Low Levels of Bisphenol-A Decrease Metabolic Rate and Boldness in Zebrafish, *Danio rerio*

By

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ABSTRACT

Human impact alters the natural environment via multiple pathways, including contamination from pollutants. This human activity may adversely impact an organism's ability to respond to environmental change. Using Bisphenol-A (BPA), a common environmental contaminant, I examined how exposure affected behavioral strategies critical for survival in a changing environment. BPA is used during plastic manufacturing, and it enters aquatic systems from wastewater streams; however, it is an endocrine-disruptor that has broad health effects from metabolism to behavior at a wide exposure range. In this study, I specifically tested whether environmentally relevant concentrations of BPA impact maximum metabolic rate and boldness in zebrafish, *Danio rerio*. I also examined activity level, optomotor response, body mass, and standard length to see if I can mechanistically explain any underlying changes caused by BPA. I treated groups of adult zebrafish for 7 days and exposed them to either 0.1% dimethyl sulfoxide (DMSO, control), a low environmentally relevant concentration of BPA (0.02 mg/L), or a 1-fold higher BPA concentration (0.2 mg/L). I found that the low exposure group experienced a decrease in maximum metabolic rate and the high exposure group showed a decrease in boldness. In other words, these changes in metabolism were not dosage dependent while the boldness results were dosage dependent. BPA had no effects on optomotor response, body mass, standard length or activity level. These results suggest that no level of BPA is safe, environmentally relevant concentrations are having an effect on adult organisms' behavior and health that could affect their survival.

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INTRODUCTION

Human activities such as housing developments, greenhouse gas emissions, and deforestation are drastically changing natural environments, leading to habitat loss, fragmentation, and exposure to unsafe contaminants. Aquatic systems are particularly vulnerable for human impact because human settlements are generally high near lakes, rivers, and estuaries (Williamson et al., 2008). They often experience habitat alterations through changes in temperature, water flow, and physical structures that may create novel and more energetically demanding conditions. Thus, the ability to navigate novel habitat conditions and regulate metabolic demands may play a large role in determining the survival of aquatic organisms in a changing environment. While physical disruptions are readily observable, human activity may also change aquatic habitats via chemical pollution. Given sufficient time, animals often adapt to structural changes in their habitats (Hoffmann and Sgrò, 2011) but being exposed to chemical pollutants may limit their ability to keep up with environmental change. For example, many chemical pollutants have been known to affect exploratory behavior and spatial learning skills of aquatic organisms (see review by Jacquin et al. 2020), which are critical for adjusting to novel environments. The impacts of chemical pollutants on organisms are multifaceted and can be more complex than it appears because exposure to a single pollutant can have effects that vary widely due to timing, duration, and concentration of exposure. Here I focus on Bisphenol-A (BPA), a common environmental pollutant, and test how its short-term exposure of environmentally relevant concentrations affects essential survival strategies of adult fish such as boldness and maximum metabolic rate. I then explore its effect on

vision and activity level of fish to understand potential mechanisms underlying these changes.

BPA is an organic chemical widely used in manufacturing polycarbonate plastics, epoxy resins, thermal paper, and canned food linings among other products (Schierow and Lister 2010). Due to high production volumes, it frequently contaminates the environment via wastewater from manufacturing plants and leaching from product processing (Flint et al. 2012). Despite its short half-life, BPA has a moderate level of bioaccumulation in the environment because of the immense amount of plastic manufacturing and human use (Corrales et al. 2015, Schierow and Lister 2010). BPA levels typically range from 0.147 to 12 μg/L (1.47×10^{-4} to 0.01 mg/L) in the U.S. and 0.01 to 44.65 μg/L (1×10^{-5} to 0.04 mg/L) throughout Asian rivers (Flint et. al., 2012; Lee et. al., 2013). While BPA exposure is not regulated in the US, a threshold of 100 μg/L (0.1mg/L) could be suggested as an allowable drinking water exposure when calculated using the U.S. Environmental Protection Agency uncertainty factor guidance (Willhite et al. 2008). Fish such as rainbow trout and zebrafish quickly process and eliminate BPA from bodily tissues, however, their constant re-exposure to BPA and its environmental persistence means it is consistently present within aquatic biota (Lindholst et al. 2003).

In human and animal models, BPA exposure at a wide range of chemical concentrations has a linear dose effect in cardiac electrophysiology (Posnack et al. 2105), hypertension (Aekplakorn et al. 2015), metabolic syndrome (Volberg et al. 2013), and

reproductive function (Grasselli et al. 2010). BPA sometimes also has non-linear dose responses, adding another level of complexity to its adverse effects (Rubin et al. 2017; Welshons et al 2003; Jones and Watson 2012), and BPA mainly affects aquatic life via estrogenic effects (Corrales et al, 2015; Flint and Wallace, 2012). For example, BPA is an estrogen agonist that disrupts the endocrine system by binding to estrogen receptors (Flint and Wallace, 2012; Pintoa et al. 2019), inducing pathways that lead to numerous effects. For example, developmental BPA exposure increases aromatase expression in the zebrafish brain which subsequently leads to behavioral changes (Le Fol et al. 2017, Cano-Nicolau et al. 2016). Developmental exposure to BPA in zebrafish larvae results in teratogenic effects such as axial muscle damage at lower concentration ranges, cardiac edema, and spinal malformations (Wang et al. 2013; Moreman et al. 2017).

Exposure to BPA may influence the chance of survival of aquatic organisms in a changing world because low dosages of BPA can result in hyperactivity in zebrafish larvae, learning deficits in adults, increased aggression between males during courtship, and disruptions in color preference patterns (Saili et al. 2011, Li et al. 2017). Novel or changing environments expose animals to diverse stressors that likely require coordinated behavioral and physiological responses. Boldness, or an organism's ability to evaluate and approach new situations, is an important behavioral strategy that ensures their survival in novel or altered environments. Boldness is a measure of an individual's propensity for risk taking and has an ecological significance because an increase in boldness means that an organism is more likely to explore and identify suitable sites in new altered environments (Wilson et al. 1994). Boldness is linked to motor and cognitive

skills and since BPA can create hyperactivity and learning deficits (Saili et al. 2011, Li et al. 2017), BPA may have negative impacts on boldness of individuals too. Attuned metabolic functions are another important factor that determine the success of individuals in novel or altered environments (Norin and Metcalfe, 2019) because they determine how much energy can be allocated for other functions such as foraging, mating, and predator evasion. However, BPA can influence an animal's ability to adjust metabolic functions according to the demands of a changing environment because of its role in metabolic syndrome (Volberg et al. 2013) and estrogen-mediated pathways of metabolism (Choi et al. 2016).

In this study, I examined the effects of environmentally relevant concentrations of BPA on metabolism and boldness, two strategies essential to cope with environmental change. I selected adult zebrafish (*Danio rerio*) as my study system. Zebrafish are small cyprinid fish native to India, where BPA surface water levels range from 38.3 to 14,800 ng/L (3.83×10^{-5} to 0.015 mg/L) throughout its rivers, lakes, and canals (Lalwani et al. 2020). I focused on environmentally relevant concentrations to highlight the potential impacts of BPA levels that could be considered "safe." I used two concentrations of BPA: a Low BPA concentration of 0.02 mg/L was near the "safe" drinking water limit (Willhite et al. 2008) and tests its effects near the upper range of BPA occurrence in Indian surface waters, while the High BPA concentration of 0.2 mg/L was 1-fold above the Low concentration. While studying exposure during early development is critical, organisms such as fish face the majority of their exposure to pollutants during adulthood mainly due to their high mobility so their exposure levels may fluctuate. Therefore, I used

a short-term exposure, i.e., 7 days on adult zebrafish. Here, I first examined the effects of BPA exposure on maximum oxygen consumption rate and boldness before testing other parameters such as activity level, visual perception, and physical size to understand the underlying biases of responses that I observed.

MATERIALS AND METHODS

Study subjects and housing

I used 270 xx wild-type adult male and female zebrafish from Rawlins Tropical Fish Farm in Lithia, Florida, United States. I housed zebrafish in 20xxx mixed-sex groups of 14 fish each in 8 L tanks. These tanks were connected to a Tecniplast Zebtec Active Blue® standalone system that uses reverse osmosis (RO) water set to standard conditions (28 ± 1^0C, 400 μS electrical conductivity, pH 7.4, 10% daily water exchange, and 14:10 light: dark photoperiod). I fed adult fish with commercial flake food (Tetramin Tropical®) twice daily prior to experimentation and once daily during BPA exposure and trials. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Arizona State University (Protocol 20-1742R).

Treating Zebrafish with BPA

I prepared 1000x concentrated stock solutions of Bisphenol-A (99% purity, Sigma-Aldrich) dissolved in 0.1% dimethyl sulfoxide (DMSO, >99.9% purity, Sigma-Aldrich). I exposed fish to one of three treatment concentrations: 0.2 mg/L Bisphenol-A

(*High BPA*), 0.02 mg/L Bisphenol-A (*Low BPA*) or 0.1% DMSO (*control*) in 20 L glass tanks. I added 10 mL of 200 mg/L BPA stock solution to 10 L of water to make the High (0.2 mg/L) BPA concentration, 10 mL of 20 mg/L BPA stock solution to 10 L of water to make the Low (0.02 mg/L) concentration, and 10 mL of DMSO to 10 L of water to make the control treatment (0.1% DMSO). I separated healthy, adult zebrafish into 15xx groups of 18 among treatments composed of equal numbers of males to females and fed them once daily during the experiment. Every day, I replaced 50% of the treatment water with fresh Tecniplast® system water and mixed in 5 mL of the stock solutions to maintain treatment concentrations. On the seventh day, I ended the treatment exposures by immersing each group of fish twice for 10 min in separate 10 L glass tanks using 5 L of Tecniplast® system water.

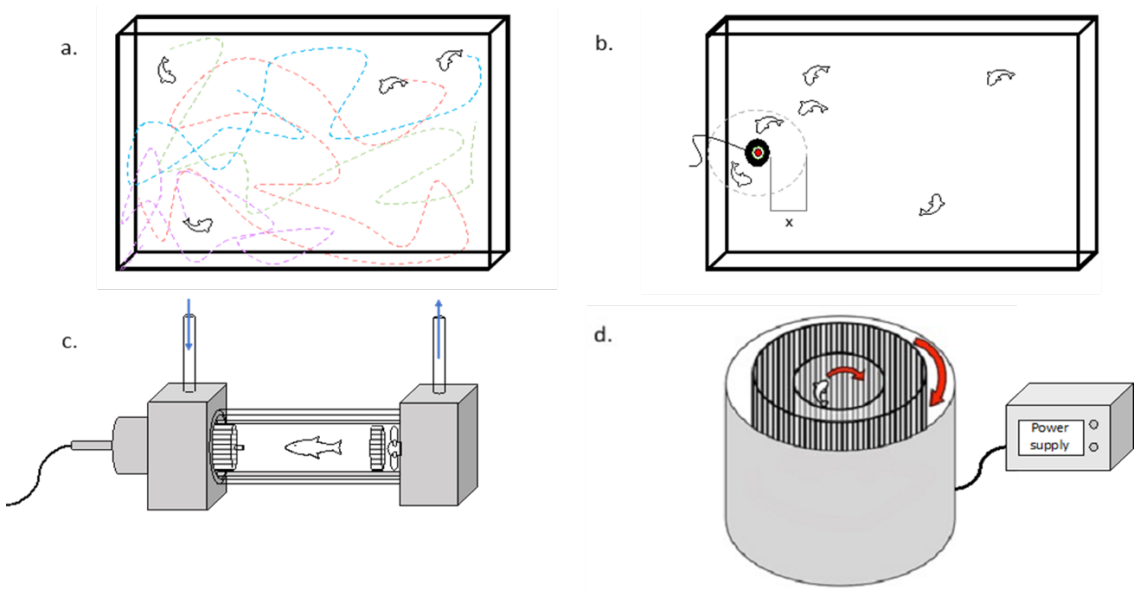

Figure 1: Assay schematic after exposing zebrafish to High BPA, Low BPA or control treatment. (a) Open arena used to test activity level by tracking the distance travelled by four

fish within 2 minutes using EthoVision® tracking software. (b) Arena used to test boldness by introducing a novel object and counting the number of individual fish that approached within 3 seconds. (c) Loligo Systems® swim tunnel used to test maximum metabolic rate of fish by measuring their oxygen consumption after swimming to exhaustion. (d) Optomotor response apparatus that features a rotating drum with interchangeable spatial frequencies. Fish show a positive response by swimming in parallel with the rotating drum.

Evaluating activity level and boldness

After ending treatment, I separated fish into 45 mixed-sex groups of 6 individuals among 20 L glass tanks holding 5 L of water and held them there overnight, totaling 15 groups per treatment. I covered the tanks on all outer sides with white plastic (Hoffmaster 114000 Plastic Table cover) and obscured the underside with white corrugated plastic (Plaskolite Corrugated Plastic Sheet). I began trials at the onset of lights by recording from above the baseline behavior of each fish group for 3 min using a webcam (Logitech HD Pro Webcam C920).

I used EthoVision XT10® (Noldus Information Technology 2013) software to track the zebrafish automatically from the video recordings (Fig.1a). The software determined the x and y coordinates of each individual every 0.03s (1790 moments/min). I then used those coordinates to calculate the distance traveled within 2 minutes. I used *Distance Traveled* as a measure of activity level from these initial recordings. EthoVision® tracked all 6 fish well in the test arenas.

I used one-way analysis of variance (ANOVA) to test the effects of different treatment conditions on the distance traveled by zebrafish. I did all calculations using the *aov* function in R (R-Development-Core-Team 2020), checking residuals for each model to confirm that the usual ANOVA assumptions were not violated.

After recording baseline behavior of zebrafish, I performed the novel object test by dropping a 1.25 cm (~ 0.6 body length) black bead with a red and white circle resembling an eye (Fun-Weevz Assorted Glass Beads) on one end of a string into one side of the tank (Figure 1b). I recorded their approach behavior to the novel object using the webcam mounted above the arena.

I counted the number of individuals out of each group of 6 that approached the novel object within one body length, within 3 s of the introduction of the novel stimulus. I used this count to identify the number of groups in which more than 50% (i.e. > 3 individuals) approached the novel object and performed Fisher's exact test to compare the number of groups meeting this criterion between each treatment and the *Control* group. I performed Fisher's exact test using *fisher.test* function in R (R-Development-Core-Team 2020).

Examining maximum metabolic rate and physical size

After the activity and boldness assays, I randomly selected one individual from each group (N = 15 for each treatment) for measures of maximum metabolic rate using a previously established protocol (Jayasundara et al. 2015). This set up consisted of a

modified Blazka-type swim tunnel respirometer (170 ml) inside a 20 L buffer tank, with a DAQ-M control device, AutoResp™ 1 software, and a fiber optic oxygen respirometer (Loligo Systems, Tjele, Denmark) (Suriyampola 2021). I maintained the buffer tank at 28°C and did not feed fish for 24 h before the experiment.

I used a funnel and tubing to move fish gently into the swim tunnel, where they acclimated for 5 min at a flow rate of 1 cm/s (0.5 body length/s) preventing the formation of hypoxic pockets. During acclimation, I used the computer to capture an image of the mid lateral view of the fish facing left. I used a ruler to measure the on-screen length of the fish from the tip of the snout to the posterior end before the caudal fin and measure 10 cm reference markings on the swim tunnel itself. I used the 10 cm reference markings to convert the on-screen measurement of the fish to its actual standard length.

I gradually increased the flow rate to 10 body length/s as determined by their standard length and maintained this speed for 10 min or until the fish collapsed, whichever occurred first. I defined "collapsed" as when the fish fell against the swim tunnel's honeycomb for two consecutive seconds and was unable to recover its upstream swimming position. If the fish did not collapse during the first 10 min of swimming, I increased the flow rate for an additional 10 min, repeating this procedure until fish collapsed. Upon fish collapse, I lowered the propeller speed to 1 cm/s (0.5 body length/s) and recorded oxygen levels during a 5-minute period at 1-minute intervals. I subtracted each reading from the initial reading taken immediately after collapse, divided by the time elapsed, and selected the largest reading as the maximum metabolic rate

(Jayasundara et al. 2015). I weighed the fish immediately after removal from the swim tunnel by using a Radwag AS 220.R2 analytical balance.

To test whether whole-organism metabolism differs between fish from different treatments, I fit an ANOVA model, testing the effect of BPA treatment on whole organism metabolic measures using the aov function in R (R Development-Core Team, 2020), and checking residuals to confirm that the usual ANOVA assumptions of homoscedasticity and normality were not violated.

Testing optomotor response

I used a circular optomotor response assay to test for differences in the reflexive visual response due to changes in underlying physiology by BPA (Suriyampola et al. 2018). I randomly selected another individual fish from the activity and boldness assay (N = 15 for each treatment) to place in a small, clear acrylic cup (7.7 cm diameter) with 2 cm of water, and placed the cup in the translucent central chamber of a circular drum (Maaswinkel & Li, 2003). The drum was lined with a printed image of black and white vertical bars that rotated clockwise, as powered by a Bodine® 24 V motor. I kept the speed of rotation at a constant 34 rotations/min and varied the band width of the bars from 0.25 cm to 1.25 cm, presenting to each fish six spatial frequencies in random order

for 20 s each. I illuminated the optomotor drum from above using LED lighting (between 1946 lx and 2073 lx).

I manually scored a positive response or nonresponse for individual fish for each of the six bandwidths. I defined a positive response as the fish swimming at least two consecutive circular turns within 20 s in the direction of the stimulus. I considered all other responses, including random swimming, being stationary and antiparallel swimming, as nonresponses (Suriyampola et al. 2018).

I used logistic regression to test for differences between treatments (X1) in terms of the proportion of fish giving a positive response (Y), while also including a repeated-measures factor for bar width (X2) since I measured each fish at six spatial frequencies. I implemented this repeated-measures logistic regression using the '*lme4*' package (Bolker & Walker, 2014) in the R statistical package (R Development-Core Team, 2020).

RESULTS

BPA exposure and maximum metabolic rate:

Zebrafish exposed to low BPA levels experience a lowered maximum metabolic rate

Using linear regression, I found that BPA treatment condition was a significant predictor of maximum metabolic rate (Fig. 2: $F_{2, 21} = 6.23$, $P = 0.01$). Zebrafish exposed to Low BPA, i.e., 0.02 mg/L, consumed less oxygen at the whole organism level (mean

oxygen consumption: 8.1 ± 0.35 mg O2/g (h)) after swimming to exhaustion. In comparison, the oxygen consumption rate of zebrafish exposed to the High BPA concentration, i.e., 0.2 mg/L, (mean oxygen consumption: 7.0 ± 0.18 mg O2/g(h)) was relatively similar to that of zebrafish in the control group (mean oxygen consumption: 6.8 ± 0.25 mg O2/g(h)). I found a significant difference between the oxygen consumption of zebrafish in the Low BPA group compared to the control ($t = -2.85$, $P = 0.01$) but the oxygen consumption of zebrafish in the High BPA group did not differ from those in the DMSO control ($t = 0.39$, $P = 0.70$).

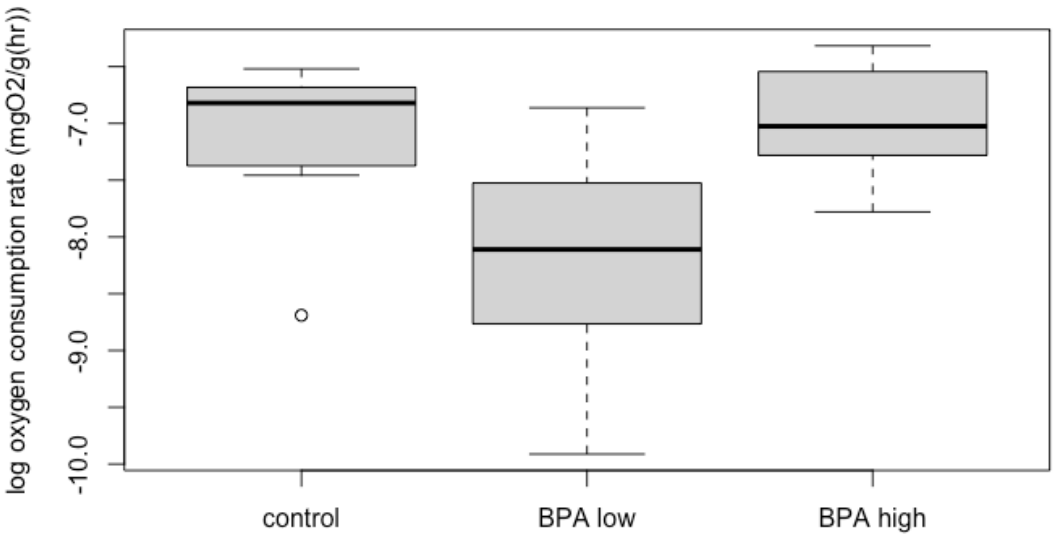

Figure 2: Maximum Metabolic Rate of Zebrafish. The log of the oxygen consumption rate of zebrafish corrected by mass. The fish exposed to low levels of BPA (0.02 mg/L) had the lowest maximum metabolic rate after swimming to exhaustion. In comparison, the fish exposed to higher level of BPA (0.2 mg/L) maintained a similar maximum metabolic rate as did zebrafish in the control group.

BPA exposure and Boldness:

Zebrafish exposed to low and high BPA levels were less bold than control fish

When I compared the number of groups with more than 50% of individuals approaching the novel object as a measure of boldness, I found that only 2 of 15 groups of fish exposed to the High BPA concentration, i.e. 0.2 mg/L, approached the object within one body length. This yielded a significant difference compared to the control treatment, in which 8 of 15 groups had more than 50% of individuals approaching the novel object (Fig. 3: Fisher's exact test: P = 0.02). However, with the Low BPA treatment, i.e. 0.02 mg/L, I did not see a significant difference in the number of groups (7 of 15 groups) with more than 50% of individuals approaching the novel stimulus compared to the control group (2 of 15 groups; Fisher's exact test: P = 0.50).

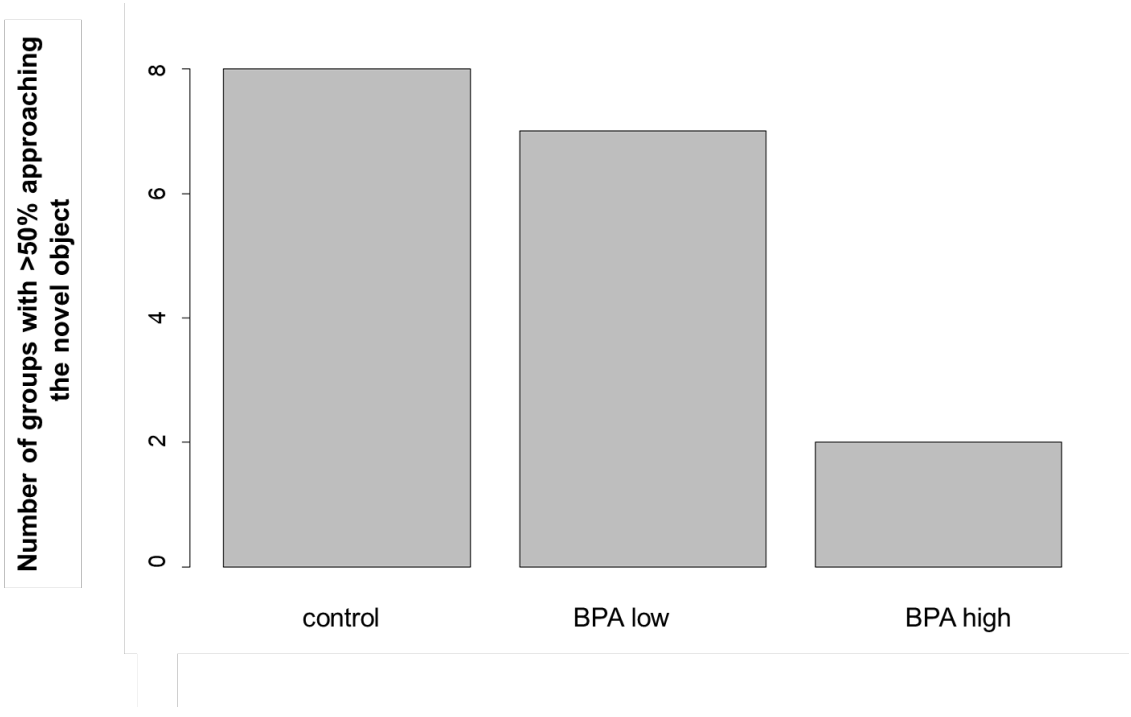

Figure 3: Boldness of zebrafish. Bar plot showing the number of groups with more than 50% of individuals approaching the novel object in one body-length distance within 3 s. Fish exposed to

High concentration of BPA (0.2 mg/L) displayed a significant decline in approach behavior, which was the measure of boldness.

BPA exposure and activity level:

Zebrafish exposed to different amounts of BPA did not differ in activity level

Zebrafish in both Low and High BPA treatments were somewhat less active compared to fish in the control group. The median distance traveled for the control group was 7128.3 cm. The median distance the Low BPA group (0.02 mg/L) traveled was 6874.7 cm and the High BPA group (0.2 mg/L) traveled a median of 6217.0 cm. However, these differences were relatively small in comparison to their variability such that the difference in activity level as measured using distance traveled was not statistically significant (Fig. 4: $F_{2,42} = 0.56$, $P = 0.57$).

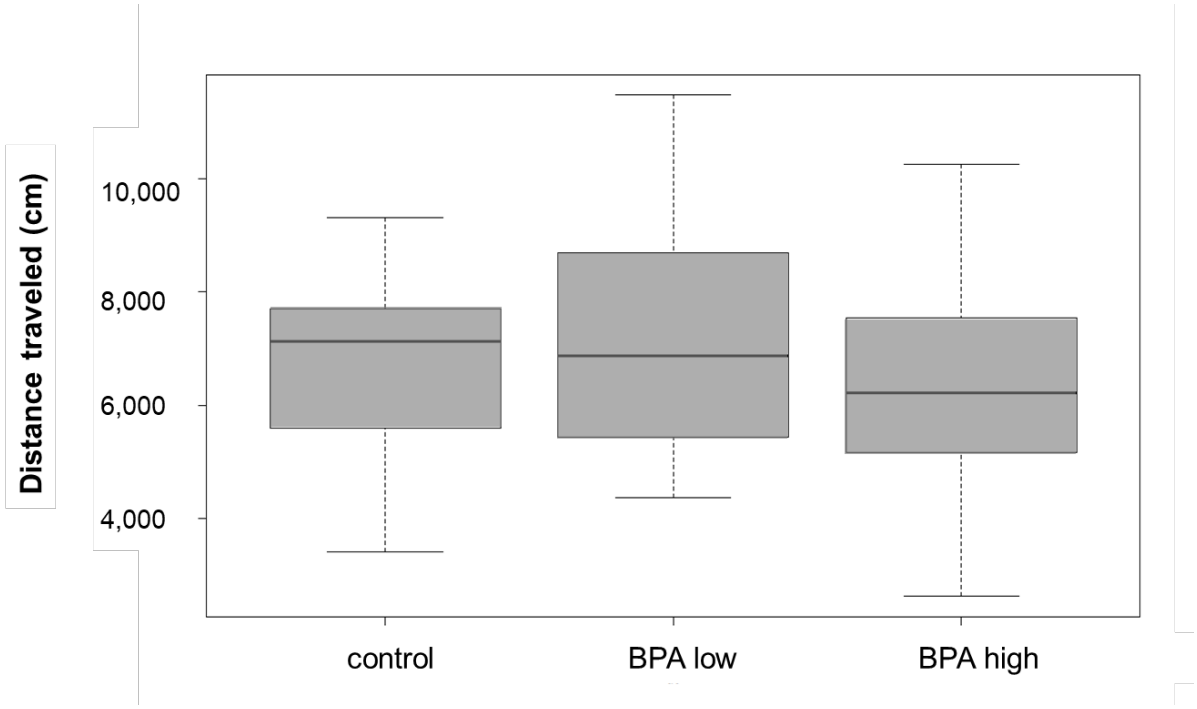

Figure 4: Distance traveled by zebrafish. Box plot showing the distance traveled of individual fish in each treatment group as tested in an open arena in centimeters. The distance traveled was tracked using EthovVision® tracking software.

BPA exposure and visual response:

Zebrafish exposed to different amounts of BPA did not differ in visual response behavior

Regardless of the treatment, zebrafish responded to the visual stimulus by swimming in the direction of the moving black and white bars. This response increased as the band width increased from 0.25 cm to 1.25 cm and repeated-measures logistic regression revealed that this effect of band width is statistically significant (Fig. 5: $z = 4.78$, $P < 0.001$). However, the exposure to BPA for 7 days did not significantly affect the response to the moving visual cue in fish exposed either to low ($z = 0.22$, $P = 0.82$) or high concentration of BPA ($z = 0.02$, $P = 0.98$).

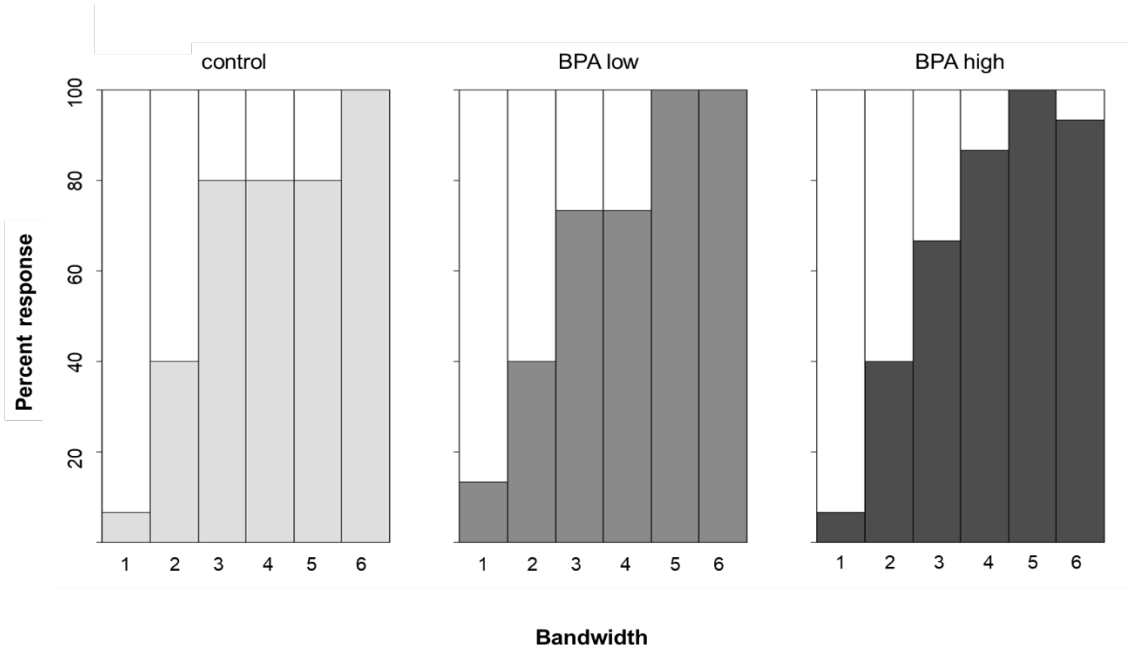

Figure 5: Optomotor response of zebrafish. Bar plots showing the percentage of individual fish that responded to optomotor test. Each bar represents the percentage of fish that responded to each bar width and the optomotor assay was performed using a rotating drum apparatus.

BPA exposure and body size:

Zebrafish exposed to different amounts of BPA did not differ in the body size of adult zebrafish

Although zebrafish that experienced High BPA concentration (0.2 mg/L) were slightly shorter (median standard length: 3.0 cm) than the fish either in the Low concentration (0.02 mg/L) or control group (median standard length in both treatments: 3.1 cm), this difference was not statistically significant in the linear regression ($F_{2,21}$ = 0.90, P = 0.42). I also found a slight decrease in body weight (median body weight: 442.4 g) in fish that were exposed to High BPA concentration compared to fish in the control group (median body weight: 458.1 g) or fish that were exposed to Low concentration of BPA (median body weight: 460.2 g). These differences resulted no statistical significance using linear regression ($F_{2,21}$ = 0.18, P = 0.84).

DISCUSSION

Maximum metabolic rate was significantly lower for the Low BPA group while the High BPA group remained similar to the control. However, boldness showed the opposite result, where zebrafish in the High BPA group were less bold than were

zebrafish in the low BPA group, which was similar to the control. These results suggest that linear or nonlinear result patterns when investigating BPA depend on what is being assessed. My results also reveal that even very low levels of BPA could alter adult organisms' behavior, affecting their chances of survival particularly in changing environments.

Metabolism is an important part of survival in the wild because fish need to be able to adapt to changes in water flow, escape from predators quickly, forage, and migrate, among other activities that can be affected by metabolism (Plaut 2001). However, higher metabolic rates have not been considered to be cost-effective and thus, animals in unpredictable environments tend to maintain low basal metabolism to offset energetic costs associated with environmental variability (Lovegrove, 2000; Norin & Metcalfe, 2019). In this study, I found that fish in the Low BPA group had lower maximum metabolic rates than did fish in the High BPA group. This suggests that zebrafish in the Low BPA group required less oxygen when swimming to exhaustion. Maximum metabolic rate is an overall measure of an organism's athleticism because it indicates the maximal rate at which oxygen can be transported from the environment to the mitochondria (Norin and Clark, 2015) and thus can affect foraging, reproduction, predator evasion and habitat navigation (Plaut 2001). Maximum metabolic rate also sets the upper limit for aerobic capacity, which in turn impacts the ability of animals to perform well in a changing environment (Norin and Clark, 2015). Our results show that fish exposed to low BPA levels have lower maximum metabolism, signifying that their

oxygen transportation to mitochondria is slow, which could make it more difficult for them to respond appropriately to environmental change.

BPA increases adipose tissue and decreases muscle mass via cell apoptosis in trunk musculature of zebrafish larvae (Wang et al. 2013), which may explain the declined oxygen consumption rates with BPA exposure. Although I did not examine adipose or muscle tissues, the lack of difference in overall body mass across treatment groups suggest that lower maximum oxygen consumption could have been driven by a different pathway other than the decrease in muscle mass. However, if fish did experience a decrease in muscle mass but also experienced an increase in adipose tissue, the whole-body mass may mask such changes in ratio and these changes would not appear in body mass measures that I collected. It is also possible that the short exposure time and the weak BPA concentrations that I used may be insufficient to create a detectable difference in body mass across treatments. Ecological factors also play a role in aerobic scope and it may be that these fish have a lower maximum metabolic rate than previous reporting because they were raised domestically and have mostly experienced stagnant water in comparison to their wild counterparts (Killen et al. 2016). These fish are also smaller than other lab raised fish fed a similar diet, which has been found to increase lipid composition (Fowler et al. 2019). These differences in size and lipid to muscle composition may lead to an overall decreased metabolic rate in all treatment groups, explaining why our numbers are smaller than what has been reported previously (Killen et al. 2016, Yuan et al. 2018).

Boldness also impacts survival because it influences exploratory behavior, which is critical for finding food resources, potential mates, and avoiding predators (Chapman et al. 2011). I found that fish exposed to high BPA concentrations were less bold in a novel object assay than were those exposed to low BPA concentrations or in the control group. Responding to a novel object could depend on their ability see the object as well their propensity to approach it. With the optomotor assay, I found that the ability to respond to a moving visual signal is similar across all three groups suggesting that there are no differences in visual capabilities of fish from treatments. I also tested the distance traveled to determine the activity level and motivational status of fish. Again, I found no difference in response across treatment groups suggesting that the decline in boldness was not triggered by differences in activity level or motivation status of fish. Lower levels of boldness as shown here could be due to an increase in anxiety. For example, zebrafish that were exposed to low levels of BPA (50 ng/L, or 5×10^{-5} mg/L) for 7 weeks spent more time in the lighted compartment while exploring a novel environment, which is an indication of increased levels of anxiety (Li et al. 2017). BPA lowers serotonin levels in the brain and decreases tyrosine hydroxylase expression, an enzyme that converts tyrosine to dopamine in the hypothalamus, resulting in elevated anxiety (Li et al. 2017), which could have accounted for the effects found in this study. However, Li et al. 2017 found that increased anxiety is coupled with a decrease in general locomotion and velocity, and this change was most significant at the 50 ng/L (5×10^{-5} mg/L) BPA exposure level but changes in locomotion were not found here at either Low (0.02 mg/L) or High (0.2 mg/L) exposures. Increased anxiety levels may slow an organisms' ability to adjust to changes in their natural habitat. The natural habitats of zebrafish undergo drastic

changes in water level and flow rate every year with the monsoon season (Bhat et al. 2015) and thus constraints on rapid adjustments to environmental changes could be detrimental for survival.

The decreases in boldness may be due to a feminization of traits by BPA, because wild female zebrafish are generally less bold than their male counterparts (Roy & Bhat, 2018). Previous work has found that low dose perinatal BPA exposure can reverse sex differentiated behavior by increasing depressive behavior in male rats and increasing anti-anxiety behavior in female rats (Chen et al. 2014). This reversal occurred after a mild stressor in which male rats showed an increase in corticosterone mRNA while female rats had increased glucocorticoid receptor mRNA. The changes in these hormonal levels after the mild stressor led to a hyperactive HPA axis in males and a hypoactive HPA axis in females (Chen et al. 2014). Similarly, another study that exposed male and female mice to BPA concentrations of 0.4 – 40 mg kg^{-1} per day for 12 weeks found that males experienced decreased testosterone in the brain and increased anxiety behaviors when evaluating their exploratory behavior and stress responses. However, female rats showed a reduction in anxiety behavior during these evaluations and did not experience changes in estrogen levels (Xu et al. 2015). Furthermore, males tend to associate more closely with their shoals than females do. Male association is stronger when shoals contain females, as was the case here, and in the presence of predators due to risk, which the novel object assay evaluated here by elevating the risk level for investigating fish (Roy & Bhat, 2018). Here, it is possible that similar action on the HPA axis and signaling may be

feminizing boldness behavior in males, skewing the results of the mixed-sex groups to appear as if BPA exposed fish were overall less bold.

The boldest fish among the three treatments were in the control group and these fish also had the highest metabolic rate, exhibiting a possible link between boldness and maximum metabolic rate, i.e. more "athleticism". This is similar to previous findings of in which proactive (bolder) zebrafish displayed more aggression, less anxiety and higher standard and maximum metabolic rates than reactive (shy) zebrafish (Yuan et al. 2018). These differences in personality translated to their survival response where proactive fish reacted more quickly to change in salinity, a stressor that could be life threatening in the wild (Yuan et al. 2018). A quick response is needed to successfully evade other stressors such as predators or habitat changes, making reactive (less bold) fish more vulnerable (Yuan et al. 2018). However, BPA exposure disrupted this pattern between aerobic scope and bolder fish because both exposed groups showed a mismatch between boldness and metabolic rate. Since boldness and aerobic metabolism are both critical aspects for survival and navigating changing environments, the effects of BPA may greatly decrease the chances of navigating environmental change, predator evasion, migration, foraging, reproduction and aerobic recovery in the wild making zebrafish more vulnerable to evading risks.

One of the interesting findings of this study is the differences in dose impacts on different survival strategies. I found that the impact of BPA on metabolism was not dose dependent but its impact on boldness was. Although maximum oxygen consumption rate

was significantly lower for fish treated with weak BPA, high BPA concentration had no impact on it suggesting the non-linear or nonmonotonic effects of BPA on metabolism. Similar patterns are not uncommon in regard to endocrine disruptors, including BPA, challenging the traditional concept of toxicology and the establishment of 'safe' doses of exposure (Vandenberg et al. 2012). Generally, natural hormones act at extremely low concentrations, displaying nonlinear relationships between hormone concentration and the number of bound receptors, and between the number of bound receptors and the maximum biological effect (Welshons et al. 2003). Hormone-mimics such as BPA also follow the same mechanisms when functioning to produce significant biological effects at relatively low concentrations. However, the influence of BPA is more cofounding as I did not see similar nonmonotonic dose-response on boldness of adult zebrafish. Here, fish exposed to high BPA concentration were less bold than were fish in control or low BPA groups. It is possible that BPA tends to have nonmonotonic effects on physiological traits such as metabolism but dose-dependent effects on behavioral responses. Future investigations should focus on understanding which traits are likely to display monotonic or nonmonotonic does-responses when exposed to BPA and other hormone-mimics.

Overall, this study reveals the complex pathways through which common pollutants may affect survival strategies of organisms, which could be pivotal in keeping up with environmental change. Adult zebrafish exposed to environmentally relevant concentrations of BPA for 7 days experienced declined oxygen consumption rate and boldness, which could adversely affect when coping with environmental change. This study also reveal that BPA could have nonmonotonic as well as monotonic dose

responses on different survival strategies, challenging the traditional concepts of toxicology and ‘safe’ doses. It also suggests that no level of BPA is safe, not even a low level, and highlight the need to investigate concentrations that are deemed safe to comprehensively understand the impacts of common pollutants.

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