THE EFFECTS OF ETHANOL ON THE PHYSICAL DEVELOPMENT, NERVOUS SYTEM DEVELOPMENT AND BEHAVIOUR OF *CAENORHABDITIS ELEGANS*

By

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ABSTRACT

In this thesis I have used the nematode worm *Caenorhabditis elegans* to study the effects of ethanol exposure on basic physical development and on specific aspects of nervous system development and behaviour. In the first part of this thesis I investigated how chronic exposure to ethanol affects the physical development and life history of worms. I found that chronic exposure to ethanol negatively impacts *C. elegans* size, rate of development, reproductive fecundity and life expectancy. I further investigated how exposure to ethanol during embryonic development affects this system by exposing eggs to ethanol for 1 hour a different time points during embryogenesis. I found that embryos exposed acutely to ethanol early in their development have a lower probability of hatching into larval worms, and those worms that do hatch display distinct dysmorphologies. In the second part of the thesis I examined how chronic exposure to ethanol affects the *C. elegans* nervous system and behaviour. In this set of experiments I found the total amount of GFP expression of two different pan-neuronal genes was decreased relative to the size of worms as a result of chronic exposure to ethanol. To determine whether this decrease in apparent size of the nervous system due to ethanol exposure affected the behavior of adult worms I examined two simple, well-studied behaviours: tap withdrawal which is mediated in part by glutamatergic neurotransmission, and the basal slowing response which is mediated by dopaminergic neurotransmission. I found that chronic ethanol exposure attenuated the tap withdrawal response, but did not affect the basal slowing response. To investigate the effects of ethanol on the tap withdrawal circuit I examined the expression patterns of two genes that are expressed in the mechanosensory circuit. I found that the expression of GLR-1::GFP,

a non-NMDA type glutamate receptor sub-unit expressed on the interneurons of the tap-withdrawal circuit, is increased as a result of chronic exposure to ethanol. I also found that the timing of the development of the post-embryonic mechanosensory cells is affected by chronic exposure to ethanol. Together these alterations in the tap circuit could have produced the behavioural changes observed. In this thesis I have characterized the effects of ethanol on the basic biology, nervous system and behaviour of *C. elegans* with the specific aim of generating data to support further investigations of the effects of ethanol on nervous system development and behaviour in this system.

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CHAPTER I

1.1 GENERAL INTRODUCTION

Alcoholism and the teratogenic effects of prenatal exposure to ethanol result in costly drains on Canada's public healthcare system. Therefore it is important to research alcohol's effects on the biology and development of model organisms in order to further our understanding of the pathology of this drug. One important area that needs to be understood further is the impact alcohol has on the natural process of development. In the report presented here, I will demonstrate the feasibility of using the nematode worm *Caenorhabditis elegans* as a model system for the study of the effects of alcohol on development and the nervous system.

Alcohol is an established teratogen meaning that in-utero exposure to it has the capacity to result in detrimental developmental abnormalities. In fact, an Institute of Medicine (Stratton, 1996) report on the issues in research on the fetal effects of drug use identified that the effects of alcohol are as severe or more severe in terms of the neuro-behavioural deficits observed than that of other teratogens such as polychlorinated biphenyls (PCBs), mercury, lead, diphenylhydantonin (DHP), X-irradiation and other recreational drugs including marijuana, cocaine and heroin.

The teratogenic effects of ethanol have been demonstrated in humans in both clinical and behavioural studies. Jones et al. in 1973 first characterized the physical, behavioural and neurodevelopmental abnormalities observed in children born to chronic alcoholic mothers. They described the same abnormalities in eight children of different ethnicity, all born to alcoholic mothers who consumed varying amounts of alcohol over the course of their pregnancy. Jones et al. found that all children displayed what have

become the three hallmark features of Fetal Alcohol Syndrome (FAS) - Craniofacial dysmorphology, pre and postnatal growth retardation and central nervous system (CNS) deficits including, retarded cognitive, motor and behavioural development. This original description of the effects of in-utero alcohol exposure has since expanded to include the many physical, behavioural and sociological issues that arise as a consequence of in-utero alcohol exposure and all are now kept under the umbrella term Fetal Alcohol Spectrum Disorder (FASD).

Developmentally, children born to alcoholic mothers show significant physical growth deficiencies postnatally; children diagnosed with FASD gained an average of 9g/day compared to 26g/day observed in children of non-alcoholic mothers (Jones et al. 1973). FAS children, on average, had cranial circumferences below the third percentile for their given gestational and postnatal age. CNS abnormalities observed included intellectual deficits ranging from mild to severe cognitive retardation, and behavioural deficiencies including perceptual motor disturbances, hyperactivity and attentional deficits (Diaz and Samson, 1980, Ernhart et al. 1995, Mattson and Riley, 1998, Riley and McGee, 2005). In composite, these three hallmark features of clinical FAS vary in severity depending the extent of prenatal exposure. It was this initial description that first highlighted the conserved abnormalities and deficits, and alerted physicians to the common features observed in the children of chronically alcoholic mothers.

Case studies and clinical studies have done well to characterize the effects of ethanol on easily quantifiable traits, however, for more precise examinations of pre-natal ethanol exposure on various physical, immune and neural processes, many mammalian model systems have been employed. The mouse, rat, guinea pig, dog and pig-tail

macaque have all been studied as models for the teratogenicity of ethanol. (Streissguth et al. 1980, Sulik et al. 1981, Cudd 2005). The use of mammalian models has allowed for more precise control over a variety of confounds within human studies- confounds such as self-reports of alcohol consumption during pregnancy, diet, the simultaneous use of other teratogenic drugs, the amount of post-natal care and most importantly the timing and dose of pre-natal ethanol exposure. Work in mammalian model systems has described the effects of ethanol on a range of behavioural and physiological processes including brain growth and neural development (e.g. Diaz and Samson, 1980, Hoffman et al, 2002, Zhou et al, 2005), immune function (Giberson et al 1992) and neuroendocrine function (e.g. Gabriel et al 1998, Zhang et al. 2005).

1.1.1 Why *C. elegans* is an Excellent Model for the Study of the Effects of Ethanol on Development:

Caenorhabditis elegans is a small, 1 mm long, soil dwelling, hermaphroditic nematode found in temperate regions. In the 1960's Sydney Brenner began using it to study the genetics of development and neurobiology (Brenner, 1974) (See Figure 1.1). Thousands of researchers have since established a database of information on many aspects of *C. elegans* life history and biology. *C. elegans* has risen to the forefront of experimental models in many different fields. It was the original pilot project for the sequencing of the human genome (Waterson and Sulston, 1995). It was the original system in which programmed cell death was first characterized by Horvitz and Sulston (1977), for which they later shared the Noble Prize in medicine with Brenner. Since its original application for detailed genetic analysis, *C. elegans* has become the model of choice in many fields of scientific study ranging from basic genetics to neural

development to learning and memory. *C. elegans* researchers have come to produce a vast database of knowledge regarding this nematode making it the ideal *in-vivo* model for experimental study.

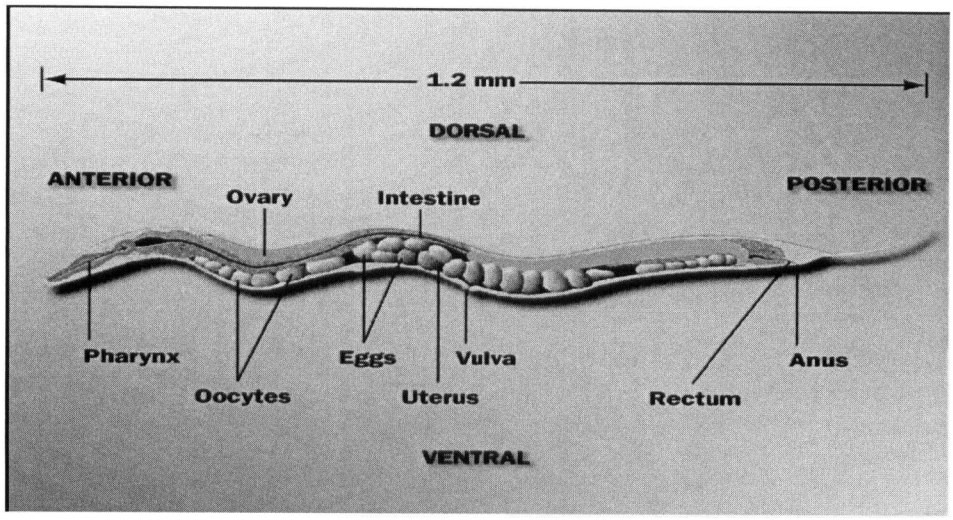


Figure 1.1 *C. elegans* Schematic Diagram
This schematic diagram portrays a hermaphrodite adult worm. Figure adapted from http://www.imsc.res.in/~sitabhra/research/neural/celegans/index.html.

C. *elegans* was originally chosen as an experimental model thanks in large part to its rapid rate of development. *C. elegans* has a 14-hour period from the time a given egg is fertilized to when it hatches (Sulston et al. 1983). After hatching, *C. elegans* passes through four larval transitions termed L1, L2, L3 and L4 over the next 36 hours of post-embryonic development on its way to reaching adulthood (Wood, 1988). Within four days of being laid as an egg, *C. elegans* will reach full reproductive maturity and is capable of producing a brood of progeny consisting of 300-350 individuals (Wood, 1988) (See Figure 1.1.1 for detailed description of the developmental timetable).

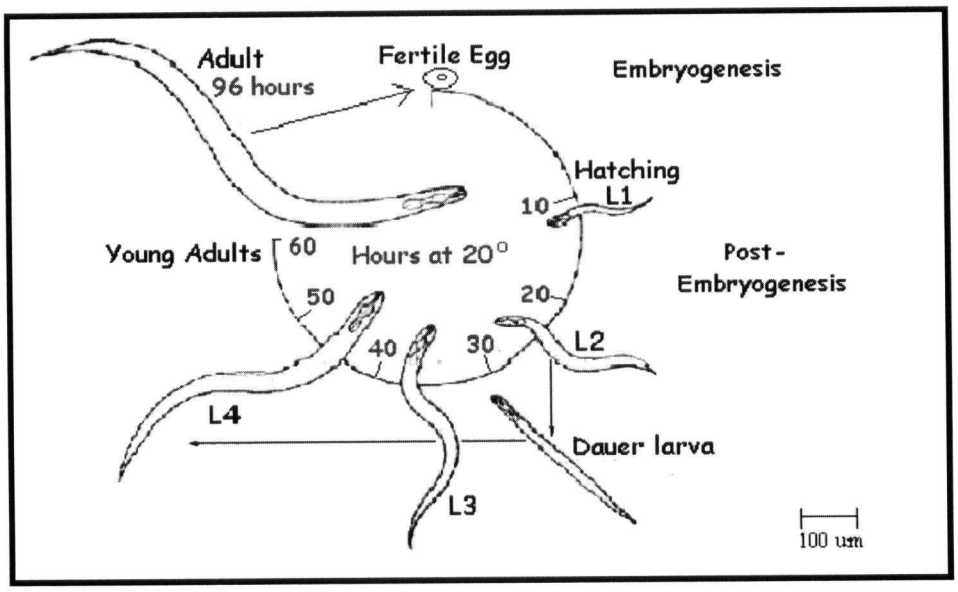


Figure 1.1.1 *Caenorhabditis* Developmental Timetable.
This figure illustrates the life cycle of the hermaphroditic nematode, *C. elegans*, from the time of embryogenesis to adulthood. The time line (in hours) represents the normal growth for *C. elegans* when it is raised under optimal conditions (20^{0C}). Adult worms will lay eggs ~3 hours after they are fertilized in-utero. This egg will hatch ~ 10-12 hours after being laid and will subsequently pass through four larval transitions, L1, L2, L3 and L4 before reaching young adulthood ~60 hours after being laid as an egg.
(Figure adapted from: www.eb.tuebingen.mpg.de/.../wurm_entw.html)

In addition to having a rapid rate of development and producing large numbers of self-fertilized progeny for high throughput study, *C. elegans* also has a small, tractable nervous system. The *C. elegans* nervous system consists of 302 identifiable neurons making ~ 5000 chemical synapses. Every neuron has been characterized and had its connectivity mapped using electron microscopy (White et al.1986). For this reason, studying the effects of ethanol on the developing neurobiology of *C. elegans* has a distinct advantage over doing so in other systems such as mammals that have millions of neurons that cannot be recognized at the level of the individual cell. *C. elegans*, with a small number of identified neurons, a transparent body and a short, well characterized

generation time, simplifies the investigation of ethanol's effects on specific aspects of neural development. In the *C. elegans* model, we have the opportunity to examine ethanol's effects on normal neural connectivity and axonal outgrowth, and the subsequent effects of these disruptions on behaviour in a system lacking the complicated interconnected CNS, immune and neuroendocrine network found in mammals. This system will allow for the identification of ethanol's specific neurotoxic effects on identified cells.

The databases of knowledge about C. *elegans* coupled with modern molecular investigative techniques contribute to the strength of this simple organism as a model for studying the effects of ethanol exposure on development. We know and understand the exact times of every cell birth and death and have a huge database regarding the timing and control of numerous developmental events. With the wealth of knowledge that exists relative to all aspects of *C. elegans* development, this system offers a unique opportunity to greatly expand our understanding of how ethanol exposure affects basic physical development, nervous system development and behaviour.

1.1.2 What We Know About Alcohol and *C. elegans*

In a study investigating the toxicity of short chain alcohols to *C. elegans*, Thompson and De Pomerai (2005) confirmed that ethanol is toxic to *C. elegans* and reported that the magnitude of toxicological consequence due to exposure to short-chain alcohols is greatest when worms are exposed to iso-butanol followed by iso-propanol, ethanol and methanol respectively. *C. elegans* expresses two ZN-containing genes for Alcohol dehydrogenase and is capable of metabolizing ethanol (Glasner et. al 1995). Studies that have specifically investigated the effects of ethanol on the behaviour of *C.*

elegans have provided critical data for our investigation. Davies et al. 2004 determined that environmental exposure to a concentration of 0.4M ethanol is sufficient to produce an internal tissue concentration of ethanol equivalent to 0.01% Blood Alcohol Concentration (BAC) in humans (Davies et al. 2004).

Davies et al. (2003) found that ethanol alters several behaviours in *C. elegans* including locomotion and egg laying. Ethanol alters these behaviour by activating the *slo*-1 gene product, a calcium activated BK potassium channel in C. elegans. Numerous loss of function *slo-1* mutant worms have been shown to be resistant to the intoxicating effects of ethanol while in contrast, *slo-1* gain of function mutant worms have been described to behave in an intoxicated manner in the absence of ethanol (Davies et al. 2003). Davis et al. (2004) also showed that *C. elegans* is capable of forming acute tolerance to ethanol. Acute tolerance to ethanol is described as a behavioural phenomenon whereby the intoxicated behaviour, suppressed locomotion, is attenuated with time despite worms being continuously exposed to ethanol (Davies et al. 2004). Differences in the ability of CB4856 (a strain showing relatively rapid development of tolerance) and N2 (a strain showing relatively slow development of tolerance) to form adaptive tolerance have been mapped to have a difference in a single gene, *npr-1*. The *npr-1* gene encodes a predicted G protein-coupled receptor in the neuropeptide Y (NPY) receptor family that can act as a receptor for two FMRFamide-related peptides (Kubiak et al., 2003; Rogers et al., 2003). Allelic variation in *npr-1* has been described such that there exists a single amino acid substitution at position 215 of NPR-1 such that the CB4856 strain carries a phenylalanine at 215 and the N2 strain carries a valine at 215. This single amino acid substitution has been demonstrated to be critically important to

mediating the intoxicating effects of ethanol in *C. elegans* (Davies et al. 2004). These two studies describing the importance of both *slo-1* and *npr-1* in mediating the effects of ethanol intoxication in *C. elegans* have improved our understanding of the molecular mechanisms through which ethanol mediates the behavioural effects seen in *C. elegans*.

The few studies involving alcohol and *C.elegans* have contributed somewhat to our understanding of ethanol's mode of action on adult worms. However, no study to date has investigated the specific effects of ethanol exposure on both the physical and nervous system development of *C. elegans*.

1.1.3 Thesis Objectives

This thesis has been written with two separate objectives. The first is to investigate the effects of ethanol on the development of *C. elegans* in order to make a contribution to the database of knowledge pertaining to this model system. The second is to present evidence supporting the use of *C. elegans* as a model system for study in the field of FASD research. To meet these separate objectives I have written a thesis that consists of two parts; the first is an examination of the effects of chronic exposure to ethanol on the physical and embryonic development of *C. elegans*. The second is an examination of more specific effects of chronic exposure to ethanol on the nervous system and behaviour of *C. elegans*. When taken together, the findings reported in these two parts each contribute to the accomplishment of these objectives.

The basic protocol followed in these studies was to expose *C.elegans* to varying concentrations of ethanol at different stages of development and then study how this exposure affected *C.elegans* development, life history and behaviour. Using a reporter gene (Green Fluorescent Protein (Chalfie et al. 1994)) GFP tagged to specific nervous

system genes) I have been able to, in part, visualize and quantify the effect ethanol exposure has on nervous system morphology and connectivity. Using this simple invertebrate system I have been able describe a comprehensive model of the effects of early ethanol exposure on physical development, neural development and behaviour. In further developing this resource rich model, we will have an opportunity to improve our overall understanding of the effects of early ethanol exposure on development. In doing so there exists the possibility that the data we generate will help in the design of behavioural and therapeutic strategies aimed at ameliorating the developmental deficits associated with early ethanol exposure in higher order systems including humans.

1.2. GENERAL METHODS

1.2.1 *C. elegans* Culture and Strains Used

Nematode strains used in this study were cultured at 20^{O}C in the presence of an abundant source of food, *E. coli*, in accordance with standard *C. elegans* culture methods (Brenner, 1974). In this study, a total of five strains were used for various experiments. The N2 strain is the original Bristol strain of Caenorhabditis elegans selected by Brenner for use as a novel genetic model (Brenner, 1974). The N2 strain is considered wild type. The N2 strain was used in all experiments detailing the physical effects of ethanol on *C. elegans*. The KP1580 (Josh Kaplan) is a transgenic strain containing chimeric non-NMDA AMPA type receptors made up of GLR-1 tagged with GFP. The *gfp* coding sequences were inserted in frame, 16 codons from the cytoplasmic tail of *glr-1* and the construct was integrated into the X chromosome of C. elegans. GLR-1::GFP is expressed in the interneurons under the control of the *glr-1* promoter (Rongo and Kaplan, 1999).

This strain was chosen for use in imaging experiments designed to investigate ethanol's effects on glutamatergic neurotransmission in *C. elegans.* The DP132 (David Pilgram) strain was used to investigate the effects of ethanol on pan neuronal GFP expression. In this strain, GFP expression starts in the early embryo and continues through adulthood in most, if not all, of the nervous system. This strain allows for pan neuronal expression due to the integration of an array carrying pDP#MMUGF12 and pRF4. pDP#MMUGD12 in an unc-119::GFP fusion that encodes 101 aa of UNC-119 and was made from the Fire lab vector pPD95.77. The UNC-119::GFP construct has been integrated in worms with along with a pRF4 plasmid containing a rol-6(su1006) mutation that results in a pronounced "roller" phenotype. This mutation results abnormal locomotion (roller phenotype) and allows for identification of worms carrying the integrated UNC-119::GFP promoter fusion. The construct was integrated using gamma rays.

The NW1229 (Joseph Culloti) is a second *C. elegans* strain expressing pan neuronal GFP. NW1229 worms contain an integrated Ex[F25B3.3::GFP; dpy-20(+)] construct in a dpy-20(-) background. In this worm GFP is tagged to F25B3.3, a gene that codes for a nervous system specific guanine nucleotide exchange factor. No information is available pertaining to the method of integration. This strain was used to confirm findings made using the DP132 strain regarding the effects of ethanol on the *C. elegans* nervous system.

The MEC-4::GFP-rol- 6 is another transgenic strain of *C. elegans* that expresses GFP in all the touch receptor neurons; ALML, ALMR, AVM, PLML, PLMR and PVM. This strain is behaviorally insensitive to mechanosensory stimulation. The GFP construct has been integrated in worms with along with a mutation resulting in a pronounced roller

phenotype. The mutation results abnormal locomotion (roller phenotype) and allows for identification of worms carrying the integrated MEC-4::GFP construct. No information regarding the integrated construct location or method of integration is available.

All strains used in this study were obtained from the *C. elegans* genomics Centre in Madison WI.

1.2.2 Ethanol Administration

In order to administer different doses of ethanol to C. elegans, I generated ethanol plates that would facilitate worms taking up ethanol from their environment (Davies, 2004). To produce an ethanol plate, standard 20ml Petri plates were filled with ~10ml of NGM agar and seeded with OP50 E and then infused with measured volume of 95% stock ethyl alcohol (UBC Chemical stores) to bring the ethanol concentration of the agar plate to desired level. In order to produce a plate with the desired ethanol concentration, the following formula was used to determine how much 95% ethanol to add to each agar plate.

$$V_{ETOH} = C_{desired} (V_{agar} + V_{ETOH}) / C_{95\% ETOH}$$

Where: V_{ETOH} is the volume of ethanol to be added in L (liters)

$C_{desired}$ is the concentration of ethanol desired in M (Molar)

V_{agar} is the volume of agar in L

$C_{95\% ETOH}$ is the concentration of the 95% ethanol in M (i.e. 16.83M)

Once calculated, the required volume of 95% ethanol was pipetted onto the seeded NGM plates. Ethanol was pipetted evenly across the plate surface to ensure even distribution of ethanol across the plate. Parafilm was used to seal the plates to prevent

ethanol from evaporating out of the NGM plate. Plates were allowed to sit for at least 2 hours to allow the ethanol to diffuse through the agar before placing worms on the plate.

In previous studies investigating the effects of ethanol on *C. elegans*, environmental concentrations of ethanol used were 0.2M, 0.4M and 0.5M (Davies et al. 2004). In these studies the 0.4M concentration was found to produce an internal tissue concentration in *C. elegans* equivalent to 0.1% Blood Alcohol Concentration (BAC) in Humans. For this reason we elected to employ these environmental concentrations for our studies as well.

To make a developmentally synchronous colony of ethanol exposed worms, 8 to 15 4-day old worms were placed on a seeded ethanol plate and allowed to lay eggs for a period of one hour. Following the one hour laying period the adult worms were picked off leaving a plate full of age matched eggs to grow up in the ethanol-laden environment. In order to account for the volatile nature of ethanol and ensure that worms were exposed to the desired concentration of ethanol throughout development it was necessary to transfer worms to a fresh ethanol plate with the desired concentration every day. Worms raised on a given ethanol plate concentration were transferred to fresh corresponding plate (i.e. the worms in the 0.2M ethanol plate were transferred to a new 0.2M ethanol plate). Worms were then followed and monitored over the course of their development and subsequent lifespan. At ~ 12 hours after hatching, individual worms from the main colony were transferred to individual ethanol plates to allow for precise monitoring of individual *C. elegans* development. This protocol was used for all exposure periods employed in the study.

1.2.3 Statistical Analysis

For statistical analysis of all recorded measurements, Statview 5.1 software was used. Data was analyzed using a univariate (one-way) ANOVA or a one tailed t-test. These techniques allowed for the statistical analysis between sample means. Following a one-way ANOVA I tested which sample means were significantly different from one another by performing all possible t-tests using Fisher's Planned least significant difference (Fisher's PLSD) post-hoc analysis. Differences between groups with P values < 0.05 were deemed to be significant.

CHAPTER II

The Effects of Ethanol on the Physical Development and Life History of *C. elegans*

2.1 INTRODUCTION

Recognizing the need to develop novel model systems to further the field of FASD research in his report, Cudd (2005) reviewed model systems in which to study the effects of alcohol on development; his summary reads:

> *"Simple animal models are useful in exploring basic science questions that relate to molecular biology and genetics that cannot be explored in higher-order animals, whereas higher-order animal models are useful in studying complex behaviors and validating basic science findings in an animal that is more like the human. Substantial progress in this field will require the judicious use of multiple scientific approaches that use different animal model systems" (pg 389).*

It is the objective of the report presented here to address this need and to develop a novel model for the study of the effects of alcohol on the developing nervous system and later behaviour. *C. elegans,* with its small number of neurons, transparent body and lack of a complicated inter-dependent immune, CNS, and neuroendocrine network appears to be a logical model for use as model system in which to study the effects of alcohol on nervous system development and later behaviour. In employing the *C. elegans* model, we have the opportunity to examine ethanol's effects on normal neural connectivity and axonal outgrowth, and the subsequent effects of these disruptions on

behaviour in a system lacking a complicated interconnected CNS, immune and neuroendocrine network.

Investigations into the effects of ethanol on the development of non-mammalian systems have been conducted. In the Japanese Medaka Fish (*Oryzias lapties*), Wang et al. (2006) observed that embryonic exposure to ethanol produces the same phenotypic features associated with pre-natal exposure to ethanol in mammalian systems. In this study, embryonic exposure to waterborne concentrations of 200-400mM was sufficient to interfere with the normal development of cartilaginous and skeletal structures within the system (Wang et al. 2006). A second fish model that has been investigated in terms of its response to embryonic exposure to ethanol is the Zebrafish (*Danio rerio*). In Zebrafish, embryonic exposure to waterborne concentrations of 2.5% and 3% ethanol was sufficient to produce significant developmental abnormalities including cyclopia (fusion of the two eyes) and a lowered heart rate (Bilotta et al. 2004). Both of these studies were initiated with the intent of establishing the Japanese Medaka and Zebrafish as simple animal models of FAS. In both cases, the major argument in favour of using the given model is that the concentration of ethanol to which developing embryos are exposed can be tightly controlled. Furthermore, it is argued that the well-timed pattern of embryonic developmental in each system allows for the identification of critical periods during development where ethanol exposure significantly impacts upon specific developmental processes (Bilotta et al. 2004, Wang et al. 2006).

In this chapter, I will describe the effects of ethanol on the development of a different, non-mammalian model system, *C. elegans*. It is the purpose of this chapter to present data supporting the use of *C. elegans* for further study of the effects of ethanol on

development. This system has the same advantages as both the Japanese Medaka Fish and Zebrafish but has many additional features such as a well-characterized neural circuitry (White et al. 1986, Bargmann, 1998), mapped and sequenced genome (Waterson and Sulston, 1995) and short generational time (Wood, 1988) that offer unique opportunities to understand the effects of ethanol on development.

In this series of experiments I investigated the effects of chronic ethanol exposure on the growth, rate of development, reproductive fecundity and longevity of a consistent population worms chronically exposed to ethanol over the course of their entire lives in a longitudinal study. I also characterized the same measurements in consistent populations of worms that were exposed to ethanol only during adulthood, or only during in-utero embryonic development. In addition, I tested the permeability of the *C. elegans* eggshell to ethanol and the effects of exposure to ethanol during ex-utero embryonic development.

2.2 METHODS

2.2.1 Body Length

Worms in the study were videotaped on the stage of a stereomicroscope (Leica Wild, Model M3Z) using a video camera (Panasonic Digital 5100) connected to a VCR (Panasonic AG1960) and television monitor (NEC PM-1271A) at 25X magnification and tracing the length of worms on to transparencies. Transparencies were then scanned into a Macintosh Apple computer using UMAX Astra 2100U scanner and software. The length of each reversal was measured in pixels using NIH image software. This process was repeated daily in order to generate the data used to identify the dose dependent effect of ethanol on *C. elegans* body length.

2.2.2 Egg-Lay Onset

C.elegans begins to lay eggs approximately 68 hours following hatch (Wood, 1988). To determine whether ethanol exposure has any affect on the timing of egg-lay onset, the time at which individual worms from the four treatments began to lay eggs was observed and recorded with the monitoring starting 68 hours after development. Individual plates were checked hourly for the presence of eggs on a dissecting microscope using dark field illumination at 25X.

2.2.3 Brood Size

During the four-five day period when adult worms are actively laying eggs, the ethanol plates on which individual worms were housed were stored in a refrigerator at 4°C following daily transfer. At 4°C will not hatch, therefore I was able to retrieve them from storage at anytime to count the number of eggs laid by individual worms. The number of eggs laid daily by each individual worm was counted on a dissecting microscope using dark field illumination at 25X. The number of eggs laid daily by each worm, in each treatment was tallied in order to determine the size of the entire brood of each individual worm laid over its lifespan.

2.2.4 Longevity

The effect of chronic ethanol exposure on *C. elegans* longevity was determined by observing and recording the day that individual worms from each of the four treatment groups died. This data allowed for the identification of the effect chronic exposure to different concentrations of ethanol had on *C. elegans* lifespan.

2.2.5 Pharyngeal Pumping

To determine whether there was a change in food intake due to exposure to ethanol I measured the rate of pharyngeal pumping in individual worms. Worms exposed to each of the treatment concentrations were recorded at 315X magnification using a Lieca MZ16F dissecting microscope. Worms were recording using Apple I-MOVIE software. The number of pharyngeal contractions made during the 20-30 second duration of time each worm was recorded was counted providing an accurate measure of the number of pharyngeal pumps made per second, per worm.

2.2.6 Effects of Early Exposure to Ethanol During In-Utero Embryonic Development.

The F1 generation of *C. elegans* that were born by worms chronically exposed to ethanol in the previous experiment was used in this phase of the study. I harvested developmentally synchronized colonies of eggs from chronically exposed worms using the same protocol described in Section 2.1 of General Methods in order to determine the effects of early in-utero embryonic exposure to ethanol on *C. elegans.* To do this, I followed the F1 generation of worms chronically exposed to ethanol in a longitudinal study and measured the same variables described in section 3.2.1 of Methods.

2.2.7. Determining Egg permeability

To determine whether or not embryonic development inside the egg would be affected by early exposure to ethanol it was necessary to determine whether or not the three-layered *C. elegans* eggshell is permeable to ethanol. To do so, 4 day old N2 worms were allowed to lay eggs for 24 hours. After 24 hours, the 4 day old worms were removed leaving a plate containing a mixture of L1 worms and unhatched eggs all at various stages of embryonic development. Eggs and young larval worms were washed

from plates by vigorously rinsing plates with 1ml of double distilled H_2O (ddH_2O) with a P1000 micropipette and centrifuged at 1500 RPM. Pellets containing a mixture of worms and eggs was then re-suspended in liquid concentrations of ethanol ranging from of 0%, 10%, 20%, 30%, 40%, 50% and 100% v/v for 1 hour. Following ethanol exposure, worms and eggs were once again centrifuged and washed twice with ddH_2O. Following re-suspension in ddH_2O, worms and eggs from each treatment group were pipetted on to fresh plates seeded with E. coli and examined to determine the lethality of ethanol exposure. Immediately following exposure the number of surviving L1s and number of eggs on each plate were counted for each treatment. 24 hours following ethanol exposure, the number of hatched eggs for each treatment group was counted. This protocol was repeated until a treatment producing 100% L1 lethality and 50% egg hatching was found.

2.2.8 Imaging Larvae Exposed to Ethanol During Ex-Utero Development.

Nomarski images of worms that were exposed to ethanol during ex-utero embryonic development were capture using a ZEISS HAL 100 inverted microscope connected to an IMAGINING Digicam camera. Worms were magnified 40X. All captured images were processed using Northern eclipse imaging software on an HP PC computer.

2.3 RESULTS

To characterize the effects of ethanol exposure on *C. elegans* I exposed worms to ethanol exogenously allowing them to take up ethanol from their environment as described by Davies et al., 2004. In these experiments I used three different doses of

ethanol, 0.1M, 0.2M and 0.4M as well as an ethanol free control group. Because of the resilient nature of the exterior cuticle that surrounds the worm, it was necessary to use these high concentrations of ethanol. Davies et al. (2004) found that exposing worms to the 0.4M environmental concentration of ethanol was sufficient to produce an internal tissue concentration of alcohol equivalent to 0.1% BAC in humans. In my experiments I tested the effects of the 0.4M ethanol concentration, half that concentration, 0.2M, and a quarter of that concentration, 0.1M. In early pilot studies, I also tested a 0.6M concentration but found that this concentration was too high and chronic exposure proved toxic to most worms rendering it useless for study.

2.3.1 EXPERIMENT SET # 1. The Effects of Chronic Ethanol Exposure on the Physical Development and Life History of *C. elegans*.

In the first sets of experiments, I examined the effects of chronic ethanol exposure on different aspects of physical development over the course of the lifespan of worms. To do this, developmentally synchronized populations of worms were exposed to the three ethanol treatments, 0.1M, 0.2M, 0.4M and a control treatment, 0.0M, from the time they were laid as eggs until the time they died (up to 21 days). During this period various measurements and observations were made. In order to control for the volatile nature of ethanol, worms were transferred to fresh ethanol environments daily over the course of this longitudinal study to ensure that they were evenly exposed to a constant concentration of ethanol. To measure the effects of chronic ethanol exposure on development, I monitored a number of variables across the lifespan of worms including, body length, the time when worms began to lay eggs, total brood size, longevity and rate of food intake.

2.3.1.1 The Effect of Chronic Ethanol Exposure on *C. elegans* Body Length

In this experiment, I observed that chronic exposure to ethanol resulted in differences in *C. elegans* body length over the course of the first 15 days of their lives. The reason for monitoring worm body length only until day 15 was that after day 15, there were too few worms remaining in any of the groups to make measuring their body lengths statistically meaningful. In this longitudinal study I found that chronic exposure to the 0.1M (n=15), 0.2M (n=15), 0.4M (n=15) and control treatments, 0.0M (n=15), produced a marked effect on the body length of *C. elegans* (Figure 2.3.1.1A). In order to more closely analyze the effect of chronic ethanol exposure on *C. elegans* body length, I compared the body lengths of worms in all groups at three time points in their lives. Worm body length was compared on day 2 to measure how chronic ethanol exposure affected worms during early larval development. Worm body length was also compared on day 4 of their lives see how chronic ethanol exposure affected worms by the time they reached full adulthood. And lastly, worm body length was compared on day 15 in order to gauge the effect of chronic ethanol at later stages of life (Figure 2.3.1.1B). In this set of experiments a repeated measures ANOVA showed significant main effects of dose ($F(3,14)=24.698$, $p<0.001$) and day ($F(3,2)= 107.997$, $p < 0.001$) on *C. elegans* body length. A significant main effect on *C. elegans* was also observed due to interaction between dose and day ($F(3,6)=6.07$, $p<0.002$; Figure 2.3.1.1B). A planned comparison using Fisher's Planned Comparison (FPLSD) between all groups on day 2 showed that the average length of worms in the 0.2M group was significantly smaller than that of worms in both the 0.0M ($p<0.05$) and 0.1M ($p<0.04$) groups. FPLSD comparisons between the 0.4M group and all other groups showed that the average body length of

worms in the 0.4M group was significantly smaller than that of worms in any of the 0.0M, 0.1M and 0.2M groups ($p<0.001$ for each comparison).

Worms are reproductively mature adults on day 4 of their lives under normal conditions. On this day, FPLSD comparisons between the 0.4M group and all other groups showed that the average body length of worms in the 0.4M group was significantly smaller than that of worms in any of the other treatment ($p<0.001$ for each comparison). FPLSD comparisons further showed that the average length of worms in the 0.2M control was significantly smaller that that of the 0.0M and 0.1M groups ($p<0.001$ for each comparison).

On day 15, after worms had begun to die under all conditions including the control condition, a different effect of chronic ethanol exposure on *C. elegans* body length was observed than what was seen on day 2 and day 4. FPLSD comparisons between the 0.0M, 0.1M and 0.2M groups on day 15 showed that the average body length of worms in the 0.0M group was significantly smaller than that of worms in the 0.1M and 0.2M group ($p < 0.025$ for each comparison). FPLSD comparison showed that the average body length of worms in the 0.4M treatment group was still significantly smaller than that of worms from all other groups on day 15 as they were on day 2 and day 4 ($p<0.001$) for each comparison.

To explain the difference between the observed affect of exposure to low doses (0.1M and 0.2M) ethanol on day 4 and day 15, I compared the length of worms on the day before they died to the day on which they died. Correlation analysis revealed that in the 0.0M treatment group there was no significant correlation between the day on which worms died and their body length on the day before death (Correlation= -.562, p =

0.1197). Correlation analysis between the day of death and body length of worms on the day before death in the 0.1M group revealed a significant correlation (Correlation= 0.331, p = 0.0499) as well as in the 0.2M group (Correlation = 0.562, p = 0.0277). In the 0.4M group, there was no significant correlation between the day of death and body length on the day before death (Correlation = 0.257, p = 0.4863). This finding suggest that chronic exposure to low doses of ethanol does not worms to grow larger over the course of their lives, but rather selectively kills smaller worms earlier in their lives.

From the observations recorded in this set of experiments, it is clear that chronic exposure to ethanol produced a significant dose dependent effect on the size of worms. It is interesting to note that while the 0.2M ethanol treatment resulted in *C. elegans* being significantly smaller than control worms early in life (day 2 and day 4), chronic exposure to low doses of ethanol, the 0.1M and 0.2M treatments, resulted in worms being significantly larger than control worms in later life (day 15).

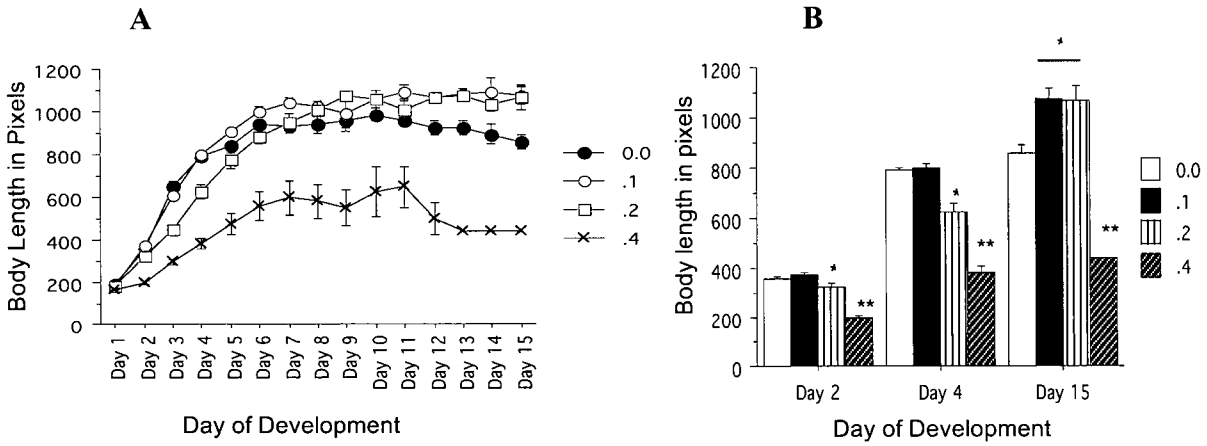


Figure 2.3.1.1. Chronic Exposure to the 0.1M, 0.2M and 0.4M Ethanol Treatments Significantly Affected the Body Length of *C. elegans* Over the Course of Their Lives (A) Chronic exposure to 0.4M ethanol results in worms growing smaller than worms in any other treatment group at any point in their lifespan. (B) Chronic exposure to the 0.2M ethanol treatment resulted in worms being significantly smaller than control 0.0M worms on day 2 and 4 but significantly larger on day 15. Chronic exposure to the 0.1M treatment resulted in worms being significantly larger than control 0.0M worms only on day 15. Chronic exposure to the 0.4M ethanol treatment resulted worms being significantly smaller than worms in any other treatment group on day 2, 4 and 15. (* and ** Indicates significance $p<0.05$).

2.3.1.2 The Effect of Chronic Ethanol Exposure on the Onset of *C. elegans* Egg-laying and Reproductive Fecundity.

The observation that worms chronically exposed to ethanol were smaller than control worms on day 4 of development suggested that chronic exposure to ethanol during development either imposed a developmental delay resulting in smaller worms at day 4, or that the ethanol exposure simply caused worms to grow smaller while continuing to mature normally. In order to address this issue, reproductive maturity, as indicated by the onset of egg-laying, was used to measure the magnitude of the developmental delay, if any, imposed by chronic ethanol exposure in this same population of worms.

Normally, worms raised at 20°C in a food rich environment will begin to lay eggs at approximately 68-70 hours after hatching (Wood, 1988). In this experiment it was found that chronic exposure to ethanol during early larval development imposed a dose dependent delay in the onset of egg laying in *C. elegans* (Figure 2.3.1.2A). A one-way ANOVA showed that chronic ethanol exposure resulted in a significant main effect of doese on the time of egg lay onset ($F(3,29)=54.921$, $p < 0.001$; Figure 2.3.1.2A). FPLSD comparisons between all groups showed that the average hour of egg-lay onset was significantly delayed by both the 0.2M (n=15) and 0.4M (n=15) ethanol treatments in comparison to the 0.1M (n=15) and 0.0M (n=15) groups ($p< 0.001$).

As well as characterizing the effects of chronic ethanol exposure on the timing of *C.elegans* egg laying, I also tested how chronic exposure to ethanol affected the probability that worms would lay eggs and I also measured the average brood size of those worms that did lay eggs from the same population of worms. In this set of experiments it was observed that chronic exposure to ethanol reduces the probability that worms would lay eggs. A one-way ANOVA showed that chronic exposure to ethanol had a significant effect on the probability worms will lay eggs ($F(3,46)=5.562$, $p < 0.025$). FPLSD comparisons showed that in a dose dependent manner, significantly fewer worms in the 0.2M (n=15) and 0.4M (n=15) treated groups initiated egg laying in comparison to worms in the 0.1M (n=15) and 0.0M (n=15) groups ($p<0.025$) for each comparison. 100% of worms in the untreated control group laid eggs while only 80% +/- 10% of worms in the 0.1M treatment group, 55% +/- 10% of worms in the 0.2M treatment group and 35% +/- 15% ever laid eggs.

Furthermore, I counted the number of eggs laid by each individual from each group that did initiate egg laying at some point in their lives and calculated the average total brood size for each of the 0.0M (n=15), 0.1M (n=12), 0.2M (n=7) and 0.4M (n=3) groups. Chronic exposure to ethanol resulted in a significant main effect of dose on the average total brood size ($F(3,37)=32.934$, $p < 0.001$; Figure 2.3.1.2B). FPLSD comparisons between all groups showed that worms in all treatment groups laid significantly fewer eggs on average than worms in the 0.0M control group ($p<0.001$) for each comparison. Worms in the both the 0.2M group and 0.4M group laid significantly fewer eggs than worms in the 0.1M group ($p<0.025$) for each comparison and worms in the 0.4M group laid significantly fewer eggs than worms in the 0.2M group ($p<0.05$).

The results presented in this set of experiments indicated that chronic ethanol exposure significantly delays the onset of egg laying (Figure 2.3.1.2A) suggesting that the reduction in size due to chronic ethanol exposure observed in Experiment 2.3.1.1 was in part due to ethanol imposing a developmental delay on *C. elegans* resulting in worms being smaller. Furthermore, when this result is coupled with the observation that chronic ethanol exposure reduces the probability that *C. elegans* will lay eggs as well as the total number of eggs laid by worms that do lay eggs, these results demonstrate that the reproductive fecundity of *C. elegans* was negatively affected by chronic exposure to ethanol.

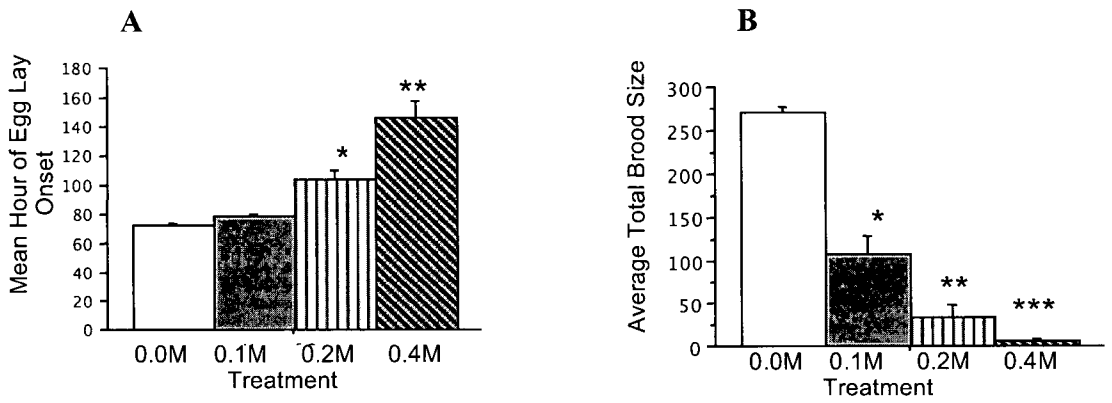


Figure 2.3.1.2 Chronic Exposure to Ethanol Produced a Significant Delay in the Onset of *C. elegans* Egg Laying.
(A) In a dose dependent manner, it took worms chronically exposed to the 0.2M ethanol treatment and 0.4M ethanol treatment longer to begin laying eggs than it did for worms in the untreated control group, 0.0M, and the 0.1M treated group (A). This delay is indicative of the overall developmental delay that occurs in *C. elegans* consequent of chronic exposure to ethanol. Chronic exposure also significantly reduced the number of eggs laid by *C. elegans* over the course of its life (B). In a dose dependent manner, worms chronically exposed to the 0.4M ethanol treatment laid fewer eggs than worms in the 0.2M group, which lay fewer eggs than worms in the 0.1M group, which lay fewer eggs than worms in the 0.0M untreated control group (B). (*, ** and *** Indicates significance $p<0.05$).

2.3.1.3 Effect of Chronic Ethanol Exposure on *C. elegans* Longevity

Chronic exposure to ethanol resulted in a marked effect on the life expectancy of *C. elegans* (Figure 2.3.1.3A). Within the same population of worms used in previous experiments, it was found that worms in the 0.4M treatment group began to die on day 4, while worms in the other two ethanol treatment groups, 0.1M and 0.2M, began to die on day 5 and 6. Worms in the control group, 0.0M did not begin to die until day 10. Although worms began to die at different point in time, all worms from all groups died before day 21. Specifically, a one-way ANOVA showed that chronic exposure to ethanol resulted in a significant effect of dose on the longevity of *C. elegans* ($F(3,46)=3.902$, $p <$

0.025). FPLSD comparisons revealed that worms in the 0.4M (n=15) treatment group lived significantly shorter lives than worms in both the control 0.0M (n=15; $p<0.001$) and 0.1M (n=15; $p<0.005$) treatment groups.

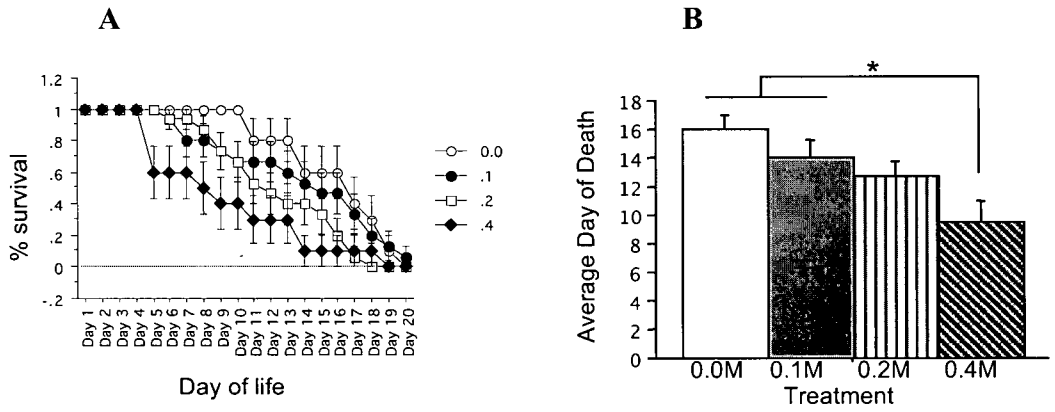


Figure 2.3.1.3 Chronic Ethanol Exposure Decreased *C. elegans* Lifespan.
(A) Chronic ethanol exposure decreased *C. elegans* longevity. Chronic exposure to ethanol results in a significant main effect on the longevity of *C. elegans* (B). Worms chronically exposed to the 0.4M ethanol treatment had a significantly lower life expectancy than worms exposed to the 0.1M and 0.0M treatments. (* indicates significance $p<0.05$)

2.3.1.4 The Effect of Chronic Ethanol Exposure on *C. elegans* Pharyngeal Pumping.

One possible reason that ethanol treated worms were smaller than control worms (Figure 2.3.1.1), is that chronic ethanol exposure may have affected *C. elegans* nutritional intake. *C. elegans* consumes food, in this case *E. coli*, by taking it in through its mouth and passing it through it pharynx where the *E. coli* is ground up mechanically by the grinder (Wood, 1988). The grinder quickly and repeatedly contracts in order to mechanically digest food before passing it further along the alimentary canal. It is possible that differences in the size of chronically exposed worms reported in Figure

2.3.1.1 might be the result of changes in food intake due to chronic exposure to ethanol. In order to determine the impact chronic ethanol exposure had on *C. elegans* nutrient intake, I measured the rate of pharyngeal pumping. If a worm is making fewer pharyngeal contractions within a given period of time it is consuming less food. I counted the number of pharyngeal contractions made by worms chronically treated with 0.0M, 0.1M, 0.2M and 0.4M ethanol. Because chronic ethanol exposure has been shown to reduce the size of worms (Figure 2.3.1.1) and delay their development (Figure 2.3.1.2), it was necessary to compare the effect of ethanol exposure on pharyngeal pumping to that of untreated size matched controls. Therefore, I also measured the number of pharyngeal contractions made by 2 day-old untreated worms. 2-day old untreated worms were found to be approximately the same size as 4 day-old 0.4M treated worms based on interpretations made from Figure 2.3.1.1 A and therefore were deemed an appropriate size matched control group.

In this experiment I used a new population of worms, not the same ones used in the previous longitudinal study. I recorded worms for a 20-30 second period of time and magnified worms by 315X while maintaining the worms in a constant field of view. The number of pharyngeal contractions made by each worm was divided by the length of time that each worm was recorded in order to calculate the number of pharyngeal contractions made per second. The number of pharyngeal contractions made per second was significantly reduced by chronic ethanol exposure in a dose dependent manner $F(3,52)=22.698$, $p < 0.001$ (Figure 2.3.1.4). FPLSD comparisons showed that 4 day-old adult worms in the 0.2M (n=13) treatment group made significantly fewer pharyngeal contractions per second than worms in the 4 day-old 0.0M (n=13; $p<0.001$) and 0.1M

(n=14; p<0.001) groups (Figure 2.3.1.4). Worms in the 0.4M (n=14) treatment group were found to make fewer contractions per second than any of the other treatment groups and the control group, $p < 0.001$ (Figure 2.3.1.4). Also, the number of pharyngeal contractions made per second by 2 day-old 0.0M (n=15) control worms was not significantly different from that of 4 day-old 0.0M controls, $p > 0.05$. This observation coupled with the finding that chronic exposure to either 0.2M or 0.4M ethanol is sufficient to reduce the number of pharyngeal contractions made per second suggests that ethanol is having a direct effect on the rate of pharyngeal pumping and the decrement in the rate of pharyngeal pumping observed in the 0.2M and 0.4M groups was no due to a secondary effect caused by the developmental delay imposed by ethanol. This observation supports the conclusion that chronic exposure to higher doses of ethanol, 0.2M and 0.4M, was sufficient to reduce the number of pharyngeal contractions made by *C. elegans.*

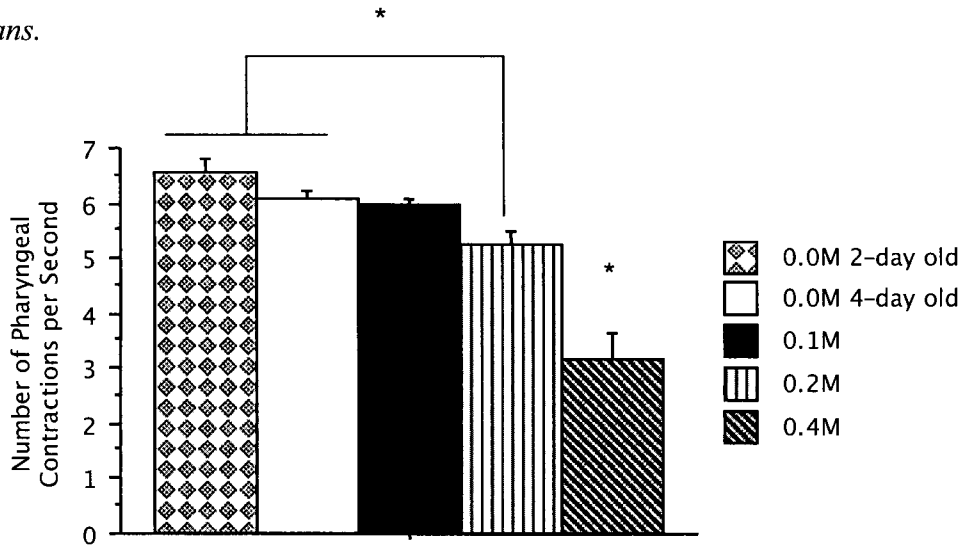


Figure 2.3.1.4 Chronic Ethanol Exposure Decreased *C. elegans* Pharyngeal Pumping.
Chronic exposure to ethanol results in a significant main effect on the number of pharyngeal contractions made pre second by *C. elegans.* Worms chronically exposed to 0.2M and 0.4M make significantly fewer pharyngeal contractions per second in comparison to worms treated with either 0.0M or 0.1M ethanol. (* and ** indicates significance, p<0.05).

2.3.1.5 Summary of Results

The results obtained from the series of experiments investigating the effects of chronic ethanol exposure on *C. elegans* development and life history have provided a comprehensive report on the ways basic worm physical development is affected. When worms are chronically exposed to ethanol there are clear functional and developmental consequences. In general, the findings reported here demonstrated that chronic ethanol exposure produced a significant developmental delay and negatively impacts reproductive fecundity and longevity in *C. elegans*.

2.3.2 EXPERIMENT SET #2. The Effects of Chronic Ethanol Exposure Beginning at the Onset of Young Adulthood on the Life History of *C. elegans*.

After completion of the experiments outlined in EXPERIMENT SET #1, it remained unclear whether the results of chronic ethanol exposure on *C. elegans* size, development, reproductive fecundity and life history were due to a specific effect of ethanol on each of these independent processes, or if they were due to a general effect ethanol exposure during early larval development may have had. To address this issue, I replicated the experiments outlined in EXPERIMENT SET #1 using a second consistent population of worms in a longitudinal study. This time I exposed worms to ethanol once they reached hour 60 of development instead of from the time they were laid as eggs. Under normal conditions, hour 60 of development marks the onset of Young Adulthood (YAD) in *C. elegans* (Wood, 1988). At this point, worms have completed all four stages of larval development and will soon begin reproducing. By allowing worms to develop normally and then exposing them to ethanol at this point in their lives, I attempted to

dissociate the effects of chronic ethanol exposure beginning at YAD from the effects of chronic ethanol exposure over the course of the entire lifespan of *C. elegans.*

2.3.2.1 The Effects of Chronic Ethanol Exposure During Adulthood on *C. elegans* Body Length.

In this experiment, I exposed developmentally synchronized populations of worms to 0.0M (n=15), 0.1M (n=15), 0.2M (n=15) and 0.4M (n=15) ethanol treatments once they had reached YAD (hour 60). I recorded the body length of all worms in all treatment groups for the first 10 days of their lives. The reason for ending the observational study at day 10 was that by this point too many worms in my 0.4M treatment group had died, leaving too few subjects for further study.

In this experiment, chronic exposure to ethanol beginning at YAD was found to produce a marked effect on *C. elegans* body length (Figure 2.3.2.1A). To more closely analyze the effect of chronic ethanol exposure on *C. elegans* body length, I compared the lengths of worms at three different time points in the *C. elegans* life cycle. In the same manner as in Experiment 2.3.1.1, I compared the length of worms treated in with all four conditions on day 2, day 4 and day 9 of their lives. The rationale for doing so was to provide a means to gauge the effects of ethanol exposure on *C. elegans* at three important points in its life history, development (day 2), adulthood (day 4) and old age (day 9).

A repeated measures ANOVA showed that chronic exposure to ethanol beginning at YAD resulted in significant main effects of dose $F(3,10)=14.07$, $p<0.006$ and day $F(3,2)= 80.833$, $p<0.001$ on the size of *C. elegans*. A repeated measures ANOVA further showed that there was a significant main effect on the size of worms due to an interaction between the day on which they were measured and dose of chronic ethanol exposure $F(3,6)=4.082$, $p<0.008$. Upon comparing the lengths of worms on day

2 of development, before any of the treatment groups had been exposed to ethanol, FPLSD comparisons between all groups showed that the average length of worms in all groups was not significantly different ($p>0.5$; n=15 for all groups) (Figure 2.3.2.1B). However, on day four, ~ 1.5 days after worms had first been exposed to ethanol, FPLSD comparisons between all groups showed that worms in the 0.0M (n=15) and 0.1M (n=14) treatment groups were significantly larger than worms in both the 0.2M (n=15) ethanol ($p<0.05$) and 0.4M (n=15) ethanol ($p<0.001$) treatment groups. Furthermore, FPLSD comparisons showed that worms in the 0.4M ethanol treatment group were significantly smaller than worms in the 0.2M ethanol treatment group ($p< 0.001$). On day 9, when the body lengths of aged worms in each of the treatment groups was compared, FPLSD comparisons between the 0.4M treatment group and all other groups showed that the average body length of worms in the 0.4M (n=2) ethanol treatment group was significantly lower than that of worms in any of the other three, 0.0M (n=6), 0.1M (n=4) and 0.2M (n=3), treatment groups ($p<0.025$). On day 9 it was interesting to observe that the average body length of worms in the 0.1M (n=4) and 0.2M (n=3) treatment groups was larger than that of worms in the control 0.0M (n=6) groups. Although there was not a significant difference between the 0.0M and 0.1M and 0.2M groups in this instance, it is interesting to note that the same trend of worms treated with lower concentrations of ethanol, 0.1M and 0.2M, results in worms being larger than control, 0.0M, worms later in life as was observed in Figure 2.3.1.1B.

The results from this experiment demonstrate that chronic exposure to ethanol beginning at YAD results in a significant impact on the body length of *C. elegans*. Specifically, it is clear that chronic exposure to 0.4M ethanol beginning at the onset of

young adulthood is sufficient to cause worms to grow significantly smaller than untreated control worms.

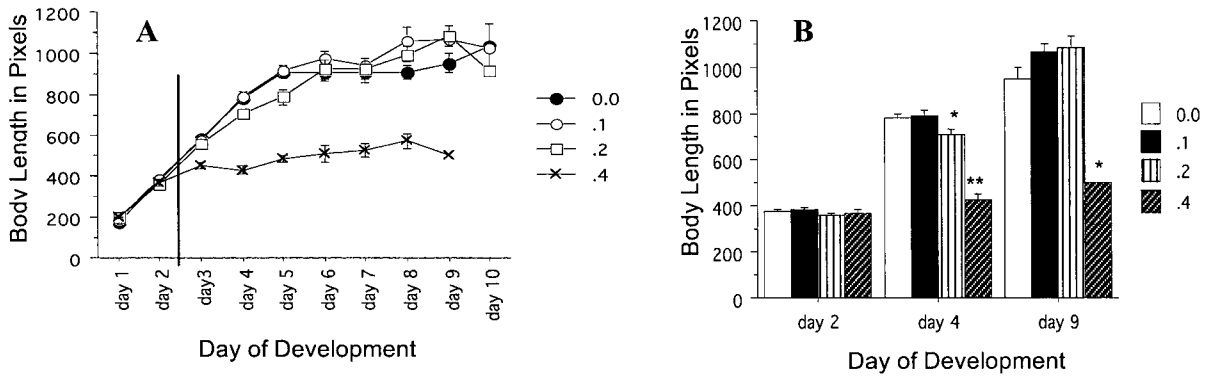


Figure 2.3.2.1 Chronic Exposure to Ethanol During Adulthood Affected *C. elegans* Body Length.
(A) Chronic exposure to the 0.1M, 0.2M and 0.4M ethanol treatments during adulthood affected the body length of *C. elegans* over the course of their lives. The line positioned between day 2 and day 3 indicates the time at which worms in the treatment groups were first exposed to ethanol. (B) Chronic exposure to the 0.2M ethanol treatment resulted in worms being significantly smaller than control 0.0M worms on day 4 but not on day 9 (B) Chronic exposure to 0.4M ethanol results in worms being significantly smaller than all other treated groups on day four and day 9 (* and ** Indicates significance $p<0.05$).

2.3.2.2 The Effects of Chronic Ethanol Exposure Beginning at Young Adulthood on the Onset of *C. elegans* Egg-laying and Reproductive Fecundity.

In this experiment, a one-way ANOVA showed that chronic exposure to ethanol beginning at the onset of YAD and reproductive development, hour 60, was sufficient to result in a main effect of dose on the hour of egg lay onset $F(3,51)=6.597$, $p<0.008$ (Figure 2.3.2.2A). FPLSD comparisons between the 0.4M (n=13) treatment group and all other groups showed that worms in the 0.4M treatment group took significantly longer to begin to lay eggs than worms in the 0.0M (n=15) group ($p<0.003$), the 0.1M group ($p<0.004$) and the 0.2M (n=14) group ($p<0.025$).

In addition to measuring the effect of chronic ethanol from the beginning of YAD onset on the timing of egg lay onset, I also measured how this exposure paradigm affected *C. elegan's* overall reproductive fecundity. To do so, I recorded the affects of exposure to 0.0M, 0.1M, 0.2M and 0.4M beginning at hour on the probability that *C. elegans* would begin to lay eggs and on the total number of eggs laid by each worm in each treatment.

I found that exposure to ethanol beginning at the onset of YAD did not affect the probability that a given worm would lay eggs (Figure 3.3.2.3A). A one-way ANOVA showed no significant difference between any of the four treatment groups, (0.0M 100% +/- 0%, n=15; 0.1M 93.3%, +/- 6.7%, n=15; 0.2M 86.7%, +/- 9.1%, n=14 and 0.4M 86.7% +/- 9.1%, n=13; $F(3,56)=.778$, $p>0.5$).

However, a count of the number of eggs laid by each worm in each treatment group that did lay eggs showed that the number of eggs laid by a given worm was affected by this exposure paradigm (Figure 3.3.2.3B). A one-way ANOVA showed a significant main effect of treatment on the total brood size of exposed *C. elegans* $F(3,72)=27.433$, $p<0.001$. FPLSD comparisons between the 0.4M treatment group (n=13) and all other treatment groups 0.0M (n=15), 0.1M (n=14) and 0.2M (n=13) showed that worms in the 0.4M treatment group laid a significantly smaller brood than all other groups ($p<0.001$) as a result of this exposure paradigm.

These results indicate that chronic exposure to the highest concentration of ethanol, 0.4M, beginning at hour 60 is sufficient to delay the onset of egg laying in *C. elegans*. Furthermore, it was observed that although chronic exposure to ethanol beginning at the onset of YAD did not significantly reduce the probability that a worm

would lay eggs, it did significantly reduce the total number of eggs that worm would lay if the worm was exposed the highest, 0.4M, ethanol treatment.

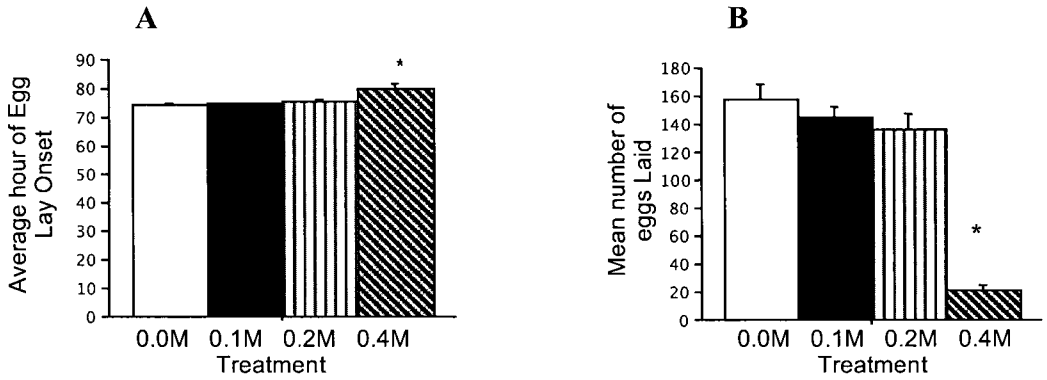


Figure 2.3.2.2 Only Chronic Exposure to the Highest (0.4M) Concentration of Ethanol Beginning at Hour 60 of Development Affected the Timing of *C. elegans* Egg-Lay Onset.
(A) Chronic exposure to 0.4M ethanol beginning at hour 60 of development resulted in exposed worms taking significantly longer to begin laying eggs in comparison to worms treated with any of the 0.0M, 0.1M and 0.2M ethanol treatments. (B) Worms exposed the highest concentration of ethanol tested in this experiment laid significantly fewer eggs over the course of their lives in comparison to worms in all other treatment groups. (* Indicates significance $p<0.05$).

2.3.2.3 The Effects of Chronic Ethanol Exposure Beginning at Young Adulthood on *C. elegans* Longevity.

In order to dissociate whether the affect of chronic exposure to ethanol on *C.elegans* longevity observed in Experiment 2.3.1.3 was due to the toxicity of chronic exposure or due to a lasting effect of exposure during larval development, I measured the survival rates for worms chronically exposed to ethanol beginning at the onset of YAD. I made a daily record of the number of worms that died in each treatment group, 0.0M, 0.1M, 0.2M and 0.4M, over the course of this longitudinal study. In this experiment, chronic exposure to ethanol beginning at the onset of YAD resulted in a marked effect on *C. elegans* life expectancy (Figure 2.3.2.3A). A one-way ANOVA showed a significant

main effect on the average day of death of *C. elegans* as a result of treatment ($F(3,48)=13.116$, $p<0.001$; Figure 2.3.2.3B). FPLSD comparisons between all treatment groups showed that worms in the 0.4M treatment group died significantly earlier in life than worms in the 0.0M ($p<0.001$), 0.1M ($p<0.001$) and 0.2M ($p<0.025$) treatment groups. FPLSD comparisons also showed that worms in the 0.2M group died significantly earlier in life than worms in both the 0.0M ($p<0.05$), and 0.1M ($p<0.02$) treatment groups. The results from this experiment indicated that chronic exposure to ethanol beginning at YAD resulted in a significant reduction in *C.elegans* longevity.

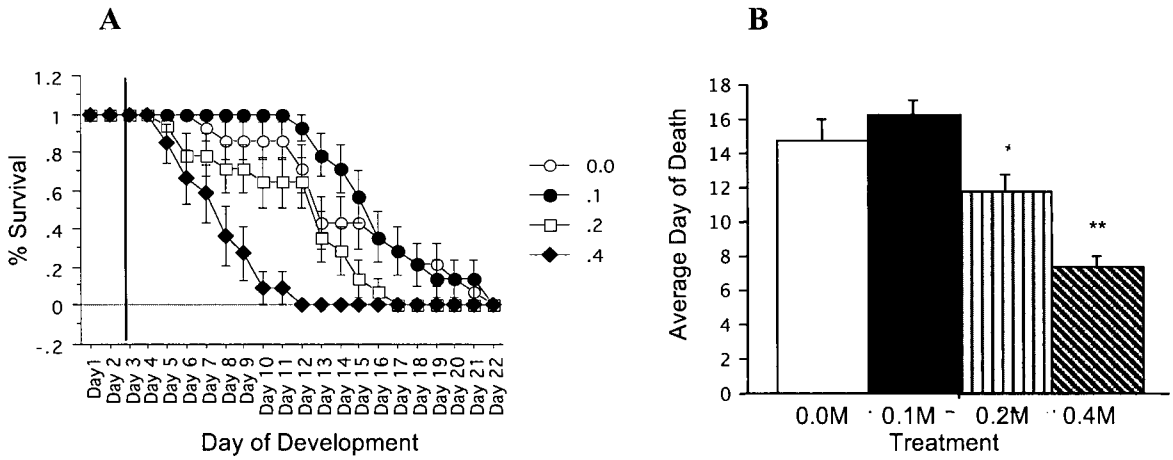


Figure 2.3.2.3 Chronic Ethanol Exposure Beginning at Young Adulthood Decreased *C. elegans* Lifespan.
(A) Chronic ethanol exposure decreased *C. elegans* longevity. The line positioned between day 2 and day 3 on the X-axis indicates the time pint when worms in the treatment groups were first exposed to ethanol. (B) Chronic exposure to ethanol results in a significant main effect on the longevity of *C. elegans*. Worms chronically exposed to the 0.4M ethanol treatment had a significantly earlier average day of death than worms in any other group. Worms exposed to the 0.2M ethanol treatment live significantly shorter lives than worms in the 0.1M and 0.2M treatment groups. (* and ** indicates significance $p<0.05$)

2.3.3 EXPERIMENT SET #3. The Effects of In-Utero Embryonic Exposure to Ethanol on the Physical Development and Life History of *C. elegans.*

After characterizing the effects of chronic ethanol exposure on adult *C. elegans* in the previous experiment sets, I investigated where there were any developmental abnormalities in the offspring born to worms chronically exposed to ethanol. After fertilization of a *C. elegans* egg occurs, in-utero embryonic development begins and the most fundamental processes pertaining to the development of a viable worm begin (Sulston, 1983).

Immediately following fertilization of an egg, the anterior posterior polarity of the embryo is established through an intricate process. After sperm entry, the maternal pronucleus resumes meiosis and pseudocleavege of the egg-membrane begins. Approximately 40 minutes after fertilization, the first cell cleavage occurs marking the beginning of founder cell establishment. The first 6 cell divisions after fertilization give rise to the asynchronous founder cells that continue to divide to give rise to the different cell and tissue types that will make up the worm. 100 minutes after the first cleavage, gastrulation begins. This process is characterized by a series of cellular migrations and further cell divisions that organize cells in such a way to allow for patterning of cell types that will go on to form the tissues and organs of the worm. Cell proliferation begins to stop ~350 minutes after the first cell division marking the beginning of morphogenesis and the formation of distinct anatomical features and tissues (Sulston et. al 1983). These processes of development are critical to the eventual viability of the *C. elegans*, therefore it was important to investigate whether in-utero exposure to ethanol during this period of time would result in any lasting effect on *C. elegans* development.

To do this experiment, four-day old worms were chronically exposed to the 0.0M, 0.1M and 0.2M from the time they hatched until they reached reproductive maturity. These chronically exposed gravid worms were subsequently transferred to 0.0M control environments and allowed to lay synchronized colonies of offspring in an ethanol free environment. Due to the developmental delay imposed by chronic exposure to the highest, 0.4M, ethanol treatment, I used five-day old worms from the chronically exposed 0.4M group to generate synchronized colonies of offspring.

Using this protocol, I generated four treatment groups- the F1 generation of worms chronically exposed to 0.0M, 0.1M, 0.2M and 0.4M ethanol. I then measured the size, timing of egg lay onset, reproductive fecundity and longevity of worms in these four treatment groups using a consistent population of worms in a longitudinal study.

2.3.3.1. The Effect of In-Utero Exposure to Ethanol on *C. elegans* Body Length

In this experiment I found that "in-utero" exposure to ethanol did not result in an observable affect on the size of *C. elegans* (Figure 2.3.3.1). In same manner as in previous experiments designed to quantify the effect of ethanol on the size of *C. elegans*, I compared the size of worms at three different points; on day 2, day 4 and day 10 of their lives. This allowed for measurement of the affect of in-utero ethanol exposure on *C. elegans* at three important points in the worm life cycle, development (day 2), adulthood (day 4) and old age (day 9) (Figure 2.3.3.1). A repeated measures ANOVA showed that in-utero exposure to 0.0M (n=15), 0.1M (n=15), 0.2M (n=15) and 0.4M (n=15) ethanol did not produce a significant main effect on the size of exposed worms $F(3,37)=1.977$, $p>0.2$, but did produce a significant main effect of day on the size of *C.* elegans

$F(3,2)=606.60$, $p<0.001$, and no significant interaction between dose and day $F(3,2)=2.408$, $p>0.05$. Because there was no obvious difference in worm body length during the first nine days of their lives as a consequence of in-utero ethanol exposure, I stopped recording worm body lengths at that time.

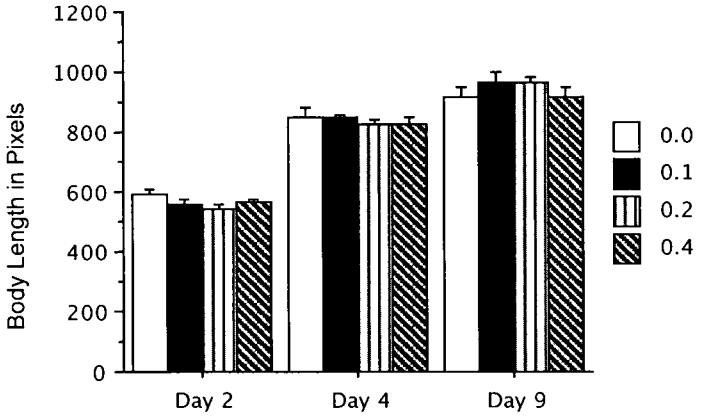


Figure 2.3.3.1 In-Utero Ethanol Exposure Had No Effect on *C.elegans* Body Length. The body lengths of the offspring of worms chronically exposed to ethanol were not affected by in-utero exposure to ethanol. On day 2, 4 and 9, the body lengths of worms exposed to 0.0M, 0.1M, 0.2M and 0.4M in-utero were not significantly different.

2.3.3.2 The Effect of In-utero Exposure to Ethanol on the Timing of Egg-Lay Onset and Reproductive Fecundity.

Worms chronically exposed to ethanol over the course of their entire lives showed both size differences (Figure 2.3.1.1) and a developmental delay (Figure 2.3.1.2A). In order to determine whether the offspring of worms chronically exposed to ethanol would be developmentally delayed as well, I measured the length of time it took the offspring of *C. elegans* chronically exposed to 0.1M (n=15), 0.2M (n=15) and 0.4M (n=15) ethanol to begin to lay eggs. Worms exposed to 0.1M, 0.2M and 0.4M ethanol in-utero did not take any longer to begin to lay eggs than untreated controls once they reached the age of

reproductive maturity (Figure 2.3.3.2A). A one-way ANOVA showed that there was no significant effect of in-utero ethanol exposure on the hour of egg-lay onset $F(3,56)=1.569$, $p > 0.2$.

This data, when coupled with the observation that offspring of worms chronically exposed to ethanol are no different from control worms in their average body length (Figure 2.3.3.1), supported the conclusion that exposure to the tested concentrations of ethanol during early in-utero embryonic development was not sufficient to produce any gross affect on the normal rate of larval development.

In addition to measuring the time it took the offspring of worms chronically exposed to ethanol to reach reproductive maturity, I also measured their total brood size of worms exposed to ethanol in-utero in order to determine if exposure to ethanol during this phase of early embryonic development resulted in lasting effects on reproductive fecundity. In this experiment, the offspring of worms chronically exposed to any of the 0.1M, 0.2M and 0.4M ethanol treatments were not significantly different from those worms in the untreated group in terms of the total number of eggs they laid over the course of their lives ($F(3,53)= 1.153$, $p > 0.3$; Figure 2.3.3.2B)

From this observation it can be concluded that exposure to the tested concentrations of ethanol during early in-utero embryonic development is not sufficient to produce any lasting gross effects on the number of eggs laid by worms that were exposed to ethanol during in-utero embryonic development.

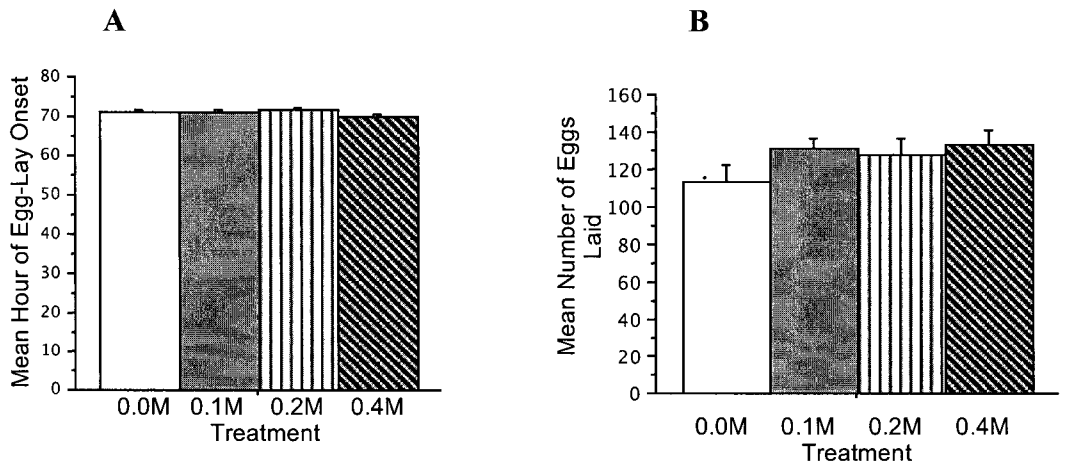


Figure 2.3.3.2 In-Utero Ethanol Exposure Had No Affect on *C. elegans* Brood Size and Reproductive Fecundity.
(A) Offspirng of worms that were chronically exposed to ethnaol did not differ from untreated controls in terms of the hour at which they began to lay eggs once they reached reproductive maturity. (B) Exposure to ethanol during in-utero embryonic development did not result in any significant effect on the number of eggs laid by worms once they reach adulthood.

2.3.3.3 The Effects of In-Utero Ethanol Exposure on *C. elegans* Longevity.

I also investigated any potential effects exposure to ethanol during embryonic may have had on *C. elegans* longevity. A one-way ANOVA showed that the average day on which the offspring of worms chronically exposed to 0.1M (n=15), 0.2M (n=15) and 0.4M (n=15) ethanol died was not significantly different from that of the offspring of untreated, 0.0M (n=15) control worms ($F(3, 51)= 0.561$, $p > 0.6$; Figure 3.3.2.4) This observation suggests that in-utero embryonic exposure to the tested concentrations of ethanol did not have any effect on *C. elegans* life expectancy.

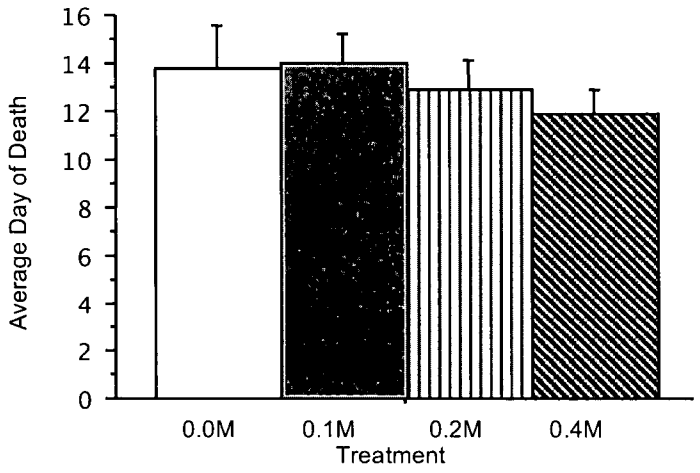


Figure 2.3.3.3 In-Utero Ethanol Exposure Had No Effect on *C. elegans* Longevity. In- utero ethanol exposure did not affect *C. elegans* longevity. The offspring of worms chronically exposed to 0.1M, 0.2M and 0.4M ethanol treatments were not significantly different from untreated controls in terms of their life expectancy.

2.3.3.4 Summary of Results

In this set of experiments, it was my objective to identify any consequences that exposure to ethanol during early in-utero embryonic development might have on *C. elegans* in later life. When taken together, the findings from this set of experiments indicate that exposure to 0.1M, 0.2M and 0.4M ethanol during the first few hours of in-utero embryonic development did not result in any gross consequences on the physical development of *C. elegans*.

2.3.4. EXPERIMENT SET #4. The Effects of Exposure to Ethanol During Ex-Utero Embryogenesis.

One possible explanation for the lack of effect of ethanol exposure on the offspring of chronically exposed worms is that approximately 20 minutes after a *C.*

elegans egg is fertilized, a tri-layered eggshell consisting of an inner vitelline membrane, a tough chitinous middle layer and an exterior layer thought to be comprised of cross-linked collagenous proteins (Chitwood and Chitwood, 1974). It is possible that this durable eggshell might have prevented ethanol from permeating the egg in EXPERIMENT SET #3 and thus protected developing embryos from the teratogenic insult of ethanol exposure.

2.3.4.1. Determining *C. elegans* Eggshell Permeability.

To further investigate the effects of ethanol on embryonic development and address this hypothesis, it was necessary to find out if there was a concentration of ethanol that was high enough to permeate the tri-layered eggshell. To do so, wild-type N2 worms were allowed to lay eggs for 24 hours resulting in a mixed population of eggs, L1 and early L2 larva being generated. This mixed population was collected and washed with various concentrations of ethanol (10%, 20%, 30% and 40%) for one hour. Control larvae were exposed to 0% ethanol (ddH_20) and for one hour as well. In this experiment I found that 1 hour exposure to 10%, 20%, 30% and 40% ethanol solutions resulted in a significant main effect on survival rates for L1 and L2 larva, ($F(4,10)=637.691$, $p< 0.001$; Figure 2.3.4.1A). Specifically, it was found that 1 hour exposure to 0% and 10% ethanol solution resulted in 0% L1 and L2 lethality, 1 hour exposure to 20% resulted in ~90% L1 and L2 lethality and 1 hour exposure to 30% and 40% resulted in 100% L1 and L2 lethality (Figure 2.3.4.1A). Planned comparison revealed larva exposed to the 0% and 10% ethanol solution have a significantly higher survival rate than those larva exposed to any of the higher concentrations, $p < 0.001$. FPLSD comparisons between groups further indicated that larvae exposed to the 20% ethanol solution had a significantly higher rate

of survival than larvae exposed to the 30% and 40% ethanol solutions, $p<0.001$ for each comparison. Thus, exposure to the 20%, 30% and 40% ethanol solutions were sufficient to kill larval *C. elegans* when were exposed for 1 hour.

If the eggshell is in fact permeable to ethanol it would be expected that exposure to the same concentrations of ethanol for the same duration of time that resulted in larval lethality would result in embryonic lethality as well. Instead, it was found that this was not the case. Despite the 20%, 30% and 40% ethanol solutions being sufficiently concentrated to kill ~100% of L1 and L2 larva, they were not strong enough to kill the same percentage of developing embryos inside exposed eggs.

As with the rate of survival following larval exposure to the 0%, 10%, 20%, 30% and 40% ethanol solutions, a one-way ANOVA showed a significant effect on the hatching probability for eggs exposed to the 0%, 10%, 20%, 30% and 40% ethanol for one hour ($F(4,10)=129.163$, $p< 0.001$; Figure 2.3.4.1B). Specifically, 100% of eggs exposed to 0% and 10% ethanol solution for 1 hour had hatched and developed into viable larva 24 hours after exposure (FIG 2.3.4.1B). Interestingly, ~50% of eggs exposed to the 20% solution, ~10% of eggs exposed to the 30% solution and ~5% of eggs exposed to the 40% solution for 1 hour hatched and developed into viable larva 24 hours after exposure where exposure to these same concentrations resulted in ~100% larval lethality (Figure 2.3.4.1A). FPLSD comparisons revealed that the probability of hatching is significantly greater for eggs exposed to the 0% and 10% ethanol solutions compared to eggs exposed to all other doses, $p < 0.001$ for each comparisons. It was also found that eggs exposed to the 20% dose had a significantly greater probability of hatching compared to eggs exposed to the 30%, $p<0.025$ and 40%, $p<0.001$ ethanol solutions.

Although this ethanol exposure protocol had a significant effect on the probability of an egg hatching, it did not have as dramatic an effect as it did on the survival rate of exposed larvae (Figure 2.3.4.1A). The data presented by this experiment suggested that the *C. elegans* eggshell is permeable to higher concentrations of ethanol and that exposure to 20% ethanol solution for 1 hour is sufficient to permeate the tri-layered *C. elegans* eggshell to produce 50% embryonic lethality in a mixed stage population of eggs.

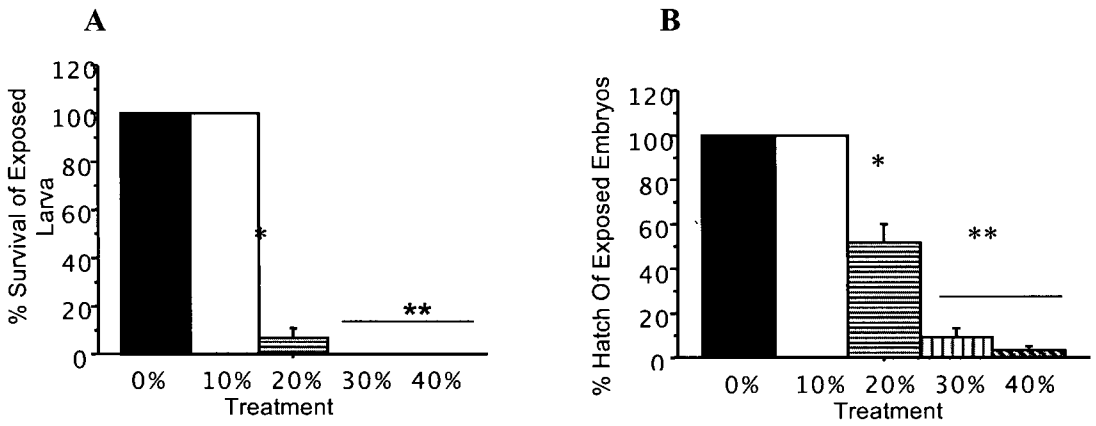


Figure 2.3.4.1 *C. elegans* Eggshells are Permeable to Ethanol.
(A) Exposing *C. elegans* larvae to high concentrations of ethanol is sufficient to produce a main effect on larval survival probability. Exposing larvae for 1 hour to 30% or 40% ethanol was sufficient to kill 100% of exposed larvae. The 10% ethanol concentration was not high enough to kill *C. elegans* larvae while ~ 10% of *C. elegans* larvae survived acute exposure to the 20% ethanol solution. (B) Acutely exposing eggs at various stages of ex-utero embryonic development to elevated concentrations of ethanol resulted in a significant main effect on hatching probability. Exposing eggs to 30% and 40% ethanol for one-hour resulted in a significantly lower probability that those eggs would hatch in comparison to eggs exposed to 20%, 10% and 0% solutions. Exposing eggs to 20% ethanol for one- hour resulted in a significantly lower probability that those eggs will hatch in comparison to eggs exposed to 10% and 0% ethanol. 100% of eggs exposed to 0% and 10% ethanol solution hatched. (* and ** indicates significance $p<0.05$).

2.3.4.2 The Effect of Acute Ethanol Exposure at Different Time Points During Ex-Utero Embryogenesis.

In the previous experiment preformed in (2.3.4.1), embryos in the mixed stage population that was exposed to ethanol were all at different developmental time points. However, it may be that the developmental stage of an embryo contributed to its hatching probability following the one-hour exposure to ethanol. To address this issue, I exposed populations of eggs, consisting entirely of embryos at known time points in ex-utero embryonic development. I exposed these populations of eggs to 20% ethanol because in the previous experiment I found that 1 hour exposure to 20% ethanol was sufficient to produce a ~50% hatching probability of exposed eggs. This concentration was comparable to an LD50 for this exposure protocol. I exposed populations of eggs that were 1hr, 2hrs, 3hrs, 4hrs, 5hrs, 6hrs, 7hrs, and 8hrs old after being laid to 20% ethanol for one-hour. I also ran a control group that consisted of a population of eggs that was exposed to 0% ethanol, ddH_20 1 hour after being laid.

The worm lays eggs approximately 3 hours after fertilization (Wood, 1988). Therefore, eggs in the 1 hr, 2 hr and 3 hr groups in this experiment were four, five and six hours past fertilization and in the stage of gastrulation. At this point, rapid cell proliferation was still occurring as well as complex cell migrations and establishment of the germ layers that would go on to differentiate into the different tissues and organs that make up *C. elegans* (Sulston et al. 1983). Eggs in the treatment groups given longer to develop (4 - 8 hrs) before exposure contained embryos that were all at different stages of morphogenesis when they were exposed to ethanol. I found that the stage of embryonic development of the egg when it was exposed to ethanol was critical to whether or not that developing embryo would survive 1 hour exposure to 20% ethanol. Eggs given less than

4 hours to develop before being exposed to 20% ethanol for 1 hour had approximately a 10-20% chance of hatching. Eggs given longer than 6 hours to develop before being exposed to 20% ethanol hatched into viable worms 100% of the time. Eggs given 5-6 hours to develop before being exposed to ethanol had approximately a 50% chance of hatching. Interestingly, eggs given 4 hours to develop had a higher probability of hatching, ~75%, than eggs given longer, 5-6 hours, to develop. A one-way ANOVA showed that exposure to ethanol resulted in a significant main effect of dose on the hatching probability of eggs at different stages of ex-utero embryonic development ($F(8,21)=8.035$, $p < 0.001$; Figure 2.3.4.2). FPLSD comparisons showed that eggs exposed to ethanol for one hour before in the first three hours after being laid had a significantly lower probability of hatching into viable worms than eggs in any of the other treatment groups, $p<0.001$ for each comparison. Interestingly, FPLSD comparisons showed that eggs in the group given 4 hours to develop before being exposed to ethanol had a significantly higher probability of hatching into viable worms than eggs given 5, $p<0.025$, or 6, $p<0.01$ hours to develop before exposure. However, when the hatching probability of eggs given 4 hours to develop before exposure was compared to that of embryos given 7 and 8 hours as well as the control group, it was found that eggs given only 4 hours to develop before exposure had a significantly lower hatching probability, $p<0.001$ for each comparison. FPLSD comparisons further showed that eggs given 5 and 6 hours to develop before being exposed to ethanol had a significantly lower hatching probability than that of eggs in the 7 hour, 8 hour and control group, $p<0.001$ for each comparison (Figure 2.3.4.2).

The observation that the time point during ex-utero embryogenesis when *C. elegans* eggs are exposed to ethanol was critical to the probability that those eggs would hatch, suggested that there are times points during ex-utero embryonic development when the developing embryo inside the egg is more or less susceptible to the teratogenic effects of ethanol. It appeared as though once eggs had made it past hour 7 of ex-utero embryonic development, the embryo inside was better able to tolerate acute exposure to ethanol.

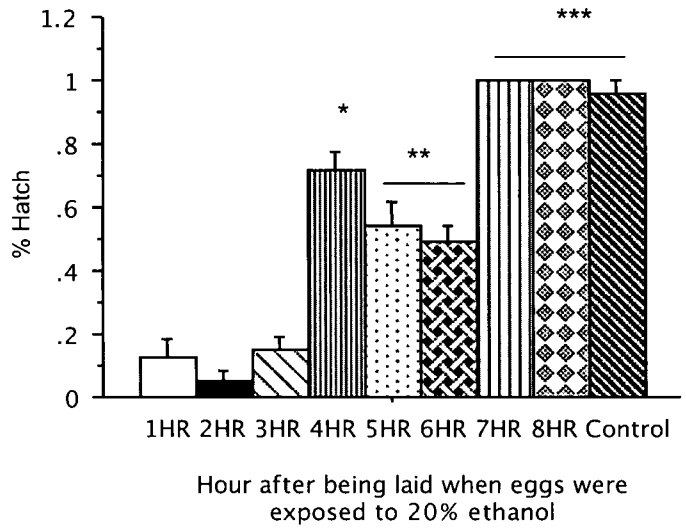


Figure 2.3.4.2 One-Hour Exposure to 20% Ethanol Differentially Affected The Hatching Probability of Eggs Exposed at Different Time Points During Ex-Utero Embryogenesis.
One-hour exposure to ethanol at different time points during ex-utero embryogenesis resulted in a significant main effect on the hatching probability of eggs. Younger eggs were affected more by exposure to ethanol than older eggs. Eggs given less than 3 hours to develop before exposure had a significantly lower hatching probability than eggs in any other treatment group. Eggs given 4 to 6 hours to develop had a significantly lower probability of hatching than eggs given more than 7 hours. (*, ** and *** indicates significance, $p<0.05$).

2.3.4.3 The Effect of Acute Exposure to 20% Ethanol During Ex-Utero Embryogenesis on the Morphology of Larval *C. elegans.*

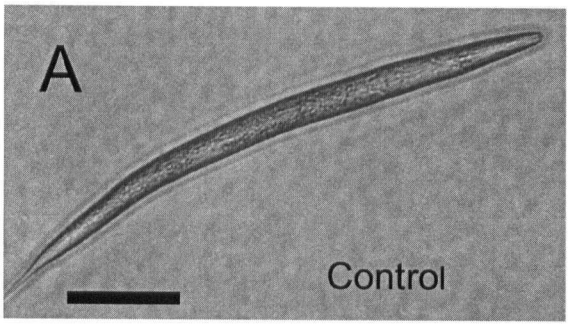
After eggs exposed to ethanol at different time point during ex-utero development hatched, I investigated the effects this exposure might have had on the newly hatched larva. In this experiment, I took Nomarski images of the few larvae from eggs that did hatch in Experiment 3.3.4.2 when they reached late staged L1 and early staged L2 larvae 24 hours after they hatched. Because so few eggs hatched from each of the groups that were exposed 1 hr, 2 hr and 3 hr after being laid, I combined the worms from these three groups into one group called "early exposed". I combined worms from the 4 hr, 5 hr and 6 hr groups in to one group called late exposed. To investigate the effects exposure to ethanol during ex-utero embryonic development may have had on larval morphology due to exposure at these two times, early or late, I magnified images 40X in order to get a clear images of larvae.

In this experiment I observed that larvae from eggs exposed to ethanol displayed marked dysmorphologies in comparison to controls (Figure 2.3.4.3). Larvae from eggs that were not exposed to ethanol during ex-utero embryonic development (Figure 2.3.4.3A) appeared to look like normal larval worms. They were smooth along the exterior cuticle and there were no obvious dysmorphologies. However, examination of the surviving larvae of eggs that were exposed to ethanol showed that regardless of whether the eggs were exposed to ethanol, early or late during embryogenesis, a sub-set of the surviving larval worms displayed clear physical dysmorphologies (Figure 2.3.4.3B-G). The severity of the dysmorphology that was incurred as a result of exposure to ethanol during embryogenesis appeared to be variable between individuals. Therefore, I made three categories to describe the physical morphology of worms that were exposed

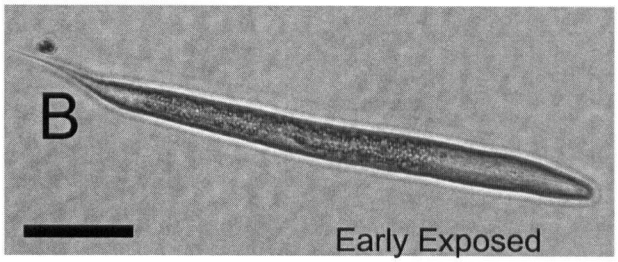
to ethanol during embryogenesis. These categories were normal, mild dysmorphology and severe dysmorpholgy. Based on a qualitative examination of worms that hatched from eggs that were exposed to ethanol either early or late during embryogenesis worms were categorized as normal, mild dysmorpholgy or severe dysmorpholgy.

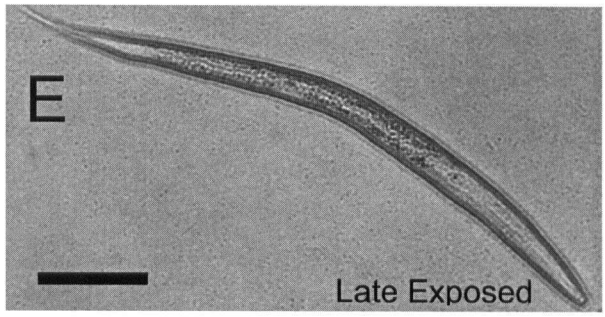
While control worms appeared smooth along the exterior cuticle, the worms that hatched from eggs that I labeled mild dysmorpholgy appeared bent and contorted along their a-p axis. As well, regions along the a-p body axis of mild dysmorpholgy worms appeared to be abnormally bulbous in some regions and overly thin in others (Figure 2.3.4.3 C and F). Worms that hatched from exposed embryos that I labeled severe dysmorpholgy appeared grossly abnormal (Figure 2.3.4.3 D and G). These worms appeared exaggeratedly bulbous and contorted in their anterior regions and overly thin in the posterior region. For worms to be labeled severe dysmorpholgy, they had to appear markedly abnormal in appearance compared to worms labeled mild dysmorpholgy.

Interestingly, despite the observation that exposure to ethanol during either early or late embryogenesis can produce such exaggerated physical dysmorphologies in *C. elegans*, this same exposure protocol was observed to leave some worms apparently normal (Figure 2.3.4.3 B and E) regardless of age when exposed. Some worms that underwent the same ex-utero exposure to ethanol were found to be no different than control worms never exposed to ethanol in terms of their physical morphology (Figure 2.3.4.3 A, B and E). Table 2.3.4.3 summarizes the percentage of worms to be labeled normal, mild dysmorpholgy and sever dysmorpholgy for each treatment group.

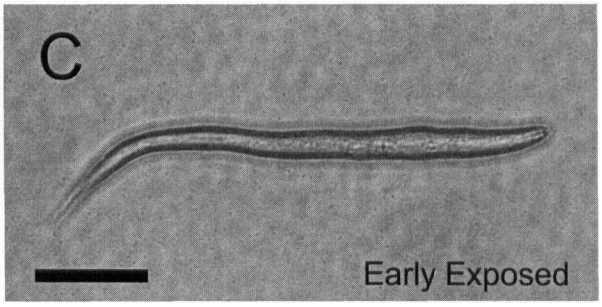


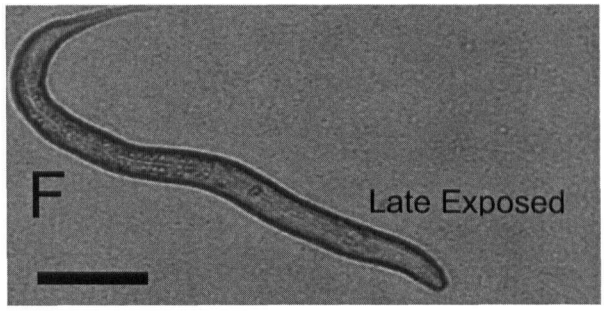
Normal



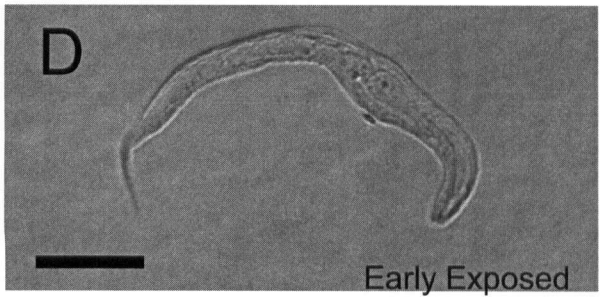


Mild Dysmorphology





Severe Dysmorphology



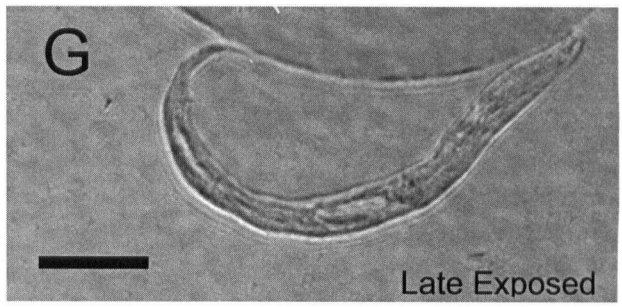


Figure 2.3.4.3 Acute Exposure to Ethanol During Ex-Utero Embryonic Development was Found to Produce a Variable Effect on the Physical Morphology of *C. elegans*. (A) Control worms never exposed to ethanol appeared smooth and healthy. (B) through (G) depict larvae from eggs that were exposed to ethanol for 1 hour during ex-utero embryonic development. Worms were classified based on a qualitative examination of their physical appearance. I subjectively evaluated the smoothness of the exterior cuticle, the appearance of bulbous regions along a-p axis and the overall quality of each specimen. Worms were classified as normal (B and E), mild (C and F) or severe (D and G) in terms of the severity of the resultant physical dysmorpholgy incurred as a consequence of exposure to ethanol during embryogenesis. (Scale bars = 500μm)

Table 2.3.4.3 Summary of Morphological Affects Due to Acute Exposure to Ethanol During Ex-utero Embryonic Development

Total Brood			**Eggs that Hatched**			
Egg Age at Time of Exposure	n	% Hatch	n	% Normal	% Mild Dysmorpholgy	% Severe Dysmorpholgy
Early Exposed Hr 1-3	n=110	13.6%	n= 22	22.7%	63.6%	13.6%
Late Exposed Hr 4-6	n=119	68.9%	n= 49	53.1%	34.7%	12.2%
Control	n=50	96.0%	n= 49	100.0%	0.0%	0.0%

2.3.4.4 Summary of Results

While no consistent phenotype resulted as a consequence of acute exposure to ethanol during ex-utero development, it was clear that this exposure paradigm impacted the normal physical morphology of worms. When this observation is taken together with the finding that ex-utero exposure to ethanol reduces the probability of embryos hatching into viable worms, it is clear that exposure to ethanol in this manner is an effective means

to expose developing *C. elegans* embryos in order to model the pre-natal ethanol exposure paradigm used in higher order systems.

2.4 DISCUSSION

In chapter II I investigated the effects of chronic exposure to ethanol on *C. elegans* development and life history. I also investigated the effects of chronic exposure to alcohol during adulthood and the effects of exposure to ethanol during in-utero and ex-utero embryonic development. In EXPERIMENT SET #1 I found that chronic exposure to ethanol resulted in a significant negative impact on *C. elegans* size, rate of development, reproductive fecundity and longevity. I further demonstrated in EXPERIMENT SET #2 that chronic exposure to ethanol only once the onset of young adulthood had been reached resulted in much the same effects on *C. elegans* size, reproductive fecundity and longevity. In terms of the effects embryonic exposure to ethanol on the later development and life history of *C.elegans,* I investigated how both in-utero and ex-utero exposure to ethanol affected *C. elegans*. In EXPERIMENT SET #3, I found that the offspring born to worms that were chronically exposed to ethanol did not show any ill effects of having been exposed to ethanol during in-utero development. In this set of experiments, no effect of in-utero ethanol exposure was observed on the size, rate of development, reproductive fecundity and longevity of *C. elegans.* In order to investigate the possibility that the *C. elegans* egg shell may have been impermeable to the concentrations of ethanol used in EXPERIMENT SET #3, I tested whether or not higher concentrations of ethanol could in fact permeate the *C. elegans* egg shell during phases of ex-utero development. In EXPERIMENT SET #4, I found that the *C. elegans* eggshell

was in fact permeable to higher concentrations of ethanol and that the time during ex-utero embryonic development a given embryo was exposed was critically important to whether or not the embryo would hatch. I also found that as a result of this exposure protocol, worms that did survive displayed a variable degree of physical dysmorphology. Larval worms ranged from having a normal appearance to looking grossly abnormal.

Taken together, the results from these four sets of experiments show that *C. elegans* is susceptible to the toxic effects of chronic ethanol exposure and that in order to model the effects of FASD in this system, it is ideal to expose embryos to high concentrations of ethanol during ex-utero embryonic development

2.4.1 Discussion of The Effects of Chronic Exposure to Ethanol

Chronic exposure to ethanol has a significant effect on the length of *C. elegans* across their lifetimes. Figure 2.3.1.1 shows that worms in the 0.0M, 0.1M and the 0.2M groups all grew throughout their lifetimes, and that both the low dose ethanol groups achieved at least the size of the control worms and in the last 4 days of the observations they appeared to be longer than the control worms. Early in development the 0.2M ethanol treatment slowed growth, but by day 15 the worms had caught up to, and appeared to surpass control worms. Careful examination of the raw data showed an interesting pattern with the 0.1M and 0.2M groups. Figure 2.3.1.3 shows that worms in 0.1M and 0.2M groups began to die sooner than worms in the 0.0M group. When I looked at which worms died first I discovered that the smallest worms in the 0.1M and 0.2M treatment groups died first. Therefore it is not that the chronic ethanol treatments caused worms to grow larger than controls, it is that the average size of surviving worms in the 0.1M and 0.2M groups were larger than worms in the control group. One explanation for this

would be that the smaller worms were less able to tolerate the insult presented by the low concentrations of ethanol and died sooner than larger worms. In contrast to the worms exposed to low doses of ethanol, the worms in the 0.4M group grew more slowly right from the first day and appeared to show asymptotic growth when they reached about half the size of control worms. However, it is interesting to note that the error bars on the 0.4M group are much larger than the error bars for any other group. The worms used in this study represent a genetically uniform population and so this difference cannot be accounted for by genetic differences between individuals. Perhaps there are variable epigenetic effects such as differences in DNA methylation, differential mRNA splicing and post-translation modifications due to ethanol exposure that influence the effects of ethanol on the size and/or survival of worms. Further study in this area would be necessary to understand this possibility.

One other possibility to account for the effect of chronic ethanol exposure observed on *C. elegans* size is that ethanol is not directly affecting the developmental pathways that mediate worm growth but rather, causes worms to take in less food. This hypothesis was investigated in experiment 2.3.1.4 where I analyzed the effect of chronic ethanol exposure on the rate of pharyngeal pumping and observed that worms chronically exposed to ethanol consume less food and presumably fewer nutrients than control worms (Figure 2.3.1.4). A comparison of the proportional difference between the rate of pumping in the 0.0M and 0.4M worms (52.8%) was remarkably similar to the proportional difference in the size of worms on day-4 between the 0.M and 0.4M group (52.6%; Fig 2.3.1.1). This observation supports the hypothesis that worms chronically treated with ethanol were smaller, at least in part, because ethanol exposure caused them

to consume less food than control worms. However, the proportional change in the brood size (96.4% Figure 2.3.3.3) and in longevity (41.6% Figure 2.3.1.5) between the 0.0M and 0.4M group are not the same as for pharyngeal pumping, suggesting that ethanol has multiple effects on development, some of which can be attributed to the effect on nutritional intake, others that cannot. Furthermore, Hansen et. al 2005, found that that dietary restriction extends *C. elegans* lifespan by down-regulating expression of key genes, including a gene required for methylation of many macromolecules. In my study I have found that ethanol exposure reduces *C. elegans* lifespan. With this finding coupled with what is known about the relationship between *C. elegans* longevity and dietary intake (Hansen et. al 2005) would suggest that more than just the reduction in the rate of pharyngeal pumping is contributing to the observed deficits in worms chronically exposed to ethanol. Further investigation of the effects of ethanol on specific developmental pathways on a molecular level would be required to further explore this issue in greater detail.

Chronic ethanol exposure had a profound effect on the time at which worms reached reproductive maturity. In Experiment 2.3.1.2 it was observed that there was a clear dose dependent delay in when worms began to lay eggs. Worms in the 0.2M treatment group took ~25 hours longer to lay eggs than worms in the control group while worms in the 0.4M treatment group took ~80 hours longer. This observation helped to address the question of whether the findings pertaining to the effect of chronic ethanol exposure on the size of worms in experiment 2.3.1.1 was due to ethanol imposing a developmental delay or if it was causing worms to stay small while continuing to develop normally. From the observation that worms take longer to reach reproductive maturity it seems

plausible that ethanol is in fact causing a developmental delay. One important issue to note is that because ethanol significantly reduced the probability that worms would lay eggs, there were fewer worms in the 0.2M and 0.4M treatment groups that I used to observe the effect of chronic ethanol exposure on the timing of egg-lay onset. The finding that exposure to ethanol during the larval stages reduces the probability that a worm will lay eggs is a further testament to the negative effects ethanol has on the development of the *C. elegans* reproductive system.

Chronic exposure to ethanol not only delayed the onset of reproduction, it also affected the overall reproductive success of *C. elegans*. In a dose dependent manner it was observed that worms exposed to 0.1M, 0.2M and 0.4M ethanol laid fewer eggs than control worms. One important caveat that must be pointed out in this experiment is that I only counted the number of eggs laid; I did not count the number of eggs that hatched into viable worms. Because I did not do this, I cannot rule out the possibility that chronic ethanol exposure not only reduces the number of eggs laid by worms but also the viability of the eggs that were laid. Regardless of this caveat, worms exposed to ethanol laid fewer eggs than control worms leading me to conclude that chronic ethanol exposure significantly reduces *C. elegans* reproductive fecundity.

When taken together, the observation that chronic ethanol exposure delays the onset of reproductive maturity and reduces the number of eggs laid by worms once they do reach reproductive maturity it is clear that ethanol interferes with *C. elegans* development in two ways. It is delaying the overall rate of worm development and it is also causing aberrant development of the worm's reproductive system. The decrease in the number of eggs laid could be the result of worms not producing as many eggs, or to worms that

produced eggs but could not efficiently lay them as a consequence of ethanol exposure. Worms that produce eggs but cannot lay them are considered to have an EGg Laying defective (EGL) phenotype that includes bloating and bursting of the cuticle (Desai and Horvitz, 1989). Although there were a few EGL worms included in this data set, close examination of the raw data used in this experiment showed that on average, all worms in the 0.2M and 0.4M treatment groups that were laying eggs laid a smaller number of eggs. It wasn't the case that some worms were laying at a normal rate while others didn't lay at all. Knowing this it seems likely that chronic ethanol exposure interferes with the development of the *C. elegans* reproductive system and reduces the normal production of eggs.

As well as affecting the size, rate of development and reproductive success of *C. elegans*, chronic exposure to ethanol also affected worms in their later life. Chronic ethanol exposure had a profound effect on the life expectancy of worms (Figure 2.3.1.3A). In this experiment it was observed that on day 5 worms in the 0.4M treatment group began to die at a rapid rate. It isn't until a few days later than worms in the 0.1M and 0.2M treatment groups begin to die. Interestingly, despite the observation that worms in the ethanol treatment groups on average died earlier than controls, there are worms in the treatment groups that are able to withstand the insult presented by ethanol and live as long as worms in the control group. In much the same manner that there was a significant degree of variability in worms' resistance to the effects of chronic ethanol exposure on their size, there also was variability in terms the resistance of *C.elegans* to the effects of chronic ethanol exposure on their rate of survival. As mentioned before, worms used in this study were from a clonal population so it is not possible that this

variability is due to genetic differences between individuals. Again, it is possible that epigenetic factors may contribute to this finding. However, further study would be required to support this theory.

2.4.2 Comparison of the Effects of Chronic Exposure to Ethanol over the Entire *C. elegans* Lifespan vs. Chronic Exposure Only Beginning at YAD

Exposing worms to ethanol once they reach YAD resulted in a significant impact on the size of worms (Figure 2.3.2.1). As in the previous experiment where worms were chronically exposed to ethanol through larval development, the 0.4M group in this experiment reached asymptotic growth at about half the size of control worms as well. However, it must be noted that while in the previous experiment worms were delayed in their rate of growth due to exposure during larval development, they continued to grow once they reached adulthood before they reached asymptotic levels. In this experiment, where worms were exposed as YADs, growth of the 0.4M group abruptly ended. In this case ethanol exposure caused worms not to grow during adulthood- they stayed at the same size as they were when they were first exposed to ethanol. This observation brings about the interesting possibility that when worms are exposed to ethanol during early larval development, they can acclimatize to the toxic nature of ethanol and continue to grow, albeit in a delayed manner, whereas worms that experience ethanol exposure for the first time as young adults cannot adapt to the high levels of this toxic substance. This theory is in line with the findings regarding the plastic nature of developing *C. elegans*. Developing worms are affected by their environment (Rose et al. 2005). It is a real possibility that in this instance, developing in the presence of low doses of ethanol helps worms tolerate exposure to ethanol in later life.

Exposure to high levels of ethanol beginning at YAD affects the immediate reproductive development of worms; worms in the 0.4M treatment group were delayed in the timing of egg-lay onset (Figure 2.3.2.2A). Worms in the 0.4M group began to lay eggs only ~10 hours after control worms compared with being delayed by ~80 hours in the previous experiment 2.3.1.2A. This observation further supports the conclusion that ethanol exposure delays development. Furthermore, it suggests that the magnitude of developmental delay is a function of the duration of ethanol exposure. In this experiment, worms were only exposed for ~9-10 hours before worms begin to lay eggs under normal conditions and the developmental delay imposed by the 0.4M treatment was not as large as the effect the 0.4M treatment had on the timing of egg-lay onset when worms were exposed to ethanol for 68-70 hours before the normal onset of egg-laying in experiment 2.3.1.2A. It would be possible to test this hypothesis by comparing the time at which that were exposed for various durations of time prior to normal egg-lay onset begin to lay eggs. The finding that ethanol exposure beginning at YAD still delays the normal onset of egg laying supports the hypothesis that ethanol exposure delays development.

However, both the total brood size and longevity of *C.elegans* were significantly reduced as a result of chronic exposure to high levels of ethanol beginning at YAD. While the observation that this exposure results in a developmental delay, this observation presents a clear example of the ongoing toxicity of ethanol exposure. The worms exposed to ethanol in this exposure paradigm were never exposed to ethanol during larval development. Therefore, the decrease in brood size and longevity observed as a result of this exposure paradigm were not due to a lasting effect of exposure during

larval development. This indicates that ethanol toxicity during adulthood is contributes to the observed effects. When taken together, the observation that chronic exposure to 0.4M ethanol delays the onset of reproductive maturity as well as reduces reproductive fecundity and longevity in worms that are exposed to ethanol beginning at YAD, it is most likely that the observed effects chronic exposure to ethanol in EXPERIMENT SET #1 are due to both the effects ethanol has on normal developmental processes as well as it toxic nature.

One finding that must be discussed is the comparison between the affect of chronic ethanol exposure on worm longevity in experiment 2.3.1.3 vs. the effect of chronic ethanol exposure in experiment 2.3.2.3. In the first experiment, worms were exposed to ethanol during larval development until their death. In the second experiment, worms were not exposed during larval development; they were only exposed from the time they reach YAD until they died. In the first experiment, worms in the 0.4M group began to die on day 4 and a few worms managed to survive as long as worms in the control condition. In the second experiment this was not the case, worms in the 0.4M group began to die on day 4 and continued to die at a rapid rate and no individuals in this group managed to survive as long as worms in the control treatment. This finding further supports the hypothesis that exposure to ethanol during early larval development, as in EXPERIMENT SET #1, was somehow protective and provided worms with a higher tolerance to the toxic affects of ethanol during later life than worms that first experienced ethanol exposure as a novelty during YAD in EXPERIMENT SET #2.

As mentioned, chronic exposure to ethanol has a profound effect on the rate at which *C. elegans* consumes food (Figure 2.3.1.4). This finding presents a serious confound that

may have affected the results of my other experiments regarding *C. elegans* development and life history in both EXPERIMENT SET#1 and #2. It is possible that the observed decrease in size, reproductive fecundity and longevity I observed were not due to ethanol having a specific effect on them, but rather, due to worms consuming less food as a consequence of being in an ethanol laden environment. It would be possible to address this issue in two different experiments. In the first I could use a strain of *C. elegans* with the "EAT" phenotype. EAT mutants have mutations in their feeding systems and do not consume as much food as wild type N2 worms (Avery, 1993). By comparing the size, timing of egg-lay onset, reproductive fecundity and longevity of several *C. elegans* EAT mutants to that of N2 worms chronically exposed to ethanol to see whether nutritional deprivation contributed to the observed findings. In the second potential experiment it would be possible to raise N2 worms on bacteria of poor nutritional quality either for their entire lifespan, or from the time they reach YAD onward and again compare the measurements of development and life history to the data collected in EPXERIMENT SETS #1 and #2 with N2 worms chronically exposed to ethanol. This line of experimentation would resolve the issues surrounding the effect of nutritional deprivation as being a potential cause of the findings made in EXPERIMENT SET#1 and #2.

2.4.3 Exposure to Ethanol During In-Utero Embryonic Development

One objective of this study was to develop a *C.elegans* model for study in the field of FASD research. Therefore I investigated whether any developmental abnormalities might arise as a consequence of exposure during in-utero embryonic development. I found no significant effects on the size, developmental rate, reproductive fecundity or longevity of the offspring of worms chronically exposed to ethanol. However, it must be

pointed out that the measurements I employed in this study were gross measurements of physical development and life history. I cannot rule out that this exposure may result in an effect that went undetected in my study.

The most obvious hypothesis for why I saw no effects of in-utero exposure to ethanol on the physical development and life history of *C. elegans* was that the concentrations of ethanol I used were too low to get into the developing egg and interfere with normal development. To test this hypothesis it was necessary to determine whether or not there exists a concentration of ethanol that can permeate the *C. elegans* eggshell.

2.4.4 Exposure to Ethanol During Ex-Utero Embryonic Development

In EXPERIMENT SET # 4 I found that exposure to 40% ethanol for one hour was sufficient to kill 100% of exposed larvae (Figure 3.4.1.1A). I also found that 40% ethanol was sufficient to kill ~95% of developing embryos inside eggs subjected to the same exposure protocol (Figure 3.4.1.1B). If this ethanol exposure was killing embryos, it was doing so because ethanol was permeating the *C. elegans* eggshell and disrupting normal development. Interestingly, in this experiment I found that 20% ethanol was sufficient to kill ~80% of larvae (Figure 3.4.1.1A) and only kill ~50% of embryos inside eggs in this exposure paradigm. For this reason I used the 20% ethanol concentration for further studies pertaining to the effects of ethanol exposure during ex-utero embryonic development. 20% v/v ethanol is the equivalent to 4.17M ethanol. This is ~10 times more concentrated than the highest, 0.4M, concentration used in previous experiments. The observation that 20% ethanol kills ~50% of embryos was comparable to an LD50.

In this first experiment, there was one serious confound. I tested a population of eggs containing embryos that were all at different stages of embryogenesis. When I exposed

populations of eggs that were all at a known time in embryonic development to 20% ethanol for one-hour, I observed that the age of embryos inside the eggs was critical to the probability that they would hatch into worms (Figure 3.4.2.2). Eggs given 3 hours or less to develop after being laid prior to being exposed to 20% ethanol had only ~10-20% chance of hatching into larval worms, eggs given 4-6 hours had ~75-50% chance of hatching and eggs given more than 7 hours to develop hatched ~100% of the time. Forty minutes after an egg is fertilized, the first cell division occurs and for the next ~1.75hrs cell proliferation begins and the founder cells will be established (Sulston et al. 1983). Approximately 2.5 hours after fertilization, gastrulation begins along with rapid cell proliferation that will continue for the next ~2hrs while the three germ layers, mesoderm, ectoderm and endoderm are established. The egg is laid ~3hrs after fertilization (Wood, 1988). Approximately 6 hours after fertilization morphogenesis begins and from the cells in the three germ layers, the differentiation of specific cell types and formation of distinct anatomical features occurs (Sulston, 1983). See figure 2.4.4 for detailed description.

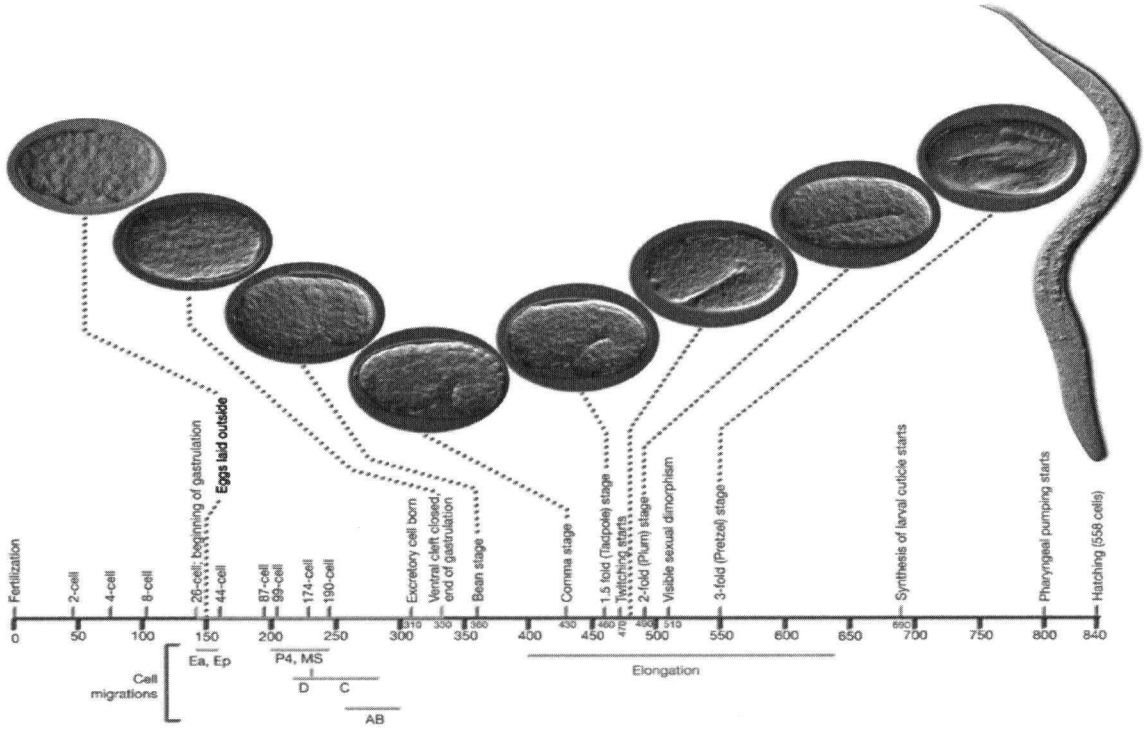


Figure 2.4.4. Timetable of Embryonic Development.
This figure depicts the timing of developmental events during embryogenesis in *C. elegans*. Figure was adapted from http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm

In the experiments reported here, the eggs that were 3 hours old when exposed to 20% ethanol contained embryos that were ~6 hours old. Therefore, eggs that were acutely exposed to 20% ethanol 1, 2 and 3hrs after being laid were exposed before the end of gastrulation and cell proliferation. It may be that exposure to ethanol during these phases of embryonic development interfered with the normal development, cell proliferation, migration and establishment of the three germ layers.

Approximately 390 minutes after fertilization, the epidermis fully matures and encapsulates the developing embryo (Wood, 1998). One possible explanation for why eggs given more than 3 hours (360 minutes post fertilization) to develop before being acutely exposed to 20% ethanol have a greater probability of hatching is that at this time the protective epidermis of the worms has fully developed and enclosed the developing

embryo. The development of the epidermis may provide a protective barrier to the developing embryo from the teratogenic insult of acute ethanol exposure.

The data reported here raise the possibility that developing embryos are more sensitive to the teratogenic insult of ethanol exposure during this critical period of embryonic development than they are at later time points in embryogenesis. This finding is of particular interest because it closely parallels observations made in both humans and other animal models. It has been shown that there are periods during the development of the nervous system that are sensitive to environmental insults including ethanol. During mammalian nervous system development, specific developmental events including cell proliferation, migration, differentiation, synaptogenesis, myelination, and apoptosis occur in a known temporal and regional pattern (Rice and Barone 2000). Exposure to ethanol during periods of in-utero nervous system development when these specific processes are occurring is known to result in specific developmental aberrations (i.e Tran and Kelly 2003, Medina et al. 2005).

After identifying that ethanol can permeate the *C. elegans* eggshell and that there appeared to be a critical period where exposure to ethanol results in embryonic lethality, I investigated the viability and morphology of worms that do survive acute exposure to 20% ethanol during ex-utero embryonic development. In this experiment I photographed the larval worms that survived exposure to ethanol during embryogenesis. Regardless of whether worms were exposed to ethanol early or late during embryogenesis, they displayed a variable degree of physical dysmorpholgy as a result of exposure. I categorized worms as normal, mildly or severely abnormal. However, it is probable that the severe dysmorpholgy that occurs as a result of embryonic exposure is a linear

function and cannot be simply parsed into three categories. Further investigation of the surviving larvae of eggs exposed to ethanol during embryonic development will be required to resolve this issue.

2.4.5 *C.elegans:* An FASD Model

The data I have reported in this chapter has illustrated the usefulness of *C. elegans* as model system for the study of the effects of ethanol on development. I have provided further support to the findings that ethanol is toxic to *C. elegans* (Thompson and De Pomerai, 2006)). I have demonstrated that exposure to ethanol results in a consistent developmental delay. I have shown that the negative effects on the physical development and life history are due to ethanol's interference with nutritional intake, interference with developmental processes and its toxic effects on the system as whole. I have investigated the effects of embryonic exposure to ethanol on *C. elegans* and in doing so have formulated an optimized exposure protocol for investigating the effects of ethanol during embryonic development. The ex-utero exposure protocol developed in this study can be used for further investigations into the specific effects of ethanol on a range of developmental processes. However, it must first be investigated whether ex-utero exposure produces a consistent developmental effect on exposed worms. It will be great interest to use this protocol to study the effects of embryonic ethanol exposure on specific processes of nervous system development. One potential application for the exposure protocol developed in this study to model FASD in *C. elegans* is genetic screening for genes involved in conferring resistance or increased susceptibility to the teratogenic effects of ethanol. It would be feasible to expose the eggs of mutagenized populations of worms to ethanol and screen for worms that either consistently display the severe

morphological abnormality or in contrast, are consistently normal and then use forward genetics to identify the mutation that confers either resistance or tolerance to the teratogenic effects of ethanol. There are ~17,000 genes in the *C. elegans* system (Waterson and Sulston, 1995) that have a high degree of homology to the ~30,000 genes in the human system. In a screen like this it would be possible to identify genes that confer resistance to the teratogenic effects of ethanol that share homology with human genes that may be involved in the manifestation of the hallmark features of FASD. In conclusion, the data presented here warrant further use of the *C. elegans* model system for studying the effects of ethanol on development.

CHAPTER III

The Effects of Chronic Ethanol Exposure on the *C. elegans* Nervous System and Behaviour

3.1 INTRODUCTION

Prenatal exposure to ethanol has been shown to produce a wide range of developmental defects in the mammalian nervous system (Voorheez and Fernandez, 1986, Miller, 1988, Bonthius and West, 1991, Kruger et al. 1993, Peoples et al. 1996, Liesi 1997, Mattson and Riley, 1998, Mattson and Riley, 1999, Ikonomidou et al. 2000, Byrnes et al., 2001, Goodlet et al. 2001, Hoffman et al. 2002, Hoffman, 2003, Olney, 2004, Jing and Li, 2004). Studies utilizing mammalian models and *in-vitro* cell culture have shown that ethanol disrupts normal neuronal proliferation (Miller, 1988, Bonthius and West, 1991, Byrnes et al., 2001, Liesi 1997) migration (Miller, 1993, Cartwright and Smith, 1995), normal apoptotic processes (Pierce et al., 1989, West et al., 1990, Smith, 1997) and cell to cell interactions (Charness et al., 1994), Most recently it was shown that ethanol exposure, *in-vitro*, reduces cell production by > 60% and eliminates growth factor-mediated cell production (Miller, 2003). It has been hypothesized that ethanol exposure disrupts the delicate balance between neuronal proliferation and death during development leading to the characteristic neural detriments associated with prenatal exposure to ethanol (Miller, 2003). Investigations into the specific mechanisms through which ethanol inhibits normal neuronal proliferation have lead to the working hypothesis that the stress response and activation of heat shock proteins induced by ethanol exposure

are key factors contributing to the observed neuronal loss and attenuation of normal differentiation processes. (Jing and Yi, 2004). In addition to properly balanced neuronal growth and proliferation, proper cell-to-cell communication and adhesion are critical aspects of normal neuronal development. Normal cell-to-cell interactions are altered by ethanol exposure (Charness et. al, 1994, Zhang et. al, 2005) in part through disruptions in Neural Cell Adhesion Molecule (N-CAM) dependent developmental events (Charness et al 1994) contributing to aberrant neural migration. The disruption of normal cell adhesion function not only contributes to physical neural disorganization but also contributes to changes in synaptic architecture and function. For example, ethanol-induced dysregulation of L1 cell adhesion molecules may lead to disruptions in NMDA receptor dependent long-term potentiation in the hippocampus (Zhang et. al, 2005). This is just one example of how ethanol exposure can result in physical aberrations leading to disruption of a cellular processes believed to mediate many cognitive functions. In-vitro, ethanol exposure interferes with neurotransmitter production/activity and receptor function (Gonzales and Hoffman, 1991, Venezuela and Fernandez, 1997) as well as other metabolic events in the nervous system (Kruger et al., 1993, Peoples et al, 1996).

Taken together, these deleterious effects of early ethanol exposure are hypothesized to be responsible for disrupting the normal cellular processes which leads to the many neuropathies associated with FASD including- neuronal-glial heterotopias, cerebellar dysplasia, agenesis of the corpus callosum, hydrocephalus and microencephaly (Jones et al, 1973, Clarren et al. 1978, Grant et. al, 1983). Brain imaging of children diagnosed with FASD has identified structural changes in various brain regions including the basal ganglia, corpus callosum, cerebellum, and hippocampus that may account for

the problem behaviors, such as alcohol and drug use, hyperactivity, impulsivity, poor socialization and communication skills (Mattson et. al, 2001)

The mammalian CNS, neuroendocrine and immune system have been shown to be a tightly linked interdependent network of processes (Besedovsky and Del Ray, 1996; Reiche et al 2004, Zhang et al. 2005) where disruption of one process results in a loss of regulatory control over the others. Prenatal ethanol exposure has been demonstrated to dysregulate normal functioning of all three facets of this interdependent processes making it difficult to identify the specific mechanisms upon which ethanol mediates its effects. For this reason it is difficult to investigate how the cellular and hormonal effects of prenatal ethanol exposure lead to the manifestation of many behavioural abnormalities. Research on animal models has provided insights into the effects of prenatal ethanol exposure on mammalian locomotory behaviour, perseverative behaviour and learning (Able and Berman 1994). Recent studies have investigated how molecular and cellular ethanol-induced changes translate into behavioural phenotypes. For example, rats prenatally exposed to ethanol perform more poorly in the elevated plus and Morris water maze (Vorheez and Fernandez, 1986, Osborn et. al, 1998). Only now are researchers beginning to characterize changes in neural activity such as decreases in hippocampal and striatal dopaminergic activity and increases in hippocampal muscarinic dependent neurotransmission that occur following pre-natal ethanol exposure (Carneiro et. al, 2005). It is clear that ethanol exposure has a broad range of molecular, cellular and behavioural consequences. However much is yet to be studied in terms of the specific targets of ethanol's neurotoxic and behavioural effects. One of the difficulties is that mammalian nervous systems are very complex; they are composed of millions to billions of

interconnected neurons that are hidden inside an opaque skull. In addition there are complex interactions between the nervous system, the neuroendocrine system and the immune system that make untangling specific effects of ethanol a challenge. The large numbers of neurons and interconnections makes it impossible to study the effects ethanol on single neurons or even groups of neurons. The opaque skull means that to study the specific physical effects of ethanol the animal must be sacrificed and dissected. The complex interactions of the nervous system, the endocrine system and the immune system make manipulations of variables very complex.

With its simple nervous system consisting of 302 identified neurons and its transparent body, *C. elegans* has the potential to elucidate the cellular effects of ethanol exposure on the nervous system in an *in-vivo* system. Furthermore, in using *C. elegans* we have the opportunity to investigate how any potential damage to the nervous system as a consequence of exposure to ethanol translates into a lasting behavioural deficit. In addition, using this system allows for the identification of ways that exposure to ethanol can result in aberrant behaviour and at the same time presents the opportunity to identify the disrupted cellular mechanism that underlies the defunct behaviour.

It is the aim of the studies reported in this chapter to investigate the effect chronic ethanol exposure has on specific aspects of the *C. elegans* nervous system and behaviour. Data presented here will further support the usefulness of the *C. elegans* model system for studying the effects of ethanol on development, and in this case nervous system development. In these studies I first investigated the global effects of ethanol exposure on the *C. elegans* nervous system. In doing so I measured the degree to which chronic exposure to ethanol affected the size of the *C. elegans* nervous system. After identifying

that the relative size of the *C. elegans* nervous system is reduced due to chronic exposure to ethanol, I investigated how this effect on the nervous system might translate into a behavioural effect. I chose to investigate the effect of chronic ethanol exposure on two well-characterized *C. elegans* behaviours; the tap withdrawal response and the basal slowing response. I chose these two behaviours because they both have been well studied, and we know the neural circuits and neurotransmitters that mediate them.

The tap withdrawal response has been characterized on behavioural, neural circuit and genetic levels in *C. elegans* (Rankin, 2003). When presented with mechanosensory stimulation in the form of a light tap on the side of the Petri dish in which a worm is dwelling, the worm will respond by reversing the direction in which it was moving (Chiba and Rankin, 1990). This behaviour has been termed the tap-withdrawal response and is mediated by glutamatergic neurotransmission (Chiba and Rankin, 1990, Rose et. al. 2002, Rose et al. 2003). The second behaviour I tested was the basal slowing response. The basal slowing response is the natural tendency for worms to move more slowly when they are transferred from a food-deprived to a food-rich environment. This behaviour is mediated by Dopamine (Sawin et al. 2000). In this set of experiments I found that the tap withdrawal response is attenuated following chronic exposure to ethanol while the basal slowing response was left intact. In an attempt to determine how ethanol affected the neural circuit underlying the tap withdrawal response, I examined the timing of the post-embryonic development of the mechanosensory neurons. To do this I used the MEC-4::GFP rol-6 strain of *C. elegans*. This strain with GFP driven by the MEC-4 promoter which is expressed in only the 6 mechanosensory neurons, ALML, ALMR, PLML, PLMR, AVM and PVM, within neural circuit that mediates the tap-

withdrawal response (See Driscoll and Kaplan, 1997 for review). This strain was used to measure the developmental delay imposed by chronic exposure to ethanol, if any, because the AVM and PVM neurons do not differentiate from the Q-blast lineage of neural precursor cells until ~ hour 20 after egg deposition (See Driscoll and Kaplan, 1997 for review). It was my objective in this phase of the study to use Epifluorescent images to measure the developmental delay, if any, in touch receptor neuron (AVM and PVM) differentiation as a consequence of early ethanol exposure. In the second experiment, I investigated the expression levels of a non NMDA glutamate receptor sub-unit that is expressed in the interneurons that mediate the *C. elegans* tap-withdrawal response (Rose et al. 2003) To do this, I measured the ratio of GLR-1::GFP expression to body length in KP1580 worms that have been engineered to express GFP tagged to GLR-1 receptors (Rongo and Kaplan, 1999).

It was the aim of the studies reported in this chapter to identify whether or not chronic exposure to ethanol results in a global effect on the *C. elegans* nervous system and to provide an example of how behavioural analysis can be used to identify more subtle effects this exposure may have on specific subcomponents the nervous system. Finally, these studies serve as examples of how *C. elegans* can be used to elucidate the specific effects of alcohol on developing nervous system components

3.2 METHODS

3.2.1 Epifluorescence Imaging:

To investigate the effects of ethanol on the expression of pan-neuronal expression of GFP within the *C. elegans* nervous system and the development of the

mechanosensory neurons I used epifluorescent images of the DP132 pan-neuronal GFP expressing worms. In the DP132 worms, GFP expression is driven throughout the nervous system behind a pUNC-119 promoter.

To quantify the ratio of total area of GFP expression to body length, I captured images of the total area of GFP expression of four day-old DP132 worms chronically exposed to either 0.0M or 0.4M ethanol and normalized it to worm body length. To do this I used an upright Zeiss Axioskop mounted with an HBO 100W/2 fluorescent light source and a Sony handycam Digital 8 camera to capture the images. Worms were anestitzed in sodium azide and mounted on glass slides with a #1 thickness Fisherbrand cover slip. Worms were magnified 100X and Images were captured using a Sony handycam camera connected to Apple computer's I-MOVIE software. Images were analyzed on a G4 Apple powerbook using Image J software where I quantified the total area of GFP expression of each worm and normalize it to the worm's body length. I calculated the length of each worm used in this experiment by measuring the body lengths of the worms from the fluorescent images in pixels using image J.

3.2.2 96 Well Micro-plate Reader Analysis of GFP Expression

In order to further quantify gross changes in pan neuronal GFP expression as a consequence of chronic ethanol exposure and to validate earlier findings in the DP132 strain, a SpectraMax Gemini XS 96 well plate reader, (Molecular Devices) was employed. In this series of experiments I also used DP132 as well as a second strain of *C. elegans,* NW1229, which expresses pan-neuronal GFP. Synchronous colonies of both strains of worms were cultured either in an environmental concentration of 0.4M ethanol or ethanol free environment. At 4 days of age, colonies were washed into eppendorph

tubes using ddH_2O and centrifuged at 1500 RPM. Worms were washed in ddH_2O twice before being separated into 200µl aliquots. 3 replicates of 200µl aliquots were placed into a 96 well plate. The 96 well plate was placed into the SpectraMax Gemini XS 96 well plate reader as per manufacturer's directions. GFP was excited at 503nm wavelength and an emission wavelength of 475nm was collected. The sum amount of fluorescent light emitted by the worms was collected and analyzed using Softmax PRO 4.0 software and quantified in arbitrary units.

The total amount of fluorescence emitted by each sample was then normalized to the mass of worms in each sample. The mass of worms used in each sample was calculated by first pre-weighing a 2 ml eppendorph tube and then adding the 200µl aliquot of worms, wicking off as much excess fluid as possible with a KIMwipe, and then weighing the eppendorph tube again to determine the difference between the two measured masses and the sample mass.

3.2.3 Tap Withdrawal Response

I measured the magnitude of the tap-withdrawal response in worms chronically exposed to ethanol. Worms were cultured for four days on plates containing 0.4M ethanol or on untreated control (0.0M) plates. At 96 hours of age, individual adult worms from each treatment group were transferred to individual Petri plates. In order to administer the mechanosensory stimulation, each individual Petri plate containing worms was placed in a holder attached to a micromanipulator resting on the stage of a stereomicroscope (Leica Wild, Model M3Z). Worms were given 2 minutes to adjust to their new environment before receiving mechanosensory stimulation in the form of a tap to the side of the Petri plate in which they were living. A single tap to the side of the

plate was administered using a copper rod attached driven under the control of a Grass S88 stimulator was used to ensure even stimulation was administered to each worm. While administering the single tap, each worm was recorded using a video camera (Panasonic Digital 5100) connected to a VCR (Panasonic AG1960) and television monitor (NEC PM-1271A). Each tap exerted approximately 1-2 newtons of force onto the plate.

After recording the reversal magnitude to tap of each worm, the length of the reversal was scored. Scoring was preformed using stop frame analysis, acetate transparencies and a fine tip marker. The reversal magnitude of each worm was scored by placing a transparency on the screen of the television monitor displaying the behavioural response and tracing the distance traveled. The acetates with the traced reversals were scanned into a Macintosh computer using UMAX Astra 2100U software. The length of each reversal was measured in pixels using NIH image software.

3.2.4 Basal Slowing Response

In this experiment, 4 day-old worms chronically treated with 0.4M ethanol or untreated controls were transferred from the food environments in which they were cultured to either an ethanol free food environment or an ethanol free no food environment.

To record the basal slowing response, worms were transferred to a Petri plate consisting a small droplet of M9 buffer (an isotonic solution) in order to wash any *E. coli* (food) from worms. Worms from each group were then transferred to either the ethanol free food-rich environment or the ethanol free food-deprived environment. Worms were then given two minutes to adjust to their new environment before their natural foraging

movements were recorded. Worms were magnified 25X on a Lieca MZ16F dissecting microscope. Worms movement was recorded using a Leica IC A trinoccular camera connected to a Macintosh powerbook computer via firewire. The movement was recording using Macintosh Apple I-MOVIE software. Each worm was recorded for ~20-30 seconds. The rate of movement for each worm in each group was scored by counting the number of body bends made by a given worm within a given period of the worm was recorded.

3.2.5 Measurement of Effects of Chronic Ethanol Exposure on the Development of the Mechanosensory Neurons.

I also used Epifluorescent imaging techniques to investigate the effects of chronic ethanol exposure on the subset of mechanosensory neurons within the *C. elegans* nervous system. The MEC-4::GFP rol-6 strain expresses GFP in only the 6 mechanosensory neurons, ALML, ALMR, PLML, PLMR, AVM and PVM. GFP expression is driven by the MEC-4 promoter and essentially serves to fill the specified neurons with GFP. The AVM and PVM neurons do not differentiate from the Q-blast lineage of neural precursor cells until ~ hour 20 of development. I used Epifluorescent images to measure the developmental delay, if any, in post-embryonic touch receptor neuron (AVM and PVM) differentiation as a consequence of early ethanol exposure. Using the MEC-4::GFP-rol-6 strain, worms were magnified 1000X and images were captured on an upright Zeiss Axioskop mounted with an HBO 100W/2 fluorescent light source and a Sony handycam Digital 8 camera connected to Apple computer's I-MOVIE software. Worms were anestitized in sodium azide and mounted on glass slides with a #1 thickness Fisherbrand cover slip. Individual frames from recorded movies were used to count GFP containing

cell bodies and record their localization in the worm in order to score differences in AVM and PVM differentiation and localization.

3.2.6 Confocal Imaging of the Effects of Chronic Ethanol Exposure on GLR-1::GFP expression.

KP1580, a strain of *C. elegans* carrying a transgene with a glutamate receptor, GLR-1, tagged with GFP was also examined with and without chronic exposure to ethanol. Confocal images were used to quantify changes of GLR::GFP in 4 day-old worms expression as a consequence of early ethanol exposure in the KP1580 strain. Worms were magnified 630X and images of both the 0.4M treated and untreated control group were obtained using BioRad Radiance Plus confocal on a an inverted Zeiss-Axiovert with DIC Optics (Bio-Rad) microscope equipped with a Krypton/Argon laser. Worms were photographed by placing them in 15μM of 0.3M 2,3-butanedione monoxime (BDM) in 10mM HEPES buffer to anestitize the worm and then mounting them on a glass slide with a 1mm cover slip. GFP was excited using a 488nm wavelength laser setting with the emitted light collected by passing through a ~510-550nm bandpass filter. The images collected were quantified using Image J 1.33 software. Optical sections were collected at 0.4 micron intervals using a 63x oil immersion lens. Total stacks of 15-20 optical sections were compiled into a single projection image in Image J. Using this technique it was possible to quantify the number of puncta and the average size of puncta and the total area of GFP expression. In this experiment, the total area of GLR-1::GFP expression was measured along the posterior ventral nerve cord of worms extending from the vulva in the mid section of the worm body to the posterior. I focused on this part of the worm for two reasons. One is that GLR-1::GFP expression is simple to quantify in the ventral cord and the second is that

previous research in the Rankin Lab has shown that level of GLR-1::GFP expression in this region are altered by experience (Rose et al. 2003, Rose et al. 2005). This total area of GLR-1::GFP expression was then normalized to the body length of *C. elegans*. The body lengths of worms were calculated by measuring their lengths in pixels in Image J.

3.3 RESULTS

3.3.1 EXPERIMENT SET #1 The Effects of Chronic Ethanol Exposure on Pan-Neuronal GFP Expression in the *C. elegans* Nervous System.

One of the primary measures of the magnitude of the insult to nervous system development imposed by early ethanol exposure described in the mammalian literature is the measurement of fetal brain to body weight ratio (Tran et al. 2000). The fact that *C. elegans* lacks a central brain, precluded me from being able to easily measure the brain to body weight ratio in this system, however, by harnessing the powerful molecular investigative tools available in the *C. elegans* systems, I was able to use two different methods to make rough estimates of the size of the *C. elegans* central nervous system relative to its body size in order to asses the effect chronic ethanol exposure had on the *C. elegans* nervous system on a global level.

3.3.1.1 Epifluorescent Imaging of Pan-neuronal GFP Expression.

DP132 is a strain of *C.elegans* that has been genetically engineered to express Green Fluorescent Protein (GFP) in all of its 302 neurons. In this strain, the UNC-119 promoter that is exclusively expressed in nervous system tissue (wormbase), drives GFP expression. The result is all 302 neurons glow green when the worm is exposed to fluorescent light. Using Epifluorescence imaging techniques and the DP132 strain, I was able to capture an image that allowed me to estimate the total size of the *C. elegans*

nervous system. To do so, the total area of GFP expression in the captured image was normalized to the body length of the individual worm.

As predicted by the results from experiment 2.3.1.1 in chapter II, chronic exposure to the 0.4M ethanol treatment resulted in worms being dramatically smaller than age matched, 0.0M, untreated controls (Figure 3.3.1.1A). Therefore, statistical analysis was done on the ratio of GFP relative to body length for worms in each group and showed that worms chronically exposed to 0.4M (n=6) ethanol had significantly smaller nervous systems relative to their size than worms in the 0.0M (n=6) group (Figure 3.3.1.1B). Specifically, a t-test showed that when chronically exposed to 0.4M (n=6) ethanol, the average ratio of total GFP expression in the nervous system to individual body length is lower than that of age matched 0.0M (n=6) treated controls $t(1,7)=2.798$, $p < 0.03$ (Figure 3.3.1.1B). This observation suggested that chronic exposure to 0.4M ethanol was sufficient to result in a significant negative impact on the expression levels of pUNC-119 GFP. I hypothesize that this reflects a decrease in the overall size of the *C. elegans* nervous system.

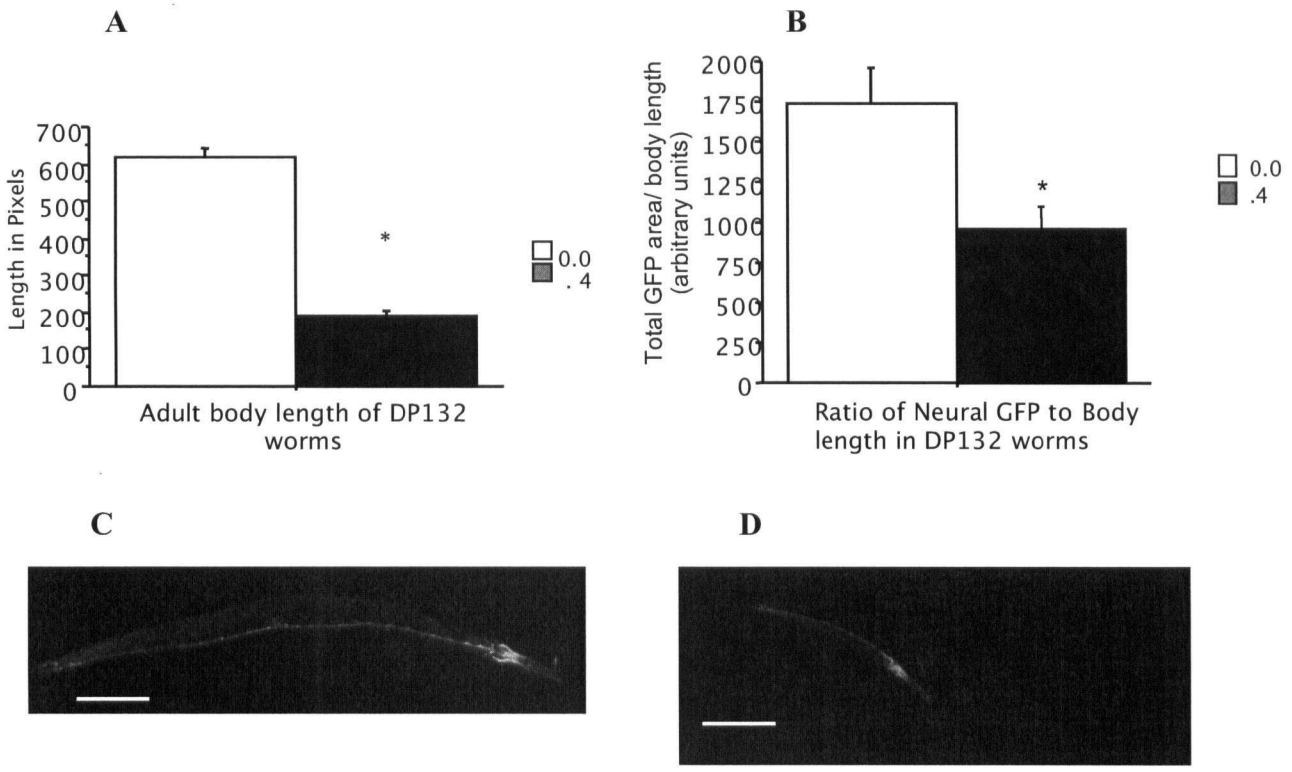


Figure 3.3.1.1 Chronic Ethanol Exposure Decreased the Ratio of Pan-Neuronal GFP Expression to Body Length in Adult DP132 Worms.
(A) Chronic exposure to 0.4M ethanol resulted in DP132 worms being dramatically smaller than control worms not exposed to ethanol at 4 days of age. (B) Epifluorescent images of DP132 worms chronically exposed to 0.4M ethanol had a significantly smaller ratio of pan-neuronal GFP expression to body length in comparison to untreated controls. (C and D) Epifluorescent image of (C) untreated and (D) treated DP132 worm magnified 100X (* indicates significance, $p<0.05$. Scale bar = 0.25mm).

3.3.1.2 Pan-neuronal GFP Fluorescence to Mass Ratio in DP132 and NW1229.

In order to further test the hypothesis that chronic ethanol exposure significantly reduced the size of the entire nervous system relative to the length of the organism, I developed a second, novel technique to measure the fluorescence intensity of GFP expression in *C. elegans*. By using a SpectraMax Gemini XS 96 well fluorescence plate reader I was able to measure the relative amount of pan-neuronal GFP expression in the

DP132 strain of *C. elegans*. The SpectraMax Gemini XS 96 well plate reader is designed to collect and measure the intensity of fluorescent light emitted by GFP when excited. Using this machine allowed for accurate quantification of the fluorescent signal emitted by large numbers of worms from each of the 0.0M and 0.4M ethanol treatment group. The SpectraMax plate reader provides arbitrary units quantifying the magnitude of fluorescence intensity. Again, these arbitrary units were normalized to the mass of measured samples in order to provide an accurate representation of the size of the fluorescent signal from *C. elegans* central nervous system relative to its body size. As with the previous measure, an un-paired t-test showed that the intensity of pan-neuronal fluorescence relative to sample mass was significantly reduced in *C. elegans* that were chronically exposed to 0.4M ethanol $t(1,3)= 6.239$, $p < 0.025$ (Figure 3.3.1.2A).

I repeated this same experiment using a second pan-neuronal GFP expressing, NW1229. As with the DP132 strain, I measured the GFP fluorescence intensity of NW1229 worms exposed chronically to either 0.0M or 0.4M gain ethanol. The arbitrary units from the SpectraMax read-out were normalized to the mass of measured samples in order to provide an accurate representation of the size of the fluorescent signal from *C. elegans* central nervous system relative to its body size. As with the previous measure, an un-paired t-test showed that the intensity of pan-neuronal fluorescence relative to sample mass was significantly reduced in the NW1229 strain as a result of chronic exposure to 0.4M ethanol ($t(1,4)= 3.103$, $p < 0.05$; Figure 3.3.1.2B).

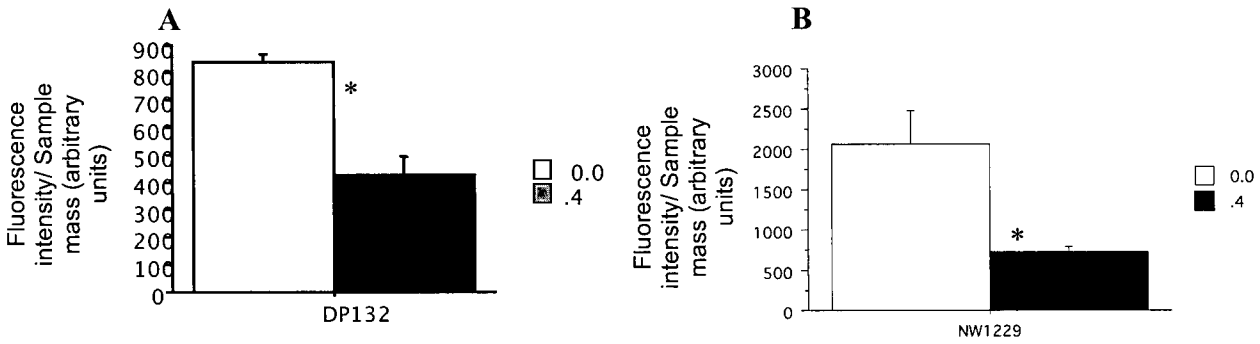


Figure 3.3.1.2 Chronic Ethanol Exposure Decreased the Ratio of Pan Neuronal GFP Fluorescence Intensity to Sample Mass.
(A) Using a SpectraMax 96 well fluorescence plate reader it was observed that DP132 worms chronically exposed to 0.4M ethanol had a significantly lower intensity of GFP fluorescence to sample mass ratio in comparison to untreated controls. (B) This same observation was made was made in a second strain of *C. elegans* that expresses GFP in a pan-neuronal manner, NW1229 (B). (* indicated significance, $p<0.05$).

3.3.1.3 Summary of Results

In experiment set #1 I identified that the expression of GFP driven by two different nervous system specific promoters is significantly reduced as a result of chronic exposure to ethanol. These findings greatly supported the working hypothesis that chronic exposure to ethanol results in an overall reduction in the mass of the *C. elegans* nervous system in the same manner as it has been shown to reduce the brain to body weight ratio in higher order systems.

3.3.2 EXPERIMENT SET #2. The Effect of Chronic Ethanol Exposure on *C. elegans* Behaviour.

After characterizing the effect of chronic ethanol exposure on the expression of two nervous system specific genes to assess the impact of chronic ethanol exposure on the neurobiology of *C. elegans,* I investigated whether this ethanol induced alteration in

the nervous system would translate into effects on *C. elegans* behaviour. Within the FASD literature, there are multiple reports of behavioural deficits that occur consequent of both chronic and prenatal exposure to ethanol. In rats these deficits include decreased foraging behaviour, increased anxiolytic and despair behaviour (Carneiro et. al 2005) and abnormal spatial learning and memory (Green 2004, Iqbal et. al, 2006., Berman and Hannigan, 2000). In humans the most common behavioural deficits that are observed as a result of prenatal exposure to ethanol include perceptual motor disturbances, hyperactivity and attentional deficits (Diaz and Samson, 1980, Ernhart et al. 1995, Mattson and Riley, 1998, Riley and Mcgee, 2005).

In developing *C. elegans* as a comprehensive model for study in the field of FASD, it is important to characterize behavioural deficits that may occur as a result of chronic exposure to ethanol. To do this, I chose to investigate the effects of chronic ethanol exposure on two well-described *C. elegans* behaviours; the tap withdrawal response, mediated by glutamatergic neurotransmission and the basal slowing response, mediated by dopaminergic neurotransmission (Sawin et al. 2000)

3.3.2.1 The Effects of Chronic Ethanol Exposure on Age Matched *C.elegans* size, Gonadal Development and Response to Tap.

One of the most well characterized behaviours observed in *C. elegans* is the worm's response to mechanosensory stimulation (Rankin, 2003). When presented with mechanosensory stimulation in the form of a light tap on the side of the Petri dish in which a worm is dwelling, the worm will respond by reversing the direction in which it was moving. This behaviour has been termed the tap-withdrawal response (Chiba and Rankin, 1990). In order to characterize the effect chronic exposure has on *C. elegans* at the behavioural level, I measured the magnitude of the tap-withdrawal response in worms

chronically exposed to ethanol. I found that exposure to ethanol affected the probability that worms would respond to tap. While 13/15 worms in the control 0.0M group responded to tap, only 8/15 worms in the 0.4M group responded to tap. I then investigated the effect ethanol had on worms that did respond to tap. In this experiment, an unpaired t-test showed that four day-old adult worms chronically exposed to 0.4M (n=8) had significantly smaller tap-withdrawal responses when worms were presented with novel mechanosensory stimulation than untreated controls (n=13) $t(1,28)=2.838$, $p < 0.025$ (Figure 3.3.2.1A).

Because ethanol affected both size (Figure 2.3.1.1) and rate of development (Figure 2.3.1.2), it is possible that the reduced response to mechanosensory stimulation was due to the fact that worms chronically exposed to 0.4M ethanol were much smaller than age matched controls. In this experiment the worms chronically exposed to 0.4M (n=13) ethanol tested in this behavioural assay were significantly smaller than those in the age matched, 0.0M (n=14) untreated control group $t(1,26)=7.051$, $p < 0.001$ (Figure 3.3.2.1B). When comparing the gonadal development between the worms treated chronically with 0.4M ethanol and the age matched untreated control worms, there was a clear delay in reproductive maturation in the 0.4M treated worms. Nomarski images of the vulval region of typical 4 day-old control worms magnified 1000X show worms that appear to be reproductively mature, gravid, adults (3.3.2.1C). Nomarski images of the gonadal region of typical 4 day-old worms chronically exposed to 0.4M ethanol show worms that lack a clearly developed vulva and show no signs of fertilized eggs indicating reproductive immaturity (Figure 3.3.2.1D).

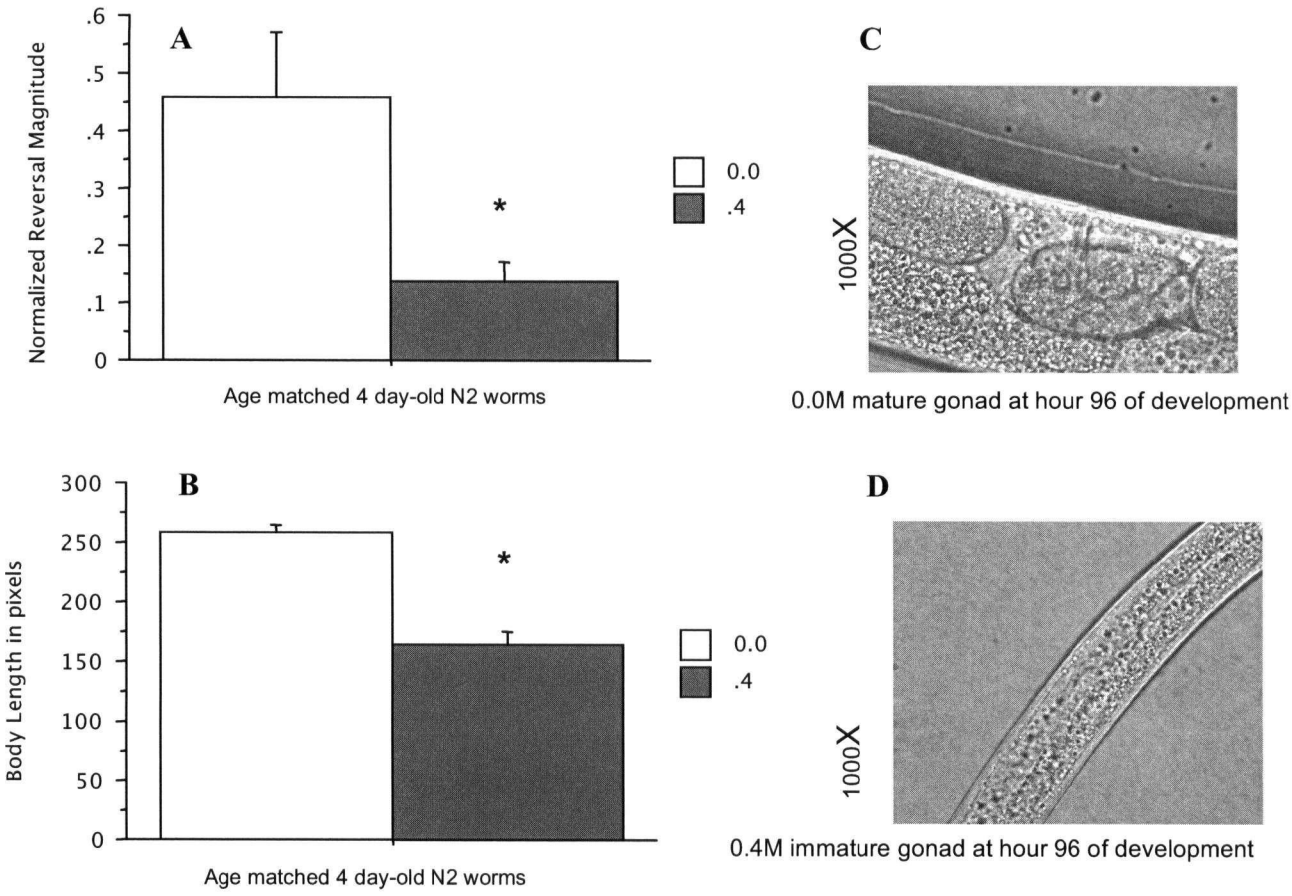


Figure 3.3.2.1 Chronic Exposure to Ethanol Affected *C. elegans* Size, Magnitude of Reversal Response and Gonadal Development.
(A) Worms chronically treated with 0.4M ethanol showed significantly smaller reversal responses to tap than age matched untreated controls. (B) The ethanol treated worms that were tested in this behavioural assay were significantly smaller from age matched untreated controls. (C and D) The worms treated with 0.4M ethanol that were used in this behavioural assay were also developmentally delayed due to treatment. Panel C and D compare the gonadal region of worms treated with either 0.4M ethanol (D) or 0.0M (C). Worms treated with 0.4M ethanol are much smaller and are not at the point of reproductive maturity.

3.3.2.2 The Effects of Chronic Ethanol Exposure on Size Matched *C.elegans* size, Gonadal Development and Response to Tap.

In order to address the issue of how the size and developmental difference between the two 96 hour groups of worms might confound the tap-withdrawal data, I ran a size-matched experiment in which size matched 2 day-old control (treated with 0.0M ethanol) worms were compared to 4 day-old 0.4M treated worms in terms of their initial response to mechanosensory stimulation. I used data from Figure 2.3.1.1 to determine the appropriate age for control worms: 4 day-old 0.4M treated worms were the same length as 2 day-old untreated control worms. In this set of experiments, 2 day-old 0.0M (n=15) untreated control worms were not significantly different from 4 day-old 0.4M (n=16) treated worms in terms of their body length ($t(1,28)=0.528$, $p > 0.05$; Figure 3.3.2.2A). Nomarski images of the developing gonad of experimental and control worms suggested that 2 day-old untreated control worms and 4 day-old 0.4M ethanol worms were at approximately the same developmental stage (Figure 3.3.2.2C and 3.3.2.2D).

Based on these data I compared the initial tap-withdrawal response of size matched 2 day-old control and 4 day-old 0.4M treated worms. In this experiment there was also a difference in the probability worms in each groups would respond to tap. Specifically, I found that 14/15 2 day-old control worms responded to tap while only 9/16 4 day-old control worms responded to tap. I then investigated the effect ethanol had on the reversal magnitude of those worms that did respond. When size-matched, the 4 day day-old 0.4M (n=9) treated worms still showed a significantly smaller average response than the 2 day-old 0.0M (n=14) control worms ($t(1,28)=2.224$, $p < 0.05$; Figure 3.3.2.2B).

This finding suggested that the reduction in the magnitude of response to mechanosensory stimulation that was observed when age-matched untreated control and

0.4M treated worms were compared was not due to the developmental delay imposed by chronic ethanol exposure, but rather, may have been due to an effect of chronic ethanol exposure on the behavioural response

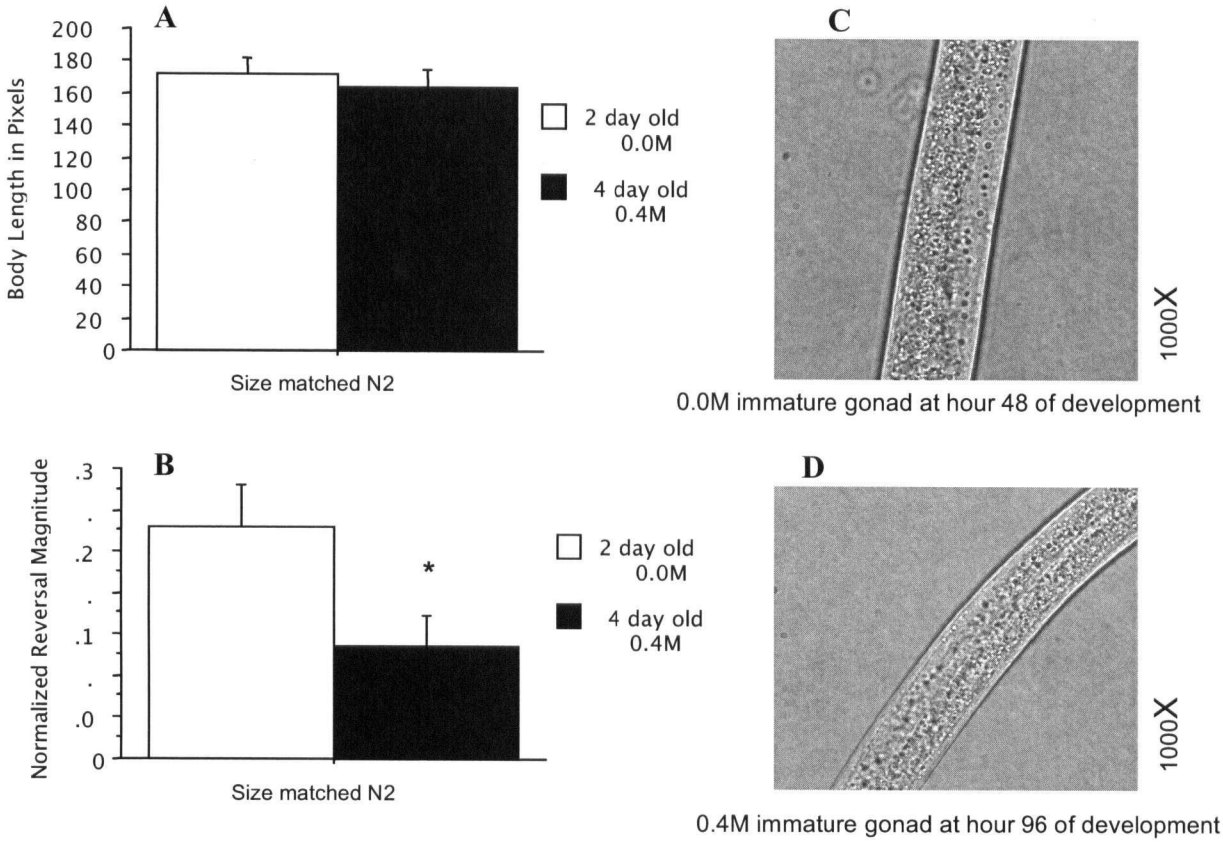


Figure 3.3.2.2 Size Matched *C. elegans* Chronically Exposed to Ethanol Still Show Significantly Smaller responses to Tap than Control Worms.
(A) The 4 day-old worms chronically treated with 0.4M ethanol that were tested in this behavioural assay were not significantly different from the 2 day-old 0.0M untreated controls. (B) The average magnitude of the initial response to mechanosensory stimulation of 4 day-old worms chronically exposed to 0.4M ethanol is significantly smaller compared to size-matched, 2 day-old untreated controls Worms treated with 0.4M ethanol used in this behavioural assay were also not developmentally delayed due to treatment. Panel C and D compare the gonadal region of 4 day old worms treated with 0.4M ethanol (D) to that of 2 day old untreated controls (C). 4 day old worms treated with 0.4M ethanol do not appear to be different in terms of their gonadal development in comparison to 2 day old 0.0M untreated controls. (* Indicates significance, $p<0.05$).

3.3.2.3 The Effect of Chronic Ethanol Exposure on the Rate of *C. elegans* Locomotion.

The rate of *C. elegans* movement when worms were in the presence of or absence of food was significantly slowed by ethanol exposure alone. In both food and no food environments, worms chronically exposed to ethanol had a lower rate of movement than control worms (Figure 3.3.2.3). A one-way ANOVA showed that exposure to ethanol had a significant effect on the number of body bend made per second by worms regardless of the whether worms were transferred from a food rich to a food rich environment or a food rich to a food deprived environment $F(3,45)=33.175$, $p<0.001$. FPLSD comparisons showed that when worms were transferred from a food environment to another food environment, worms chronically exposed to 0.4M ethanol (n=11) made fewer body bends per second than 0.0M untreated controls (n=13), $p < 0.02$ (Figure 3.3.2.3A). Again, when transferred from a food environment to a no food environment, worms chronically treated with 0.4M ethanol (n=11) made significantly fewer body bends per second than 0.0M untreated controls (n=11), $p < 0.001$ (Figure 3.3.2.3B). Thus, chronic ethanol exposure decreased the normal rate of movement in *C. elegans* regardless of whether or not worms were in the presence of food.

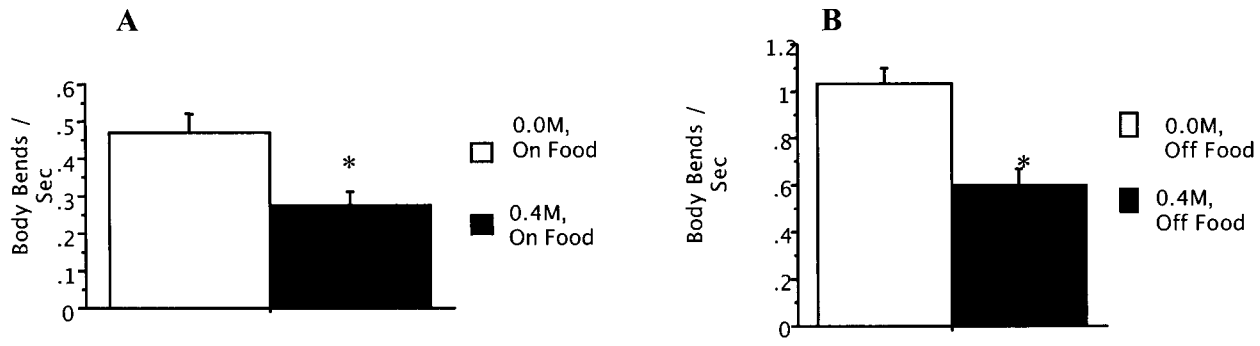


Figure 3.3.2.3 Chronic Exposure to Ethanol Exposure Decreases *C. elegans* Rate of Movement.
Regardless of whether *C. elegans* is in the presence or absence of food, worms treated with 0.4M ethanol move less than untreated controls. In the presence of food, worms treated with 0.4M ethanol move significantly less than untreated controls (A). When in an absence of food, worms treated with 0.4M ethanol still move significantly less than untreated controls (B). (* indicates significance, $p<0.05$).

3.3.2.4. The Effect of Chronic Ethanol Exposure on the *C. elegans* Basal Slowing Response.

When *C. elegans* is in a food-rich environment, i.e. an agar Petri-plate seeded with *E. coli*, its tendency to move around on the plate is far less than if it were to in a food-deprived environment (Sawin et al. 2000). The basal slowing response describes the natural tendency for worms to move less when they are transferred from a food environment to another food environment than they would if they were transferred a from food environment to a no food environment. I investigated the effects of chronic ethanol exposure on the basal slowing response of *C. elegans*. In this experiment, 4 day-old worms chronically treated with 0.4M ethanol or untreated controls were transferred from a food environment to either an ethanol free food environment or an ethanol free no food environment.

Although the average rate of movement both on and off food was reduced as consequence of ethanol exposure (Figure 3.3.2.3), the basal slowing response of *C.*

elegans was not affected by chronic exposure to ethanol (Figure 3.3.2.4). After re-plotting and analyzing the data from experiment 3.3.2.3 I found that regardless of ethanol treatment, worms in both the 0.4M and 0.0M treatment groups still moved less when they were transferred from a food rich to a food rich environment than they did when they were moved from a food rich to a food deprived environment. FPLSD comparisons showed that worms chronically exposed to 0.4M ethanol made fewer body bends per second when they were transferred from a food rich to a food rich environment than they did when transferred from a food rich to a food deprived environment ($n=11$ for both groups), $p < 0.002$. This same basal slowing response was observed in untreated controls. Untreated 0.0M worms made fewer body bends per second when transferred from a food rich to a food rich environment ($n=13$) than they did when transferred from a food rich to a food deprived environment ($n=11$, $p < 0.001$; Figure 3.3.2.4). Worms in the 0.0M group made 45.4% fewer body bends per second after being moved from one food to another food environment than they did after being moved from a food environment to a no food environment. Worms in the 0.4M group made 45.7% fewer body bends per second after being moved from one food to another food environment than they did after being moved from a food to a no food environment. This finding supports the hypothesis that chronic exposure to ethanol did not attenuate the basal slowing response in *C. elegans*.

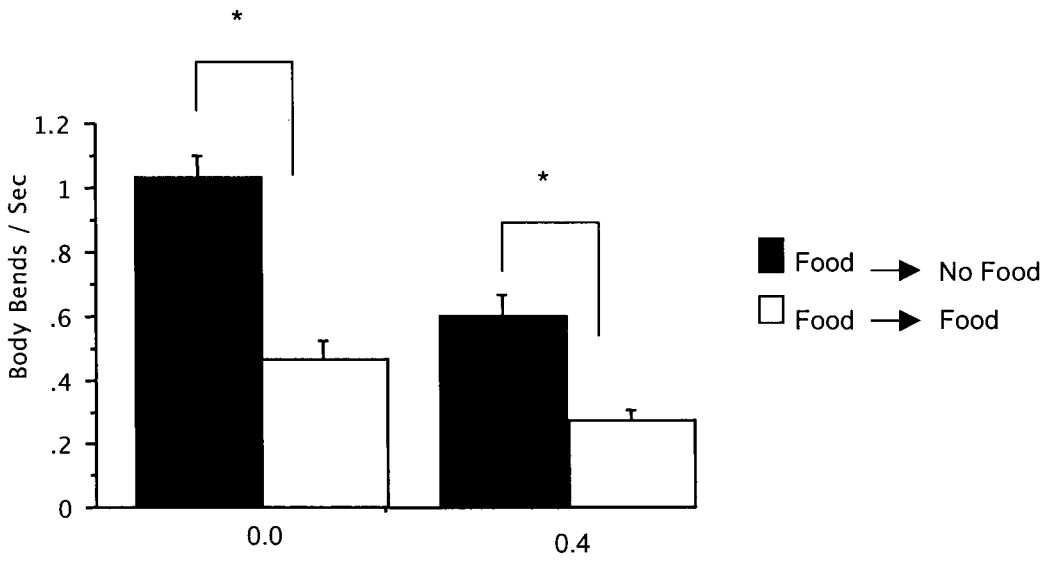


Figure 3.3.2.4 *C. elegans* Chronically Exposed to Ethanol Still Show a Basal Slowing Response.
The basal slowing response of *C. elegans* is not affected by chronic exposure to ethanol. Worms treated with 0.4M ethanol move significantly less when placed in a food rich environment than they do when placed in a food deprived environment. Worms not treated with ethanol, 0.0M, also move significantly less when placed in a food rich environment than they do when placed in a food deprived environment. (* indicates significance, $p<0.05$). This data is re-plotted from figure 3.3.2.3

3.3.2.5 Summary of Results

In this set of experiments I found that the *C. elegans* tap withdrawal response was attenuated as a result of chronic ethanol exposure while the basal slowing response was left intact. The finding that one of these behaviours, the tap-withdrawal response, was attenuated by exposure to ethanol while the other, the basal slowing response, remained intact, lead to the hypothesis that ethanol may be somehow altering the mechanosensory circuit to produce the observed behavioural deficit. Therefore, in the next set of experiments I examined the effect of chronic ethanol exposure on two genes expressed in the neurons that mediate the tap withdrawal response.

3.3.3 EXPERIMENT SET #3 The Effects of Chronic Ethanol Exposure on the *C. elegans* Mechanosensory circuit.

The *C. elegans* tap-withdrawal response is mediated by an interconnected group of 6 mechanosensory neurons and four pairs of interneurons connected to a pool of motor neurons that execute the response (Wicks and Rankin, 1997). The major neurotransmitter that has been implicated in modulating the tap withdrawal response is glutamate (Rose et al. 2002, Rose et al. 2003, Rose et al. 2005). To address the hypothesis that ethanol somehow alters normal signaling within the mechanosensory circuit to produce the decreased tap withdrawal response observed, I investigated the effect of chronic ethanol exposure on the *C. elegans* mechanosensory circuit on two different levels; first on the level of the cell and second on the level of the gene. In the first experiment, I assessed differences in the time at which the AVM and PVM mechanosensory neurons differentiated from the Q-blast lineage of neural precursor cells. AVM and PVM differentiate into functional neurons during post-embryonic development unlike the other four mechanosensory neurons (ALML, ALMR, PLML and PLMR) that differentiate during embryonic development (See Driscoll and Kaplan, 1997 for review). Until AVM and PVM differentiate, ~20 hours after worm are laid as eggs, the neural circuit that mediates the tap-withdrawal response remains incomplete. Using the MEC-4::GFP-rol-6 strain of *C. elegans,* it was possible to visualize the mechanosensory neurons to assess the effect chronic exposure to ethanol had on the timing of the tap-withdrawal circuit development by counting the number of GFP expressing cells at different time points. In the second experiment I investigated the effect of chronic ethanol exposure on the expression of s glutamate receptor sub-unit, *glr-1.* The tap withdrawal response is driven

by electrical signaling from the mechanosensory neurons on to the interneurons and is modulated by glutamatergic neurotransmission from the mechanosensory neurons on to the same interneurons within the touch circuit (Rose et al. 2003). Previous research has shown that the GLR-1 non-NMDA glutamate receptor sub-unit plays a critical role in both the long-term memory (Rose et al. 2002) and developmental plasticity (Rose et al., 2005) of the tap withdrawal response. I examined differences in the level of GLR-1::GFP expression within the interneurons that comprise the mechanosensory circuit using the KP1580 strain of worms that expresses GFP fused to non-NMDA GLR-1 receptor sub-unit. Using this strain I was able to visualize and quantify relative changes in GLR-1::GFP expression as a consequence of chronic ethanol exposure.

3.3.3.1 The Effect of Chronic Ethanol Exposure on the Timing of AVM and PVM Differentiation.

To measure the effect of chronic ethanol exposure on the timing of AVM and PVM differentiation, I counted the number of visible GFP containing mechanosensory neurons in MEC-4::GFP-rol-6 worms chronically exposed to either 0.4M or 0.0M ethanol. I counted the number of neurons at two different time points, 26 hours and 96 hours after worms were laid as eggs (Table 3.3.3.1). In this experiment I found that 26 hours after worms were laid as eggs, 93.3% of worms in the 0.0M (n=30) and 96.8% of worms in the 0.4M (n=31) had only four visible GFP containing neurons, ALML/R and PLML/R. At hour 96 I found that 100% of 0.0M (n=27) control worms had 6 visible GFP containing cells including AVM and PVM while only 66.7% of 0.4M (n=26) worms had 6 visible GFP containing cells. This data suggested that chronic exposure to 0.4M ethanol delayed the development of mechanosensory neurons in some worms.

Table 3.3.1.1 Summary of The Effect of Chronic Ethanol Exposure on the Timing of AVM and PVM Differentiation

Hour After Hatch	Treatment	Number of Visible Mec-4::GFP Cells		
		4	5	6
Hr 26	0.0M (n=30)	93.3%	6.7%	0.0%
	0.4M (n=31)	96.8%	3.2%	0.0%
Hr 96	0.0M (n=26)	0.0%	0.0%	100.0%
	0.4M (n=27)	18.5%	14.8%	66.7%

3.3.3.2 The Effect of Chronic Ethanol Exposure on GLR-1::GFP Expression.

Using the KP1580 strain I was able to visualize expression of the glutamate receptor sub-unit GLR-1 in the *C. elegans* nervous system. By measuring GLR-1::GFP using both Confocal imaging techniques and the SpectraMax plate reader, I investigated the impact chronic ethanol exposure had on the expression of those glutamate receptor sub-units.

Despite observations that on a global level the overall fluorescence level to sample size ratio of the DP132 pan-neuronal GFP strains was significantly decreased as a consequence of chronic treatment with 0.4M ethanol in experiment 3.3.1.2 using the SpectraMax plate reader, I did not see this same decrease in the KP1580 strain (Figure 3.3.3.2A). Data from the SpectraMax plate reader showed that chronic exposure to the 0.4M ethanol treatment did not reduce the total amount of GLR-1::GFP expression relative to body mass $t(1,3)=-0.764$, $p > 0.05$ (Figure 3.3.3.2A). If anything, chronic

exposure to the 0.4M ethanol treatment appeared to cause a small increase in the relative amount of GLR-1::GFP expression.

To further investigate this phenomenon, confocal images of the posterior ventral nerve cord of individual KP150 worms chronically exposed to 0.4M ethanol over the course of their lives were compared to that of age matched controls (Figure 3.3.3.2B). In *C.elegans*, GLR-1::GFP is expressed in individual worms is not expressed in a unfirom patterns along the process. In these worms, GLR-1::GFP is expressed in individual puncta along the ventral nerve cord. An un-paired comparison of the ratio of total area of GFP expression to body length between worms chronically exposed to 0.4M ethanol and untreated controls showed that the chronic 0.4M treatment significantly increased the relative amount of GLR-1::GFP expression in the posterior ventral nerve cord $t(1,28)=-2.0744$, $p < 0.05$. Furthermore, chronic exposure to 0.4M ethanol also proved to increase the density of GFP expressing puncta along the ventral nerve cord (Figure 3.3.3.2C). An unpaired comparison showed that 0.4M treated worms expressed significantly more GLR-1::GFP puncta along their nerve cords $t(1,48)=2.070$, $p<0.02$.

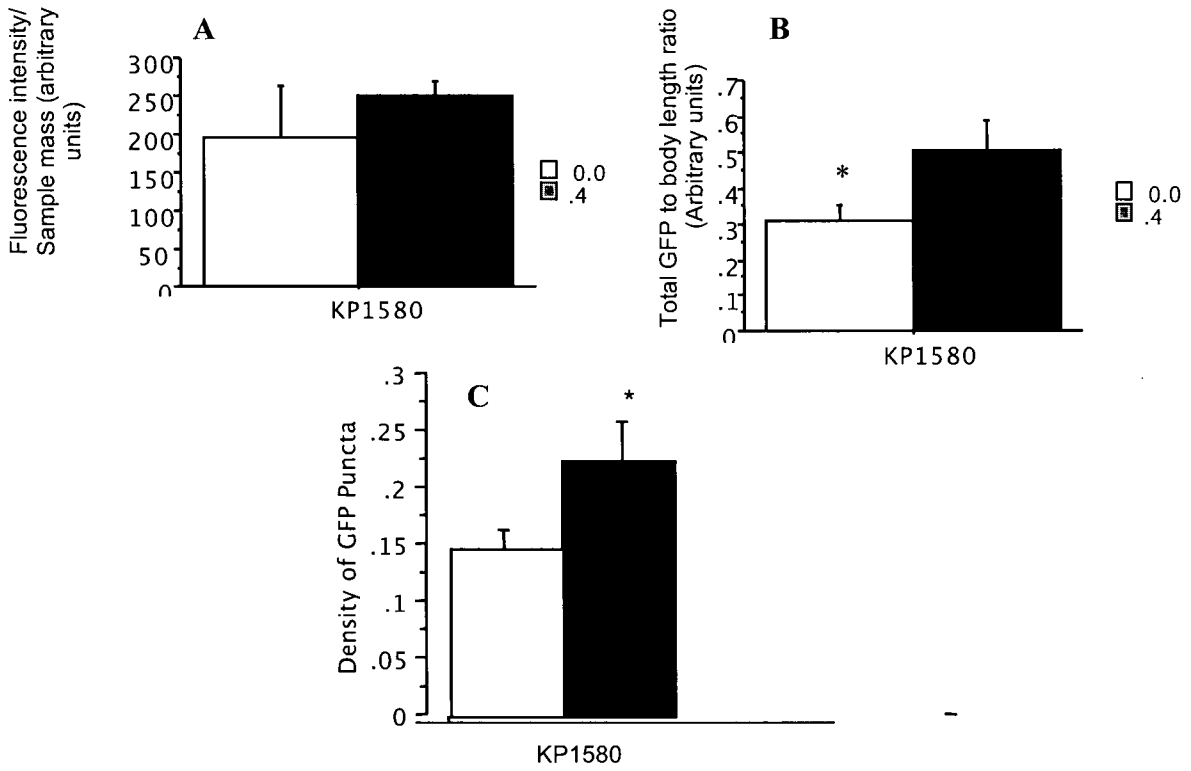


Figure 3.3.3.2 Chronic Ethanol Exposure Decreased the Ratio of GLR-1::GFP Expression to Body Length in KP1580 worms.
(A) Using the SpectraMax 96 well fluorescence plate reader it was found that chronic exposure to 0.4M ethanol did not result in KP1580 worms having a significantly lower GLR-1::GFP fluorescence to mass ratio than untreated controls. (B) Confocal images of the posterior ventral nerve cord of KP1580 worms chronically exposed to 0.4M ethanol revealed a significant main effect on the ratio of GLR-1::GFP expression to body length in comparison to untreated controls. Worms chronically exposed to 0.4M ethanol had a significantly larger ratio than untreated controls. (C) Chronic exposure to 0.4M ethanol resulted in worms expressing significantly more GLR-1::GFP puncta along the ventral nerve cord. (* indicated significance $p < 0.05$).

3.4 DISCUSSION

In this chapter I report a series of experiments designed to characterize the effects of chronic ethanol exposure on the *C. elegans* nervous system and behaviour. I investigated the global effect ethanol exposure had on the *C. elegans* nervous system and

found that the expression levels of GFP driven by two nervous system specific genes was decreased as a result of chronic ethanol exposure using two different investigative techniques (Figure 3.3.1.1 and Figure 3.3.1.2). This finding supports the hypothesis that chronic exposure to ethanol results in a nervous system specific effect on *C. elegans* in much the same manner it results in a lowered brain to body weight ratio in higher order model systems. After finding that chronic ethanol exposure altered the *C. elegans* nervous system I investigated two well-studied behaviours to determine whether they showed deficits resulting from chronic exposure to ethanol. I found that the tap-withdrawal response was attenuated as a result of chronic exposure to ethanol (Figure 3.3.2.1 and Figure 3.3.2.2). I also found that although ethanol exposure lead to worms that moved more slowly than control worms both on and off food (Figure 3.3.2.3), worms exposed to ethanol still showed a proportionate basal slowing response (Figure 3.3.3.4). In order to investigate potential causes of the decreased tap-withdrawal response I investigated ethanol's effects on the mechanosensory circuit that co-ordinates the tap withdrawal response as well as the expression of a specific post-synaptic component, GLR-1, that is required for the glutamatergic neurotransmission that mediates the necessary signaling. In this set of experiments I found that chronic exposure to ethanol affected the development of the mechanosensory circuit (Table 3.3.3.1) and that the expression of GLR-1::GFP was increased in KP1580 worms chronically exposed to ethanol (Figure 3.3.3.2).

3.4.1 Discussion of Findings From Experiment Set #1.

In this set of experiments I found that the expression of GFP driven by two different pan-neuronal promoters decreased following chronic exposure to ethanol. For

this set of experiments, I employed two different techniques, Epifluorescent imaging and the SpectraMax plate reader. I chose these two techniques because in both the NW1229 and DP132 strains GFP is promoter driven were engineered to express throughout the cell bodies and processes of all neurons. Thus, these two constructs were useful for identifying a change in the size of the entire nervous system as a result of chronic exposure. When measured with the SpectraMax plate reader, GFP intensity of the control DP132 group was much less than that of the control NW1229 group, suggesting that the promoter driving GFP expression is stronger than the pUNC-119 promoter driving GFP expression in the DP132 strain.

In this set of experiments I developed a novel way to examine the effects of a drug, in this case, ethanol on the *C. elegans* nervous system using the SpectraMax plate reader. The SpectraMax plate reader has previously been used to measure changes in GFP fluorescence intensity in cell-culture (Parker et al. 2004), but to my knowledge has not before been used to measure a change in GFP fluorescence intensity in *C. elegans*. The validity of measurements made by the SpectraMax plate reader was confirmed using Epifluorescent and Confocal imaging techniques. Both these microscopy techniques have been used to quantitatively measure changes in gene expression before (Rose et al. 2005). The findings from the Epifluorescence (Figure 3.3.1.1) and Confocal imaging (Figure 3.3.3.2) analysis support further use of the SpectraMax plate reader for the analysis of changes in GFP fluorescence intensity.

In these experiments I used two different techniques to try and estimate the size of the effect of chronic ethanol exposure on the size of the *C. elegans* nervous system and in

both instances generated data supporting the hypothesis that the relative size of the *C. elegans* nervous system is reduced due to chronic exposure to ethanol.

3.4.2 Discussion of Findings From Experiment Set #2

This set of experiments was designed to investigate the effects of chronic exposure to ethanol on two different behaviours; the tap withdrawal response and the basal slowing response. In the first experiments I found that the tap withdrawal response is attenuated as a result of exposure (Figure 3.3.2.1). First, I found that 4-day old 0.4M treated worms had smaller initial responses to tap than age-matched untreated controls. To address the possibility that 0.4M treated worms had smaller reversal magnitudes due to the fact they were smaller than age matched controls, I then compared the reversal magnitudes of size matched 2 day-old untreated control worms to that of 4 day-old 0.4M treated worms. In this experiment I again found that 4-day old 0.4M treated worms had smaller reversal responses than size matched 2 day-old controls (Figure 3.3.2.2). This finding suggests that the developmental delay imposed by chronic ethanol exposure was not solely responsible for the observed attenuation of the tap withdrawal response in the first experiment. This finding instead suggests that chronic exposure to ethanol had an effect on the nervous system and thus on the behaviour.

However, there are confounds in this experiment that remain unaccounted for. By size matching 2 day-old control worms to 4 day-old 0.4M treated worms, I cannot be sure that I am comparing worms that are at the same developmental stage, I can only make a rough approximation. To fully address this issue I would have to compare the reversal responses of developmentally stage-matched worms. To do this I would need to characterize the exact magnitude of delay caused by chronic exposure to ethanol at each

stage of development. One way to developmentally match worms treated with 0.4M ethanol to untreated control worms would be to determine the effect chronic ethanol exposure has on the timing each larval transition. The passage of *C. elegans* from one larval stage to another is marked by the shedding of the cuticle that surrounds the worm as well as a pronounced change in the structure of the gonad (Wood, 1988). A second way to developmentally stage worms exposed chronically exposed to ethanol would be to use a GFP marker that is expressed in the gonad and carefully characterize the gonadal development of worms chronically treated with 0.4M ethanol and compare the rate of development to that of untreated controls.

In Experiment 2.3.1.5 I found that chronic exposure to ethanol decreased the rate of pharyngeal pumping in worms. This may have lead to a decrease in energy intake that may have affected the 0.4M treated worms' response to tap. As mentioned in the discussion of Chapter I, two different experiments could be designed to dissociate the effects of ethanol on the nutritional intake of worms those directly produced by ethanol exposure. In the first I could compare the responses to tap of several strains of *C. elegans* with the "EAT" phenotype (Avery, 1993) to that of N2 worms exposed to ethanol to see whether nutritional deprivation contributed to the observed findings. In the second experiment it would be possible to raise N2 worms on bacteria of poor nutritional quality for their entire lifespan and then compare their responses to tap to that of N2 worms chronically exposed to ethanol. Both these potential experiments would help to dissociate the effect ethanol is having on the nutritional intake of worms from the specific effect it is having on worms to cause the observed behavioural deficit.

In these studies I have not examined the effects of chronic ethanol exposure on the musculature of *C. elegans*. It is also possible that chronic exposure to ethanol may lead to abnormal muscle structure and/or function in *C. elegans*. If such were the case, this could be another possible explanation for the observed behavioural deficit; worms do not have the muscle strength to respond to tap. This issue could be addressed by examining the effects of chronic ethanol exposure on *C. elegans* muscle physiology using a muscle promoter driven GFP expressing strain of worm. This would be a simple way to assess whether ethanol has an effect on the musculature of *C. elegans*.

I observed that although chronic ethanol exposure lead to worms that made fewer body bends per second than control worms (Figure 3.3.2.3), the basal slowing response was left intact. In these experiments, regardless of ethanol treatment, the proportional decrease in the number of body bends made per second after worms were transferred from a food to a food environment compared to the number of body bends made per second after they were moved from a food to a no food environment was not significantly different regardless of ethanol treatment (Figure 3.3.2.4). The tap withdrawal response is initiated by electrical signaling between gap junctions within the mechanosensory circuit. But this electrical signaling is modulated by glutamatergic neurotransmission (Rose et al. 2003). In contrast, the basal slowing response is governed by dopaminergic neurotransmission (Sawin et. al, 2000). This finding suggests the possibility that chronic ethanol exposure may be having a specific effect on the worm nervous system by altering one neurotransmitter system, glutamate, and not the other, dopamine. This can be further investigated by studying the effects of ethanol exposure on the expression of GFP

attached to other genes involved in glutamatergic and/or dopaminergic neurotransmission.

3.4.3 Discussion of Findings From Experiment Set #3

This series of experiments was designed to investigate the potential causes of the observed attenuation of the tap-withdrawal response on the level of the cell and the gene. In the first set of experiments I found that chronic exposure to ethanol affected the post-embryonic development of the mechanosensory circuit. However, the severity of the effect of ethanol on the development of the mechanosensory circuit cannot be fully assessed from the observations made in this experiment. In order to fully measure the effect of chronic ethanol exposure on the timing of AVM and PVM differentiation and establishment of the mechanosensory circuit, it would be necessary to mark the exact time that AVM and PVM first appeared in control worms and then compare the length of time it takes for the same observation to be made in worms chronically exposed to 0.4M ethanol. However, the observation that ~ 67% of 4 day-old MEC-4::GFP-rol-6 worms had six GFP expressing neurons including AVM and PVM (Table 3.3.1.1) would suggest the possibility that same proportion of worms used in the tap withdrawal experiments 3.3.2.1 and 3.3.2.2 would have had all six mechanosensory neurons as well. In experiments 3.3.2.1 and 3.3.2.2 it was observed that 8/15 (~54%) and 9/16 (~56%) of worms reversed to tap respectively. One explanation for why the remaining worms in those two treatment groups did not respond to tap was that they lacked functional AVM and PVM neurons.

In experiment 3.3.3.1 I observed that ~67% of MEC-4::GFP-rol-6 had visible AVM and PVM neurons. Despite AVM and PVM being present at hour 96 of this

experiment, there is no way to state whether or not those neurons were functional. In addition to differentiating in the presence of ethanol, AVM and PVM also had to extend processes in order to connect with the interneurons to form a functional mechanosensory circuit. In this study, the morphology and connectivity of these neurons were not analyzed. There still exists the possibility that although ~67% worms had AVM and PVM present by day-4 of development, these two neurons were not fully functioning. This issue could be addressed on a surface level by comparing the morphology of the extended processes from AVM and PVM in worms chronically treated with ethanol, or it could be addressed more accurately by comparing the electrophysiological activity of these two neurons between control worms and worms chronically treated with ethanol. Although it appears as though the development of the mechanosensory circuit is affected by chronic exposure to ethanol, the extent to which this alteration contributes to the observed behavioural deficits cannot be determined until further experimentation is conducted.

One way to assess the post-embryonic delay in mechanosensory circuit development imposed by chronic ethanol exposure is to compare it to the delay in the development of the reproductive system. At hour 96 of development the gonadal region of worms exposed to 0.4M ethanol was comparable to that of 2 day-old 0.0M control worms (Figure 3.3.2.1). However, at hour 96 of development, ~ 67% of MEC-4::GFP-rol-6 worms had the same number of mechanosensory neurons (6) as 96 hour untreated controls. When comparing these two findings it appears that chronic exposure to ethanol is delaying the development of the reproductive system more than the development of the mechanosensory circuit. This observation would suggest that chronic exposure to

ethanol can have differential effects on the development of different organ systems within *C. elegans*.

To further investigate the potential causes of the attenuation of the tap-withdrawal response due to chronic ethanol exposure, I investigated the expression level of a gene that plays a critical role in the mechanosensory circuit. Specifically, I quantified changes in the expression of GLR, a non-NMDA type glutamate receptor sub-unit, tagged to GFP. I found that chronic exposure to ethanol resulted in an increase in GLR-1::GFP experiment 3.3.3.2. Although there was an observed change in the expression of a specific component of the glutamatergic pathway, I cannot be sure that this resulted in a change in the efficacy of signal strength between mechanosensory neurons and interneurons. To do so would require measuring a change in the excitatory response of worm interneurons as a direct consequence of chronic exposure to ethanol using electrophysiological analysis.

The observation that GLR-1::GFP expression increases as a result of chronic ethanol exposure might provide a possible explanation for why worms that show decreased tap withdrawal responses do not respond to tap. However, in two papers published by the Rankin Lab investigating the *C. elegans* tap withdrawal response, there was a correlation between the level of *glr-1* expression and the magnitude to the tap withdrawal response (Rose et al., 2003, Rose et al. 2005). The data presented in this report is the first example where an increase in *glr-1* expression does not correlate with an increased response to tap. From this finding I would hypothesize that the change in *glr-1* expression due to chronic ethanol exposure is not the underlying cause of the attenuated tap withdrawal response observed. Instead it is more likely that the changes in

the post-embryonic development, connectivity and functioning of the mechanosensory neurons due to ethanol exposure contributed to the observed behavioural deficit.

In this set of experiments I only looked at two components of the neural circuit underlying the response to tap and found that ethanol alters both. Although it would seem more plausible that the effect of chronic ethanol exposure on the development and connectivity of the mechanosensory circuit is the underlying cause for the observed change in behaviour it is still possible that there are nervous system components including the interneurons, motorneurons and proteins within in the signaling cascade that mediate the behaviour that are affected by exposure to ethanol that also contribute to the attenuated behavioural.

These two experiments provide further evidence supporting the use of *C. elegans* as a model for study in the field of FASD research. In these experiments I have identified a behaviour affected by chronic ethanol exposure and have relied on an established database of knowledge to investigate some of the specific cellular mechanisms that ethanol is interfering with to produce the behavioural deficit. As mentioned in the discussion of Chapter I, *C. elegans* shares a great deal of genetic homology with humans (Waterson and Sulston, 1995). In this set of experiments I have provided an example of how it is possible to use this system to identify specific nervous system gene products that are affected by ethanol exposure to cause aberrant behaviour. It is important to use findings made in simple systems such as the ones made here as pilot data for future studies in higher order systems including mammals.

One other point that needs to be discussed in concluding this chapter is the variable nature of the effects ethanol has on the *C.elegans* behaviour. In the set of

experiments preformed in this chapter I found that ethanol exposure increased the expression of a nervous system specific gene, in this case, *glr-1,* while decreasing the expression of a second nervous system specific gene, *unc-119.* Also, I observed that chronic exposure to ethanol appears to slow the development of the reproductive system more so than the development of the mechanosensory circuit. On a behavioural level, I found that chronic exposure to ethanol attenuated one behaviour, the tap withdrawal response and left a second behaviour, the basal slowing response, intact. Ethanol exposure does not appear to result in a consistent affect, an increase or a decrease, in the expression of all nervous system specific genes, nor does it produce a consistent attenuation or enhancement of all behaviours. Rather, the data presented in this report suggests that ethanol exposure appears to effect different genes and behaviours in different ways. For this reason, in future experiments it will be important to investigate the effects of chronic ethanol exposure on different aspects of *C. elegans* nervous system function and behaviour without the preconceived notion that exposure to ethanol results in a consistent effect.

Chapter IV

GENERAL DISCUSSION

4.1 Discussion of the Significance of Thesis Findings

This thesis was written with the aim of achieving two separate objectives. The first was to investigate the effects of ethanol on the development of *C. elegans* in order further our understanding of the effects of ethanol on biological systems. The second was to investigate the feasibility of using *C. elegans* as a model system for study in the field of FASD research. To meet these separate objectives I examined the effects of ethanol on *C. elegans* at two separate levels. In the first part of this study (Chapter II) I investigated the effects of ethanol exposure at different life stages on the basic physical development and life history of *C. elegans.* In the second part of this study (Chapter III) I engaged in a more specific examination of the effect of ethanol on the neurobiology and behaviour of *C. elegans*. In the two separate investigations in this thesis I worked towards meeting the stated objectives of this study. In the second chapter of the thesis I showed that chronic exposure to ethanol affects the basic development and life history of *C. elegans.* The findings reported in Chapter II are significant in the sense that they contribute to our overall understanding of how *C. elegans* is affected by this drug. Also, prior to this thesis, it was not known whether or not the *C. elegans* eggshell was permeable to ethanol. We now know that it is and have a better understanding of the concentrations of ethanol that will permeate the eggshell and affect the developing embryo inside. The findings from Chapter II also contribute to meeting the second objective of this thesis, developing *C. elegans* as a model for study in the field of FASD. In this chapter I developed a protocol for exposing developing *C. elegans* embryos to ethanol that is sufficient to

produce a marked effect on the physical morphology of the larvae that hatch from eggs that were exposed during embryonic development. This finding is of significance due to its potential applications for later study. In Chapter II I only gave a brief description of how exposure to ethanol during ex-utero embryonic development affected *C.elegans*. It will now be important to further characterize the effects of this exposure protocol on *C. elegans*. It will be important to do so by fully describing the observed dysmorphologies and identifying whether there are consistent developmental abnormalities incurred as a consequence of exposure. It will also be of great importance to investigate how this exposure protocol affects the later development of *C. elegans*. In my study I did not follow worms exposed to ethanol during ex-utero embryonic development in a longitudinal study. In future experiments it would be important to do so in order to characterize what effect this exposure protocol has on the later development and life history of *C. elegans*. In addition, it will be of great interest to examine any effects this exposure protocol has on the development of the *C. elegans* nervous system using the broad spectrum of investigative techniques that exist to do so. In conclusion the findings from Chapter II have made a significant contribution to our understanding of how ethanol affects *C. elegans* as well as opened the door to many future experiments investigating the effects of embryonic exposure to ethanol in this system.

In Chapter III I examined the effects of ethanol on specific aspects of the nervous system and behaviour of *C. elegans*. In this chapter, as in Chapter II, I have reported data that contributes to meeting each of the stated objectives of this thesis. In this Chapter III I showed that the SpectraMax fluorescence plate reader can be used to quantify changes in GFP fluorescence intensity in *C. elegans* and confirmed the validity of this data using

an established microscopy techniques. The development of this novel technique is significant in that it can be used in future studies in which it is necessary to measure changes in GFP fluorescence intensity in this system.

In addition to developing a novel investigative technique, I also showed that the developing nervous system is sensitive to the effects of ethanol and that the effects of ethanol on the developing nervous system correlate with behavioural deficiencies in this system. In doing so I provided an example of how *C. elegans* can be used in the field of FASD research. Here I presented a behavioural effect of ethanol and simultaneously identified that ethanol produced disruptions in the cellular mechanisms mediating the behaviour. This is a prime example of this system can be used to describe the behavioural effects of ethanol while at the same time investigate the disrupted cellular mechanisms that cause the observed behavioural effects. The findings presented in Chapter II, as with the findings from Chapter I, contribute data that clearly serves to meet the two stated objectives of this thesis- furthering our understanding of the effects of ethanol on *C. elegans* and developing *C. elegans* as a model for study in the field of FASD research.

The most important contribution this thesis makes is the development of a novel protocol with which to study the effects of ethanol on the developing nervous system. In Chapter I I developed a novel method for exposing developing *C. elegans* eggs to ethanol resulting in a clear affect on the normal development of the embryo inside. In Chapter II I provided an example of how *C. elegans* can be used for the study of the effects of ethanol on the neurobiology and behaviour. In the future it will be possible to examine

the effects of embryonic exposure on the nervous system development and later behaviour of *C. elegans.*

Using this simple invertebrate system I have been able describe a comprehensive model of the effects of early ethanol exposure on physical and neural development and behaviour. The findings reported here open the door to a new line of study aimed to further characterize the effects of ethanol on this developing system. In further developing this resource rich model, we will have an opportunity to improve our overall understanding of the effects of early ethanol exposure on development. The justification for doing so lies in the possibility the data we generate and findings we make will provide insight into how ethanol affects the development and nervous system of higher order species. Using findings made in this model as pilot data for further study in higher order systems, there exists the possibility that we will be able to assist in the development of behavioural and therapeutic strategies aimed at ameliorating the deficits associated with early ethanol exposure in higher order systems including humans.

4.2 Constraints, Limitations and Applications

In this study I have only scratched the surface of an investigation into the effects of ethanol on the physical development, nervous system development and behaviour in *C. elegans*. It is important to recognize that my study has been a sampling of the effects of ethanol on a number of different aspects of *C. elegans* biology rather than an in depth investigation of one single aspect. From the data presented here, no broad sweeping conclusions can be made with regard to the effects of ethanol on the *C. elegans* nervous system. It can only be stated that ethanol does affect *C. elegans* nervous system

development and behaviour and that further investigations into exactly how ethanol exposure does so are warranted.

Furthermore, despite the fact that *C. elegans* and humans share a great deal of genetic homology, it is important to keep in mind that this does not necessarily mean that exposure to ethanol will result in the same physiological response. A given finding made in *C. elegans* does not necessarily predict that the same finding will be made in humans. Humans are not a clonal population of individuals, but rather have a broad range of individual differences. Humans, unlike laboratory worms live in a complex environment where they are not simply affected by controlled variation in their environment. It is important to recognize that FASD in humans is the result of a combination of effects that ethanol exposure produces on the basic physiology, immune function, endocrine system and nervous system development of developing fetuses. *C. elegans* is a simple invertebrate that cannot be used to fully appreciate the effects of ethanol on the development of complex organism such as humans.

The importance and significance of findings made through the study of *C. elegans* in this field will come from the high throughput nature of studies employing *C. elegans*. In the future it will be possible to use *C. elegans* to test many hypotheses regarding the effects of ethanol on different aspects of biology in pilot studies in order to identify which hypothesis are worth investigating in greater depth in higher order systems. In conclusion, despite the fact that *C. elegans* lacks the complexity of higher order systems such as rodents and humans, further study of the effects of ethanol on this system are warranted by virtue of the fact it can be used to explore hypotheses that are either not testable in higher order systems or too costly to investigate. Insight into the

ways ethanol affects development in *C. elegans* may lead to novel therapeutic approaches in treatment of children exposed to ethanol prenatally.

References

1) Abel, E.L and Berman, R.F. 1994. Long-term behavioural effects of prenatal alcohol exposure in rats. *Neurotoxicology and Teratology.* 16(5); 467-470.

2) Avery, L. 1993. The genetics of feeding in *Caenorhabditis elegans. Genetics.* 133: 897-917.

3) Bargmann, C.I. 1998. Neurobiology of the *Caenorhabditis elegans* genome. *Science.* 282; 2028-2033.

4) Besedovsky HO, del Rey A. 1996. Immune-neuro-endocrine interactions: facts and hypotheses. *Endocrinology Review.* 17(1):64-102.

5) Berman, R.F. and Hannigan, J.H. 2000. Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. *Hippocampus.* 10(1): 94-110.

6) Billota, J., Barnett, J.A., Hancock, L and Saszik, S. 2004. Ethanol exposure alters Zebrafish development: A novel model of fetal alcohol syndrome. *Neurotoxicology and Teratology.* (e-pub ahead of print)

7) Bonthius, D.J. and West, J.R. 1991. Permanent neuronal deficits in rats exposed to alcohol during the brain growth spurt. *Teratology.* 44; 147-163.

8) Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics, 77, 71-94.

9) Byrnes, M.L., Reynolds, J.N. and Brien, J.F. 2001. Effect of prenatal ethanol exposure during the brain growth spurt of the guinea pig. *Neurotoxicology and Teratology.* 23; 355-364.

10) Cartwright, M.M. and Smith, S.M. 1995. Stage-dependent effects of ethanol on cranial neural crest cell development: partial basis for the phenotypic variations observed in fetal alcohol syndrome. *Alcohol Clinical and Experimental Research.* 19(6):1454-62.

11) Cartwright, M.M, and Smith, S.M. 1995. Increased cell death and reduced neural crest cell numbers in ethanol-exposed embryos: partial basis for the fetal alcohol syndrome phenotype. *Alcohol Clinical and Experimental Research.* 19(2):378-86.

12) Carneiro, L.M., Diogenes, J.P, Vasconcelos, S.M., Argao, G.F., Noronha, E.C., Gomes, P.B. and Viana, G.S. 2005. Behavioural and neurochemical effects of rat offspring after prenatal exposure to ethanol. *Neurototxicology Teratology.* (Epub ahead of print)

13) Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. 1994. Green fluorescent protein as a marker for gene expression. *Science.* 263; 802-805.

14) Charness, M.E., Safran, R.M. and Perides, G. 1994. Ethanol inhibits Neural Cell-Cell adhesion. *Journal of Biological Chemistry.* 269(2); 9304-9309.

15) Chiba, C. and Rankin, C.H. 1990. A developmental analysis of spontaneous and reflexive reversals in the nematode *Caenorhabditis elegans. Journal of Neurobiology.* 21: 543-554.

16) Chitwood, B.G. and Chitwood, M.B. 1974. *Introduction to nematology.* University Park Press. Baltimore Maryland.

17) Clarren, S.K., Alvord,E.J., Sumi, S.M., Streissguth, A.P. and Smith D.W. 1978. *Journal of Pediatrics.* 92; 64-67.

18) Cudd, T.A. 2005. Animal model systems for the study of alcohol teratology. *Experimental biological medicine.* 230(6); 389-393.

19) Davies, A.G., Bettinger, J.C., Theile, T.R., Judy, M.E. and McIntire, S.L. 2004. Natural variation in the *npr-1* gene modifies ethanol responses of wild strains of *C. elegans. Neuron.* 42; 731-743.

20) Davies A.G., Shimomura-Pierce, J.T., Kim, H., VanHoven, M.K., Theile, T.R., Bonci, A., Bargmann, C.I. and McIntire S.L. 2003. A central role of the BK Potassium channel in behavioural responses to ethanol in C. elegans. *Cell.* 115: 655-666.

21) Desai, C. and Horvitz, H.R. 1989. *Caenorhabditis elegans* mutants defective in the functioning of the motor neurons responsible for egg-laying. *Genetics.* 121:703-721.

22) Diamond, I. and McIntire, S.L. 2002. Alcohol Neurotoxicity. In diseases of the nervous system: Clinical Neuroscience and Therapeutic Principles, J.C. McAurthur, ed. (Cambridge: Cambridge University Press), pp 1814-1826.

23) Diaz and Samson. 1980. Impaired Brain growth in neonatal rats exposed to ethanol. *Science.* 208 (16): 751-753.

24) Driscoll, M. and Kaplan, J. 1997. Mechanotransduction. In Riddle, Blumenthal, Meyer and Preiss (Eds.), The Nematode *Caenorhabditis elegans* II (pp. 645-678). Cold Spring Harbor Laboratory: Cold Spring Harbor.

25) Ernhart et al. 1995. Neonatal diagnosis of Fetal Alcohol Syndrome (FAS): Not necessarily a hopeless prognosis. *Alcoholism: Clinical and Experimental Research.* 19 (6): 1550-1557.

26) Gabriel, K., Hoffman, C., Glava, M. and Weinberg, J. 1998. The hormonal effects of alcohol use on the mother and fetus. *Alcohol health and research world.* 22(3); 170-177.

27) Giberson, P.K. and Weinberg, J. 1992. Fetal alcohol syndrome and functioning of the immune system. *Alcohol health and research world.* 16(1); 29-38.

28) Glasner JD, Kocher TD and Collins JJ (1995) *Caenorhabditis elegans* contains genes encoding two new members of the Zn- containing alcohol dehydrogenase family . *Journal of Molecular Evolution.* 41;46-53.

29) Gonzales, R.A. and Hoffman, P.L.. 1991. Receptor-gated ion channels may be selective CNS targets for ethanol. *Trends in Pharmacological Science.* 12(1);1-3.

30) Goodlett, C.R and Horn, K.H. 2001. Mechanisms of alcohol-induced damage to the developing nervous system. *Alcohol Research Health.* 25(3):175-84.

31) Grant, K.A., Choi, E.Y. and Samson, H.H. 1983. Neonatal ethanol exposure: effects on adults behaviour and brain growth. *Pharmacology Biochemistry and Behaviour.* 18(1); 331-336.

32) Green JT. (2004) The effects of ethanol on the developing cerebellum and eyeblink classical conditioning. Cerebellum. 3(3):178-87.

33) Hansen M., Hsu, A.L., Dillin, A. and Kenyon, C. 2005. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a Caenorhabditis elegans genomic RNAi screen. *PLoS Genet.* 1(1):119-28.

34) Hoffmann, C.E., Simms, W., Yu, W.K. and Weinberg, J. 2002. Prenatal ethanol exposure in rats alters serotonergic-mediated behavioural physiological function. *Psychopharmacology.* 161; 379-386.

35) Hoffman, H.A. 2003. Functional Genomics of Neural and behavioural plasticity. *Genomics and Plasticity.* 272-281.

36) Ikonomidou, C., Bittigau, P., Ishimaru, M.J., Wozniak, D.F., Koch, C., Genz, K., Price., M.T., Stefovska, V., Horster, F., Tenkova, T., Dikranian, K. and Olney, J.W. 2000. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 287(5445); 947-948.

37) Iqbal, U., Rikhy, S., Dringenberg, H.C., Brien, J.F. and Reynolds, J.N. 2006. Spatial learning deficits induced by chronic prenatal ethanol exposure can be

overcome by non-spatial pre-training. *Neurotoxicology and Teratology.* 28(3):333-41.

38) Jing, H. and Li, Y. 2004. Effects of ethanol on mouse brain embryonic development and Heat Shock Protein 73 expression. *Toxicology in-vitro.* 18(5); 601-607.

39) Jones, K.L., Smith, D.W., Ulleland, C.N. and Streissguth, A.N. 1973. Pattern of malformation in offspring of chronic alcoholic mothers. *The Lancet.* Saturday June 9th;1267-1271.

40) Kruger, H,, Wilce,P.A. and Shanley, B.C. 1993. Ethanol and protein kinase C in rat brain. *Neurochemistry International.* 22(6):575-581.

41) Kubiak, T.M., Larsen, M.J., Nulf, S.C., Zantello, M.R., Burton, K.J., Bowman, J.W., Modric, T. and Lowery, D.E. 2003. Differential Activation of "social" and "solitary" variants of the *Caenorhabditis elegans* G-protein coupled receptor NPR-1 by its cognitive ligand AF9. *Journal of Biological Chemistry.* 278: 33724-33729.

42) Liesi, P. 1997. Ethanol-exposed central neurons fail to migrate and undergo apoptosis. *Journal of Neuroscience Research.* 48; 439-448.

43) Mattson S.N., Riley E.P. 1998. A Review of the Neurobehavioural Deficits in Children with Fetal Alcohol Syndrome or Prenatal Exposure to Alcohol. *Alcohol Clinical and Experimental Research* 22 (2): 279-294

44) Mattson, S.N. and Riley, E.P. 1999. Implicit and explicit memory functioning in children with heavy prenatal alcohol exposure. *Journal of International Neuropsychology.* 5; 462-471.

45) Mattson, S.N., Schoenfield, A.N. and Riley, E.P. 2001. teratogenic effects of alcohol on brain and behaviour. *Alcohol research and Health.* 25(3); 189-191

46) Medina, A.E., Krahe, T.E. and Ramoa, A.S. 2005. Early alcohol exposure induces persistent alteration of cortical columnar organization and reduced orientation selectivity in the visual cortex. *Journal of Neurophysiology.* 93(3):1317-25.

47) Miller, M.W. 1988. Effects of prenatal exposure to ethanol on the development of the cerebral cortex: I. Neuronal Generation. *Alcoholism: Clinical and Experimental Research.* 12(3); 440-449.

48) Miller, M.W. 1993. Migration of cortical neurons is altered by gestational exposure to ethanol. *Alcoholism: Clinical and Experimental Research.* 17(2); 304-314.

49) Miller, M.W. 2003. Balance of cell proliferation and death among dynamic populations: A mathematical model. *Journal of neurobiology.* 57(2); 172-182.

50) Olney, J.W. 2004. Fetal alcohol syndrome at the cellular level. *Addiction Biology.* 9(2):137-49.

51) Parker, L.H., Schmidt, M., Jin, S.W., Grray, A.M., Beis, D., Phan, T., Frantz, G., Palmieri, S., Hillan, K., Stainieer, D.Y.R., de Suavage, F. J. and Ye, W. 2004. The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. *Nature.* 458: 754-759.

52) Peoples, R.W., Li, C. and Weight, F.F. 1996. Lipid vs protein theories of alcohol action in the nervous system. *Annual Review of Pharmacological Toxicology.* 36:185-201.

53) Pierce, D.R. and West, J.R. 1986. Blood alcohol concentration: A critical factor for producing Fetal Alcohol Effects. *Alcohol.* 3; 269-272.

54) Pierce, D.R., Goodlet, C.R. and West, J.R. 1989. Differential neuronal cell loss following early postnatal alcohol exposure. *Teratology.* 40; 113-126.

55) Rankin, C. H. (2003) From Gene to Identified Neuron to Behavior, *Nature Reviews Genetics*, 3, 622-630.

56) Reiche, E.M.V., Nunes, S.O.V., Morimoto, H.K 2004. Stress, depression, the immune system and cancer. *The Lancet- Oncolgy.* 5; 617-625.

57) Rice, D. and Barone, S. Jr. 2000. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. *Environmental Health Perspectives.* Jun;108 Suppl 3:511-33.

58) Riley, E.P. and McGee, C.L. 2005. Fetal alcohol spectrum disorders: An overview with emphasis on changes in brain and behaviour. *Experimental biological medicine.* 230(6); 357-365.

59) Rogers, C., Reale, V., Kim, K., Chatwin, H., Li, C., Evans, P. and De Bono, M. 2003. Inhibition of *Caenorhabditis elegans* social feeding by FMFRaide-related peptide activation of NPR-1. *Nature Neuroscience.* 6; 1178-1185.

60) Rongo C. and Kaplan, J.M. 1999. CaMKII regulates the density of central glutamatergic synapses *in-vivo. Nature.* 402: 195-199.

61) Rose, J. K. & Rankin, C.H. (2001) Analyses of Habituation in C. elegans, *Learning and Memory*, 8, 63-69.

62) Rose, J.K., Kaun, K.R. and Rankin, C.H. 2002. A New group-training procedure for habituation demonstrates that pre-synaptic glutamate release contributes to long-term memory in *Caenorhabditis elegans. Learning and Memory.* 9; 130-137.

63) Rose, J.K., Kaun, K.R., Chen, S.H. and Rankin, C.H. 2003. GLR-1, a non-NMDA glutamate receptor homolog, is critical for long-term memory in *Caenorhabditis elegans. Journal of Neuroscience.* 22;23(29):9595-9.

64) Rose, J.K,, Sangha, S., Rai, S., Norman, K.R. and Rankin C.H. 2005. Decreased sensory stimulation reduces behavioural responding, retards development and alters neural connectivity in *Caenorhabditis elegans. The Journal of neuroscience.* 25(31): 7159-7168.

65) Roselle, G.A. 1992. Alcohol and the immune system. *Alcohol health and research world.* 16-22.

66) Sawin, E.R., Ranganathan, R. and Horvitz, H.R. 2000. *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. *Neuron.* 26:619-631.

67) Simmons, R.W., Wass, T., Thomas, J.D. and Riley, E.P. 2001. Fractionated simple and choice reaction time in children with prenatal exposure to ethanol. *Alcohol clinical and Experimental Research.* 26; 1412-1419.

68) Smith, S.M. 1997. Alcohol-induced cell death in the embryo. *Alcohol Health and Research World.* 21(4); 287-297.

69) Streissguth et al. 1980. Teratogenic effects of alcohol in humans and laboratory animals. *Science.* 209 (18): 353-361.

70) Stratton, K., Howe, C., Battaglia, F. Editors (1996). Fetal Alcohol Syndrome: Diagnosis, Epidemiology, Prevention, and Treatment Report of a study by a committee of the Institute of Medicine, Division of Biobehavioral Sciences and Mental Disorders.. National Academy Press, pg 33-50.

71) Sulik, K.K., Johnston, M.C. and Webb, M.A. 1981. Fetal alcohol syndrome: Embryogenesis in a mouse model. *Science.* 214; 936-938.

72) Sulton, J.E. and Horvitz, H.R. 1977. Post-embryonic cell lineages of the nematode *Caenorhabditis elegans. Developmental Biology.* 78:577-597.

73) Sulston, J.E., Schierenberg, E., White, J.G. and Thomson, J.N. 1983. The embryonic cell lineage of the nematode *Caenorhabditis elegans. Developmental Biology.* 100: 64-119.

74) Thompson, G. and De Pomerai, D.I. 2005. Toxicity of Short-Chain Alcohols to the Nematode *Caenorhabditis elegans:* A Comparison of Endpoints. *Journal of Biochemistry and Molecular Toxicology.* 19(2): 87-95.

75) Tran, T.D., Croinse, K., Marino, M.D., Jenkins, W.J. and Kelly, S.J. 2000. Critical periods for the effects of alcohol exposure on brain weight, body weight, activity and investigation. *Behavioural brain research.* 116; 99-110.

76) Tran, T.D., Kelly, S.J. 2003. Critical periods for ethanol-induced cell loss in the hippocampal formation. *Neurotoxicology and Teratology.* 25(5):519-28.

77) Venezuela, C.F. 1997. Alcohol and neurotransmitter interactions. *Alcohol health and research world.* 21(2); 144-148.

78) Vorhees, C.V. and Fernandez, K. 1986. Effects of short-term prenatal alcohol exposure on maze activity, and olfactory orientation performance in rats. *Neurobehavioural toxicology and teratology.* 8; 23-28.

79) Wang, X., Williams, E., Haasch, M.L. and Dasmahapatra, A.K. 2006. Japanese Medaka (*Oryzias latipes):* developmental model for the study of alcohol teratology. *Birth Defects Research* (part B) 77: 29-39.

80) Waterson, R. and Sulston J.E. 1995. The genome of *Caenorhabditis elegans. Proceedings of the National Academy of Sciences.* 92: 10836-10840.

81) West, J.R., Goodlet, C.R., Bonthius, D.J., Hamre, K.M. and Marcussen, B.L. 1990. Cell population depletion associated with fetal alcohol brain damage: Mechanisms of BAC-dependent cell loss. *Alcoholism: Clinical and Experimental Research.* 14(6); 313-818.

82) Wicks, S.R. and Rankin, C.H. 1997. Effects of tap withdrawal response habituation on other withdrawal behaviors: the localization of habituation in the nematode *Caenorhabditis elegans. Behavioural Neuroscience.* 111(2):342-53.

83) White, J.B., Southgate, E., Thomson, J.N. and Brenner, S. (1986). The structure of the nervous system of Caenorhabditis elegans. Philosophical Translations of the Royal Society of London (B. Biological Sciences), 314, 1-340

84) Wood, W.B. (1988). Introduction to C. elegans biology. In Wood, W.B. (Ed.), The Nematode *Caenorhabditis elegans* (pp. 1-16). Cold Spring Harbor Laboratory: Cold Spring Harbor.

85) Zhang, X., Silwowka, J.H. and Weinberg, J. 2005. Prenatal alcohol exposure and fetal programming: Effects on neuroendocrine and immune function. *Experimental biological medicine.* 230(6); 376-388.

86) Zhang, J., Campbell, R.E., Ting, A.Y. and Tsien, R.Y. 2002. Creating new fluorescent probes for cell biology. *Nature Reviews Molecular Cell Biology.* 3(12):906-18.

87) Zhou, F.C., Sari, Y. and Powrozek, T.A. 2005. Fetal alcohol exposure reduces serotonin innervation and compromises development of the forebrain along the serotonergic pathway. *Alcohol Clin Exp Res.* 29(1):141-9