Retinoic acid prevents alteration of neuronal morphology and locomotion of zebrafish embryos induced by ethanol exposure during gastrulation

by

Jannatul Ferdous

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences
University of Alberta

Abstract

Ethanol (EtOH) exposure during development has been shown to lead to deficits in fine and gross motor control. In this study I used zebrafish embryos in the gastrulation stage to determine the developmental effects of a brief, 5-hour exposure to EtOH. I treated fertilized zebrafish eggs from 5.25 hours post fertilization (hpf) to 10.75 hpf, with 10 mM, 50 mM, 100 mM EtOH or 150 mM EtOH. Embryos were removed from the EtOH wash at the end of gastrulation and were allowed to develop. They were then examined for general animal morphology, the c-start reflex behavior, Mauthner cell morphology and motor neuron morphology in 2-day old embryos. EtOH treated animals exhibited a minor but significant increase in gross morphological deformities compared with untreated fish. Locomotor studies showed that EtOH treatment resulted in an increase in the average angular velocity of the tail bend during the escape response, and an increase in the peak speed of the tail. Furthermore, there was a marked increase in abnormally directed escape behaviors, with treated embryos showing greater incidences of c-starts in inappropriate directions. Immunolabelling of the Mauthner cells, which are born during gastrulation, showed them to be significantly smaller in fish treated with 100 mM EtOH and 150 mM EtOH compared with untreated animals. Immunolabelling of primary motor neurons using the anti-znp1antibody, showed no significant effect on axonal branching of primary motor neurons, whereas secondary motor axons possessed a greater number of branches in ethanol treated fish compared with controls. Together these findings indicate that ethanol exposure during gastrulation leads to alterations in behavior and neuronal morphology.

The vitamin A derivative, RA, has been shown to prevent FASD in a number of preparations. Therefore, I determined whether co-treatment of alcohol and retinoic acid could

prevent the effects of alcohol exposure during gastrulation. I exposed zebrafish embryos to either ethanol (150 mM) alone, retinoic acid (10^{-9} M) alone, or a combination of retinoic acid (10^{-9} M) plus ethanol (150 mM) for 5 hours from 5 hpf to 10.75 hpf (gastrulation). Ethanol treatment during gastrulation altered a range of features in embryonic zebrafish. Importantly, I found that co-treatment with retinoic acid prevented all of the effects of ethanol including survival, body length, M-cell morphology and escape response movements. Treatment with RA alone had no significant effect on any of these parameters. Together these findings show that ethanol exposure during the brief period of gastrulation has a significant effect on neuronal morphology, and that this can be prevented with retinoic acid co-treatment

Acknowledgements

First and foremost I would like to express my sincere gratitude towards my supervisor and mentor throughout my Master's program, Dr. Declan Ali. Without his constant support and guidance I couldn't have finish this one. I would like to thank him for providing me the opportunity to develop my academic interests and understand the importance of toxicology research. I also want to thank my supervisory committee member, Keith Tierney, for his invaluable insights and advice.

I would like to thank all the members of Ali lab, whom I have had a chance to work with, Birbikram Roy, Marcus Cunninghum, Kazi Tanveer Ahmed, Rajarshi Mukherjii, Md. Ruhul Amin and Md Shah Sufian for their excellent support and expertise. It's hard to find good friends in co-workers and I was fortunate enough to get some. Thanks to the previous summer students who used to bring fresh air in the lab and were delight to work with, Shubham, Savanna and Whitney.

My journey was a very eventful and memorable one, as I had both my children- Mrinmoyee and Oronyo during the course. I thank them as well for being my courage and motivation towards completion. Finally I want to thank my husband, Kazi, for being my constant support and our families for having confidence on me. Thank you!

Table of Contents

List of Tables

List of Figures

1. Introduction

1.1. Fetal Alcohol Spectrum Disorder (FASD)

Alcohol (Ethyl alcohol, ethanol, or EtOH), through its teratogenic properties can affect fetal development in many ways. Ethanol is lipid and water soluble, and when it is consumed by pregnant females it rapidly crosses the placental membrane and may affect the fetus (Idanpaan-Heikkila, Jouppila et al. 1972). Ethanol can cause significant damage to the fetus (Jones 1975, Sokol, Delaney-Black et al. 2003). The most severe disorder that results from prenatal ethanol exposure (PNEE) is Fetal Alcohol Syndrome (FAS).

The study of the relationship between maternal alcohol consumption during pregnancy and its effects on the fetus remained largely out of focus until the 1970's- when Drs. Kenneth Lyons Jones and David Wayne Smith coined the term Fetal Alcohol Syndrome (FAS) (Jones 1975, Jones and Smith 1975), describing it as an array of physiological and intellectual abnormalities among children. FAS is a disorder characterized by facial dysmorphologies such as midfacial hypoplasia, wide spaced eyes, a smooth philtrum, growth retardation and CNS dysfunction resulting in cognitive, motor and behavioral problems (Sokol, Delaney-Black et al. 2003). The extent of the damage caused by ethanol can lead to a wide variability in the severity and symptoms associated with PNEE. The disorders that result from PNEE are now grouped under the umbrella term Fetal Alcohol Spectrum Disorders (FASD), which encompasses the physiological, neurological, behavioral and cognitive features displayed by individuals exposed to alcohol by maternal consumption during pregnancy. The prevalence of FASD is variable for different populations ranging from 2–7 per 1000 in the United States (May, Gossage et al. 2009) to ~100 per 1000 children in South Africa (Oliver, Blank et al. 2013). Populations of first-grade schoolchildren (~6.5–7.8 y old) estimated to be affected by FASD are as high as 20–50 per 1000

in the United States and some Western European countries (May, Gossage et al. 2009). In Canada, the estimated prevalence of FASD is 9 cases per 1000 live births (Health Canada, 2006) and in Canadian aboriginal communities it ranges from 7.2 to 190 cases per 1000 live births (Chudley, Conry et al. 2005). However, the true prevalence of FASD is uncertain because it is underreported due to a lack of awareness, a lack of resources, and a lack of trained and skilled diagnostic professionals (Oliver, Blank et al. 2013) (Health Canada, 2006).

The effects of PNEE on the brain of the developing child differ based on factors such as- the amount of alcohol consumed by the mother, the peak blood alcohol level attained, the duration of the exposure and when the exposure occurred (Chudley, Conry et al. 2005). Neurological damage leading to symptoms such as seizures, poor coordination and balance, and visual motor difficulties (Centre for Disease Control, 2005) (Chudley, Conry et al. 2005) are quite common in FASD patients. Also, impaired cognitive functioning displayed in individuals with no facial abnormalities reinforces the idea of variability among the effects of pre-natal exposure (Mattson and Riley, 1998). As a result, many children with FASD do not qualify for special help since traditional cognitive testing does not take into account other neurocognitive deficits but focuses more on criteria like IQ (Green 2007). Individuals with FASD who display an average IQ often present deficits in planning, sequencing, reasoning and the ability to inhibit and learn from consequences (Kodituwakku, Kalberg et al. 2001, Kodituwakku, May et al. 2001). Neural regions more commonly affected in FASD individuals include the ventricles, the corpus callosum, the anterior commissure, the basal ganglia and the cerebellum (Riley and McGee 2005).

The number of individuals diagnosed with prenatal alcohol exposure-related disabilities has increased significantly over the last few decades, in part due to improvements in our ability

to detect the disorder due to better social awareness and medical diagnostic capabilities (Chudley, Conry et al. 2005, Goh, Chudley et al. 2008, Astley 2013). Surprisingly, in North America as many as 30% of women continue to drink during pregnancy, with approximately 5% engaging in "binge" drinking (Ethen, Ramadhani et al. 2009). This may occur for various reasons: being ignorant of potential consequences, addiction, or unawareness of pregnancy. Further, changes in social attitudes and drinking patterns among women of childbearing age also may contribute to these growing numbers.

According to the National Institute of Alcohol Abuse and Alcoholism (NIAAA), a binge drinking event occurs when the consumption of alcohol raises one's blood alcohol concentration (BAC) to 0.08% or above, within a 2-hour time frame (National Institute of Alcohol Abuse and Alcoholism, 2004). For an average woman, this equates to approximately 4 standard drinks in two hours or less. A recent study conducted by the United States Center for Disease Control suggested that binge drinking prevalence, frequency, and intensity was found to be highest in women aged 18-24 years of age (Center for disease control, 2012). Therefore, if a woman is a binge drinker, there is a high probability that she will continue to do so after becoming pregnant (Ethen, Ramadhani et al. 2009), in part because many women do not realize they are pregnant in the first trimester and continue to binge drink during this period (O'Leary, Bower et al. 2010, O'Leary, Nassar et al. 2010).

1.2. Retinoid Acid Rescue of Ethanol Toxicity

Ethanol exposure during embryogenesis alters RA biosynthesis and signalling (Duester 1991, Leo and Lieber 1999, McCaffery, Koul et al. 2004). These findings have supported a link between FASD and RA signalling in early development (Sulik, Johnston et al. 1981, Duester

1991, Pullarkat 1991, Leo and Lieber 1999, McCaffery, Koul et al. 2004). Moreover, it has been demonstrated that application of RA can prevent the harmful effects of alcohol on ocular and limb development (Duester 1991, Keir 1991), lending further support to the notion that the harmful effects of alcohol may occur through alteration of the RA signalling pathway.

Vitamin A is essential throughout life and its influence is most critical during pregnancy and childhood when cells proliferate and differentiate in response to different levels of vitamin A. It is involved in fetal development, organogenesis, organ homeostasis, cell and neuronal growth and differentiation, development of the central nervous system and limb morphogenesis (Durston, Timmermans et al. 1989, Maden, Ong et al. 1990, Wagner, Thaller et al. 1990, Duester 1991, West, Chen et al. 1994, Kumar, Singh et al. 2010). Retinoic acid is an active derivative of Vitamin A. Vitamin A can directly be converted to all-trans-retinol, by a two-step reaction involving retinal dehydrogenase (an enzyme that experiences ethanol inhibition) (Figure 1). Retinol can then be converted to retinoic acid (RA) by alcohol dehydrogenases (ADHs) and aldehydye dehydrogenase (ALDHs) enzymes (reviewed by (Rhinn and Dolle 2012). RA binds to nuclear Retinoic Acid Receptors (RARs) which form heterodimeric combinations with Retinoid X Receptors (RXRs). The RAR-RXR heterodimers bind to specific genomic sequences: retinoic acid response elements (RAREs), located in the enhancer region of RA target genes (Duester 2008, Niederreither and Dolle 2008) (Figure 1). Activation of RARs induces chromatin remodeling, which allows a pre-initiation complex to assemble and facilitates transcription (Dilworth, Fromental-Ramain et al. 2000, Rhinn and Dolle 2012). Some of the genes modulated by RA signaling include Cdx1, Cyp26a1, Drd2, Fgf8, Hnf1b, homeobox genes (Hox), Pax6, Wnt8a etc (Cunningham and Duester 2015).

Alcohol consumption leads to a reduction in the levels of vitamin A because alcohol degradation requires the activity of the enzymes used for vitamin A production. EtOH detoxification by converting it into acetic acid requires the function of ADHs and ALDHs, which are also used for RA biosynthesis. Thus, it is likely that EtOH acts as a competitive inhibitor of the RA biosynthesis pathway (Duester 1991, Deltour, Ang et al. 1996). When retinol is not oxidized to RA, it accumulates in nonhepatic tissues, such as in the kidney and the lungs and may cause morphologic defects. Ethanol exposure also causes decreased expression of RAR and its DNA-binding activity, whereas RXR expression increases in rat cerebellum (Kumar, Singh et al. 2010). When ethanol exposed Zebrafish embryos are supplemented with low levels of RA (10^{-9} M), some of the morphological defects caused by alcohol exposure are prevented (Marrs, Clendenon et al. 2010). On the other hand, excessive consumption of retinoic acid leads to increased levels of fetal anomalies, suggesting there is a range of concentrations for which this nutrient is appropriate (Zachman and Grummer 1998).

1.3. Animal Models of FASD

Following the inaugural publications on FAS in humans on FAS in humans (Jones and Smith 1973), a number of animal models were developed to explore the teratogenic effects of EtOH (Riley and McGee 2005). Model organisms serve as powerful research tools and allow rigorous control of the factors one wants to investigate. Indeed, animal models have been used to discover and analyze a range of biological mechanisms and factors associated with and influencing alcohol abuse. Different model organisms are studied to understand mechanisms by which embryonic ethanol exposure disrupts development. Ethanol toxicity studies using certain animal models have the advantage of being precisely controlled, while human studies rely on self-reporting. Moreover, drinking is often associated with tobacco or drug use, which

independently have been shown to affect fetal development (Cudd 2005). Variables such as nutrition and genetics are difficult to control in human studies, whereas, animal studies allow for the collection of a greater array of dependent variables. However, animal models present some differences compared to humans and thus the investigator must be aware of these differences in order to reach meaningful conclusions. First of all, brain and CNS development occurs at different times during gestation (Dobbing and Sands 1979). Structures are more vulnerable to damage by ethanol exposure when they are undergoing early or rapid development. For example, in rats, the majority of brain development occurs after birth while non-human primates and sheep experience brain development earlier in pregnancy than humans (Cudd 2005). Therefore, ethanol exposure must be timed precisely depending on the structure of interest because brain regions develop at different times during gestation and the rate of brain growth varies at different times during gestation. Structures that are rapidly growing or first emerging are far more vulnerable to alcohol than at later times when development has slowed or is complete. Secondly, no animal model has shown all the diagnostic criteria of FAS, resulting in the necessity for multiple models and careful selection of the appropriate model (Cudd 2005). Additionally, animal models do not display the same intelligence and behaviours as humans, although behavioural abnormalities such as feeding difficulties and attention deficit has been observed in animals exposed to ethanol prenatally (Driscoll, Streissguth et al. 1990, Cudd 2005).

Animal models used for FASD studies include rodents such as rats and mice (Becker, Diaz-Granados et al. 1996), larger mammalian models such as pig and sheep (Brien, Clarke et al. 1985, Richardson, Patrick et al. 1985), and non-mammalian models such as zebrafish (*Brachydanio rerio*), round worm (*Caenorhabditis elegans*), and fruit fly (*Drosophila melanogaster*) (Laale 1971, Ranganathan, Davis et al. 1987, Bupp Becker and Shibley 1998,

Dhawan 1999, Su, Huang et al. 2001, Reimers, Hahn et al. 2004, Thompson and de Pomerai 2005). Although each model system has its own advantages and disadvantages, recent studies have explored zebrafish (*Danio rerio*) (Carvan, Loucks et al. 2004, Arenzana, Carvan et al. 2006, Bradfield, West et al. 2006, Dlugos and Rabin 2007, Li, Shah et al. 2007) as a model to study ethanol-mediated developmental toxicity.

1.4. Zebrafish Ecology

Zebrafish (*Danio rerio*), are a small freshwater tropical teleost fish that are native to the Ganges river and found in East India, Bangladesh, Nepal and Myanmar. They (family Cyprinidae) were first introduced into the laboratory by the late George Streisinger in the 1970s (Grunwald and Eisen 2002) and are a great model for studying development, with a balance of complexity and simplicity. After their introduction by Streisinger, they gained more wide spread popularity in the 1990s (Page 1990), and studies utilizing transgenic techniques began using zebrafish. Zebrafish is a small creature of only 3–5 cm in length which reproduces robustly. These properties make it easy to maintain a large number of animals in a relatively small space, a prerequisite for carrying out large-scale studies. Zebrafish offers other benefits such as external fertilization and development, small size which minimizes cost and waste volume for drug and toxicant studies, and are useful for examining chemical toxicity during prenatal development. In fact, it has been suggested to be an efficient compliment over other vertebrate models (Guo 2004). Furthermore, it is possible to take a forward genetic approach to investigate vertebrate-specific processes affecting development and disease.

Zebrafish are readily available, inexpensive and are easy to care for in large numbers. They are highly prolific when healthy (Lele and Krone 1996, Zhao, Xie et al. 2012) and females

are capable of spawning year-round. They reproduce every 2-3 weeks, where each clutch may contain many tens of eggs and adulthood is reached approximately 3 months after egg fertilization. Zebrafish eggs are large (~0.7mm at fertilization), optically transparent and easy to manipulate. The embryos develop into swimming larva in 48-72 hours post fertilization (hpf), and they exhibit stereotypical escape response behaviors as early as 27 hpf. The eggs are permeable to a range of pharmacological agents, including ethanol. Zebrafish are economical and are thus more feasible to be used in high throughput screening studies (Miscevic, Rotstein et al. 2012).

1.5. Zebrafish Embryonic Development

To understand the teratogenic effects of alcohol we need to understand the different stages of embryonic development. In zebrafish there are seven distinct periods of embryogenesis- the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods (Kimmel, Ballard et al. 1995). The newly fertilized eggs are at the zygotic period, which lasts about 40 minutes. The cleavage period from 0.75 hpf - 2.25 hpf denotes the second to the seventh metasynchronous cell cycles, ending in 64-cell stage embryos. The blastula period from 2.25 hpf - 5.25 hpf encompasses development from the eighth zygotic cell cycle, or 128-cell stage. In this period embryo go through asynchronous cell cycles, begins midblastula transition and epiboly, and creates a border between the yolk cell and the blastodisc. During the gastrula period (5.25 hpf - 10 hpf), epiboly continues and morphogenetic cell movements of involution, convergence and extension produce the primary germ layers and the embryonic axis. The M-cell is born during this period, around 8 hpf -10 hpf (Mendelson 1986). The segmentation period (10 hpf - 24 hpf) marks the beginning of primary organogenesis and somitogenesis through which a basic vertebrate body plan emerges. At the pharyngula phase (24 hpf - 48 hpf) the body

straightens up from the curved state, fins develop, and pigment cells differentiate and melanophors begin to form characteristic body stripes. The circulatory system, consisting of a beating heart and a closed set of channels also forms at this stage. The embryos are encased in the chorion and hatch out asynchronously during the hatching period (48 hpf - 72 hpf). They continue to grow and morphogenesis of many rudimentary organs nears completion by the end of day 3 (72 hpf). During the early larval period, after 72 hpf and onwards, the morphogenesis of the hatched larva is complete and the swim bladder becomes inflated.

The primordial central nervous system of zebrafish begins to develop during gastrulation. Towards the end of gastrulation the neural plate becomes thick and well defined. Ten distinct folds or neuromeres are formed along the anterior part of the neural plate that will eventually develop into the brain. The posterior cells of the neural plate give rise to the trunk spinal cord. Other CNS structures, such as hypothalamus, cerebellum, rudimentary structures of the eyes and optic primordia are formed later in the segmentation period. In humans, gastrulation takes place between third and fourth week of embryogenesis and alcohol consumption during this period may have a profound effect on an infant's health.

Morphogenesis of the neural tube closely parallels neuronal differentiation. The first neurons to arise in the nervous system are referred to as primary neurons, which rapidly develop long axons, and form a simple neural network that mediates the early embryonic movements such as spontaneous movement of the trunk and tail, and an avoidance response to tactile stimuli (Grunwald, Kimmel et al. 1988, Metcalfe, Myers et al. 1990). Different types of identifiable primary neurons, including interneurons of the hindbrain and spinal cord (Mendelson 1986, Kuwada, Bernhardt et al. 1990), motoneurons (Eisen, Myers et al. 1986, Myers, Eisen et al. 1986) and sensory neurons (Metcalfe, Myers et al. 1990) have been identified as appeared from

very early stages of development. The earliest interneurons to originate in zebrafish are a pair of M-cells, which belong to the reticulospinal neuron (RSN) group and play a key role in the escape response of the organism (Zottoli 1978, Kimmel, Sessions et al. 1981, Mendelson 1986). RSNs arise as a single cluster in the midbrain and seven separate clusters in the hindbrain, with each cluster sitting in one neuromere (Lee, Eaton et al. 1993). The RSNs, together with other descending neurons of the brain stem regulate sensorimotor coordination and control the turning and swimming behaviours in zebrafish (Eaton, Lee et al. 2001, Gahtan, Sankrithi et al. 2002). Arising at about 7.5 hours post fertilization (hpf) during the gastrulation period, M-cells are the first of the RSNs to appear in the nervous system, closely followed by their serial homologs MiD2cm and MiD3cm, which appear at 9.5 hpf (Mendelson 1986). The M-cell has two large crescent shaped dendrites, one projecting laterally (the lateral dendrite) and the other projecting ventrally (the ventral dendrite) (Kimmel, Sessions et al. 1981). Additional finer dendrites arise from these two prominent dendrites and the cell body.

1.6. Zebrafish Mauthner Cell

In the Ali lab, the C-turn escape response of zebrafish is used as a model system for movement. This locomotor response is ideal for our studies because it relies on a simple reflex arc that is present as early as 27 hours post-fertilization (hpf) (Saint-Amant and Drapeau 1998), and which consists of sensory neurons associated with sensing sound and pressure, and large interneurons called the Mauthner cell and its homologs, MiD2cm and MiD3cm, motor neurons and muscle cells. The stereotypical escape response causes the fish to turn away from a stimulus directed at either the tail or the head (in doing so, forming the eponymous “C” shape) and swim rapidly away. The Mauthner neurons (and its homologs) that mediate this response can be identified by their large cell bodies located in rhombomere 4 (5 and 6 respectively) of the

hindbrain (Kimmel et al., 1995). The electrical and synaptic activity associated with these neurons can be recorded as early as 24 hpf, allowing researchers to examine the populations of neurotransmitter receptors that underlie Mauthner cell activity (Roy and Ali 2013, Roy, Ferdous et al., 2015). In 48-72 hpf embryos, the rapid, short latency (<10 ms) C-turn response is mediated primarily by the Mauthner cells, and so we have focused on these neurons in this study.

M-cells receive sensory inputs from the somatosensory, visual, acousto-vestibular and lateral line systems, with each type of afferent projecting to one of the prominent dendrites (Figure 2). The first sensory inputs to the M-cells form around 18 hpf from somatosensory trigeminal ganglion cells, which are also some of the first sensory neurons to arise in the nervous system around 14 hpf (Kimmel, Warga et al. 1990, Metcalfe, Myers et al. 1990). Peripheral axons from the trigeminal ganglia, located between the eye and the otic vesicle, innervate the skin of the head and rostral part of the yolk sac, while the central axons project to the hindbrain. M-cells also receive somatosensory input from the Rohon-Beard neurons in the spinal cord that innervate the skin of the trunk and project axons to the hindbrain around 19 hpf (Metcalfe, Myers et al. 1990). Transmission of somatosensory stimuli to M-cells is taken over by the neurons of dorsal root ganglia (DRG) as the Rohon-Beard cells start to die off (Lewis and Eisen 2003, Kohashi and Oda 2008). Bipolar neurons of these ganglia begin to differentiate at 36 hpf and appear in the spinal cord by 45 hpf (Bernhardt, Chitnis et al. 1990, An, Luo et al. 2002, Reyes, Haendel et al. 2004). Input from the ears (otoliths) is relayed by the acoustic-vestibular ganglion (VIIIth cranial nerve) to the M-cells beginning at 23 hpf (Kimmel, Warga et al. 1990). Input from the lateral line neurons arrive last at around 25 hpf and are believed to modulate the M-cell's response to visual and auditory input (Faber and Korn 1975, Chang, Lin et al. 1987). The

sensory inputs onto M-cells terminate as electrical, chemical or mixed synaptic contacts (Kimmel, Warga et al. 1990).

The M-cells relay sensory information to motor centers in the brain and spinal cord. The pair begins to extend axons in the segmentation period (18 hpf), concurrently with the arrival of axonal growth cones from trigeminal sensory neurons. The axons cross the midline and descend on the contralateral side to extend the entire length of the spinal cord. The axons send collaterals to the spinal motor neurons that form chemical synapses with the primary and secondary motor neurons and descending interneurons (Celio, Gray et al. 1979, Fetcho 1992, Fetcho and O'Malley 1995, Svoboda and Fetcho 1996). The descending interneurons are electrically coupled to two to four motor neurons (Celio, Gray et al. 1979, Gahtan, Sankrithi et al. 2002). Thus, activation of an M-cell results in synchronous activation of the spinal motor neurons, leading to contraction of the body and tail musculature that drives the escape response of the fish (Fetcho and O'Malley 1995).

The escape response, an evasive behavior that animals use to flee from predators, is essential for the survival of the animal (Eaton, Bombardieri et al. 1977). The response consists of two stages; in the first stage the animal makes a C-bend to orient the body away from the stimulus. This is followed in the second stage by swimming movements away from the perceived threat (Eaton, DiDomenico et al. 1988). The M-cells are critically involved in the first stage of the escape response (Zottoli 1977, Eaton, Lee et al. 2001) and are responsible for activation of short-latency C-start (SLC) responses occurring within 5-6 ms of detection of the stimulus (Liu and Fetcho 1999, Burgess and Granato 2007, Kohashi and Oda 2008). Fish where M-cells were ablated lacked SLC and showed long-latency C-start (LLC) responses characterized by an increased latency and turn duration, and decreased angular velocity (Liu and Fetcho 1999,

Kohashi and Oda 2008). In freely swimming zebrafish, tactile stimuli to the head activates the M-cells and its homologs, producing a large escape response (Foreman and Eaton 1993). A stimulus to the tail activates only M-cells that produce a rapid but small response. M-cells usually fire only one action potential when activated followed by a temporary inhibition (Zottoli 1977, Nakayama and Oda 2004). Inhibition occurs due to activation of inhibitory synaptic inputs onto the M-cells and increased conductance of potassium currents (Furukawa and Furshpan 1963, Zottoli 1977, Nakayama and Oda 2004, Brewster and Ali 2013). Inhibitory commissural interneurons synapse onto both M-cells and contribute to the feed forward inhibition, whereas inhibitory collateral neurons provide recurrent inhibition to the ipsilateral M-cell.

1.7. Zebrafish as a Model for FASD

A number of studies have explored the use zebrafish embryos as a model for embryonic alcohol exposure. (Bilotta, Saszik et al. 2002, Bilotta, Barnett et al. 2004, Carvan, Loucks et al. 2004, Lockwood, Bjerke et al. 2004, Arenzana, Carvan et al. 2006, Bradfield, West et al. 2006, Matsui, Egana et al. 2006, Dlugos and Rabin 2007, Li, Shah et al. 2007, Tanguay and Reimers 2008). After embryonic exposure to ethanol, zebrafish larvae exhibit morphological abnormalities, some of which are similar to identified phenotypes for humans with FASD such as ocular deficits, facial deformities, and cardiac malformations (Bilotta, Saszik et al. 2002, Bilotta, Barnett et al. 2004). Gross morphological effects of EtOH exposure on zebrafish embryos include pericardial edema (accumulation of fluid in the pericardial sac of the heart), axial malformations (clubbed/misshapen extremities), otolith defects, axial blistering and delayed development. Other effects include reduced eye diameter, craniofacial and skeletal deformities, cardiac malformations, altered heart rate, hyperactivity, microcephaly and reduced length of the fish (Bilotta, Saszik et al. 2002, Bilotta, Barnett et al. 2004, Carvan, Loucks et al.

2004, Lockwood, Bjerke et al. 2004, Arenzana, Carvan et al. 2006, Matsui, Egana et al. 2006, Dlugos and Rabin 2007, Li, Shah et al. 2007, Tanguay and Reimers 2008). The severity of the abnormal phenotype is dependent on both the stage of exposure and the dose of the ethanol treatment. For example, Bilotta, Barnett et al. (2004) found that ethanol exposed zebrafish embryo exhibits smaller eyes, larger heads and yolks, and malformations in the heart. With increasing concentration from 1.5% to 2.9% (v/v), the severity of the zebrafish's abnormalities and mortality rate increased. Also, slight physical differences in morphology between the embryos exposed to 1.5% ethanol and to the control solutions from 0 to 8 hpf were detected (Bilotta, Barnett et al. 2004). Together, these findings indicate there is a dose-dependent relationship between ethanol exposure and morphological abnormalities. Furthermore, the stage specificity was also certain from the fact that ethanol exposure during the first 24 hours of development affected the zebrafish more than any later period to ethanol exposure.

Ethanol exposure has also been shown to cause behavioural and neuronal changes in zebrafish embryos. Carvan, Loucks et al. (2004) reported an altered startle response and learning and memory deficits in zebrafish as a result of early exposure to low concentrations of EtOH. They also noticed increased cell death in the CNS. When exposed to 1% EtOH from 22 to 24 hpf, 6-month old zebrafish exhibited an altered behaviour in response to images of conspecifics and predators, where the exposed fish did not make an attempt to join conspecifics and did not swim away from an image of a predator (Fernandes and Gerlai 2009), demonstrating that brief exposure to EtOH can have lasting behavioural effects. Early EtOH exposure has also been shown to induce defects in optic nerves and motor neurons (Parng, Roy et al. 2007). EtOH exposure may also be related to the loss of Sonic hedgehog (Shh) signal transduction (Li, Shah et

al. 2007), which is important for the normal development of motor neuron progenitors and somites.

1.8. Research objective, Rationale and Hypothesis

The purpose of my thesis was to examine the effects of embryonic ethanol exposure on the locomotive behavior and the cells involved in locomotion. Additionally, I wanted to test whether retinoic acid supplementation was able to prevent the adverse effects caused by ethanol exposure. Therefore, I divided my research objectives into two main parts as follows:

Objective 1: To determine if ethanol exposure during gastrulation has an effect on Mauthner cell morphology as this cell is born during gastrulation and is involved in the escape response.

Rationale: Several studies have explored the effects of early EtOH exposure on the visual system of the zebrafish specifically, since 90% of children diagnosed with FAS display some kind of ocular problem and various concentration and exposure window has been used over the years (Bilotta, Saszik et al. 2002, Bilotta, Barnett et al. 2004, Carvan, Loucks et al. 2004, Arenzana, Carvan et al. 2006, Matsui, Egana et al. 2006, Dlugos and Rabin 2007). Previous studies by our lab exposed embryos to relatively high concentrations of ethanol (0.5% - 3%, which roughly corresponds to ~86 mM – ~514 mM EtOH) between 8 hours post fertilization (hpf) and 24 hpf (Sylvain, Brewster et al. 2010, Sylvain, Brewster et al. 2011), and found that this exposure paradigm had a significant effect on motor neuron and muscle fiber morphology. In this study I sought to reduce the ethanol

exposure by lowering the concentration of ethanol to values that more closely approximate blood alcohol concentrations (BAC) during alcohol consumption.

Hypothesis: Ethanol exposure during gastrulation will alter Mauthner cell development and alter the zebrafish escape response.

Objective 2: To determine if retinoic acid can prevent the effect of ethanol on both behavior and neuronal morphology.

Rationale: Potential relationships has been established between FASD and retinoic acid (RA) signaling by different researchers (Sulik, Johnston et al. 1981, Duester 1991, Pullarkat 1991, Leo and Lieber 1999, McCaffery, Koul et al. 2004), which suggests that ethanol exposure during embryogenesis alters RA biosynthesis and signaling. RA rescue experiments were also used to test its role in ethanol-induced defects during gastrulation, ocular and limb development (Duester 1991, Keir 1991). When ethanol exposed Zebrafish embryos are supplemented with low levels of retinoic acid (10^{-9} M), retinoic acid supplementation seems to correct those dysmorphologic signs compared with the ethanol-exposed group, but the rescued phenotype was not compatible to the control, which had not been exposed to either ethanol or retinoic acid (Marrs, Clendenon et al. 2010).

Hypothesis: RA co-treatment with ethanol will prevent the adverse effect on M-cell morphology as well as escape behavior.

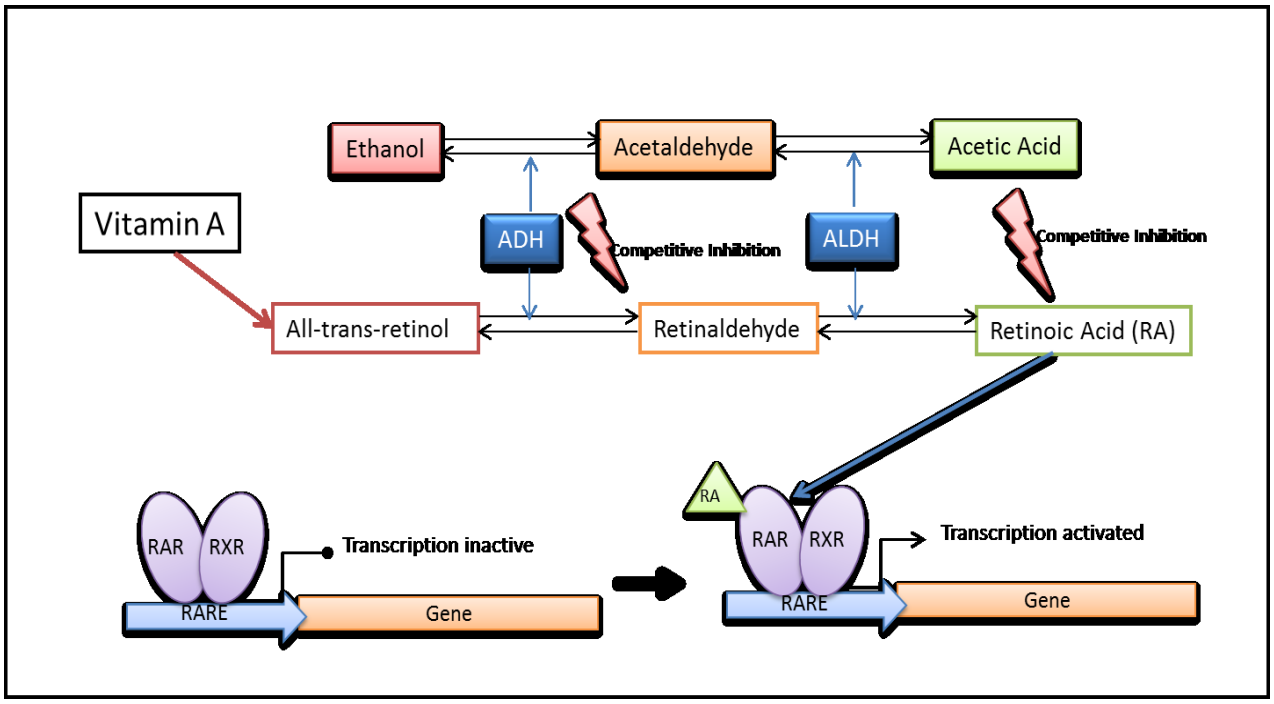
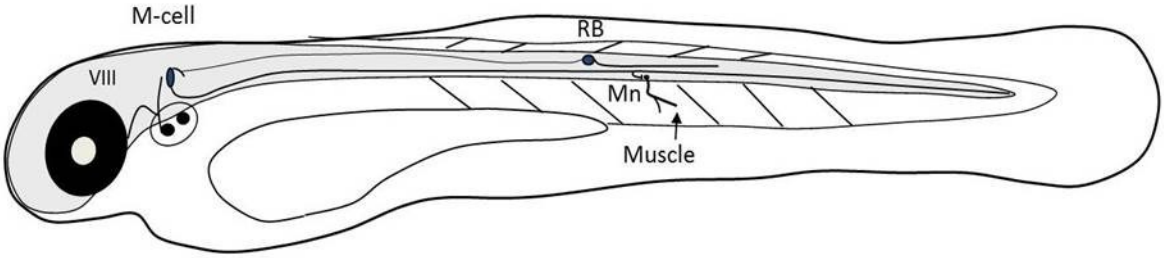

Ethanol
Acetaldehyde
Acetic Acid
Vitamin A
ADH
Competitive Inhibition
ALDH
Competitive Inhibition
All-trans-retinol
Retinaldehyde
Retinoic Acid (RA)
RAR
RXR
RARE
Transcription inactive
Gene
RA
RAR
RXR
RARE
Transcription activated
Gene

Figure 1. Schematic representation of Vitamin A to retinoic acid induced transcriptional activation and competitive inhibition by ethanol. ADH-Alcohol dehydrogenase enzyme; ALDH-Aldehyde dehydrogenase enzyme; RAR- Retinoic acid receptor; RXR- Retinoid X receptor; RARE- Retinoic acid response elements


M-cell
RB
VIII
Mn
Muscle

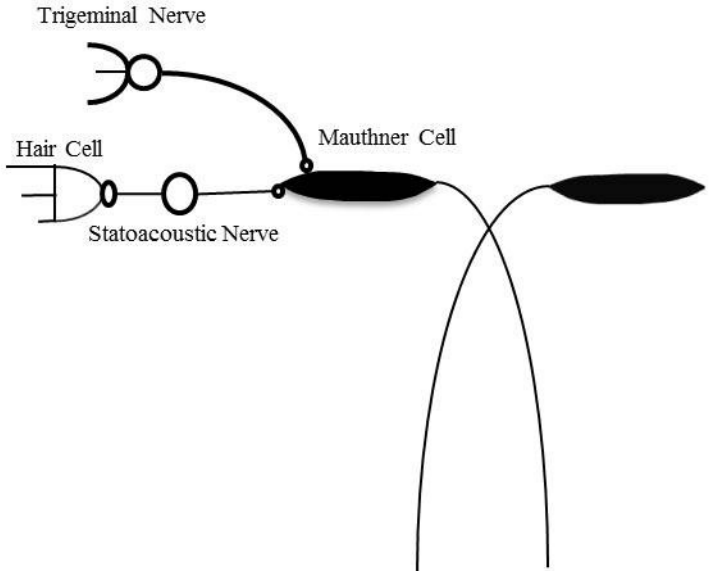

Trigeminal Nerve
Mauthner Cell
Hair Cell
Statoacoustic Nerve

Figure 2. Mauthner cells are the largest neurons in the zebrafish CNS and are involved in the c-start escape reflex. They are born at 8 hours post fertilization (hpf), during gastrulation, and are located in rhombomere 4 of the hindbrain. M-cells receive sensory information from a number of sources including the eighth cranial nerve, the trigeminal nerve and acousticovestibular inputs.

2. Materials and Methods

2.1. Animal Care

Wild type zebrafish (*Danio rerio*) were housed at the University of Alberta Aquatic Facility. For breeding, 3 to 5 adults, usually consisting of 3 females and 2 males, were placed in breeding tanks the evening before eggs were required. The following morning, fertilized eggs were collected from the breeding tanks, usually within 30 mins of fertilization, around 8.30 am. A 12h light/dark cycle and 28.5°C temperature was set for housing the embryos and larvae in incubators.

2.2. Ethanol and Retinoic Acid Exposure

Embryos were exposed to various concentrations of ethanol (0 mM (control), 10 mM, 50 mM, 100 mM and 150 mM) during the period of gastrulation, which occurs between 5.25 hours post fertilization (hpf) to 10.75 hpf (1.15 pm to 6.45 pm) and at 12-17 hpf (8 pm to 1 am), the period just after gastrulation, by adding anhydrous EtOH (Commercial Alcohols) to the Embryo Media (EM). The chosen concentrations of ethanol could be related to blood alcohol concentrations (BAC) in human following drinking (Table 1). At the end of the EtOH exposure period, the fertilized eggs were washed several times in fresh EM, and then allowed to develop at 28.5 C until 2 days post fertilization (dpf). For the retinoic acid rescue experiments, 10^{-9} M RA (controls), or 10^{-9} M RA + 150 mM ethanol were used as exposure media. In immunohistochemical studies, pigment formation was blocked by adding 0.003% phenylthiourea (PTU) dissolved in egg water at 24 hpf. All protocols were carried out in compliance with

guidelines described by the Canadian Council for Animal Care (CCAC) and the University of Alberta.

Embryo Media contained 13.7 mM NaCl (BDH), 4.1 mM $NaHCO_3$ (BDH), 1.0 mM $MgSO_4$ (BDH), 0.98 mM CaCl2 (Fisher Scientific), 0.54 mM KCl (BDH), 0.044 mM KH_2PO_4 (Sigma-Aldrich), and 0.025 mM Na_2HPO_4 (BDH). Penicillin-Streptomycin and the antifungal agent Methylene Blue (Sigma-Aldrich) were added to the EM solution. RA (10 mM) stock solution (Sigma-Aldrich) was prepared by dissolving 50 mg RA into 16.64 ml of DMSO. 10^{-9} M RA was chosen as optimal concentration for treatments that minimized its toxic effect and displayed maximum rescue phenotypes as previously described (Marrs, Clendenon et al. 2010, Sarmah and Marrs 2013).

Table 1: Comparison of exposed ethanol to BAC and drink frequency in human

Concentrations used	**Blood Alcohol Concentration (BAC)**	**Drinks per hour**
10 mM	~0.012%	1/5
50 mM	~0.06%	1
100 mM	~0.03%	2
150 mM	~0.18%	3

2.3. Embryo Imaging and Morphological Observations

Embryos were imaged at 2 dpf using a Lumenera Infinity2-1R colour microscope camera mounted on a dissecting microscope. All immunohistochemical imaging were done on a Zeiss LSM confocal microscope and photographed under a 20x objective. Images were compiled using Zeiss LSM Image Browser software and are shown as z-stack compilations of the trunk unless specified.

The number of fish still alive and the number of fish that had hatched out of the chorion was recorded on each day until 5 dpf. Gross deformities were observed at 2 dpf where body length of the fish, the number of larvae exhibiting pericardial edema, axial malformations was counted for each treatment. Measurements of embryo length were done using a microscope eyepiece equipped with a micrometer. Morphological observations were performed using a dissecting microscope; embryos were placed in a 16-well plate with one embryo per well and anesthetized in 0.02% MS222.

2.4. Immunohistochemistry

Embryos (48 dpf) were fixed in 2% paraformaldehyde for 1–2 h and washed with 0.1 M PBS-T (phosphate buffered saline (PBS) and 0.5% Tween-20; Hanington et al., 2008) every 15 minutes for 2 hours. PBS consisted of (in mM): 150 NaCl, 8 NaH_2HPO_4, 2 $NaH_2PO_4 \cdot 2H_2O$, and had a p^H of 7.2. The preparations were then permeabilized for 30 min in 4% Triton-X 100 containing 2% BSA (bovine serum albumin) and 10% goat serum. Tissues were incubated for 48 hours at 4°C in either mouse monoclonal anti-3A10 (Developmental Studies Hybridoma Bank (DHSB)), to identify M-cells, or mouse monoclonal anti-znp-1 (Trevarrow, Marks et al. 1990), which targets primary motor axons, or mouse monoclonal anti-zn-8 (Trevarrow, Marks et al.

1990) (DHSB), which identifies secondary motor neurons, mouse monoclonal F59 (Crow and Stockdale 1986), to identify red muscle cells, or mouse monoclonal F310 (Crow and Stockdale 1986) for targeting white muscle cells (Table-2).

Table 2. Antibodies Used

Antibody	**Source**	**Primary antibody concentration**	**Secondary antibody concentration**	**Epitope**	**References**
3A10	DSHB*	1:250	1:1000	Mauthner cell (M-cell)	Trevarrow et al. (1990)
znp-1	DSHB*	1:250	1:1000	Primary motor neuron axons	Trevarrow et al. (1990)
zn-8	DSHB*	1:250	1:1000	Secondary motor neuron axons	Fashena and Westerfield (1999)
zn-12	DSHB*	1:250	1:1000	Sensory neurons	Metcalfe et al. (1990)
F59	DSHB*	1:50	1:1000	Myosin heavy chain, red muscle cells	Crow and Stockdale (1986)
F310	DSHB*	1:50	1:1000	Myosin light chain, white muscle cells	Crow and Stockdale (1986)

All primary antibodies were diluted at 1:250 in PBS. Tissues were washed in PBS twice every 15 minutes for 2-3 hours and then incubated for 4 hours at room temperature in the secondary antibody, Alexa Fluor® 488 goat anti-mouse IgG, (Molecular Probes, Life Technologies), at a dilution of 1:1000. Preparations were then washed for 7 h in PBS, followed by mounting in Mowiol (Calbiochem (Mowiol 4-88 catalogue number 475904)) mounting media for viewing.

2.5. Mounting Media- Mowiol Preparation

To prepare the mounting media 2.4 g Mowiol was added to 6 g glycerol followed by 6 ml water. The mixture was stirred for several hours at room temperature. The mixture was then heated to 50°C for 30 min with addition of 12 ml of 0.2M phosphate buffer, ph 7.4 and constant mixing. The mixture was allowed to cool after the Mowiol dissolved, and 0.1% n-propyl gallate (Sigma P3130) was added to it, and then cleared by centrifugation at 5000x g for 15 min. Finally they were aliquoted in cryovials and stored at –20°C. Stock solutions are stable for up to 2 weeks at +4°C.

2.6. Low-melting Point Agarose (LMPA) Behaviour Recording

2 dpf zebrafish embryos were immobilized for behavioural observation in 2% low-melting point (26°C-30°C) agarose (LMPA) dissolved in embryo medium (Sigma-Aldrich 2-Hydroxyethylagarose, type VII low gelling temperature). The LMPA was made up in 50 mL

aliquots and left to dissolve in a 66° C water bath for approximately one hour. After the LMPA was fully dissolved, 7-10 embryos were transferred to a 35mm petri dish in a small amount of embryo medium and the LMPA was cooled to 32°C in an ice bath. The embryo media was then replaced with 2 mL of LMPA, which was then left to solidify. In order to observe embryo movements, the LMPA was cut away from the embryo tails while leaving the heads embedded in the gel and embryo media was added to the petri dish to ensure that the larvae remained immersed in solution. Embryo stimulation was performed using a pulse of basic 2% phenol red (Sigma-Aldrich) dissolved in egg water ejected from a Picospritzer II (General Valve Corporation); the pulse lasted approximately 15-20 milliseconds (ms) with a pressure of approximately 35-55 pounds per square inch (psi) of pressure. Borosilicate glass micropipettes (Sutter Instrument; O.D.: 1.2mm, I.D.: 094mm, 10cm length) were pulled using a Flaming/Brown Sutter Instrument pipette puller (model P-97; heat: 459, Vel: 70, Time: 200) [program 50]. The picospritzer pipette was positioned as close as possible to the embryo's otolith without contacting the embryo. The tail movements were then recorded using an AOS video camera (AOS S-PRI 1995; 1250 FPS; shutter speed: 800 us; 2700 frames per sequence) mounted on a dissecting microscope.

The same procedure was followed for recording the free-swimming behavior where the embryos were kept in a small petridish immersed in embryo media and stimulated with both head and tail stimulus.

2.7. Quantification of Behavior

The video recordings were transferred and analyzed using a Motion Analysis Software, ProAnalyst® Professional Edition (Xcitex Inc). The following behavioral parameters were quantified: angle of c-bend, time to maximum c-bend, angular velocity, time to correct, following c-bend, instantaneous peak speed and acceleration. Quantification of all measures was conducted after calibration of ProAnalyst® by inputting the actual dimensions of the test plate and thus results are expressed in mm.

2.8. Analysis and Statistics

Acquired images of tissues labelled with Zn8, Znp-1, F59 and F310 were analysed using Imaris software package (Bitplane Scientific Software, South Windsor, CT, USA). For each fish examined three hemi-segments were randomly chosen for measurements. For analysis using F59 and F310, the angle was determined after tracing the outline of the segments, using Imaris, In case of F310, measurements was performed on one image slice taken midway through the white muscle, due to the thickness of the white muscle layer. With Zn8 and Znp-1 labelled images, for each hemi-segment examined, the number of fibers was counted, and the length and width of three fibers were measured, where the width of the fibers was measured at three locations along their length and averaged. Measurements were not averaged per fish or per hemi-segment. The fibers measured were chosen based on their position within the hemi-segment, where a fiber from near the centre line, the middle and the outer edge of the hemi-segment were analyzed. For all immunohistochemistry experiments, at least three fish were examined per trial and at least

three trials were conducted for each antibody used. Negative controls were also conducted and no unusual background was detected (data not shown).

All values are reported as means ± SEM (standard error of the mean). Significance was determined using a one-way ANOVA followed by a Tukey post-hoc multiple comparisons test where appropriate ($p < 0.05$). When normality tests failed, the Dunn's method of comparison was performed followed by a Kruskal–Wallis one-way ANOVA on ranks. Statistical analysis was done using using the statistical package associated with GraphPad prism and Microsoft Office Excel 2007.

3. Results

3.1. Ethanol Exposure Study During Gastrulation:

3.1.1 Gross Morphology

Exposure to ethanol resulted in a number of gross morphological changes to zebrafish embryos that were readily apparent. Embryos treated with 10, 50, 100 and 150 mM ethanol exhibited morphological defects compared to untreated embryos (Figure 3, 4). For instance, there was a higher incidence of axial malformation (curved tails) in treated embryos (Figure 3, 4E).

I examined the proportion of animals that were alive on each day following ethanol treatment to determine if ethanol adversely affected survival. I found that the proportion of embryos that were alive after 24 hours was not significantly different between groups (Figure 4A; N=10-12 experiments; $p<0.05$). But as time progressed, the proportion of live animals per group differed in an ethanol-dependent manner. For instance, embryos treated with 150 mM ethanol had the lowest level of survival (59%) by day 5, compared with untreated animals in which approximately 82% of the larvae survived (Figure 4A). These results indicate that treatment of zebrafish embryos (during gastrulation) with 50, 100 or 150 mM EtOH significantly reduced larval survival.

I found that there was a significant difference in the rate of hatching between treatments, such that embryos exposed to 100 and 150 mM ethanol experienced lower rates of hatching compared with either the controls or the 10 mM exposed embryos (Figure 4B; $p<0.05$). For instance, by day 4, 89.5 ± 0.1% of the untreated animals had hatched whereas only 64 ± 0.1% of the 150 mM ethanol treated fish had hatched (Figure 4B).

The effects on morphology was dose-dependent as those treated with 150 mM ethanol exhibited more severe deformities compared with control (Figure 4E; $p<0.05$). Embryos treated with 150 mM ethanol had significantly shorter bodies (2.70 ± 0.05 mm, n=45) compared with untreated (2.91 ± 0.02 mm, n=61) and 10 mM ethanol treated embryos (2.91 ± 0.03 mm, n=79) (Figure 4C; $p<0.05$).

3.1.2. Locomotor Responses

To determine if ethanol exposure altered the locomotor response, I examined the escape response by puffing solution onto the head and filming the embryo's response. This mechanosensitive stimulus has been previously shown to activate M-cells in 1-3 dpf embryos (Kohashi and Oda, 2008). Figure 5 shows still images obtained from videos of locomotor responses acquired at a frame rate of 1250 frames per second. The sharp c-bend of the escape response was usually completed within 30 ms after the start of the movement in untreated embryos (control, Figure 5). Quantification of the bend angle, the average angular velocity, the time to maximum bend, time to relax, peak speed and peak acceleration of the tail are shown in Figure 6, 7, 8 and 9. I found that fish treated with the higher ethanol concentrations exhibited a significantly greater bend of the trunk compared with controls (Figure 5, 6A; $p<0.05$). I found that the maximum bend angle of untreated fish was 202 ± 11 degrees (n = 22). The bend angle was 252 ± 8 degrees (n = 30) in 100 mM ethanol treated fish and 265 ± 17 degrees (n = 15) in 150 mM ethanol treated fish (Figure 6A). Interestingly, the time of the response, from the initial movement to the point at which maximum bend angle was achieved was not different among the groups (Figure 7A; $p>0.05$). Thus, the average angular velocities of 100 mM ethanol treated fish (8.8 ± 0.4

degrees/ms, n = 30) and 150 mM ethanol treated fish (9.2 ± 0.6 degrees/ms, n = 15) were significantly greater than controls (7.1 ± 0.4 degrees/ms, n = 17) (Figure 6B; $p<0.05$). Even though the time to the maximum bend angle was not different between groups, the time to relax (or to return to the initial starting position) was significantly greater both in 100 mM ethanol treated fish (29 ± 3 ms, n = 30) and 150 mM ethanol treated fish (35 ± 1.7 ms, n = 15) compared to the untreated (19 ± 1.5 ms, n = 21) ones (Figure 7B; $p<0.05$).

I examined the speed and acceleration of the tail to determine if ethanol treatment altered these parameters. A measurement of the peak instantaneous speed obtained from a single experiment is shown in Figure 8A. There was no difference in the mean peak speed recorded from control and 100 mM EtOH-treated fish (Figure 8B) show no obvious difference in the peak speed, but when I analyzed the files on a frame by frame basis, the instantaneous peak speed attained by the ethanol treated fish was significantly greater than controls (Figure 9A). This indicates the instantaneous peak speed attained by the tail movement is subject to variables more than the mean peak speed in the ethanol treated fish. For instance, the 150 mM ethanol treated group attained instantaneous peak speeds of 0.191 ± 0.007 mm/ms (n=15) and it was 0.197 ± 0.006 mm/ms (n=40) in 100 mM ethanol treated group compared with control speeds of 0.168 ± 0.005 mm/ms (n=32); $p<0.05$) (Figure 9A). Peak acceleration of the tail trended towards higher values in both the 150 mM and 100 mM ethanol group but was not significantly different from any other treatment (Figure 9B; $p>0.05$).

Finally, I noted that some of the ethanol treated embryos exhibited aberrant turn directions and turned towards the stimulus rather than away from it. Quantification of these responses indicated that about 40% of the embryos treated with 150 mM ethanol and 30% of the 100 mM ethanol responded inappropriately by turning toward the stimulus compared with 8% (1

of 12 embryos) in the controls (Figure 9C). Taken together, these results suggested that ethanol treatment during gastrulation altered the c-start escape behavior.

3.1.3. Neuronal and Muscle Fibre Morphology

My locomotor results suggested that cells involved in the c-start response may be affected by EtOH treatment. Therefore, I examined the morphology of one of the key pairs of neurons involved in generating the escape response; the M-cells. To do this, I immunolabelled embryos with the anti-3A10 antibody that labels M-cells and other primary neurons which contain the 3A10 neurofilament. I focused my attention on embryos treated with 100 mM and 150 mM ethanol since these groups showed the greatest differences in morphology and behavior throughout the earlier parts of the study. The staining patterns were similar but the M-cells of ethanol treated groups appeared to be smaller in size compared with the controls. To obtain estimates of cell size, I measured the width of the cell at its widest point, and the axonal diameter immediately before the axons crossed (arrowhead Figure 10A, B). The width of the cell body in controls was 7.7 ± 0.5 μm (n = 16) while it was 5.3 ± 0.4 μm (n = 16) in 100 mM and 5.7 ± 0.4 μm (n = 22) in 150 mM ethanol treated groups (Figure 10A-C; $p<0.001$). The axon diameter was 3.0 ± 0.1 μm in controls (n=18), 2.2 ± 0.1 μm (n = 16) and 2.3 ± 0.1 μm (n = 22) in 100 mM and 150 mM treated groups, respectively (Figure 10A, B, D; $p<0.05$). These findings indicate that ethanol treatment during gastrulation had a significant effect on cell morphology.

To determine if ethanol treatment affected other neurons involved in locomotion, I imaged spinal cord motor neurons projecting from the spinal cord to the muscle. As locomotive defects were observed starting at 100 mM EtOH, I used this concentration for the immunohistochemical comparison. Immunohistochemical labelling of primary and secondary

motor neurons (anti-znp-1 and anti-zn8 respectively) showed no obvious differences in the staining patterns between treated and untreated fish (Figure 11, 12). However, when I counted the number of branches emanating from axons of motor neurons in the trunk, I found that there was a significant increase ($p<0.05$) in the number of branches emanating from the secondary motor axons of fish exposed to 100 mM EtOH (7.4 ± 0.3 branches, n=15) compared with controls (6.1 ± 0.3 branches, n=18) (Figure 12B). There were no differences in the number of branches emanating from primary motor axons. Taken together, our results show that ethanol treatment of zebrafish embryos during gastrulation resulted in significant effects on motor neuron morphology.

Finally, I sought to determine if muscle fibers were also affected by early EtOH exposure. Therefore, muscle fibers were labelled with antibodies directed against slow tonic (red) and fast twitch (white) muscle myosin (F59 and F310, respectively; Figure 13, 14). No apparent deformities in structure or orientation of either type of muscle fibers could be seen visually, suggesting that EtOH exposure do not affect muscle fibers. Therefore I did not proceed with any further quantification of these fibers.

3.2. Ethanol Exposure Study After Gastrulation

Brain Structures are more vulnerable to damage by ethanol exposure when they are undergoing early and/or rapid development. (Dobbing and Sands 1979). For example, in rats, the majority of brain development occurs after birth while in non-human primates and sheep, brain development occurs earlier in pregnancy compared with humans (Cudd 2005). Therefore, ethanol exposure must be timed precisely depending on which structure is being studied. One of

my goals was to examine the effects of ethanol toxicity on the M-cell because this cell is born during gastrulation. The M-cell is born around 8 hpf -10 hpf during the gastrula period (Mendelson 1986). As a control experiment, and to study the effects of ethanol exposure after the M-cells were already born, I examined the escape response behavior and M-cell morphology of embryos that were exposed to ethanol from 12 hpf to 17 hpf, during the segmentation period (10 hpf -24 hpf).

3.2.1. Locomotor Responses

I examined the escape response of 2 dpf embryos that were previously exposed to ethanol from 12 to 17 hpf. Quantification of the bend angle, the time to maximum bend, the average angular velocity, time to relax, peak speed and peak acceleration of the tail are shown in Figure 15. None of these parameters were significantly different from controls after exposure to either 100 mM or 150 mM ethanol.

3.2.2. Neuronal Morphology

The M-cells of embryos exposed to ethanol from 12 to 17 hpf showed no significant differences when compared with controls (untreated embryos) (Figure 16). The diameter of M-cells of animals in the treated group was 8.8 ± 0.3 μm (n=28), while in controls it was 8.7 ± 0.3 μm (n=20) ($p>0.05$). The diameter of the M-cell axon was also not affected following ethanol exposure (Figure 16). These data suggest that exposure specifically during gastrulation may be more impactful then exposure at some other times.

3.3. Retinoic Acid

The vitamin A derivative, retinoic acid has been shown to prevent FASD in a number of preparations (Duester 1991, Deltour, Ang et al. 1996, Zachman and Grummer 1998, Marrs, Clendenon et al. 2010, Sarmah and Marrs 2013, Cunningham and Duester 2015). 10^{-9} M retinoic acid has shown to prevent ethanol induced effects in zebrafish embryos, but did not show any teratogenic effect itself (Marrs, Clendenon et al. 2010). To test whether retinoic acid (10^{-9} M) can prevent the developmental defects induced by ethanol exposure during gastrulation in zebrafish, I exposed fertilized embryos during the gastrulation period (5.25 hpf to 10.75 hpf) to either embryo media (control), 150 mM ethanol, 10^{-9} M RA, or a combination of ethanol (150 mM) and RA (10^{-9} M). At the end of the gastrulation period, embryos were washed several times in embryo media and were then allowed to develop until 2 dpf when they were examined further in morphological, electrophysiology and locomotion studies.

3.3.1. General Morphology

Co-treatment of RA with ethanol appears to partially prevent the effects of ethanol (Figure 17 A-D). For instance, embryos exposed to ethanol exhibited a significant rise in pericardial edema (36 ± 10%, n=6 experiments) and axial malformation (29 ± 8%, n=6 experiments) (Figure 17F, G; $p<0.05$) compared with controls (6 ± 3% for pericardial edema and 2 ± 2% for axial malformation; n=6 experiments). Co-treatment with RA prevented the effects of ethanol (Figure 17F, G; $p>0.05$), and application of RA alone did not result in malformations that were significantly different from controls (Figure 17F, G; $p>0.05$). When I looked into their body lengths I found that exposure to alcohol resulted in a small but significant reduction in body

length (Figure 17E; $p<0.05$; n=43-45 fish) which was prevented by co-treatment with RA (Figure 17E, $p>0.05$; n=43-45 fish). Specifically, I found that control embryos had a body length of 2.90 ± 0.03 mm (n=43), while alcohol treated embryos had a body length of 2.70 ± 0.05 mm (n=45), and embryos co-treated with ethanol + RA had a body length of 2.91 ± 0.04 mm (n=43). Embryos treated with RA alone had a body length of 2.91 ± 0.04 (n=39).

To ascertain the overall health of the embryos, I examined hatching and survival rates, and found that embryos treated with alcohol died at a significantly higher rate compared with controls (Figure 18A; $p>0.05$; n=12 experiments and 465-645 fish), whereas embryos co-treated with ethanol and RA survived as well as controls (Figure 18A; n=12 experiments and 445-465 fish). Ethanol treated embryos hatched at lower rates than controls, or ethanol + RA treated embryos (Figure 18B; $p>0.05$; n=9 experiments and 460-647 fish). These findings suggest that alcohol exposure during gastrulation adversely affects embryonic development, and that co-application with RA prevents the ethanol-induced deficits.

3.3.2. Locomotor Responses

To examine escape movements, I embedded embryos in low-melting point agar, freed the tails and stimulated the animals with a mechanical stimulus to the head. I quantified parameters such as bend angle, angular velocity, maximum speed and maximum acceleration, as I had done previously. I found that embryos treated with 150 mM EtOH plus 10^{-9} M retinoic acid exhibited movement parameters that were the same as untreated controls. In other words, co-treatment with RA prevented the effects of EtOH. Figure 19 shows still images obtained from

videos acquired at a frame rate of 1250 frames per second. The sharp c-bend of the escape response was usually completed within 30 ms of movement initiation (control, Figure 19).

The maximum bend angle was significantly higher; 265 ± 20 degrees (n = 14) in 150 mM ethanol compared with untreated embryos 195 ± 11 degrees (n = 26) (Figure 20A; $p<0.05$). The maximum bend angle of animals co-treated with ethanol and RA was 205 ± 16 degrees (n = 20), which was not significantly different from controls ($p<0.05$). The time of the response, from the start of the movement to the point at which maximum bend angle was achieved was not different among the groups (Figure 20B; $p>0.05$). Thus, the average angular velocity of 150 mM ethanol treated fish (9.3 ± 0.6 degrees/ms, n = 14) was significantly greater than controls (7± 0.4 degrees/ms, n = 26) (Figure 20C; $p<0.05$). Co-treatment with RA prevented the effects of 150 mM ethanol and resulted in an angular velocity of (6.8 ± 0.6 degrees/ms, n = 20). I measured the time for the trunk to relax after the c-bend. This was also prevented by RA co-treatment and had a value of 28 ± 2 degrees/ms (n = 20), which was significantly greater than the 150 mM ethanol treated fish (35 ± 1.8 degrees/ms, n = 14) compared to the untreated (19 ± 1.5 degrees/ms, n = 25) ones (Figure 20D; $p<0.05$).

Next, I examined the maximum or peak speed and peak acceleration of the tail to determine if RA co-treatment affected any of these parameters. However, none of these values were significantly different from each other (Figure 21A, B; $p>0.05$). Some of the ethanol treated embryos exhibited aberrant turn directions and turned towards the stimulus rather than away from it. Quantification of these responses showed that about 20% (3 of 15 embryos) of embryos treated with 150 mM responded inappropriately by turning toward the stimulus, compared with 8% (1 of 12 embryos) of the controls (Figure 21C). Embryos co-treated with 10^{-9} M RA and 150 mM ethanol showed normal responses, where only 1 of 20 animals responded inappropriately

(Figure 21C). 10^{-9} M RA treatment alone had no detrimental effect on the direction of the C-start reflex.

3.3.3 Mauthner Cell Morphology

My behavioral results suggested that neurons involved in the c-start response might be affected by EtOH treatment. Therefore, I examined M-cell morphology by immunolabeling the M-cell with anti-3A10. The staining patterns were similar between the groups, but the M-cells of 150 mM ethanol treated groups appeared to be smaller than those of the controls (Figure 22 A-D).

To obtain estimates of cell size, I measured the width of the cell at its widest point, and the axonal diameter just proximal to decussation (arrowhead Figure 22A, B, C, D). The width of the cell body in controls was 7.7 ± 0.5 μm (n = 16) while it was 5.7 ± 0.4 μm (n = 22) in 150 mM ethanol treated groups (Figure 22A-E; $p<0.05$). When measured, the width was significantly greater in both 10^{-9} M RA control (8.5 ± 0.5 μm, n = 18) and 150 mM EtOH+10^{-9} M RA co-treatment (8.0 ± 0.6 μm, n = 16) ($p<0.05$). My immunohistochemical data showed thinner, and sometimes wispy-looking axons in ethanol treated animals, and therefore I examined the diameter of the M-cell axons in all groups. I found that the axon diameter was significantly smaller in embryos that had been exposed to ethanol during gastrulation and that this was prevented by RA co-treatment. For instance, it was 3.0 ± 0.1 μm in controls (n=18) while 2.3 ± 0.1 μm (n = 22) in 150 mM treated group (Figure 22F; $p<0.05$). In both 10^{-9} M RA control (3.7 ± 0.1 μm, n = 18) and 150 mM EtOH+10^{-9} M RA co-treatment (3.2 ± 0.2 μm, n = 16) the axon diameter was greater than the EtOH group ($p<0.05$). These findings indicate that ethanol

treatment during gastrulation has a significant effect on M-cell morphology, and that co-treatment with RA prevented the effects of ethanol.

Overall, my results show that a brief exposure to ethanol, only during gastrulation, altered numbers of developmental features in zebrafish embryos. In particular, embryos showed reduced survival, increased morphological malformation, aberrant neuronal morphology and altered locomotive response. However, when embryos were co-treated with retinoic acid, the adverse effects of ethanol exposure were prevented.

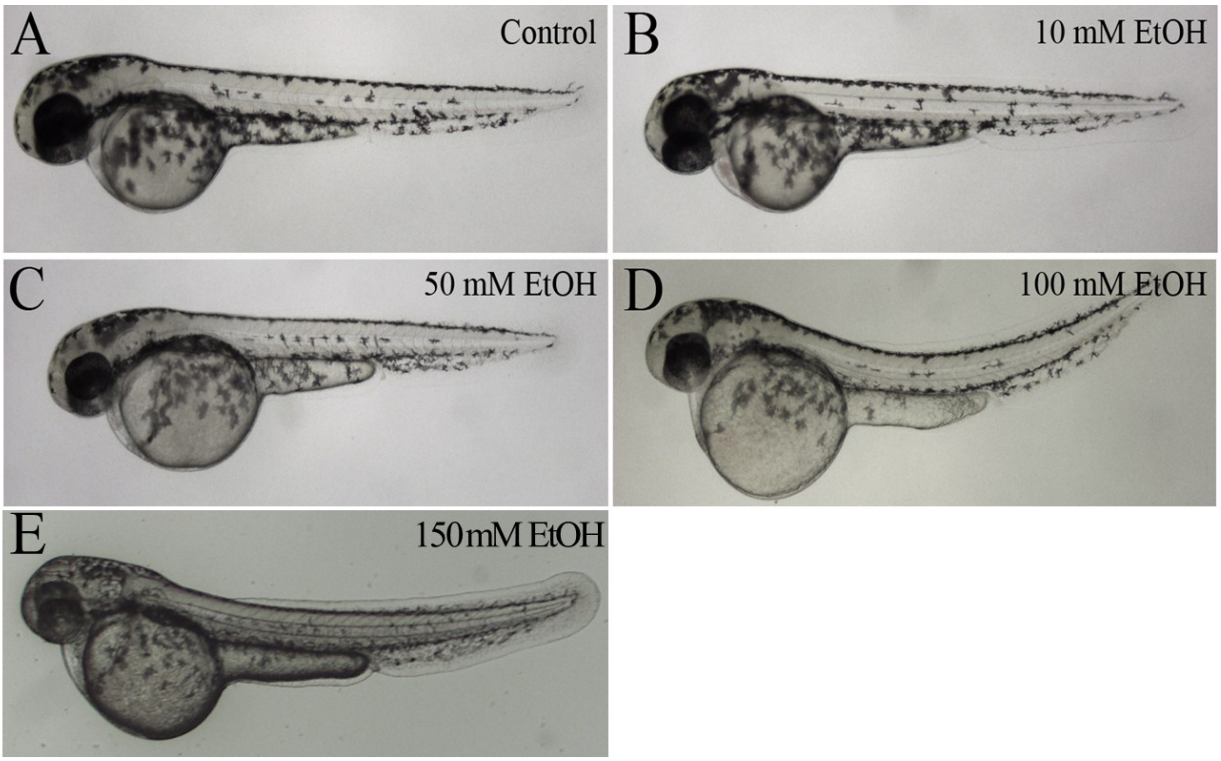

A
Control
B
10 mM EtOH
C
50 mM EtOH
D
100 mM EtOH
E
150mM EtOH

Figure 3. Effect of ethanol exposure on zebrafish embryos. Embryos were exposed to 0% (A; control), 10 mM (B), 50 mM (C), 100 mM (D) or 150 mM (E) EtOH from 5.25 hpf to 10.75 hpf and then allowed to develop in normal embryo media. Images were taken at 48-52 hpf.

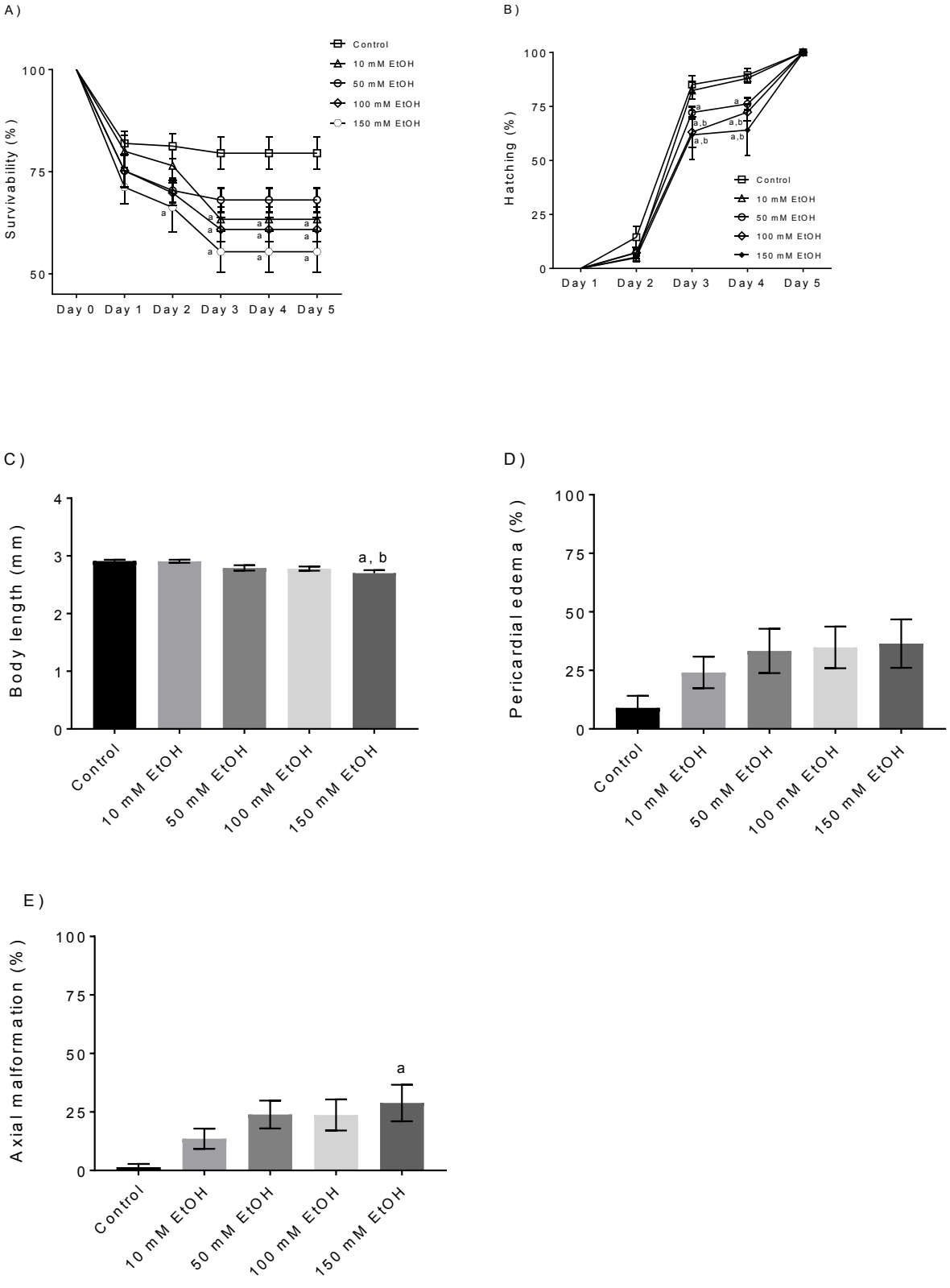

A)
Survivability (%)
100
75
50
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Control
10 mM EtOH
50 mM EtOH
100 mM EtOH
150 mM EtOH
B)
Hatching (%)
100
75
50
25
0
Day 1
Day 2
Day 3
Day 4
Day 5
C)
Body length (mm)
4
3
2
1
0
a, b
Control
10 mM EtOH
50 mM EtOH
100 mM EtOH
150 mM EtOH
D)
Pericardial edema (%)
100
75
50
25
0
E)
Axial malformation (%)
100
75
50
25
0
a

Figure 4. Effect of EtOH exposure on survival, hatching and gross morphology. (A, B) Line graph showing the percentage of survived embryos and embryos that hatched within the first 5 days after egg fertilization (N=10-12 batch). (C) Bar graph showing the body lengths of fish in different treatments (n = 61, 79, 60, 73 and 45 for controls, 10 mM, 50 mM, 100 mM and 150 mM EtOH treated fish respectively). (D) Bar graph showing the percentage of fish exhibiting pericardial edema (N = 7, 8, 7, 7 and 6 batches for controls, 10 mM, 50 mM, 100 mM and 150 mM EtOH treated fish respectively). (E) Bar graph showing the percentage of fish exhibiting axial malformation (N = 6, 7, 7, 8 and 6 batches for controls, 10 mM, 50 mM, 100 mM and 150 mM EtOH treated fish respectively). [a] Significantly different from controls on the same day, $p<0.05$; [b] significantly different from 10 mM EtOH treated embryos on the same day, $p<0.05$.

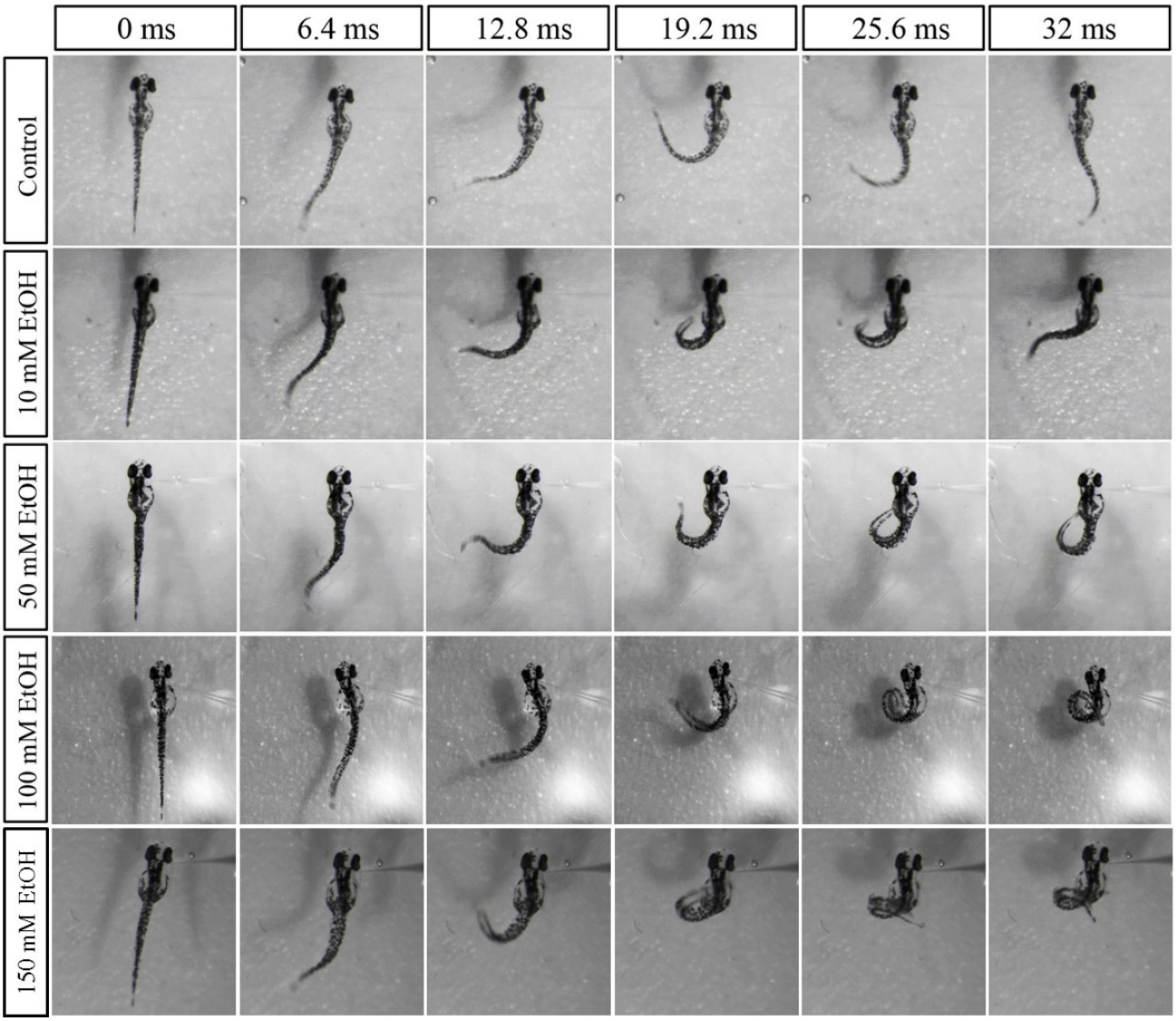
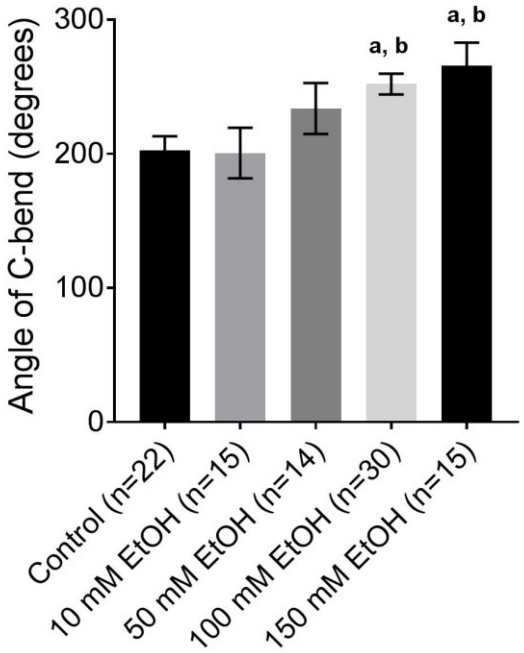

0 ms
6.4 ms
12.8 ms
19.2 ms
25.6 ms
32 ms
Control
10 mM EtOH
50 mM EtOH
100 mM EtOH
150 mM EtOH

Figure 5. Zebrafish embryos undergo a c-start bend in response to a jet of water directed at the head just behind the eyes. The head of each embryo is constrained in agar, with a small hole cut out just behind the eye region to stimulate the fish. The trunk is free to move so that the locomotor response can be examined. Still frame images were acquired every 800 μsec over the full duration of the response but every eighth image is shown for clarity. The picospritzer is located to the top right in every image. Embryos were treated for 5 hours between 5 hpf and 10 hpf with embryo media (controls; n = 22), 10 mM EtOH (n = 15), 50 mM EtOH (n = 14), 100 mM EtOH (n = 30) or 150 mM EtOH (n = 15).

A)


Angle of C-bend (degrees)
300
200
100
0
a, b
a, b
Control (n=22)
10 mM EtOH (n=15)
50 mM EtOH (n=14)
100 mM EtOH (n=30)
150 mM EtOH (n=15)

B)

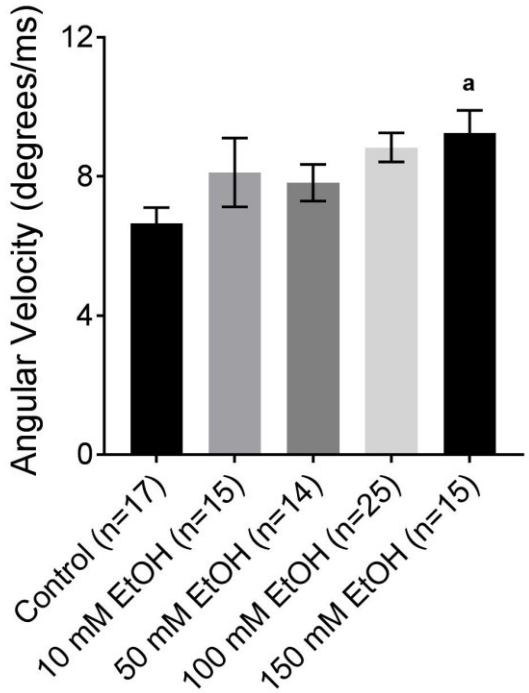
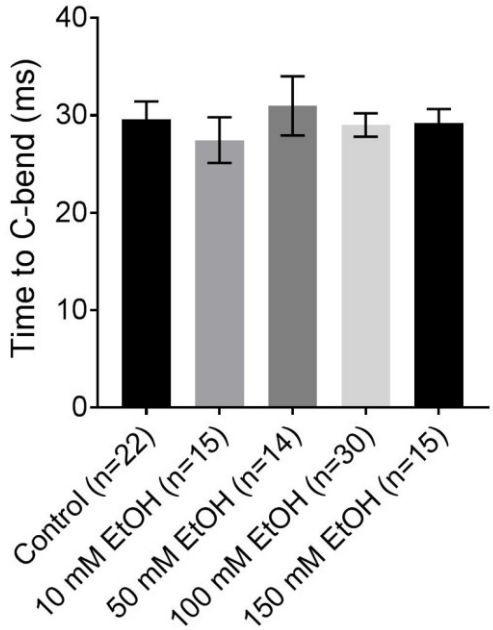

Angular Velocity (degrees/ms)
12
8
4
0
a
Control (n=17)
10 mM EtOH (n=15)
50 mM EtOH (n=14)
100 mM EtOH (n=25)
150 mM EtOH (n=15)

Figure 6. Quantification of the c-bend in response to a stimulus towards the head in 5.25 hpf -10.75 hpf embryo. (A) Bar graph shows the maximum angle of bend for controls (n = 22), 10 mm EtOH (n = 15), 50 mM EtOH (n = 14), 100 mM EtOH (n = 30) and 150 mM EtOH (n = 15). (B) Bar graph showing the angular velocity obtained by dividing the maximum bend angle by the time to bend for controls (n = 17), 10 mm EtOH (n = 15), 50 mM EtOH (n = 14), 100 mM EtOH (n = 25) and 150 mM EtOH (n = 15). [a] significantly different from controls, $p<0.05$; [b] significantly different from 10 mM EtOH, $p<0.05$.

A)


Time to C-bend (ms)
40
30
20
10
0
Control (n=22)
10 mM EtOH (n=15)
50 mM EtOH (n=14)
100 mM EtOH (n=30)
150 mM EtOH (n=15)

B)

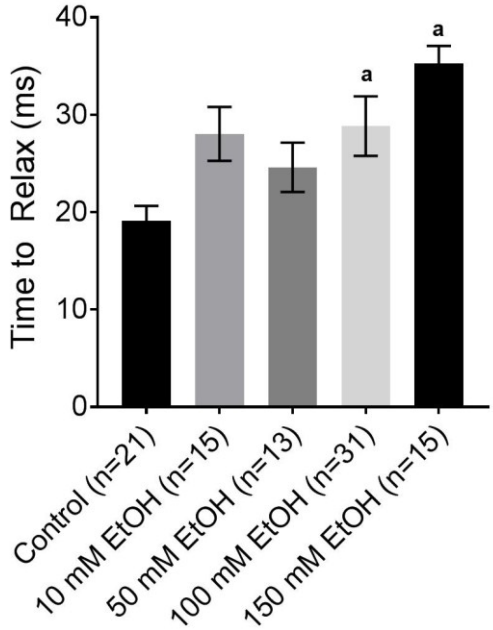

Time to Relax (ms)
40
30
20
10
0
a
a
Control (n=21)
10 mM EtOH (n=15)
50 mM EtOH (n=13)
100 mM EtOH (n=31)
150 mM EtOH (n=15)

Figure 7. Quantification of time in 5.25 hpf -10.75 hpf embryo (A) Bar graph showing time for the c-bend to occur from the moment the fish starts to respond, to the maximum point of curvature of the tail for controls (n = 22), 10 mm EtOH (n = 15), 50 mM EtOH (n = 14), 100 mM EtOH (n = 30) and 150 mM EtOH (15). (B) Time taken for the tail to move from the end of the c-start to the second bend of the tail for controls (n = 21), 10 mm EtOH (n = 15), 50 mM EtOH (n = 13), 100 mM EtOH (n = 31) and 150 mM EtOH (n = 15). [a] significantly different from controls, $p<0.05$.

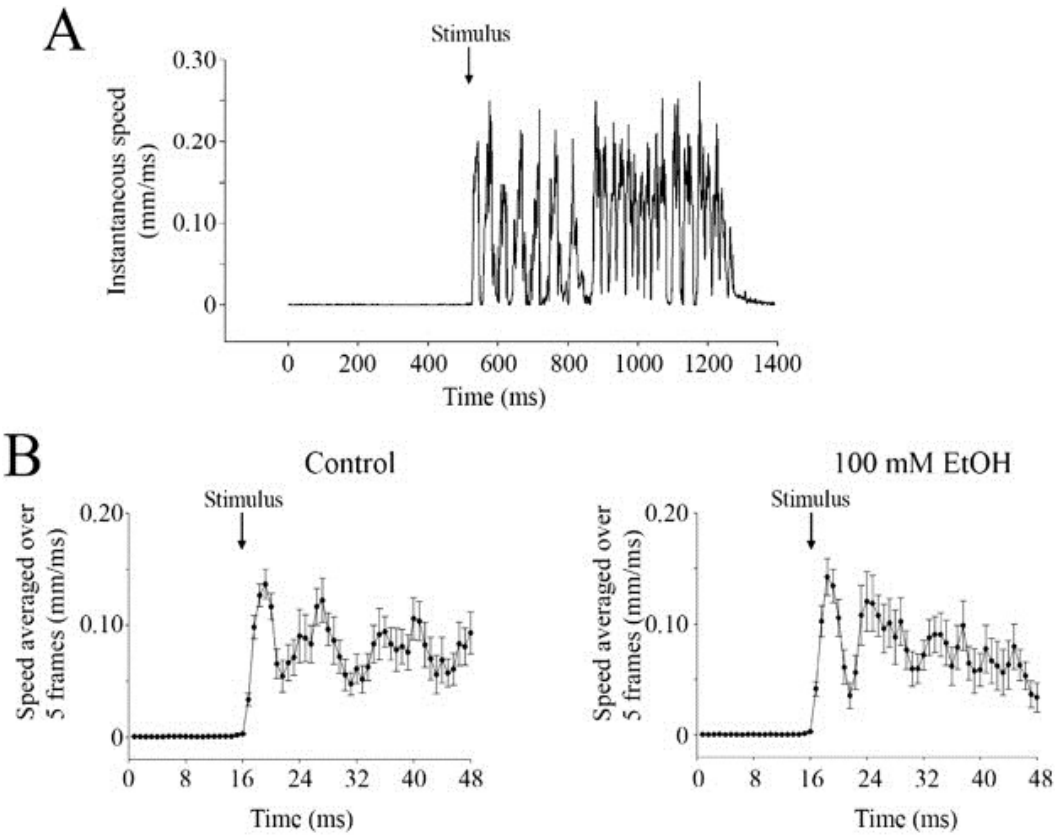
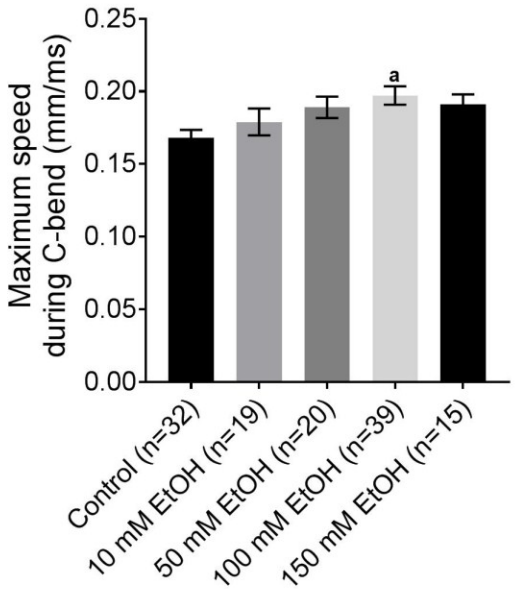
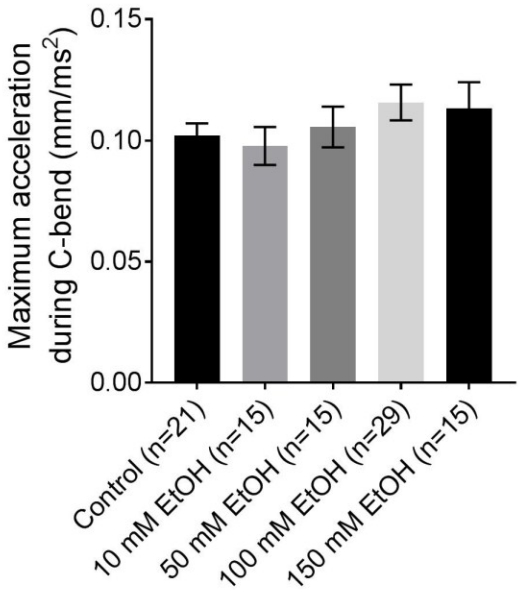

A
Stimulus
Instantaneous speed (mm/ms)
0.30
0.20
0.10
0
0 200 400 600 800 1000 1200 1400
Time (ms)
B
Control
100 mM EtOH
Stimulus
Speed averaged over 5 frames (mm/ms)
0.20
0.10
0
0 8 16 24 32 40 48
Time (ms)

Figure 8: Measurements of speed and acceleration of the tail following a stimulus to the head. (A) An example of a raw measurement of instantaneous speed of the tail prior to and immediately following a stimulus (arrow). (B) Instantaneous speed averaged over 5 frames for control (n = 32) and 100 mM EtOH-treated (n = 40).

A)


Maximum speed during C-bend (mm/ms)
0.25
0.20
0.15
0.10
0.05
0.00
a
Control (n=32)
10 mM EtOH (n=19)
50 mM EtOH (n=20)
100 mM EtOH (n=39)
150 mM EtOH (n=15)

B)


Maximum acceleration during C-bend (mm/ms^2)
0.15
0.10
0.05
0.00
Control (n=21)
10 mM EtOH (n=15)
50 mM EtOH (n=15)
100 mM EtOH (n=29)
150 mM EtOH (n=15)

C)

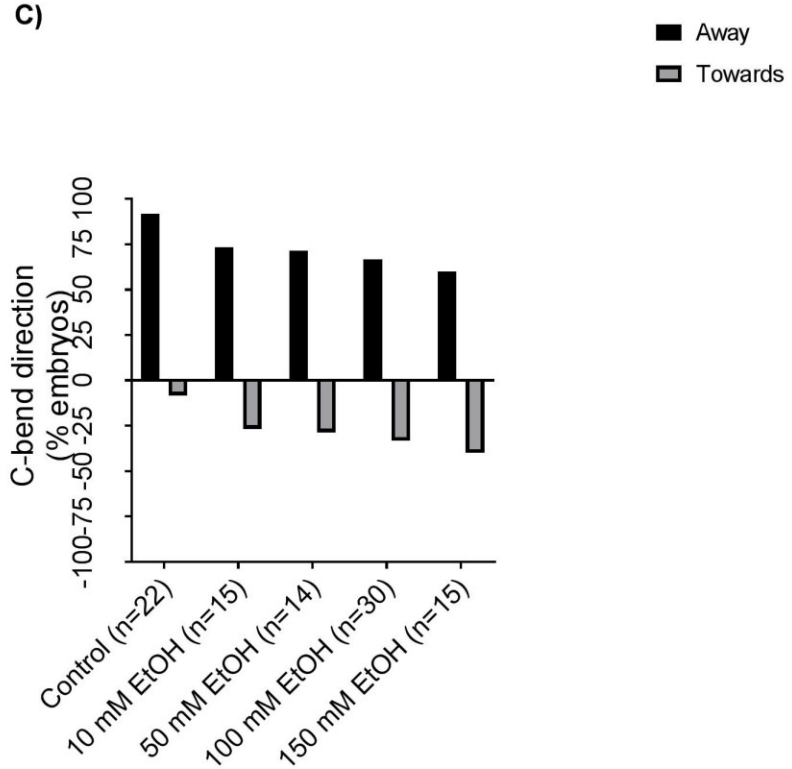
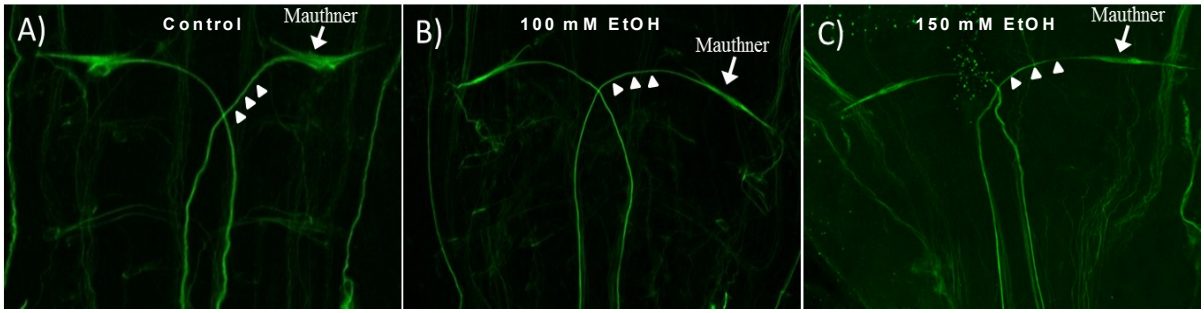
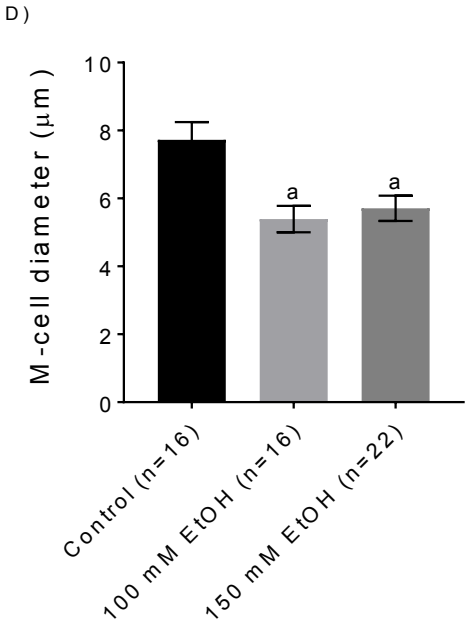

Away
Towards
C-bend direction (% embryos)
100 75 50 25 0 -25 -50 -75 -100
Control (n=22)
10 mM EtOH (n=15)
50 mM EtOH (n=14)
100 mM EtOH (n=30)
150 mM EtOH (n=15)

Figure 9. Quantification of the c-bend in response to a stimulus to the head in 5.25 hpf - 10.75 hpf embryo. (A) Bar graph shows the peak instantaneous speed attained during the c-bend for control (n = 32), 10 mM (n = 19), 50 mM (n = 20), 100 mM (n = 39) and 150 mM (n = 15). (B) Shows the peak acceleration attained during the c-bend for control (n = 21), 10 mM (n = 15), 50 mM (n = 15), 100 mM (n = 29) and 150 mM (n = 15). (C) Shows the direction of movement for controls (n = 22), 10 mm EtOH (n = 15), 50 mM EtOH (n = 14), 100 mM EtOH (n = 30) and 150 mM EtOH (15). The appropriate direction is always away from the stimulus. [a] significantly different from controls, $p<0.05$.


A)
Control
Mauthner
B)
100 mM EtOH
Mauthner
C)
150 mM EtOH
Mauthner


D)
M-cell diameter (µm)
10
8
6
4
2
0
a
a
Control (n=16)
100 mM EtOH (n=16)
150 mM EtOH (n=22)

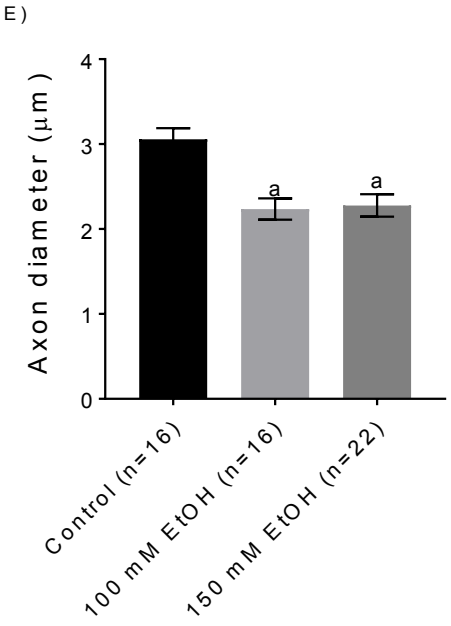

E)
Axon diameter (µm)
4
3
2
1
0
a
a
Control (n=16)
100 mM EtOH (n=16)
150 mM EtOH (n=22)

Figure 10. Immunohistochemical identification of the Mauthner cell (anti-3A10 antibody) in untreated controls (A; n = 16), 100 mM EtOH treated embryos (B; n = 16) and 150 mM EtOH treated embryos (C; n = 22) at 5.25 hpf – 10.75 hpf. Arrows point to the Mauthner cell body while the arrowheads point to the proximal axon immediately prior to the cross over point. (D, E) Measurements of the cell body diameter at its widest point (n = 14) and the axonal diameter (arrowheads, n = 12); [a] significantly different from controls, $p<0.05$.

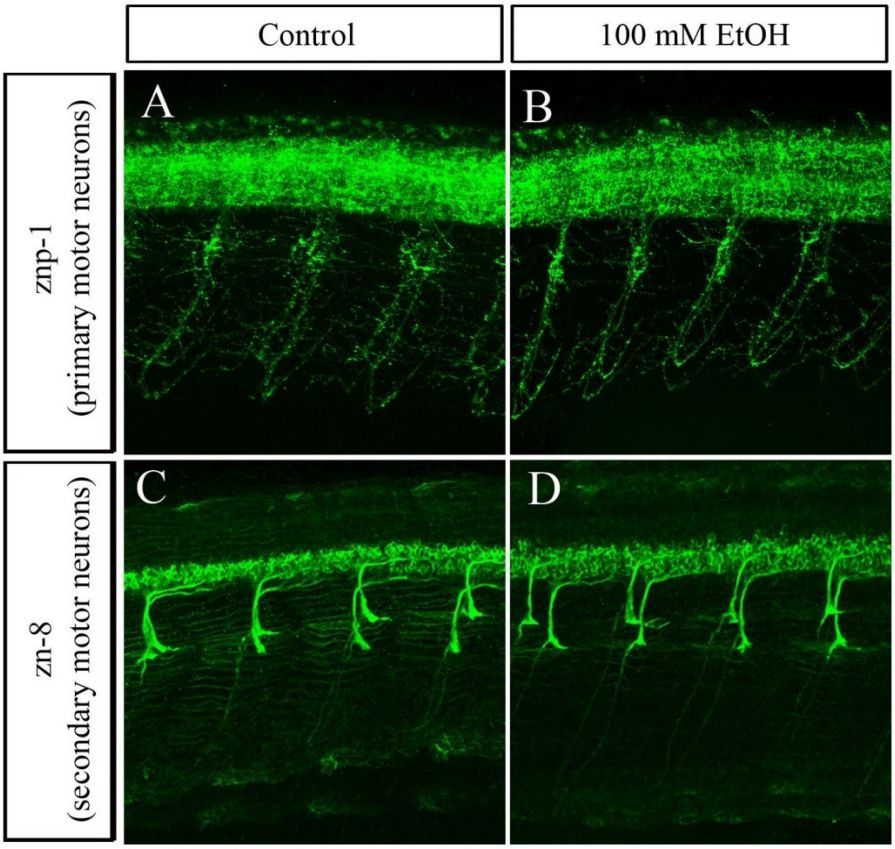
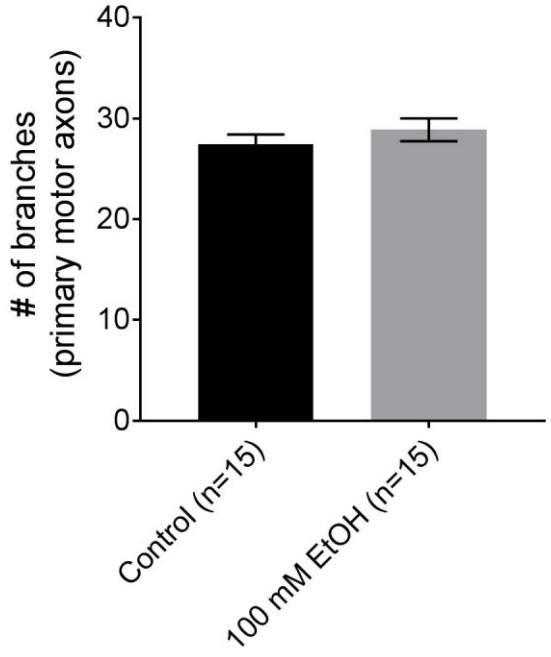

Control
100 mM EtOH
znp-1
(primary motor neurons)
zn-8
(secondary motor neurons)
A
B
C
D

Figure 11. Motor neuron morphology in 48 hpf embryos from untreated and 100 mM EtOH treated embryos. (A, B) Anti znp-1 identifies primary motor neurons (controls, n = 31; 100 mM EtOH, n = 27). (C, D) Anti zn-8 identifies secondary motor neurons (controls, n = 23; 100 mM EtOH, n = 29).

A)


# of branches
(primary motor axons)
40
30
20
10
0
Control (n=15)
100 mM EtOH (n=15)

B)

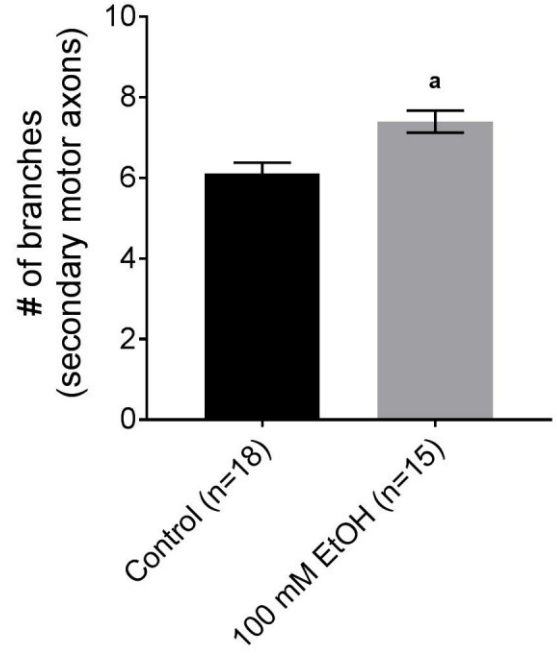

# of branches
(secondary motor axons)
10
8
6
4
2
0
a
Control (n=18)
100 mM EtOH (n=15)

Figure 12. Measurement of number of brunches emanating from the primary axon and secondary axon. (A) Bar graphs showing the number of branches emanating from the primary axon in control (n = 15) and 100 mM EtOH (n = 15) treated fish. (B) Bar graphs showing the number of branches emanating from the secondary axon in control (n = 18) and 100 mM EtOH (n = 15) treated fish. [a] denotes significantly different from controls, $p<0.05$.

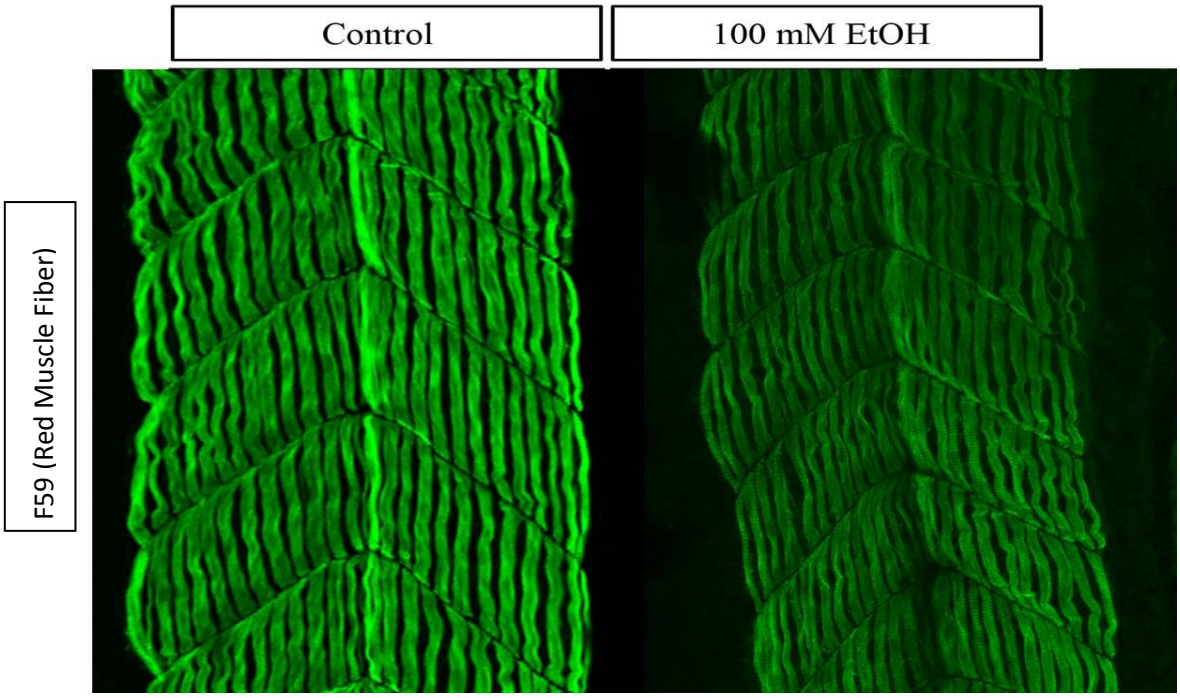

Control
100 mM EtOH
F59 (Red Muscle Fiber)

Figure 13. Red muscle fibre morphology in zebrafish larvae at 2 dpf following exposure to 0mM (n = 19) and 100 mM (n = 15) EtOH and labelled with F59.

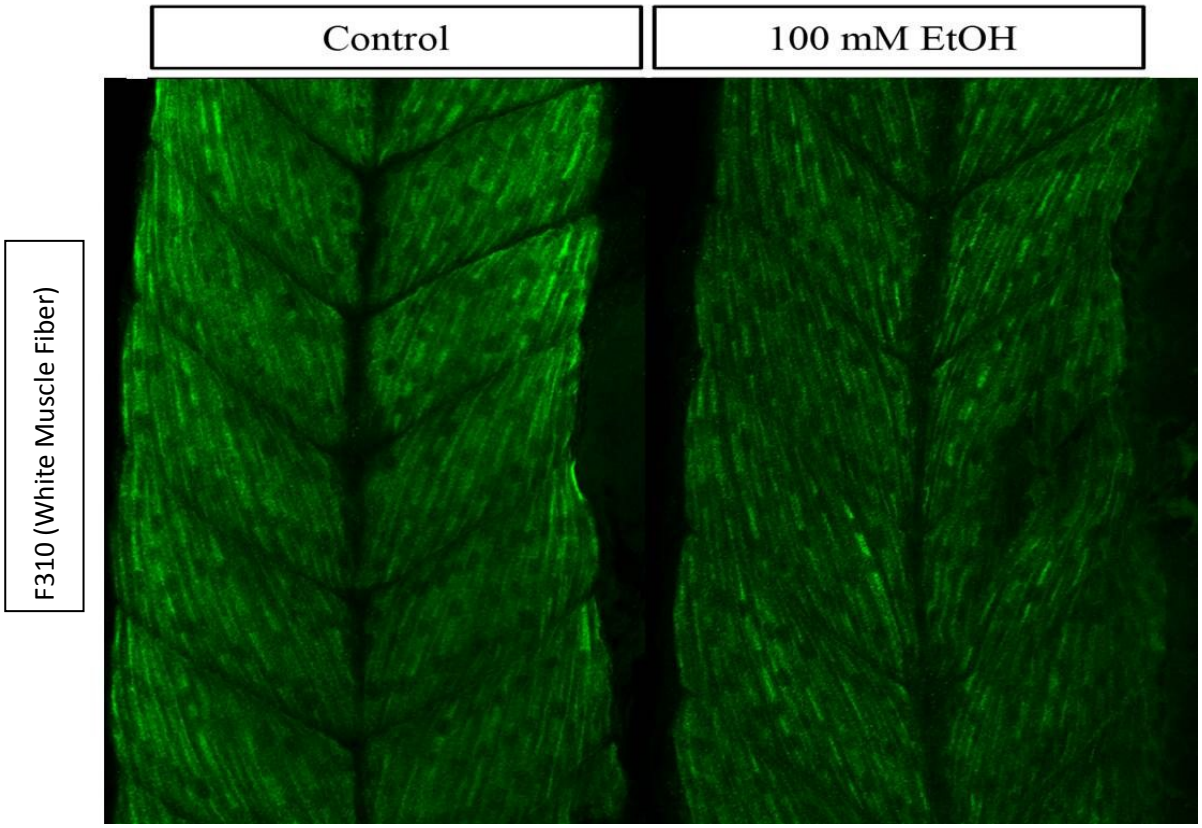

Control
100 mM EtOH
F310 (White Muscle Fiber)

Figure 14. White muscle fibre morphology in zebrafish larvae at 2 dpf following exposure to 0mM (n = 19) and 100 mM (n = 14) EtOH and labelled with F59.

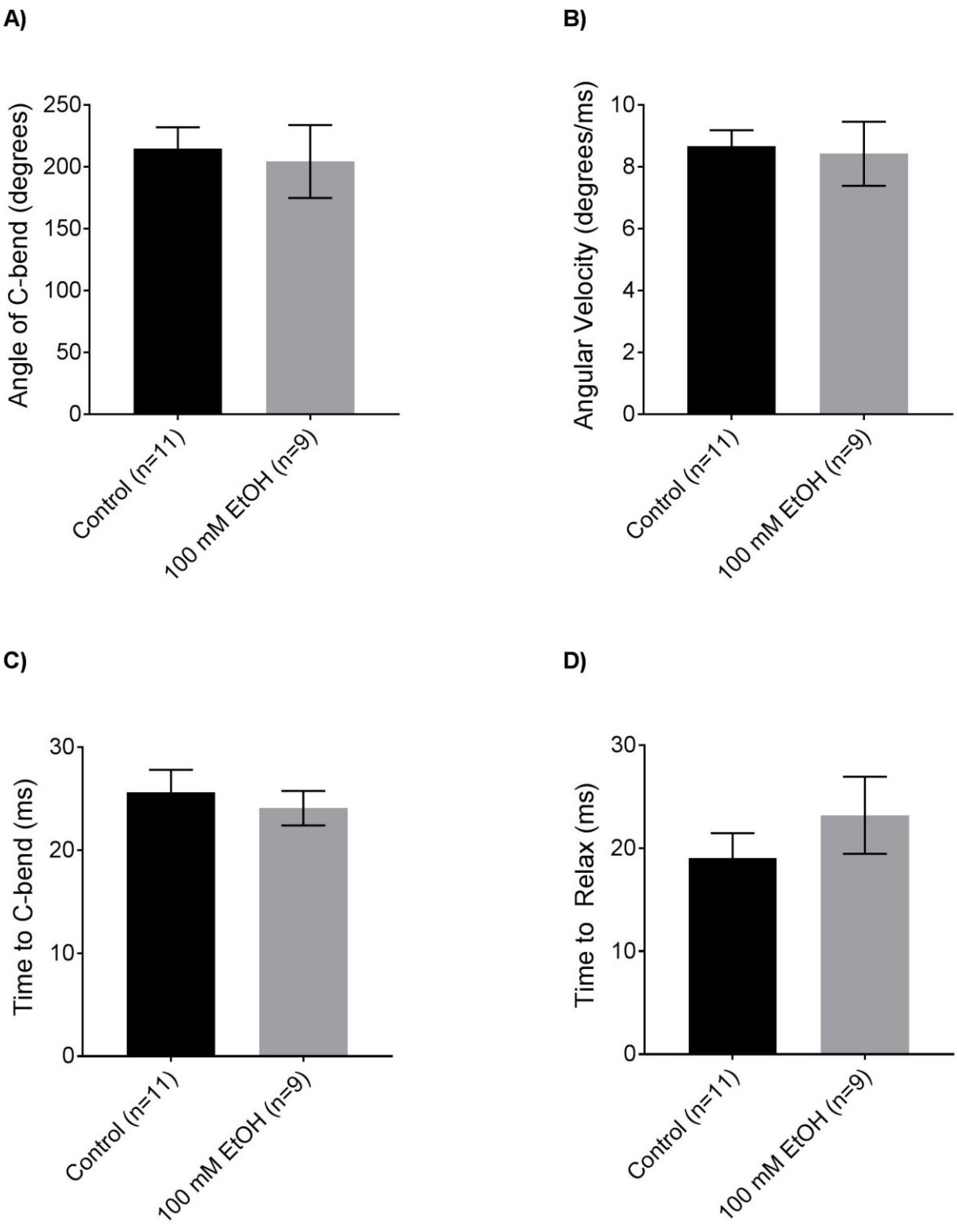

A)
Angle of C-bend (degrees)
250
200
150
100
50
0
Control (n=11)
100 mM EtOH (n=9)
B)
Angular Velocity (degrees/ms)
10
8
6
4
2
0
Control (n=11)
100 mM EtOH (n=9)
C)
Time to C-bend (ms)
30
20
10
0
Control (n=11)
100 mM EtOH (n=9)
D)
Time to Relax (ms)
30
20
10
0
Control (n=11)
100 mM EtOH (n=9)

Figure 15. Quantification of escape behavior in 12 hpf -17 hpf zebrafish embryo. Bar graph showing (A) maximum angel of the c-bend, (B) average angular velocity, (C) time to c-bend and (D) time taken for the tail to move from the end of the c-start to the second bend of the tail for control(n = 11) and 100 mM EtOH (n = 9).

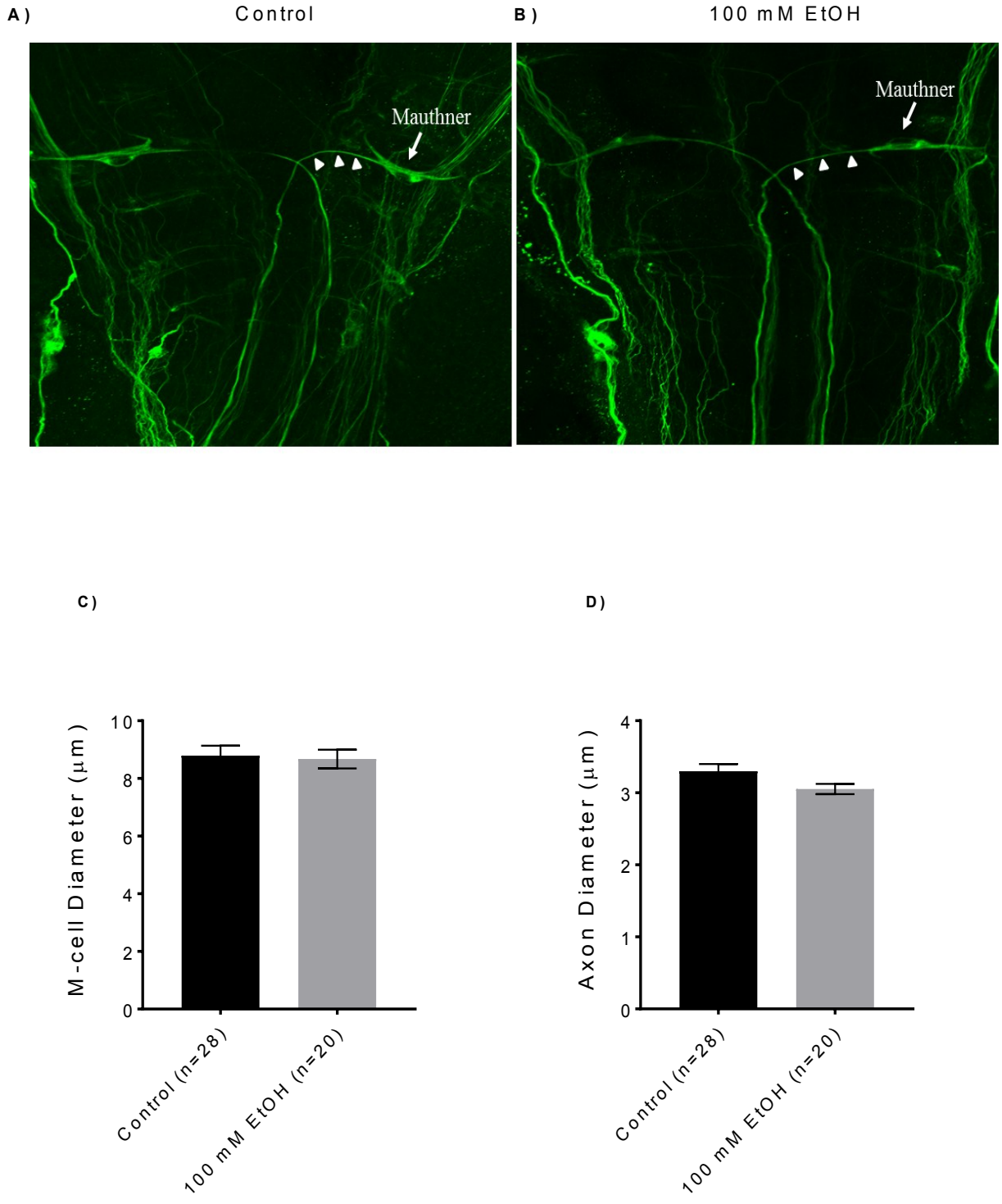

A)
Control
B)
100 mM EtOH
Mauthner
Mauthner
C)
M-cell Diameter (µm)
10
8
6
4
2
0
Control (n=28)
100 mM EtOH (n=20)
D)
Axon Diameter (µm)
4
3
2
1
0
Control (n=28)
100 mM EtOH (n=20)

Figure 16. Immunohistochemical identification of the Mauthner cell (anti-3A10 antibody) in untreated controls (A; n = 28), 100 mM EtOH treated embryos (B; n = 20) at 12 hpf – 17 hpf. (C, D) Measurements of the cell body diameter at its widest point and the axonal diameter (arrowheads).

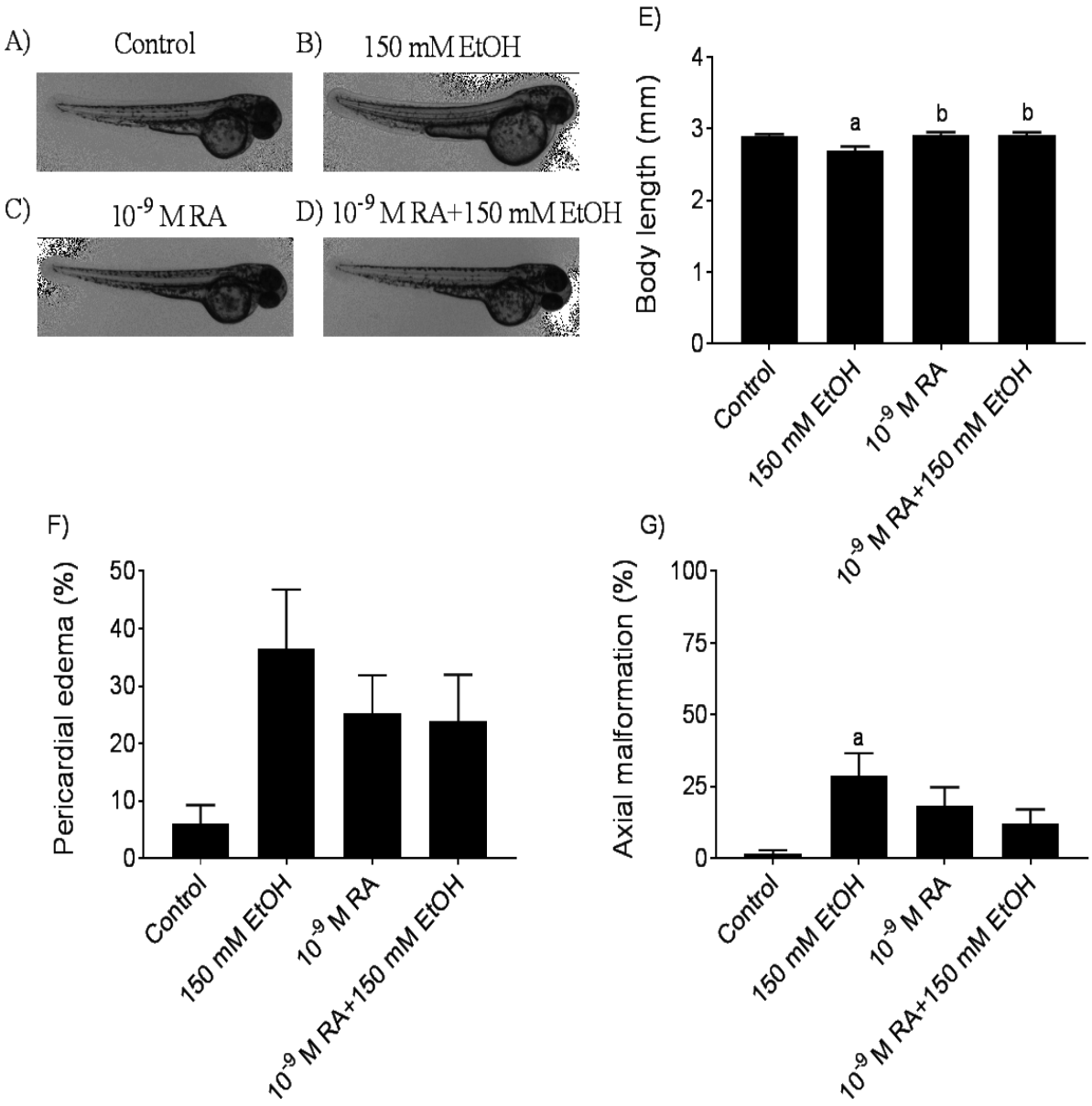
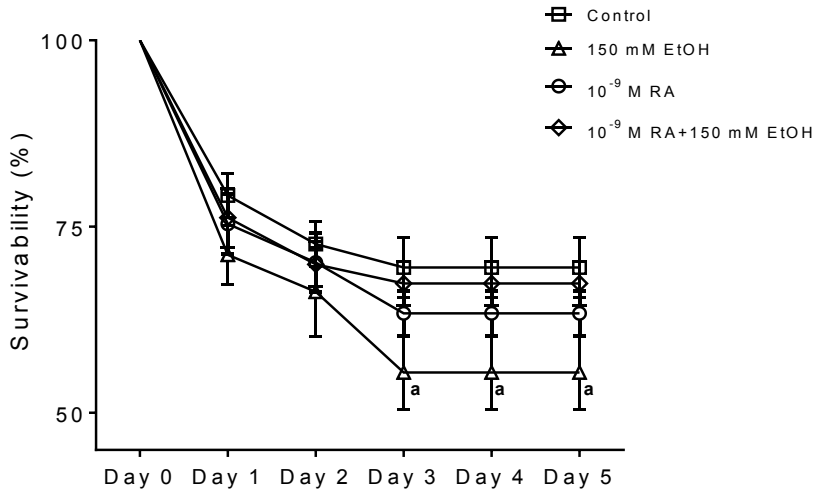

A)
Control
B)
150 mM EtOH
C)
10-9 M RA
D) 10-9 M RA+150 mM EtOH
E)
Body length (mm)
a
b
b
Control
150 mM EtOH
10-9 M RA
10-9 M RA+150 mM EtOH
F)
Pericardial edema (%)
Control
150 mM EtOH
10-9 M RA
10-9 M RA+150 mM EtOH
G)
Axial malformation (%)
a
Control
150 mM EtOH
10-9 M RA
10-9 M RA+150 mM EtOH

Figure 17. Effect of RA and EtOH exposure on gross morphology. (A-D) 2 dpf embryos were imaged after exposure to Control (A), 150 mM EtOH (B), 10^{-9} M RA (C), and 10^{-9} M RA+150 mM EtOH (D) during gastrulation. (E) Bar graph showing the body lengths of fish in different treatments (n = 43, 45, 39 and 43 for controls, 150 mM EtOH, 10^{-9} M RA and 150 mM EtOH+10^{-9} M RA treated fish respectively). (F) Bar graph showing the percentage of fish exhibiting pericardial edema (N = 6 batches). (G) Bar graph showing the percentage of fish exhibiting axial malformation (N = 6 batches). [a] Significantly different from controls on the same day, $p<0.05$; [b] significantly different from 10 mM EtOH treated embryos on the same day, $p<0.05$.

A)


Control
150 mM EtOH
10-9 M RA
10-9 M RA+150 mM EtOH
Survivability (%)
100
75
50
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
a
a
a

B)

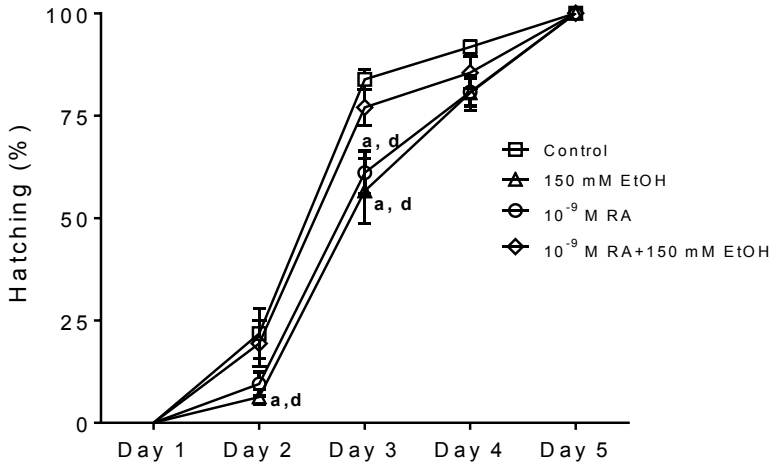

Hatching (%)
100
75
50
25
0
Day 1
Day 2
Day 3
Day 4
Day 5
a, d
a, d
a,d
Control
150 mM EtOH
10-9 M RA
10-9 M RA+150 mM EtOH

Figure 18. Effect of RA and EtOH exposure on hatching and survivality. (A, B) Line graph showing the percentage of hatched embryos and embryos that survived within the first 5 days after egg fertilization (N=12 batches for survival and N=9 batches for hatching). [a] Significantly different from controls on the same day, $p<0.05$; [d] significantly different from 10^{-9} M RA+150 mM EtOH treated embryos on the same day, $p<0.05$.

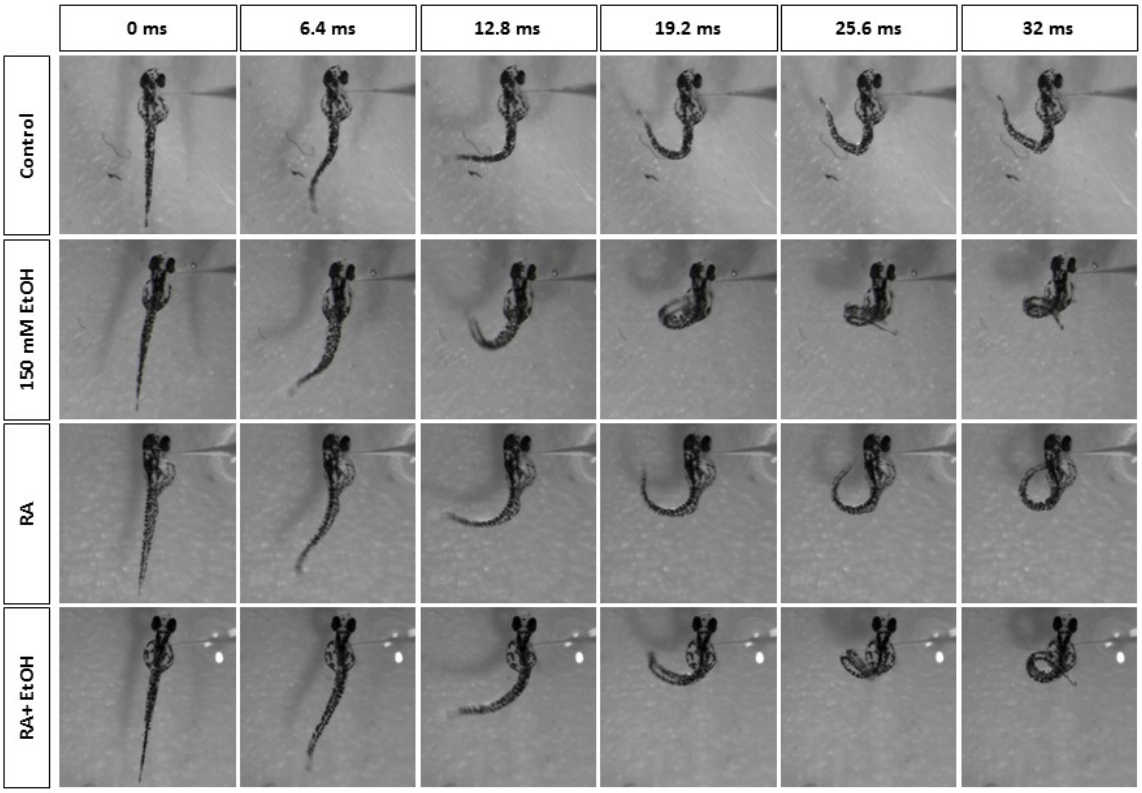

0 ms
6.4 ms
12.8 ms
19.2 ms
25.6 ms
32 ms
Control
150 mM EtOH
RA
RA+EtOH

Figure 19. Zebrafish embryos undergo a c-start bend in response to a jet of water directed at the head just behind the eyes. The head of each embryo is constrained in agar, with a small hole cut out just behind the eye region to stimulate the fish. The trunk is free to move so that the locomotor response can be examined. Still frame images were acquired every 800 μsec over the full duration of the response but every eighth image is shown for clarity. The picospritzer is located to the top right in every image. Embryos were treated for 5 hours between 5 hpf and 10 hpf with embryo media (controls; n = 26), 150 mM EtOH (n = 14), 10^{-9} M RA (n = 15) and 150 mM EtOH+10^{-9} M RA (n = 20).

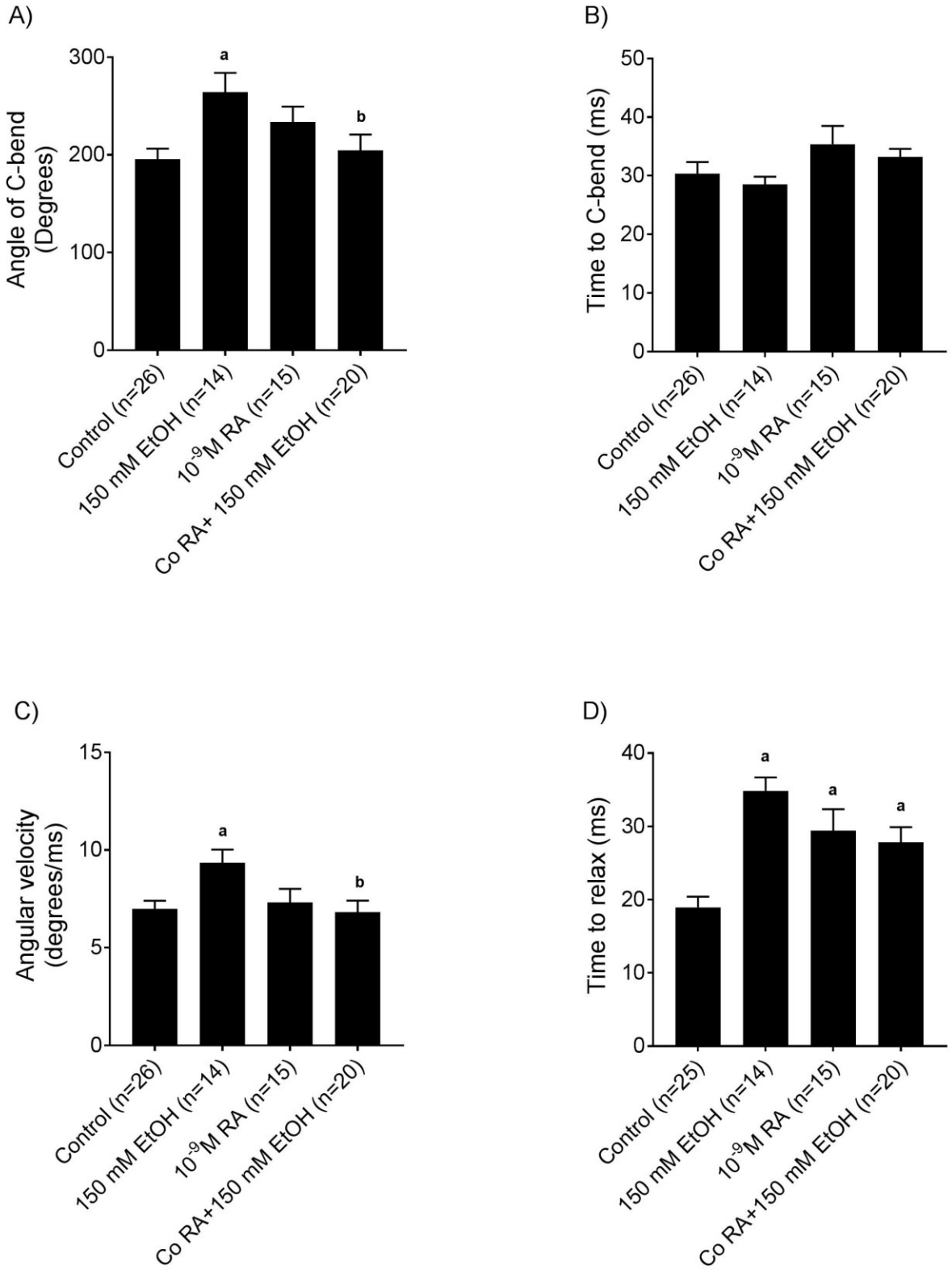

A)
Angle of C-bend (Degrees)
300
200
100
0
a
b
Control (n=26)
150 mM EtOH (n=14)
10-9M RA (n=15)
Co RA+ 150 mM EtOH (n=20)
B)
Time to C-bend (ms)
50
40
30
20
10
0
Control (n=26)
150 mM EtOH (n=14)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)
C)
Angular velocity (degrees/ms)
15
10
5
0
a
b
Control (n=26)
150 mM EtOH (n=14)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)
D)
Time to relax (ms)
40
30
20
10
0
a
a
a
Control (n=25)
150 mM EtOH (n=14)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)

Figure 20. Quantification of the c-bend for the retinoic acid rescue experiment in response to a stimulus towards the head in 5.25 hpf -10.75 hpf embryo. (A) Bar graph shows the maximum angle of bend for controls (n = 26), 150 mM EtOH (n = 14), 10^{-9} M RA (n = 15) and 150 mM EtOH + 10^{-9} M RA (n = 20). (B) Bar graph showing the angular velocity obtained by dividing the maximum bend angle by the time to bend. (C) Bar graph showing time for the c-bend to occur from the moment the fish starts to respond, to the maximum point of curvature of the tail. (D) Time taken for the tail to move from the end of the c-start to the second bend of the tail. [a] significantly different from controls, $p<0.05$; [b] significantly different from 150 mM EtOH, $p<0.05$.

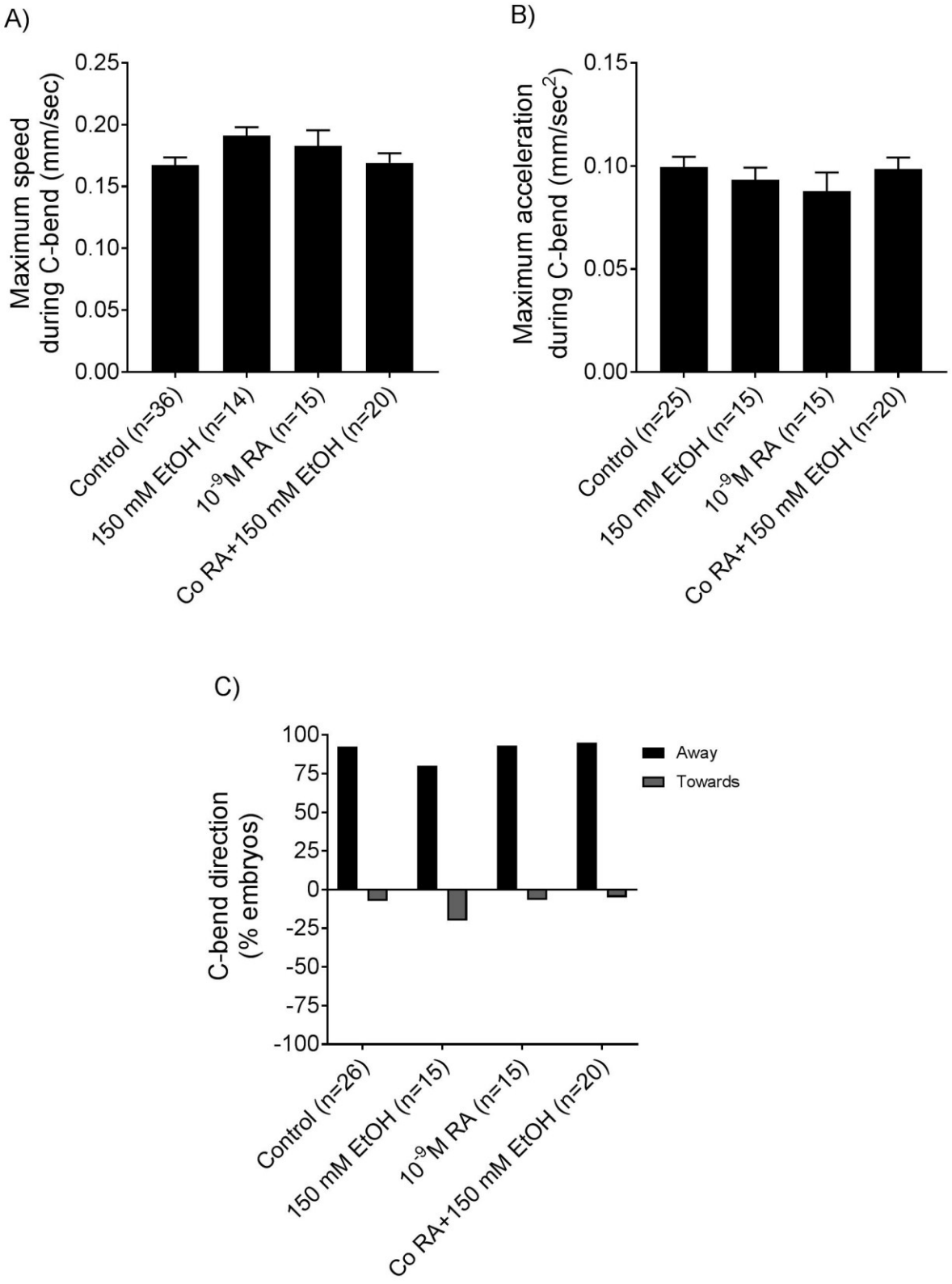
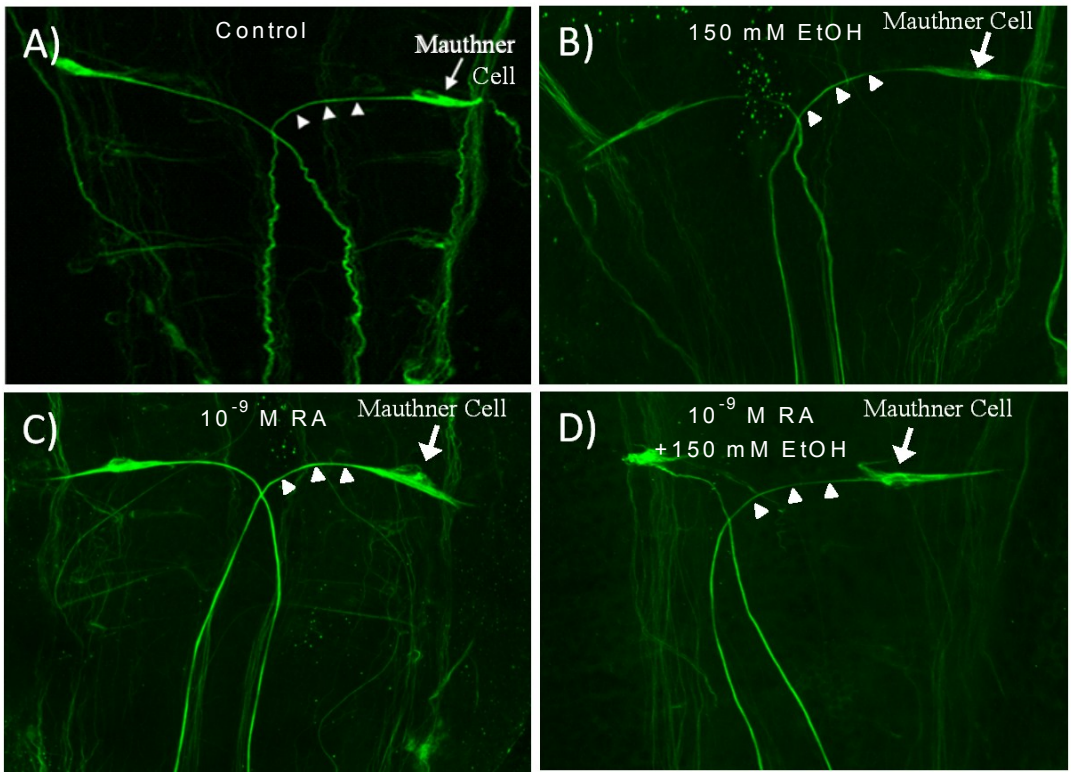
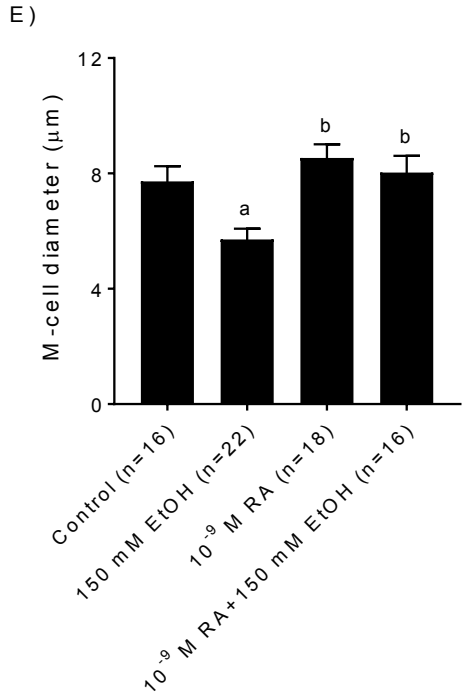

A)
Maximum speed during C-bend (mm/sec)
0.25
0.20
0.15
0.10
0.05
0.00
Control (n=36)
150 mM EtOH (n=14)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)
B)
Maximum acceleration during C-bend (mm/sec2)
0.15
0.10
0.05
0.00
Control (n=25)
150 mM EtOH (n=15)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)
C)
C-bend direction (% embryos)
100
75
50
25
0
-25
-50
-75
-100
Away
Towards
Control (n=26)
150 mM EtOH (n=15)
10-9M RA (n=15)
Co RA+150 mM EtOH (n=20)

Figure 21. Quantification of the c-bend for the retinoic acid rescue experiment in response to a stimulus towards the head in 5.25 hpf -10.75 hpf embryo. (A) Bar graph shows the peak instantaneous speed attained during the c-bend for controls (n = 36), 150 mM EtOH (n = 14), 10^{-9} M RA (n = 15) and 150 mM EtOH + 10^{-9} M RA (n = 20). (B) Shows the peak acceleration attained during the c-bend for control (n = 26), 150 mM EtOH (n = 14), 10^{-9} M RA (n = 15) and 150 mM EtOH + 10^{-9} M RA (n = 20). (C) Shows the direction of movement for control (n = 26), 150 mM EtOH (n = 15), 10^{-9} M RA (n = 15) and 150 mM EtOH + 10^{-9} M RA (n = 20). The appropriate direction is always away from the stimulus.


A)
Control
Mauthner Cell
B)
150 mM EtOH
Mauthner Cell
C)
10-9 M RA
Mauthner Cell
D)
10-9 M RA
+150 mM EtOH
Mauthner Cell


E)
M-cell diameter (µm)
12
8
4
0
a
b
b
Control (n=16)
150 mM EtOH (n=22)
10-9 M RA (n=18)
10-9 M RA+150 mM EtOH (n=16)

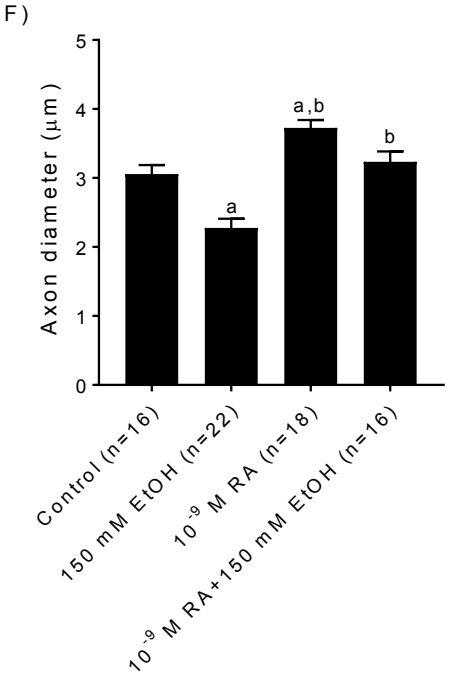

F)
Axon diameter (µm)
5
4
3
2
1
0
a
a,b
b
Control (n=16)
150 mM EtOH (n=22)
10-9 M RA (n=18)
10-9 M RA+150 mM EtOH (n=16)

Figure 22. Immunohistochemical identification of the Mauthner cell (anti-3A10 antibody) in untreated controls (A; n = 16), 150 mM EtOH (B; n = 18), 10^{-9} M RA (C; n = 22) and 150 mM EtOH+10^{-9} M RA treated embryos (D; n = 16). Arrows point to the Mauthner cell body while the arrowheads point to the proximal axon immediately prior to the cross over point. (E, F) Measurements of the cell body diameter at its widest point and the axonal diameter. [a] significantly different from controls, $p \leq 0.05$; [b] significantly different from 150 mM EtOH, $p \leq 0.05$.

4. Discussion

4.1. General Findings

It is well-established that prenatal or early embryonic exposure to alcohol has a detrimental effect on developing organisms (Jones and Smith 1973, Jones 1975, Kalberg and Buckley 2007, Sylvain, Brewster et al. 2010, Sylvain, Brewster et al. 2011, Shan, Boutin et al. 2015, Ferdous, Mukherjee et al. 2017). To my knowledge, very few studies have focused specifically on exposure to alcohol only during gastrulation (Sulik, Johnston et al. 1981). My study primarily sought to investigate how brief EtOH treatment of zebrafish embryos during gastrulation leads to alterations in animal behavior and morphology of the M-cell and motor neurons. My objectives were two-fold. First, I wanted to determine if ethanol exposure altered excitatory synaptic activity associated with the M-cell. Second, I wanted to determine if retinoic acid could prevent the effects of ethanol exposure during gastrulation. M-cells first appear around 8 hpf (during gastrulation) and project an axon contralaterally down the spinal cord. They are involved in the escape response and when the fish is stimulated by sound or touch, the M-cells become activated by electrical and chemical synaptic transmission. I particularly focused on cells involved in locomotion because motor deficits and altered reflex responses have been observed in young humans, rodents and zebrafish that were exposed to EtOH during development (Driscoll, Streissguth et al. 1990, Carvan, Loucks et al. 2004, Kalberg and Buckley 2007). Specifically, I found that exposure to 50 mM, 100 mM or 150 mM EtOH, between 5.25 hpf and 10.75 hpf, resulted in a small increase in physical abnormalities by the time of hatching and differences in the startle response when compared with untreated embryos. Furthermore, treatment with 100 mM and 150 mM EtOH resulted in smaller M-cell bodies and axons. There were no obvious changes in the morphology of either primary or secondary motor neurons. I also

investigated the effects on muscle morphology but found no change in the appearance of white or red muscle fibres.

Besides looking at the adverse effects of EtOH exposure, in this experimental model the extent of the rescue phenotype was determined, particularly during the gastrulation stage of zebrafish development. Previously published work demonstrated the effect of various concentrations of ethanol, which were shown to alter the general morphology, escape behavior and neuronal morphology of the zebrafish embryo when animals were exposed during gastrulation (Shan, Boutin et al. 2015, Ferdous, Mukherjee et al. 2017). In this study, retinoic acid co-treatment with ethanol prevented the altered phenomena.

The main findings can be summarized as follows: ethanol exposure during gastrulation altered gross morphology of 48 hpf embryos, survival and body length, and leads to a higher incidence of cardiac edema and axial malformation. Moreover, ethanol exposure changes the morphology of M-cells. In locomotor studies, the angle of the C-start response is altered and the relaxation time is lengthened following alcohol treatment. Co-treatment of ethanol and retinoic acid prevented all of the effects of ethanol exposure, while treatment with retinoic acid alone had very little or no effect on many of the parameters I investigated. These findings show that ethanol exposure during gastrulation has an impact on the developing embryo. However this can be prevented by co-treatment with retinoic acid. Thus, even brief exposure to alcohol may be detrimental to the embryo and can alter both neuronal morphology and locomotion.

4.2. Gross Morphology

The results presented in this study indicate that EtOH exposure resulted in physical deformities of the embryo such as shorter trunks, pericardial edema and axial malformations. In humans, FASD is characterized by pre- and post-natal growth deficiencies, ocular deficits, cranial and facial abnormities, as well as joint and cardiac anomalies (Kenneth L. Jones 1973, Jones 1975). When exposed to EtOH prenatally, rodents display very similar neurobehavioral and physiological effects compared to children with FASD, including altered brain development and poor motor coordination (Driscoll, Streissguth et al. 1990). Zebrafish embryos exposed to 1% EtOH did not display a significant increase in physical deformities, whereas fish exposed to 1.5% EtOH showed an increase in pericardial edema. Furthermore, 1.5% EtOH treatment resulted in only a few, mild deficits in motor neuron and muscle fiber morphology. This is mirrored in the hatching rate and swimming movements of larvae exposed to 1.5% EtOH when compared to larvae exposed to 2% and 2.5% EtOH. Other studies have reported comparable effects in zebrafish morphology when using similar EtOH concentrations over slightly different exposure windows (Bilotta, Barnett et al. 2004, Arenzana, Carvan et al. 2006). Another study reported mortality rates of 50% or more at 5 dpf when zebrafish were exposed from 3 to 24 hpf to EtOH concentrations over 380 mM {approximately 2.2%;(Reimers, Flockton et al. 2004)}. In this study, I found that there were significantly higher levels of mortality and lower hatching rates in ethanol-treated embryos compared with untreated animals.

It is important to note that although zebrafish have been used in several studies as a model for FASD, the timing, the length and the ethanol concentration used in the studies vary greatly, making it difficult to compare results between studies. For example, Arenzana, Carvan et al. (2006) exposed embryos to 1.5% and 2.4% EtOH from 4.7 hpf to 24 hpf, while Dlugos and

Rabin (2007) exposed embryos to concentration between 0.1% and 1.0% v/v from 0 hpf to 72 hpf. Interestingly, although zebrafish display defects that are similar to other species as a result of early ethanol exposure, the doses required to produce these defects in zebrafish embryos are remarkably higher. One possible explanation is that the chorion impedes the flow of ethanol from the surrounding medium to the embryo. Secondly, a human fetus may be exposed to alcohol concentrations that are higher than the mother's blood alcohol level due to the lack of alcohol dehydrogenase in the embryo (Yelin, Schyr et al. 2005).

Zebrafish embryos, like all cold-blooded vertebrates, absorb nutrients and oxygen through the skin (Matsui, Egana et al. 2006). Ethanol also passes through the skin and increase the blood alcohol level of the embryos. With the intact chorion of zebrafish embryo, Reimers, Flockton et al. (2004) measured internal ethanol concentrations that were around 30% that of the exposure concentration after 4 h of exposure. Li, Yang et al. (2007) have found that after an exposure to 2% EtOH for 6 h (4.25-10.25 hpf), with the chorion intact, zebrafish blood alcohol content only reached 0.034 gdL-1. This concentration is lower than the legal blood alcohol content limit for operating a motor vehicle in Canada (0.08 gdL-1). In my study I used concentrations of ethanol that more closely approximate BAC levels experienced during drinking episodes. Binge drinking is defined as the consumption of at least 4 drinks in an hour (Bala, Marcos et al. 2014) and a single episode can lead to a blood alcohol concentration (BAC) of ~0.16-0.2% (~35 mM – 44 mM) that returns to normal in about 24 hours (Bala, Marcos et al. 2014). Several laboratories have measured zebrafish embryonic ethanol concentrations after various exposure paradigms and have obtained differing results, likely due to the variety of exposure times and testing methods. For instance, Fernandes and Gerlai (2009) found that 2 hrs of ethanol exposure at 24 hpf results in about 1/25 the external alcohol reaching the embryo,

while Reimers, Flockton et al. (2004) determined that the embryonic ethanol dose was about 1/4-1/3 of the external concentration for intact chorionated fish when exposed and sampled at 24 hpf. Yet another study showed that dechorionated embryos took up about 60-70% of the waterborne ethanol concentration within 2 hrs of exposure (Bradfield, West et al. 2006). Based on these findings we make a conservative estimate that approximately 20-25% of the waterborne alcohol concentration probably reached the embryos. Therefore, we estimate that an exposure of 10 mM EtOH equates to an embryonic dose of ~2 mM EtOH (0.012%), while an exposure of 100 mM equates to an embryonic dose of 20 mM EtOH (0.12%). Thus, exposure to 10 mM to 150 mM EtOH may approximate a BAC resulting from 1-4 drinks every hour. Taken together, this suggests that moderate alcohol exposure over the period of gastrulation may have a detrimental effect on neuronal morphology.

4.3. EtOH Behavior

Recent molecular and pharmacological studies have demonstrated that alcohol and particularly ethanol, directly acts on specific membrane proteins, such as receptors, ion channels, and signaling pathways (Harris 1999, Krystal, Staley et al. 2006, Harris, Trudell et al. 2008, Spanagel 2009). These proteins include glutamate NMDA receptors, γ-aminobutyric acid A (GABA-A) receptors, glycine, 5-hydroxytryptamine-3 (5-HT3), and nicotinic acetylcholine (nACh) receptors, as well as L-type Ca2+ channels and G protein-activated inwardly rectifying K^+ channels (Vengeliene, Bilbao et al. 2008). Alcohol indirectly affects various neurotransmitter/ neuropeptide systems (Vengeliene, Bilbao et al. 2008), which leads to typical acute behavioral effects of alcohol that range from disinhibition to sedation. In addition, alcohol activates some

specific signaling pathways and results in alterations of both gene expression and the neuroadaptations underlying alcohol-related disorders (Ron and Jurd 2005, Yao, Fan et al. 2008, Neasta, Ben Hamida et al. 2010). As a result of repeated alcohol intake, the long-lasting cellular and neurophysiological changes that trigger alcohol-seeking behavior become apparent in the brain reinforcement system (Yao, Fan et al. 2008, Neasta, Ben Hamida et al. 2010). Whether this behavioral response transforms into addictive behavior eventually depends on genetic and environmental factors (Spanagel 2009).

Behavioral assessment tools for measuring adult zebrafish behavior have been developed and tested by a number of laboratories. For example, Levin, Chrysanthis et al. (2003) developed a spatial discrimination learning task for zebrafish and have used this technique to assess the effects of chlorpyrifos exposure on developing zebrafish. Behavioral measures like activity and aggression measures are also being used to assess the effects of ethanol on zebrafish behavior (Gerlai, Lahav et al. 2000). Zebrafish represents a useful model for examining basic questions about the effects of embryonic exposure to ethanol on development with few experimental confounds. Many studies find significant effects on motor function that arise from alcohol consumption during pregnancy (Mattson and Riley 1998). The deficits range from mild defects in fine and gross motor control to kinetic tremors, reduced grip strength and alterations in motor speed, precision and timing of movement (Marcus 1987). It is interesting that the behavioral consequence of ethanol exposure in our study was a slight but significant increase in the bend angle and the peak velocity of the tail during the startle reflex. Notably, the average time to maximum bend angle was not different between treatments although there was a trend towards slightly shorter times in the treated groups. This translates into a significantly greater angular velocity in the ethanol treated groups compared with controls. Ethanol can induce hyperactivity

in larval or adult zebrafish at relatively lower concentrations (~1-2% in the larva and 0.25-0.5% in the adults), but it leads to decreases in activity at higher doses (Gerlai, Lahav et al. 2000, Lockwood, Bjerke et al. 2004). My findings differ from these studies in that we exposed the gastrulating embryos for a brief period of time and then examined their activity a day and a half after ethanol removal. Thus, the effects of ethanol were relatively long-lasting and were not simply reversed upon wash out. The startle reflex itself was strong, implying that not only were the synaptic connections between cells functioning well, but the trunk musculature contracted in a fairly robust manner. Moreover, the primary and secondary motorneurons appear to be morphologically normal.

4.4. Neuronal Morphology

The Mauthner cell is born around 8 hpf during the gastrulation period and starts to project an axon around 17-18 hpf (Kimmel, Hatta et al. 1990). The first afferent contacts arise from trigeminal ganglia around the time of axon outgrowth but the synapses do not appear to be functional until around 24 hpf (Kimmel, Hatta et al. 1990, Ali, Buss et al. 2000). Thus, ethanol exposure of the M cell at the time of cell birth may alter its normal developmental program, resulting in its smaller size. A number of studies have found that treatment with growth factors such as fibroblast growth factor (Fgf), sonic hedgehog (Shh), retinoic acid (RA), folic acid (FA) and antioxidants are capable of reducing or negating the effects of ethanol exposure (Reimers, La Du et al. 2006, Loucks and Ahlgren 2009, Marrs, Clendenon et al. 2010, Sarmah and Marrs 2013, Zhang, Ojiaku et al. 2013). Additionally, it has been suggested that ethanol may disrupt the post-translational cholesterol modification of genes that respond to Shh (Li, Yang et al. 2007).

The exact mechanisms by which these factors prevent the effects of ethanol remain unknown, but may be as straightforward as a direct inhibition of the signal transduction pathways for these compounds. The M cell typically fires once following stimulation (Liu and Fetcho 1999, Nakayama and Oda 2004, Brewster and Ali 2013). This single action potential induces a unilateral contraction of the contralateral trunk musculature by activation of two main pathways. The first is a direct pathway in which the single Mauthner action potential induces a suprathreshold potential in primary motorneurons, while the second pathway entails direct activation of a descending interneuron which itself monosynaptically activates both primary and secondary motoneurons. Immediately after firing an action potential, a short latency inhibition is fed back onto both M-cells to ensure that only a single response is elicited in the appropriate cell. If this inhibition does not occur, the M-cell may fire repetitively or both M-cells may fire together leading to an ineffective startle reflex (Faber, Fetcho et al. 1989). An exaggerated response such as the one we have seen likely occurs due to a small increase in motor neuron and muscle fiber activation. This in turn may occur through alterations in the fine control of either the excitatory or inhibitory circuits.

In a previous study, embryos were exposed to much higher ethanol concentrations (0.5%-3%) for 16 hours (from 8 hpf to 24 hpf). In that study, a number of significant changes to synaptic physiology associated with trunk neuromuscular junctions (NMJs) were noted, but only at the higher ethanol concentrations. Therefore, it is unlikely that there would be major alterations at the NMJ given the robust startle reflex in this study. I found no major effects on primary or secondary motor neurons in this study. Primary neurons are born around 9-16 hpf whereas secondary motor neurons start to be born around 14 hpf until after 25 hpf (Myers, Eisen et al. 1986). Thus, even though some primary motor neurons were exposed to ethanol during cell

birth, I did not detect any obvious morphological differences. It is interesting that I found an effect on the number of branches of secondary motor neurons, which were born after ethanol removal. Currently I am unable to explain this finding except that it highlights the potential long-term and lingering effects of ethanol exposure even after its removal.

4.5. Rescue with Retinoic Acid

I found that co-treatment of retinoic acid with ethanol prevented the effects of ethanol treatment alone. Among the 2 classes of retinoic acid receptors RARs and RXRs, binding of retinoic acid to both of them are essential for regulation of cell and neuronal differentiation which then allows the transcription of limb, brain and nervous system development (Zachman and Grummer 1998, Marrs, Clendenon et al. 2010). Embryonic exposure to high concentrations of ethanol alters the expression of RAR and RXR in the cerebellum (Kumar, Singh et al. 2010), underscoring the importance of maintaining an adequate supply of RA throughout pregnancy. Indeed, there is a lot of evidence linking the levels of RA, retinol and FASD (Zachman and Grummer 1998, Young, Giesbrecht et al. 2014) (reviewed by Ballard, Sun et al. (2012)). Alcohol can play an important role in inhibiting retinoic acid production mechanism in most parts of the brain. The retinoic acid mediated pathway could be a major mechanism of the visible expression of FASD. When retinol is not oxidized, it will accumulate in non-hepatic tissues, such as in the kidney and the lungs (Zachman and Grummer 1998). It has been suggested that the morphologic defects commonly seen in individuals with deficiency may also be due to an excess amount of retinol accumulating in non-hepatic tissues (West, Chen et al. 1994). Therefore, for proper

growth and development, maintaining a balance of retinol and retinol metabolism is essential, which can be disturbed due to maternal consumption of alcohol (Young, Giesbrecht et al. 2014).

Xenopus embryos exposed to ethanol exhibit a higher incidence of physical malformations that resemble those of individuals with FASD (Yelin, Schyr et al. 2005, Yelin, Kot et al. 2007). It is well established that RA signaling is decreased in the presence of alcohol, in part because alcohol competes for enzymes that are responsible for the production of RA, such as alcohol dehydrogenase and acetaldehyde dehydrogenase (McCaffery, Koul et al. 2004, Kot-Leibovich and Fainsod 2009). Alcohols have a direct effect on retinoic acid metabolism through direct inhibition of alcohol or acetaldehyde dehydrogenase (McCaffery, Koul et al. 2004). This inhibition decreases the amount of retinoic acid available to produce the effects on neural development. Supporting the theory that ethanol inhibits acetaldehyde, a unique study has been conducted by Marrs, Clendenon et al. (2010) of zebrafish exposed to ethanol in utero (Ballard, Sun et al. 2012). This study helps to determine the pathophysiologic concentration of ethanol required to induce developmental changes in individuals with FASD and shows morphological defects using that concentration. Retinoic acid supplementation corrected the dysmorphologic signs compared with the ethanol-exposed group. However, they were not fully rescued to the level of unexposed organisms (Marrs, Clendenon et al. 2010). In another study retinoic acid was tested specifically to see if it can reverse ethanol-induced cardiac defects in zebrafish and during cardiogenesis, co-supplementation of retinoic acid with ethanol reversed small eye and body length defects but could not rescue heart edema (Sarmah and Marrs 2013). In this study, while I used concentrations of ethanol and retinoic acid similar to what have been used by Sarmah and Marrs (2013), I only exposed embryos during the 5-hour gastrulation period, as opposed to the

21-hour exposure in previous studies. In this case I found that supplementation with RA has effectively rescued most of the ethanol-induced defects.

An excess of RA can be harmful to CNS development, and consumption of abnormal concentrations of RA can produce fetal anomalies similar to FASD deficiencies (Zachman and Grummer 1998, Napoli 2011). Moreover, an abundance of RA can affect learning and cognitive abilities, in addition to nervous system development and neuron specification (Napoli 2011). These findings suggest the importance of retinoic acid in FASD normalizing cell formation. To address this issue, further research is required to determine a defined amount of retinoic acid that can be consumed to alleviate alcohol-induced effects without producing other negative side effects. Although these studies showed beneficial effects with retinoic acid supplementation, they failed to consider the consequences of consuming excess RA during pregnancy. Carefully designed dietary intervention studies are needed to investigate and determine the specific amount of RA required in FASD because excess amounts of retinoic acid produce morphologic defects.

4.6. Conclusion and Future Studies

FASD is a complex, multifactorial, and intriguing disorder. The consequences that can ensue from alcohol consumption vary on the spectrum from producing little to no effect to fetal mortality. Because numerous metabolic derangements can develop as a result of alcohol consumption during pregnancy, it is critical to find ways to minimize and reduce the physical and neurological malformations that develop in the fetus. My findings suggest that acute exposure to moderate concentrations of alcohol may have a detrimental effect on embryos. Such exposures have the potential to lead to alterations in behavior, locomotion, and importantly to

neuronal morphology and/or function. It is quite likely that alcohol exposure during cell birth in particular, rather than during gastrulation per se, is a key aspect of these results. Thus, these findings suggest that alcohol consumption early in the first trimester when neurons are being born may have a detrimental and significant impact on neuronal health.

The information garnered yielded mixed results regarding the impact of supplementation. Consequently, nutrient supplementation is only a part of a total strategy to ameliorate the impact of FASD. Regardless of alcohol consumption, prenatal supplementation must be carefully planned to avoid the risk of human pregnancy complications. Single nutrients should be more carefully monitored. Because FASD is the result of multiple metabolic impairments, supplementation with 1 nutrient may not be effective to fully reverse the damage induced by alcohol consumption. Some future research in this area may focus on-

- The primary focus of future studies should be to determine the optimal amount of a nutrient needed to reduce a specific detrimental outcome of FASD and on the long term effects of such supplementation. If results from these studies produce beneficial effects, then it would be prudent to examine the combined effects of multiple nutrients, which may have beneficial and synergistic effects (Young, Giesbrecht et al. 2014).

- Future studies, including epigenetics, will shed more light on the potential protective and preventive roles of retinoic acid and other vitamins in reducing the abnormalities associated with the development of fetal alcohol syndrome.

- Further research in this area is warranted because it may lead to substantial progress in our understanding of normal and abnormal embryonic development at the molecular level.

5. References Cited

Ali, D. W., R. R. Buss and P. Drapeau (2000). "Properties of miniature glutamatergic EPSCs in neurons of the locomotor regions of the developing zebrafish." Journal of neurophysiology **83**(1): 181-191.

An, M., R. Luo and P. D. Henion (2002). "Differentiation and maturation of zebrafish dorsal root and sympathetic ganglion neurons." J Comp Neurol **446**(3): 267-275.

Arenzana, F. J., M. J. Carvan, 3rd, J. Aijon, R. Sanchez-Gonzalez, R. Arevalo and A. Porteros (2006). "Teratogenic effects of ethanol exposure on zebrafish visual system development." Neurotoxicol Teratol **28**(3): 342-348.

Astley, S. J. (2013). "Validation of the fetal alcohol spectrum disorder (FASD) 4-Digit Diagnostic Code." J Popul Ther Clin Pharmacol **20**(3): e416-467.

Bala, S., M. Marcos, A. Gattu, D. Catalano and G. Szabo (2014). "Acute binge drinking increases serum endotoxin and bacterial DNA levels in healthy individuals." PLoS One **9**(5): e96864.

Ballard, M. S., M. Sun and J. Ko (2012). "Vitamin A, folate, and choline as a possible preventive intervention to fetal alcohol syndrome." Med Hypotheses **78**(4): 489-493.

Becker, H. C., J. L. Diaz-Granados and C. L. Randall (1996). "Teratogenic actions of ethanol in the mouse: a minireview." Pharmacol Biochem Behav **55**(4): 501-513.

Bernhardt, R. R., A. B. Chitnis, L. Lindamer and J. Y. Kuwada (1990). "Identification of spinal neurons in the embryonic and larval zebrafish." J Comp Neurol **302**(3): 603-616.

Bilotta, J., J. A. Barnett, L. Hancock and S. Saszik (2004). "Ethanol exposure alters zebrafish development: a novel model of fetal alcohol syndrome." Neurotoxicol Teratol **26**(6): 737-743.

Bilotta, J., S. Saszik, C. M. Givin, H. R. Hardesty and S. E. Sutherland (2002). "Effects of embryonic exposure to ethanol on zebrafish visual function." Neurotoxicol Teratol **24**(6): 759-766.

Bradfield, J. Y., J. R. West and S. E. Maier (2006). "Uptake and elimination of ethanol by young zebrafish embryos." Neurotoxicology and teratology **28**(5): 629-633.

Bradfield, J. Y., J. R. West and S. E. Maier (2006). "Uptake and elimination of ethanol by young zebrafish embryos." Neurotoxicol Teratol **28**(5): 629-633.

Brewster, D. L. and D. W. Ali (2013). "Expression of the voltage-gated potassium channel subunit Kv1.1 in embryonic zebrafish Mauthner cells." Neurosci Lett **539**: 54-59.

Brewster, D. L. and D. W. Ali (2013). "Expression of the voltage-gated potassium channel subunit Kv1.1 in embryonic zebrafish Mauthner cells." Neuroscience letters **539**: 54-59.

Brien, J. F., D. W. Clarke, B. Richardson and J. Patrick (1985). "Disposition of ethanol in maternal blood, fetal blood, and amniotic fluid of third-trimester pregnant ewes." Am J Obstet Gynecol **152**(5): 583-590.

Bupp Becker, S. R. and I. A. Shibley, Jr. (1998). "Teratogenicity of ethanol in different chicken strains." Alcohol Alcohol **33**(5): 457-464.

Burgess, H. A. and M. Granato (2007). "Sensorimotor gating in larval zebrafish." J Neurosci **27**(18): 4984-4994.

Carvan, M. J., 3rd, E. Loucks, D. N. Weber and F. E. Williams (2004). "Ethanol effects on the developing zebrafish: neurobehavior and skeletal morphogenesis." Neurotoxicol Teratol **26**(6): 757-768.

Celio, M. R., E. G. Gray and G. M. Yasargil (1979). "Ultrastructure of the Mauthner axon collateral and its synapses in the goldfish spinal cord." J Neurocytol **8**(1): 19-29.

Chang, Y. T., J. W. Lin and D. S. Faber (1987). "Spinal inputs to the ventral dendrite of the teleost Mauthner cell." Brain Res **417**(2): 205-213.

Chudley, A. E., J. Conry, J. L. Cook, C. Loock, T. Rosales, N. LeBlanc and D. Public Health Agency of Canada's National Advisory Committee on Fetal Alcohol Spectrum (2005). "Fetal alcohol spectrum disorder: Canadian guidelines for diagnosis." CMAJ **172**(5 Suppl): S1-S21.

Crow, M. T. and F. E. Stockdale (1986). "The developmental program of fast myosin heavy chain expression in avian skeletal muscles." Dev Biol **118**(2): 333-342.

Cudd, T. A. (2005). "Animal model systems for the study of alcohol teratology." Exp Biol Med (Maywood) **230**(6): 389-393.

Cunningham, T. J. and G. Duester (2015). "Mechanisms of retinoic acid signalling and its roles in organ and limb development." Nat Rev Mol Cell Biol **16**(2): 110-123.

Deltour, L., H. L. Ang and G. Duester (1996). "Ethanol inhibition of retinoic acid synthesis as a potential mechanism for fetal alcohol syndrome." FASEB J **10**(9): 1050-1057.

Dhawan, R., Dusenbery, D. B., and Williams, P. L. (1999). "Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode Caenorhabditis elegans." Journal of Toxicology and Environmental Health Association **58**: 451-462.

Dilworth, F. J., C. Fromental-Ramain, K. Yamamoto and P. Chambon (2000). "ATP-driven chromatin remodeling activity and histone acetyltransferases act sequentially during transactivation by RAR/RXR In vitro." Mol Cell **6**(5): 1049-1058.

Dlugos, C. A. and R. A. Rabin (2007). "Ocular deficits associated with alcohol exposure during zebrafish development." J Comp Neurol **502**(4): 497-506.

Dobbing, J. and J. Sands (1979). "Comparative aspects of the brain growth spurt." Early Hum Dev **3**(1): 79-83.

Driscoll, C. D., A. P. Streissguth and E. P. Riley (1990). "Prenatal alcohol exposure: comparability of effects in humans and animal models." Neurotoxicol Teratol **12**(3): 231-237.

Duester, G. (1991). "A hypothetical mechanism for fetal alcohol syndrome involving ethanol inhibition of retinoic acid synthesis at the alcohol dehydrogenase step." Alcohol Clin Exp Res **15**(3): 568-572.

Duester, G. (2008). "Retinoic acid synthesis and signaling during early organogenesis." Cell **134**(6): 921-931.

Durston, A. J., J. P. Timmermans, W. J. Hage, H. F. Hendriks, N. J. de Vries, M. Heideveld and P. D. Nieuwkoop (1989). "Retinoic acid causes an anteroposterior transformation in the developing central nervous system." Nature **340**(6229): 140-144.

Eaton, R. C., R. A. Bombardieri and D. L. Meyer (1977). "The Mauthner-initiated startle response in teleost fish." J Exp Biol **66**(1): 65-81.

Eaton, R. C., R. DiDomenico and J. Nissanov (1988). "Flexible body dynamics of the goldfish C-start: implications for reticulospinal command mechanisms." J Neurosci **8**(8): 2758-2768.

Eaton, R. C., R. K. Lee and M. B. Foreman (2001). "The Mauthner cell and other identified neurons of the brainstem escape network of fish." Prog Neurobiol **63**(4): 467-485.

Eisen, J. S., P. Z. Myers and M. Westerfield (1986). "Pathway selection by growth cones of identified motoneurones in live zebra fish embryos." Nature **320**(6059): 269-271.

Ethen, M. K., T. A. Ramadhani, A. E. Scheuerle, M. A. Canfield, D. F. Wyszynski, C. M. Druschel, P. A. Romitti and S. National Birth Defects Prevention (2009). "Alcohol consumption by women before and during pregnancy." Matern Child Health J **13**(2): 274-285.

Faber, D. S., J. R. Fetcho and H. Korn (1989). "Neuronal networks underlying the escape response in goldfish. General implications for motor control." Annals of the New York Academy of Sciences **563**: 11-33.

Faber, D. S. and H. Korn (1975). "Inputs from the posterior lateral line nerves upon the goldfish Mauthner cells. II. Evidence that the inhibitory components are mediated by interneurons of the recurrent collateral network." Brain Res **96**(2): 349-356.

Ferdous, J., R. Mukherjee, K. T. Ahmed and D. W. Ali (2017). "Retinoic acid prevents synaptic deficiencies induced by alcohol exposure during gastrulation in zebrafish embryos." Neurotoxicology **62**: 100-110.

Fernandes, Y. and R. Gerlai (2009). "Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish." Alcohol Clin Exp Res **33**(4): 601-609.

Fernandes, Y. and R. Gerlai (2009). "Long-term behavioral changes in response to early developmental exposure to ethanol in zebrafish." Alcoholism, clinical and experimental research **33**(4): 601-609.

Fetcho, J. R. (1992). "Excitation of motoneurons by the Mauthner axon in goldfish: complexities in a "simple" reticulospinal pathway." J Neurophysiol **67**(6): 1574-1586.

Fetcho, J. R. and D. M. O'Malley (1995). "Visualization of active neural circuitry in the spinal cord of intact zebrafish." J Neurophysiol **73**(1): 399-406.

Foreman, M. B. and R. C. Eaton (1993). "The direction change concept for reticulospinal control of goldfish escape." J Neurosci **13**(10): 4101-4113.

Furukawa, T. and E. J. Furshpan (1963). "Two inhibitory mechanisms in the Mauthner neurons of goldfish." J Neurophysiol **26**: 140-176.

Gahtan, E., N. Sankrithi, J. B. Campos and D. M. O'Malley (2002). "Evidence for a widespread brain stem escape network in larval zebrafish." J Neurophysiol **87**(1): 608-614.

Gerlai, R., M. Lahav, S. Guo and A. Rosenthal (2000). "Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects." Pharmacol Biochem Behav **67**(4): 773-782.

Gerlai, R., M. Lahav, S. Guo and A. Rosenthal (2000). "Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects." Pharmacology, biochemistry, and behavior **67**(4): 773-782.

Goh, Y. I., A. E. Chudley, S. K. Clarren, G. Koren, E. Orrbine, T. Rosales, C. Rosenbaum and F. S. T. Taskforce for Development of (2008). "Development of Canadian screening tools for fetal alcohol spectrum disorder." Can J Clin Pharmacol **15**(2): e344-366.

Green, J. H. (2007). "Fetal Alcohol Spectrum Disorders: understanding the effects of prenatal alcohol exposure and supporting students." J Sch Health **77**(3): 103-108.

Grunwald, D. J. and J. S. Eisen (2002). "Headwaters of the zebrafish -- emergence of a new model vertebrate." Nat Rev Genet **3**(9): 717-724.

Grunwald, D. J., C. B. Kimmel, M. Westerfield, C. Walker and G. Streisinger (1988). "A neural degeneration mutation that spares primary neurons in the zebrafish." Dev Biol **126**(1): 115-128.

Guo, S. (2004). "Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish?" Genes Brain Behav **3**(2): 63-74.

Harris, R. A. (1999). "Ethanol actions on multiple ion channels: which are important?" Alcohol Clin Exp Res **23**(10): 1563-1570.

Harris, R. A., J. R. Trudell and S. J. Mihic (2008). "Ethanol's molecular targets." Sci Signal **1**(28): re7.

Idanpaan-Heikkila, J., P. Jouppila, H. K. Akerblom, R. Isoaho, E. Kauppila and M. Koivisto (1972). "Elimination and metabolic effects of ethanol in mother, fetus, and newborn infant." Am J Obstet Gynecol **112**(3): 387-393.

Jones, K. L. (1975). "The fetal alcohol syndrome." Addict Dis **2**(1-2): 79-88.

Jones, K. L. and D. W. Smith (1973). "Recognition of the fetal alcohol syndrome in early infancy." Lancet **302**(7836): 999-1001.

Jones, K. L. and D. W. Smith (1975). "The fetal alcohol syndrome." Teratology **12**(1): 1-10.

Kalberg, W. O. and D. Buckley (2007). "FASD: what types of intervention and rehabilitation are useful?" Neurosci Biobehav Rev **31**(2): 278-285.

Keir, W. J. (1991). "Inhibition of retinoic acid synthesis and its implications in fetal alcohol syndrome." Alcohol Clin Exp Res **15**(3): 560-564.

Kenneth L. Jones, D. W. S. (1973). "RECOGNITION OF THE FETAL ALCOHOL SYNDROME IN EARLY INFANCY." The Lancet **302**(7836): p999–1001.

Kimmel, C. B., W. W. Ballard, S. R. Kimmel, B. Ullmann and T. F. Schilling (1995). "Stages of embryonic development of the zebrafish." Dev Dyn **203**: 253-310.

Kimmel, C. B., K. Hatta and W. K. Metcalfe (1990). "Early axonal contacts during development of an identified dendrite in the brain of the zebrafish." Neuron **4**(4): 535-545.

Kimmel, C. B., S. K. Sessions and R. J. Kimmel (1981). "Morphogenesis and synaptogenesis of the zebrafish Mauthner neuron." The Journal of comparative neurology **198**(1): 101-120.

Kimmel, C. B., R. M. Warga and T. F. Schilling (1990). "Origin and organization of the zebrafish fate map." Development **108**(4): 581-594.

Kodituwakku, P. W., W. Kalberg and P. A. May (2001). "The effects of prenatal alcohol exposure on executive functioning." Alcohol Res Health **25**(3): 192-198.

Kodituwakku, P. W., P. A. May, C. L. Clericuzio and D. Weers (2001). "Emotion-related learning in individuals prenatally exposed to alcohol: an investigation of the relation between set shifting, extinction of responses, and behavior." Neuropsychologia **39**(7): 699-708.

Kohashi, T. and Y. Oda (2008). "Initiation of Mauthner- or non-Mauthner-mediated fast escape evoked by different modes of sensory input." J Neurosci **28**(42): 10641-10653.

Kot-Leibovich, H. and A. Fainsod (2009). "Ethanol induces embryonic malformations by competing for retinaldehyde dehydrogenase activity during vertebrate gastrulation." Dis Model Mech **2**(5-6): 295-305.

Krystal, J. H., J. Staley, G. Mason, I. L. Petrakis, J. Kaufman, R. A. Harris, J. Gelernter and J. Lappalainen (2006). "Gamma-aminobutyric acid type A receptors and alcoholism: intoxication, dependence, vulnerability, and treatment." Arch Gen Psychiatry **63**(9): 957-968.

Kumar, A., C. K. Singh, D. D. DiPette and U. S. Singh (2010). "Ethanol impairs activation of retinoic acid receptors in cerebellar granule cells in a rodent model of fetal alcohol spectrum disorders." Alcohol Clin Exp Res **34**(5): 928-937.

Kuwada, J. Y., R. R. Bernhardt and N. Nguyen (1990). "Development of spinal neurons and tracts in the zebrafish embryo." J Comp Neurol **302**(3): 617-628.

Laale, H. W. (1971). "Ethanol induced notochord and spinal cord duplications in the embryo of the zebrafish, Brachydanio rerio." J Exp Zool **177**(1): 51-64.

Lee, R. K., R. C. Eaton and S. J. Zottoli (1993). "Segmental arrangement of reticulospinal neurons in the goldfish hindbrain." J Comp Neurol **329**(4): 539-556.

Lele, Z. and P. H. Krone (1996). "The zebrafish as a model system in developmental, toxicological and transgenic research." Biotechnol Adv **14**(1): 57-72.

Leo, M. A. and C. S. Lieber (1999). "Alcohol, vitamin A, and beta-carotene: adverse interactions, including hepatotoxicity and carcinogenicity." Am J Clin Nutr **69**(6): 1071-1085.

Levin, E. D., E. Chrysanthis, K. Yacisin and E. Linney (2003). "Chlorpyrifos exposure of developing zebrafish: effects on survival and long-term effects on response latency and spatial discrimination." Neurotoxicol Teratol **25**(1): 51-57.

Lewis, K. E. and J. S. Eisen (2003). "From cells to circuits: development of the zebrafish spinal cord." Prog Neurobiol **69**(6): 419-449.

Li, P., S. Shah, L. Huang, A. L. Carr, Y. Gao, C. Thisse, B. Thisse and L. Li (2007). "Cloning and spatial and temporal expression of the zebrafish dopamine D1 receptor." Dev Dyn **236**(5): 1339-1346.

Li, Y. X., H. T. Yang, M. Zdanowicz, J. K. Sicklick, Y. Qi, T. J. Camp and A. M. Diehl (2007). "Fetal alcohol exposure impairs Hedgehog cholesterol modification and signaling." Laboratory investigation; a journal of technical methods and pathology **87**(3): 231-240.

Liu, K. S. and J. R. Fetcho (1999). "Laser ablations reveal functional relationships of segmental hindbrain neurons in zebrafish." Neuron **23**(2): 325-335.

Lockwood, B., S. Bjerke, K. Kobayashi and S. Guo (2004). "Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening." Pharmacology, biochemistry, and behavior **77**(3): 647-654.

Lockwood, B., S. Bjerke, K. Kobayashi and S. Guo (2004). "Acute effects of alcohol on larval zebrafish: a genetic system for large-scale screening." Pharmacol Biochem Behav **77**(3): 647-654.

Loucks, E. J. and S. C. Ahlgren (2009). "Deciphering the role of Shh signaling in axial defects produced by ethanol exposure." Birth defects research. Part A, Clinical and molecular teratology **85**(6): 556-567.

Maden, M., D. E. Ong and F. Chytil (1990). "Retinoid-binding protein distribution in the developing mammalian nervous system." Development **109**(1): 75-80.

Marcus, J. C. (1987). "Neurological findings in the fetal alcohol syndrome." Neuropediatrics **18**(3): 158-160.

Marrs, J. A., S. G. Clendenon, D. R. Ratcliffe, S. M. Fielding, Q. Liu and W. F. Bosron (2010). "Zebrafish fetal alcohol syndrome model: effects of ethanol are rescued by retinoic acid supplement." Alcohol **44**(7-8): 707-715.

Matsui, J. I., A. L. Egana, T. R. Sponholtz, A. R. Adolph and J. E. Dowling (2006). "Effects of ethanol on photoreceptors and visual function in developing zebrafish." Invest Ophthalmol Vis Sci **47**(10): 4589-4597.

Mattson, S. N. and E. P. Riley (1998). "A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol." Alcoholism, clinical and experimental research **22**(2): 279-294.

May, P. A., J. P. Gossage, W. O. Kalberg, L. K. Robinson, D. Buckley, M. Manning and H. E. Hoyme (2009). "Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies." Dev Disabil Res Rev **15**(3): 176-192.

McCaffery, P., O. Koul, D. Smith, J. L. Napoli, N. Chen and M. D. Ullman (2004). "Ethanol increases retinoic acid production in cerebellar astrocytes and in cerebellum." Brain Res Dev Brain Res **153**(2): 233-241.

Mendelson, B. (1986). "Development of reticulospinal neurons of the zebrafish. II. Early axonal outgrowth and cell body position." The Journal of comparative neurology **251**(2): 172-184.

Metcalfe, W. K., P. Z. Myers, B. Trevarrow, M. B. Bass and C. B. Kimmel (1990). "Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish." Development **110**(2): 491-504.

Miscevic, F., O. Rotstein and X. Y. Wen (2012). "Advances in zebrafish high content and high throughput technologies." Comb Chem High Throughput Screen **15**(7): 515-521.

Myers, P. Z., J. S. Eisen and M. Westerfield (1986). "Development and axonal outgrowth of identified motoneurons in the zebrafish." The Journal of neuroscience : the official journal of the Society for Neuroscience **6**(8): 2278-2289.

Myers, P. Z., J. S. Eisen and M. Westerfield (1986). "Development and axonal outgrowth of identified motoneurons in the zebrafish." J Neurosci **6**(8): 2278-2289.

Nakayama, H. and Y. Oda (2004). "Common sensory inputs and differential excitability of segmentally homologous reticulospinal neurons in the hindbrain." The Journal of neuroscience : the official journal of the Society for Neuroscience **24**(13): 3199-3209.

Nakayama, H. and Y. Oda (2004). "Common sensory inputs and differential excitability of segmentally homologous reticulospinal neurons in the hindbrain." J Neurosci **24**(13): 3199-3209.

Napoli, J. L. (2011). "Effects of ethanol on physiological retinoic acid levels." IUBMB Life **63**(9): 701-706.

National Institute on Alcohol Abuse and Alcoholism (NIAAA) (2004). "NIAAA Council Approves Definition of Binge Drinking". NIAAA Newsletter (3)

Neasta, J., S. Ben Hamida, Q. Yowell, S. Carnicella and D. Ron (2010). "Role for mammalian target of rapamycin complex 1 signaling in neuroadaptations underlying alcohol-related disorders." Proc Natl Acad Sci U S A **107**(46): 20093-20098.

Niederreither, K. and P. Dolle (2008). "Retinoic acid in development: towards an integrated view." Nat Rev Genet **9**(7): 541-553.

O'Leary, C. M., C. Bower, S. R. Zubrick, E. Geelhoed, J. J. Kurinczuk and N. Nassar (2010). "A new method of prenatal alcohol classification accounting for dose, pattern and timing of exposure: improving our ability to examine fetal effects from low to moderate alcohol." J Epidemiol Community Health **64**(11): 956-962.

O'Leary, C. M., N. Nassar, J. J. Kurinczuk, N. de Klerk, E. Geelhoed, E. J. Elliott and C. Bower (2010). "Prenatal alcohol exposure and risk of birth defects." Pediatrics **126**(4): e843-850.

Oliver, J. A., M. D. Blank, K. J. Van Rensburg, D. A. MacQueen, T. H. Brandon and D. J. Drobes (2013). "Nicotine interactions with low-dose alcohol: pharmacological influences on smoking and drinking motivation." J Abnorm Psychol **122**(4): 1154-1165.

Page, L. M. (1990). "Zebrafish as developmental models." Science **250**(4986): 1320.

Parng, C., N. M. Roy, C. Ton, Y. Lin and P. McGrath (2007). "Neurotoxicity assessment using zebrafish." J Pharmacol Toxicol Methods **55**(1): 103-112.

Pullarkat, R. K. (1991). "Hypothesis: prenatal ethanol-induced birth defects and retinoic acid." Alcohol Clin Exp Res **15**(3): 565-567.

Ranganathan, S., D. G. Davis and R. D. Hood (1987). "Developmental toxicity of ethanol in Drosophila melanogaster." Teratology **36**(1): 45-49.

Reimers, M. J., A. R. Flockton and R. L. Tanguay (2004). "Ethanol- and acetaldehyde-mediated developmental toxicity in zebrafish." Neurotoxicology and teratology **26**(6): 769-781.

Reimers, M. J., M. E. Hahn and R. L. Tanguay (2004). "Two zebrafish alcohol dehydrogenases share common ancestry with mammalian class I, II, IV, and V alcohol dehydrogenase genes but have distinct functional characteristics." J Biol Chem **279**(37): 38303-38312.

Reimers, M. J., J. K. La Du, C. B. Periera, J. Giovanini and R. L. Tanguay (2006). "Ethanol-dependent toxicity in zebrafish is partially attenuated by antioxidants." Neurotoxicology and teratology **28**(4): 497-508.

Reyes, R., M. Haendel, D. Grant, E. Melancon and J. S. Eisen (2004). "Slow degeneration of zebrafish Rohon-Beard neurons during programmed cell death." Dev Dyn **229**(1): 30-41.

Rhinn, M. and P. Dolle (2012). "Retinoic acid signalling during development." Development **139**(5): 843-858.

Richardson, B. S., J. E. Patrick, J. Bousquet, J. Homan and J. F. Brien (1985). "Cerebral metabolism in fetal lamb after maternal infusion of ethanol." Am J Physiol **249**(5 Pt 2): R505-509.

Riley, E. P. and C. L. McGee (2005). "Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior." Exp Biol Med (Maywood) **230**(6): 357-365.

Ron, D. and R. Jurd (2005). "The "ups and downs" of signaling cascades in addiction." Sci STKE **2005**(309): re14.

Roy, B. and D. W. Ali (2013). " Patch Clamp Recordings from Embryonic Zebrafish Mauthner Cells" J Vis Exp. **79**, e50551.

Roy, B., J. Ferdous and D.W. Ali (2015). "NMDA receptors on zebrafish Mauthner cells require CaMKII-α for normal development" Dev Neurobiol **75**(2):145-62.

Saint-Amant, L. and P. Drapeau (1998). "Time course of the development of motor behaviors in the zebrafish embryo." J Neurobiol **37**(4): 622-632.

Sarmah, S. and J. A. Marrs (2013). "Complex cardiac defects after ethanol exposure during discrete cardiogenic events in zebrafish: prevention with folic acid." Developmental dynamics : an official publication of the American Association of Anatomists **242**(10): 1184-1201.

Sarmah, S. and J. A. Marrs (2013). "Complex cardiac defects after ethanol exposure during discrete cardiogenic events in zebrafish: prevention with folic acid." Dev Dyn **242**(10): 1184-1201.

Shan, S. D., S. Boutin, J. Ferdous and D. W. Ali (2015). "Ethanol exposure during gastrulation alters neuronal morphology and behavior in zebrafish." Neurotoxicol Teratol **48**: 18-27.

Sokol, R. J., V. Delaney-Black and B. Nordstrom (2003). "Fetal alcohol spectrum disorder." JAMA **290**(22): 2996-2999.

Spanagel, R. (2009). "Alcoholism: a systems approach from molecular physiology to addictive behavior." Physiol Rev **89**(2): 649-705.

Su, H. M., M. C. Huang, N. M. Saad, P. W. Nathanielsz and J. T. Brenna (2001). "Fetal baboons convert 18:3n-3 to 22:6n-3 in vivo. A stable isotope tracer study." J Lipid Res **42**(4): 581-586.

Sulik, K. K., M. C. Johnston and M. A. Webb (1981). "Fetal alcohol syndrome: embryogenesis in a mouse model." Science **214**(4523): 936-938.

Svoboda, K. R. and J. R. Fetcho (1996). "Interactions between the neural networks for escape and swimming in goldfish." J Neurosci **16**(2): 843-852.

Sylvain, N. J., D. L. Brewster and D. W. Ali (2010). "Zebrafish embryos exposed to alcohol undergo abnormal development of motor neurons and muscle fibers." Neurotoxicol Teratol **32**(4): 472-480.

Sylvain, N. J., D. L. Brewster and D. W. Ali (2011). "Embryonic ethanol exposure alters synaptic properties at zebrafish neuromuscular junctions." Neurotoxicol Teratol **33**(2): 313-321.

Tanguay, R. L. and M. J. Reimers (2008). "Analysis of ethanol developmental toxicity in zebrafish." Methods Mol Biol **447**: 63-74.

Thompson, G. and D. I. de Pomerai (2005). "Toxicity of short-chain alcohols to the nematode Caenorhabditis elegans: a comparison of endpoints." J Biochem Mol Toxicol **19**(2): 87-95.

Trevarrow, B., D. L. Marks and C. B. Kimmel (1990). "Organization of hindbrain segments in the zebrafish embryo." Neuron **4**(5): 669-679.

Vengeliene, V., A. Bilbao, A. Molander and R. Spanagel (2008). "Neuropharmacology of alcohol addiction." Br J Pharmacol **154**(2): 299-315.

Wagner, M., C. Thaller, T. Jessell and G. Eichele (1990). "Polarizing activity and retinoid synthesis in the floor plate of the neural tube." Nature **345**(6278): 819-822.

West, J. R., W. J. Chen and N. J. Pantazis (1994). "Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage." Metab Brain Dis **9**(4): 291-322.

Yao, L., P. Fan, Z. Jiang, A. Gordon, D. Mochly-Rosen and I. Diamond (2008). "Dopamine and ethanol cause translocation of epsilonPKC associated with epsilonRACK: cross-talk between cAMP-dependent protein kinase A and protein kinase C signaling pathways." Mol Pharmacol **73**(4): 1105-1112.

Yelin, R., H. Kot, D. Yelin and A. Fainsod (2007). "Early molecular effects of ethanol during vertebrate embryogenesis." Differentiation **75**(5): 393-403.

Yelin, R., R. B. Schyr, H. Kot, S. Zins, A. Frumkin, G. Pillemer and A. Fainsod (2005). "Ethanol exposure affects gene expression in the embryonic organizer and reduces retinoic acid levels." Dev Biol **279**(1): 193-204.

Young, J. K., H. E. Giesbrecht, M. N. Eskin, M. Aliani and M. Suh (2014). "Nutrition implications for fetal alcohol spectrum disorder." Adv Nutr **5**(6): 675-692.

Zachman, R. D. and M. A. Grummer (1998). "The interaction of ethanol and vitamin A as a potential mechanism for the pathogenesis of Fetal Alcohol syndrome." Alcohol Clin Exp Res **22**(7): 1544-1556.

Zhang, C., P. Ojiaku and G. J. Cole (2013). "Forebrain and hindbrain development in zebrafish is sensitive to ethanol exposure involving agrin, Fgf, and sonic hedgehog function." Birth defects research. Part A, Clinical and molecular teratology **97**(1): 8-27.

Zhao, S., P. Xie, G. Li, C. Jun, Y. Cai, Q. Xiong and Y. Zhao (2012). "The proteomic study on cellular responses of the testes of zebrafish (Danio rerio) exposed to microcystin-RR." Proteomics **12**(2): 300-312.

Zottoli, S. J. (1977). "Correlation of the startle reflex and Mauthner cell auditory responses in unrestrained goldfish." J Exp Biol **66**(1): 243-254.

Zottoli, S. J. (1978). "Comparison of Mauthner cell size in teleosts." J Comp Neurol **178**(4): 741-757.