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The Effects of Chronic Ethanol Exposure on *Caenorhabditis elegans* Germline

Andrea Zegarra

Submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biology from the Department of Biological Sciences of Seton Hall University
December 2021

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Abstract

Ethanol is the most commonly abused drug in the world. Alcohol consumption increases a number of health risks, morbidity and mortality, and chronic and acute diseases. Some health risks include high blood pressure, stroke, liver disease, cancer, and can include mental health and social problems. Chronic alcohol consumption can lead to learning and memory problems, along with alcohol dependence. Additionally, alcohol consumption has an impact on ovarian reserve, steroid hormone production, sperm quality, fecundity, and fertility treatments. The purpose of this study was to analyze the effects of ethanol exposure on fertility of the N2 wild type as measured by the number of progeny along with the effects of chronic ethanol consumption on the mitotic germline of L4 *Caenorhabditis elegans*. In addition, the effects of alcohol consumption were analyzed in two mutant strains: GC1373and GC1374. GC1373 has additional mutations in the glp-1 gene and notch pathway. With mutations in these functionalities, the GC1373 strain has a reduction in the differentiation of all germ stem cells. GC1374 only has the reduction of function mutation, on the pk1417 allele, this strain only produces half the number of adult germline stem cells. Worms were treated with 0 mM, 200 mM, 300 mM and 400 mM ethanol concentrations for 7 days, after L4 stage is reached, and the effects assayed by progeny counting, ethanol absorbance, mitotic germ cell counting. Our results demonstrate that chronic ethanol exposure causes lasting effects on the *C. elegans* germline. Chronic ethanol exposure also decreased the progeny counts of the mutant strains GC1373 and GC1374. The results presented support previous work performed on various animal models indicating chronic ethanol exposure decreased the reproductive abilities; acute exposure, for 15 minutes or 120 minutes, does not cause lasting effects on the progeny of *C. elegans* nor on the birth rate of other animal models. Our data suggests that chronic ethanol exposure causes lasting damage to the *C. elegans*

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germline.

Key words: C. elegans, ethanol, fertility, disinhibition, stem cell.

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Introduction

Overview and Life Cycle

Caenorhabditis elegans are small, free-living soil nematodes that are widely used as a genetic model system, as well as a model for studying aging (Tissenbaum, 2015), disease (Apfeld and Alper, 2018), developmental biology (Alberts, et al., 2002), neuroscience (Sengupta and Samuel, 2009) and more. At least 60% - 80% of human genes (Kaletta and Hengartner, 2006), and 40% of the genes associated with human diseases have clear orthologs in the *C. elegans* genome (Culetto and Sattelle, 2000; Shaye and Greenwald, 2011). *C. elegans* have a rapid life cycle and are easily raised under laboratory conditions (Corsi et al., 2015). The worms appear semitransparent which makes possible observation of all cells in the living animal using differential interference contrast (DIC) microscopy (Corsi et al., 2015).

A hermaphroditic *C. elegans* adult is capable of self-fertilization. The adults lay eggs at the 24-cell embryonic stage within an impermeable eggshell. Eggs hatch with larva at the L1 stage of development. In culture, larva eat a lawn of OP50 *E. coli* bacteria as they develop into successive larval stages (see Figure 1). The L1 larval stage lasts for 12 hours after with the organism undergoes its first molt to emerge at the L2 larval stage. In the late L3/early L4 stage of larval development the somatic gonad precursor cells start to form the gonad sheaths, the spermathecae and the uterus. In the L4 stage, gonadogenesis, which begins around 7 hours post hatch, has completed. Meiosis in the germline commences at the L3/L4 adult molt stage where germline stem cells differentiate in spermatocytes. Sperm production ends in the late L4 stage. The remaining germline cells continue to undergo meiosis and strictly generate oocytes. At the end of each developmental phase the animal undergoes lethargus, a period of sleep-like inactivity

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which ends with molting of the previous cuticle (Raizen et al., 2008). Once stages L1 through L4 are completed, a mature hermaphrodite adult will lay its first eggs (Figure 1; Corsi et al., 2015).

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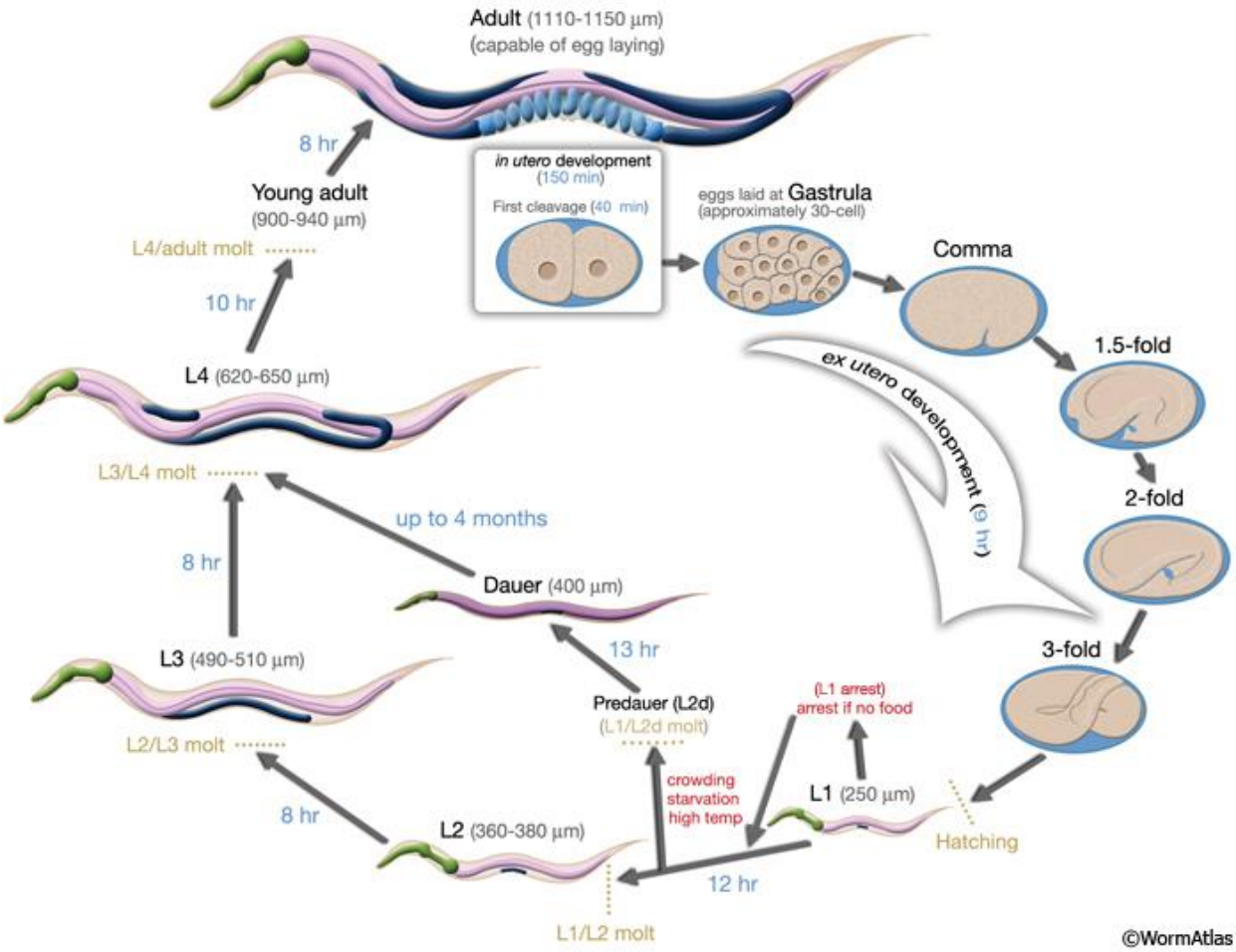

Figure 1. The life cycle of *C. elegans*. An adult lays ex utero developmental egg which hatches after 9 hours and begins the L1 stage. If food sources are depleted or there is overcrowding, the worm will enter pre-Dauer as shown prior to L2. The stages of growth are L1 through L4 until a young adult and ultimately, an adult emerges. Image reproduced from WormAtlas with permission.

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Approximately 12 hours after the L4 animal molts, an adult hermaphrodite is capable of producing progeny for a period of two to three days or until all the sperm stored in the spermathecae have been used for fertilization. Overall, *C. elegans* cultures take about three days at 25°C to develop from fertilized eggs to adults capable of producing gametes.

C. elegans Germ Cell Development

Wild-type *C. elegans* can be categorized into two sexual forms: self-fertile hermaphrodites and cross-fertile males. Males arise infrequently within a population (0.1% - 0.2%) through spontaneous non-disjunction in the hermaphrodite germline. Under laboratory conditions, males are generated through heat-shocking worms in the L4 stage of development (Lints and Hall, 2009). Hermaphrodites are the predominate sex with a population of (approximately 99.5%). In terms of fertilization, hermaphrodites lay up to 300 self-fertilized eggs; however, if mated with males, hermaphrodites can produce approximately 1000 offspring, indicating sperm as a limiting factor in self-fertilization over their lifespan of 12 to 18 days (Riddle et al., 1997).

The gonad of a hermaphrodite forms an ovotestis hybrid that produces haploid amoeboid sperm that are stored within the spermatheca in the L4 and near the adult stage of the germline in which it switches over to producing much larger oocytes. Through meiotic prophase and gametogenesis, similar to an assembly line, germ cells switch from proliferative cells to meiotic development becoming sperm by the end of the L4 stage (Pazdernik et al., 2013). Therefore, the hermaphroditic adults are females whose gonads temporarily produce sperm before they produce oocytes (Corsi et al, 2015; Figure 2). The distal end of the gonad is covered by a somatic distal tip cell that sheaths the mitotically proliferating germline, called the proliferative zone, as indicated by yellow in Figure 3. Just below the U-shaped region of the gonadal arm there are five

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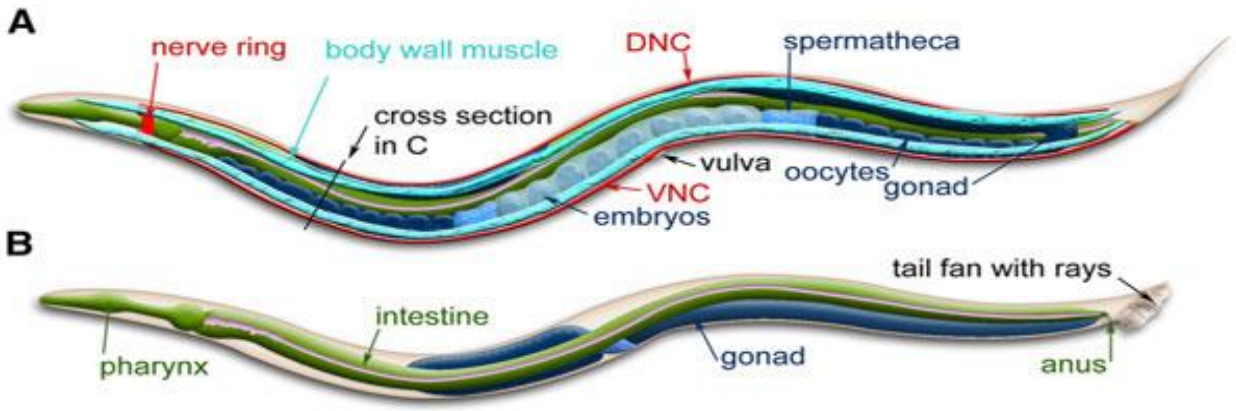

Figure 2. *C. elegans* anatomy differences. Major anatomical structures of a hermaphrodite (A) and male (B) from the lateral perspective are shown. **A.** Hermaphrodites have amoeboid sperm stored within the spermatheca and will switch over to oocyte production where they will develop embryos. **B.** Males will only have a gonad to produce sperm cells from their germline. Image reproduced from WormAtlas with permission.

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pairs of gonadal sheath cells that surrounding the mature oocytes leading them towards the spermatheca which contains mature sperm (Hubbard, 2007; Pazdernik et al., 2013). The green region represents the germ cells in meiotic prophase I stage as they begin to transition through the U-shaped region of the gonad into developing oocytes. The developing oocytes, as shown by the blue/purple region, surrounded by sheath cells in pairs of thick and thin filaments. The sheath cells contract to drive ovulation towards the spermatheca. The darker blue region indicates the spermatheca proceeded by the clear embryos within the uterus (Pazdernik et al, 2013).

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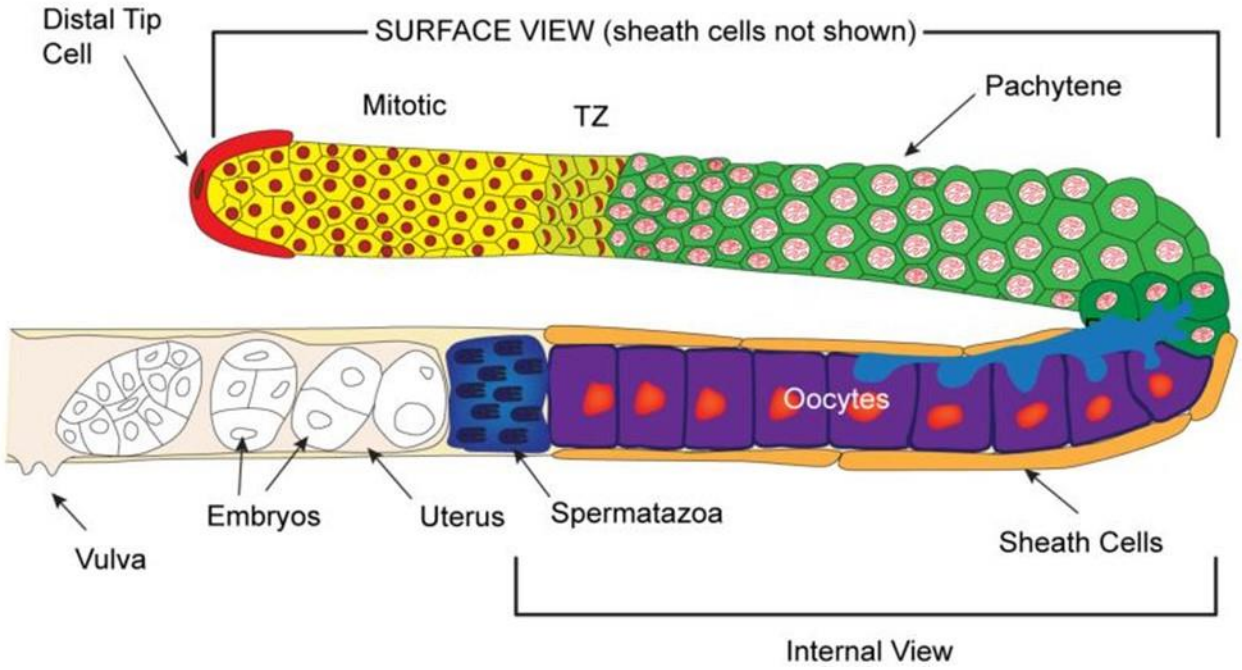

Figure 3. *C. elegans* schematic of adult hermaphrodite and gonad development. The diverse colors indicate the areas of gonadal development. Through an assembly line like manner, germ cells switch to sperm while remaining cells that switch in the L4 stage and adulthood will become oocytes. Image reproduced from WormAtlas with permission.

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Ethanol Exposure, Reproduction, and Infertility

Alcohol consumption in humans can result in serious health problems including alcoholic-related liver disease (ALD) (Tan et al., 2020), alcohol misuse disorder, and infertility. Both chronic and acute ethanol exposure affect ovarian reserve (Li et al., 2012), steroid hormone production (Srivastava et al., 2015), sperm quality (Grover et al., 2014), fecundity and the effectiveness of assisted fertilization (Rooney and Domar, 2014); Van Heertum and Rossi, 2017).) Alcohol is a well-studied compound with a broad range of physiological and behavioral effects on animals and humans alike (Bettinger et al., 2004). Models such zebrafish and rat have been used to look at the effects of chronic and acute ethanol consumption.

Zebrafish studies have reported the effects of both chronic and acute alcohol exposure on embryonic development and adult behavior. A negative impact of chronic alcohol exposure on fecundity was found in both male and female parents (Dewari et al., 2019). Ethanol exposed males displayed severe effects of chronic ethanol exposure on fecundity in comparison to their female counterparts. Fecundity is the measurement of fertility and is defined as the physiological maximum potential reproductive output of an individual over the course of their lifetime (Bradshaw and McMahon, 2008). Ethanol-exposed parents had a decrease in fecundity when placed in 0.5% ethanol infused water. Chronic alcohol exposure significantly decreases fecundity and negatively affects reproductive capacity of both parent zebrafish. When zebrafish are withdrawn from alcohol, they completely recover fecundity.

Similarly, rodent models show behavioral changes when exposed to chronic high doses at all steps of gestation (Schambra et al., 2015). Akomolafe and colleagues (2017) found rodents containing high blood ethanol content produced fetuses with a variety of defects. The study found that after 21 days of chronic ethanol exposure; prostate gland antioxidant enzyme, seminal

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vesicle, epididymis, and testis activities decreased in ethanol-treated rodents in comparison to the normal controls. Alcohol exposure has been shown to induce changes in the epigenome of sperm in exposed male animals, and these epimutations are inherited in the offspring (Chastain and Sarkar, 2017).

In *C. elegans*, exposure to chronic ethanol delays the ability of egg-laying and results in fewer eggs being laid by a single parent worm (Davies et al., 2004). In over 40% of exposed worms infertility is caused by the failure of worms to lay fertilized eggs so that offspring hatched within the parent's body. Chronic ethanol exposure directly impairs the egg-laying ability of the worms, but not the egg's viability. Another study determined that chronic ethanol exposure during larval development not only decreases the reproductive fecundity and longevity of the parent worm, but also delayed physical growth and onset of reproductive maturity along with overall development (Davis et al., 2018). In the same study, acute exposure at various periods of development resulted in decreased probability of exposed eggs hatching. The progeny capable of hatching displayed physical dysmorphologies. Davis and colleagues reported a significant effect on the reproductive abilities of multiple models due to chronic ethanol exposure. Acute exposure to ethanol results in dose-dependent decreases in the rates of locomotion and egg laying (Davies et al., 2015).

Ethanol and Signaling in C. elegans

Ethanol exposure modifies the expression of genes and methylation profiles in embryonic stem cells (DiRocco et al., 2019). Human embryonic stem cells, particularly those that differentiate into hepatocytes, are impaired by alcohol-mediated inhibition of the mitogen-activated protein kinase and extracellular signal regulated kinase (MAPK/ERK) along with the WNT signaling pathways. The MAPK/ERK signaling pathway takes part in integrating the

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external signals from the mitogens such as epidermal growth factor which promotes cell growth and proliferation in many mammalians cell types (Zhang and Liu, 2002). The WNT signaling pathway regulates cell fate determination, cell migration, cell polarity, neural patterning, and organogenesis during the stages of embryonic development (Komiya and Habas, 2008). Ethanol exposure stimulates the gene expression changes in the human embryonic stem cell derived cortical neurons.

As shown in Figure 4, ethanol can function as a solvent for carcinogens to enter cells. Tumor cells begin to form as ethanol increases the activation of multiple pro-carcinogens present in alcoholic beverages (Seitz et al., 2007). As ethanol oxidizes and binds to proteins forming mutagenic adducts which ultimately leads to the immune suppression that allows for an easier disperse of tumor cells. However, cancer stem cells have the ability to initiate the growth of tumorigenic stem-like cells and drive tumor growth (Di Rocco et al., 2019). These cells are similar to embryonic stem cells as they have the capability to self-renew and to differentiate into multiple cell types.

mTOR-mitochondria-ROS plays a key role in regulating stem-cell quiescence and self-renewal. When mTOR affects the stem cells, it leads them to impaired differentiation. It has been noted that ethanol exposure activates the NLRP3 inflammation in induced pluripotent stem cells. Inflammation has long been known to damage tissue through alcohol-mediated means. It is important to note the role of the mTOR pathway when influenced by ethanol leads to impaired differentiation and stem cell failure (Di Rocco et al., 2019).

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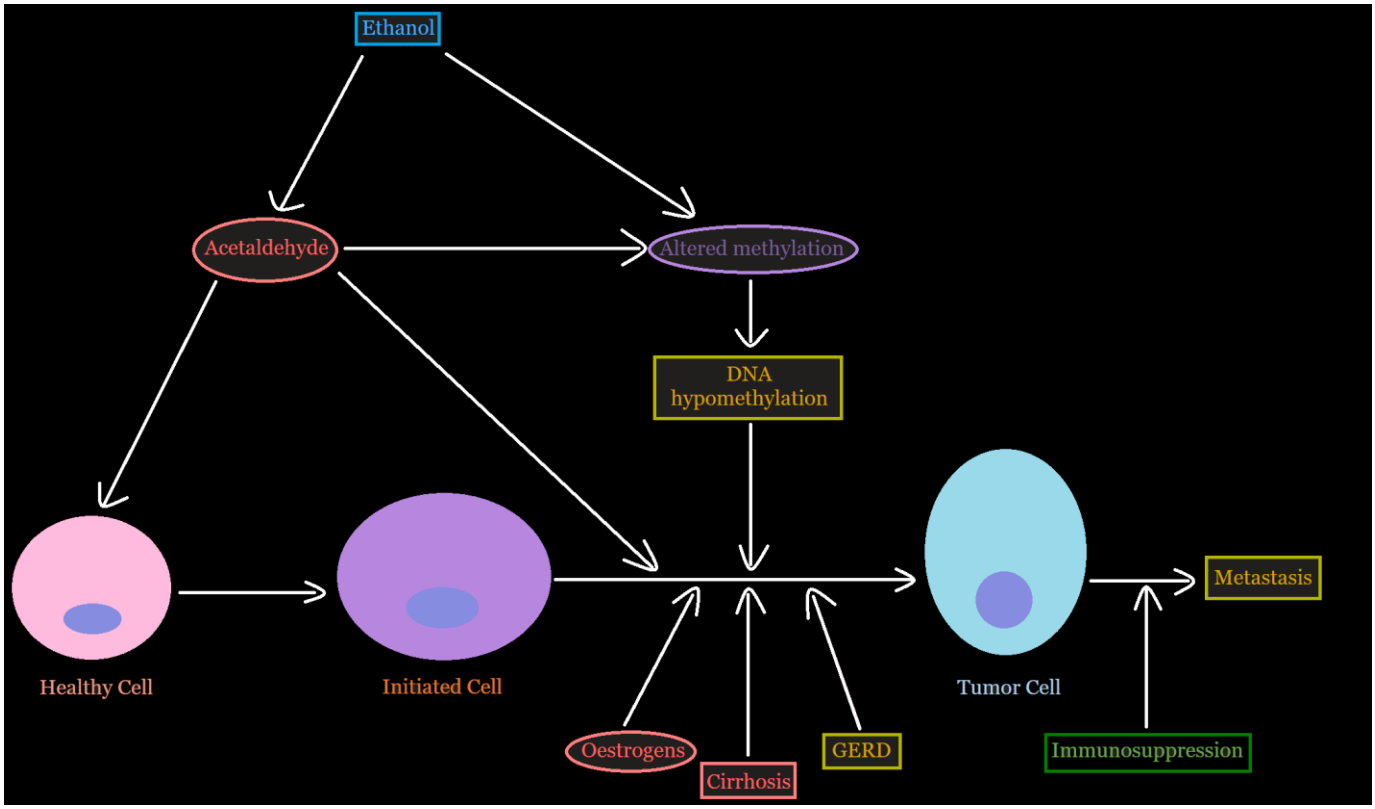

Figure 4. Mechanisms indicating the effect of ethanol on a variety of cell processes. Ethanol is processed by alcohol dehydrogenase to acetaldehyde, a carcinogen, which binds to DNA. After Seitz et al., 2007.

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Intoxication and Disinhibition in C. elegans

Intoxication is defined as clinically significant behavioral or psychologic changes following the use of a substance (Byrne and Kirschner, 2018). Disinhibition is defined as normally suppressed behaviors that are displayed following intoxication (Topper et al., 2014). Rodent studies have reported disinhibition responses to ethanol such as altered locomotor patterns, including transient increases in total movement during acute intoxication as well as increased grooming behaviors and overall movement attributing by the lack of coordination caused by ethanol consumption (Topper et al., 2014). As the levels of ethanol concentration increase so do the levels of disinhibition which is also noted in other studies regarding C. elegans. Studies found when exposed to 500 mM of ethanol, the C. elegans had a decline in locomotion and other factors, which ultimately lead to immobility after 30 minutes (Davies et al., 2015). Similarly in rodent models, animals exposed to isolation stress displayed anxiety behaviors as indicated by reduced time spent in plus-maze tests. Consumption of ethanol relieved anxiety behaviors (Pohorecky, 2008).

Goal of the Current Work

It has been well established that chronic, as well as acute, alcohol exposure damages tissues and impedes organ function; however, the role of ethanol exposure and its deteriorating effects on stem-cell properties and population have just begun to be investigated in worms. It has been noted that alcohol susceptibility can be dependent on target cells which can be influenced by the dose along with the duration of exposure. The goal of the current work was to analyze the effect of ethanol exposure on the mitotic progenitor germline in *C. elegans*.

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Methods

NGM Culture

C. elegans stocks were obtained from the I Genetics Center (University of Minnesota) and maintained using standard procedures. The strains used in this study were: N2, GC1413 rrf-1(pk1417) I; naSi2(mex-5p::H2B::mCherry::nos-2 3′UTR) II; teIs113(pie-1p::GFP::H2B::zif-1 3′UTR) V , GC1373 rrf-1(pk1417) I ; glp-1(e2141) III; hjSi20 [myo-2p::mCherry::unc-54 3′UTR] IV ; zuIs70 [end-1p::gfp::caax; unc-119(+)] V , and GC1374 rrf-1(pk1417) I; hjSi20 [myo-2p::mCherry::unc-54 3′UTR] IV; zuIs70 [end-1p::gfp::caax; unc-119(+)] V.

C. elegans were maintained on Nematode Growth Media (NGM) agar plates at 25 degrees C. NGM agar stock was made by combining 2.25 g of NaCl (US Biological, Salem MA), 12.75 mL agar (US Biological, Salem MA), 1.95 g peptone (US Biological, Salem MA) and 750 mL of distilled H_2O, then swirled to mix thoroughly. The media was autoclaved for 40 minutes and cooled for 15 minutes. Once cooled, 18.75 mL 1M KPO_4 buffer pH 6.0 (108.3 g KH_2PO_4, 35.6 g K_2HPO_4, 1 L H_2O) (US Biological, Salem MA) was added directly to the media using a serological pipette. Lastly, 750 µL 1M $CaCl_2$, $MgSO_4$ and 5 mg/mL cholesterol were added. Using semi-sterile procedures, stock plates were poured by pipetting 8 mL of media into 35 mm plates 12-well plate (3 ml/well). The plates were kept at room temperature until solidified approximately 24 hours. Once solidified, they were kept in sterile bins at 4°C.

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Strain Maintenance

Strain	**Description**
N2	Wild type *C. elegan* strain
GC1413	The mutant strain contains reduction of function mutation at the pk1417 allele; RNAi is most impactful in the germline, contains germline and germline stem cell markers.
GC1373	The mutant strain containing a reduction of function glp-1/Notch pathway at the pk1417 allele.
GC1374	The mutant counterpart of GC1373 containing only a reduction of function mutation at the pk1417 allele.

Culture Maintenance

Each strain was maintained at 20°C for 24 hours. One strain of *C. elegans* was used per well plate. Lab conditions included feeding of OP50 *E. coli* bacteria. Serological pipettes were used to seed the *E. coli* OP50 liquid culture. Luria Broth (LB) is a nutritionally rich media used for bacteria culture. LB was made from 1.55 g of Luria Broth powder (U.S Biological) dissolved in 100 mL of deionized H_2O. The media was autoclaved for 30 minutes to sterilize. After 24 hours, the LB media was inoculated with OP50 *E. coli* and incubated at 37°C for 24 hours.

A nickel-sized amount of *E. coli* was added per 35 mm petri plate and one drop was added per well. The plates required 24 hours at room temperature to set and form a lawn of bacteria on top of the NGM agar. The *C. elegans* were added directly onto freshly made plates and stored at 20°C to allow the worms to crawl and procreate. The remaining plates were stored at 4°C in sterile plastic storage boxes for up to 2 to 3 weeks. A picking method, which uses a worm picker to pick single worms on a pasture pipette with a platinum wire tip, was used to

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transfer individual worms to designated plates. Chunking was used to maintain overall strains throughout the duration of the experiments to have a multitude of worms on hand.

Ethanol infused E. coli

The NGM agar plates were seeded with OP50 bacteria and set to dry for 24 to 48 hours, depending on the amount of lawn growth. The ethanol infused agar plates were optimized by calculating an appropriate dosage per mM of ethanol in 8 mL of NGM plates. The negative control plate had 0 µL/8mL; while the remainder of the amounts per concentrations were 94 µL/8mL, 140 µL/8mL, and 187 µL/8mL, respectively. The ethanol was micropipetted surrounding the OP50 *E. coli* lawn to absorb and incorporate with the worm food at each concentration. Approximately, 5 to 20 plates were used per individual trials at various concentrations of ethanol, allowing 2 hours for the ethanol to be thoroughly absorbed into the *E. coli.*

Chronic Ethanol Exposure

C. elegans were seeded onto the ethanol infused *E. coli* plates to ensure chronic exposure was attained. Worms were exposed to concentrations of 200 mM, 300 mM, and 400 mM ethanol. Once dried, a single parent worm was transferred to fresh ethanol infused plates every 48 hours as their progeny begin to hatch and grow in the media. Frequent transfers ensured that the parent worm would not be confused with the progeny and that the progeny do not produce their own progeny. Progeny were then counted and totaled for each concentration evaluated.

Acute Ethanol Exposure

Plates were infused with ethanol as described above. Worms were treated with 300 mM and 500 mM ethanol. The plates were set to dry for 2 hours at room temperature to prevent the ethanol from evaporating or drying out Once set, 15 worms were placed on the 3 plate

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concentrations, 5 worms per treatment for 15 minutes. The worms were then transferred to fresh NGM plates without any alcohol to determine the effects of short-term ethanol exposure. Progeny was then counted and compared to the results of chronic ethanol exposure.

Progeny Count

Following exposure to ethanol, worms were placed in a 20°C incubator for 48 hours, checked at 24-hour intervals. After 48 hours, the parent worms will have laid eggs that have either hatched into L1 larvae or remained unhatched. *C. elegans* with progeny on their plates were transferred to a fresh ethanol infused NGM plate to keep them exposed to ethanol. Upon hatching, the worm plates without the parent worm were placed under a dissecting microscope and counted. Each larva was individually vacuumed off the plates to ensure that progeny was not counted multiple times. Unhatched eggs did not count towards the progeny count. Progeny was counted for 5 – 7 days. Prism was used to determine statistical significance (Welsh's t-test).

DAPI Staining

4',6'-diamidino-2-phenylindole (DAPI) was used to stain the germline nuclei. A hatch off was performed on both N2 and GC1413 *C. elegans* strains, to ensure the worms were all at the same stage of their life cycle. A hatch off is a procedure in which the various stages of *C. elegans* are washed off the NGM plates using M9 buffer leaving behind unhatched eggs behind. This ensures the *C. elegans* will all grow at the same rate. These worms were incubated at 20°C for 2 to 3 hours until most of the eggs had hatched. The worms were washed off the plate using M9 Buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 L diH_2O) (US Biological, Salem MA) and placed onto 0 mM and 300 mM plates. The plates were checked under a dissecting microscope to ensure that there were at least 200 worms present per plate. The worms were incubated at 20°C and checked periodically for approximately 60 hours or until they

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reached L4 stage of development. The worms were then washed off again using M9 Buffer. The worms were placed into low retention Eppendorf tubes (Fisher Scientific, Pittsburgh PA). The worms were centrifuged for 2 minutes at 1000 rpm. The M9 supernatant was carefully removed and 750 µL of 100% ethanol was added directly to the pellet and incubated at room temperature for 3 minutes. The worms were centrifuged at 1000 rpm for 2 minutes and the ethanol supernatant was removed. A drop of VectaShield Antifade Mounting Media+DAPI (Vector Labs, Burlingame CA) was added to the Eppendorf tubes and were kept at 4°C, until ready to be imaged under a confocal microscope.

Alcohol Reagent Assay

In order to confirm that the worms were absorbing ethanol, an alcohol reagent assay was performed using MedTest Dx (Pointe Scientific, Canton MI). The sample size was at least 300 worms per treatment concentration. The worms were kept on ethanol infused NGM plates until they reached the L4 stage of development. The L4 *C. elegans* were washed off using M9 buffer and placed into low retention Eppendorf tubes. The worms were centrifuged for 1 minute at 1000 rpm to form a pellet and the supernatant was extracted and discarded. If the worms were not assessed right away, they were placed in -80°C until they were ready to be used in the assay. If frozen, the specimens were thawed on ice. Each concentration (0 mM, 300 mM, 500 mM) had 1 mL of alcohol reagent added to the Eppendorf tubes. Negative controls vials contained NaCl, and positive control vials contained 100% ethanol. The Eppendorf tubes were incubated for 5 minutes at 30°C. Prior to transferring the worms from tubes to well plates, the tubes were vortexed for 10 seconds to resuspend the pellet. The specimens were transferred to a 12-well plate and the absorbance was read at 340 nm.

Microscopic Imaging of Worm Locomotion

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Individual treated worms were located on the 35 mm plates by scanning at low magnification on a Tritech Research dissecting microscope. For the control sample, five untreated L4 larvae were placed on a fresh *E. coli* lawn and allowed to roam the media for approximately 5 minutes. Images were taken using an AmScope digital camera attached to the eye piece of the dissecting microscope. This procedure was repeated for the treatment concentrations of 200 mM, 300 mM, and 400 mM.

Confocal Laser Scanning Microscopy (CLSM)

Slides were scanned using a 10X objective lens to locate the worms mounted on the slide. Once the worm was identified and the vulva located the magnification was switched to a 40X oil immersion lens. Samples were excited with a 405 nm LED laser. Image stacks were collected by setting the top and bottom points of the volume and optimizing the slice overlap. Images were collected at 8 microseconds dwell time per pixel at a 1388x1040 resolution. Once collected, the image stacks were visualized and rendered as maximum intensity projections using the Fiji ImageJ software (PUT FIJI WEBSITE).

Image Processing for Germline Cell Count using ImageJ

The images generated by the CLSM were analyzed using the image J software. The image stack was initialized under the cell counter setting. The images were analyzed using the cell counter setting under the type four marker. The germline stem cells were counted across each image stack to confirm their state and development. The markers were tallied and saved as a separate cell counter image.

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Results

Treatment with Increasing Ethanol Doses Produces a Disinhibitory Effect on L4 C. elegans

Figure 5 shows the disinhibitory effect of increasing concentrations of ethanol on L4 *C. elegans*. The sinusoidal motion of untreated worms can be seen in Figure 5A as traces within the medium. This natural movement is the same whether in water or in soil. Figure 5B shows the change in the sinusoidal pattern with worms treated with 200 mM ethanol. The effect on coordination is observable approximately three minutes following treatment. At this concentration, the sinusoidal motion is present but occurs at a lower amplitude and higher frequency than untreated worms. The trails left behind the L4 stage larvae in Figure 5B show the lack of coordination when compared to Figure 5A. After approximately three minutes of ethanol exposure at 300 mM ethanol (Fig. 5C) larval sinusoidal motion decreased to a low amplitude and low frequency. Eventually, the body no longer produced any movement, but the head of the L4 larva continued to move forward and backwards but the worm was unable to continuing moving through the media. Approximately one minute after treatment with 400 mM ethanol (Figure 5D) the worms folded and cease moving.

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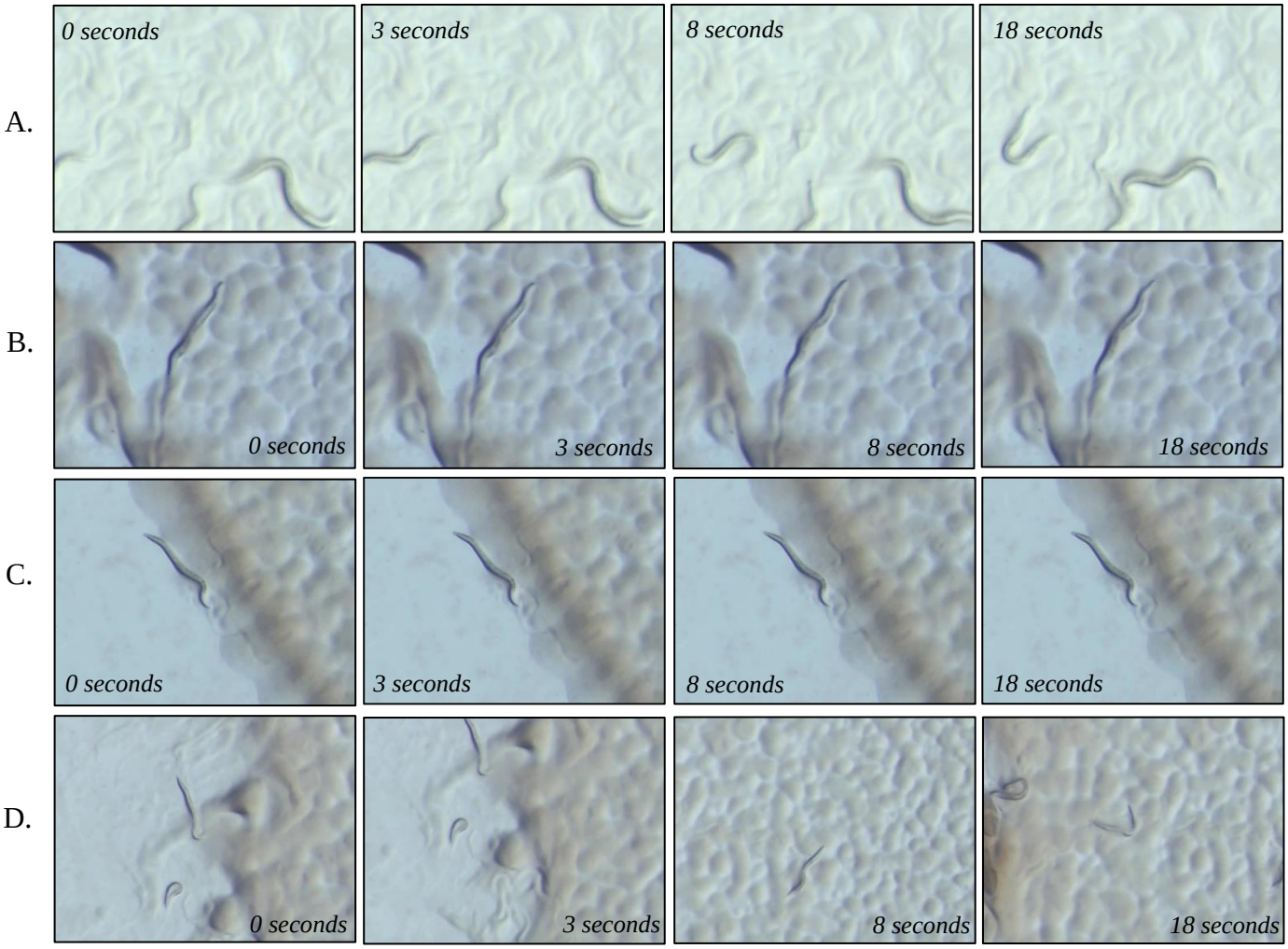

Figure 5. These images were taken from videos which demonstrate the disinhibitory effects of ethanol on the L4 nematodes. **A.** The control *C. elegans* demonstrate standard sinusoidal motion across the NGM agar at the time stamps of 0, 3, 8 and 18 seconds. **B.** At 200 mM, the *C. elegans* can still produce a sinusoidal motion; however, the waveform is modified from the control which is noted at the given time stamps. **C.** At 300 mM, the worms can no longer produce a sinusoidal motion; the images used at the given time stamps indicate lack of motion as the worm did not move. **D.** At 400 mM, the *C. elegans* folded onto themselves and ceased movement as noted by the time stamps, which was done by moving the plate around. Images were taken on a dissecting microscope using AmScope by Andrea Zegarra.

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Treatment with Increasing Concentrations of Ethanol Causes a Decline in Progeny Production in L4 C. elegans

The L4 nematodes were treated with 0 mM (control) 200 mM, 300 mM, and 400 mM concentrations of ethanol for the entirety of each experiment. The period from day 1 to day 3 allows the L4 larvae to further develop into fully grown adults, lay eggs and for those eggs to hatch. Progeny were counted on days 4, 5, 6 and 7. In order to ensure hatchlings were not counted more than once, a vacuum suction method was used; the hatchlings were suctioned off and counted. Only viable offspring were used to determine the total progeny count from the L4 stage to adulthood. Figure 6 shows the number of progeny for increasing concentrations of ethanol. The 0 mM concentration resulted in an average progeny count of 233 hatchlings. As the concentrations were increased, the progeny production decreased. At 200 mM, the developing *C. elegans* produced an average of 188 hatchlings. The concentrations of 300 mM and 400 mM produced the least amount of progeny across all the experimental trials with an average of 92 and 20 hatchlings, respectively. When comparing the various concentrations to the control, both 300 mM and 400 mM had a statistically significant difference.

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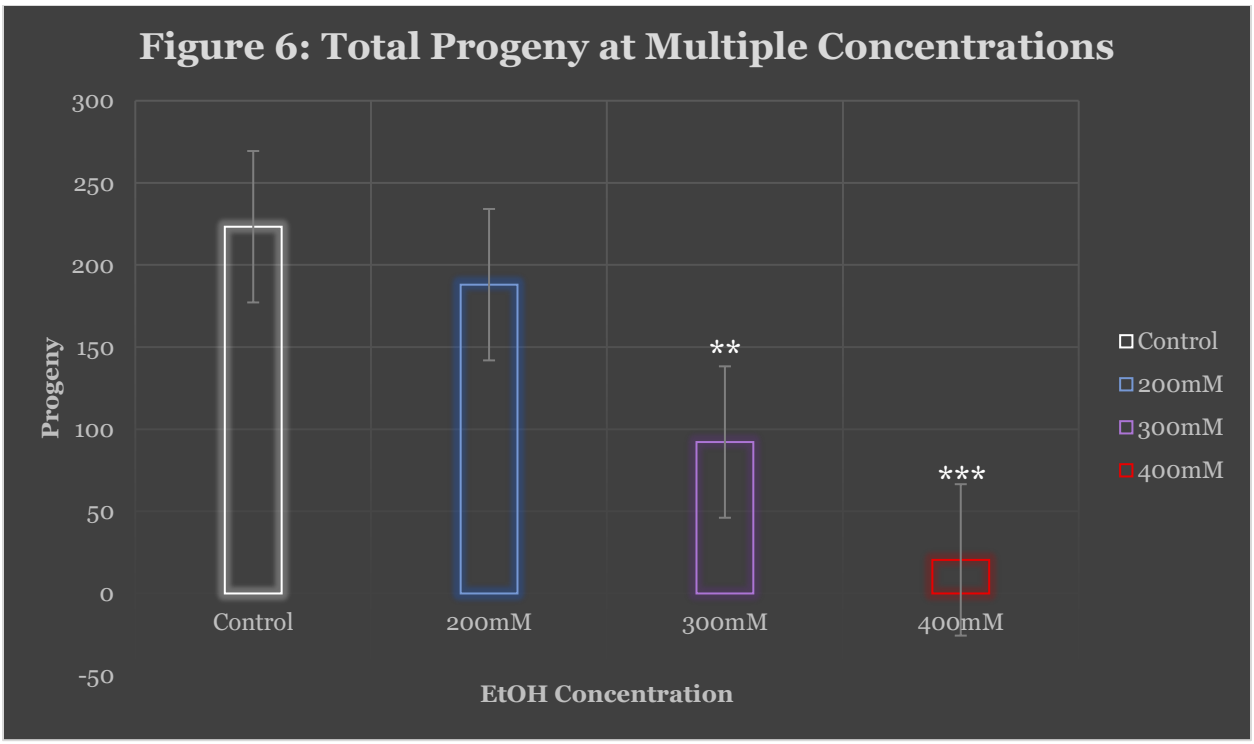

Figure 6: Chronic ethanol exposure was assessed across 6 experiment replicates at various concentration (0 mM, 200 mM, 300 mM, and 400 mM). After chronic exposure, the *C. elegans* had reduced fertility represented by the number of progeny throughout their life span. M Both 300 mM and 400 mM had a statistically significant difference with of p-values 0.0026 and 0.0002, respectively.

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Ethanol Absorbance Assay Demonstrates Ethanol Consumption in L4 C. elegans

In order to determine their level of intoxication, 300 larvae were hatched and grown to the L4 stage of development. These *C. elegans* were exposed to ethanol at 0 mM (control), 300 mM and 500 mM concentrations for 10 to 12 hours, or until the L4 larvae reached the young adult phase. Ethanol absorbance was measured using the MedTest Dx assay. This assay determines the concentration of ethanol present in the *C. elegans* relative to concentration of alcohol reagent absorbed. The negative control was an NaCl solution, and the positive control was 100% ethanol. As shown in Figure 7, the average absorbance reading for the NaCl and 100% , versus 100% ethanol were 0.0 AU and 2.27 AU, respectively. The controls had an average absorbance reading of 0.047 AU. The absorbance readings for the 300 mM and 500 mM were 1.03 AU and 1.67 AU, respectively. A significant difference was seen between the negative control and the 300 mM and 500 mM concentrations. This data, along with images showing disinhibition (see Figure 5) serves as confirmation that L4 *C. elegans* were intoxicated and consuming ethanol infused within the *E. coli* throughout these experiments.

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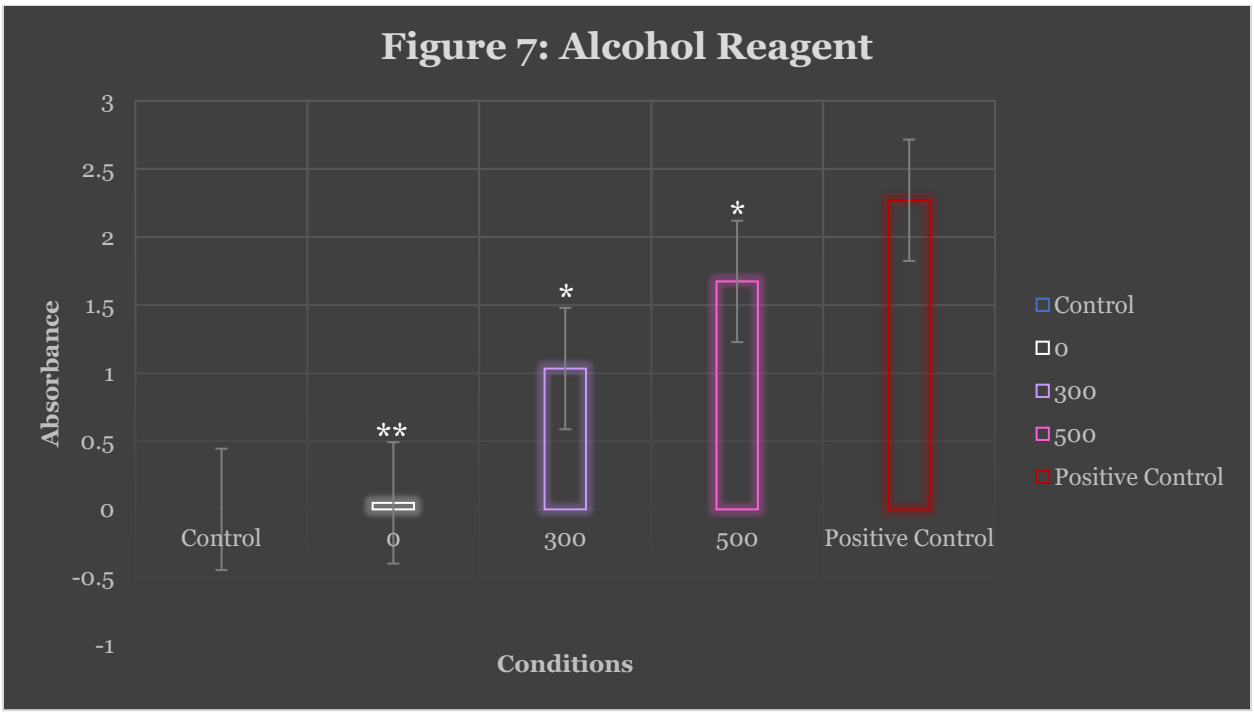

Figure 7: Two concentrations were used to determine the ethanol content that the L4 larva consumed. After 12 hours of ethanol exposure, the assay shows the absorbance for the young adult worms increasing when compared to the control group of NaCl and the absorbance reading of 0 mM ethanol *C. elegans*. Welch's t-test identified a p-value of 0.0016 for 0 mM, 0.0305 for 300 mM and 0.0498 for the 500 mM concentration.

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Chronic Exposure to 300 mM Ethanol Directly Affects the Mitotic Germline

Twelve L4 larvae were treated with 300 mM ethanol over a two-day period which allowed the larvae to reach the adult stage. The adults' nuclei were stained with DAPI and viewed by confocal laser scanning microscopy (CLSM). Figure 8 shows the effect of ethanol exposure on the germline. Figures 8B and 8D show the method for counting mitotic nuclei. Mitotic nuclei were marked with purple as they were counted. Figures 8A and 8B show the adult germline of control worms (0 mM). Figures 8C and 8D show the effects of 300 mM ethanol on the mitotic germline.

As shown in Figure 9, control worms had an average mitotic cell count of 175. The *C. elegans* treated with 300 mM ethanol showed a decrease in the average number of mitotic germline cells relative to controls (110) Using Welch's t-test for analyzing both conditions, it was determined that there was a significant difference of $P<0.0001$.

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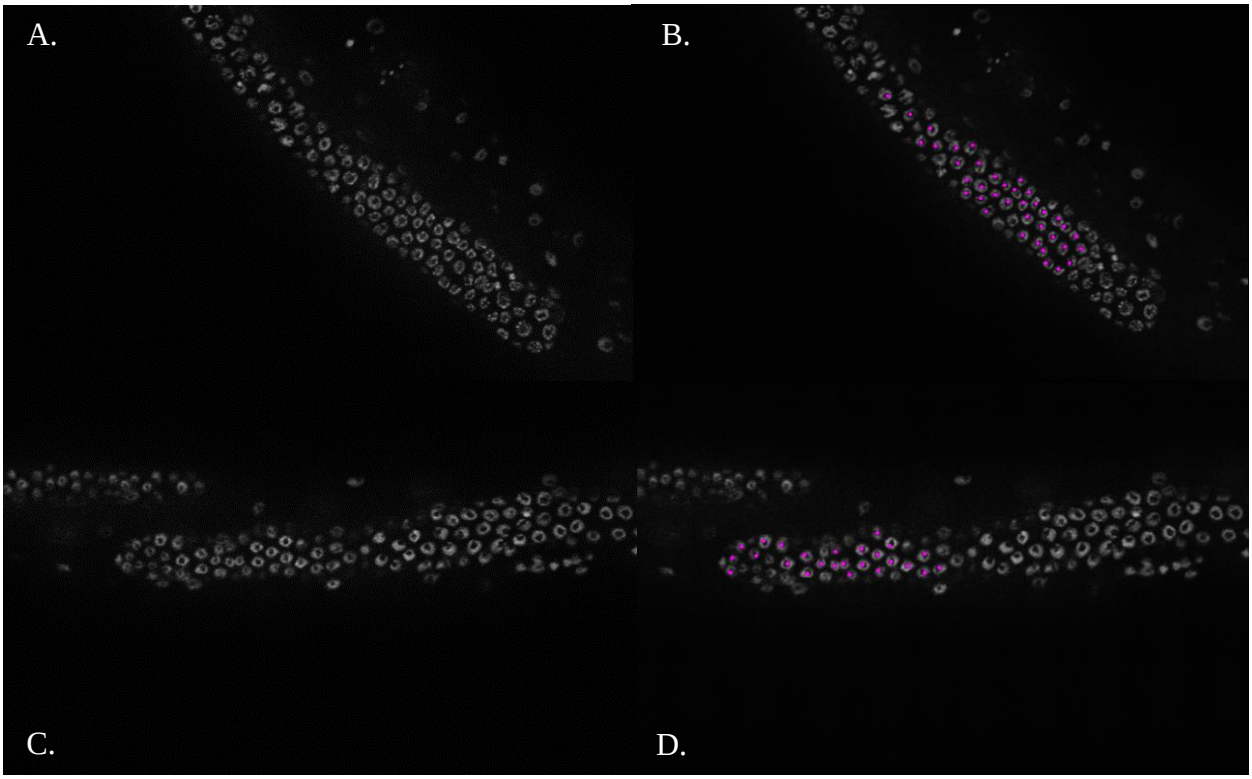

Figure 8. A and **B** indicate the germline of 0mM *C. elegans* along with the number of cells within on stack pinned in purple. While **C** and **D** indicate the germline of 300 mM *C. elegans* under the same conditions revealing less mitotic cells present after chronic ethanol consumption.

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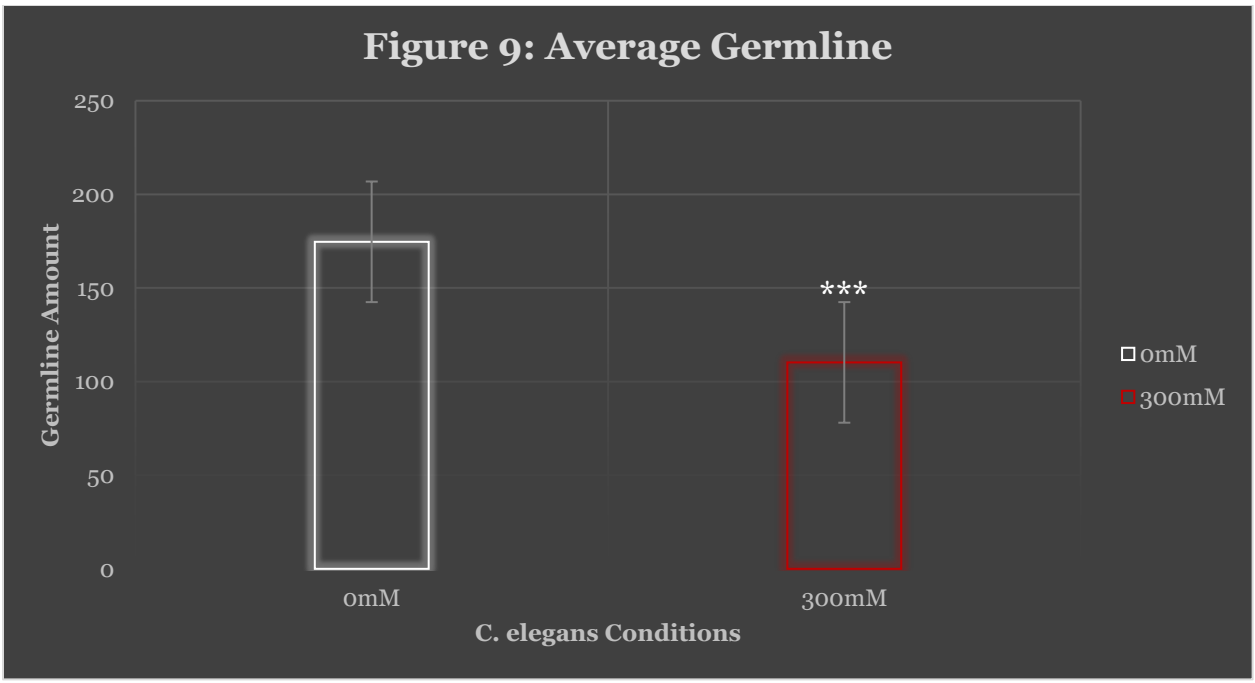

Figure 9: At 0 mM, the *C. elegans* had an average mitotic germline of 175 cells. At 300 mM, the mitotic germline average dropped to 110 Using Welch's t-test for analyzing both conditions, it was determined that there was a significant difference of $P<0.0001$.

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Treatment with 300 mM Promotes a Decline in Progeny Production in L4 GC1373/GC1374 C. elegans

Chronic exposure was evaluated in 4 replicates with 12 *C. elegans* per trial. GC1373 is a mutant strain for the glp-1/Notch pathway and contains an effective RNAi which is restricted to the germline. GC1373 contains a reduction of function mutation which enables them to produce half the number of adult germline progenitors, which leads to less overall progeny production. In *C. elegans*, glp-1/notch activity prevents germline stem cell differentiation. However, GC1374 is the mutant counterpart containing only a reduction of function mutation.

GC1373/GC1374 L4 *C. elegans* were treated with 0 mM, and 300 mM ethanol. The progeny was counted on days 5 and 7. The parent worm was observed on day 6 and transferred to fresh agar plates if there was a considerable number of eggs present on the plate. The number of progeny was determined across the replicates at the end of the 7th day. Figure 10 shows the effect of chronic ethanol exposure on progeny count. Control GC1373 mutants had an average of 44 hatchlings, while the worms exposed to 300 mM of ethanol produced an average of 14 hatchlings.

As shown in Figure 11, GC1374 *C. elegans* produced an average of 153 progeny, far greater than GC1373, but still less than the average wild-type *C. elegans* amount. The treated GC1374 *C. elegans* produced an average of 75 progeny. Although there was a difference in the amount of progeny, there was no statistical significance between the 0 mM and 300 mM concentrations.

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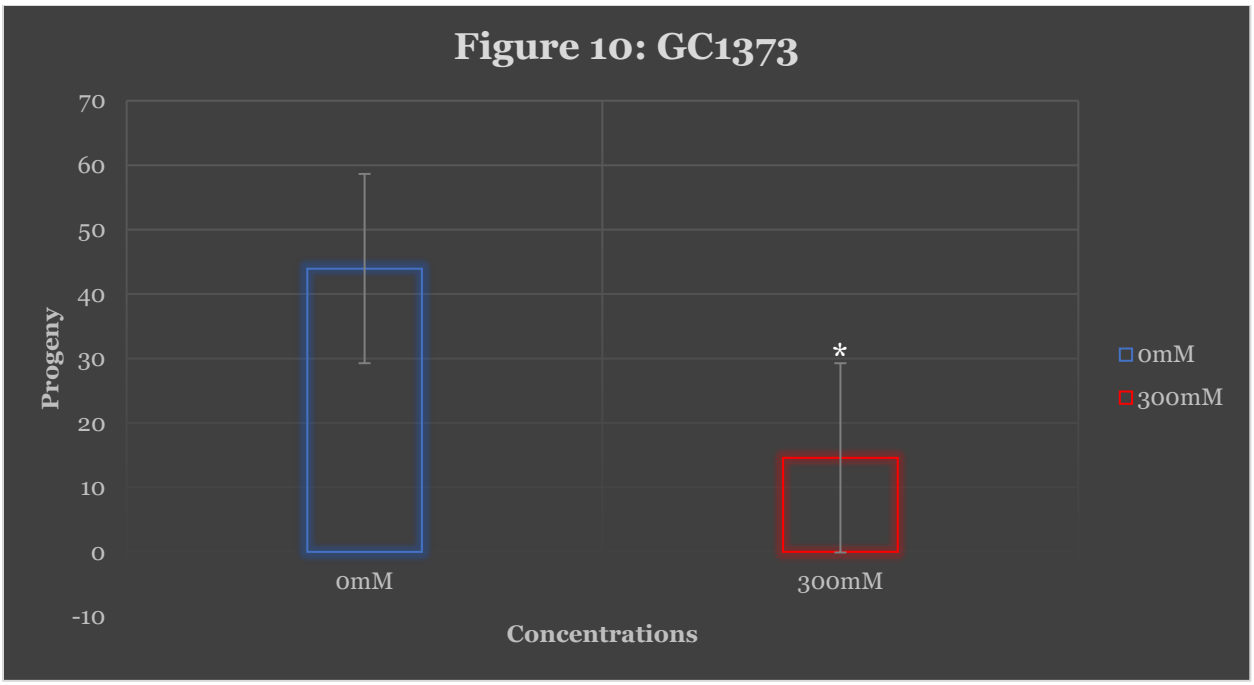

Figure 10: Chronic exposure on the notch mutant was done in four replicates with twelve worms per trial. In the mutant strain, GC1373 a Welch's t-test determined it to be more prone to a decline in progeny production; due to the reduction of function mutation with a statistically significant difference of p-value=0.037.

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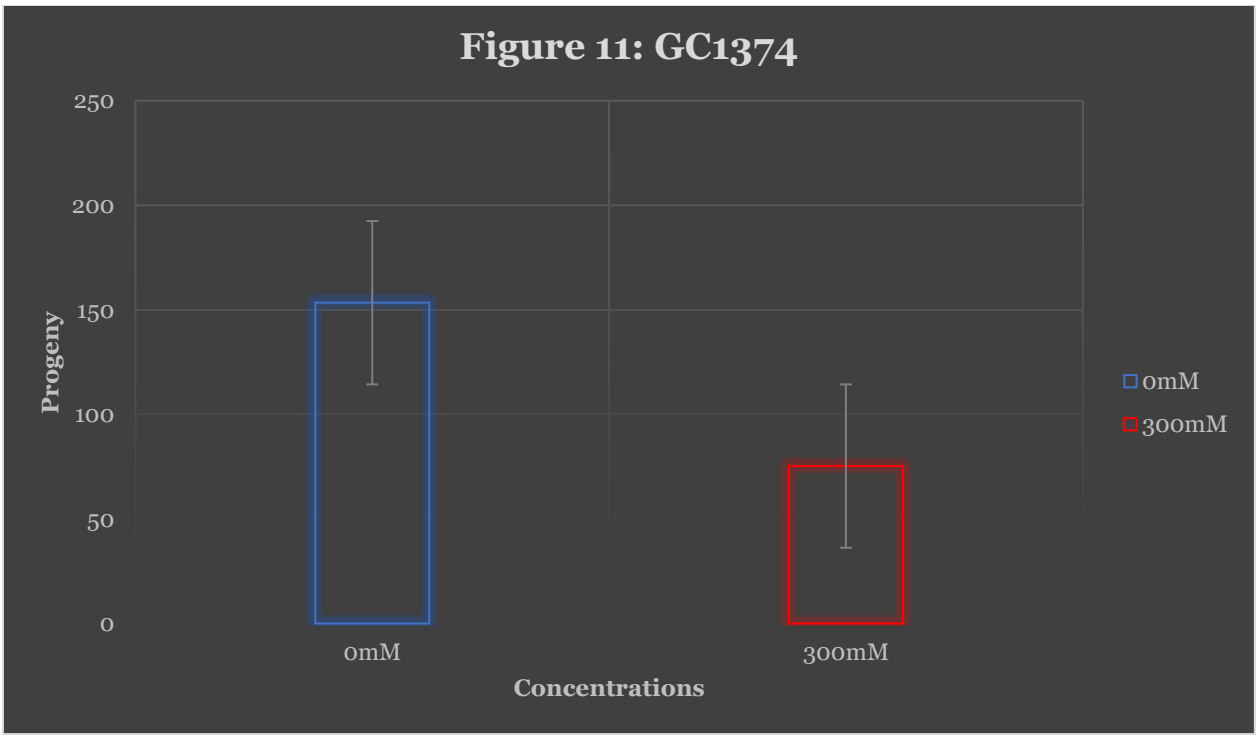

Figure 11: In the GC1374 strain, although there is a difference in amount of progeny, there is no statistical significance between the 0 mM and 300 mM concentrations of GC1374 with a p-value of 0.093.

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Discussion

Ethanol Disinhibition Responses in Wild Type L4 C. elegans

In the current work, we demonstrated that increasing concentrations of ethanol induced increasing disinhibition of locomotion in *C. elegans*. Previous studies found *C. elegans* exposed to a concentration of 500 mM ethanol on semi-moist agar plates, demonstrated a gradual decline in locomotion, feeding and egg-laying behaviors; eventually, leading to immobility after 30 minutes (Davies et al., 2015). The high dose resulted in an internal ethanol concentration relevant to human consumption and disinhibition in rodent models (Alaimo et al., 2012). Topper and colleagues (2014) demonstrated that *C. elegans* exposed to ethanol through an immersive method, in which the worm was immersed in ethanol and NGM solution, displayed behaviors rarely observed in water. The study showed that ethanol induced fits of crawling and other crawling associated behaviors such as foraging and reversals, which require coordinated motion.

Topper concluded that ethanol should be viewed as specifically disinhibiting crawling behaviors. Ethanol consumption was determined using an ethanol absorbance which read the concentrations of ethanol within the *C. elegans* after chronic ethanol consumption (Davies et al., 2004). Upon exposure to increasing doses of ethanol, the L4 *C. elegans* exhibit disinhibition effects within 5 minutes of ethanol exposure. As the *C. elegans* consumed the ethanol, the sinusoidal motion decreased and caused changes in the amplitude and frequency of the movement. The results found in our study confirm that of previous studies which interpret disinhibition effects strictly maintained to crawling. The amount of ethanol given to the *C. elegans* determines the effect disinhibition has on the crawling behavior.

Ethanol Impact on Wild Type L4 Mitotic Germline

Previous studies have reported that low doses of ethanol have the capability to elongate

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the life span of L1 larva (Castro et al., 2012). *C. elegans* exposed to ethanol from the gastrula until larval stage 4 (L4) develop up to 1122 differently altered genes (Patananan et al., 2015). This indicates that different pathways are activated depending on the ethanol concentration and the stage at which the *C. elegans* are exposed. Garaycoechea and colleagues (2018) determined endogenous aldehydes are a ubiquitous source of DNA damage that impair blood production. They determined some damage occurs within the hematopoietic stem cells which affects other aspects such as aging and mutagenesis. It has been previously reported that ethanol can lead to genomic instability in rodent models. Garaycoechea examined hematopoietic cells for evidence of broken chromosomes. Hematopoietic stem cells mutated by aldehydes were functionally compromised and displayed myeloid bias (Garaycoechea et al, 2018). Ethanol exposure alters gene expression and methylation profiles in embryonic stem cells (Liu et al., 2009).

Due to the undifferentiated nature of the mitotic germ cells, they are more sensitive to ethanol (Chastain and Sarkar, 2017). The germline is the only part of the *C. elegans* cell lineage that proliferates for the life of the organism. The maintenance of germ cell proliferation is accomplished through interactions with a distal tip cell as mentioned previously. Ethanol induces changes in the epigenome of sperm in exposed male animals (Chastain and Sarkar, 2017).

In *C. elegans*, a finite number of sperm are produced early in the *C. elegans* lifespan; in most other animals, including humans, sperm are produced throughout the lifespan with a finite number of eggs being produced. The current work found that chronic ethanol consumption commencing at the L4 stage of life decreased the amount of mitotic germ cells. When comparing the untreated *C. elegans* to the 300 mM treated L4 larva, the germline cells were greater than the treated. The potential effect ethanol had on the germline coincides with the results demonstrated within the hatchlings of the various concentrations. As the concentration of ethanol increased,

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the amount of germline cells decreased along with the number of viable hatchlings.

Ethanol and the impact on Wild Type and Mutant Strain Progeny Production

Previous studies have found delays in onset egg-laying upon ethanol exposure (Yu et al., 2011). *C. elegans* on average lay up to 300 eggs in their lifetimes. Yu and colleagues determined that long-term ethanol exposure reduced the number of eggs laid in the life cycle of the *C. elegans*; the lowest amount being that of 21 eggs. At the highest concentration in the study, Yu found that 40% of the worms either became infertile or developed bags, which is a condition in which the offspring hatches within the body of the parent. The data suggested chronic ethanol exposure impairs the egg-laying process. The current work supports this hypothesis, and our data demonstrates that chronic ethanol exposure effects the germline of the *C. elegans* and not just the general characteristic of egg-laying. Our study found that as ethanol concentrations increased, the number of offspring the *C. elegans* decreased.

This factor was also noted in mutant strains GC1373 and GC1374. GC1373 is the mutant strain containing a reduction of function glp-1 (Roy et al., 2018). Glp-1 engages in several processes within *C. elegans* such as multicellular organism development, negative regulation of stem cell differentiation and positive regulation of cell population proliferation (Arnaboldi, 2021). This is due to the GC1373 is ability of reduce a function which causes differentiation of all germ stem cells (Roy et al., 2018). Due to the mutation present within GC1373, the mutant strain produced less than half of the progeny when compared to the GC1374, which only contains a reduction of function mutation. The chronic ethanol consumption led the mutant strain to decrease the number progeny produced versus the untreated counterpart.

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Conclusion

The goal of the current work was to determine the effect of chronic ethanol consumption on the *C. elegans* germline. Our focus was to measure the number of progeny produced in the life cycle of the *C. elegans* along with the total amount of germ cells present after chronic ethanol exposure. Our results indicate that in wild-type worms chronic ethanol exposure results in a decrease in eggs laid and a decrease in germline stem cells.

Additionally, chronic ethanol exposure at the wild type and mutant L4 larva stage has a statistically significant effect on the number of cells produces and progeny laid. The mutant strains containing a reduction of function mutation had a lower progeny count when exposed to chronic ethanol. Our results indicate that in the mutant strains chronic ethanol exposure decreases the ability of the parent worm to lay eggs.

Future Work

Future work would include acute exposure to determine the likelihood of a *C. elegan*s recovering from the effects of ethanol which could lead to the analysis of acute versus chronic exposure. Further studies could determine the effect of chronic exposure to ethanol from the L1 stage on the development of adult animals. This would allow us to follow the development of animals from hatchling to progeny production, the L4 molt, mid-L4, late L4, adult molts and first round of progeny production. Studies using SLO-1 mutant strain could determine the effect of chronic ethanol exposure on the fertility of the mutant animals. SLO-1 shown to be essential for ethanol mediated behavioral phenotypes including rate of egg laying. The studies would demonstrate facilitate our understanding of the damage caused to the germline by chronic ethanol consumption.

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