Running head: DEVELOPMENTAL CHANGES IN ZEBRAFISH

Developmental changes in zebrafish embryos exposed to bisphenol A leeched from commonly used plastics

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Acceptance of Senior Honors Thesis

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Abstract

Bisphenol A, an estrogen mimicker, is found in the commonly used plastic, polycarbonate. Zebrafish embryos were raised in water that had been heated in polycarbonate water bottles by either microwaving for 5 minutes, or boiling for one hour. Near total mortality was seen in 7 days for the embryos growing in bottle-microwaved water, and 9 days for those raised in bottle-boiled water. Zebrafish embryos were also grown in the following known concentrations of BPA: 2mg/l, 14mg/l, 16mg/l, 50mg/l, 135mg/l, and 235mg/l. A stepwise relationship between concentration of BPA and embryo lifespan was seen, and the lowest observed adverse effect level was at 14mg/l.

Background

Bisphenol A – A description

Bisphenol A (BPA) is a benzene derived monomer commonly used in the production of polycarbonate plastics, popular due to their high strength and clarity. These plastics, identified with the Plastic Identification Code "07" (Figure 1A), may be found in a wide range of consumer products including Macbooks, Tupperware, pacifiers, disposable and reusable water bottles, eyeglass lenses, compact discs, DVDs, baby bottles, piping, and thousands of other products. Due to rising demand of polycarbonate products, bisphenol A production has increased significantly in the last few years, reaching a global production of 6.4 billion pounds in 2003 (Burridge, 2003). As one of the most widely produced chemicals of the 21st century, bisphenol A has gathered a growing level of attention from the media, policy makers and affected corporations. To provide a balanced perspective on this controversial issue, a review of the research from BPA's discovery up to the present follows with an attempt to incorporate every significant source in the history of this infamous molecule.

Early research on BPA

Bisphenol A was discovered in 1891 when a Russian chemist named A.P. Dianin mixed two parts phenol with one part acetone (the A of BPA) in the presence of acid (Dianin, 1891). Almost no further research on BPA was documented until E.C. Dodds and W. Lawson stumbled upon BPA's estrogenic activity while conducting a study of estrogenic structures in the late 1930s and early 1940s. Dodds and Lawson compared estrogenic activity in rats of various organic compounds, by subcutaneous injection. The researchers found that chemicals with the greatest estrogenic activity followed the

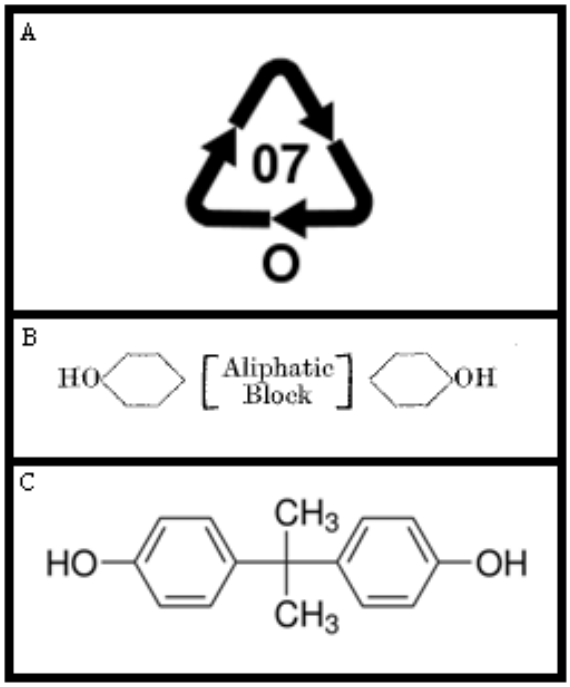

Figure 1 (A) This code (or the variant "PC") appears on polycarbonate plastics. (B) This is the general structure for estrogen-mimicking molecules depicted by Dodds et al., (1944). (C) The structure of BPA bears a striking resemblance to the estrogenic conformation described in Figure 2.

pattern depicted in Figure 1B, of two phenolic rings with a slightly branched aliphatic section dividing them (Dodds et al., 1944). This structure closely resembles BPA's structure (Figure 1C), and so it should come as no surprise that Dodds and Lawson found estrogenic activity in BPA (Dodds & Lawson, 1936).

At the time, the link between BPA and estrogen activity went unnoticed, and scientific literature remained largely silent on the *in vivo* effects of BPA for the next 60 years. In the meantime, research on industrial applications of BPA expanded: from a 1915 patent on an improved production method of BPA (Beatty, 1915), to its involvement in the development of polycarbonate plastics and epoxy resins in the 1950s. The secret was out: polycarbonate's thermal strength, physical rigidity, impact resistance, and high transparency formed the ideal polymer for hundreds of applications, and over the next 50 years BPA worked its way deep into American lives. Perhaps it was this unchecked expansion in the use of polycarbonate that eventually inspired a renewed interest in its *in vivo* effects.

Bisphenol A – Research in the Polycarbonate Era.

Only occasionally throughout the 1960s and 1970s did BPA research leave the realm of industrial application to concern health safety issues, and these few exceptions were mostly allergy-based (Gaul, 1960; Thorgeirsson & Fregert, 1977). It was not until the 1980s that interest in potential health effects of BPA arose, first mentioned briefly by M. Manson in an epoxide study as possibly toxic (1980). Then in 1987, Morrissey et al. conducted high dose BPA toxicity studies in mice and rats by exposing pregnant mothers to increasing levels of BPA. High BPA exposure increased adult mortality and fetal resorptions, and reduced weight gain in both maternal and fetal mice. This study

concretely demonstrated a toxic effect of BPA, but again went largely unnoticed, possibly due to the tremendously high BPA concentrations under which the studies were conducted. During this time, the Environmental Protection Agency (EPA) set the BPA exposure limit at 50µg BPA / kg body weight / day, based on the previously described paper by Morrissey et al., and a 1982 study (National Toxicology Program, 1982) examining the effects of BPA fed to lab rats. This number was based on the lowest observable adverse effect level (LOAEL), and then divided by a factor of 1,000 to account for uncertainty due to some extreme human sensitivities (10), uncertainty in the relation between animal effects and human effects (10), and uncertainty in relation between duration of exposure and toxicity (10) (Environmental Protection Agency, 1993).

Over the next decade research into BPA effects were again largely ignored, though some investigation into its concentration in waterways was conducted (Dorn, Chou, & Gentempo, 1987). Then, in 1997, a study came out revealing low-dose exposure effects in rats (vom Saal et al., 1997). Based on the theory that low doses of biologically active substances can have completely different effects from high doses, researchers found that concentrations as low as 20µg/kg/day increased adult prostrate weight, while concentrations of 200mg/kg/day or above shrunk the prostrate. This study's LOEAL was lower than the previous LOEAL by a factor of 2,500, and fell underneath the bounds of the EPA's daily human exposure limit, meaning that they found adverse effects at a level below the EPA's already very conservative limit. These findings prompted a storm of original research examining low-dose effects of BPA *in vitro* and *in vivo*.

Current trends – Research from 1997 to the present

Between 1997 and 2007 over 100 studies on the *in vivo* biological effects, metabolic rates, and resultant developmental defects were conducted in mice, rats, sheep, and fish. Of these studies, 51 linked BPA to changes in brain structure and physiology, or behavioral changes. In that same time period, 22 reports showed an alteration in male reproductive organs and/or lower sperm count; 17 studies described changes in female reproductive organs, along with an earlier/faster puberty; 8 cases indicated a change in metabolism, and 7 observed an altered immune system (Richter et al., 2007).

Accompanying this expansion in the knowledge of biological effects of BPA was a growing look at normal and worst-case human exposure levels, as well as sources of exposure. Research indicates that BPA may leech from consumer polycarbonate plastics during everyday use. Furthermore, the volume of BPA leeching has been found to increase with use, to a maximum leeching attained around 50 consecutive days of use (assuming normal washing) (Bredey, 2003). In 2003, it was found that 93% of children and adults had Bisphenol A in their urine (Calafat,Ye, Wong, Reidy, & Needham, 2003). Knowledge like this has pushed BPA through 100s of studies in the last ten years, examining its biological effects.

While estrogenicity had been first noted under high-dose BPA exposures in lab rats in the 1930s, it has only recently gained attention in the media, due to a recent explosion in the already extensive consumer use of BPA, and the aforementioned low-dose studies linking BPA to developmental defects. The effects of growing public concern can be seen in the retail industry. Two years ago, BPA could be found in most pacifiers and baby bottles. Today due to market pressures, many retailers (most notably,

Wal-Mart) have pulled BPA-containing products from their shelves. These actions have not yet reduced average individual BPA exposure to below what many researchers would consider safe. Recent studies have proposed that the current FDA exposure maximum of 50µg/kg/day is not low enough for the general population and (because BPA is most closely linked to developmental defects) for infants in particular (Davis et al., 2008).

Zebrafish

The zebrafish (*Danio rerio*) is a small fish about 6cm in length, characterized by a series of five pigmented stripes running the entire length of each side of its body. Each female lays between 45 and 200 eggs per day, depending on conditions. Once fertilized, the zebrafish embryo rapidly develops, within a protective chorion, from a single cell situated atop a large ball of yolk, to the thousand cell blastoderm only four hours later. By the end of 24 hours, organogenesis has begun, the major organs have visibly formed, and the embryo has begun to adopt a more familiar shape. At about 48 hours post fertilization, the embryo hatches, breaking free of the chorion, and begins swimming and eating. Within 90 days, the hatchling will have reached adulthood and will be ready to lay its own eggs. One of the most remarkable aspects of this process is that the embryo and surrounding chorion remain transparent for the first several days of development, allowing the entire developmental process to be observed from fertilization to the first several days after hatching. Over the last ten years, these characteristics have made zebrafish a popular vertebrate model for a growing list of applications in developmental research. Their short life-cycle and size makes them an ideal target for genetic screening. (Wolpert et al., 2007) The zebrafish's hardiness makes them excellent stress test subjects, as they can survive fairly severe environmental changes without succumbing, surviving

long enough to show developmental defects. Finally, zebrafish are easy and inexpensive to raise, requiring only filtered water, and a minimal investment in fish food, making them an ideal animal model for research labs with limited funding. All of these characteristics have contributed to making zebrafish the model of choice in this study.

Method

Maintenance of zebrafish

Zebrafish were obtained from Petsmart (Lynchburg, Virginia) and raised in a computer controlled incubation chamber. Ideal breeding conditions were maintained to ensure a maximum yield. The zebrafish received fourteen hours of daylight and ten hours of darkness every night. The temperature and humidity were kept at 28.5°C and 61%, respectively. A triple filtration system consisting of two physical filters and a UV radiation lamp was put into place to guarantee a clean environment for the fish. Two 80-gallon tanks were stocked with fifty fish each. Marbles on the tank floor ensured that the adults did not scavenge their offspring. The embryos were harvested daily from the tanks, about forty five minutes after the morning feeding, while the eggs were still in the 2-16 cell stages. Collection was done by siphon with a 3 foot piece of flexible tubing.

Preparation of water bottles

Two polycarbonate-containing Eddie Bauer brand sports water bottles labeled "FDA compliant," "Dishwasher safe," and "microwave safe" were purchased from Wal-Mart. Each of these bottles was then subjected to conditions to simulate 45 days of use, as described by Bredey, Fjeldalz, Skjevraky, & Herikstady, (2003). The bottles were first subjected to 10 minutes of intense scrubbing, with wire brushes and hot water and soap. Scrubbing was followed by an hour of boiling, in which each bottle was filled with water,

and then placed in a beaker of water heated on a hotplate. Temperature remained around 98° throughout the boiling. Upon completion, the bottles were subjected to seven additional rounds of scrubbing-boiling.

Microwave tests

Water from the zebrafish tanks was used to fill a sports bottle prepared in the manner described above. The nearly-full bottle was microwaved on HIGH for five minutes and then allowed to cool for two minutes. Water from this bottle was then poured into a Petri dish (volume: 40ml) and allowed to cool to room temperature. Another Petri dish was filled with tank water, microwaved for 30 seconds on high, and cooled to room temperature. A final dish was filled with tank water to serve as a control. Embryos were added to each dish and placed into the incubation chamber.

Boiled water bottle tests

The second water bottle prepared above was filled with zebrafish tank water. It was then boiled for an hour on a hotplate, inside of a beaker, in the same way as described above. Upon completion of boiling, water from this bottle was poured into a Petri dish and allowed to cool to room temperature. Finally, fish embryos were added to this dish, and to a control, and both were placed in the incubation chamber.

Tests with known concentrations of BPA

97% pure BPA was purchased from Sigma-Aldrich and added to 1% dimethyl sulfoxide (DMSO) to produce the following known concentrations of BPA: 235 mg/l, 125 mg/l, 50 mg/l, 16 mg/l, 14 mg/l, and 2 mg/l. Zebrafish embryos, harvested within 2 hours post fertilization, were added to Petri dishes containing each of these concentrations of BPA. A control population was also raised in 1% DMSO.

Zebrafish embryo maintenance and observations

Zebrafish embryos were raised in sterile, 40ml Petri dishes. Their water was changed once every 3-4 days, and the embryos were fed every other day, post hatching. Embryos were tracked for approximately ten days, or until all members of the study population were deceased. Observations were made using a *Leica EC4* dissection microscope with digital imaging capabilities. Photographs of representative and unique embryos were taken using the microscope in conjunction with the Leica LAS-EZ digital imaging program.

Results

Results of microwave tests

Zebrafish growing in water that had been microwaved in BPA-containing water bottles exhibited a 7% survival rate after 10 days, while the control sample possessed a 60% survival rate in the same timeframe (Figure 2). After the first 7 days, no additional mortality was observed, and the study was considered complete. Embryos raised in microwaved Petri-dish water did not exhibit any differentiation from the control, unless microwaving was done long enough to visibly denature the shape of the plastic itself.

Results of boiled water bottle tests

Zebrafish raised in water that had been previously boiled in a BPA-containing water bottle experienced a steady drop in number. This study was run parallel to a control and to a group of embryos raised in the microwaved water described above. Total mortality of the boiled water embryos was reached within 10 days (Figure 3). Mortality of the microwaved water embryos was seen within 7 days. A 40% decrease in the control population was observed; however, this had almost completely leveled off by the 6^{th} day.

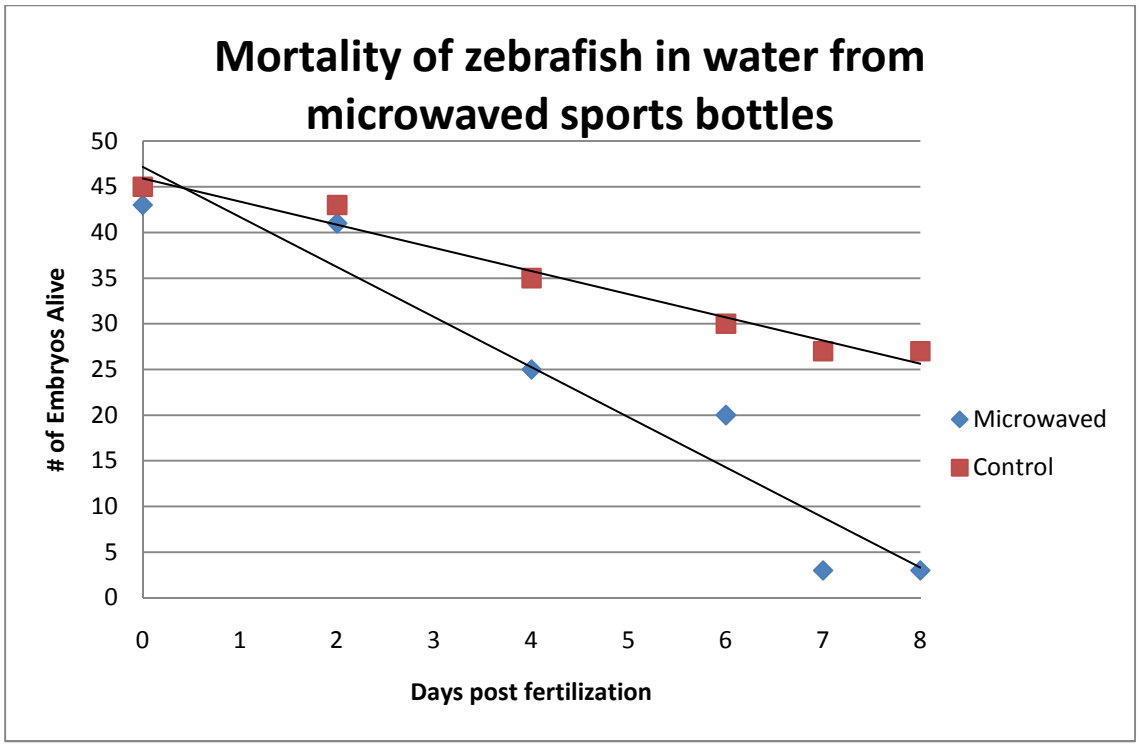

Figure 2: The surviving number of zebrafish embryos growing in water that had been microwaved in a BPA-containing bottle can be seen here, compared with a control population. Mortality of 93% of the study group was attained by 8 days post fertilization, while the control group experienced a 40% mortality in the same period. Best fit lines are given for both groups. Notice that both groups level off in the last two days.

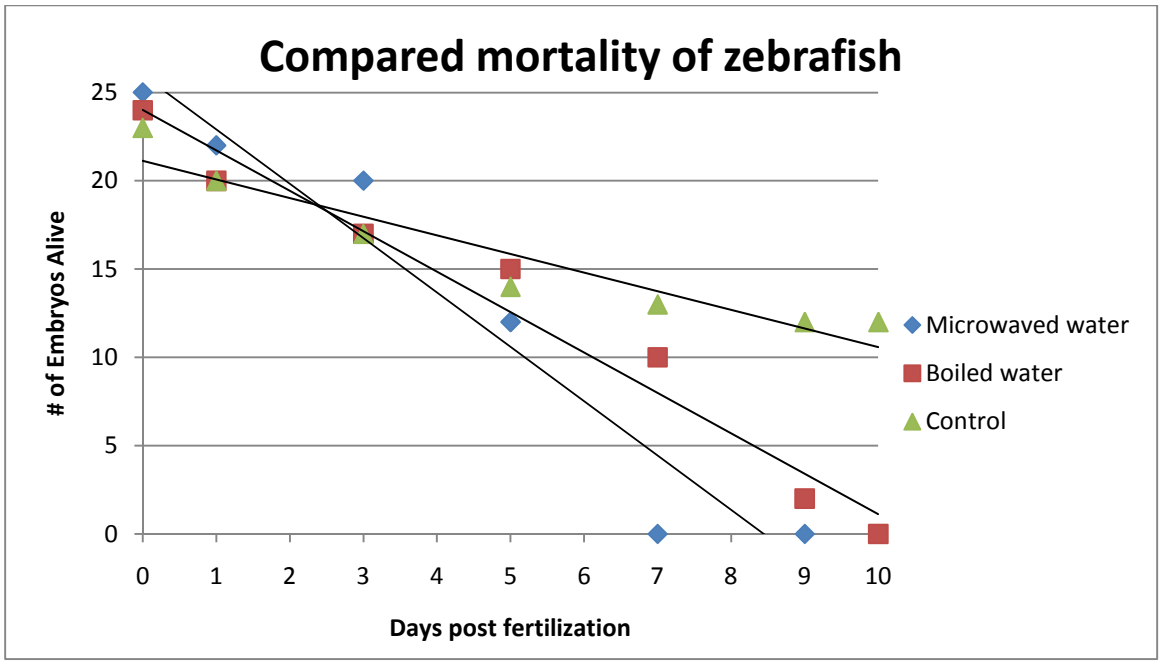

Figure 3: The survival rate of zebrafish embryos growing in boiled and microwaved bottle water can be seen here, compared with a control population. Total mortality was again attained by 8 days post fertilization in the microwaved water group, while mortality was not complete in the boiling group until day 11. The control group again experienced a 40% mortality in the same period. Best fit lines are given for all groups. Notice that while a steady downward trend is seen in the two study groups, the control population follows a more asymptote-like trend, almost completely leveling off by day 7.

Observed effects of known concentrations of BPA in vivo

Zebrafish embryos grown in 235mg BPA/l all died within 24 hours of their initial exposure (which was done within 2 hours post fertilization). Embryos in 135mg BPA/l were run in parallel to this study, and total mortality was seen at 48 hours post fertilization. Zebrafish growing in 50mg BPA/1 exhibited some minor kinking of body structure at 24 hours post fertilization. At 48 hours, heartbeat was non-existent, and blood had begun pooling on the ventral side of the embryos (Figure 4). Additionally, pigment formation was suppressed, as can be seen by comparison with a control embryo (Figure 5). By 72 hours post fertilization, total mortality was witnessed in the 50mg BPA/l group.

At 16mg BPA/l, pigment formation had not begun at the 24 hour stage, and a comparison is given in Figures 6 and 7. At 48 hours, blood pooling and a significantly lower pulse was seen (Figures 8 and 9). Heart rate was measured at 50 beats per minute in the test group, while the control population had a pulse of 125 beats per minute, which is normal for this stage of development (Barrionuevo & Burggren, 1999). By 72 hours post fertilization, 80% of the initial population was gone, however the remaining embryos survived until 5 days post fertilization, growing and developing pigmentation, in spite of a reduced heart rate. A study was done at 14mg BPA/l to confirm these findings, and the results were similar, with the same developmental defects and with a slightly higher surviving percentage of embryos. Figure 10 provides a summary of these results, emphasizing the step-like nature of the effect of increased BPA concentration on zebrafish mortality.

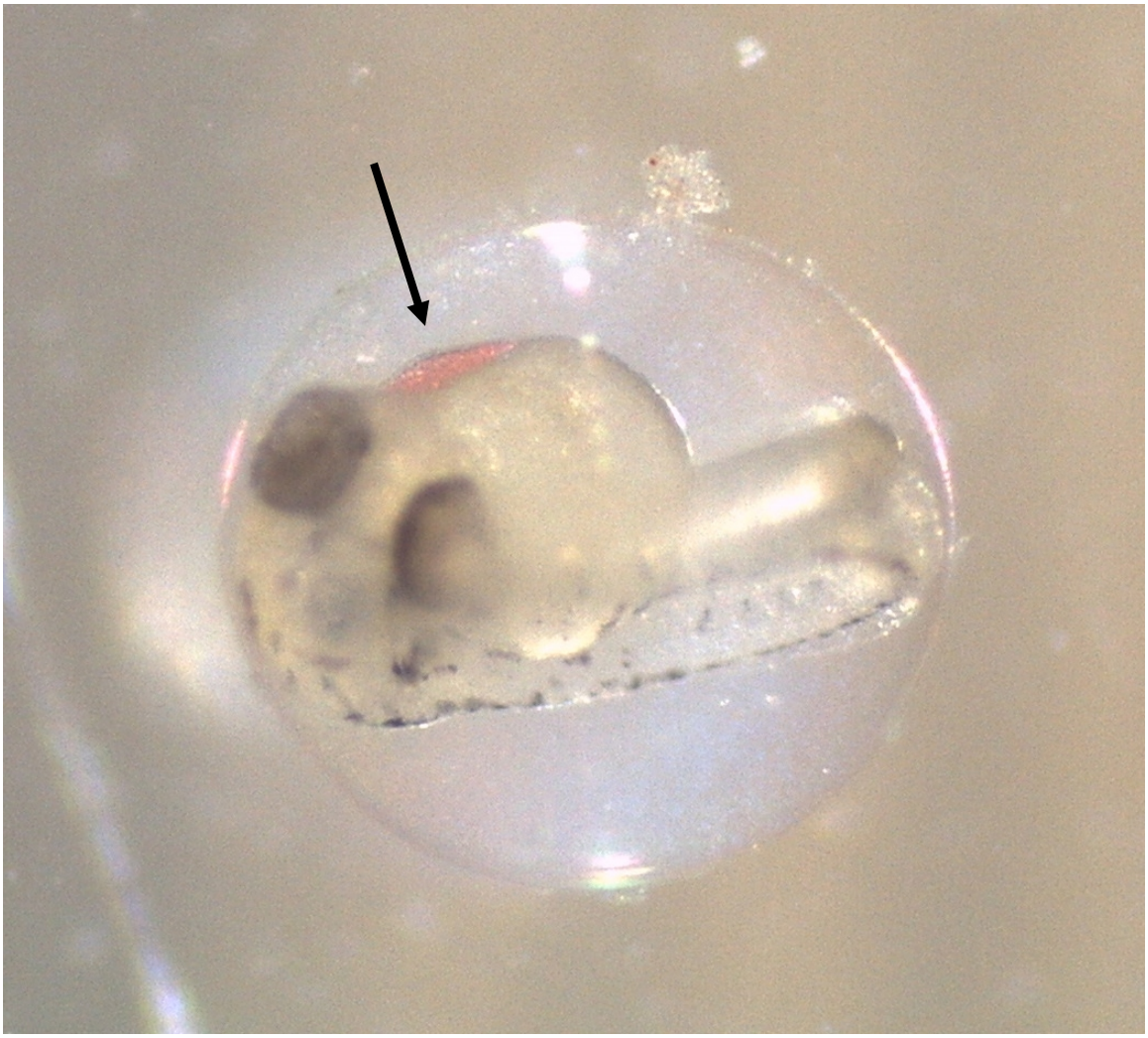

Figure 4: A zebrafish embryo grown in 50mg BPA/l is pictured here at 48 hours post fertilization. The arrow points out the blood pooling which is presumably caused by a stopped (or nearly stopped) heart. Decreased pigment formation should also be evident upon comparison with the control example (Figure 5). More difficult to see at this angle, but also significant is a minor neck kink.

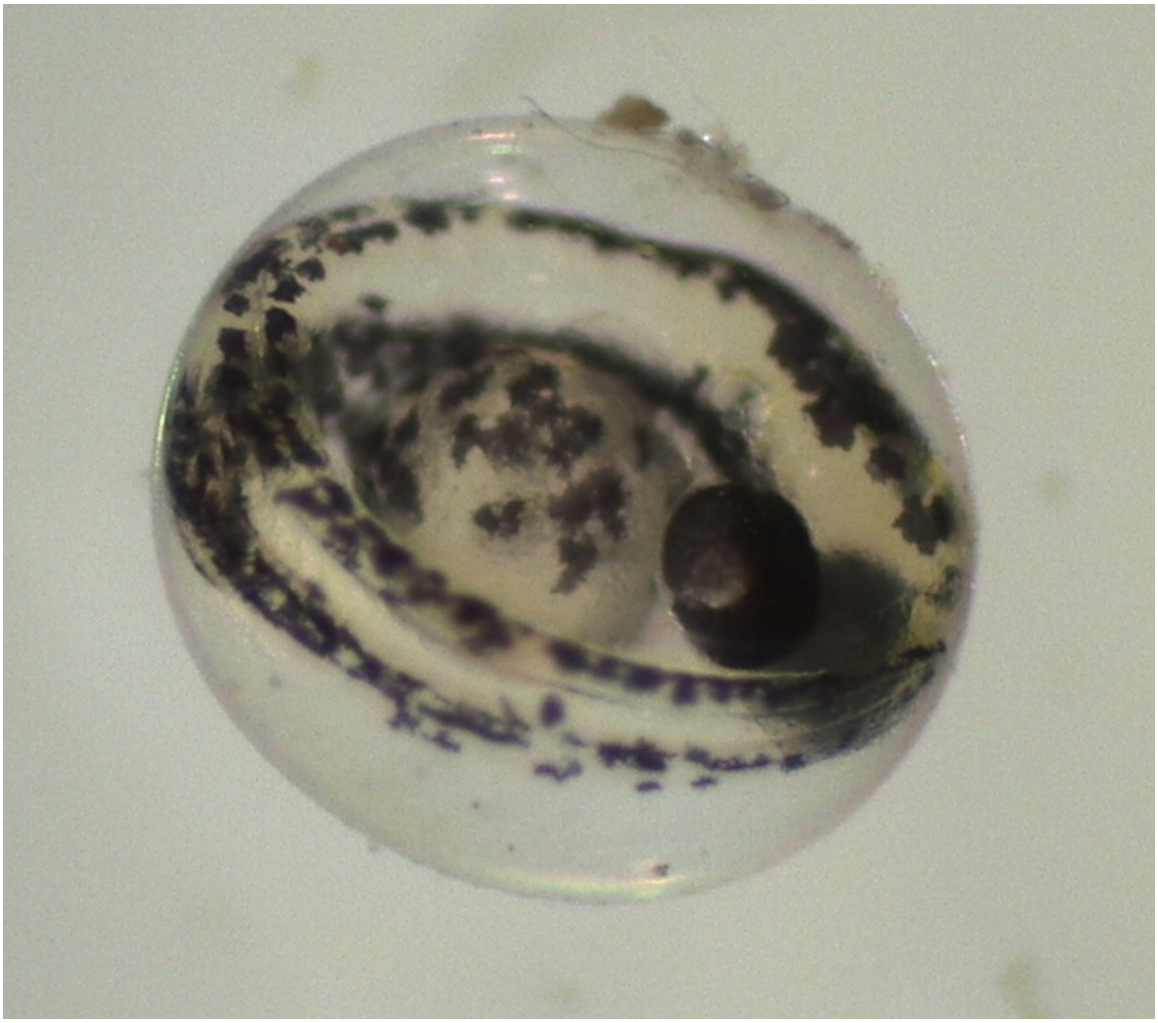

Figure 5: This is a control embryo from the 50mg BPA/l study at 48 hours post fertilization. A comparison of this with the previous embryo reveals a lower level of pigment formation in the study group.

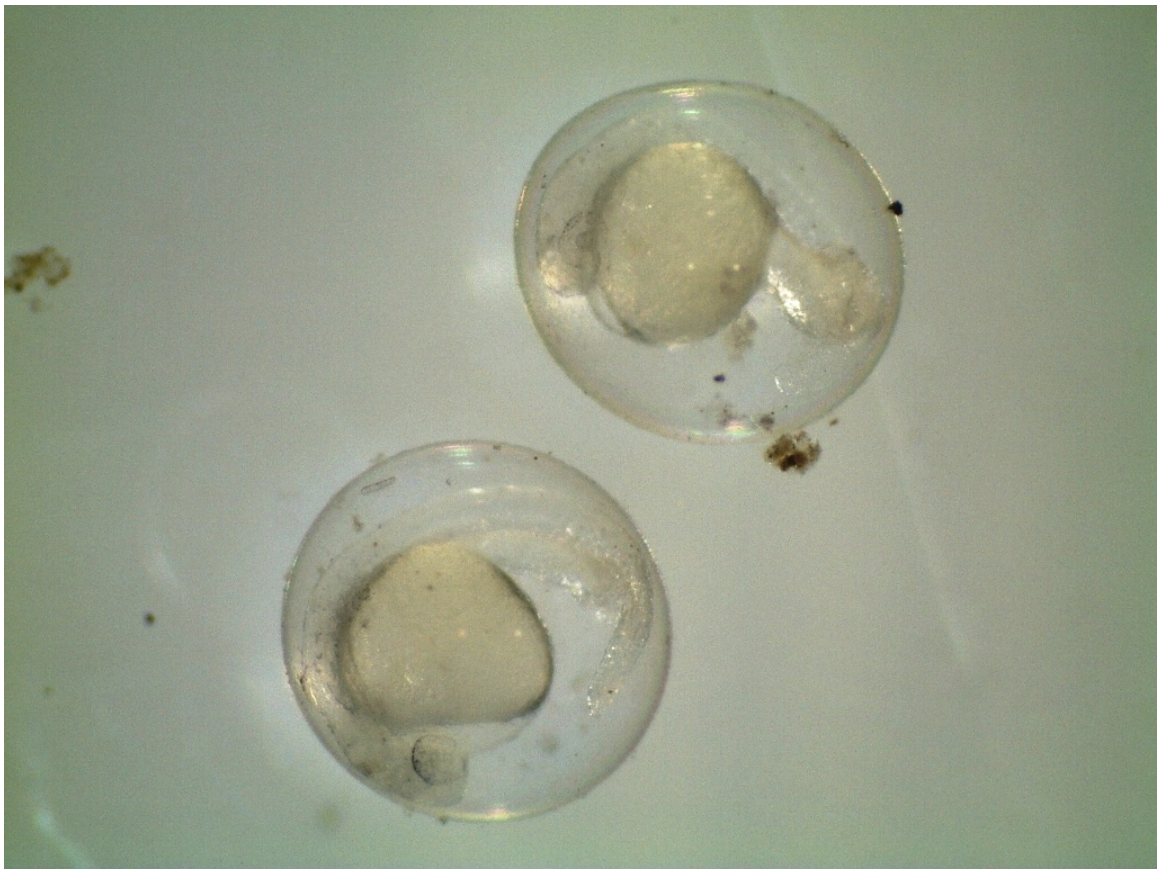

Figure 6: The 24 hour stage 16mg BPA/l embryos pictured here display almost a total lack of pigment formation, with both the trunk and the eyes being nearly devoid of color. Additionally, tail kinking has begun.

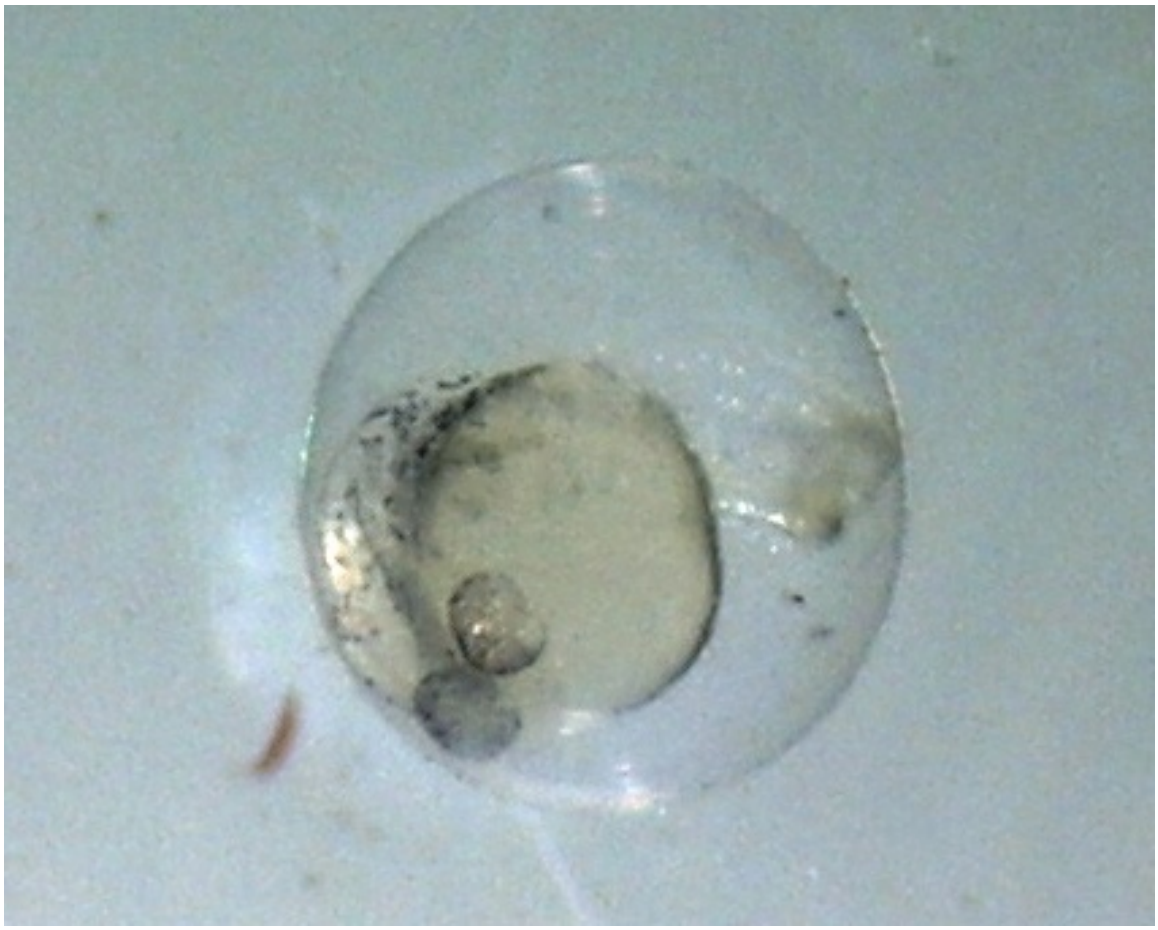

Figure 7: This control embryo from the 16mg BPA/l study at 24 hours post fertilization exhibits normal onset of pigment formation along the trunk and in the eyes.

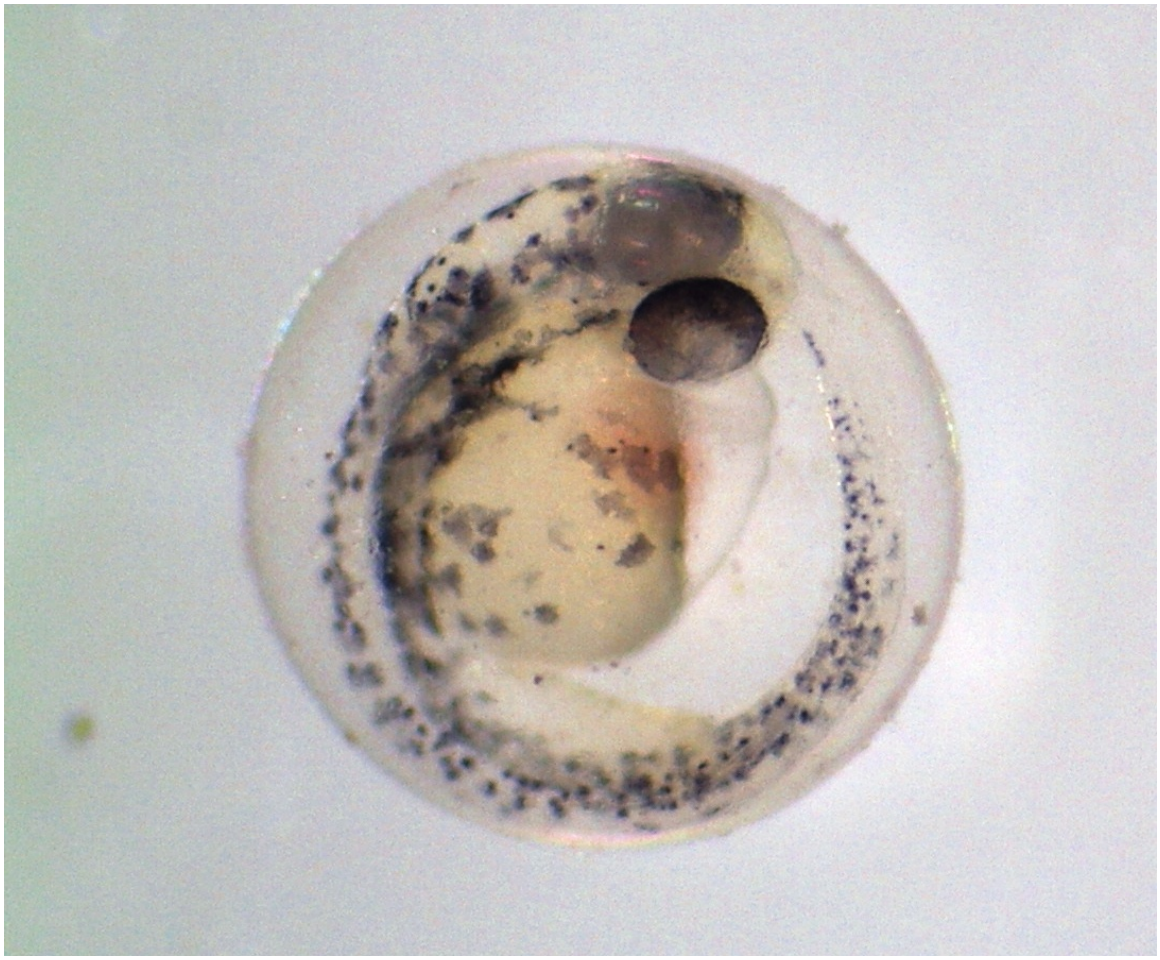

Figure 8: This embryo is from the 16 mg BPA/l population at 48 hours post fertilization. The edema seen here is not as severe as in the 50mg BPA/l population (Figure 4), and the heart is actually beating, although much slower than normal for this stage of development. Pigment formation is more progressed than in the 50mg embryos at this stage, but is less advanced than seen in the control at the same stage (Figure 9).

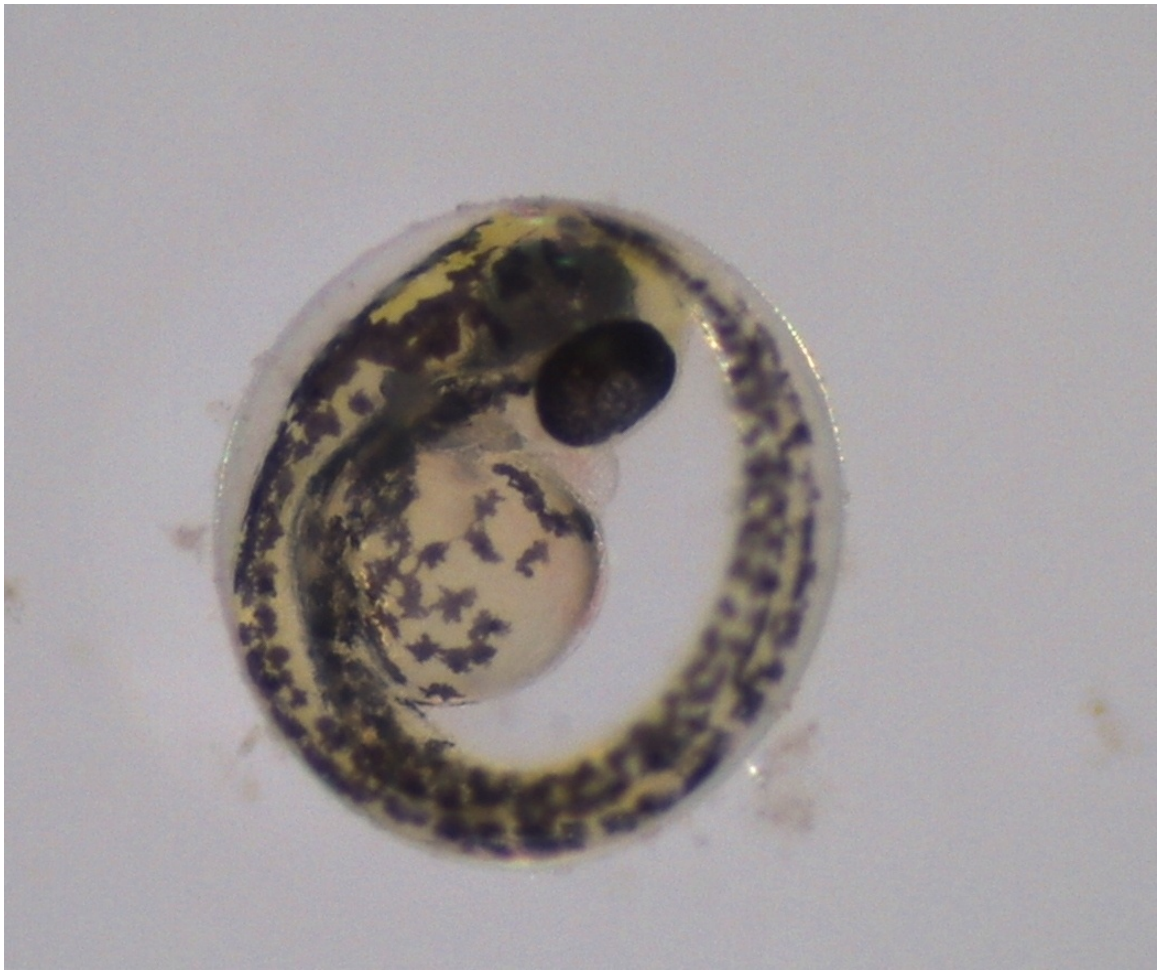

Figure 9: An embryo from the control population run alongside the 16mg BPA/l study group is given here for comparison with the previous picture. Note not only how much darker, but how much more thoroughly spread the pigmentation is on this embryo than it is in the study group at this stage (Figure 8).

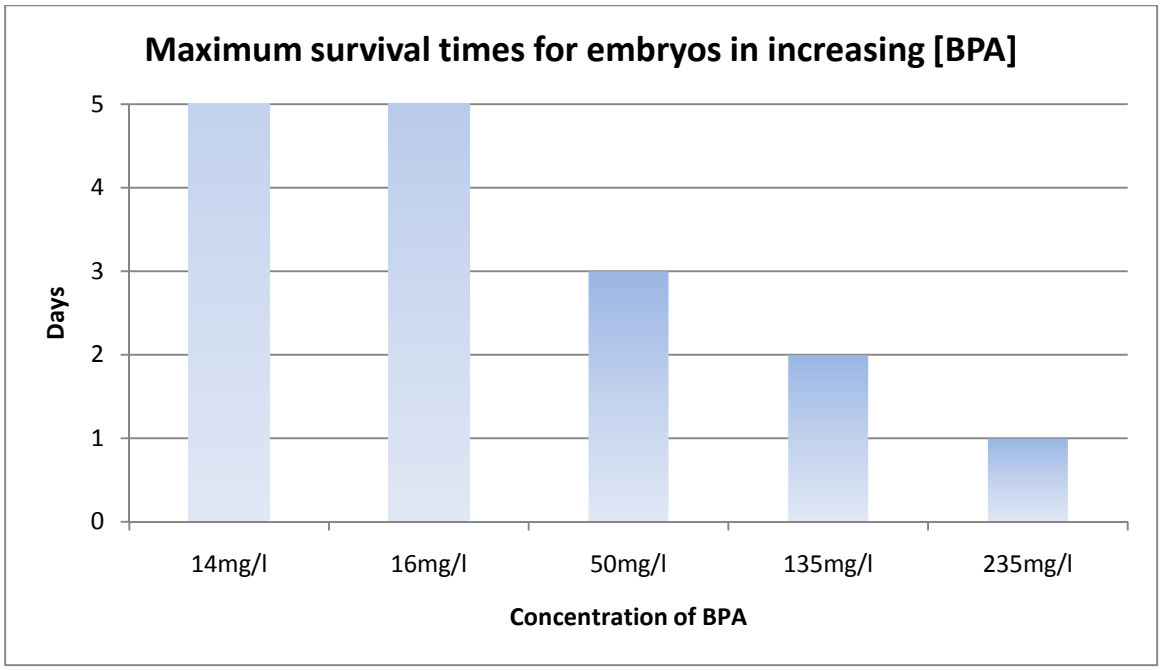

Figure 10: The number of days before total mortality is given here for embryos grown in varying concentrations of BPA. Note the stepwise pattern in the 50, 135, and 235 mg BPA/l groups, with maximum survival time decreasing by 1 day as concentration increases by a factor of about two. Following this pattern, it is possible that an embryo raised in a concentration of BPA between 25-30mg/l might expect a maximum lifespan of 4 days, though this was not tested. Not pictured are the embryos raised in 2mg BPA/l who showed no deviation from the control population.

Zebrafish embryos raised in 2mg BPA/l showed no developmental differences from the control population. Similarly, embryos growing in 1% DMSO exhibited no deviations from normal development (results not shown).

Obstacles to research

Ideally, yields of 50 to 100 eggs were expected each day, though this did not remain consistent throughout the entire time of research. At this project's beginning, while the BPA-Water studies were being conducted, daily embryo counts fell within the ideal range. However, shortly after returning from a period of research inactivity (due to Christmas break), egg production levels fell to almost nothing, and remained at this point for two weeks without improvement. During this time, several of the adult fish appeared emaciated, in spite of being well fed consistently throughout this time. Death of these fish quickly followed. Ammonia and nitrite tests were run in search of a cause for this turn of events, but revealed normal levels for both. A proposed cause was the introduction of a parasitic infection to the fish, via the blood worms they were being fed along with their normal fish flakes in an attempt to vary their diet. While there was no evidence to support this theory, in the interest of safety and in order to ensure continuation of research all the fish were quarantined, the fish tanks were bleached, dechlorinated, and stocked with new fish, and the blood worms were removed from the fish's diet.

After this procedure, production of embryos was near-ideal for about one month. Then a decline to between 8 and 20 embryos per day was observed, followed by a total cessation of embryo production. This resulted in a lowering of the numbers to work with during all research conducted in the last few weeks.

Discussion

Observed trends in embryos raised in bottle-heated water

It was found that embryos raised in water heated in a BPA-containing bottle by boiling reached total mortality within 10 days post fertilization, while those raised in water heated by microwaving succumbed within 7-8 days. That 5 minutes of microwaving produced similar (and perhaps even more intense) results as an hour of boiling is remarkable and raises concerns about the safety of polycarbonate dishes intended for storing liquid foods (such as soups) for reheating and consumption. A 2008 worst-case scenario study of BPA migration from polycarbonate bottles estimated that concentrations as high as 521μg/l might be obtained after 6 days of heating at 70°C (Cao & Corrivaeu, 2008). This study, however, failed to take into consideration the 35-fold increase in BPA migration after 50 days of use (Bredey et al., 2003), nor did it consider the effects of soap residues, whose basicity might also increase migration. Finally, this study suggests that migration of BPA from microwaved plastics may be even greater than migration seen after an hour of boiling at 100°C. Heat is directly related to BPA migration, with 100°C increasing migration rate by 55 times that of room temperature (Lea, Carlsona, Chuaa, & Belcher, 2008). It is not unreasonable to think that a used polycarbonate container with a soap residue microwaved for at least 10 minutes might produce more adverse levels of BPA than the study listed above. This raises cause for concern because the above scenario describes a typical use of many polycarbonate food containers. Worst-case studies of microwaved BPA-containing food containers should be conducted to ensure consumer safety.

On the embryos treated with known concentrations of BPA

A stepwise pattern was observed in which decreasing the concentration of BPA by about half (actual range: .64 – .37) increased the maximum possible survival time by one day. This held true from 235mg BPA/l, the minimum concentration necessary to ensure total mortality within 24 hours, to 14mg BPA/l, the LOAEL – the lowest point at which negative effects were seen. At this level a 60% decrease in heart rate led to a slow death over the course of five days. No adverse effects were visible at 2mg BPA/l, corresponding with a previous toxicity study in which the LOAEL was found to be 16.75mg BPA/l, and the lowest no effect level was 2mg BPA/l (Duana, Zhua, Zhua, Yaoa, & Zhu, 2008).

In each of the 14mg BPA/l, 16mg BPA/l, and 50mg BPA/l studies, increasing BPA concentration was associated with an increasing level of edema, a decreasing heart rate, and a decrease in pigment formation in the embryos. It is possible that a decrease in heart rate led to pooling of blood and other fluids, causing the various tissues of the embryos to be starved of necessary nutrients, leading to a decrease in pigment formation, and ultimately resulting in death. However, it is also possible that edema increased pressure on the heart, lowering heart rate and leading to the same nutrient starvation described above. Current research indicates that BPA increases oxidative stress during development, destroying lipids, proteins, DNA, and mitochondria (Chitra, Latchoumycandane, & Mathur, 2003). Oxidative stress may also result in ATP depletion, thus inhibiting metabolic pathways (Guerin, P., Mouatassim, S., & Menezo, 2001). From these findings, it could be postulated that BPA-induced depletion of ATP might inhibit the pathway responsible for providing energy to the heart, resulting in edema, nutrient

starvation, and death. Future research, in which potentially lost ATP is artificially replaced by injection, along with BPA, might confirm whether this is indeed the mechanism of BPA in the developing zebrafish embryo.

On the safety of DMSO as a future solvent in zebrafish studies

Because BPA is somewhat hydrophobic, a safe, aprotic solvent was necessary to develop known concentrations of BPA. A previous zebrafish toxicity study had used ethanol as a solvent (Duana et al., 2008). However, an earlier study had indicated that low concentrations of ethanol may have a significant fetal alcohol effect upon zebrafish (Bilotta, Barnett, Hancocka, & Saszik, 2004). 1% DMSO was used as a solvent, and it was found to have no adverse effects when compared against a control population of embryos. DMSO might continue to be used as a safe, organic alternative to ethanol in future zebrafish toxicity studies.

On problems encountered during this research project

The data concerning the obstacles to research encountered during this project are provided as a caution to future researchers. When selecting food for zebrafish, a varied diet is preferred, as variety tends to increase embryo production. However, ensuring quality food, free of infectious agents, is of the highest importance. Potentially, the ideal diet for these kinds of studies would be a commercially prepared meal of processed food.

Comparing the heated water bottle results with the known concentrations

Bredey (2003) suggested that the concentration of BPA in the boiled water bottle should be about 8.4µg/l, well below the LOAEL. In both heated water bottle studies, the control group population experienced a 40% decrease in number, while in the known concentration studies, the control typically decreased by only about 10%. The greater

decrease in population of the control group in the heated bottle studies suggests that the zebrafish in these studies experienced a more adverse environment than those in the known concentration studies. This disparity might be correlated to the difference in numbers used in the studies – approximately 50 embryos were used in each of the heated bottle experiments, while the known concentration studies were done with closer to 10 embryos. To make up for the difference in statistical significance, many of the lower count studies were repeated. Higher population density could result in increased competition for food and higher accumulation of waste, leading to nitrogen poisoning. That the control population diminished over time does not undermine the results derived from the study groups, because both the test and control groups underwent the same conditions. Rather, this suggests that BPA, when in conjunction with other environmental stresses, may play a more significant part in inducing mortality than when acting on its own. This proposal has been confirmed by a joint toxicity study, conducted by Duana et al. (2008). BPA administered along with another toxin, pentachlorophenol, increased the observed developmental defects in zebrafish from those levels seen in pentachlorophenol on its own, even though the level of BPA given was lower than the levels needed to cause defects independently.

Conclusion

A few simple points may be learned from this report. Microwaving may lead to plastic degradation and thus higher levels of BPA migration than experienced in otherwise normal use. A follow-up study, exploring the concentrations of BPA leeched from polycarbonate exposed to varying levels of microwave activity, could verify these findings.

Adverse effects, resulting in total mortality, were detected in embryos raised in 14mg BPA/l and embryos raised in water from the heated bottles. While it is unlikely that the concentrations of BPA in both of these studies was the same, it is possible that the greater environmental stress suffered by the embryos in the bottle studies worked in conjunction with BPA to cause higher mortality than would normally be seen, resulting in mortality levels expected only in much higher concentrations of BPA than present in the bottles. This suggests that BPA may play a contributing role in developmental defects, even when it is below the level needed to induce defects on its own.

Heart rate in the developing zebrafish decreased as BPA concentration increased. This change may be due to BPA-induced depletion of ATP, and disruption of heart metabolic pathways, leading to edema, reduced pigment development, and ultimately death. This pattern was seen in BPA concentrations down to 14mg/l which, if concentration in the water is considered the same as concentration in the embryo, may be translated to 14mg/kg body weight. This concentration is less than one third of that used in establishing the current BPA exposure limit. Combined with the above findings, this study contributes to a growing body of work suggesting that the currently established BPA exposure limit needs to be re-examined.

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