Abstract

Sub-lethal toxic effects of Bisphenol A on *Caenorhabditis elegans*: the role of stress resistance genes in BPA-induced growth inhibition

by Zailene Flood

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Director: Xiaoping Pan

DEPARTMENT OF BIOLOGY

Endocrine disrupting chemicals (EDCs) have become of concern for a variety of health issues. Bisphenol-A (BPA) is a widely studied EDC and has been characterized by its estrogen-like effects. BPA is a prevalent component in polycarbonate plastics, having one of the highest production volumes in the world. Humans are exposed to BPA in products such as water bottles, dental sealants and on the inside of food and beverage cans. Due to frequent human exposure to BPA, research on the resulting biological effects is highly significant. In this study, we utilized the model organism *Caenorhabditis elegan*s (*C. elegans*) to investigate potential impacts of BPA on growth, reproduction, locomotion and feeding behaviors and gene expressions. At dosage of 1.0 µM of BPA exposure from L1 stage to adulthood, the worm's body size was significantly reduced. To test the hypothesis that BPA exposure may associated with obesity risk, we conducted Oil Red O staining to test fat storage in worms exposed to 0.1 and 1.0 µM of BPA exposure from L1 stage to adulthood, using glucose as a positive control. However, our results show that the body fat storage decreased when exposed to BPA at both tested concentrations. For behavior assays, worms experienced a decrease in locomotion speed when exposed to high concentration of BPA (1.0 µM) and a stimulation of locomotion speed at low concentration BPA exposure (0.1 µM). Attainment levels of worms were significantly decreased in the high concentration treatment group. At the first 8 hrs of observation, less than 10% of 1.0 µM of BPA treated worms were able to reach the food source. Additionally, high BPA exposure (1.0 µM)

decreased egg productions throughout the egg-laying period. The brood size of 1.0 μM treated worms was reduced to 7% of control. In contrast, low BPA treatment group (0.1 μM) significantly increased early egg laying between 65-96 hr period after L1. The expression of 3 (*egl-10*, *sod-1* and *old-1*) selected genes were affected significantly. Changes in gene expression were more evident at high dosage than at the relatively low level. *Egl-10* and *old-1* genes implicated in egg-laying and stress resistance was upregulated by BPA. *Sod-1* associated with protecting cells from oxidative damaged was down regulated. Other tested genes were *cat-4, egl-5, egl-19, egl-44, egl-46, egl-47, pink-1, age-1, old-1* and *ric-3*. Together these results suggest BPA-associated reproductive toxicity and neurobehavioral deficits.

Sub-lethal toxic effects of Bisphenol-A on *Caenorhabditis elegans*: the role of stress resistance genes in BPA-induced growth inhibition

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Zailene Flood

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Zailene Flood

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by

Zailene Flood

APPROVED BY:

DIRECTOR OF DISSERTATION:____

Xiaoping Pan, PhD

COMMITTEE MEMBER:____

Baohong Zhang, PhD

COMMITTEE MEMBER:____

Mary Farwell, PhD

COMMITTEE MEMBER:____

Myon-Hee Lee, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY:

____

Jeff McKinnon, PhD

DEAN OF THE GRADUATE SCHOOL:

____

Paul J. Gemperline, PhD

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INTRODUCITON: PROJECT DESCRIPTION

Nature of the Problem

Bisphenol A, a chemical widely used to make polycarbonate plastics, is a growing public concern regarding its potential effects on the brain, behavior and reproduction. BPA acts as an endocrine-disrupting compound that mimics the naturally occurring estrogen hormone (Fig.1). It can interfere with hormone synthesis, hormone receptor expression and alter gene activities in target tissues (Diamanti-Kandarakis et al., 2009). The primary source of exposure to bisphenol A is through the diet (Shelby, 2008). This is alarming due to the fact that general population exposer to BPA occurs on a daily basis. My goal is to discover what potential consequences may arise in the future after increasingly recurrent exposure to BPA.

More than 1 million pounds of BPA are released into the environment annually (Shelby, 2008). In particular, due to physical properties such as durability, transparency and weightlessness, BPA containing plastics are found in a variety of common household products. Common uses include food and beverage cans, dental sealants, sunglasses, compact disc and baby bottles (Li et al., 2013). It is equally important to understand how safety and human health are impacted as a result of prolonged exposure. The National Toxicology Program (NTP) expressed concern ranging from “negligible to some concern” for potential exposures (Shelby, 2008). The FDA’s current assessment is that BPA is safe at low levels (Shelby, 2008). To address uncertainties about the safety of BPA more research is needed to determine if BPA poses a risk to human health.

Literature Review

The scientific literature evaluating the toxic effects of bisphenol A in laboratory animals are expanding (Shelby, 2008). However, only a very small number of studies have looked at associations between bisphenol A exposure and disorders of reproduction or developmental effects utilizing *Caenorhabditis elegans* as a model organism.

Two previous studies done by Allard et al and colleagues helped to gain insights into the effect of BPA on reproduction by exposing worms to BPA medium, delivering a continuous high dose of BPA throughout the worms development and reproductive periods (Allard & Colaiacovo, 2010; Allard & Colaiacovo, 2011). Results revealed that an exposure to 1 mM BPA in the plate medium corresponds to an internal concentration of 2 µg/g (2ppm) detected from worm extract (Allard & Colaiacovo, 2011). This is the range of the internal concentrations reported for several rodent models and human serum levels following occupational exposure. A marked reduction in the number of eggs laid and embryonic viability were observed suggesting meiotic impairment (Allard & Colaiacovo, 2011). To address reproductive impairments, four parameters were tested: BPA concentration (100 µM, 500 µM, or 1 mM), vehicle (DMSO or ethanol), cholesterol concentration (5µg/mL, 0.5µg/mL, or none), and culture method (liquid or plate). Interestingly, cholesterol is thought to mask the effect of endocrine-disrupting chemicals in C. *elegans* and was omitted from the medium. The most consistent results were low overall toxicity, as judged by both growth and behavior, was seen in worms exposed to BPA dissolved in ethanol at a final concentration of 1mM for 4 days (Allard & Colaiacovo, 2010). However, a six fold reduction in the mean number of eggs laid and a dramatic increase in embryonic lethality was observed (Allard & Colaiacovo, 2010). The mechanism of reproductive toxicity of BPA is still unexplained, but was suspected to be related to hormonal activity. A 2009 study suggested that exposure of placental cells to low doses of BPA ranging from 0.0002 to 0.2 µg/ml may cause

detrimental effects in association with high blood pressure, intrauterine growth limitations and miscarriages (Benachour & Aris, 2009).

Kohra et al exposed two and half-day old worms to 10 and 0.1 μM BPA and recorded attainment levels (the number of worms reaching the food source divided by the total number of worms on the Petri plate) at 2,4,6,8 and 24hrs. Additionally, experimental plates of two controls, one containing only medium and the other containing dimethyl sulfoxide (DMSO), were tested. There was no evidence of altered attainment levels between the two controls. However, at 10 μM treated group the attainment levels decreased significantly compared to the untreated control in all observations (Shinya et al., 2002). Similarly, at a lower concentration of 0.1 μM group, the attainment levels decreased significantly at 2, 4, 6 and 8 hrs (Shinya et al., 2002). Comparing attainment levels between both concentrations showed no significant difference. Thus foraging behavior of C. *elegans* in response to the environment is an interesting test endpoint for chemical hazard assessment.

Emerging evidence has linked BPA to the worldwide obesity epidemic, particularly among children. Obesity is known to result in several adverse health effects including, but not limited to type 2 diabetes, insulin resistance, hypertension, coronary heart disease and liver and kidney disease (Li et al., 2013). In countries with differing dietary styles and physical activity, the increasing prevalence of obesity suggests the existence of exposure to environmental risk factors, collectively termed "environmental obesogens." One such important potential obesogen is BPA. Exposure to BPA has been shown to suppress the release of adiponectin, an adipocyte-specific hormone that increases insulin sensitivity (Li et al., 2013).Therefore, there is a biological plausibility that BPA could lead to insulin resistance and increased susceptibility to obesity and metabolic syndromes. In a recent study, Li et al conducted a population-based study to examine the relationship between urine BPA and obesity in school-age children. Spot urine

samples and anthropometric measurements were collected. Results indicated high urine BPA levels (≥2 μg/L), associated with overweight female student's ages 9-12, but not in males (Li et al., 2013). BPA is an environmental estrogen which could accelerate females' pubertal development and weight gain. This study provided some evidence for an association between BPA exposure and obesity-related outcomes in childhood. However, literature is limited on this association (Li et al., 2013).

Research conducted by Nomura et al, on *C. elegans* provided a more efficient means of studying the lipid metabolism because of the ease with which fat can be visualized in its body. Nomura and colleagues investigated the ability of *C. elegans* to accumulate body fat following the consumption of excess calories and the mechanism it uses to metabolize fat. The *C. elegans* were grown on media containing glucose (0, 0.1, 2, 5, 10, 50 and 100 mM) or 10 mM of various types of sugars (fructose, mannose, galactose and xylose) and monitored for changes in body fat using the Nile red staining and monitored for the expression of *sbp-1*, which facilitates fat storage in *C. elegans*. After adding either glucose or fructose to culture dishes containing *C. elegans*, there was a considerable increase in fluorescence from the Nile red staining, indicating an increase in intracellular fat (Nomura, Horikawa, Shimamura, Hashimoto, & Sakamoto, 2010). Additionally, *sbp-1* was strongly expressed in the intestine. The fat content started increasing with 2 mM glucose and continued to rise with increasing concentrations of glucose in a dose dependent manner (Nomura et al., 2010). In order to test if s*bp-1* regulates fatty acid synthesis, *sbp-1* knockdown was tested via RNAi feeding and then stained. Results indicated a "clear" or relatively transparent body without fat storage. Nile red analysis indicated a decrease in the amount of fat stored in *C. elegans* also supports findings from other assays which indicated decreased body size and delayed growth. Additionally, the number of eggs laid decreased considerably (Nomura et al., 2010).

More recently in December 2013, Tseng et al and colleagues demonstrated phthalates, a common endocrine disruptor, inducing neurotoxicity affecting locomotion behaviors through oxidative stress in *C. elegans (Tseng, Yang, Yu, Li, & Liao, 2013)*. The most commonly used phthalates are bis(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), and diisobutyl phthalate (DIBP), which are used as plasticizers, solvents, and additives in numerous consumer products, such as vinyl flooring, food containers, cosmetics, pharmaceuticals, and children's toys (Schettler, 2006; Wormuth, Scheringer, Vollenweider, & Hungerbuhler, 2006). For body bend analysis, L4-stage wild-type worms were exposed to various concentrations of commonly used phthalates (DEHP (2 and 20 ppm), DBP (500 and 1000 ppm), and DIBP (100 and 1000 ppm)) for 24 h at 20°C. The results showed that all of the examined concentrations of DEHP, DBP, and DIBP caused significant reductions in the number of body bends compared with those of non-exposed control worms (Tseng et al., 2013). Similarly, a substantial decrease in head thrashing occurred in worms exposed to DEHP (2 and 20 ppm), DBP (500 and 1000 ppm), and DIBP (1000 ppm), compared with non-exposed control worms (Tseng et al., 2013). Moreover, while L4-larval stage nematodes were exposed to DEHP, DBP, and DIBP for 24 h, a significant decrease in reversal frequency was observed in worms in all examined concentrations of DEHP, DBP, and DIBP, compared with the non-exposed control worms (Tseng et al., 2013). When L4-larval stage nematodes were exposed to DEHP at a concentration of 2 ppm, significant ($P<0.001$) body bend, head thrash, and reversal frequency defects were observed, whereas the adult nematodes required higher concentrations of DBP (500 ppm) and DIBP (100 ppm) exposure to exhibit similar defects (Tseng et al., 2013). The results indicate that the endocrine disruptor phthalates DEHP, DBP, and DIBP can cause locomotion behavior defects in *C. elegans* so it will be interesting to determine if BPA affects behaviors as well.

Hypothesis

Exposure to BPA will cause various sublethal effects including growth and reproduction inhibition, abnormal locomotion behaviors, and affect key genes involved in these processes.

Objectives

- Evaluate the impact of BPA exposure on growth and major fat stores
- Investigate locomotion and foraging behaviors following BPA exposure
- Evaluate reproduction rates following BPA exposure
- Analyze the expression of selected genes following BPA exposure and study the gene functions in response to BPA exposure.

METHODS/MATERIALS

Organism

Caenorhabditis elegans are free-living, soil-dwelling, roundworms commonly used in research as model organisms for the study of gene regulation and function (Hubbard & Greenstein, 2005). *C. elegans* were the first multicellular organisms to have their entire genome sequenced with more than 20,000 genes identified (Hubbard & Greenstein, 2005). They have 60-80% homology with the human genome, a short life cycle (approximately 3 days), and life span (approximately 2-3 weeks), and are also easily cultured and maintained. Observing complex developmental processes in *C. elegans* such as embryogenesis and morphogenesis is convenient. Their transparent body and genetic tractability makes them easy to manipulate by adding, removing, or altering specific genes (Fig. 2). As a food source, *C. elegans* use *Escherichia coli* (*E.Coli* OP50), which is easily cultured. *C.elegans* are used as great models for toxicological studies for an extensive amount of environmental toxicants (Leung et al., 2008). *C.elegans* life cycle consist of four larval stages (L1-L4) before reaching the adult stage (Fig.3). Due to high level of conserved genes and gene pathways from *C.elegans* to humans, data collected in *C.elegans* can be indicative for higher organisms. *C.elegans* can be hermaphrodites, males and females and can therefore self-cross fertilize.

Assays

Growth and Locomotion

The synchronized, wild type N2, L1 stage worms were cultured on Nematode Growth Medium (NGM) media including a control and two treatment groups containing 0.1 µM and 1.0 µM BPA, respectively. The treatment last for 2.5 days until adulthood (65 hours), once embryos appeared in the body. Three replicates were performed for each treatment. Two and a half days later, five worms were collected from each of three replicates and transferred onto each of 3 BPA free tracking petri dishes, previously seeded with *E. coli* OP50. Using a worm tracking system, five minute-videos were taken per replicate per treatment and analyzed by the Wormlab software. Nine behavioral parameters were used as toxicity endpoints, including: mean body area, body length, wavelength, body width, bending angle, omega bends, reversals ratio, smoothed forward movement speed and smoothed backward movement speed. Each endpoint is defined based on Wormlab software in Table 1 (MFB, 2010).

Reproduction

L1 worms were exposed to 0.1 µM or 1.0 µM BPA on NGM agar for 65 hr. After 65 h worms were collected and transferred onto new treatment matched petri dishes, previously seeded with OP50. At a fixed time for three consecutive days, the worms were transferred onto new treatment matched plates seeded with OP50 to allow egg laying. On the day of transfer, the eggs and larvae on plate previously occupied by the worms, was counted using a light microscope.

Gene Expression

For gene expression, there were 5 biological replicated for each group (control, 0.1 μM and 1.0 μM BPA). Several plates of wild type N2 worms were synchronized. Over 2,000 L1 worms were plated onto each plate. Both treatment and control plates were incubated at 20°C for ~65 hours (L1-early adult). Each plate was washed several times into 15ml falcon tubes. The tubes were centrifuged and washed 3× with M9 and the supernatant was discarded. Worm pellets were then transferred into 2ml microfuge tubes, centrifuged, frozen with liquid nitrogen and stored in a -80°C freezer until RNA extractions were performed. RNA extractions were performed (see detailed methodology for procedure process) on each of the 15 frozen tubes of worms (5 for control, 5 for low BPA treatment, 5 for high BPA treatment). RNA quality of each tube was measured using the NanoDrop ND-1000 Micro-Volume UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and based on the absorbance ratios of 260/280 and 260/230 (the 3 best replicates were chosen). Next, I performed reverse transcription (see detailed methodology for procedure process) to obtain cDNA using TaqMan microRNA Reverse Transcription kit from Applied Biosystems (Foster City, CA). The RT product was then used to perform qRT-PCR (see detailed methodology for procedure process) on 96-well-plate using the 7300 Real-Time PCR System (Applied Biosystem) using the SYBR Green PCR master mix from SuperArray Bioscience Corp. (Frederick, MD). 13 genes were analyzed: *cat-4, egl-5, egl-10, egl-19, egl-44, egl-46, egl-47, pink-1, sod-1, age-1, old-1, oxi-1, ric-3* using *tba-1* as a reference gene. The detailed description and classification information of these genes is listed on Table 2.

Feeding Behavior

Feeding behavior testing was conducted as described by Kohra (Shinya et al., 2002). L1 worms were cultured on experimental plates consisting of two concentrations (0.1μM and 1.0 μM) of BPA, and two controls, one containing only medium and the other containing ethanol

(EtOH), which the same amount was used as the solvent for BPA. Each plate was previously seeded with *E. coli* OP50. The set-ups were then incubated at 20°C for 60 h, and then the worms were transferred to chemical free 10-cm plates for the feeding behavior assay. On the latter, *E.coli* was grown circularly within a 0.5cm radius from the center of the plate. Each exposed worm was rinsed three times with M9 buffer and transferred one-by-one onto the chemical free plates. Each plate was loaded with 7 worms evenly placed 4 cm from the center. Ten replicates were performed for each treatment (70 worms total per treatment). At time points 2, 4, 6, 8, and 24 hr of incubation at 20°C, the number of worms that reached the central *E. coli* colony was counted using a light microscope. The attainment level of *C. elegans* was obtained by dividing the number of worms that reached the food source by the total number of worms on the plate.

Oil Red O staining

Oil-Red-O staining for major fat storage was conducted as descried by O'Rourke (O'Rourke, Soukas, Carr, & Ruvkun, 2009). Oil red O staining represents a validated method to study the regulation of long-term energy stores (O'Rourke et al., 2009). We put to use oil red O staining as a facile method to study fat mass in *C. elegans*. Oil red O staining correlates, in all cases tested, with biochemically measured triglyceride mass. Using glucose as a positive control, synchronized L1 larva were bred on NGM plates containing 2mM of glucose. One gram of glucose was dissolved into 10 mL of water and 90 µL was placed directly on top of the agar until dry. A total of 200-300 day-1 synchronized adult worms were separately washed from control and treatment plates with 1x PBS. Worms were washed three times with 1x PBS pH 7.4 and allowed to settle by gravity. To permeabilize the cuticle, worms were resuspended in 120 µl of PBS to which an equal volume of 2x MRWB buffer containing 2% paraformaldehyde (PFA) was added. 2x MRWB buffer: 160 mM KCl, 40 mM NaCl, 14 mM Na2EGTA, 1 mM

spermidine-HCl, 0.4 mM spermine, 30 mM Na-PIPES pH 7.4, 0.2% ß-mercaptoethanol). Samples were gently rocked gently for 1h in a 1.5 mL centrifuge tube at room temperature (allowing animals to rock inside the volume, without spreading the 240µl volume over the whole tube). Animals were allowed to settle by gravity, buffer was removed using pipette, and worms were washed three times with 1x PBS to remove PFA. Worms were then resuspended in 5 mL of 60% isopropanol and incubated for 15 minutes in a 15 mL centrifuge tube at room temperature to dehydrate. Oil-Red-O is prepared as follows: a 0.5g Oil-Red-O powder /100 mL isopropanol stock solution (100 %) equilibrated by rocking for several days was freshly diluted to 60% with water and rocked again for at least 1h, then filtered with 0.45 or 0.22µm-filter. After allowing worms to settle, isopropanol was removed, 1 mL of 60% Oil-Red-O stain was added, and animals were incubated overnight with rocking gently. Dye was removed after allowing worms to settle, and 200 µL of 1x PBS 0.01% Triton X-100 was added. Animals were mounted and imaged with a Leica colour camera outfitted with DIC optics.

RNAi by Feeding

The technique of RNAi, coupled with the availability of the complete genomic sequence of *C. elegans* (*C. elegans* sequencing consortium, 1998), has made possible the rapid study of gene function, both on a single gene level and at a global scale (Ahringer, 2006). Exposing *C. elegans* to dsRNA causes a reduction in the level of mRNA for the corresponding endogenous gene (Bargmann, 2001). RNAi plates were prepared by adding 25 mg Ampicillin and 240 mg IPTG (Isopropyl-β-D-thiogalactopyranoside) to 1L of cooled NGM media before pouring plates. The 30 µL of 250 mg/mL Ampicillin was added to 30 mL 2xYT media. 2xYT buffer: 10 g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, add H_2O for a total volume of 500 mL. Target RNAi bacteria (*sod-1* and *old-1*) were inoculated in 2xYT solution and incubated at 37° C at 225 rpm for 14-16 hours. RNAi bacteria were collected by centrifuging at 4,400 rpm for 10 minutes.

The supernatant was removed and 450 µL of supernatant was saved for future use. The 450 µL of supernatant was added back into the tube to suspend the bacteria. The 100 µL of each bacterium was added to RNAi plates and plates were kept in a dark space at room temperature for 2 days to dry. L1 of F0 worms were cultured on this media for two and half days followed by synchronization. The F1 generation was then cultured on experimental plates consisting of two concentrations (0.1µM and 1.0 µM) of BPA until adulthood, and an average length of 50 worms per treatment was measured using Leica software.

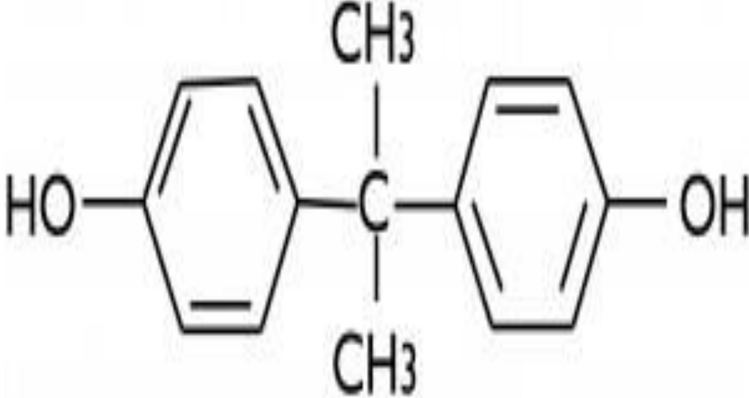


Fig.1. Chemical structure for bishenol A (BPA)

Fig.2. Translucent body of *C.elegan*

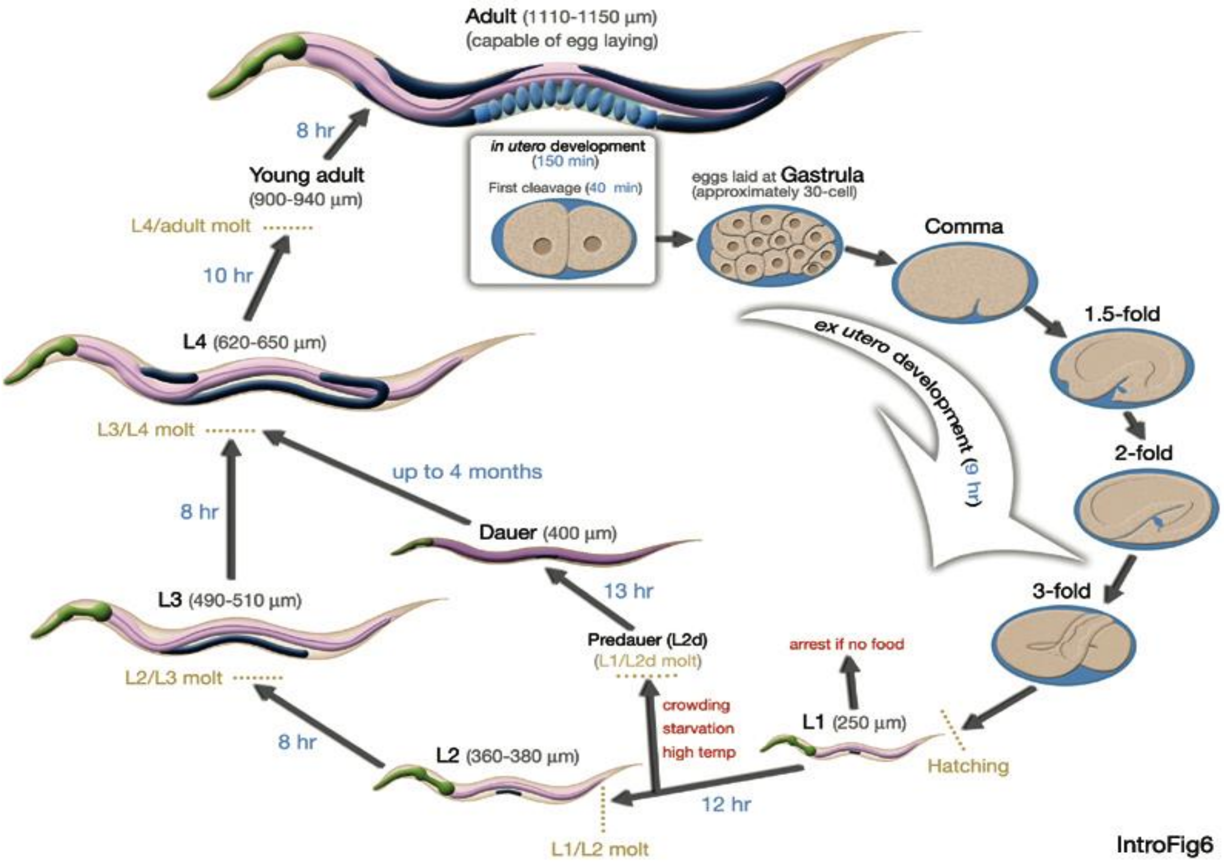


Fig.3. *C. elegans* life cycle at 22˚C (artwork by Altun and Hall, © Wormatlas)

Length		Distance from head to tail along central axis (red central line)
Width		Distance along the cross section of the worm averaged over its entire body from head to tail.(blue cross-section lines)
Area		Space covered by green external contour.

Wavelength		Distance between negative and positive inflection points
Smoothed Speed		A three-frame moving average speed smoothed over a 20 second span. The moving average speed is the instantaneous velocity along the worm's central line averaged over a number of frames.
Bending angle		The angle between the centroids of both the head and the tail.
Omega bend		Occurs when the worm makes an omega-shaped movement.

Table 1: Definitions of endpoints calculated by the Wormlab MBF software

Gene symbol	Locus tag	Gene description	Egg-laying/ reproduction	Stress/Life Span	Development/ Cell Fate	Muscle & Neuron	Reference
cat-4	F32G8.6	abnormal CATecholamine distribution	√			√	(Hardaker, Singer, Kerr, Zhou, & Schafer, 2001)
egl-5	C08C3.1	Egg Laying defective	√		√		(Kalis, Murphy, & Zarkower, 2010; Nicholas & Hodgkin, 2009)
egl-10	F28C1.2	Egg Laying defective	√			√	(Chase, Pepper, & Koelle, 2004; van der Linden, Simmer, Cuppen, & Plasterk, 2001)
egl-19	C48A7.1	Egg Laying defective	√			√	(Frokjaer-Jensen et al., 2006)
egl-44	F28B12.2	Egg Laying defective	√		√		(Wu, Duggan, & Chalfie, 2001)
egl-46	K11G9.4	Egg Laying defective	√		√		(Wu et al., 2001)
egl-47	C50H2.2	Egg Laying defective	√			√	(Moresco & Koelle, 2004)
pink-1	EEED8.9	PINK (PTEN-INduced Kinase) homolog	√	√			(Samann et al., 2009)
sod-1	C15F1.7	SOD (superoxide dismutase)	√	√			(Doonan et al., 2008; Shibata, Branicky, Landaverde, & Hekimi, 2003)
age-1	B0334.8	AGEing alteration		√	√		(Ayyadevara et al., 2009)
old-1	C08H9.5	Overexpression Longevity Determinant		√			(Murakami & Johnson, 2001)
oxi-1	Y39A1C.2	Oxidative stress Induced		√			(Camon et al., 2003; Yanase & Ishi, 1999)
ric-3	T14A8.1	Resistance to Inhibitors of Cholinesterase				√	(Halevi et al., 2002; Shteingauz, Cohen, Biala, & Treinin, 2009)

Table 2: Description and classification of 13 selected genes

Statistical Analysis

For statistical analysis, the IBM SPSS Statistics 20 software for Windows 7 was used. To determine statistical differences between treatment and control groups in apoptosis cell counts and gene expression fold changes the statistical test analysis of variance (ANOVA) was used. If treatment groups were statistically significant at $p < 0.05$ level, least significant difference (LSD) multiple comparisons were carried out to compare means among groups.

Project Relevance

Given concern about BPA, and the ongoing evaluation and studies on its safety, providing additional information and addressing uncertainties would yield supporting evidence of adverse effects contributing to the medical field, environment and public. Further investigation using *C. elegans* will provide additional insight into the toxicity of chemicals including environmental pollutants to the reproductive and neurological effects in humans at the molecular level. Additionally, this study will help agencies, such as the National Toxicology Program and FDA’s National Center for Toxicological Research, better regulate production of BPA.

RESULTS

Impact of Bisphenol A on growth

BPA treated two and a half-day old N2 worms had an average length of 1100 ± 22.4, 700 ± 21.9, and 500 ± 23.1 µm for the control, 0.1 µM, and 1.0 µM treatments respectively (Fig 4). There was a significant difference in body length when comparing the high concentration treatment group to both the control and low concentration treatment group. Findings in body width, area, and wavelength show consistent results with body length as they are dependent upon one another. As the concentration increased, the values for all four parameters decreased ($P<0.05$). BPA treated N2 worms had an average width of 150, 70, 65 µm; average area of 22000, 8000, 4000 μm^2; and average wavelength of 520, 290, 240 µm for the control, 0.1 µM, and 1.0 µM treatments respectively (Figs.5, 6, 7). The wavelength showed a significant difference when comparing the high concentration treatment group to both the control and low concentration treatment. The body area and body width showed significant differences in both treatment groups compared to the control, but not between low and high treatments.

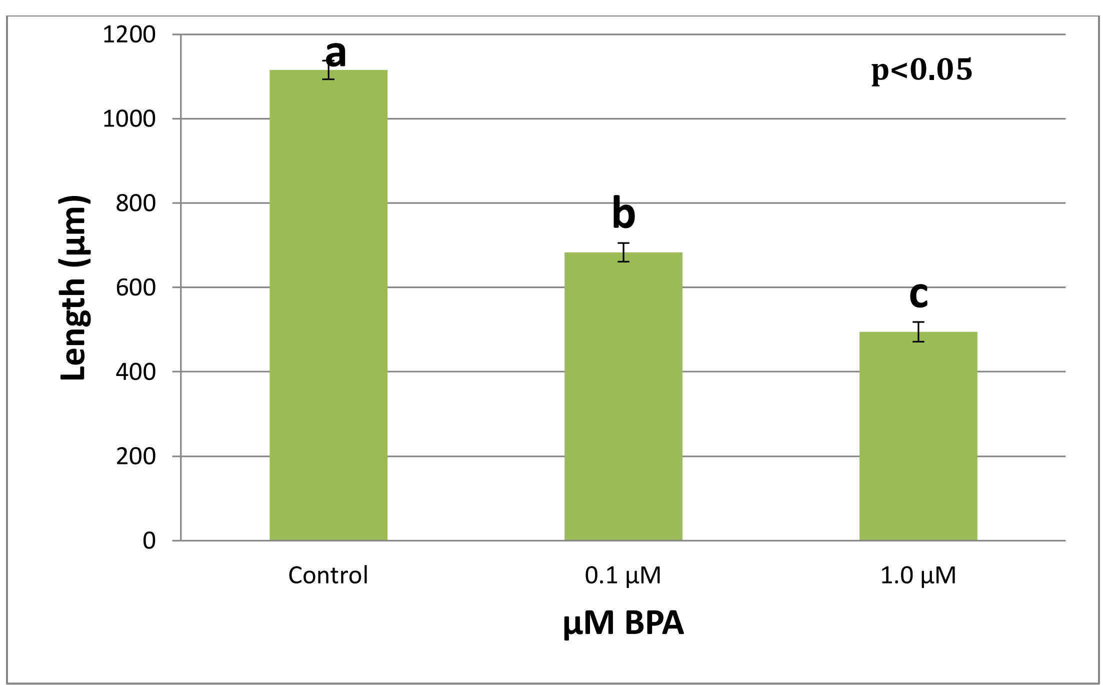


Fig.4. Average body length of N2 worms after L1-Adult dosing. Different letters denote statistically significant differences. Error bars indicate standard deviations of 15 worms tracked in 3 individual experiments. N= 15

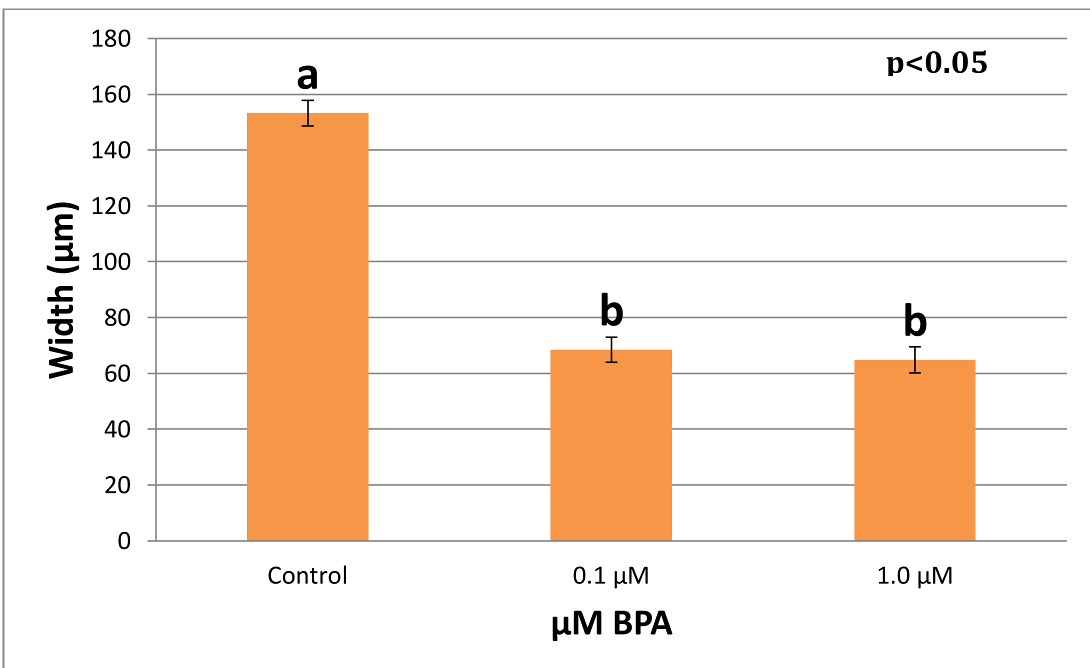


Fig.5. Average body width of N2 worms after L1-Adult dosing. Different letters denote statistically significant differences. Error bars indicate standard deviations of 15 worms tracked in 3 individual experiments. N= 15

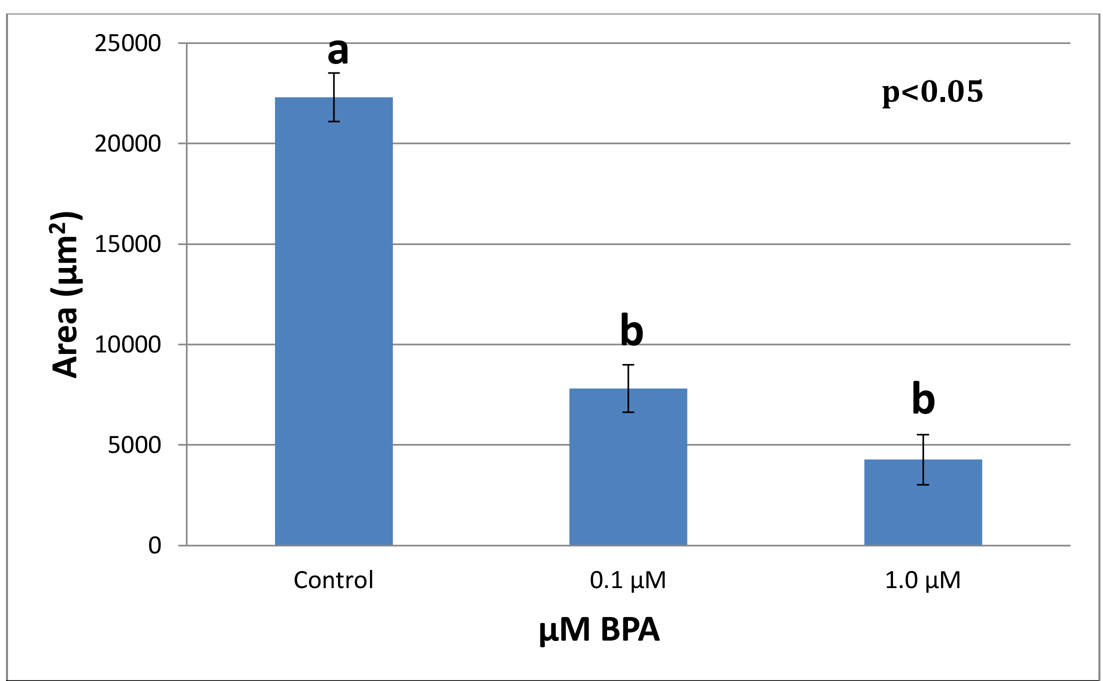


Fig.6. Average body area of N2 worms after L1-Adult dosing. Different letters denote statistically significant differences. Error bars indicate standard deviations of 15 worms tracked in 3 individual experiments. N= 15

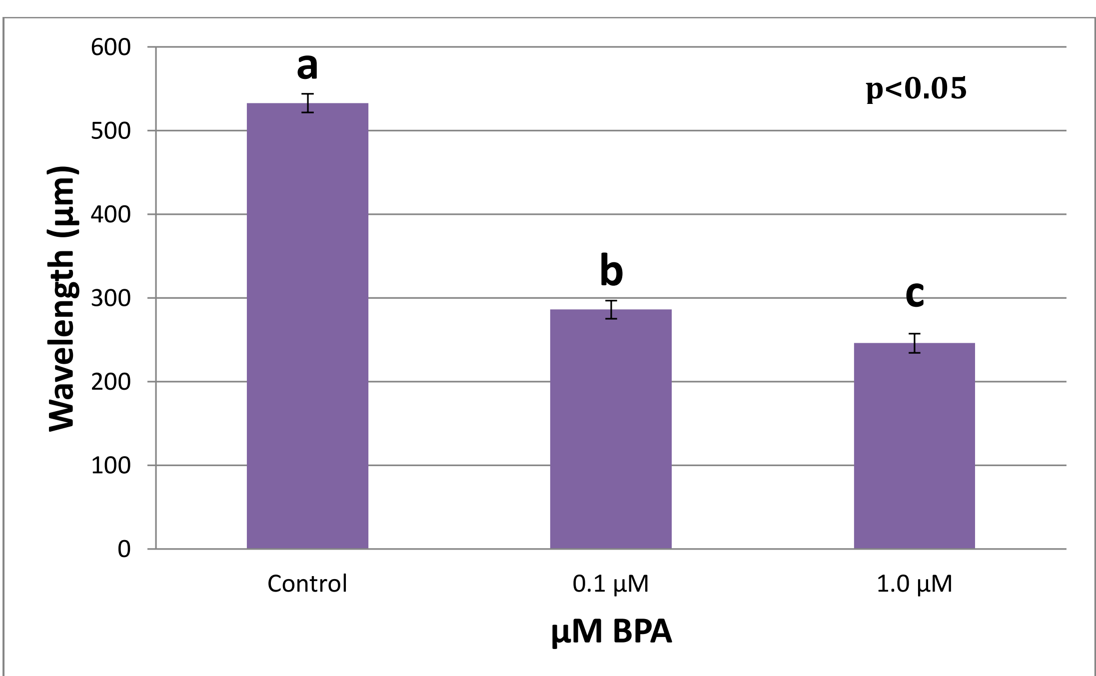


Fig.7. Average body wavelength of N2 worms after L1-Adult dosing. Different letters denote statistically significant differences. Error bars indicate standard deviations of 15 worms tracked in 3 individual experiments. N= 15

Impacts of Bisphenol A on *C. elegans* locomotion

A dose-dependent and statistically significant decrease in the average reversals was observed in worms exposed to low and high BPA concentrations (Fig.8). There were no statistical differences in the bending angle or omega bends (Fig. 9, 10). However, the average values of reversals and bending angles were higher in the control than in BPA treatment groups, while the higher concentration treated worms did the most omega bends.

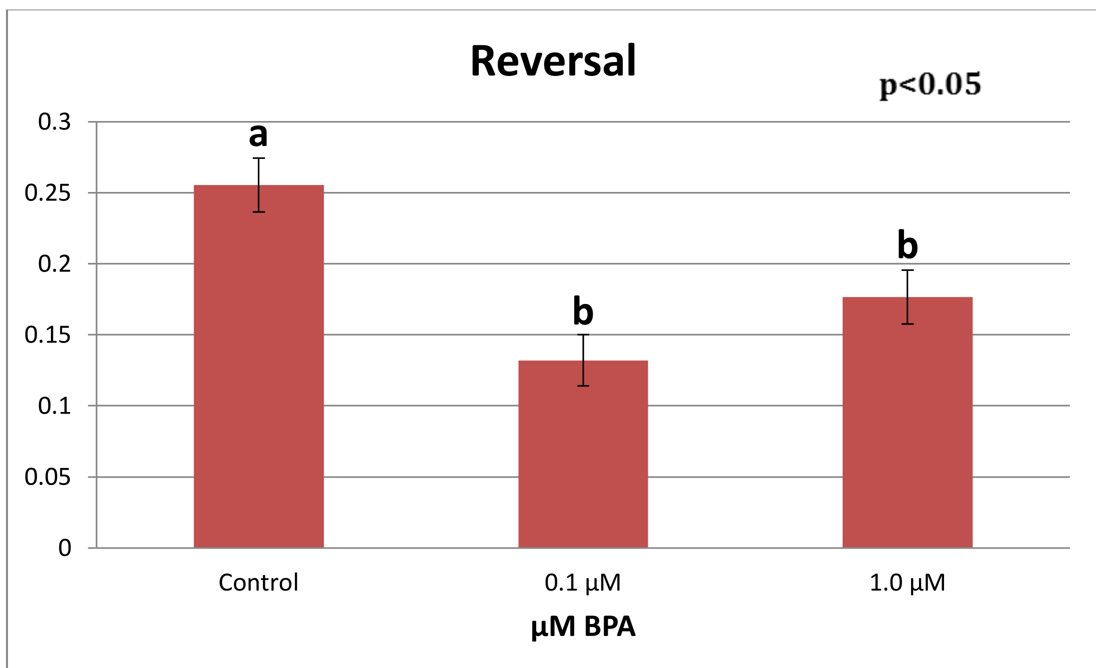


Fig.8. Reversal behavior in adult hermaphrodite *C. elegans* as a function of BPA exposure. The y-axis represents a ratio calculated from the proportion of time that worms move backward. Different letters correspond to statistically significant differences ($p<0.05$). N = 15

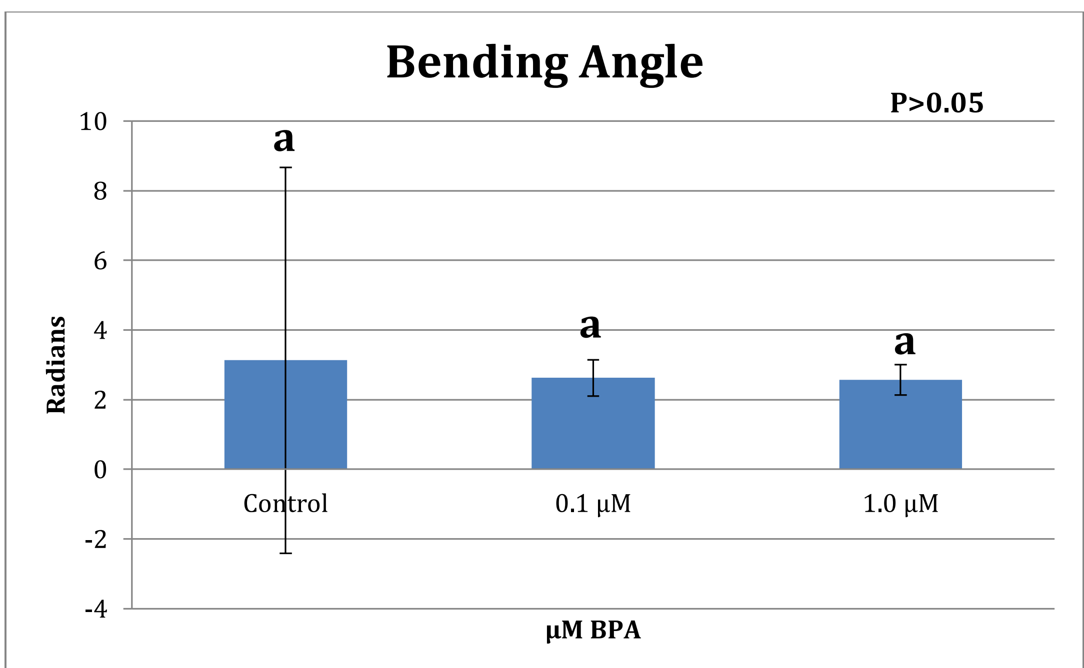


Fig.9. Bending angel in adult hermaphrodite *C. elegans* as a function of BPA exposure. The y-axis represents the average bending angle represented by radian values. Different letters correspond to statistically significant differences ($p<0.05$). N=15

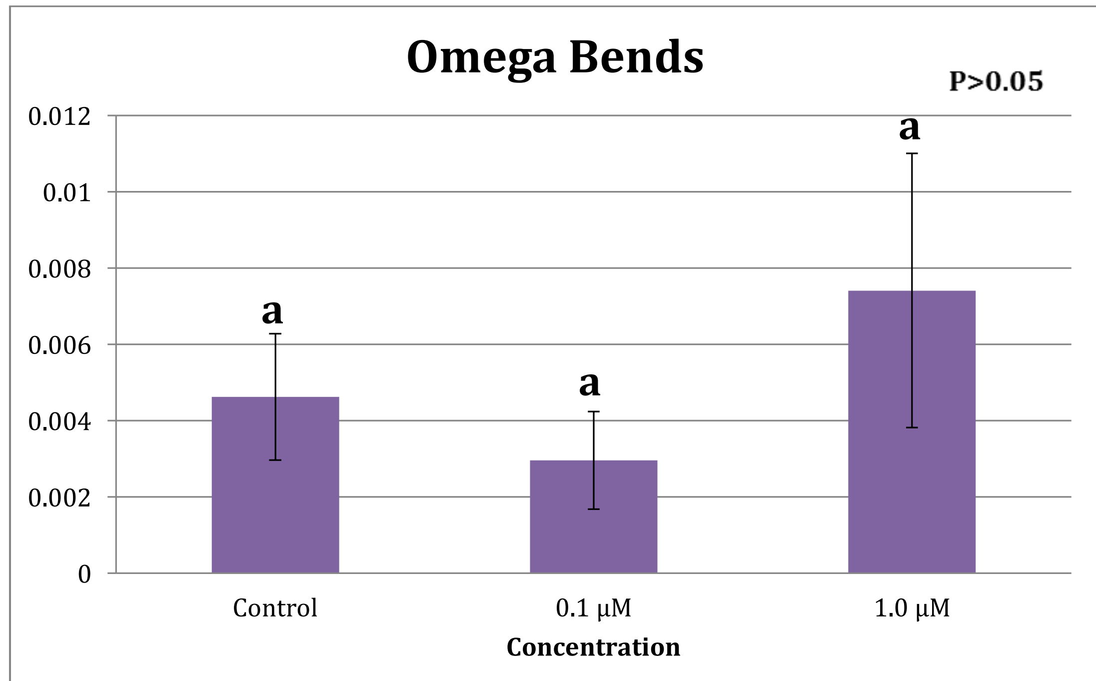
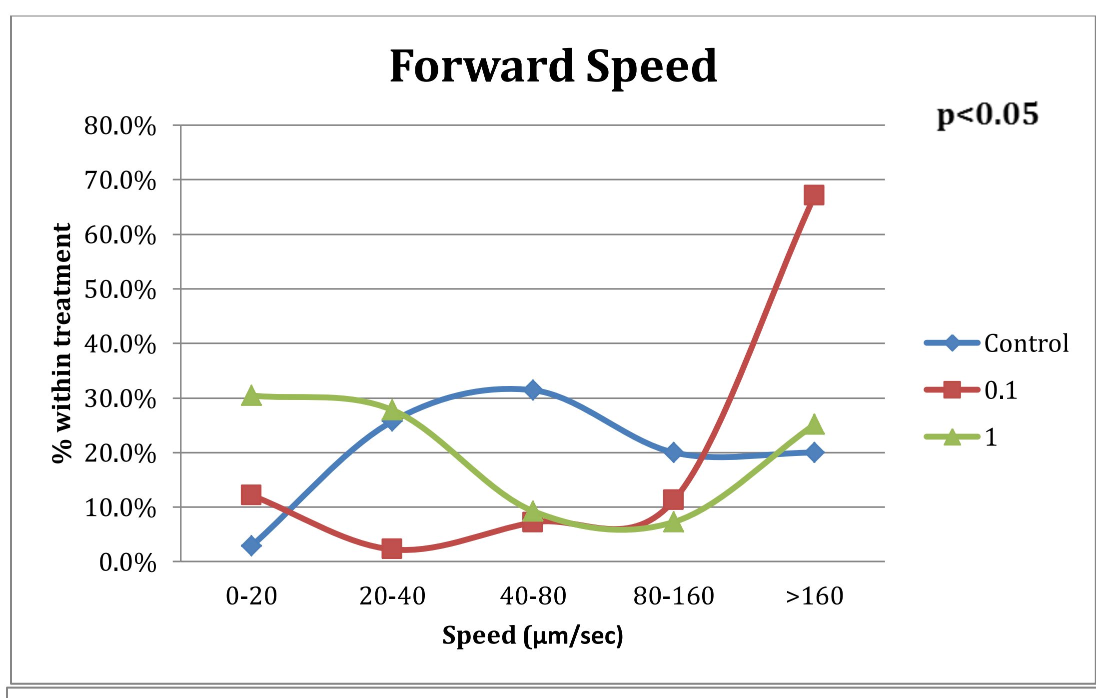


Fig.10. Omega bends in adult hermaphrodite *C. elegans* as a function of BPA exposure. The y-axis represents a ratio calculated from the proportion of times the worms perform the omega bend. Different letters correspond to statistically significant differences with respect to the control ($p<0.05$). N=15

Effect of BPA on locomotion speed in *C. elegans*

A stimulation of locomotion at 0.1µM BPA treated group was seen; 67.1% of the worms traveled at forward speeds of >160 µm/s and 68.7% at backward speeds of >160 µm/s at 0.1 µM (Fig. 11). The control group had its highest percentage of worms distributed at low speed ranges of between 20-80 µm/sec, with 57.1% in the forward direction and 61.4% in the reverse. Only 2.9% and 5.7% of total worms display the lowest control locomotion speed between 0-20 µm/s for forward and reverse speeds, respectively. At high BPA treatment, most worms display a low locomotion speed of between 0-40 µm/s, with 58.3% of worms traveling in the forward direction and 57.8% in the reverse direction. In summary, a majority of worms experienced a decrease in locomotion speed when exposed to high BPA concentration and increase in speed when exposed to low BPA concentration as compared to the control.



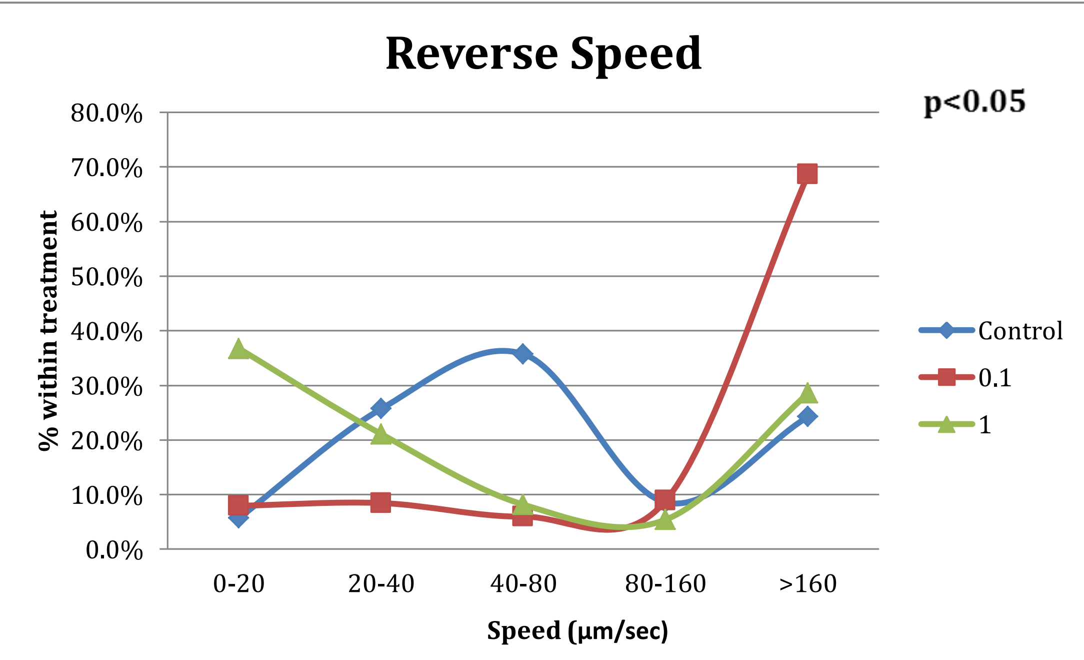


Fig.11. The impact of BPA on the forward and backward locomotion speed (μm/s) in adult *C. elegans* N2 hermaphrodites. Different letters correspond to statistically significant differences with respect to control. ($P<0.05$).The x-axis represents speed (μm/s) divided into 5 ranges. The y-axis represents a ratio calculated from the percentage of worms in each speed range. N=15

Effects of BPA on reproduction

Worms were dosed from L1 for 65 h until adulthood, and then transferred to new BPA containing plates for continuing reproduction assay. At 89 and 96 hours after L1, there was a significant difference in egg production among control and the 1.0 μM treatment group. The average number of eggs laid per worm at 89 h for control was approximately 152 and for those exposed to high concentration BPA was significantly reduced to 12 ($p < 0.05$; Fig.12). Similarly, at 96 h time point, an average of 192 control group eggs were counted and those exposed to high concentration BPA was significantly reduced to 14 ($p < 0.05$, Fig.12). However, no significant difference among control and 0.1 μM BPA was noted at any time point. The 0.1 μM dosed group produced slightly more eggs compared to the control during 65-113 h post-L1 with a total average of 451 in 0.1 μM group verses 420 in the control. The number of eggs laid from 120-137 h after L1 dropped until no eggs were observed. At 120 h the control, low dose and high dose produced 44, 24 and 0 eggs per worm respectively. By 137 h no more eggs were produced.

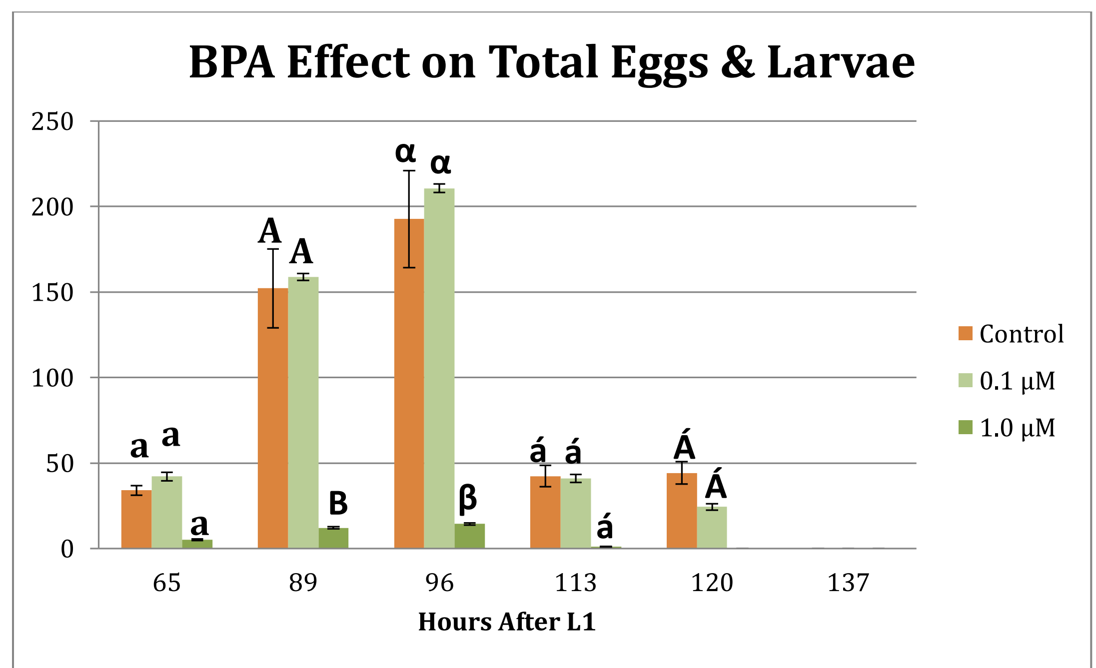


Fig.12. The impact of BPA on reproduction, represented by the number of eggs plus larvae produced during the time interval between the parent worms transfer. Worms were dosed from L1for 65 hours and then moved to NGM agar plate with food and the same concentration of BPA to allow egg-laying. The x-axis represents the time period in hours after L1. The y-axis represents the average number of eggs plus larvae produced per worm over time (n=15 per treatment). Different letters in the same group of bars at each time point correspond to statistically significant differences at $p<0.05$ level.

Effects of BPA on Feeding Behavior

As shown in Fig. 13, there was no difference in ($p>0.05$) altered attainment levels between the untreated control and the EtOH vehicle control. When the *C. elegans* were exposed to 1.0 µM BPA, the attainment levels decreased significantly compared to the untreated control at 2, 6, 8, and 24 hr. There was also significant difference in attainment level compared to control when exposing to 0.1 µM BPA at 4 and 6 hr. A comparison between the attainment levels of the organism treated with 0.1 µM and 1.0 µM showed a significant difference at all time points except 6 hr.

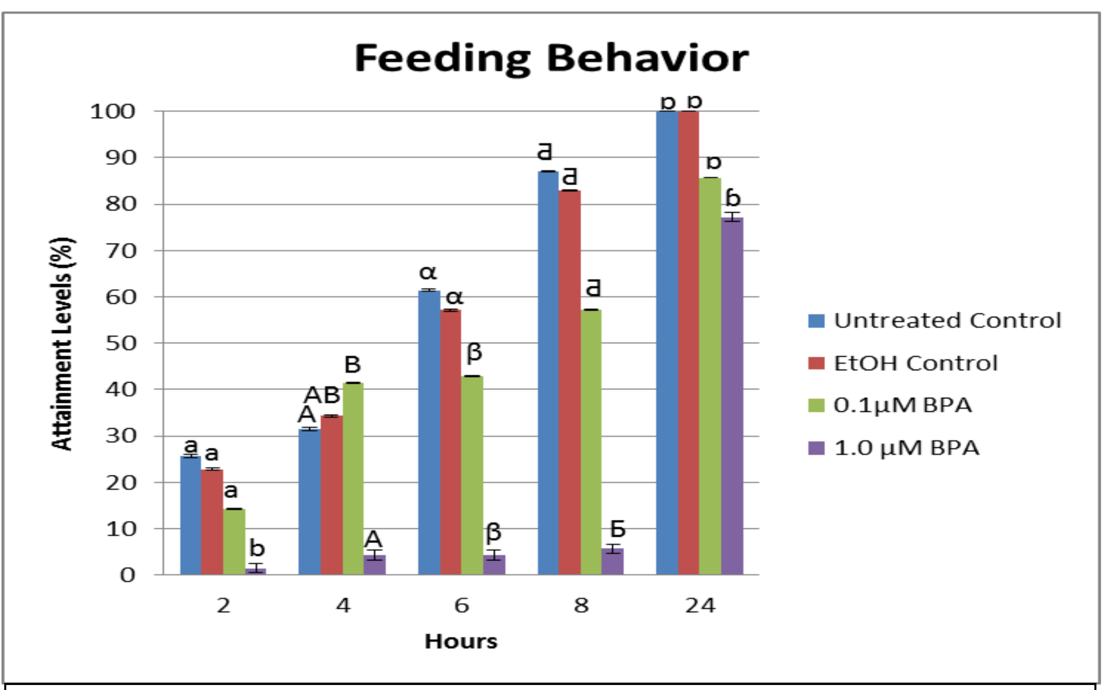


Fig.13. Influence of BPA in attainment level of *C. elegans.* The x-axis represents the time, in hours, used to measure the number of nematodes reaching the *E.coli* colony. The y-axis represents the attainment levels (obtained by dividing the number of worms that reached the food source by the total number of worms on the plate). Different letters correspond to statistically significant differences ($p<0.05$). N=70

Effects of BPA on Gene Expression

This study identified the effect of BPA on the expression levels of 13 genes selected for testing, including *cat-4, egl-5, egl-10, egl-19, egl-44, egl-46, egl-47, pink-1, sod-1, age-1, old-1, oxi-1, ric-3* using *tba-1* as reference gene which is required partially for normal embryonic development (Fig. 14). Of the 13 selected genes, 9 are related to egg-laying, 5 are stress-response related gene and 2 incorporate both functions (Table 2). Gene expression experiments indicated that an important egg-laying related gene *egl-10* was significantly up-regulated at 1 µM BPA treated group. We also found that *old-1* which plays a role in stress resistance and regulation of adult lifespan was significantly up-regulated at 1 µM BPA while *sod-1* known to protect cells from oxidative damage was down-regulated at both BPA treated groups. This indicated BPA

may affect the stress-defense system and results in life-span defects. Fig. 11 shows the fold change in gene expression of the 13 tested genes varying from 12.5-fold down-regulation (*sod-1*) to 14.9-fold up-regulation (*egl-10*). Statistical analysis indicated that there were 3 tested genes expressed aberrantly in response to at least one dosage of BPA. These three genes are *egl-10, sod-1* and *old-1*. Among the 3 genes, 2 (*egl-10* and *old-1*) were significantly up-regulated, 1 gene (*sod-1*) was significantly down-regulated. Gene regulation was active at the high concentration BPA treatment group; 3 (*egl-10*, *old-1,* and *sod-1*) and 1 (*sod-1*) were differentially expressed at 1.0 μM and 0.1 μM BPA treatment groups, respectively.

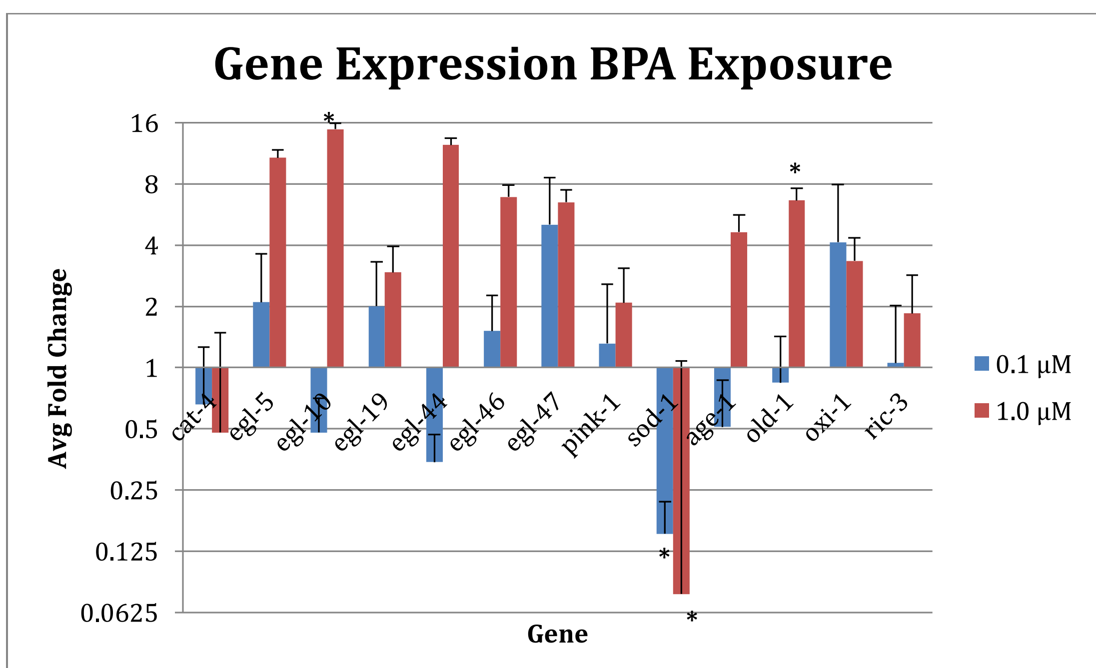


Fig.14 Gene expression of selected genes following exposure. X-axis represents different genes, y-axis is the expression fold change compared with control (1: control; > 1 means up-regulation and < 1 means down-regulation) .TBA-1 is the reference gene. * represent statistically significant differences at $p < 0.05$ level.

Effects of BPA on fat storage

Here we show staining of *C. elegan* major fat stores using the Oil Red O (O'Rourke et al., 2009). We used worms exposed to glucose as a positive control and monitored for changes in body fat

as BPA concentration increased. Fig.15 A shows an increase in fat storage, following the consumption of glucose, in comparison to the control (Fig.15B). However, as the concentration of BPA increased the amount of fat accumulation decreased (Fig. 15C, 15D) when compared to the control.

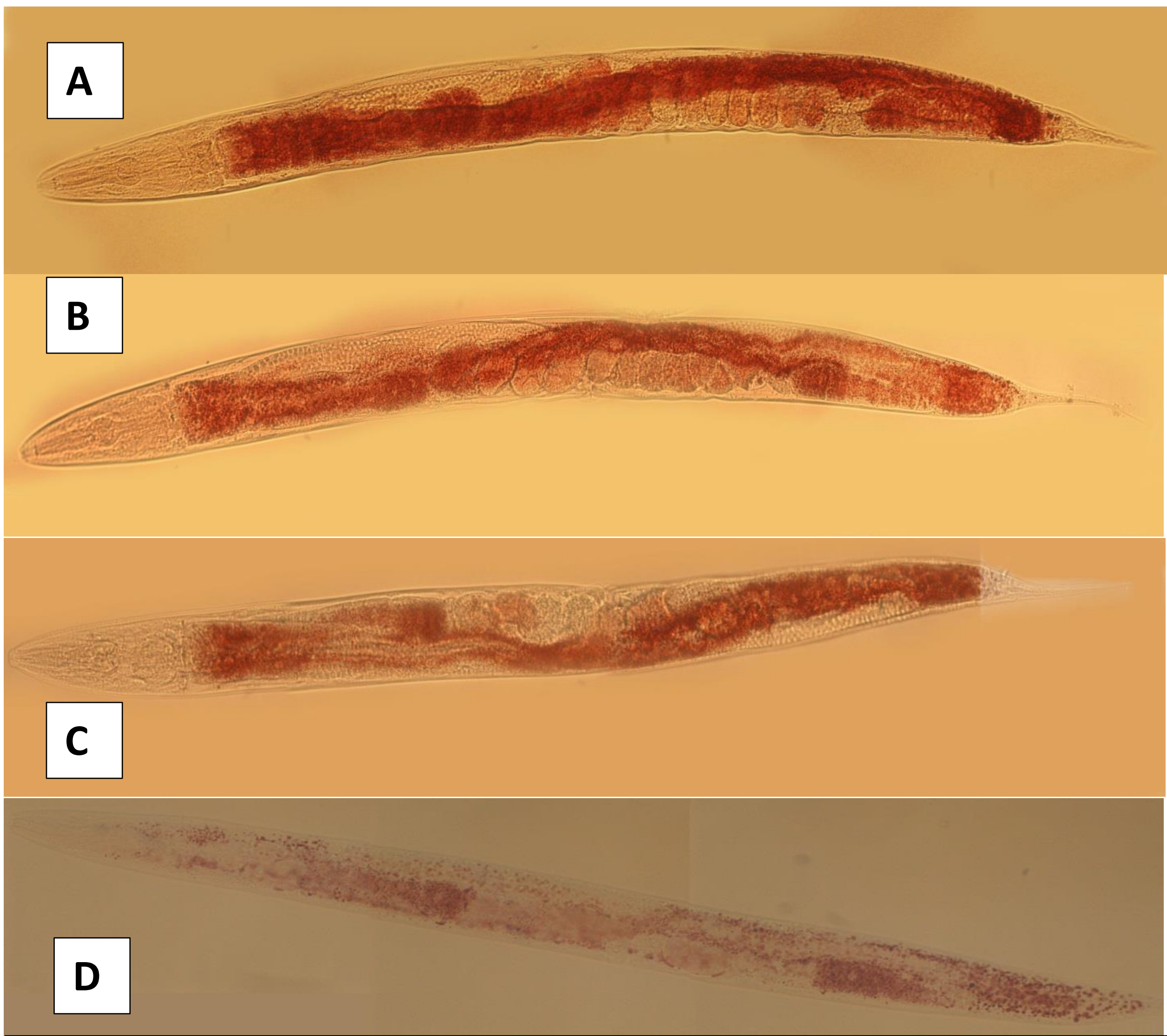
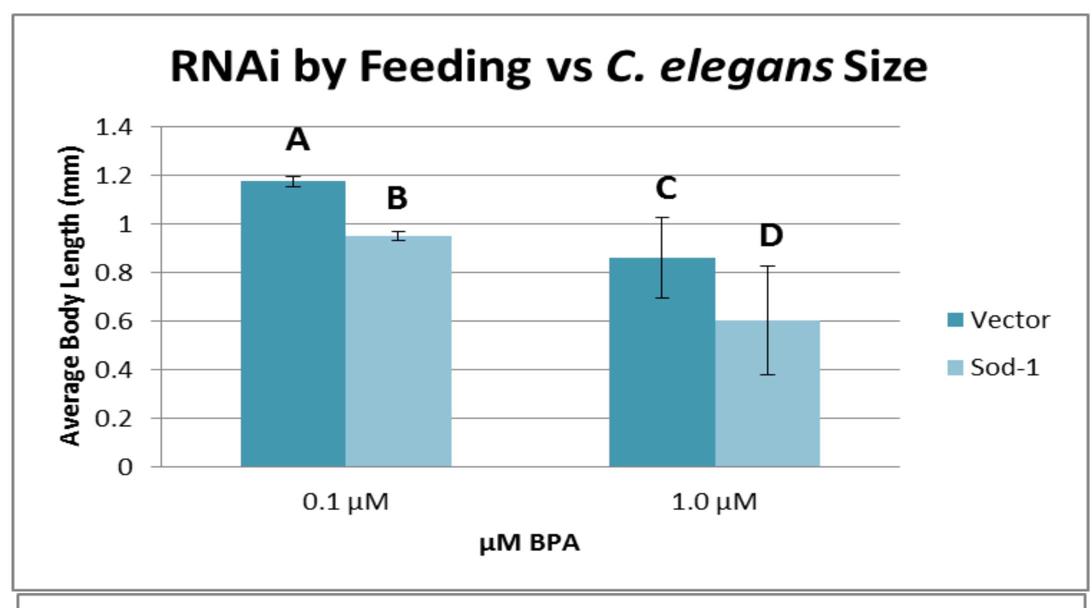


Fig.15. BPA (Dose L1-L4). Images of stained fat stores in *C. elegans* represented by O Red Oil stains. A) Positive control using glucose diet. B) N2 worms (control). C) 0.1 µM BPA. D) 1.0 µM BPA

Roles of *sod-1* and *old-1* in growth regulation in response to BPA (RNAi by Feeding)

Based on the significant results of the gene expression, we next assayed the functions of genes involved in stress response (*sod-1* and *old-1*) by RNAi feeding (Fig.16). In worms exposed to low concentration BPA, the average body length of the vector N2 worms was 1170 µm. In comparing the 0.1 µM vector to the *old-1* gene knockdown, there was an increase in length. However, comparing the 0.1 µM vector to the *sod-1* gene knockdown, there was a decrease in length. In worms exposed to high concentration BPA, there was a decrease in length for both *old-1* and *sod-1* gene knockdown. All results were statistically significant.



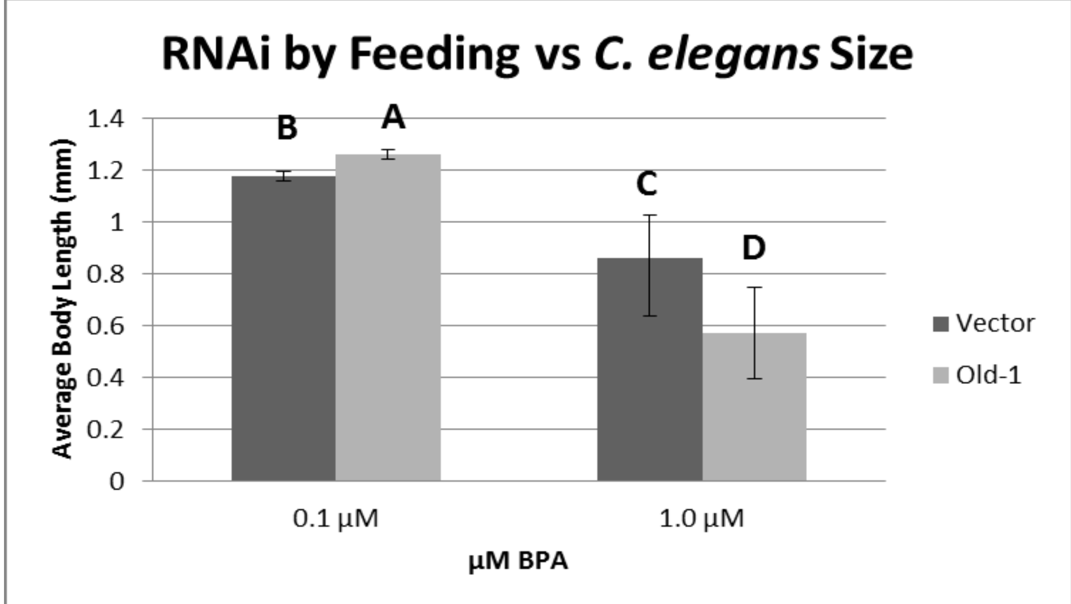


Fig.16. Average length of N2 worms after RNAi feeding (n = 50), followed by L1-Adult BPA dosing. Different letters denote statistically significant differences.

DISCUSSION

Chronic BPA exposure at the high concentration (1.0 μM) causes BPA-induced growth inhibition. Since BPA exposure in humans occurs at various ranges and last for a prolonged period of time, it is important to investigate effects of chronic exposure to high concentrations of BPA. Based on the available data the primary source of exposure to biphenol A for most people is through diet. The highest estimated daily intake of bisphenol A in the general population occurs in infants and children and there is some indication that exposure to bisphenol A is increasing (Shelby, 2008). The median levels of BPA in human urine doubled from 1.3 μg/L to 2.7 μg/L from 1988-2004 (Shelby, 2008).

In this study we observed a dose-dependent decrease in length, width, area and wavelength when L1 stage *C. elegans* were exposed to low (0.1 μM) and high (1.0 μM) concentrations of BPA for 2.5 days. This suggests that exposure to a range of levels of bisphenol A can reduce growth in *C. elegans*.

At approximately 65 hrs posthatch at 20°C, *C. elegans* begin egg laying and remain fertile until egg laying ends ~128 h post-hatching (Altun & Hall, 2009). We incorporated these time intervals for comparison of reproductive effects. We found that the majority of eggs laid for each treatment was a ~96 h after L1 and by ~137 h after L1 no eggs were produced. These findings are consistent with the fact that egg laying maximal of wt *C. elegans* occurs ~31 h after egg-laying begins (Altun & Hall, 2009). Chronic exposure to 1.0 μM BPA significantly inhibited egg production (Fig. 12) from 24 hours after egg laying began until the egg laying maximum. Conversely, exposure to 0.1 μM BPA significantly induced egg laying at ~96 h and remained slightly greater than the control at 65, 89 and 113 h after L1. At 72 h (The time point 137 hr post L1) after the start of egg laying, no more eggs were observed, although, however 1.0 μM BPA

exposed worms stopped egg laying at ~120 h time point as compared to the control and 0.1 µM treatment groups which still laid 44 and 24 eggs, respectively. Taken together, exposure to micromolar concentrations of 1.0 µM BPA, decreases egg productions throughout *C. elegans* reproductive period.

Speed can be calculated as wavelength x oscillation frequency. Therefore, the wavelength and speed are directly proportional. This is consistent with our data, where a decrease in speed in 1.0 µM BPA was associated with a decrease in wavelength. The high concentration treatment group showed BPA-induced systemic toxicity as it was negatively affected in all the locomotive indices. Most of the worms had minimal forward and reverse speeds (0-20µm/s) at 1.0 µM BPA treated group. In normal food-replete conditions, worms tend to be “dwelling,” a behavior with frequent reversals and increased turn angles. This was not observed in our case, rather the number of reversals significantly decreased at 1.0 µM BPA treated group. This suggests that BPA exposure impacts locomotion and consequently affects worm dietary consumptions. It was reported that slow locomotion speed is associated with impaired neuronal network formed by interneurons AVA, AVB, AVD, and PVC (Leung et al., 2008).

We then investigated the feeding behaviors of *C. elegans* after exposure to BPA by measuring their attainment levels (the number of worms reaching the food source divide by the total number of worms on the plate). Before 24 hr time point, more than half of the worms at 1.0 µM BPA treated group did not reach the food source, suggesting inadequate feeding. This data supports and lends a probable explanation for reduced body growth.

We also investigated the expression patterns of 13 genes. Many of these selected genes are known to regulate functions including: movement, mating, foraging, cell migration, egg laying and stress response (Altun & Hall, 2009; Kim et al., 2001; Wu et al., 2001) The *egl* family

gene *egl-10* along with two others (*sod-1* and *old-1*) exhibited significant changes. E*gl-10* functions to encode an RGS protein which modulates motor neuron functions related to egg-laying (Moresco & Koelle, 2004; Wu et al., 2001). We found that at 1.0 μM BPA, *egl-10* exhibited a significant 14.9-fold up-regulation, suggesting *egl-10* is among those sensitive to high dose BPA exposure. This may suggest an adaption mechanism to compensate the reproduction deficits. Additionally, we tested if the oxidative stress-responsive genes are changed in *C. elegans* following BPA exposure. We found that *old-1*, playing a role in stress resistance and regulation of adult lifespan was significantly up-regulated while *sod-1*, known to protect cells from oxidative damage had a 12.5-fold down-regulation. This indicated BPA may affect the stress-defense system and results in life-span defects.

To determine whether *old-1* and *sod-1* is involved in regulating growth in response to BPA exposure, we evaluated their effect with knockdown *old-1* and *sod-1.* In worms exposed to 0.1 and 1.0 μM BPA there was a decrease in length in the *sod-1* loss of function mutants. These results were consistent with the gene expression data and indicated a possible involvement of *sod-1* in growth regulation. Studies have shown lack of *sod-1* results in increased age-related muscle mass loss (sarcopenia), macular degeneration, and shortened lifespan among others (Muller, Lustgarten, Jang, Richardson, & Van Remmen, 2007). The transcription of *old-1* is upregulated in response to heat, UV light, and starvation (Murakami & Johnson, 2001). Our experiment indicated that old-1 is also upregulated in response to BPA treatment. However, our results did not support that *old-1* is related to growth regulation.

Indicated by Oil Red O fluorescence and consistent with our growth results, we found that increasing BPA concentration shows a decreasing amount of body fat. We used glucose as a positive control to illustrate that fat accumulation increased with the intake of excess calories (Fig.15A). *C. elegans* fat stores following exposure to 0.1 μM and 1.0 μM BPA showed an

evident decrease in the amount of body fat. Contrary to previous studies, our observations suggest that BPA exposure at tested concentrations is not associated with increased fat storage/obesity in worms.

GENERAL METHODS

C.elegans **Treatments**

All chemicals were obtained from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). BPA suspension was made in 5 mL ethanol and ultrapure water. N2 strain was synchronized and eggs were collected (Brenner, 1973) (see detailed methodology). Eggs were allowed to hatch as L1 worms without food. N2 L1s were kept at 20°C on an NGM agar medium with OP50 as food source. For the treatment, agar dose plates were made by mixing BPA solution into the agar medium to make a low and high concentration (0.1 μM and 1.0 μM BPA). Exposure time for both treatment and control (unexposed worms of the same living conditions) was from the L1 to adult stage (~72 hr).

DETAILED METHODOLOGY

Synchronization Process

The synchronization and collection process is as follows, NGM plates containing the N2 worms were washed 3x with M9 solution and transferred into 15 ml centrifuge tubes. These tubes were centrifuged, supernatant discarded, and 5 ml M9 added, then centrifuged again. This step was repeated 2 more times to wash the worms. After the third wash the supernatant was discarded, 5 ml of synchronization solution (0.675 mL NaOH , 1.25 mL bleach and 4.375 mL distilled water) was added, and tubes were gently hand shaken for 5-8 minutes. Tubes were then centrifuged, supernatant discarded, 5 ml of M9 added, and tubes centrifuged again. The synchronization process will lyse the worms, killing them, and leaving behind eggs left inside and outside of the body to be collected. The remaining eggs inside the tubes with M9 will be incubated at 20° C on a shaker for 12-18 hours, where they will hatch as L1s.

RNA Extraction

RNA was extracted using a mirVANA miRNA isolation Kit. To begin, each of 10 frozen tubes containing all the worms were thawed on ice for 10 minutes. 600 µl of Lysis/Binding Buffer was added to all 10 tubes and each tube was sonicated, to disrupt and homogenize the tissue, using an ultrasonic converter on ice. Following sonication, 60 µl of RNA homogenate additive was added to each tube and each tube was vortexed for 30 seconds and placed on ice for 10 minutes. After ice incubation, each tube received 600 µl of Acid-Phenol Chloroform then vortexed for 60 seconds. Tubes were then centrifuged for 5 minutes (10.000 rpm) and the aqueous phase of each tube was transferred into 10 new 2 ml centrifuge tubes (this process was repeated X2). The final aqueous phase of each tube was transferred into a new tube and 1.25 volume of 100% ethanol was then added to each tube. Each of the lysate/ethanol mixtures were then transferred to filter cartridges in new tubes and centrifuged for ~15 seconds (10,000 rpm).

Each filter cartridge of each tube was then washed with miRNA wash solution 1 and 2/3 provided in kit. Next, each filter cartridge of each tube was transferred into new tubes and 50 µl of pre-heated (95°C) nuclease-free water was applied to each filter. Each tube containing filter cartridges were then centrifuged for 5 seconds (5,000 rpm) to recover RNA product. Finally, each RNA product was quantified using a NanoDrop ND-100 spectrophotometer and immediately stored in the -80 ºC freezer until time for reverse transcription.

Reverse Transcription (RT)

After NanoDrop analysis of the RNA samples, I chose the 4 best treatment and 4 best control RNA samples to use for RT-PCR (8 tubes). RT-PCR was performed using a TaqMan® MicroRNA Reverse Transcription Kit. 8 new 0.5 ml microfuge tubes were collected. Several calculations were made to determine how much nuclease free water, RNA sample, and master mix would be added to each the microfuge tubes. The master mix contained calculated volumes of components provided by the kit (RNase inhibitor, 100 mM dNTPs, 10X Reverse Transcription Buffer, Multiscribe™ Reverse Transcriptase, and primer mix (not provided in kit)). After each tube received calculated volumes of nuclease-free water, RNA sample, and the master mix, the tubes were mixed gently and centrifuged for 10 seconds (200 rpm). The tubes were then incubated on ice for 5 minutes and loaded into the thermal cycler for reverse transcription. The thermal cycler process includes 30 minutes at 16°C, 30 minutes at 42°C, 5 minutes at 85°C, and will hold at 4°C. After RT-PCR, each tube, now containing RT product or cDNA, was removed from the thermal cycler and each tube received 80 µl of DNase-free water. The tubes were then mixed by vortexing and immediately stored at -20°C until needed for qRT-PCR.

Quantitative Real-Time PCR (qRT-PCR)

Previously made RT-PCR products and purchased SYBR® Green dye were placed on ice to thaw. Using a 384-well plate microplate, following a written template, I loaded each

individual well with 5.5 µl RNase DNase-free water, 7.5 µl SYBR® Green dye, and 1 µl RT-PCR product. I then loaded each well with 1 µl of primer solution (20 µl forward, 20 µl reverse, 60 µl water) specific to the genes of interest. For each RT-PCR product there were 3 technical replicates. Once the plate was fully loaded it was covered and sealed tightly with a film to prevent evaporation of any well samples. The plate was then centrifuged to ensure each sample mixture is settled on the bottom of each well. Next, the plate was loaded into the qRT-PCR machine to undergo one 10 minute cycle at 95°C (enzyme activation), and 45 PCR cycles including 15 seconds at 95°C (denaturation of DNA), and 60 seconds at 60°C (DNA annealing and extension).

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