hr static renewal. Due to the short half-life of BPA, a new 20 mg BPA/L standard was made daily, diluted, and replaced in the vials.

Severity Index:

Observations of individual embryos were made daily. A published atlas of normal medaka development (Iwamatsu, 1994) was used to determine if embryos were developing normally. Recorded abnormalities were given a daily severity index (SI) score as described in Villalobos *et al* (Villalobos, et al., 2000). Briefly, abnormalities in individual embryos were recorded each day and a severity index score was calculated (Equation 1) on a scale ranging from 0 = no abnormalities observed, to a maximum of 11.5 = death (Table 3). Those unfamiliar with embryological lesions are encouraged to read Sharp (1990) as it provides pictures and definitions of embryological lesions. However, the results section of this paper provides pictures of cardiovascular lesions (Figures 1 and

2). When more than one lesion was observed in an embryo, SI scores for each lesion were summed. Observations were recorded for a period of 9-d, at which time surviving embryos were euthanized with MS-222 (Sigma Chemical Co).

Equation 1. Severity Index computation, from Villalobos *et al* (2000)

$$SI = \left[\sum_{i=1}^{n} (CR \times E_i) + \sum_{j=1}^{n} (CV \times E_j) + \sum_{k=1}^{n} (SK \times E_k) + \sum_{l=1}^{n} (OA \times E_l) \right] \div \text{embryos per dose}$$

Where E_i= no. embryos with craniofacial (CR) value, E_j= no. embryos with cardiovascular (CV) value, E_k= no. embryos with myoskeletal (SK) value, and E_l= no. embryos with other anomaly (OA) value.

Table 3. Scoring guide for the assessment of a Severity Index according to gross abnormalities observed in the medaka early life (embryonic and/or larval) stage assay (Villalobos *et al.*, 2000).

Cranio-facial value - CR	Observed effect	Cardio-vascular value - CV	Observed effect	Myo-skeletal value - SK	Observed effect	Other anomaly value-OA	Observed effect
0	No observable lesion	0	No observable lesion	0	No observable lesion	0	No observable lesion
1	Minor, transient defect in structure or size (i.e. less expansion of brain, less head width, less eye pigmentation)	1	Bradycardia, Arrhythmia, Enlarged heart, Mild pericardial edema	1	Minor spinal curvature, Minor, delayed development (smaller body width /size at early stages)	1	Minor, transient yolk sac edema (yse) Changes in gall bladder coloration
2	Moderate anomaly in structure or size, not compromising survival (i.e. deformed jaw) Synophthalmia, Exophthalmia	2	Moderate edema (i.e. pericardial, peritoneal), sometimes reversible Hemostasis, Some hemorrhaging	2	Marked (one or multiple) spinal curvatures (i.e. lordosis, kyphosis, scoliosis)	2	Delayed and/or prolonged hatch (i.e. > 24 h), Abnormal (i.e. head first) hatch Moderate yse
3	Severe anomaly in structure or size, irreversible and often compromising survival (microphthalmia, microcephalia)	3	Severe edema, in most cases irreversible Subepidermal (include eye) edema, Extended hemorrhaging	3	Growth reduction within proportion of body size or length (stunting), that may be compensated at a later time	3	Diminished yolk resorption, No swim bladder inflation Larval weakness
4	Anencephaly Anophthalmia Cyclopia	4	Tubular heart (extreme edema), Acardia Brain hemorrhaging	4	Complete stunted development (no proportion in body size/length) Body opacity	4	Larval failure to thrive (loss of equilibrium, lack of mobility, permanent recumbency)
						11.5	Mortality (embryo / larval)

96-hour larval mortality experiment

The major differences between the larval exposure and the embryo exposure were the use of a solvent to make stock solutions, duration of exposure, and the only endpoint assessed was mortality. 250-ml I-Chem jars (Nalge Nunc International Corp; Rochester, NY) were used to hold the newly hatched (<24-hr) medaka larvae for this static daily renewal test. 1 ml of ethanol was used to rapidly dissolve the BPA into 1 liter of ERM. The stock solution was used to make the dilution to 200 µg/L, resulting in medaka being exposed to only 0.1% ethanol. A control, solvent control, and 200 µg/L BPA were tested. There were 3 replicates of each test solution. Each jar contained 150 ml of test solution and ten larvae, for a total of 30 individuals tested per solution. This was a 24-hr static renewal exposure, with new stock solution and dilutions made daily. Jars were kept in a 25°C water bath. Larvae were also checked daily for mortalities and were fed *ad lib* with TetraMin baby food.

Statistical Analysis

Differences in SI values among treatments were examined in SAS (SAS, 1996) by the repeated measures function (proc mixed) using an autoregressive correlation pattern, with each vial serving as the replicate. The rationale for this statistical analysis was that: the same endpoint was being measured daily (severity of deformity) and daily measurements were not independent of one another (i.e. if an embryo exhibited hemostasis on day 6, on day 7 the embryo would probably still exhibit hemostasis). Effects of treatments on the number of

embryos expressing deformities at day 9 follows the binomial distribution which was used to determine if there were differences in deformity rates between controls and the greatest concentration (200 µg/L). Day 9 was chosen for the analysis to provide the most biologically meaningful differences. Differences in larval mortality rates per replicate between after 96-hr of exposure data of the larvae was tested for statistical differences in mortality with a t-test (SAS, 1996).

RESULTS

Embryo Exposure

No deformities were observed until after day 3 (Fig. 3). Statistical analysis revealed that on days 5-8 the SI of embryos exposed to 200 µg/L BPA were significantly greater than controls ($p < 0.05$). However, by day 9, embryos exposed to 200 µg/L BPA were no longer significantly different from those exposed to 0 µg/L BPA. There were no significant differences in SI between embryos exposed to BPA over all 9 days ($p = 0.13$). A regression of SI, on day 9, as a function of BPA concentration, was not significant ($r^2 = 0.04$, $p > 0.05$).

Most embryos exhibited no deformities (Table 4). Most deformities observed were cardiovascular in nature (Figures 1 and 2). Although by day 9 embryos exposed to 200 µg/L BPA had a 24% rate of deformity (Table 4), there was not a significant difference between this group and the controls ($p = 0.0994$).

Larval exposure

Only one larva died during the 4-d exposure period. This individual was from the solvent control group. There were no differences in larval mortality among treatments ($p > 0.05$). Long-term effects were not assessed since larvae were not grown out to adulthood.

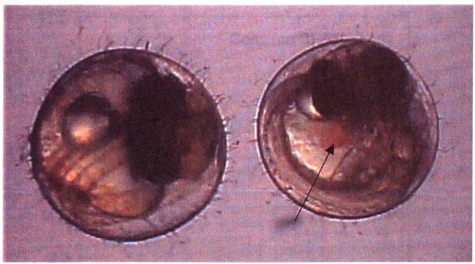


Figure 1. Hemorrhage

Embryo on the right was exposed to 200 µg BPA/L and has a large hemorrhage (arrow) at the left duct of Cuvier region. The hemorrhage appears as a large diffuse red coloring below the eye. The embryo on the left is a normally developing control individual of the same age.

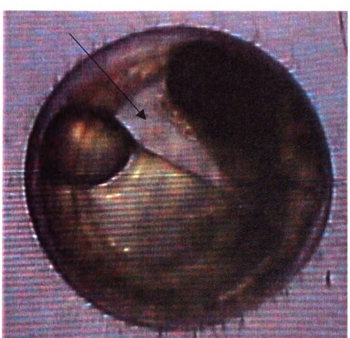


Figure 2. Pericardial Edema

This embryo was exposed to 200 µg BPA/L and has a severe pericardial edema (arrow). A pericardial edema is a sac of fluid surrounding the heart region. It appears as a clear wedge between the head and yolk sac.

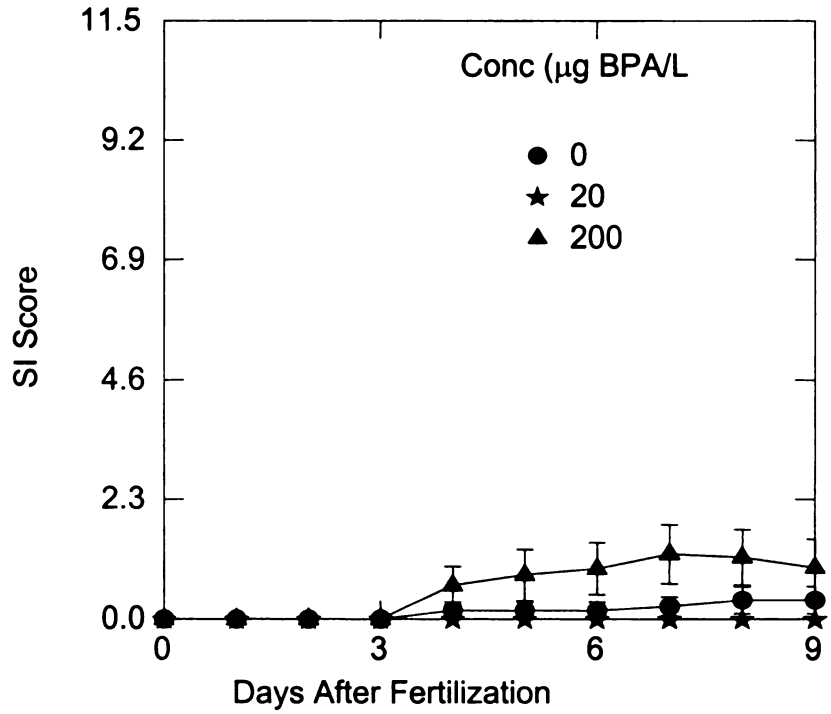


Figure 3. Average Severity Index scores with error bars over time.

Table 4. Number of embryos with deformities for each concentration tested. Sample size is 25 individuals per concentration.

Concentration (µg/L)	# deformed	% deformed
0	2	8
20	0	0
200	6	24

DISCUSSION

Larval exposure

Survival of newly hatched medaka larvae was not affected by exposure to 200 µg/L of BPA after 96-hr. This result is consistent with previous acute toxicity testing with freshwater fish, which reported LC_{50}s in the mg/L range (Table 5). Environmentally relevant concentrations of BPA do not appear to be acutely toxic to medaka or fathead minnows (*Pimephelas promelas*) (Table 5).

Advantages and disadvantages of scoring system

Computing a severity index is a very time intensive process. It involves daily monitoring of each embryo. In addition, the scorer must be experienced at determining abnormalities and then assigning them to a mild, moderate, or severe category. Depending on the chemical and dose tested, there may be numerous zeros (embryos with no abnormalities) at the end of the exposure. If this occurs, the data will not be normally distributed. However, non-parametric versions of the repeated measures test (based on rank scores) produces similar results. Even though the repeated measures procedure has some difficulties, there are several advantages to using the daily severity index. Using this index allows for quantification of differences between embryos and treatments instead of just anecdotal observations. In addition, changes over time (when toxicity begins to occur and potential healing) can be determined. However, if this amount of discrimination is not needed, then assessing the embryos on the final day of exposure would be more efficient and easier to analyze statistically. In

addition, the severity index data on day 9 yielded very similar p-values to the binomial analysis of the day 9 data.

Table 5. Comparison of results of freshwater fish species exposed to BPA.

Fish species	Test type	Endpoint	Results (µg/L)	Reference
Medaka	48-h LC_{50}	Mortality	15,000	(Staples *et al.*, 1998)
	Larval 96-hr LC_{50}	Mortality	> 200	This paper
	embryo life stage	Abnormal development	> 200	This paper
Fathead minnow	96-hr LC_{50} static	Mortality	4,700	(Alexander *et al.*, 1988)
	96-hr LC_{50} flow through	Mortality	4,600	(Alexander *et al.*, 1988)

Embryo exposure

Embryos exposed to 200 µg/L BPA did not exhibit abnormalities until after day 4, and between days 4-8, the severity index score of embryos was significantly greater than for lesser concentrations (Figure 3). The lower 200 µg/L SI scores on day 9 were a result of individual embryos healing. There was a lessening in the severity of observed hemorrhages, the resulting lower SI scores were responsible for a non-significant value on day 9. Results of other exposure studies, in which medaka embryos have been exposed to various toxicants, indicate stage-specificity of abnormalities. Medaka embryos exposed to TCDD (Wisk and Cooper, 1990) and a PCN mixture (Halowax 1014) (Villalobos, et al., 2000) did not exhibit lesions until day 4 or 5. It is interesting to note that day 4 coincides with formation of the liver, and consequently it has been hypothesized that the mechanism of action of resulting deformities is metabolism by cytochrome P-450 enzymes.

Since all organs are developed by day 9, the medaka embryo may be metabolizing and/or excreting BPA more quickly than it is being exposed. Although the pathways of BPA metabolism by bacteria have been well documented (Lobos, et al., 1992, Spivack, et al., 1994), little has been done to investigate how BPA is metabolized in fish species. This study did not attempt to determine if the embryos were metabolizing the BPA or which enzyme system may be responsible for the metabolism of BPA. Studies that investigate possible metabolism may give additional insight into the mechanism of action for the embryological deformities that were observed.

Since the route of exposure was waterborne instead of injection, it can be argued that the chorion protected the developing embryo by decreasing exposure. However, the chorion is semi-permeable. Previous work with TCDD indicated that medaka egg waterborne exposure and egg nanoinjection yielded similar LC/LD_{50}s (Wright, et al., 1997). Recently, zebrafish (*Danio rerio*) embryos were used to study the uptake and clearance of C^{14} labeled atrazine. They found that in a waterborne exposure protocol, atrazine penetrated the chorion quickly followed by a slowing to steady state (Wiegand, et al., 2000). Within 3-hr after the medium was changed to atrazine-free water, 75% of the absorbed atrazine was released from the embryo to the medium (Wiegand, et al., 2000). As atrazine has a similar Log K_{ow} (2.75) and water solubility (30 mg/L) as BPA (Wiegand, et al., 2000), waterborne exposures to hydrophilic compounds (such as BPA) may be more meaningful than a nanoinjection protocol.

This study indicates that BPA does result in embryonic deformities at environmental concentrations, but that the medaka embryo is capable of healing these deformities prior to hatching. Furthermore, environmentally relevant concentrations of BPA do not cause acute lethality to medaka larvae. Therefore, fishes in the wild, with similar or lesser sensitivity to BPA, may be affected similarly by BPA.

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