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Bisphenol A induces reproductive toxicity by antagonizing cholesterol uptake at the StAR mitochondrial transmembrane transporter in C. elegans

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Bisphenol A induces reproductive toxicity by antagonizing cholesterol uptake at the StAR mitochondrial transmembrane transporter in *C. elegans*

A thesis submitted in partial satisfaction

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in Environmental Health Sciences

by

Katherine Elise Gibbs

2018

ABSTRACT OF THE THESIS

Bisphenol A induces reproductive toxicity by antagonizing cholesterol uptake at the StAR mitochondrial transmembrane transporter in *C. elegans*.

by

Katherine Elise Gibbs

Master of Science in Environmental Health Sciences

University of California, Los Angeles, 2018

Professor Michael D. Collins, Chair

Endocrine-disrupting compounds (EDCs) are found in numerous products and due to their structural similarity to endogenous hormones, they can interfere with many aspects of the endocrine system. Bisphenol A (BPA) is one such EDC commonly used as an intermediate in the production of several plastic polymers, including polycarbonate and epoxy resins. Here, we examined the role of BPA as a reproductive toxicant using three strains of *Caenorhabditis elegans:* N2 (wild-type), *Strl-1* F52F12.7(ok3347) I (StAR) and *tspo-1* C41G7.9(tm5526) (TSPO). The StAR and TSPO mutants have a mutation that inactivated the StAR or TSPO mitochondrial membrane transporter respectively. A combination of one cholesterol concentration (0 μg/ml cholesterol, 0.5 μg/ml cholesterol or 5 μg/ml cholesterol) with a BPA concentration (0 μM BPA, 100 μM BPa or 500 μM BPA) allowed us to analyze how varying cholesterol and BPA concentrations affect fertility, the germline and

cholesterol uptake in the mitochondria. We found that cholesterol antagonizes the reproductive toxicity of BPA in wild-type (N2) *C. elegans* and BPA may induce its reproductive toxicity through the StAR transporter as the StAR mutants did not show signs of reproductive toxicity with increased exposure to BPA. These results indicate that cholesterol rescues the reproductive effects of BPA and that StAR may be an important component of this mechanism.

The thesis of Katherine Elise Gibbs is approved.

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2018

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INTRODUCTION

BPA

Endocrine-disrupting compounds (EDCs) have been found in numerous products ranging from personal care products and fragrances to pharmaceuticals and plastics (Colborn et al., 1993). Due to their structural similarity to endogenous hormones, EDC's can interfere with many aspects of the endocrine system including, but not limited to, hormone synthesis, storage, receptor recognition, and post receptor activation (Choi et al., 2004; Crisp et al., 1998; DeRosa et al., 1998; Goldman et al., 2000). EDC's have sparked alarm as they can induce their toxic effects at very low levels (below the no-observed-adverse-effect level) which is not common among other toxicants (Schönfelder et al., 2002). Many environmentally-prevalent pollutants including polychlorinated biphenyls (PCBs), dioxins, polyaromatic hydrocarbons (PAHs), phthalates, pesticides, heavy metals (arsenic, cadmium, lead and mercury) as well as bisphenol A (BPA) act as EDCs which is a concern for the health of the environment and humans (Colborn et al., 1993; Hodges et al., 2000; Iavicoli et al., 2009). More specifically EDCs have been linked to many adverse human health effects including mutagenicity, immunotoxicity, carcinogenicity, neurotoxicity and various reproductive, developmental, physiological and hormonal effects which lead to decreased sperm count, impaired fertility, irregular menstrual cycle and infertility (Balabanič et al., 2011; Choi et al., 2004; Schantz and Widholm 2001). This paper will specifically look at the role of the EDC Bisphenol A (BPA), 2,2-bis-4-hydroxyphenyl propane, as a reproductive toxicant using the model *Caenorhabditis elegans*.

Approximately 4 million metric tons of BPA are produced annually making it one of the highest production chemicals in the world. It is commonly used as an intermediate in the production of several plastic polymers, including polycarbonate and epoxy resins commonly found in water bottles, dental fillings, receipt paper, cans, among others (Shelby et al., 2008; Vandenberg et al., 2007). Aging, heating, and contact with acids and bases, such as detergent, cause the BPA polymers to break apart, a process commonly referred to as "leaching." These detached monomers can seep into food, dust and/or saliva exposing anything that comes into contact with them, including humans, to their harmful effects (Can et al., 2005; Kang et al., 2006; Lenie et al., 2008; Vandenberg et al., 2007; vom Saal and Hughes, 2005; Welshons et al., 2006). Once exposed via inhalation or ingestion, BPA travels through the enterohepatic flow and into the bloodstream. BPA is conjugated to glucuronide in the digestive system and excreted via the urinary system. Consistent with the general toxicokinetic pathway, BPA has been detected in blood, saliva, and human tissues as well as in 95% of human urine samples in the United States (Arakawa et al. 2004; Calafat et al., 2005; Lang et al., 2008; Ouchi and Watanabe, 2002; Yang et al. 2003). BPA is also detected in mammary tissue, placental tissue, breast milk, amniotic fluid, and ovarian follicular fluid (Ikezuki et al., 2002; Lenie et al., 2008; Schönfelder et al., 2002; Vandenberg et al., 2007, 2009). The presence of BPA in these tissues and fluids used primarily for reproduction justifies the need to research the reproductive toxicity of BPA.

Rodent studies have found that BPA exposure induces early onset puberty, changes in weight gain, early vaginal opening and ovarian morphological abnormalities, such as cystic ovaries, cystadenomas, hemorrhagic follicles, and large antral-like

follicles, in the offspring (Honma et al., 2002; Hartshorne, 1997; Howdshell et al., 1999). Sheep studies have found that prenatal BPA exposure, at levels close to that seen in human maternal circulation, result in low birth weight and prolonged first breeding season of the offspring (Savabieasfahani, 2006).

While animal studies have detected physical changes to the offspring of BPA exposed mothers, cell studies have investigated BPA's direct effect on developing oocytes. Hunt et al. (2003) found that administration of 20-100 ng/g body weight of BPA for 5-7 days in prepubertal mouse pups led to a dose-related significant increase in the number of metaphase II oocytes with misaligned chromosomes (Hunt et al., 2003; Susiarjo et al., 2007). Thus, BPA's endocrine-disrupting activity seems to disrupt meiosis, the cell division process that creates sperm or eggs, which leads to aneuploidy in maturing oocytes. Eichenlaub-Ritter et al. (2008) found a similar trend of reduction in the yield of maturation competent oocytes from ovaries of female mice exposed *in vivo* to 20 or 40 ng/g BPA, from 52.2 ± 9.6 oocytes to 48.8 ± 10.0 oocytes and 47.9 ± 7.8 oocytes, respectively, but the reduction did not reach statistical significance as reported in the Hunt et al. study (2003). Eichenlaub-Ritter et al. (2008) also found a trend for more oocytes in telophase I in the 40 ng/g BPA group compared to the control (3.5% versus 1.9%), but did not detect an increase in numbers of telophase I oocytes in the 100 ng/g BPA group compared to the control. Can et al. (2005), evaluated the effects of BPA on cell cycle progression in mouse cumulus-oocyte complexes (COCs) which were exposed to 10 or 30 mM of BPA for either 0-8 or 8-18 hours. During the first half of meiotic division (meiosis-I), hours 0-8, 10 mM BPA- treated cells showed a slight delay in progression, and they mostly reached the stage of M-I (74%, $P < 0.05$) while a lower rate

of cells (61%, $P < 0.001$) reached M-I when treated with 30 mM BPA. When cells were exposed to BPA during the transition from M-I to M-II, which occurs between hours 8–10 in mice, 39% ($P < 0.001$) and 53% ($P < 0.001$) of cells remained in M-I with 10 and 30mM BPA exposure, respectively, while nearly all (94%) of the controls transitioned to M-II. Lenie et al. (2008) confirmed these findings in as oocytes exposed to 3nM of BPA were able to pass through metaphase II, but displayed chromosomal abnormalities in mature oocytes while exposure to 30 nM of BPA induced a checkpoint-based block in meiotic maturation caused by congression failure. BPA's ability to induce toxicity to oocyte cell cycle progression at concentrations ranging from 3nM to 30mM as outlined in the studies above make it a cause for concern as BPA doesn't follow a clear dose-response.

The abnormalities in development and oocyte maturation outlined above may be linked to BPA's ability to interfere with the endocrine system. Peretz et al. (2010) found that exposure to 440 μM BPA not only significantly decreased antral follicle growth, but also significantly decreased estradiol, estrone, testosterone, androstenedione, DHEA-S and progesterone levels compared with DMSO controls. In a follow up study, Peretz et al., (2013) found that mouse antral follicles exposed to BPA (10 μg/mL and 100 μg/mL) significantly decreased progesterone levels 24 to 96 hours after exposure and significantly decreased androstenedione, testosterone, and estradiol levels 72 to 96 hours after exposure compared to DMSO controls. Other toxicological *in vivo* studies indicate that BPA doses from 20 to 400 μg/kg/d can disrupt normal physiology by interfering with endogenous hormones in rodents, nonhuman primates, and cell culture test systems (Rochester et al., 2013). There are many possible mechanisms BPA can act on the

endocrine system including interference at the estrogen receptors, androgen receptor, estrogen-related receptors, upregulation of the aryl hydrocarbon receptor, and genetic and epigenetic modifications (Accocina et al., 2015). This study will specifically examine BPA's role in the transport of cholesterol into the mitochondria via the steroidogenic acute regulatory protein (StAR), a conserved step in the synthesis of steroid hormones in mammals and other hormones in *C. elegans*.

StAR and TSPO Transporters

Steroidogenic acute regulatory protein (StAR), a cytosolic protein with a mitochondrial targeting signal, is the rate limiting factor in the delivery of cholesterol (Ikonen, 2006). StAR is located on the outer mitochondrial membrane (Bose et al., 2002) where it transports cholesterol to the intermembrane space. The peripheral-type benzodiazepine receptor (PBR), located on the inner membrane, transports cholesterol to the matrix where it undergoes steroidogenesis (Papadopoulos, 2004). P-450 side chain cleavage enzyme (P450scc/CYP11A1) catalyzes three distinct reactions, 20α-hydroxylation, 22-hydroxylation and scission of the 20,22 carbon-carbon bond, which results in the transformation of cholesterol to pregnenolone (Miller et al., 1988; Miller et al., 1999). Pregnenolone is then used to synthesize other hormones such as progesterone, androstenedione, testosterone, and estradiol. StAR expression significantly increased not only after luteal cells were exposed to progesterone (Rekawiecki et al., 2005), but also in humans with enhanced free cholesterol (Caballero et al., 2008). This positive correlation between StAR, cholesterol levels and progesterone levels further strengthens the evidence for StAR's possible role in cholesterol transport and steroid hormone synthesis.

Exposure to BPA (10 and 100 μg/mL) significantly decreased not only expression of Cyp11a1 and StAR, but also levels of androstenedione, testosterone, and estradiol suggesting that Cyp11a1 and StAR are important factors in steroidogenesis (De Coster and Larabeke, 2012; Peretz et al., 2010; Peretz et al., 2013). All these effects were reversed when BPA was removed indicating that BPA can increase or prevent the catabolism of steroid hormones (Peretz et al., 2013, Tabb et al., 2006). Although StAR seems to be the predominant OMM cholesterol transporter, Transcolator protein (TSPO) has also been shown to bind and translocate cholesterol from the OMM to the IMM (Isspo et al., 2013). While the relationship between BPA and StAR have been addressed above, there are currently no studies that look at the relationship between BPA and TSPO.

While it is clear BPA somehow interacts with StAR to suppress hormone synthesis, there are no studies that look at the possible antagonistic relationship of BPA and cholesterol in the use of the StAR or TSPO transporters and the downstream effect on reproductive function. Our study hopes to fill these research gaps by using the model *Caenorhabditis elegans.*

Caenorhabditis elegans

Caenorhabditis elegans is a highly utilized model for reproductive toxicity studies for many reasons. The nematodes are relatively easy and cheap to maintain when compared to animal and mammalian models as they have a short life cycle (~4 days at 20°C), so data can be collected on a whole, living organism at the same rate as on cells in culture (Ferreira et al., 2017). Their short life cycle coupled with their ability to lay

around 250 to 350 eggs through self-fertilization makes them an excellent model for reproductive toxicity studies as researchers have large numbers to gauge reductions in brood size and progeny growth (Boyd et al., 2010; Ferreira et al., 2017). Additionally, the transparent cuticle of the worms aids in visualization of the symmetrically arranged U-shaped bi-lobed gonad without dissection (Ferreira et al., 2017).

The overall reproductive system of *C. elegans* is displayed as a temporal-spatial gradient in the symmetrically arranged bi-lobed gonads (Ferreira et al., 2017; Zetka and Rose, 1995). Each lobe is U-shaped, starting from the center of the worm's body, extending towards either the tail or mouth of the worm and then looping back to a common uterus and vulva. Each of the different stages of germline development can be identified by the nuclei location along the germline as well as their morphology. The germline nuclei initially display a compact circular morphology through the mitotic zone (Ferreira et al., 2017). While under the influence of the distal tip cells (DTC) germline nuclei exit the mitotic zone and enter the transition zone (leptotene and zygotene of Prophase I) where the nuclei become crescent shaped due to chromosome pairing and the initiation of synaptonemal complex formation (synapsis). The germline nuclei then enter the pachytene zone where the nuclei resemble "tracks" or "spaghetti". These tracks represent the pairs of homologous chromosomes connected by a number of proteins that make up the synaptonemal complex. Cellular DNA damage and exposure to an environmental stressor, such as BPA, during meiosis can trigger genome integrity checkpoints and induce apoptosis (Ferreria et al., 2017; Gartner et al., 2000). These apoptotic germ cell nuclei appear in the pachytene zone as a round, bubble under DIC microscopy and emit intense fluorescence following acridine orange staining and

visualization under the fluorescein isothiocyanate (FITC) filter (Ferreria et al., 2017). During diplotene and diakinesis, the final stages of prophase I, chromosomes condense further and the germline nuclei can be visualized in a single file (Ferreira et al., 2017). Cellularization occurs during diakinesis to form oocytes containing six pairs of homologous chromosomes. The oocytes will be fertilized as they pass through the spermatheca and then expelled into the worm's uterus where early embryogenesis begins. Early embryos become encapsulated with a chitinous shell and laid in the environment through the vulva. These canonical changes to the nuclei can be visualized by DAPI nuclear staining to assess germline health and function (Ferreira et al., 2017).

In regards to cholesterol transport in *C. elegans*, nematodes are unable to synthesize cholesterol and require it in their diet (Kurzachalia and Ward, 2003). In nature, *C. elegans* receive their cholesterol from animal feces or yeast/plant remnants found in the soil. In the lab, this is an advantage when studying sterol biosynthesis as the researcher can regulate the amount of cholesterol *C. elegans* can use for steroidogenesis (Hieb and Rothstein, 1968**;** Chitwood and Lusby, 1991). Matyash et al. (2001) found that cholesterol mostly accumulates in the pharynx, nerve ring, excretory gland cell, gut and germline cells. They also suggest that accumulation of cholesterol in oocytes may be dependent on the LDL yolk receptor RME-2 as they found lower levels of dehydroergosterol (DHE), a fluorescent cholesterol analog, in the oocytes of *rme*-2 mutants. *C. elegans* grown on plates depleted of cholesterol displayed defects in molting (Yochem et al., 1999) and inhibition of growth and reproduction (Chitwood et al., 1984). Cholesterol levels in gonads in particular influence signal transduction of the Ras/MAP-

kinase pathway which accelerate meiotic cell cycle progression via exit from the pachytene arrest (Church et al., 1995; Scheel et al., 1999).

Hypothesis

Preliminary research has shown that exposure to increasing levels of BPA leads to a decreased brood size when low levels of cholesterol are present. However, exposure to high levels of cholesterol rescues the germline defects as brood size does not decrease as BPA levels increase. Here, our study is going to look further into this possible antagonistic relationship between BPA and cholesterol specifically at the site of the StAR mitochondrial transporter. **We hypothesize that BPA and cholesterol uptake are antagonistic at the StAR transporter and that BPA germline toxicity can be phenocopied by inhibiting mitochondrial cholesterol transport in *C. elegans*.**

To evaluate this relationship we will conduct a fertility assessment for N2 (wild-type) and two mutants, *Strl-1* F52F12.7(ok3347) I (StAR) and *tspo-1* C41G7.9(tm5526) (TSPO), as well as conduct a germline morphology examination and cholesterol assay on extracted mitochondria. While the fertility assessment and germline morphology examination will give us an idea of the phenotypic reproductive toxicity effects, the cholesterol assay on extracted mitochondria will give us a possible mechanism to how BPA acts as a reproductive toxicant. We will have nine different exposures each with either 0 µg/ml cholesterol, 0.5 µg/ml cholesterol or 5 µg/ml cholesterol and 0 µM BPA, 100 µM BPA or 500 µM BPA. The nine exposures are: 1) 0 µg/ml cholesterol and 0 µM BPA 2) 0 µg/ml cholesterol and 100 µM BPA 3) 0 µg/ml cholesterol and 500 µM BPA 4)

0.5 µg/ml cholesterol and 0 µM BPA 5) 0.5 µg/ml cholesterol and 100 µM BPA 6) 0.5 µg/ml cholesterol and 500 µM BPA 7) 5 µg/ml cholesterol and 0 µM BPA 8) 5 µg/ml cholesterol and 1000 µM BPA 9) 5 µg/ml cholesterol and 500 µM BPA. The nine exposure groups will let us determine how a range of cholesterol and BPA interact.

METHODS

Materials and Methods

Animal maintenance: Bristol N2 *C. elegans* were used as the wild type and maintained on the Nematode growth medium (NGM) at 20°C. For exposure, eggs obtained from the sodium hypochlorite treated gravid worms were placed on the NGM plates mixed with 0 (0.1% ethanol), 100 µM, and 500 µM BPA combined with 0 (0.1% ethanol), 0.5 µg/ml, and 5 µg/ml cholesterol. Ethanol was used as the vehicle since both cholesterol and BPA were dissolved in ethanol. Exposure lasted from the birth to the beginning of reproduction (72 hours) of the tested *C. elegans*. Strains with mutation on *Strl-1* F52F12.7(ok3347) I and *tspo-1* C41G7.9(tm5526) I were subjected to the same assays with N2 to identify the critical mitochondrial cholesterol transporter that BPA relied on to exhibit its toxicity.

Fertility Assessments

To assess the fertility damage caused by the exposure, we counted the total the number of eggs laid by each nematode during its entire reproductive period, the number of larvae and the number of offspring that survive to adulthood. Embryonic lethality was calculated as the percentage of eggs laid divided by the number of larvae. Larval lethality

was calculated as the percentage of larvae divided by the number of adults. Brood size is the total number of progeny that survive to adulthood.

Germline Apoptosis Assay

The germline apoptosis assay was performed following the protocol mentioned before. After reaching adulthood, worms were incubated in M9 solution (5.8 g Na_2HPO_4, 3.0 g KH_2PO_4, 5.0 g NaCl, 0.25 g $MgSO_4$ in 1 L dH2O) with 25 µg/ml of Acridine Orange (AO) for two hours at room temperature (Ferreria et al., 2017). AO specifically stains the engulfed apoptotic germline nuclei and emit 525 nm fluorescence after excitation, which makes the apoptotic nuclei visible under the Fluorescein isothiocyanate (FITC) filter of the fluorescent microscope. The total number of apoptotic nuclei in the pachytene zone of the posterior gonad for each worm was counted. Apoptotic index was calculated as the percentage of apoptotic nuclei divided by the total number of nuclei in the pachytene zone.

Germline Morphology Examination

To examine the morphological changes after exposure, DNA staining, following a previous protocol (Ferreria et al., 2017) was performed with DAPI on the young adult worms fixed by the Carnoy's fixation. Images from the posterior gonad of each worm were captured and the number of nuclei in each developmental stage was counted.

Preparation of Worms

To synchronize the worms we bleached N2, *Strl-1* F52F12.7 (ok3347) or *tspo-1* C41G7.9 (tm5526) worms by exposing collected worms to bleach solution (7.5 ml 10 N NaOH, 6 ml Chlorox, 36.5 ml dH2O) for 3 minutes to kill all stages of worm except the eggs. After washing the bleached worms with M9 solution 3 times, we transferred the eggs to Nematode growth medium (NGM) plates of the given exposure without *E. coli* OP50 bacteria. After 24 hours in the 20 °C incubator, we transferred the arrested L1 worms to new plates of the same given exposure with OP50 bacteria (Matyash et al., 2001). 72 hours after the double synchronization in a 20 °C incubator, when the adult worms just started laying eggs, we gently washed the worms off the plates with M9 solution as to not disrupt the agar (containing cholesterol and/or BPA). These worms were transferred to a 50 ml conical tube and then spun down in the centrifuge at 3000x g for 1 min (Brake = 1). We let the remaining worms gravity settle for 10 minutes. The pellet of worms was transferred to a new 50 ml conical tube via pasteur pipette and filled with M9. The wash was repeated three more times to ensure bacteria and agar were not in our samples. The worms were then aliquoted into 2 ml tubes so that each tube had about 400 µl (~10,000, worms). Let worms gravity settle and remove excess supernatant.

Preparation of Isolation Solutions

Reagents from the Sigma® Mitochondria Isolation Kit (Catalog Number: MITOISO1) were used throughout the mitochondria extraction. 72 ml 1x Extraction Buffer A (EBA) containing 0.25 mg/ml Trypsin (14.4 ml 5x EBA stock + 57.6 ml ddH2O + 1.44 ml trypsin (12.5 mg/ml)) was prepared and 64 ml 1x EBA (without

trypsin) (12.8 ml 5x EBA stock + 51.2 ml ddH2O) was made for the extraction. 40 ml Worm Lysis Buffer (WLB) (32 ml 1 M sucrose + 400 µl 100 mM EDTA (100 mM) + 400 µl Tris-HCl (1 M, pH 7.4)) + 7.2 ml H2O) was made for suspension and storage of the mitochondria. All reagents were kept on ice. 60 ml of 1M sucrose solution was made (50.8 ml 1.02M sucrose, 600 µl 100 mM EDTA, 600 µl Tris-HCl (1 M, pH 7.4)). 60 ml of 2 M sucrose solution was made (50.8 ml 2.04 M sucrose, 600 µl 100 mM EDTA, 600 µl Tris-HCl (1 M, pH 7.4)).

Isolation of Mitochondria from Whole Worms

To perform the extraction we used the Sigma® Mitochondria Isolation Kit (Catalog Number: MITOISO1). All reagents and worms were kept on ice throughout the procedure. Each 2 ml tube of worms was suspended in in 1.3 ml EBA with trypsin and then incubated on ice for 3 min. The tubes were then centrifuged at 3000x g for 1 minute to pellet the worms and the supernatant was aspirated. The pellet was then washed with 1.0 ml EBA with trypsin and incubated on ice for 20 minutes. 320 µl albumin solution (50 mg/ml) was added to each tube to quench the proteolytic reaction. The tubes were centrifuged at 3000x g for 1 minute to pellet the worms and then the supernatant was aspirated. The pellet was washed with 0.7 ml EBA (without trypsin), the tubes of similar groups were combined and the tubes were centrifuged at 3000x g for 1 minute to pellet the worms. Supernatant was aspirated and then the pellet was washed again with 1.4 ml EBA (without trypsin) and centrifuged at 3000x g for 1 minute to pellet. Each 2 ml sample was homogenized using a cold dounce homogenizer (300 strokes). Homogenizer was rinsed between groups with soap, cold deionized water and EBA (without trypsin). Newly homogenized samples were transferred to new 2 ml tubes and 100 µl of the

homogenate from each group collected and stored (sample A) for further analysis. The 2 ml tubes of homogenate were then centrifuged at 600x g for 5 minutes. 100 µl of supernatant (crude extracts) was collected from each sample for further analysis (sample B). The rest of the supernatant was transferred to a new 2 ml tube and centrifuged at 11000x g for 10 min. 100 µl of the supernatant (mitochondrial supernatant) was collected from each sample for further analysis (sample C). The rest of the supernatant was discarded and the pellet was suspended in 300 µl of WLB. 5 ml of the 2 M sucrose solution was added to each of the 9 10.4 ml polycarbonate centrifuge bottle (with cap assembly) and 5 ml of the 1 M sucrose solution was overlaid on the 2 M sucrose solution to create a gradient. 300 µl of samples in WLB was then gently added via pasteur pipette to the top of each tube. The tubes were then centrifuged in the Type 40 ultracentrifuge at 35,000 rpm for 90 minutes at 4 °C. We transferred the brown band (mitochondria) in the middle of the sucrose gradient to new 10.4 ml polycarbonate centrifuge bottle (with cap assembly). The samples were diluted with 3 volumes cold 1 x PBS and centrifuged at 21,000 rpm for 30 minutes at 4° C. The pellet was resuspended in 100 µl 1 x PBS (sample D) and stored at -20 °C with the other samples for further analysis.

Preparation of Cholesterol Assay Reagents

The Amplex® Red Cholesterol Assay Kit from InvitrogenTM (Molecular Probes®) was used for the cholesterol assay. The cholesterol standard curve was created by diluting 5 µl 2 mg/ml cholesterol reference with 20 µl 1x Reaction Buffer to create 25 µl of 400 µg/ml cholesterol. 6 µl 400µg/ml was diluted into 144 µl 1x Reaction Buffer to create 150 µl of 16 µg/ml cholesterol. 150 µl 1x Reaction Buffer was added to create 300

µl of 8 µg/ml cholesterol. 2-fold serial dilutions were continued to create a cholesterol standard curve of 0.125, 0.025, 0.5, 1, 2, 4, and 8 µg/ml. 23 µl of 3.0% H202 was dissolved into 977 µl deionized H20 to make the 20 mM H202 positive control. 1x Reaction Buffer was used as the negative control. 5ml of 300 µM Amplex® Red working solution was made by adding 50 µl Horseradish peroxidase (HRP), 50 µl cholesterol oxidase stock solution, 5 µl cholesterol esterase stock solution, 75 µl Amplex® Red reagent stock solution to 4.82 mL of 1x Reaction Buffer.

Cholesterol Assay

Pipette 50 µl of the diluted samples and controls into separate wells of a 384-well black/clear plate. Add 50 µl Amplex® Red working solution to each well to begin the reaction. The plate was incubated for 30 minutes or longer at 37 °C protected from light. The FlexStation at the Molecular Screening Shared Resources (MSSR) center at UCLA was used to read the fluorescence of each sample at an excitation wavelength of 565 nm and an emission wavelength of 595 nm.

Preparation of Protein Assay Reagents

The BioRad Colorimetric Protein Assays kit was used for this assay. A protein standard curve was made by adding 0.5 ml of the 3 mg/ml protein stock to 0.5ml deionized H20 (1 ml 1.5 mg/ml protein). Add 0.8 ml H2O to 0.2 ml of the 1.5 mg/ml to make 1 ml 0.3 mg/ml protein. Continue these 5-fold serial dilutions to make a curve with 0.0024, 0.012, 0.06, 0.3 and 1.5 mg/ml protein. Deionized H2O was used as the negative control.

Protein Assay

5 µl of standards or samples were pipetted into wells of a 96-well black/clear plate. 25 µl of Reagent A' were added into each well followed by 200 µl Reagent B into each well. The plate was incubated for 15 minutes at room temperature. Absorbance was measured by the Invision at the MSSR at 75 nm.

JC-1 and MITO-ID Staining

About 500 eggs were placed on agar plates with exposure of varying levels of cholesterol and/or BPA after being bleached. 100 L4 stage *C. elegans* were synchronized about 48 hours after the bleaching and transferred to new agar plates with the same exposure. After 24 hours, when the *C. elegans* were adults, 50 were transferred to a 2 ml conical tube with 500 µl of M9 and 0.5 µl JC-1 staining. Either 1 µl 0.1M SA (final concentration of 200 µM), 0.5 µl 10uM FCCP solution (final concentration of 10nM) or 0.5 µl of 10 mM CCCP solution (final concentration of 10 µM) was added as the positive control. Each tube was wrapped in foil and incubated on a rotator in the dark for 3 hours. Tubes were centrifuged for 2 minutes at 350 g to pellet worms. Supernatant was removed to ~100 µl and 10 µl of the pelleted *C. elegans* were mounted on a 2% agar pad. The germlines were observed under fluorescence using FITC and TxRed channels. FITC was set first, ensuring there was no saturation, then we applied the same exposure time to the TxRed channel. Different exposure times were used for each worm in the same group and across groups. For the MITO-ID staining the procedure was the same as JC-1 except for the exposure time was 30 minutes instead of 3 hours.

RESULTS

N2 Fertility Assessment and Germline Morphology

We first assessed whether exposure to varying levels of BPA and cholesterol would alter the worms' fertility. The overall fertility of the worms was examined by assessing four metrics; egg number, embryonic lethality, larval lethality and brood size. The number of eggs laid by each adult N2 *C. elegan* was used to determine if exposure to varying levels of BPA and cholesterol would alter the worms ability to produce and lay eggs. In the no cholesterol groups, we found that there was a general decrease in egg number as BPA levels increased. With 0.5 μg/ml cholesterol exposure the egg number did not decrease as BPA levels increased, however, there was a significant increase between the no cholesterol/500 μM BPA and 0.5 μg/ml cholesterol/500 μM BPA ($p<0.01$). With 5 μg/ml cholesterol exposure the egg number again did not decrease as BPA levels increased. There was also a significant increase between the no cholesterol/500 μM BPA to 5 μg/ml cholesterol/500 μM BPA ($p<0.01$) (Figure 1A). Cholesterol's ability to restore egg number suggests that cholesterol antagonizes the reproductive toxicity of BPA.

Embryonic lethality was assessed to determine the percentage of eggs that do not hatch. When no cholesterol is present, there is a significant increase in embryonic lethality as BPA levels increase from no BPA to 500 μM BPA ($p<0.00001$) and from 100 μM BPA to 500 μM BPA ($p<0.01$). With 0.5 μg/ml cholesterol exposure, the percent embryonic lethality did not increase with BPA exposure and embryonic lethality significantly decreased from no cholesterol/500 μM BPA to 0.5 μg/ml cholesterol/500 μM BPA ($p<0.01$). With 5 μg/ml cholesterol exposure, the percent embryonic lethality

again did not increase with BPA exposure, but it significantly decreased from no cholesterol/500 μM BPA to 5 μg/ml cholesterol/500 μM BPA($p<0.01$) (Figure 1B). The trend of decreased embryonic lethality with cholesterol exposure suggests that cholesterol antagonizes the reproductive toxicity of BPA.

Larval lethality was then assessed which is an indicator of how many hatched larvae don't make it to adulthood. When no cholesterol is present, BPA causes a significant increase in larval lethality from no BPA to 500 μM BPA ($p<0.0001$) and from 100 μM BPA to 500 μM BPA ($p<0.01$). With 0.5 μg/ml cholesterol exposure, the percent larval lethality does not increase with BPA exposure, but significantly decreased between no cholesterol/500 μM BPA and 0.5 μg/ml cholesterol/500 μM BPA ($p<0.01$). With 5 μg/ml cholesterol exposure, the percent larval lethality again did not increase with BPA exposure, but it did significantly decrease between no cholesterol/500 μM BPA and 5 μg/ml/500 μM BPA ($p<0.05$) (Figure 1C), suggesting that cholesterol antagonizes BPA.

We finally examined brood size which assesses how many larvae reach adulthood. We found that without cholesterol, brood size significantly decreased with exposure to 500 μM BPA ($p<0.01$). Cholesterol exposure at 0.5 μg/ml ($p<0.01$) and 5 μg/ml ($p<0.01$) significantly restores brood size compared to no cholesterol even when BPA is present at 500 μM BPA (Figure 1D). Overall, egg number, embryonic lethality, larval lethality and brood size give us an idea of the overall fertility of the N2 *C. elegans* and that cholesterol was able to antagonize the reproductive toxicity induced by BPA in all four assessments.

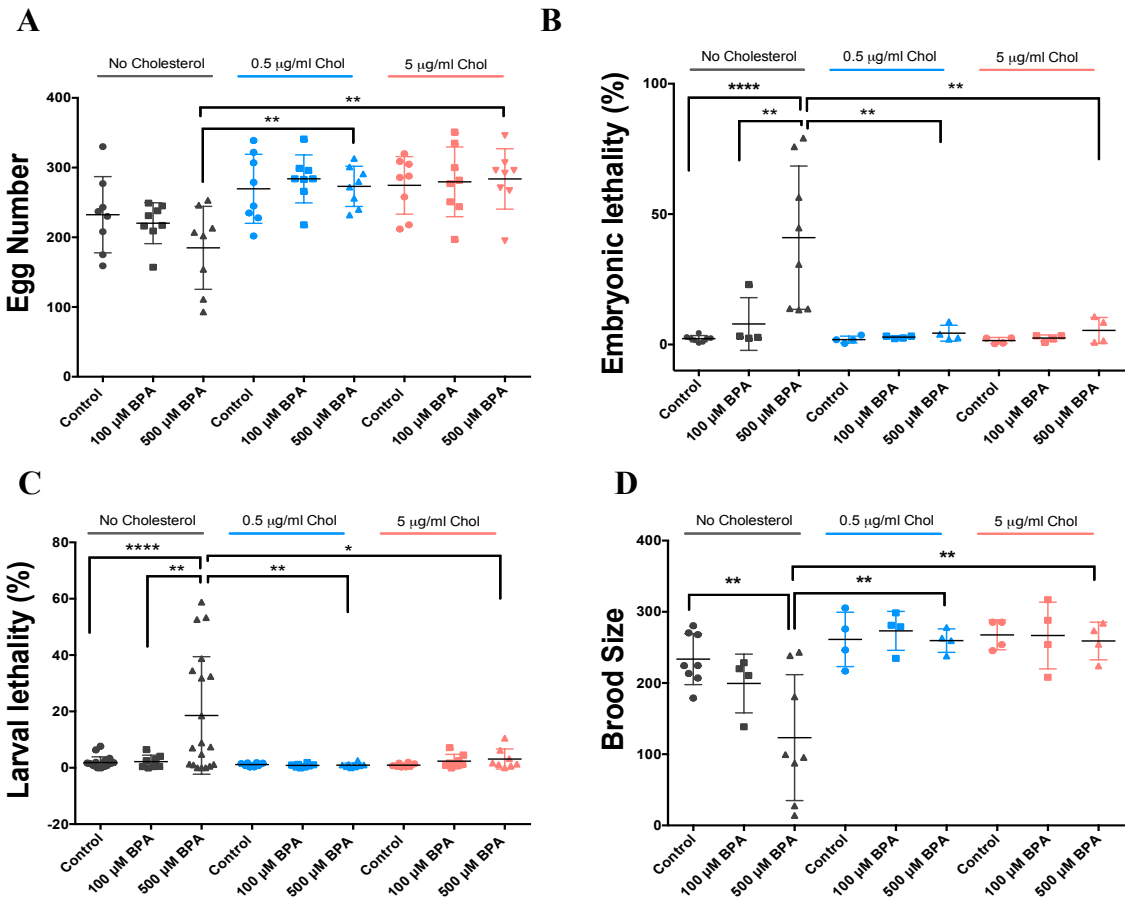


Figure 1. *Exogenous cholesterol antagonizes reproductive toxicity of BPA.* N2 *C. elegans* were grown on NGM plates with varying levels of BPA and cholesterol for the duration of their life. (A, B, C, D) Reproductive toxicity was assessed via fertility assessment. (A) The total number of eggs laid by each individual worm. (B) Embryonic lethality is the percent of eggs that do not hatch. (C) Larval lethality is the percent of larvae that do not reach adulthood. (D) Brood size is the number of offspring that reach adulthood. N = 4 – 8 for each group. Two-way ANOVA with Sidak's multiple comparison test. Significance only shown between groups with similar cholesterol or BPA levels. *p< 0.05, **p<0.01 and ****p<0.0001. Experiments performed in partnership with Yichang Chen.

We then conducted a germline morphology examination of the N2 *C. elegans* which showed us the damage to the germline itself through quantification of gaps and the apoptotic nuclei. Gaps demonstrate the frequency of germline nuclei loss caused by the given exposure. We found that when no cholesterol is present, the frequency of gaps is

significantly higher in combination with 500 μM BPA compared to no BPA ($p<0.0001$). Exposure to 5 μg/ml cholesterol/500 μM BPA significantly reduced the number of gaps compared to no cholesterol/500 μM BPA ($p<0.01$) (Figure 2A-B). This again demonstrates that cholesterol antagonizes the reproductive toxic effects of BPA.

Apoptotic index is the quantification of apoptotic germline nuclei in every 100 pachytene nuclei (Supplementary Figure 1). We found that when no cholesterol is present, the apoptotic index significantly increases from no BPA to 500 μM BPA ($p<0.00001$) and from 100 μM BPA to 500 μM BPA ($p<0.05$). Exposure to 5 μg/ml cholesterol/500 μM BPA significantly reduces the apoptotic index compared to no cholesterol/500 μM BPA ($p<0.0001$) (Figure 2C-D). The germline morphology results coupled with the fertility assessment results support our hypothesis that cholesterol antagonizes the reproductive toxicity of BPA.

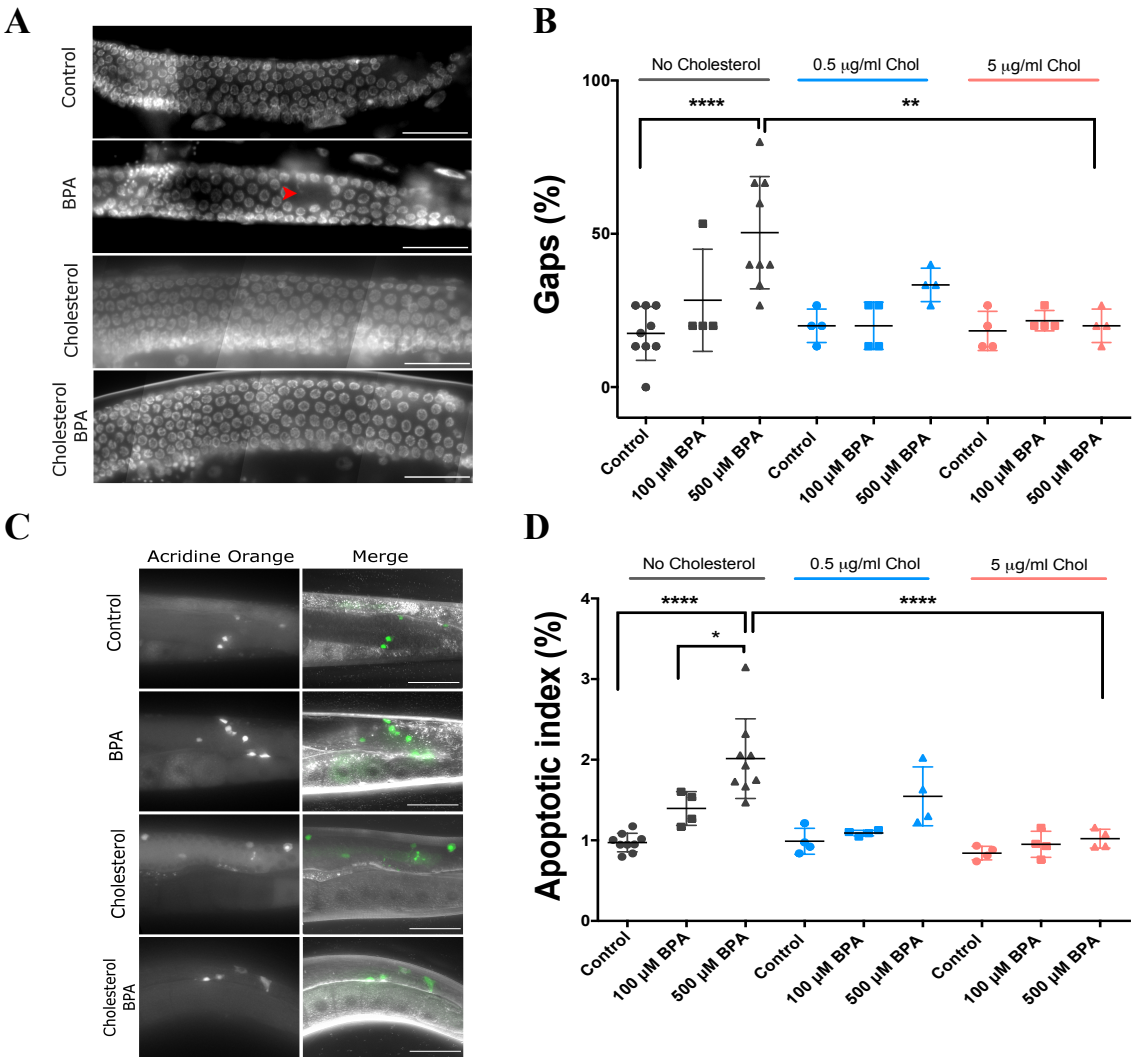


Figure 2. *Exogenous cholesterol antagonizes reproductive toxicity of BPA*. (A) Low-magnification images of DAPI-stained pachytene region of the gonad from age-matched hermaphrodites exposed to vehicle control (0.1% ethanol), 500 µM BPA, 5µg/ml cholesterol and the combination of these two. Red arrow is an area with a gap which indicates germline nuclei loss (Scale bar,10 µm). (B) The frequency of germline nuclei loss (gap) caused by exposures was examined with DAPI staining. (C) Low-magnification images of AO-stained gonads from age-matched hermaphrodites exposed to vehicle control (0.1% ethanol), 500 µM BPA, 5µg/ml cholesterol and the combination of these two. Merge is of AO stain and 40x DIC image (Scale bar, 25 µm). No staining is necessary for visualization of worm under DIC. (D) Quantification of apoptotic germline nuclei in every 100 pachytene nuclei was calculated for each exposed worm with AO staining. N = 4 – 9 for each group. Two-way ANOVA with Sidak's multiple comparison test. Significance only shown between groups with similar cholesterol or BPA levels. *p< 0.05, **p<0.01 and ****p<0.0001. DAPI and AO performed by Yichang Chen.

Cholesterol Assay

To determine how cholesterol antagonizes the reproductive toxicity of BPA, we decided to look at the mitochondria to determine if the amount of cholesterol being transported across the mitochondrial membrane differed based on BPA exposure. To do this, we conducted a mitochondrial extraction followed by a cholesterol and protein assay of the samples taken of the total worm (minus the cuticle) and samples of pure mitochondria. Figure 3A is the results of the cholesterol assay for samples containing the total worm (minus the cuticle). While there were no significant changes between groups, there was a general trend of increased cholesterol in the total worm as BPA levels increase (Figure 3A). There was no clear relationship between BPA and cholesterol in the mitochondria itself (Figure 3B). When we took the ratio of cholesterol in the mitochondria to the total worm (minus the cuticle) there was a decrease in cholesterol as BPA levels increase (Figure 3C). Although not significant, this trend could indicate that BPA is somehow interfering with the uptake of cholesterol inside the mitochondria. The assay, however, was detecting cholesterol in the no cholesterol group. Since *C. elegans* can't produce endogenous cholesterol, no cholesterol should be present when the worms aren't exposed to cholesterol. We ran another experiment where we did not expose the *C. elegans* to cholesterol for two full generations. This group was compared to *C. elegans* exposed to 5 μg/ml cholesterol for only one generation and another group exposed to 5 μg/ml cholesterol for two generations (Supplementary Figure 2). We still detected cholesterol in both groups exposed to no cholesterol. Thus, other assays or techniques should be utilized.

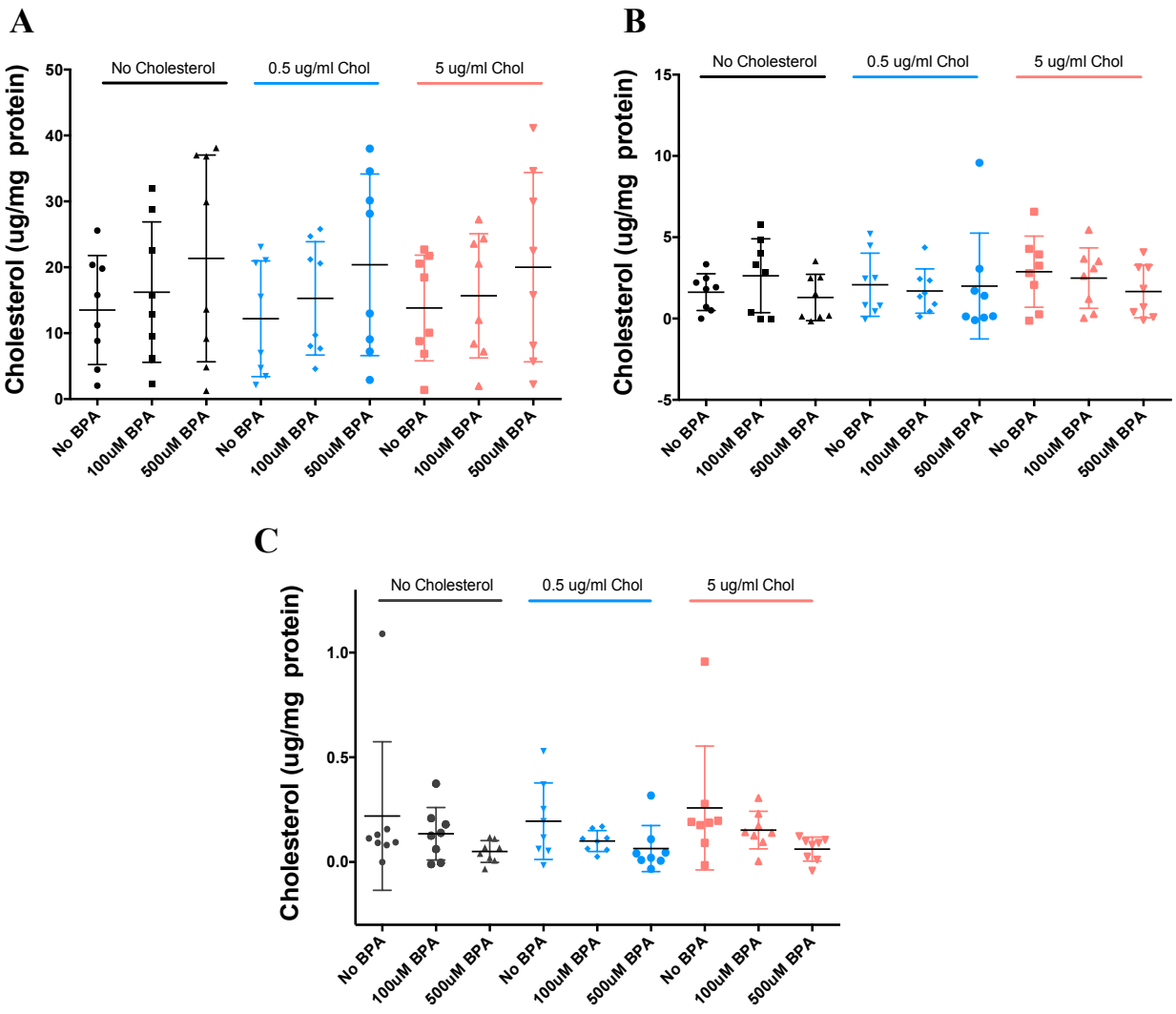


Figure 3. *BPA impairs uptake of cholesterol into the mitochondria.* (A) Cholesterol levels normalized to protein levels found in the total worm (minus cuticle) samples containing everything except the mitochondria and cuticle of *C. elegans.* (B) Cholesterol levels normalized to protein levels found in extraction samples containing pure mitochondria of *C. elegans.* (C) Ratio of cholesterol found in the mitochondria samples to cholesterol found in samples containing everything except the mitochondria and the cuticle of *C. elegans.* N = 8 for each group. Two-way ANOVA with Sidak's multiple comparison test. Significance only shown between groups with similar cholesterol or BPA levels.

StAR Fertility Assessment and Germline Morphology

A fertility assessment and germline morphology examination were then done on StAR mutants which do not have a functional StAR mitochondrial transmembrane transporter and thus lack the ability to pass cholesterol into the mitochondria via this transporter. We hypothesized that comparing the results of StAR with those of N2 would improve our understanding of how BPA induces its reproductive toxicity seen in the N2 *C. elegans.* The overall fertility of the StAR mutants was assessed as described above in the N2 results. StAR mutants did not show a significant change in egg number between cholesterol and BPA exposures (Figure 4A). There was also not a significant change in embryonic lethality or larval lethality with varying BPA and cholesterol levels (Figure 4b-c). Brood size also did not show significant changes among various BPA and cholesterol concentrations (Figure 4D). The germline morphology examination was also conducted as described above in the N2 results. The percentage of gaps and apoptotic nuclei normalized to total pachytene germline nuclei number (Supplementary Figure 3) also did not change significantly between and cholesterol exposures (Figure 5). BPA did not induce reproductive toxicity in the StAR mutants indicating that BPA may rely on the mitochondrial transmembrane protein StAR to elicit its' toxicity.

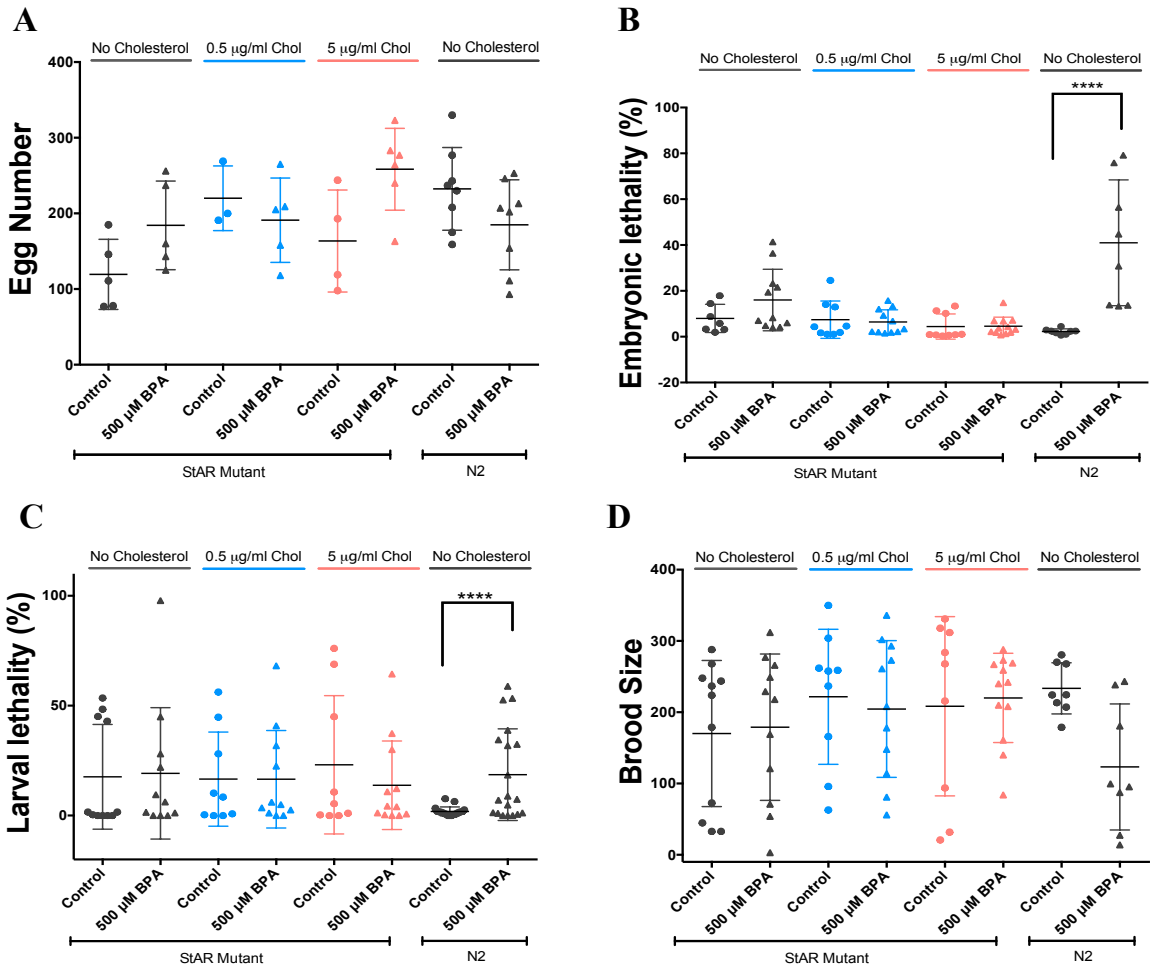


Figure 4. *BPA relies on the mitochondria transporter StAR to exhibit its reproductive toxicity.* StAR *C. elegans* were grown on NGM plates with varying levels of BPA and cholesterol for the duration of their life. (A, B, C) Reproductive toxicity was assessed via fertility assessment. (A) The total number of eggs laid by each individual worm. (B) Embryonic lethality is the percent of eggs that do not hatch. (C) Larval lethality is the percent of larvae that do not reach adulthood. (D) Brood size is the number of offspring that reach adulthood. N = 5 – 12 for each group. Two-way ANOVA with Sidak's multiple comparison test. Significance only shown between groups with similar cholesterol or BPA levels. ****p<0.0001. Experiments performed in partnership with Yichang Chen.

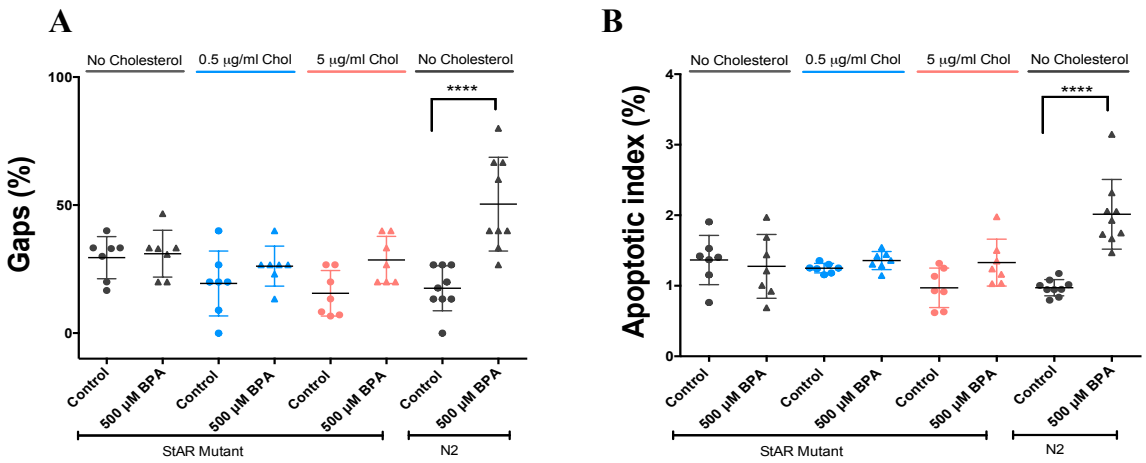


Figure 5. *BPA relies on the mitochondria transporter StAR to exhibit its reproductive toxicity.* (A) The frequency of germline nuclei loss (gap) caused by exposures was examined with DAPI staining (B) Quantification of apoptotic germline nuclei in every 100 pachytene nuclei was calculated for each exposed worm with AO staining. N = 7– 9 for each group. Two-way ANOVA with Sidak's multiple comparison test. Significance only shown between groups with similar cholesterol or BPA levels. ****p<0.0001. DAPI and AO performed by Yichang Chen.

TSPO Fertility Assessment and Germline Morphology

A similar fertility assessment a germline morphology examination was then conducted on TSPO mutants, which do not have a functional TSPO mitochondrial transmembrane transporter and thus lack the ability to pass cholesterol or other compounds into the mitochondria via this transporter. We hypothesized that comparing the results of TSPO with those of N2 and StAR would improve our understanding of how BPA induces its' reproductive toxicity seen in the N2 *C. elegans*. The overall fertility of the TSPO mutants was assessed as described above in the N2 results. TSPO mutants did not show a significant change in egg number with varying BPA and cholesterol exposures (Figure 6A). There was also not a significant increase in embryonic lethality

between exposure groups although there was a strong increase in embryonic lethality in the no cholesterol/500 μM BPA group (Figure 6B).

Larval lethality was significantly higher in the no cholesterol control and no cholesterol with 500 μM BPA ($p<0.01$). Cholesterol exposure at 0.5 μg/ml with 500 μM BPA ($p<0.01$) and 5 μg/ml with 500 μM BPA ($p<0.05$) significantly reduces larval lethality compared to no cholesterol with 500 μM BPA exposure (Figure 6C). Brood size did not significantly change between exposures although again there was a dramatic decrease in brood size in the no cholesterol/500 μM BPA group (Figure 6D). Together, these results suggest that cholesterol is antagonizing BPA even with a nonfunctional TSPO transporter.

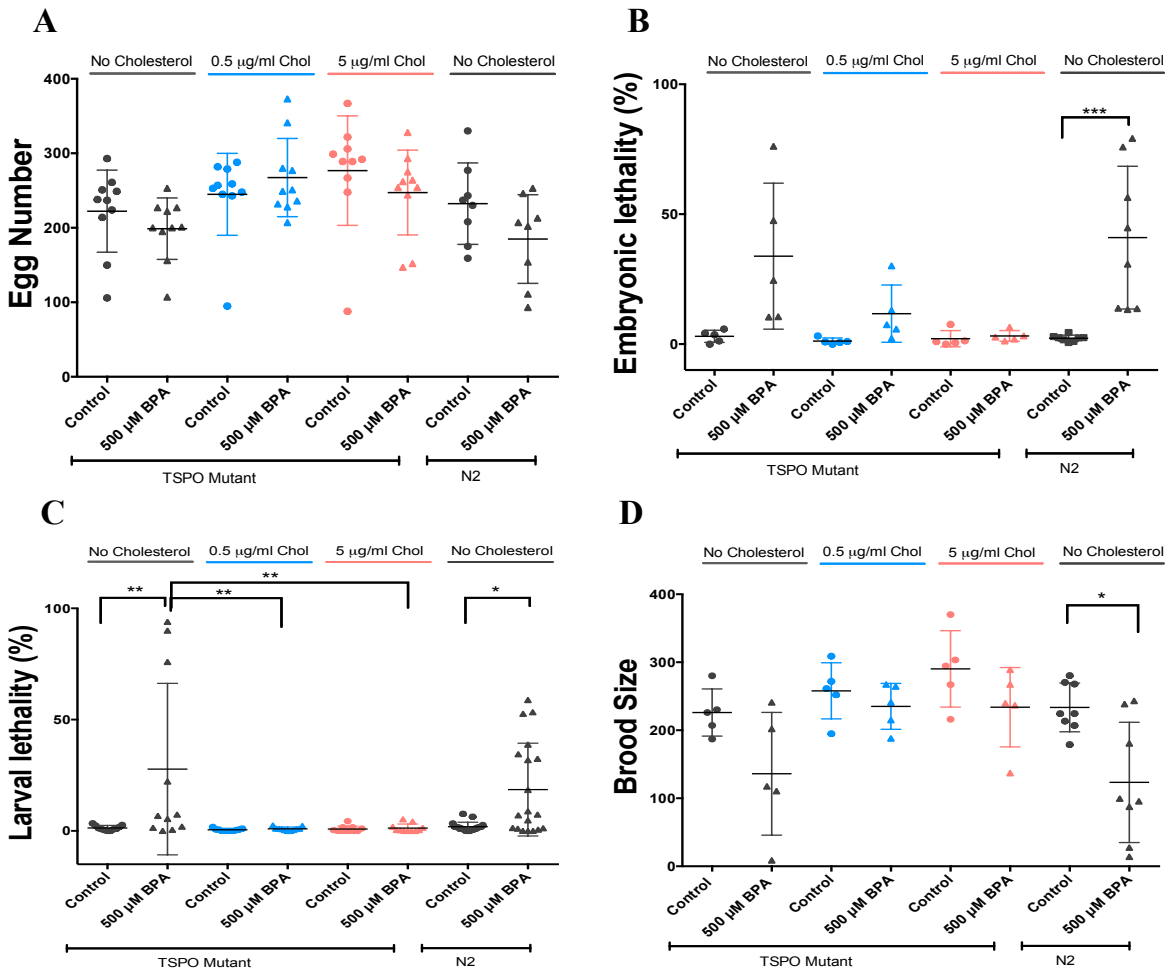


Figure 6. *BPA does not rely on mitochondria transporter TSPO to exhibit its reproductive toxicity.* TSPO *C. elegans were* grown on NGM plates with varying levels of BPA and cholesterol for the duration of their life. (A, B, C, D) Reproductive toxicity was assessed via fertility assessment. (A) The total number of eggs laid by each individual worm. (B) Embryonic lethality is the percent of eggs that do not hatch. (C) Larval lethality is the percent of larvae that do not reach adulthood. (D) Brood size is the number of offspring that reach adulthood. N = 5 – 11 for each group. Two-way ANOVA with Sidak's multiple comparison test. *p< 0.05, **p<0.01 and ***p<0.001. Experiments performed in partnership with Yichang Chen.

The germline morphology examination was also conducted as described above in the N2 results. In the no cholesterol group gaps significantly increased with 500 µM BPA exposure compared to no BPA (p<0.0001) (Figure 7A). The percentage of apoptotic

nuclei normalized to total pachytene nuclei number (Supplementary Figure 4) significantly increased between the no cholesterol without BPA group and the no cholesterol with 500 µM BPA ($p<0.05$) (Figure 7B). Overall, the significant increase in larval lethality, gaps and apoptotic nuclei coupled with the similar response in N2 worms suggest that TSPO is not needed for BPA to induce reproductive toxicity.

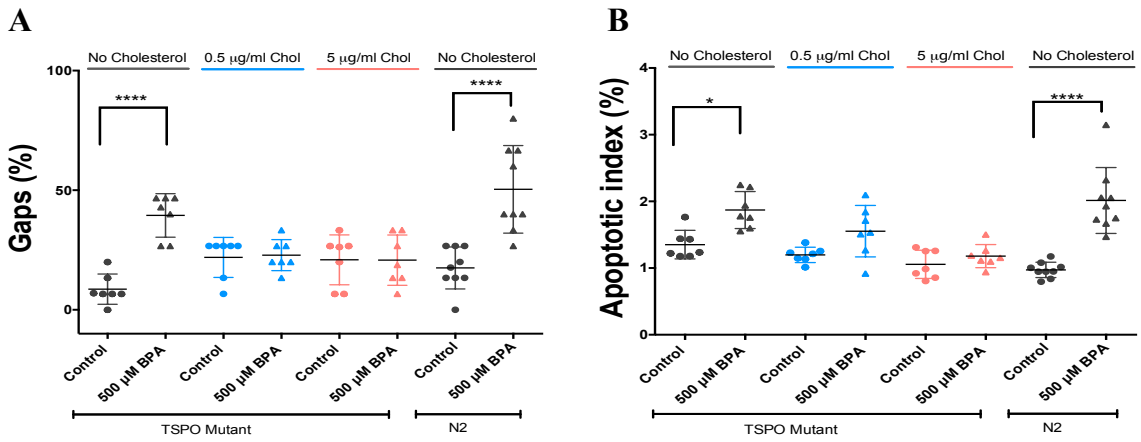


Figure 7. *BPA does not rely on mitochondria transporter TSPO to exhibit its reproductive toxicity.* (A) The frequency of germline nuclei loss (gap) caused by exposures was examined with DAPI staining. (B) Quantification of apoptotic germline nuclei in every 100 pachytene nuclei was calculated for each exposed worm with AO staining. N = 7– 9 for each group. Two-way ANOVA with Sidak's multiple comparison test. *p< 0.05 and ****p<0.0001. DAPI and AO performed by Yichang Chen.

JC-1 and MITO-ID

JC-1 and MITO-ID are dyes that stain the mitochondrial membrane potential. Green fluorescence represents a depolarized mitochondrial membrane while red fluorescence represents a stable mitochondrial membrane. Both dyes are usually applied to look at the mitochondria membrane potential in cell models and neither of these dyes has been tried on a whole organism. The dye seemed to only be able to penetrate the outer cuticle of the worm or the intestine due to the worm consuming the dye. Neither

JC-1 nor MITO-ID reliably dyed our area of interest, the germline nuclei. During image capture, we also were had to use different exposure times for each individual worm (even within the same chemical exposure group) because some worms were stained more or less than others based on how much they consumed. Figure 8A shows the data from the trials we did with all of the cholesterol and BPA exposures along with exposures to positive controls such as sodium azide, FCCP and CCCP in either the plates (P) or in the liquid media along with the JC-1 exposure (L). Figure 8B shows our attempt to determine if liquid exposure to the positive control CCCP prior to JC-1 or MITO-ID would give us a better results as we would give the positive control time to induce its toxicity prior to the dye being added. This method also gave us very inconsistent results for our positive controls, which we would expect to be less than 1 indicating a higher rate of depolarization.

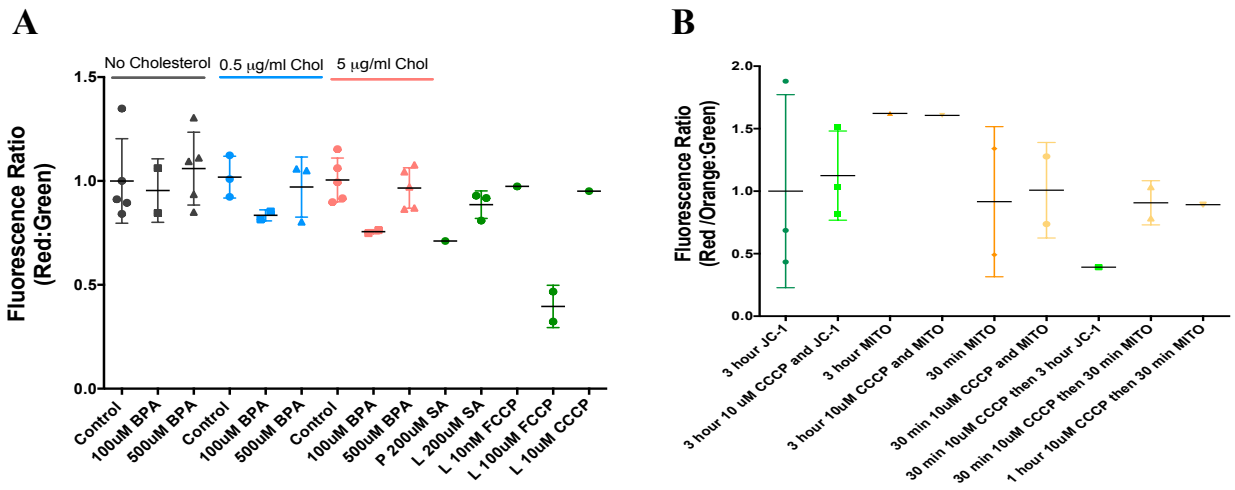


Figure 8. *JC-1 and MITO-ID staining were not effective at showing the mitochondria membrane potential of mitochondria in the germline of a whole organism.* (A) JC-1 was tested with varying concentrations of positive controls sodium azide, FCCP and CCCP in different media. P = exposure in plate, L = exposure in liquid. N = 1 – 5. (B) *C. elegans* grown on NGM plates with 5µg/ml cholesterol were stained with JC-1 or MITO-ID for varying time intervals. The positive control was 10 µM CCCP. N = 1 – 3.

DISCUSSION

Two mutants, *Strl-1* F52F12.7(ok3347) I (StAR) and *tspo-1* C41G7.9(tm5526) (TSPO), were used to determine if BPA is interfering with cholesterol uptake at the site of one or both of these two mitochondrial transmembrane proteins. Fertility assessments, germline morphology examinations and cholesterol assays were used to test our hypothesis that BPA and cholesterol uptake are antagonistic at the StAR transporter and that BPA germline toxicity can be phenocopied by inhibiting mitochondrial cholesterol transport in *C. elegans*. Overall, our results suggest that cholesterol rescues the reproductive toxicity induced by BPA exposure in N2 *C. elegans*.

There were no significant changes to overall fertility, assessed by egg number, embryonic lethality, larval lethality and brood size, in the StAR mutants regardless of exposure to varying concentrations of BPA and cholesterol. The germline morphology examination supported these results as there were no significant changes to the percent of gaps or the apoptotic index between groups. The results from the fertility assessment and germline morphology examination indicates that the StAR mitochondrial membrane transporter may be necessary for BPA to induce reproductive toxicity.

The TSPO mutant had an increased trend of larval lethality between the no cholesterol/no BPA and no cholesterol/500 μM BPA. There was also a decreased trend in brood size between no cholesterol/no BPA and no cholesterol/500 μM BPA. The germline morphology examination showed that the percent gaps and apoptotic index significantly increased between the same groups, no cholesterol/no BPA and no cholesterol/500 μM BPA. The reproductive toxicity induced by BPA for larval lethality,

percent gaps and apoptotic index reduced back to baseline levels when cholesterol at both the 0.5 μg/ml and 5 μg/ml cholesterol demonstrating that cholesterol antagonizes the reproductive toxicity of BPA. This relationship was also seen in the in N2 fertility assessment and germline morphology examination demonstrating that the TSPO mitochondrial membrane transporter may not be necessary for BPA to induce reproductive toxicity.

The results from the fertility assessments and germline morphology examinations for N2, StAR mutants and TSPO mutants support our hypothesis that BPA and cholesterol uptake are antagonistic at the StAR mitochondrial membrane transporter. The cholesterol assay and JC-1 and MITO-ID staining were used to verify that these phenotypes were actually due to the transport or lack of transport of cholesterol across the mitochondrial membrane.

We looked into the possible interference of BPA and cholesterol by conducting mitochondria extractions and cholesterol assays to determine the levels of cholesterol inside the mitochondria and outside the mitochondria. The results show a general trend that cholesterol levels outside the mitochondria increase as BPA levels increase. This is seen across all three cholesterol levels, suggesting that cholesterol is somehow inhibited from entering the mitochondria, possibly by BPA. The ratio of cholesterol levels inside the mitochondria to outside the mitochondria also support this as there seems to be less cholesterol found inside the mitochondria compared to outside. The presence of any cholesterol in the no cholesterol group is concerning as *C. elegans* cannot synthesize endogenous cholesterol. A cholesterol assay testing the reagents in the mitochondria extraction process prior to conducting these eight repeats revealed that some of the

reagents, such as sucrose and bacteria, react to the assay. We were hopeful that washing the samples with non-reactive reagents would solve this problem, but cholesterol is still present. In a follow up experiment worms were not exposed to cholesterol for two generations, one generation or provided cholesterol for both generations. The worms not exposed to cholesterol for two generations were laying eggs and reproducing just as the other two groups and cholesterol was still detected following the mitochondria extraction and cholesterol assay. These findings showed that the worms are most likely getting cholesterol through the agar in the NGM plates as no other reagent in the plates or mitochondria extraction process could contain cholesterol. Although we were using an agar with no cholesterol listed in the ingredients, further experiments should be done using different types of agar to see if the results we found can be verified with an adequate negative control.

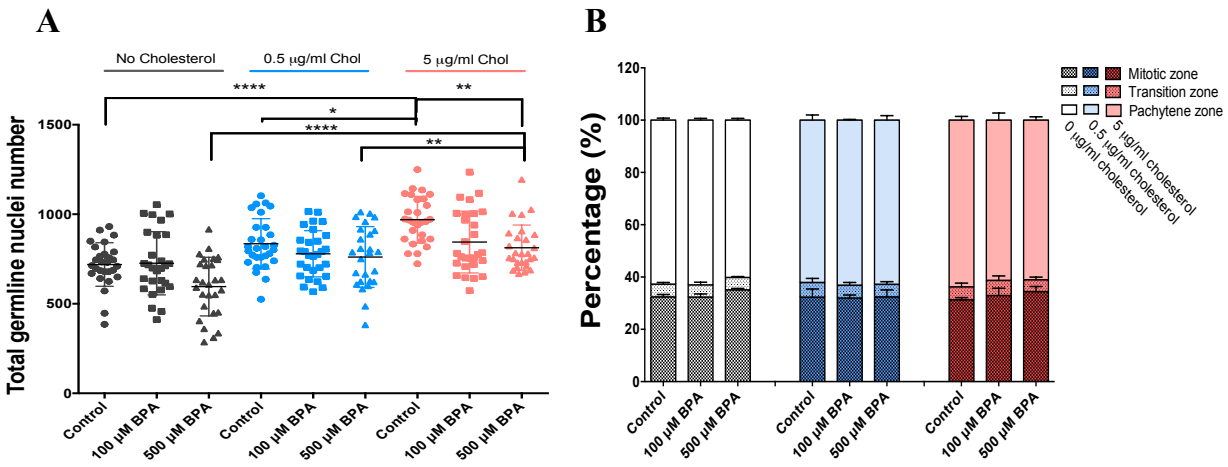
JC-1 and MITO-ID were conducted to determine if BPA alters the mitochondrial membrane potential of the germline cells in *C. elegans*. I ran into many limitations troubleshooting both JC-1 and MITO-ID. First, both JC-1 and MITO-ID have only been conducted to determine the mitochondrial membrane potential in a cell model and neither has been documented to work in a whole living organism, such as *C. elegans.* In a cell model the dye only has to penetrate the cell membrane, however, in a whole organism the dye has to travel through many layers of cells to stain the mitochondria of the germline cells. Second, the dye seemed to either stain the cuticle of the worm or the gastrointestinal tract from consumption of the dye in the exposure media when and not the germline. This builds on the first limitation that the dye does not seem strong enough to penetrate multiple layers of cells but rather gets absorbed by the first layer of cells it

contacts. Third, exposure times for FITC and TxRED varied for every single worm (even in the same exposure group). I initially attempted to set the same exposure time for all worms, but some images were highly saturated while others were not visible. This forced me to alter the exposure time for every single worm to ensure we would have enough images to assess the stains. Fourth, I tried a variety of positive controls at different concentrations and for different exposure times, but nothing seemed to give us consistent, reliable results. For these reasons I decided not to include the JC-1 and MITO-ID results in the final published piece, but I did want to highlight it in my manuscript as I did most of the work for this portion of the project.

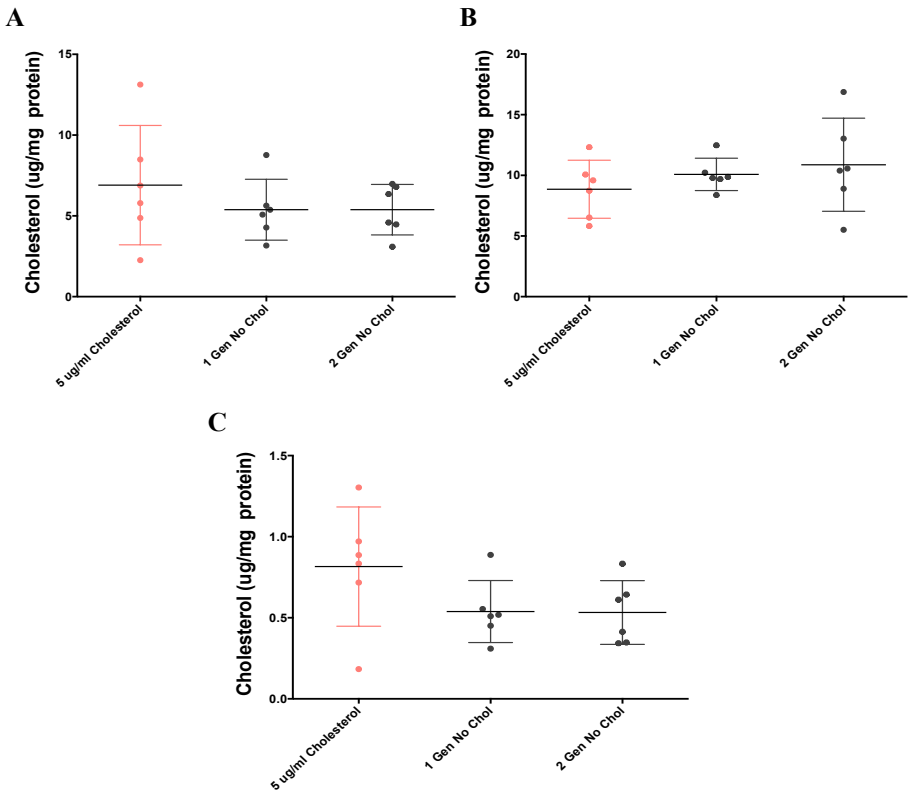
Further experiments should be done looking at the role of StAR in the reproductive toxicity of BPA. Samardzija et al. (2018) found that StAR mRNA levels increased and progesterone levels decreased with exposure to BPA in rat granulosa cells. While this supports our findings that BPA is somehow interacting with StAR, the exact mechanism and downstream effects of this interaction are unclear. Experiments should be done to clarify BPA's action at the StAR mitochondrial membrane transporter to determine if this is a main pathway BPA elicits endocrine disruption. Also, producing StAR/TSPO double mutants may give us a better idea if both are necessary for the transport of cholesterol or if there are other possible mitochondrial membrane transporters shuttling cholesterol. It is also unclear if cholesterol and/or BPA diffuse through the mitochondrial membrane. Further experiments should be done to examine all possible modes of cholesterol and BPA transportation into the mitochondria.

This research builds on the case against the use of BPA in products such as water bottles, receipt paper, and food can linings (Shelby et al., 2008; Vandenberg et al., 2007). BPA has been shown to cause abnormalities in oocyte development and maturation (Can et al., 2005; Eichenlaub-Ritter et al., 2008; Hunt et al., 2003) while also interfering with endogenous hormone synthesis in humans, rodents, nonhuman primates, and cell culture test systems (Galloway et al., 2010; Peretz et al., 2010; Rochester et al., 2013;). These harmful properties of BPA coupled with its ubiquitous presence in the environment, as approximately 4 million metric tons of BPA are produced annually and over 95% of the population have detectable levels of BPA, make it an important public health concern (Calafat et al., 2004; Shelby et al., 2008; Vandenberg et al., 2007). Therefore, future work should be conducted on: (1) confirming the antagonistic relationship of cholesterol and BPA possibly at the site of the StAR mitochondrial transmembrane transporter through cholesterol assays and new staining methods and (2) identifying new possible mitochondrial transmembrane transporters that could be transporting BPA into the mitochondria and ultimately inducing its' toxic effects on reproduction.

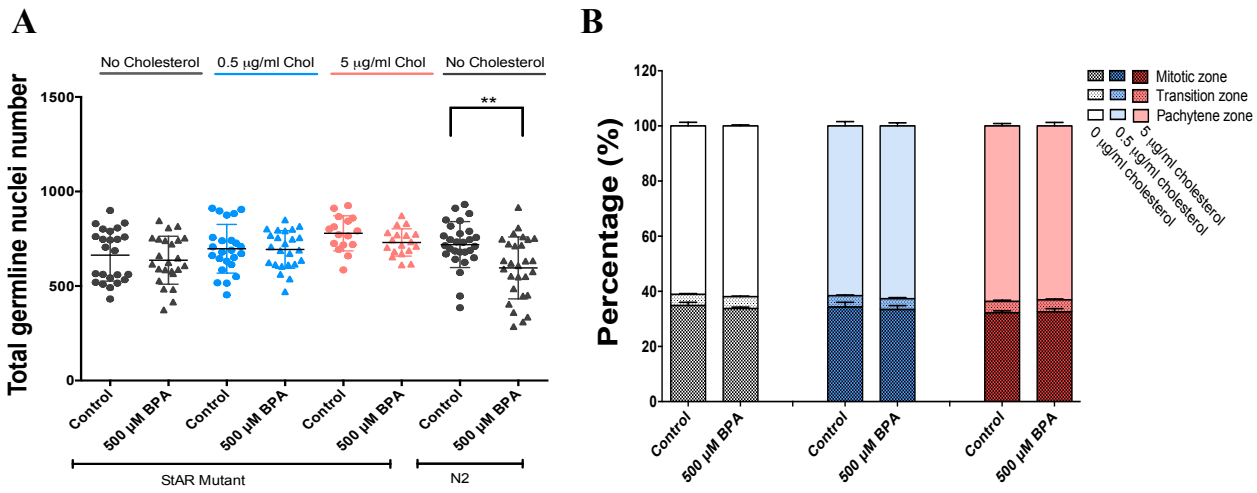
SUPPLEMENTARY FIGURES



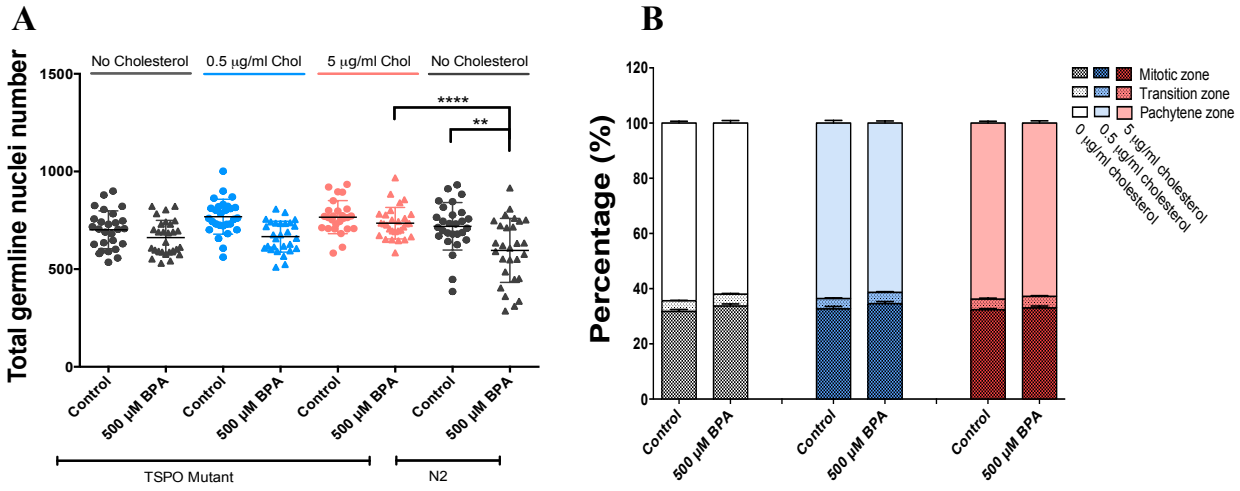
Supplementary Figure 1. *Total gernline nucli number increases with increased levels of cholesterol in N2 C. elegans.* DAPI was used to stain the germline nuceli to determine the total number of germline nuclei and the percentage in the mitotic, transition and pachytene zones. N= 28. Two-way ANOVA with Sidak's multiple comparison test. *p< 0.05, **p<0.01 and ***p<0.001 ****p<0.0001.



Supplementary Figure 2. (A) Cholesterol levels normalized to protein levels found in extraction samples containing everything except the mitochondria and cuticle of *C. elegans*. (B) Cholesterol levels normalized to protein levels found in extraction samples containing pure mitochondria of *C. elegans*. (C) Ratio of cholesterol found in the mitochondria samples to cholesterol found in samples containing everything except the mitochondria and the cuticle of *C. elegans*. N = 8 for each group. Ordinary One-Way ANOVA.



Supplementary Figure 3. *Cholesterol levels do not increase the germline nuclei number in StAR C. elegans.* DAPI was used to stain the germline nuceli to determine the total number of germline nuclei and the percentage in the mitotic, transition and pachytene zones. N = 16 - 28. Two-way ANOVA with Sidak's multiple comparison test. *p< 0.05, **p<0.01 and ***p<0.001 ****p<0.0001.



Supplementary Figure 4. *Cholesterol levels do not increase the germline nuclei number in TSPO C. elegans.* DAPI was used to stain the germline nuceli to determine the total number of germline nuclei and the percentage in the mitotic, transition and pachytene zones. N = 28. Two-way ANOVA with Sidak's multiple comparison test. *p< 0.05, **p<0.01 and ***p<0.001 ****p<0.0001.

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