Xenoestrogenic effects of BPA on *Xenopus laevis* tadpoles

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ABSTRACT

Xenoestrogens commonly found in household items such as adhesives, protective coatings, and plastics are also in our waterways (Staples et al. 1998). These estrogen-like chemicals have the potential to feminize humans along with wildlife. In order to test the potentially feminizing effects of xenoestrogens, 2.7 µg/L of 17ß-estradiol (E_2) was used as the positive control. Bisphenol A (BPA) dosages of 1 µg/L, 950 µg/L, and 1900 µg/L were administered to the water, in which *Xenopus laevis* tadpoles stages 50 to 55 were housed. Two experiments were conducted. In the first I treated tadpoles with E_2, 1 µg/L, and 1900 µg/L BPA for sixteen days. In the second I treated tadpoles with 950 µg/L BPA for fourteen days. Sex ratios were determined, by inspection of gross gonadal morphology via dissection using a dissecting microscope to assess the potential developmental disruptions that may occur with exposure to E_2 and BPA. Vitellogenin (Vg) levels were determined in tadpole homogenates via ELISA. Since Vg synthesis does not naturally occur among males, any traces of Vg were hypothesized to be due to feminization. Serum from adult females was tested to verify the Vg assay. In the first experiment (E_2-BPA group), BPA and E_2 did not affect tadpoles' weight, development, survivorship, circulatory system, or Vg synthesis. In the second experiment (Intermediate BPA group), BPA did not affect tadpoles' sex ratio, development, weight, but did affect Vg synthesis in males.

INTRODUCTION

Xenoestrogens

Numerous environmental chemicals disrupt development and induce endocrine changes in animals; several are estrogenic. These estrogen-mimicking chemicals, known as xenoestrogens, can interrupt the normal functions of sex steroids in both males and females

by inducing feminization in all classes of vertebrates (Kloas et al. 1999). Xenoestrogens found in pesticides, herbicides, and industrial solvents, are accumulating at an alarming rate. The likelihood of humans and wildlife being exposed to these substances is increasing and can negatively affect susceptible species. Most notably, many of our water supplies are contaminated.

Xenoestrogens in Water Systems

Our waterways contain over 900 types of anthropogenic organic compounds. For example, sediments collected in Commencement Bay in Tacoma, WA hold high levels of toxins that can lead to diseases, such as liver carcinomas, in bottom-dwelling fish (Malins et al. 1984). Furthermore, seven sewage-treatment work (STW) effluents in Britain containing several xenoestrogens, including estrone and 17 alpha-ethynylestradiol, triggered feminization of male rainbow trout (Desbrow et al. 1998). Additionally, bisphenol A, bisphenol F, and butyl benzyl phthalate were found in surface water, sediments, and sewage treatment plant effluents throughout Germany (Fromme et al. 2002).

Though many weak estrogenic chemicals are found in effluents, mixtures of xenoestrogens are considerably more potent than individual xenoestrogens (Sumpter and Jobling 1995). Since most waterways contain various xenoestrogens, enhanced estrogenic effects can occur. Bisphenol A (BPA) is a commonly found xenoestrogen in waterways (Sumpter and Jobling 1995).

BPA

BPA can bind and activate human estrogen receptors; thus, it is an endocrine disrupting chemical (EDC) (Dodds and Lawsome 1938). BPA is also an industrial chemical synthesized by condensation of two phenol groups and one acetone molecule (Figure 1).

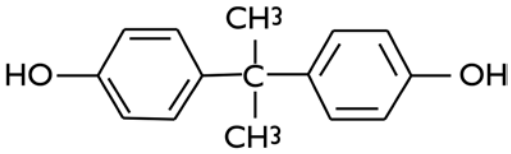


Figure 1. **Chemical structure of bisphenol A ($C_{15}H_{16}O_2$).** BPA is a carbon-based synthetic compound. Melting point is 158 ºC. The US Food and Drug Administration (FDA) identified possible hazards to fetuses, infants, and young children ("FDA Study BPA").

BPA was first synthesized and reported to have estrogen-mimicking effects on the female reproductive system in the 1930s (Dodds 1936 as cited in Rochester 2013). The EDC is used as an intermediate in the production of resins and polymers such as polycarbonate and epoxy resins (Ballesteros-Gomez et al. 2009). Polycarbonate is used in reusable plastic bottles, feeding bottles, plates, goblets, cups, microwave ovenware, storage containers, among a plethora of other food-related items. Epoxy resins are used for internal coating of food and beverage cans to protect against rusting, corrosion, and direct contact between the metal can wall and the food or beverage (Cao et al. 2011). Canned food is considered the primary source of BPA for humans, because BPA can migrate into foods during storage and processing at elevated temperatures (Cao et al. 2011). BPA was also found in food samples packaged in glass, plastic, paper, and laminated paperboard or polyethylene carton (Tetra Pak). Concentrations ranged from 0.1 to 1.28 ng/g with an average concentration of 0.46 ng/g (Geens et al. 2010).

3% of polycarbonate and 10% of the epoxy resins are used in food-related materials; the rest are found in a wide array of supplies and products such as coatings, pipes, dental sealants, nail polishes, and flame-retardant ingredients (Ballesteros-Gomez et al. 2009). The widespread applicability makes manufacturing and processing BPA an important economic factor as well as a major source of BPA released into the environment. A total of 2.8 million metric tons of BPA was produced in 2002, and an estimated 5.5 million metric tons was

produced in 2011 (Bailin et al. 2008 as cited in Rochester 2013). The production, handling, and transportation of BPA discharges dust emissions. In 1993, an estimated 109 tons of BPA were released into the air, surface water, and wastewater, with an additional 0.085% of the total BPA produced being recycled, landfilled, or incinerated (Staples et al. 1998).

Because BPA is widely used in indoor applications and consumer products, there is a great risk of the EDC leaching into household dust thus causing contamination (Loganathan and Kannan 2011). House dust containing BPA is a common source of BPA ingestion, especially in young children due to their more frequent hand-to-mouth contact (Calafat et al. 2008). While BPA can bind to and activate the human estrogen receptor, it can also interact with other endocrine receptors such as thyroid hormone receptors and peroxisome proliferator-activated receptor gamma (Diamanti-Kandarakis et al. 2009). BPA was also classified as a reproductive toxin substance of category 3 with a half-life of 2.5 to 4 days (Staples et al. 1998). A category 3 reproductive toxicant means that evidence from animal studies on BPA causes concern for human fertility and for developmental toxic effects in humans (Staples et al. 1998). In short, the amount of BPA released into the environment poses a substantial risk to both humans and wildlife.

In contrast to these studies, the European Food Safety Authority (EFSA) published a risk assessment on BPA in 2006 and concluded the tolerable daily intake (TDI) for humans was 50 µg/kg body weight/day. Human exposure through food is lower than the TDI. Thus, the EFSA believed there was no need to decrease the amount of BPA in food (EFSA 2010). According to the Federal Drug Administration (FDA), BPA is benign at the current levels found in foods. BPA entering an adult or child is rapidly metabolized and eliminated in humans ("FDA Study BPA"). FDA's National Center for Toxicological Research (NCTR)

found BPA in food passed from pregnant mothers to the fetus was so low it was undetectable ("Bisphenol A Application").

While the FDA deems BPA to be safe, the organization has been supportive of industrial efforts to find alternative chemicals in manufacturing products ("FDA Study BPA"). Internationally, the FDA joined Health Canada in encouraging Canadian industries to refine manufacturing methods for production of infant formula in can linings to minimize migration of BPA ("Bisphenol A Application"). Furthermore, the European Union banned BPA in plastic infant feeding bottles to safeguard infants and protect the general population's health ("Bisphenol A Application").

Aside from governmental research, a plethora of scientific experiments provides growing evidence that supports the notion of BPA exposure adversely affecting both humans and animals. BPA exposure has been linked to multiple health problems, including reproductive disorders, diabetes, and cardiovascular disease ("Bisphenol A Application"). Women recruited from the Massachusetts General Hospital (MGH) Fertility Center undergoing *in vitro* fertilization treatments showed higher urinary BPA in correlation with lower serum E_2 and oocyte yield per cycle (Ehrlich et al. 2012). Therefore, BPA negatively impacted female fertilization. Similarly, males working in BPA and epoxy resin manufacturing companies were found to have lower quality sperm with decreased mobility and ability to fertilize eggs (Li et al. 2011 as cited in Rochester 2013). Urinary BPA was also positively correlated with BMI in eight to eleven-year-olds (Perera et al. 2012 as cited in Rochester 2013). Furthermore, perinatal and postnatal mice exposed to low-dose (1 µg/mL) and high dose (10 µg/mL) BPA showed increases in obesity and hyperlipidemia (Miyawaki et al. 2007).

Tadpoles

In times of declining worldwide populations of amphibians, it is important to investigate the effects of BPA on *Xenopus laevis* tadpoles (Levy et al. 2004). *X. laevis* are a good indicator species for a variety of reasons. First, *X. laevis* can be generalized to represent all amphibians living in our environment. Agricultural and industrial wastewater containing EDCs such as BPA can infiltrate the breeding grounds of many amphibian species. Consequently, tadpoles may be exposed to BPA at some time during development (Levy et al. 2004). Second, *X. laevis* have permeable skin susceptible to environmental changes, the effects of which can be monitored through abnormal growth during many stages preceding metamorphosis (Pancharatna et al. 2010). Third, they can be cultured with relative ease. Last, because tadpoles are vertebrates, effects in *X. laevis* are relevant to humans who may also be affected by xenoestrogens (Kloas et al. 1999). Thus, observing the effects of BPA on *X. laevis* can shed light on the potentially harmful chemicals found in our water systems.

X. laevis in embryonic stages exposed to commonly found xenoestrogens such as nonylphenol, octyl phenol, and methoxychlor revealed increased mortality, morphologic deformations, frequent apoptosis and altered neural crest formations in the tail bud stage of the embryos. Not only did exposure to these environmental xenoestrogens reveal harmful effects, but it also exposed a suppressive effect on metamorphosis (Bevan et al. 2002). Larvae, stage 52, kept in dechlorinated water, reached stage 56 or 62 with no hindrance. However, larvae, stage 52, immersed in 10^{-5} M BPA for 21 days showed a retardation of metamorphosis by 1-2 stages. The effects of BPA were found to be concentration-dependent (Iwamuro et al. 2006). Thus, it is imperative to continue exploring the complicated effects of BPA and discover the potential implications on humans.

Hypothesis

17ß-Estradiol (E_2) is a sex steroid produced by female vertebrates with a half-life of approximately ten days (Kloas et al. 1999; Jurgens et al. 2002). E_2 targets the liver, which produces Vg, a precursor of egg-yolk proteins. Vg serves as an indicator of sexually mature females in all egg-laying vertebrates (Kloas et al. 1999). The potentially feminizing effects of BPA was measured using E_2 as a positive control. Because amphibian larval development is hormone-dependent, BPA was hypothesized to mimic E_2 and in this way elicit feminization of male tadpoles. Sex ratios and Vg synthesis were observed to quantify as a bioindicator of feminization. Thus, I hypothesized that xenoestrogens found in our waters, like BPA, would feminize humans along with wildlife at large.

METHODS

Animal Maintenance

250 tadpoles (Stage 50 for Experiment 1 (E_2-BPA group); Stage 55 for Experiment 2 (Intermediate BPA group)) were purchased from eNasco in Fort Atkinson, WI and fed tadpole chow from Xenopus I, once every other day. 17 x 28.5 cm tanks using dechlorinated water treated with Amquel were maintained at 25±1 ºC and 12:12 LD. The water was changed every three days (Pancharatna et al. 2010).

E_2-BPA group: Chemicals

X. laevis stage 50 (when tadpoles become sensitive to estrogen (Miyata and Kubo 2000)) tadpoles were treated with varying concentrations of BPA and E_2 (Figure 2).

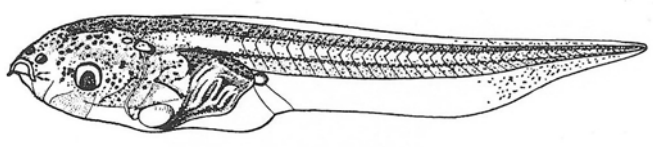


Figure 2. **Stage 50.** Closely examining development of limb buds is a sound indicator for stage (Nieuwkoop and Faber 1994).

3.8 mg BPA were dissolved in 100 µL ethanol (EtOH). Stock solutions of 20 µg/L of "low" BPA and 100 µg/L of "high" BPA were diluted in 2.0 L of dechlorinated water. BPA concentrations of 1 µg/L and 1900 µg/L were used based on environmental amounts of BPA found in waterways in Germany (± 1 order of magnitude) (Fromme et al. 2002).

Stock solution of 2.0 µg/L of E_2 was prepared by adding 2.7 mg of E_2 to 1000 µL of EtOH. E_2 concentration of 2.0 µg/L was added to 2 L dechlorinated water. This concentration was based on a study done on *X. laevis* by Pickford et al. 2003.

Intermediate BPA group: Chemicals

Tadpoles (Stage 50 – 55) were exposed to intermediate concentrations of BPA. Due to delayed shipments, some tadpoles arrived at later stages (Stage 55). Because only 50 tadpoles were ordered, 25 tadpoles were controls while the other 25 were treated with BPA.

9.5 mg of BPA were dissolved in 300 µL EtOH. Stock solution of 60 µg/L was diluted in 2 L of dechlorinated water. BPA concentration of 950 µg/L was based on the average of the previous BPA concentrations used in the E_2-BPA group.

E_2-BPA group: Chronic exposure to BPA and E_2

A total of 200 tadpoles were used for this experiment. Each plastic tank contained 50 tadpoles and was divided into the following systems:

- 50 tadpoles at Stage 50 was exposed to 1 µg/L BPA
- 50 tadpoles at Stage 50 was exposed to 1900 µg/L BPA
- 50 tadpoles at Stage 50 was exposed to 2.7 µg/L E_2 (positive control)
- 50 tadpoles at Stage 50 was exposed to only dechlorinated water (control)

Intermediate BPA group: Chronic exposure to BPA

A total of 50 tadpoles were used for this experiment. Each plastic tank contained 25 tadpoles:

- 25 tadpoles at Stages 50 - 55 were exposed to 950 µg/L BPA
- 25 tadpoles at Stages 50 – 55 were exposed to only dechlorinated water (control).

E_2-BPA group: Sex Ratio

In order to test the effects of BPA and E_2 on feminization of tadpoles, sex was determined by gonadal morphology via dissection using a dissecting microscope (Figure 3).

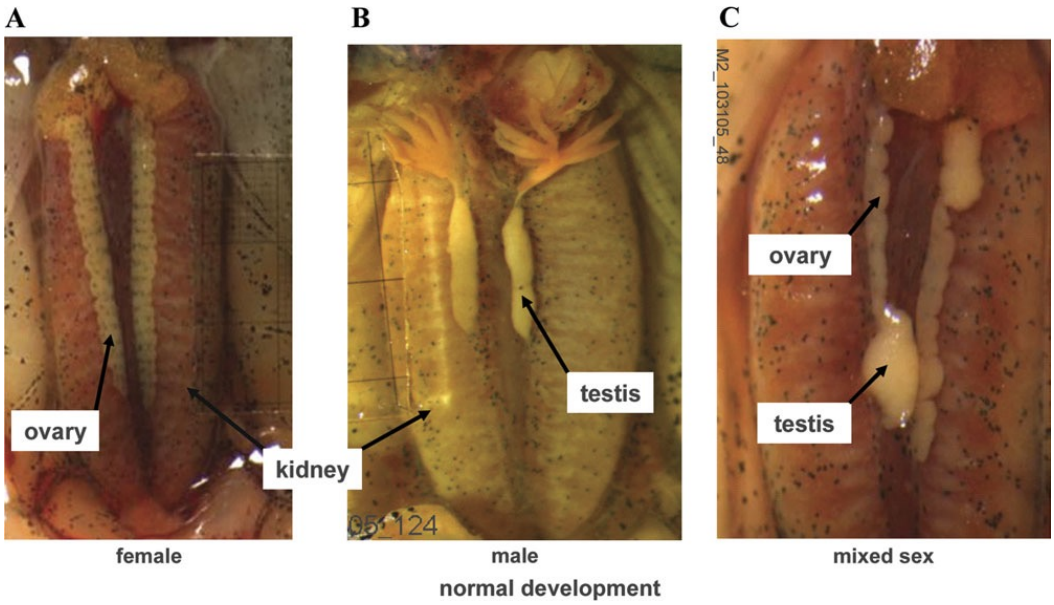


Figure 3. **Gonads of normal stage 66 males and females vs. stage 66 males exposed to 0.2 µg/L of E_2.** a) Arrows point to ovaries, which contain bumps and are longer compared to b) testis, which are smooth and shorter in length. c) Shows hermaphroditism (Kloas et al. 2009).

Sex determination was done via dissection of gonadal morphology as soon as the E_2 group had 11 tadpoles left. The control group was reduced to 1 tadpole when dissections took place. After dissections, each tadpole was homogenized in 80 µL PBS. The homogenate was then centrifuged at 13,000 x g for 10 minutes, and 80 µL of supernatant were reserved and stored at -20 ºC until ELISA determinations.

Intermediate BPA group: Sex Ratio

Gonadal morphology was determined via dissection after two weeks of BPA treatment. After dissections, each tadpole was homogenized in 200 µL PBS. The homogenates were centrifuged at 13,000 x g for 10 minutes, and 100 µL of supernatant were reserved and stored at -20 ºC until ELISA determination.

Stages and Development

Tadpole stages were determined based on limb bud formation. Tadpoles that fell into two different stages had either a plus (+) or minus (-). More advanced stages were denoted plus signs (stage + 0.5) and earlier stages were given minus signs (stage – 0.3). The plus and minus signs were subjective annotations to identify tadpole stages.

The circulatory system development was assessed using three categories: absent, beginning, and developed. ‘Absent' circulatory systems revealed no visible circulatory system, ‘beginning' circulatory systems showed some blood vessels though not fully established as the ‘developed' circulatory systems.

Vitellogenin (Vg)

Serum Vg levels was measured using an ELISA (Vitellogenin Enzyme-Linked Immunosorbent assay from K. Selcer Laboratory Duquesne University, Pittsburgh, PA). Control from a vitellogenic adult female serum underwent the following serum dilutions:

		Antigen	Buffer
#1s	1:100	10 uL Serum	+990 uL PBS
#2s	1:1000	50 uL #1s	+450 uL PBS
#3s	1:10000	50 uL #2s	+450 uL PBS
#4s	1:100000	50 uL #3s	+450 uL PBS

The diluted serum and the tadpole homogenates were placed into a microtiter plate so that the antigen could attach to the wells. Incubated for an hour and a half on a rotary shaker, wells were washed several times with PBS buffer before adding the 100 µL of the blocking

buffer (5% milk in PBS), which was then incubated for 30 minutes. After washing once more with PBS, 100 µL of the primary antibody (Rabbit Anti-Xenopus Vitellogenin #498) were added to each well and incubated overnight. After removing the primary antibody and washing several times with PBS, 100 µL of the secondary antibody (Goat Anti-Rabbit IgG) were added to each well and was incubated in a shaker for two hours. The secondary antibody was removed and washed several times with PBS again. After washing, 100 µL of the developer (Bio-Rad TMB Peroxidase Substrase Kit) were added into each well. After the addition of the developer, the plate was incubated for 1 hour and 45 minutes before stopping the reaction with 100 µL of sulfuric acid. Results were quantified using a plate reader at a wavelength of 450 nm.

Data Analysis

Data for stages, weights, and Vg levels were analyzed using an ANOVA and the StatView SE statistical package. Chi Square was used to analyze circulatory system development and sex ratios between treatments. Further, a 2-way ANOVA was used to compare sex and treatment to mean Vg levels using StatView SE in the Intermediate BPA group. Significant levels for all these tests were set at 5% ($p < 0.05$).

RESULTS

E_2-BPA group

Because the control group was reduced to one tadpole on termination day, it provided little information about untreated animals. The final weights of animals treated with E_2 and BPA were not different (Figure 4; $p = 0.33$). Weights for the estradiol, low BPA, and high BPA were 0.076 ± 0.01 g, 0.074 ± 0.01 g, and 0.088 ± 0.01 g, respectively.

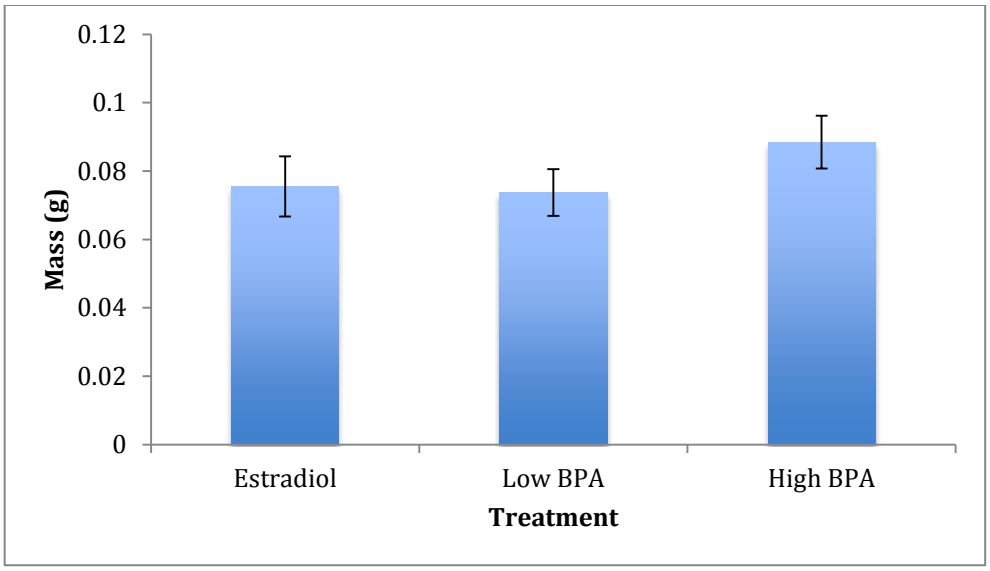


Figure 4. **Total body net weights of animals exposed to 3 treatments.** 2 µg/L estradiol, 1 µg/L BPA, and 1900 µg/L BPA groups had 13, 16, and 17 tadpoles, respectively. Error bars represent standard error of the mean (SEM).

BPA did not affect development as assessed by final stages (Figure 5; $p = 0.77$). Stages for estradiol, low BPA, and high BPA were 51.477 ± 0.3, 51.213 ± 0.2, and 51.282 ± 0.2, respectively.

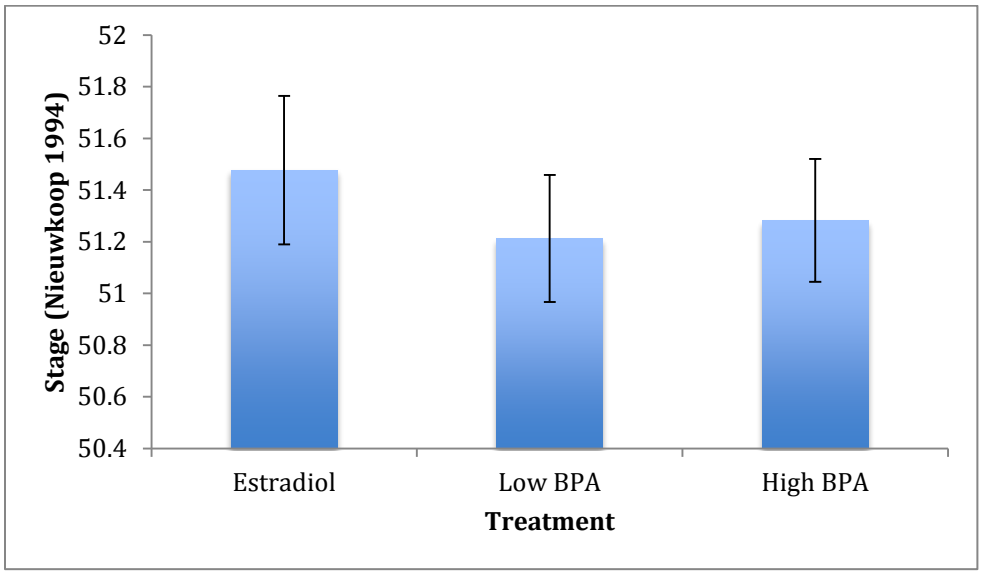


Figure 5. 2 µg/L estradiol, 1 µg/L BPA, and 1900 µg/L BPA groups had 13, 16, and 17 tadpoles, respectively. Vertical bars represent SEM.

Circulatory system development was not affected by treatment ($X^2 = 1.76$, df = 4; p = 0.78). Treatment also did not affect the survivorship of tadpoles (Figure 6; $X^2 = 0.82$, df = 2, p = 0.67).

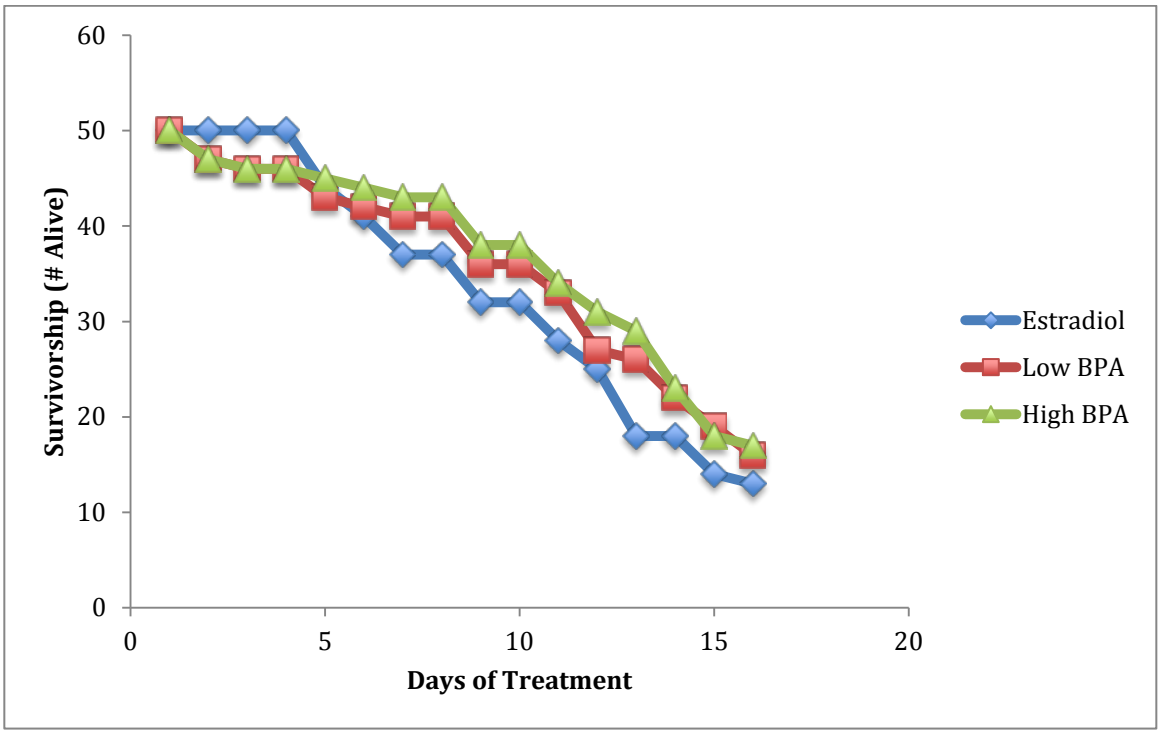


Figure 6. **Survivorship of tadpoles during the 16 days of treatment.** 2 µg/L estradiol, 1 µg/L BPA, and 1900 µg/L BPA groups had 13, 16, and 17 tadpoles, respectively.

Vg levels were not affected by treatment (Figure 7; p = 0.94). Vg absorbance for estradiol, low BPA, and high BPA were 0.292 ± 0.03, 0.307 ± 0.03, and 0.302 ± 0.04, respectively.

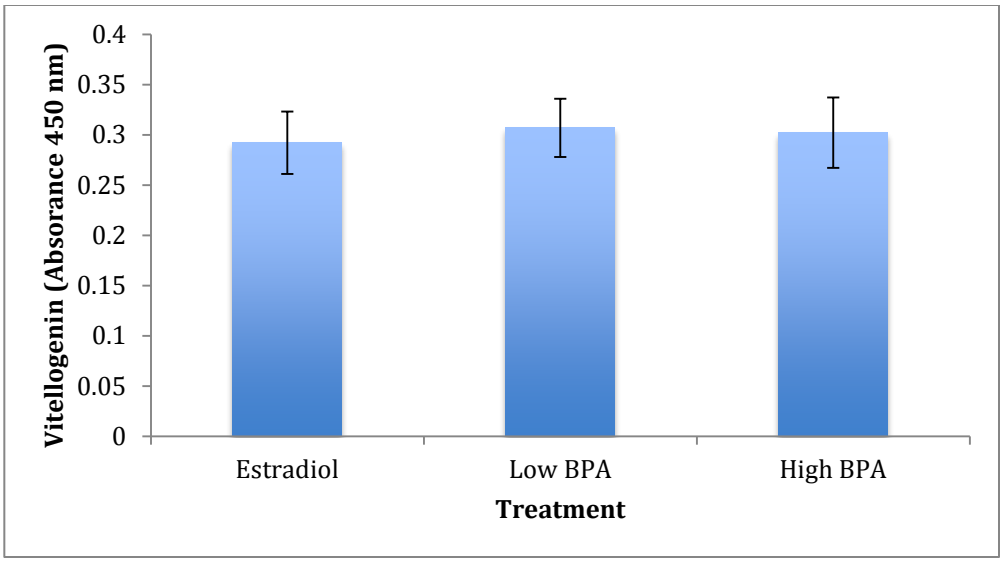


Figure 7. **Vg absorbance (450 nm)** 2 µg/L estradiol, 1 µg/L BPA, and 1900 µg/L BPA groups had 13, 16, and 17 tadpoles, respectively. Vertical bars represent SEM.

Intermediate BPA group

BPA did not affect weights (Figure 8; p = 0.86). Weights for the control and BPA groups were was 0.286 ± 0.02 g and 0.279 ± 0.04 g, respectively.

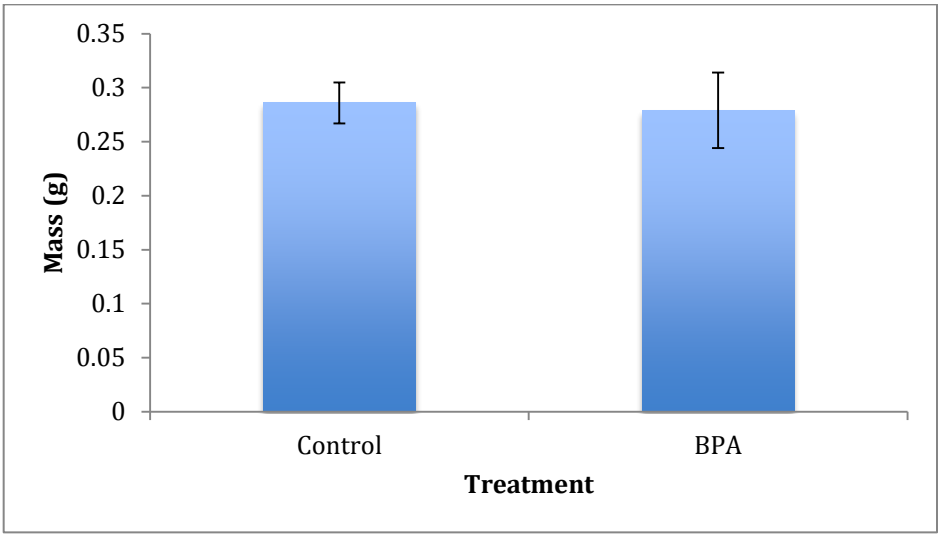


Figure 8. **Total body net weights of animals exposed to 2 treatments.** The control and 950 µg/L BPA groups had 13 and 15 tadpoles, respectively. Error bars represent SEM.

BPA also did not affect developmental stages (Figure 9; p = 0.23). Stages for the control and BPA group were 55.162 ± 0.6 and 54.180 ± 0.5, respectively

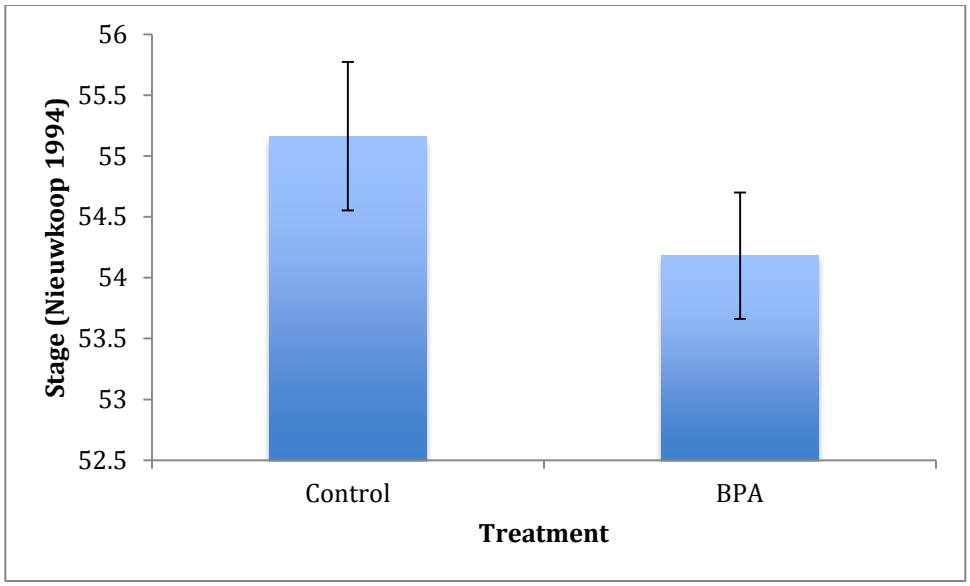


Figure 9. Control and 950 µg/L BPA groups had 13 and 15 tadpoles, respectively. Vertical bars represent SEM.

Sex was assessed as male, female, or indeterminate for the control and BPA groups. Treatment did not affect gender ($X^2 = 4.435$, df = 2; p = 0.109). Treatment did not affect Vg levels (Figure 10; p = 0.78). The absorbance of the control and BPA groups were 0.269 ± 0.05 and 0.256 ± 0.01, respectively.

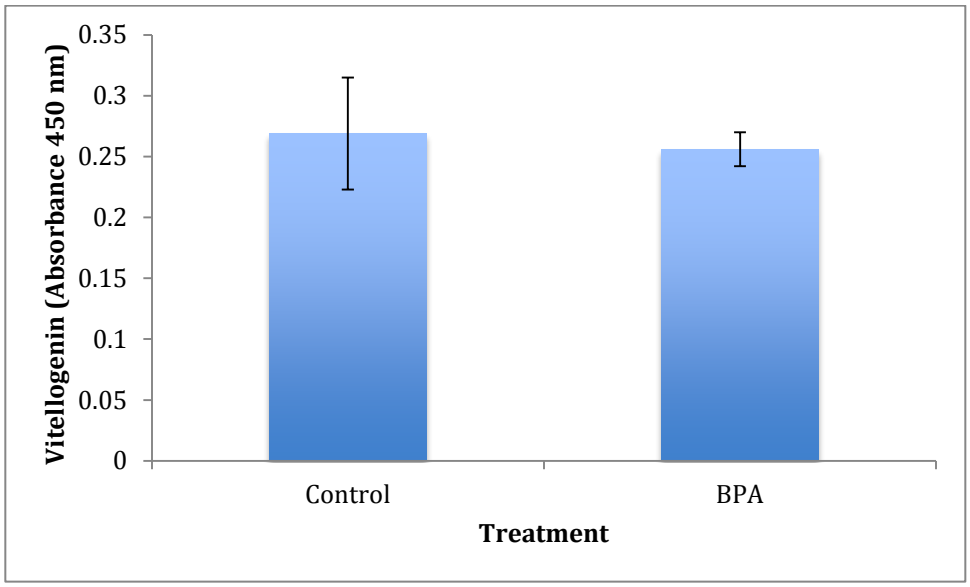


Figure 10. **Vg absorbance (450 nm).** Control and 950 µg/L BPA groups had 13 and 15 tadpoles, respectively. Vertical bars represent SEM.

Vg levels affected sex and treatment (Figure 11; p = 0.001). One control male had significantly higher levels of Vg (Figure 11; p = 0.005). However, there were no significant differences between female and indeterminate sexes (Figure 11; p = 0.146). The absorbance for control females, males, indeterminate, treated female, males, and indeterminate sexes were 0.171 ± 0.02, 0.677 ± 0.00, 0.281 ± 0.05, 0.331 ± 0.00, 0.300 ± 0.04, and 0.237 ± 0.01, respectively.

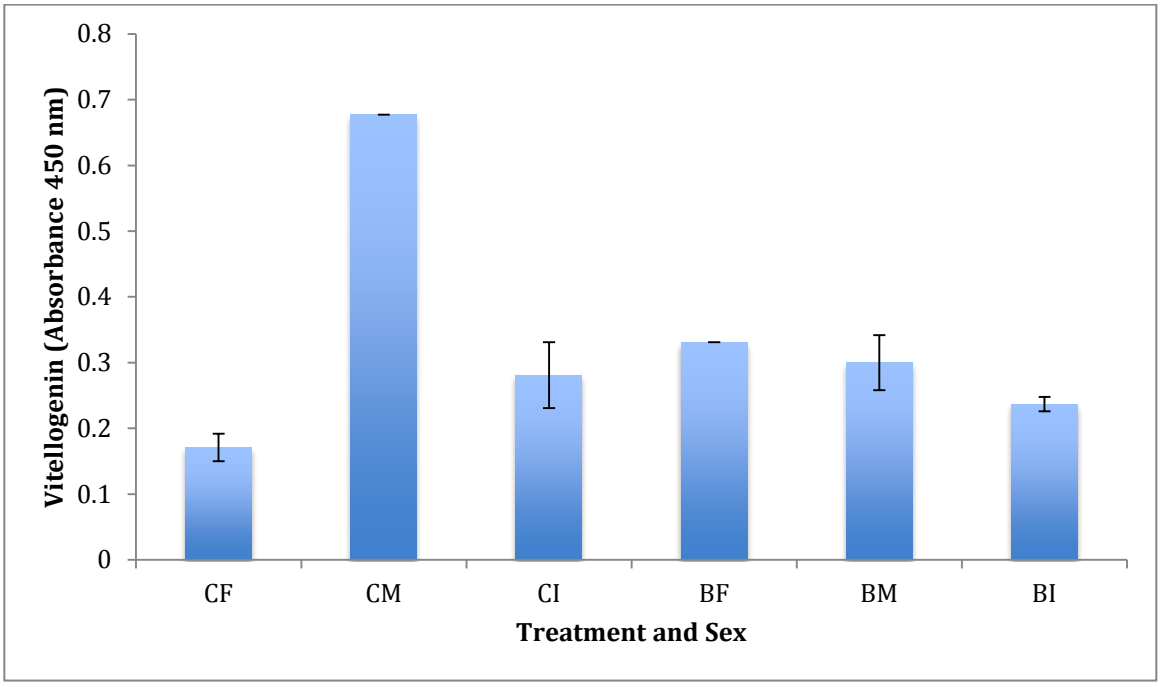


Figure 11. **Vg absorbance (450 nm).** 'C' represents control, 'F' is female, 'M' is male, 'I' is indeterminate, and 'B' is BPA. The control females, male, and indeterminate sexes had 5, 1, and 7 tadpoles, respectively. The BPA females, male, and indeterminate sexes had 1, 3, and 11 tadpoles, respectively. Vertical bars represent SEM.

DISCUSSION

BPA did not significantly increase weight of the tadpoles compared to controls in either experiment (Figure 4; Figure 8). In humans, higher urinary BPA was associated with increased BMI and waist circumference in eight to eleven-year-olds and in individuals 40 or older (Perera et al. 2012 as cited in Rochester 2013; Galloway et al. 2010). Similarly, in

mice, BPA concentrations were correlated with obesity and hyperlipidemia (Miyawaki et al. 2007). Tadpole weights may not have been affected by BPA due to many reasons. For instance, in an *in vitro* study of human adipose tissue, there was no association between BMI and adipose tissue BPA concentration. The negative correlation indicated that increased urinary or serum BPA was not due to increased adipose stores of BPA (Fernandez et al. 2007 as cited in Rochester 2013). Thus, reasons explaining the correlation between BPA and body weight may vary. Race, ethnicity, and sex can also alter BPA and body weight. In children between the ages of six and nineteen, higher urinary BPA was associated with obesity. However, examining race or ethnicity revealed the relationship between high BPA and obesity was only seen in Caucasians (Trasande et al. 2012). Another study with nine-year-old children found girls that had increased prenatal exposure to BPA had decreased BMI and body fat. However, boys with increased prenatal exposure had a positive association with increased BPA and BMI, waist circumference, fat mass, and obesity (Harley et al. 2013). All things considered, the associations between BPA and body weight are complex. Examining how BPA affects metamorphosis may reveal more information on how BPA impacts overall development of tadpoles.

EDCs such as BPA slow tadpole metamorphosis (Iwamuro et al. 2006). For instance, stage 52 tadpoles kept in dechlorinated water reached stages 56 or 62 with no hindrance; however, stage 52 tadpoles treated with 2280 µg/L BPA for three weeks were developmentally delayed by several stages. Further, the effects of BPA were concentration-dependent: 2280 µg/L or 22,829 µg/L BPA blocked thyroid hormone (T_3), thus shortening the tails of tadpoles (Iwamuro et al. 2006). However, my results did not concur with the previous findings. I found no significant differences in the final stages of BPA-treated

tadpoles and controls (Figure 5; Figure 9). My treatments may not have elicited the expected results due to the concentrations of BPA used. A recent study suggests that BPA follows a biphasic or U-shaped dose-response relationship where low dosages can elicit a stronger reaction to endocrine disruption than higher concentrations (Calabrese 2001 as cited in Levy et al. 2004). In living animals, one explanation for a biphasic dose response is because the threshold concentration must be exceeded before a physiological effect appears in the organism. In other words, in higher concentrations, when the effective dosage is passed, metabolizing enzymes may suppress adverse health effects. In rainbow trout, high concentrations of BPA might be correlated to increased metabolism of BPA due to detoxification enzyme activities increasing (Lindholst et al. 2000). In my study, concentrations of 1 µg/L, 950 µg/L, and 1900 µg/L BPA were used, which may have been either too high or low to affect the development of tadpoles. Furthermore, in the E_2-BPA group, using the circulatory system as a bioindicator of overall development, there were no significant differences between E_2 and BPA. Concentrations may not have been within the critical range to show any developmental disruptions. Thus, correlation between BPA and development is also complex, and requires further research in regards to ecological relevance of endocrine disrupting dosages that can potentially impact humans and wildlife.

BPA did not appear to affect the sex ratio of the tadpoles. However, several studies reveal xenoestrogens, such as BPA, having the potential to interrupt normal functions of sex steroids. In both males and females, BPA induces feminization in almost all classes of vertebrates (Kloas et al. 1999). In male rainbow trout, feminization was triggered due to xenoestrogens found in water effluents (Sumpter and Jobling 1995). For tadpoles, since amphibian larval development is hormone-dependent, both E_2 and BPA treatments were

hypothesized to induce feminization of males to become females. Sex ratio differences were observed in 1950 when all E_2 treated *X. laevis* tadpoles underwent sex reversal (Levy et al. 2004). However, for my experiment, BPA did not change gonadal morphology, which can be due to a plethora of reasons. Testicular and ovarian tissues are not visually distinguishable until tadpoles reach stage 55 (Levy et al. 2004). Unfortunately, due to an unexpected amount of deaths, termination was premature; thus, the average tadpole stage was below 55. It is possible that even if feminization were occurring, the majority of tadpoles were simply too young to make an observable distinction between sexes. Further, 22.8 µg/L BPA consistently feminized tadpoles while 228 µg/L and 2.28 µg/L BPA showed inconsistent feminization (Levy et al. 2004). Therefore, using 1 µg/L, 950 µg/L, and 1900 µg/L BPA may have been outside the critical range of BPA concentration to elicit feminization. In other words, the BPA concentrations used may have been in an ineffective dosage range along the biphasic dose response curve. Moreover, *X. laevis* have a sensitive period for the induction of sex reversal between stages 50 and 52 (Miyata et al. 1999). If treated tadpoles were either younger or older than the sensitive window, treatments would not have been effective.

Another indicator of feminization is by examining Vg production. Vg synthesis occurs in tadpole livers as early as stage 53 (Knowland 1978). Additionally, 22.8 µg/L BPA induced Vg synthesis in *X. laevis* tadpoles (Kloas et al. 1999). In my experiment, Vg appeared in the serum of some animals of each group; however, treatment did not affect levels of absorption (Figure 7; Figure 10; Figure 11). The highest Vg level came from a control male, which could have been due to errors made in running the ELISA, or may have been a misidentification of sex. Though the majority of tadpoles showed Vg, this could have been due to several reasons. For example, the average tadpoles were stage 54, thus too young to

determine via gonadal morphology, but old enough to produce Vg. Therefore, it is impossible to conclude whether Vg synthesis was due to the female to male ratio being larger or if BPA was causing the feminization of male tadpoles.

Future Research and Conclusion

Due to the lack of current knowledge about BPA uptake, metabolism, and excretion in anuran tadpoles, further experimentation is needed. In this study, some key follow-up experiments would be to look at a broader range of dosages to identify critical concentrations that potentially cause feminization. Another vital aspect of the study was the termination stage of tadpoles. Because the majority of tadpoles were too young to show visible gonads, keeping the tadpoles alive long enough to determine the sex could have drastically changed the results.

In brief, BPA has the potential to act as a feminizer in the environment. Massive quantities of BPA are released into the ecosystem through human manufacturing, and both humans and wildlife are susceptible to exposure (Staples et al. 1998). In the present study, the concentrations of BPA did not affect the parameters that were measured. Further studies unhindered by tadpole death and broader dosages of BPA can potentially reveal feminization of tadpoles. Ultimately, these studies can show the endocrine disrupting effects that BPA has on vertebrates.

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