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Developmental age strengthens barriers to ethanol accumulation in zebrafish

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Abstract

Fetal Alcohol Spectrum Disorders (FASD) describes a wide range of phenotypic defects affecting facial and neurological development associated with ethanol teratogenicity. It affects approximately 1 in 100 children born in the United States each year. Genetic predisposition along with timing and dosage of ethanol exposure are critical in understanding the prevalence and variability of FASD. The zebrafish attributes of external fertilization, genetic tractability, and high fecundity make it a powerful tool for FASD studies. However, a lack of consensus of ethanol treatment paradigms has limited the interpretation of these various studies. Here we address this concern by examining ethanol tissue concentrations across timing and genetic background. We utilize headspace gas chromatography to determine ethanol concentration in the *AB*, *flil:EGFP*, and *Tu* backgrounds. In addition, we treated these embryos with ethanol over two different developmental time windows, 6–24 hours post fertilization (hpf) and 24–48 hpf. Our analysis demonstrates that embryos rapidly equilibrate to a sub-media level of ethanol. Embryos then maintain this level of ethanol for the duration of exposure. The ethanol tissue concentration level is independent of genetic background, but is timing-dependent. Embryos exposed from 6–24 hpf were 2.7–4.2-fold lower than media levels, while embryos were 5.7–6.2-fold lower at 48 hpf. This suggests that embryos strengthen one or more barriers to ethanol as they develop. In addition, both the embryo and, to a lesser extent, the chorion, surrounding the embryo are barriers to ethanol. Overall, this work will help tighten ethanol treatment regimens and strengthen zebrafish as a model of FASD.

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Keywords

fetal alcohol spectrum disorders; zebrafish; ethanol tissue concentration

Introduction

Fetal Alcohol Spectrum Disorders (FASD) describes a wide array of neurological defects and morphological malformations associated with ethanol teratogenicity (Elliott et al., 2008). This array of ethanol-induced defects can be attributed to many different factors, including the timing and dosage of ethanol administration and genetic background (Riley, Infante, & Warren, 2011; Sulik, 2005). The complex interplay of these variables in humans makes understanding FASD challenging, necessitating the use of animal models.

Amniote model systems have been used by numerous labs to study various aspects of FASD. Consistent with what is found in early exposure in humans, particularly during gastrulation, binge levels of alcohol cause profound teratogenesis. Recently, even later exposures to ethanol have been shown to cause developmental anomalies (Lipinski et al., 2012). In addition, the availability of many mouse mutants has prompted an analysis of potential gene-ethanol interactions, particularly with the Sonic Hedgehog pathway, which has been implicated in ethanol teratogenesis (Ahlgren, Thakur, & Bronner-Fraser, 2002; Hong & Krauss, 2012; Loucks & Ahlgren, 2009; Sulik, 2005). In sum, this work has shown that these models are useful for understanding the influences of ethanol exposure on embryonic outcome.

The zebrafish is ideally suited for studying the effects of timing, dosage, and genetics on ethanol teratogenesis. Zebrafish are genetically tractable, highly fecundate, and fertilize externally, allowing ethanol to be consistently administered to precisely staged embryos. Additionally, the developmental programs regulating embryonic development are conserved between amniotes and fish (Sheehan-Rooney, Pálinskásová, Eberhart, & Dixon, 2010; Swartz et al., 2011). Thus, findings in zebrafish can help guide the analyses of FASD in humans (McCarthy et al., 2013).

Zebrafish have been used to examine the teratogenic effects of ethanol (Ali, Champagne, Alia, & Richardson, 2011; Bilotta, Barnett, Hancock, & Saszik, 2004; Blader & Strähle, 1998; Dlugos & Rabin, 2003; Gerlai, Lahav, Gou, & Rosenthal, 2000; Gerlai, Lee, & Blaser, 2006; Li et al., 2007; Lockwood, Bjerke, Kobayashi, & Guo, 2004; McCarthy et al., 2013; Pan, Chatterjee, & Gerlai, 2012; Reimers, Flockton, & Tanguay, 2004; Stockard, 1910; Zhang, Ojiaku, & Cole, 2013; Zhang, Turton, Mackinnon, Sulik, & Cole, 2011). However, there is a lack of consensus regarding ethanol treatment regimens and how these regimens would relate to humans. In mammalian systems, blood alcohol levels directly correlate to tissue levels (Adalsteinsson, Sullivan, Mayer, & Pfefferbaum, 2006; Flory, O'Malley, Grant, Park, & Kroenke, 2010; Quertemont, Green, & Grant, 2003). However, there is some inconsistency in reported tissue levels in ethanol-exposed zebrafish embryos. To address the concern of tissue ethanol concentrations, we used headspace gas chromatography to determine ethanol concentrations across the variables of timing and genetic background. In this study, we demonstrate that the zebrafish embryo has some

barrier to the accumulation of ethanol. This results in ethanol concentrations approximately 3–6-fold less than the medium, depending on embryonic age. In our study, we examined ethanol concentrations after an early exposure (6–24 hpf) and a later exposure (24–48 hpf). At 24 hpf, embryos contained 2.7–4.2-fold less ethanol than the medium, while the embryos contained 5.8–6.2-fold less at 48 hpf. Further, we found that genetic background did not alter ethanol tissue concentrations. Collectively, our results help elucidate the tissue accumulation of ethanol in exposed zebrafish and show that genetic background is not a common source of variation in tissue levels of ethanol.

Materials and methods

Zebrafish (*Danio rerio*) care and use

All embryos were raised and cared for using established IACUC protocols (Westerfield, 1993) approved by the University of Texas at Austin. Three strains were utilized in this study, AB, Tubigan (Tu, in the text), and *Tg(fli1:EGFP)^{y1}* transgenic embryos (Lawson & Weinstein, 2002; *fli1* in the text). Embryos were treated with 1% ethanol diluted in embryo media from either 6–24 hpf or 24–48 hpf. Embryos were then gathered for determination of tissue ethanol concentrations.

Embryo volume and weight calculations

In order to calculate tissue concentrations of ethanol in zebrafish embryos, the average volume of an embryo is needed. Because embryo volume is small, we used displacement of a relatively large volume of water to reduce error. 0.5 mL microcentrifuge tubes (Fischer Scientific) were filled with 250 μ L (250 mg) of water using a P200L Pipetman pipette (Gilson, Inc.) and a fill line was marked representing 250 μ L. The water was removed and 10 embryos were placed into the tube at a time, which was then refilled to the 250- μ L marked line. This process eliminates the concern about fluid clinging to the embryos as any carryover was included in the final measurement. All of the water was then carefully removed from the samples; any sample where an embryo was pipetted was not used. While it is impractical to remove all residual water in all of our treatments, we estimated the residual liquid by using a Kimwipe[®] to remove liquid stuck to the embryo and microcentrifuge tube sides in 10 samples of 24 hpf embryos (10 embryos per sample). Based on our weight measures we were able to determine that there was approximately 0.067 μ L of residual water, per embryo. This is a small fraction of the calculated volume for the embryos (see results) and, importantly, would be similar across all treatments. The weight of the final water sample was subtracted from the initial 250 mg of water weight. Volume was directly determined from the weight of the removed water.

To determine dry weight of the embryos, the 10 embryos previously measured for volume were placed on pre-weighed glass coverslips (22 mm $\times$ 22 mm) and baked at 70 $^{\circ}$ C for 2 h. The samples were allowed to cool and were weighed (Mettler-Toledo XS64; accurate to 0.01 mg). Baking the embryos at 70 $^{\circ}$ C for longer than 2 h did not further decrease the weight of the embryos, demonstrating that baking for 2 h completely dried the embryo samples. The P200L Pipetman used for these analyses was calibrated with a systemic error (representing accuracy) of 0.09 μ L and a random error (representing precision) of 0.03 μ L.

(<http://www.pipetman.com/RequestLiterature.aspx> - Verification Procedures for Accuracy & Precision). The pipette and the Mettler-Toledo scale are calibrated multiple times a year.

Measurement of ethanol concentration using headspace gas chromatography

To determine ethanol tissue concentration relative to media exposure, headspace gas chromatography (GC) was used. Embryos, either with their chorions intact or removed prior to ethanol treatment, were exposed to media ethanol at 1% from 6–24 or 24–48 hpf. Samples were taken at 24 hpf for the early exposure period and 48 hpf for the later exposure period. Samples consisted of 10 pooled embryos that were rinsed once for 1 sec in fresh media lacking ethanol to eliminate ethanol adhering to the exterior of the samples, and then treated with 50 μ L of Pronase (2 mg/mL – Roche Applied Science) for 10 min to aid in removal of the chorion in the chorionated samples. Dechorionated embryos were also treated with pronase for consistency. 450 μ L of 5 M NaCl was then added to each sample and the samples were then vortexed for 10 min to homogenate the embryos. Silica beads were added to the 48-hpf-old embryos to aid in homogenization. For each sample, an aliquot of 2 μ L was transferred to a 2-mL GC vial and sealed with a PTFE silicon septum and plastic cap. For each time point, 6 treated and untreated embryo samples were also collected and homogenized. In addition, 6 media reference samples were collected and diluted 10-fold in 5 M NaCl and aliquotted to a 2-mL GC vial and sealed. A Varian CP 3800 gas chromatograph with flame ionization detection and a Varian CP 8400 headspace autosampler, heated to 58 °C, were used to analyze the concentrations of ethanol in the homogenized samples. The stationary phase was an HP Innowax capillary column (30 m $\times$ 0.53 mm $\times$ 1.0 μ m film thickness) and helium was the mobile phase. Resulting ethanol peak heights were recorded using Varian Star Chromatography Workstation software. The GC signal-to-noise ratio was 7 and calibration was achieved using external standards from 0.3125 to 40 mM ethanol in 5 M NaCl as described in Doyon et al., 2003. The accuracy and precision of the calibration was verified against a 1% ethanol standard in triplicate and was within 0.013% of the targeted 1% ethanol concentration with a coefficient of variation of 0.018. Quantification of ethanol concentrations of all samples was performed by comparing peak heights obtained from the Varian Star Chromatographic analysis with the external standards.

Statistical analysis

Ethanol concentrations in embryo samples were compared to one another, untreated controls, and media using a 1-way ANOVA with a Tukey's *post hoc* test and a student's *t* test in Graphpad Prism v5.02 (Graphpad Software Inc., La Jolla, CA).

Results

Zebrafish were first used to examine the teratogenic effects of ethanol as early as 1910 (Stockard, 1910), and more recent work has expanded upon this idea (Ali et al., 2011; Bilotta et al., 2004; Blader & Strähle, 1998; Dlugos & Rabin, 2003; Gerlai et al., 2000; Gerlai et al., 2006; Li et al., 2007; Lockwood et al., 2004; McCarthy et al., 2013; Pan, Chatterjee, & Gerlai, 2012; Reimers, Flockton, et al., 2004; Zhang et al., 2013; Zhang et al., 2011). However, the myriad of ethanol treatment regimens used in these studies creates a

lack of consensus in ethanol administration. This wide range of ethanol regimens makes it difficult to assess pharmacologically relevant tissue concentrations of ethanol.

To determine tissue concentrations in the zebrafish embryo it is necessary to know the volume of the embryo. The embryo, which consists of the blastoderm and yolk, is surrounded by the extraembryonic fluid, which is encased by the chorion (Fig. 1). Previous work estimated embryonic volume assuming the embryo (blastoderm + yolk) was spherical (Hagedorn, Kleinhans, Artemov, & Pilatus, 1998). Using this model of a spherical embryo, Hagedorn et al. (1998) calculated the volume of the embryo using the prolate spheroid formula ($V = 4/3\pi ab^2$, a = major and b = minor semi-axes) to be 0.2 μL . However, even at very early stages the embryos are not perfectly spheroidal, which complicates this previous measure of embryo volume. In our work, the embryos are tested at 24 and 48 hpf, when the embryos are not spheroidal in shape. To more accurately measure embryonic volume, we measured water displacement of embryos removed from their chorion (embryo) and embryos within their chorion (embryo + extraembryonic fluid). Water displacement eliminates discrepancies due to changes in embryo shape and size over developmental time. To examine if manual error in pipetting may have influenced embryo volume measures, we repeated these volume measures 100 times using a single sample of 10 embryos. We were able to show that the average volume measure equaled our reported volume measures and only had a standard deviation (S.D.) of 0.2 μL , ruling out pipetting as the source of the error in our sample measures. In addition, we examined the accuracy of our water displacement by measuring the volume of a piece of pure copper wire, which has a density of 8.96 g/cm^3 . Using 0.15 g of copper wire, we were able to calculate the volume of the wire from the density to be approximately 17 μL . Our displacement method calculated the volume to be 17.1 μL , S.D. = 2.8 μL ($n = 50$). Collectively, these results demonstrate that our technique is both highly precise and accurate.

Using water displacement, we determined that at 24 and 48 hpf the volume of an embryo was 1.1 ($n = 14$; 10 embryos per sample) and 1.4 μL ($n = 13$; 10 embryos per sample), respectively, while the volume of an embryo + extraembryonic fluid was 2.0 μL ($n = 15$; 10 embryos per sample) (Table 1), making the extraembryonic volume 0.9 and 0.6 μL at 24 and 48 hpf, respectively. Thus, the embryo increases slightly in volume between 24 and 48 hpf while the volume of the extraembryonic fluid decreases. These results are consistent with previous work using water displacement to assess embryo volume (Lockwood et al., 2004) and are higher than those observed using the spherical model. This increase would be expected since the embryo is growing in size during the developmental time windows we test.

These volume calculations allow us to understand the partitioning of ethanol in the embryo and extraembryonic fluid. Previous work suggests that ethanol equilibrates in total embryonic water indicating that ethanol concentrations will be equal between blood and tissues and cells (Harris, Trudell, & Mihic, 2008). Work from Hagedorn et al. (1997) looking at water permeability in the embryo demonstrated that 73.6% of the embryo is water. Similarly, Bradfield, West, & Maier (2006) used an indirect analysis to show that water comprised ~78% of the embryos. At 24 hpf, the embryo volume is 1.1 μL . Thus, using the 73.6% value, the water fraction of the embryo is 0.8 μL . By 48 hpf, the volume of

the embryo has increased to 1.4 μL , with a water fraction of 1 μL (Table 1). These differences will prove critical in understanding the dynamics of embryonic ethanol tissue concentration.

In our analyses, we used a 1% (171 mM) ethanol media concentration to identify dominant modifiers of ethanol teratogenicity (McCarthy et al., 2013, Swartz et al., 2013). We chose to assess the rate of ethanol accumulation and loss via headspace gas chromatography. We exposed embryo samples within the chorion ($n = 3$; 10 embryos per sample) to 1% ethanol and measured ethanol concentration at 5, 10, 15, 20, and 30 min. Embryos were then removed from the ethanol and ethanol concentrations were measured 5, 10, 15, 20, and 30 min after washout. The embryos plateaued at approximately 50 mM ethanol after 5 min of initial exposure. Embryos returned to baseline levels of ethanol concentration within 5 min of ethanol removal (Fig. 2). These results suggest that zebrafish embryos rapidly equilibrate to ethanol concentration.

Timing and genetic background are known to be important factors in ethanol teratogenicity. We tested three different genetic backgrounds that we frequently use, *AB*, *flil*, and *Tu* (Table 2), for differences in ethanol accumulation. Embryos were exposed to ethanol in two independent time intervals: 6–24 hpf and 24–48 hpf, covering gastrulation/early organogenesis and later organogenesis, respectively (for each genetic background at each time point, $n = 6$; 10 embryos per sample). In all experimental paradigms, there is no significant difference in ethanol media concentration (data not shown, for each genetic background at each time point, $n = 6$). In 6–24 hpf treatments, *AB*, *flil*, and *Tu* embryos contained 40.7, 53.0, and 58.7 mM ethanol. For this same time period the embryo + extraembryonic fluid averaged 75.3, 85.7, and 91.2 mM ethanol, respectively. Therefore, the embryos themselves contained 36–46% less ethanol than the embryo + extraembryonic fluid. Because there is variation in the concentration of ethanol in the embryo media, we calculated ratios for comparison across groups (Fig. 3 and Table 3). The results for *AB* embryos produced an embryo to media concentration ratio of 24%, while embryo + extraembryonic fluid had a ratio of 48%. The embryo to media ratios for *flil* were 35% for embryo and 58% for embryo + extraembryonic fluid. *Tu* embryo to media ratios were 37% and 56% for embryo and embryo + extraembryonic fluid, respectively. A 1-way ANOVA demonstrated that there was no significant difference between genetic backgrounds in the same treatment groups (Statistical analyses results summarized in Table 4; ANOVA). However, when the embryos from one genetic background were compared to their embryo + extraembryonic fluid counterparts, significant differences were observed (t test – Table 4).

To confirm that the quick wash step in our protocol did not lower the ethanol concentration in the embryos, we measured ethanol concentration in embryos without washing ($n = 5$; 10 embryos per sample). We show that this rapid wash step does not decrease ethanol concentrations compared to unwashed embryos (Fig. 4). These data, combined with the water volume assessments previously examined, suggest that ethanol similarly partitions between the embryo and the extraembryonic fluid across backgrounds at 24 hpf.

By 48 hpf (24–48 hpf ethanol exposure), the ethanol concentrations for embryo and embryo + extraembryonic fluid had dropped across all backgrounds (Table 2). In 24–48 hpf

treatments, *AB*, *fli1*, and *Tu* embryos contained 26.7, 29.8, and 27.8 mM ethanol, respectively. Embryo + extraembryonic fluid had 61.5, 72.3, and 77.6 mM ethanol, respectively. By 48 hpf, the embryo contains 38–44% of the ethanol within the chorion, very similar to what is observed by 24 hpf. However, when the embryo to media ratios are calculated there is a decrease in ethanol concentration (Fig. 3 and Table 3). The embryo to media ratio for *AB*, *fli1*, and *Tu* was 16, 18, and 17% for embryos, while the ratio for embryo + extraembryonic fluid was 36, 43, and 46% for *AB*, *fli1*, and *Tu*, respectively. Again, there was no significant difference in embryo or embryo + extraembryonic fluid ethanol concentration between the three genetic backgrounds (ANOVA – Table 4). Significant differences were observed between embryo and embryo + extraembryonic fluid in each genetic background (*t* test – Table 4). The decrease in ethanol concentration coupled with the increase in the water volume of the embryo suggests an increase in removal of ethanol at 48 hpf. Comparing the tissue concentrations of each treatment group across the two time points showed that only the embryos for each genetic background were significantly different, while embryos + extraembryonic fluid were not significantly different (*t* test – Table 4). To show that the difference in volume of embryos at the two time points does not contribute to this temporal significant difference, we reanalyzed the embryos' concentrations by using a single volume measure. We calculated the concentrations of both 24 and 48 hpf embryos using the volume of 24 hpf or 48 hpf embryos. These results did not differ from our results using the proper volume of each time point.

Collectively, our results show that tissue ethanol concentrations do not equal media concentrations, but are about 30% of media concentrations. In addition, no significant difference in tissue ethanol concentration across genetic backgrounds was observed. However, there are significant differences between embryo and embryo + extraembryonic fluid as well as between the two time points of embryos in individual genetic backgrounds. While these results are consistent with the ethanol concentration determined by other groups (Flentke, Klinger, Tanguay, Carvan, & Smith, 2014; Reimers, Flockton, et al., 2004; Zhang et al., 2013), these data suggest that there are two levels of barriers to full ethanol equilibrium with the media. The first is at the level of the chorion, while the second is set up between the embryo and the extraembryonic fluid.

Discussion

The zebrafish is gaining use as a model for Fetal Alcohol Spectrum Disorders. Zebrafish eggs are fertilized externally, making it possible to precisely regulate the timing and dosage of ethanol. This along with the genetic resources available makes the zebrafish system a powerful additional model system to study the teratogenic effects of ethanol on development.

In rodent models, the tissue concentration of ethanol follows the blood alcohol concentration (Harris et al., 2008); however, in zebrafish it is not possible to obtain blood samples from early embryos. Currently there is a lack of consensus for zebrafish ethanol treatment regimens and a poor understanding of ethanol tissue concentrations in these regimens. A wide range of treatment times and dosages, from 0.5% to 10% ethanol using both binge and chronic treatments, has been used throughout the FASD zebrafish literature (Ali et al., 2011;

Bilotta et al., 2004; Blader & Strähle, 1998; Dlugos & Rabin, 2003; Gerlai et al., 2000; Gerlai et al., 2006; Li et al., 2007; Lockwood et al., 2004; McCarthy et al., 2013; Pan et al., 2012; Reimers, Flockton, et al., 2004; Zhang et al., 2013; Zhang et al., 2011). It is difficult to assess the pharmacologically relevant effects of ethanol on zebrafish development without understanding the tissue level ethanol concentration. This understanding will help establish a consensus in zebrafish ethanol regimens and enable researchers to correlate the effects of ethanol across species.

To understand the effects of ethanol at a particular development time, it is essential to know how quickly ethanol equilibrates between the media and embryonic tissues. Work in rodents has demonstrated that ethanol equilibrates in a matter of minutes in brain tissues (Adalsteinsson et al., 2006; Quertemont et al., 2003). We found that zebrafish embryos reached equilibration within 5 min, the shortest time interval tested. Upon washout, the embryos returned to baseline concentration levels within 5 min, also the shortest time interval tested. We confirmed that with such a rapid rate of equilibrium, washing the embryos prior to processing did not lower the ethanol concentration within the embryos, further strengthening our experimental paradigm. Collectively, our work along with recent work from Flentke et al. (2014) demonstrate that zebrafish reach a rapid equilibrium that is maintained for the duration of exposure.

Ethanol exposure times have varied substantially in the literature (Ali et al., 2011; Bilotta et al., 2004; Blader & Strähle, 1998), yet the effects of embryonic stage on ethanol accumulation have not been examined. We examined exposure times from 6–24 hpf and 24–48 hpf, correlating with gastrulation/early organogenesis and later organogenesis, respectively. Following a 6–24 hpf ethanol treatment, the ratio of embryo to media ethanol concentration across genetic backgrounds ranged from 24–37%, consistent with the ethanol concentration determined by other groups (Flentke et al., 2014; Reimers, Flockton, et al., 2004; Zhang et al., 2013). Though the embryo is increasing in volume from 24 to 48 hpf, the concentration of ethanol in the embryonic tissue is significantly decreasing. By 48 hpf, the embryos' ethanol concentration was 16–17% of the ethanol concentration of the media. In addition, this decrease is not affected by the increase in embryo volume. Collectively, these results demonstrate that from a similar ethanol media dosage, the tissue levels of ethanol drop substantially over developmental time.

The decrease in the tissue levels of ethanol could be due to several factors. First, several alcohol dehydrogenases have been identified in zebrafish, *adh5*, *adh8a*, and *adh8b*, all three of which can process ethanol (Dasmahapatra, Doucet, Bhattacharyya, & Carvan, 2001; Reimers, Hahn, & Tanguay, 2004). In addition, other enzymes such as the cytochrome P450, Cyp2E1, may play a role in ethanol metabolism (Passeri, Cinaroglu, Gao, & Sadler, 2009). The embryo may be simply expressing more of these enzymes that process ethanol or expressing the enzymes that process ethanol at a higher rate. Second, there could be non-enzymatic processes removing the ethanol. Flentke et al. (2014) suggest this export of ethanol could be performed by aquaglyceroporin family members as observed in yeast (Teixeira, Raposo, Mira, Lourenço, & Sá-Correia, 2009). Members of the aquaglyceroporin family have been identified in zebrafish but their ability to export ethanol has not been characterized (Hamdi et al., 2009). Third, it is possible that as the embryo continues to

develop a physical barrier, perhaps the periderm or epidermis could be expanding and/or strengthening. In support of a physical barrier affecting the ethanol concentration, cutting the embryo in half results in tissue concentrations equal to that of the media (Ikeda et al., 2013). Any one or a combination of these embryonic processes could result in the decrease in tissue levels of ethanol between 24 and 48 hpf.

In addition to embryonic processes, the chorion could be a barrier to ethanol accumulation. To determine if the chorion acts as a barrier, we measured ethanol concentrations of the embryo + extraembryonic fluid. The concentration of ethanol within embryos + extraembryonic fluid is higher than that observed for dechorionated embryos (Table 3). This could simply be due to ethanol passing freely through the chorion but being impeded from accumulating in the embryo, as discussed above. If this were the case, then the ethanol concentration in the extraembryonic fluid would equal that of the media. Then the expected concentration of ethanol in embryo + embryonic fluid would be determined by adding the relative proportions of ethanol expected in the embryo (no chorion levels) and ethanol in the media. At 24 and 48 hpf the embryo consists of approximately 50% and 61% water by volume, respectively. In each instance, we failed to reach the expected proportion based on an assumption of free diffusion across the chorion (Table 5), although we are within 4 to 14% of the expectation. Therefore, based on our calculations the chorion does generate a small barrier to free diffusion.

Our data show that the embryo and the chorion are barriers to ethanol accumulation, with the embryo being the predominant barrier. Collectively, our result suggests that embryonic enzymatic activity is partly the cause of this effect. Because the embryos are in ethanol for either 18 or 24 h, the effects of low permeability would be negated, leaving degradation or active export as likely causes. Indeed, protein-mediated facilitated diffusion has been proposed to be involved in the concentration gradient between the media and zebrafish tissue (Flentke et al., 2014).

In addition, our work also demonstrates that this equilibrium state is independent of genetic background. Previous ethanol work in zebrafish has used multiple genetic backgrounds, including AB, WIK, long-fin striped, blue long-fin, and *flil* (Ali et al., 2011; Dlugos & Rabin, 2003; Gerlai et al., 2000; Gerlai et al., 2006; Lockwood et al., 2004; McCarthy et al., 2013; Pan et al., 2012; Reimers, Flockton, et al., 2004; Zhang et al., 2013; Zhang et al., 2011). Specifically, work from Dlugos & Rabin (2003) and Pan et al. (2012) demonstrated that different strains had different sensitivities to ethanol exposure. When assessed for ethanol tissue concentrations, all three strains had comparable levels of ethanol. This suggests that while sensitivity to ethanol can vary from strain to strain, ethanol tissue concentrations remain consistent.

Our data fit with the growing body of literature that in zebrafish, as well as *C. elegans* and fruit flies, the developing embryos do not contain the same tissue concentration of ethanol to which they are exposed (Alaimo et al., 2012; Robinson et al., 2012). This work differs from previously published zebrafish data in that we are examining multiple parameters that have not been previously examined (Table 6). We exposed three different backgrounds of zebrafish to a single ethanol dose over two time points to explicitly look at the role of

developmental age and genetic background on ethanol accumulation. In addition, we sought to determine where barriers to ethanol accumulation might exist in the zebrafish. We used headspace gas chromatography to more precisely analyze these ethanol levels in tissue. This method directly measures ethanol levels within the tissue and has been used in ethanol analysis in zebrafish (Bradfield et al., 2006) and fruit flies (Robinson et al., 2012). Enzymatic measures rely on alcohol dehydrogenase processing to produce a secondary analyte to measure. Enzymatic rates could hamper the quantification of absolute ethanol levels.

While there are differences both in the treatment and quantification regimens employed in the studies listed in Table 6, there is a growing consensus in recent work. Overall, the data suggest that the ethanol tissue concentration is a fraction of that in the media. Our data suggest that the early embryo maintains ethanol concentrations approximately 25–35% that of the media and this value decreases over developmental time. We demonstrate that both the embryo and chorion act as barriers to ethanol accumulation in the embryo. It is not well understood what creates these barriers to ethanol equilibrium, but it raises the question, do blood alcohol levels in mammalian systems represent absolute tissue levels or are there barriers between blood and tissue that lower this equilibrium state?

Understanding the pharmacokinetics of ethanol in zebrafish will enable researchers to standardize ethanol treatment regimens and establish the standard for the continued use of zebrafish as a model for FASD. The ability to tightly regulate ethanol timing and dosage to developing embryos allows work to understand the genetic and cellular mechanisms of ethanol teratogenicity. This understanding combined with the conservation of development between human and zebrafish (Bakkers, 2011; Bandmann & Burton, 2010; Swartz, Sheehan-Rooney, Dixon, & Eberhart, 2011) will greatly improve our understanding of FASD.

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Figure 1.

A schematic of an embryo within its chorion. The embryo consists of the blastoderm (cellular portion of the embryo) and yolk surrounded by an extraembryonic fluid, which is all encased by the chorion.

Figure 2.

Rate of ethanol equilibration. Ethanol rapidly equilibrates in embryos + extraembryonic fluid and plateaus at approximately 50 mM ($n = 3$; 10 embryos per sample). Equilibration occurs within 5 minutes of ethanol exposure. Ethanol washes out of embryos + extraembryonic fluid in an equally rapid fashion, within 5 minutes.

Figure 3.

Ratio of ethanol samples to media. Ethanol concentration for each strain was divided by the media samples of that strain and then averaged. At 24 hpf, embryos contained roughly 24–37% of media levels, while embryo + extraembryonic fluid was 48–56%. By 48 hpf the ratios had decreased across all backgrounds, with embryos containing 16–17% ethanol and embryo + extraembryonic fluid containing 36–46% ethanol. In all conditions, $n = 6$; 10 embryos per sample.

Figure 4.

Comparison of ethanol concentration of washed and unwashed embryo samples. The quick 1 second wash (described in Materials and methods) does not decrease ethanol concentrations in embryo + extraembryonic fluid samples compared to embryo + extraembryonic fluid samples that do not undergo the quick wash ($n = 5$; 10 embryos per sample).

Table 1

Embryonic and Extraembryonic Water Volumes

	Total Volume ^{a,b}	% of Total Volume	Water		
			Embryonic Volume ^a	Extraembryonic Volume ^a	% in Extraembryonic
Embryo + extraembryonic ^c	2 ± 0.2				
Embryo 24 hpf ^d	1.1 ± 0.1	57%	0.8	0.9	49.5%
Embryo 48 hpf ^e	1.4 ± 0.2	68%	1.0	0.6	61.3%
					50.5%
					38.7%

^aVolume is in μ L.

^bValues are reported as $\pm$ standard error of the mean (S.E.M.)

^c $n = 15$; 10 embryos per sample

^d $n = 14$; 10 embryos per sample

^e $n = 13$; 10 embryos per sample

Table 2

Ethanol Tissue Concentration (mM)

	6–24 hpf^a		
	<i>AB</i>	<i>Fli</i>	<i>Tu</i>
Embryo			
Media	168.8 ± 1.5	159 ± 11.9	160.5 ± 1.9
Untreated Embryos	2.3 ± 0.8	4.3 ± 1.3	3.7 ± 1.4
Treated Embryos	40.7 ± 4.5	53 ± 2.9	58.7 ± 10.0
Embryo + extraembryonic			
Media	157.5 ± 4.3	152.5 ± 9.2	162.3 ± 2.6
Untreated Embryos	2.7 ± 1.2	2.5 ± 0.7	3.7 ± 1.6
Treated Embryos	75.3 ± 5.1	85.7 ± 4.5	91.2 ± 4.9
	24–48 hpf^a		
	<i>AB</i>	<i>Fli</i>	<i>Tu</i>
Embryo			
Media	165.8 ± 3.5	169.7 ± 2.1	162.3 ± 1.3
Untreated Embryos	1.3 ± 0.6	0.3 ± 0.3	0
Treated Embryos	26.7 ± 2.4	29.8 ± 1.3	27.8 ± 3.1
Embryo + extraembryonic			
Media	170.8 ± 2.3	166.7 ± 5.5	169.8 ± 1.3
Untreated Embryos	1.0 ± 0.5	0.7 ± 0.4	0
Treated Embryos	61.5 ± 11.4	72.3 ± 10.2	77.7 ± 5.3

^aIn all conditions, *n* = 6; 10 embryos per sample^bValues are reported as ± S.E.M.

Table 3^a Ratio of ethanol – sample to media^b

	<i>AB</i>	<i>fli</i>	<i>Tu</i>
Embryo			
6–24 hpf^c	0.24 ± 0.02	0.35 ± 0.04	0.37 ± 0.07
24–48 hpf	0.16 ± 0.01	0.18 ± 0.01	0.17 ± 0.02
Embryo + extraembryonic			
6–24 hpf	0.48 ± 0.04	0.58 ± 0.05	0.56 ± 0.03
24–48 hpf	0.36 ± 0.07	0.43 ± 0.06	0.46 ± 0.03

^a In all conditions, $n = 6$; 10 embryos per sample^b Values are reported as ± S.E.M.^c Window of ethanol exposure

Table 4

^a 1-Way ANOVA

Condition	Comparison	Fstatistic	pvalue ^b
24 hpf Embryo	$AB \times Fli \times Tu$	2.1	0.2
24 hpf Embryo + Extraembryonic Fluid	$AB \times Fli \times Tu$	3	0.7
48 hpf Embryo	$AB \times Fli \times Tu$	1.4	0.3
48 hpf Embryo + Extraembryonic Fluid	$AB \times Fli \times Tu$	0.8	0.5
<i>t</i> Test ^c			
Condition	Comparison	tstatistic	pvalue
24 hpf AB	Embryo $\times$ Embryo + Extraembryonic Fluid	5	0.0006
24 hpf fli	Embryo $\times$ Embryo + Extraembryonic Fluid	3.3	0.009
24 hpf Tu	Embryo $\times$ Embryo + Extraembryonic Fluid	2.6	0.02
48 hpf AB	Embryo $\times$ Embryo + Extraembryonic Fluid	2.9	0.02
48 hpf fli	Embryo $\times$ Embryo + Extraembryonic Fluid	4.3	0.002
48 hpf Tu	Embryo $\times$ Embryo + Extraembryonic Fluid	7.8	< .0001
AB Embryo	24 hpf $\times$ 48 hpf	2.9	0.02
AB Embryo + Extraembryonic Fluid	24 hpf $\times$ 48 hpf	1.5	0.2
fli Embryo	24 hpf $\times$ 48 hpf	3.9	0.003
fli Embryo + Extraembryonic Fluid	24 hpf $\times$ 48 hpf	1.8	0.1
Tu Embryo	24 hpf $\times$ 48 hpf	2.9	0.02
Tu Embryo + Extraembryonic Fluid	24 hpf $\times$ 48 hpf	2.4	0.04

^a In all conditions, $n = 6$; 10 embryos per sample.^b A p value .05 is significant.^c A student's t test was used.

Table 5

Calculation of Expected Extraembryonic Ethanol Concentration

24 hpf	Expected ^a	Observed ^b	Difference from expected ^c
$.50^d \times [.24]^e + .50^f \times [1]^g =$	0.62	0.48	0.14
$.50 \times [.35] + .50 \times [1] =$	0.68	0.58	0.10
$.50 \times [.37] + .50 \times [1] =$	0.69	0.56	0.13
48 hpf			
$.61 \times [.16] + .39 \times [1] =$	0.49	0.36	0.13
$.61 \times [.18] + .39 \times [1] =$	0.49	0.43	0.06
$.61 \times [.17] + .39 \times [1] =$	0.49	0.46	0.03

^a Expected result of embryo + extraembryonic fluid if there is no barrier to ethanol at the chorion. Here the extraembryonic fluid ethanol concentration will be equal to the media, 1% ethanol.

^b Observed result of embryo + extraembryonic fluid

^c Difference between the expected and observed results

^d Percentage of water in the embryo at either 24 or 48 hpf

^e Concentration of ethanol in the embryo at either 24 or 48 hpf

^f Percentage of water in the extraembryonic fluid at either 24 or 48 hpf

^g Expected percentage concentration of ethanol in the extraembryonic fluid

Table 6

Study	Stages of Exposure ^a	Duration of Exposure	Ethanol Dosage ^b	Number and Duration of Washes ^c	Assay ^d	Embryonic Ethanol Concentration ^e
Ali et al., 2011	4.5–60 hpf	1 h	1700 mM	1X for 30 sec	MRS	0.86%
Bradfield et al., 2006	4 hpf	20–360 min	50 or 500 mM	3X	HGC / NMR	70%
Dlugos & Rabin, 2003	Adult	2 h/2 wks	43–171 mM	NA	Enzyme	1–3 µg/mg [#]
Flenke et al., 2014	6 hpf	1–8 h	50–300 mM	1X	Analox	35%
Li et al., 2007	4.3–5.3 hpf	6 h	43–342 mM	NA	Enzyme	0.71–7.4 mM
Lockwood et al., 2004	7 dpf	1–20 min	257–513 mM	1X for 30 sec	Enzyme	25–71 mM
Reimers Flockton et al 2004	3 hpf	21–45 h	339 mM	2X	Enzyme	31–35%
This Study	6–48 hpf	18–24 h	171 mM	1X	HGC	24–37%
Zhang et al., 2013	6–72 hpf	6–24 h	86–342 mM	1X–3X	Enzyme	32%

^aDevelopmental stages when ethanol was added. hpf – hours post fertilization; dpf – days post fertilization.

^bReported as mM concentration.

^cNumber and duration of washes (where listed) to remove excess ethanol from the embryo before embryo processing.

^dAssays used to assess embryonic ethanol concentration. MRS – Magnetic Resonance Spec.; HGC – Headspace Gas Chromatography; ¹⁴C – Radiometry; Enzyme – Ethanol assay kit measuring NADH production using Alcohol dehydrogenase; Analox – analyte analyzer.

^eReported as % v/v or mM concentration except #. Reported as µg ethanol/mg brain wet weight.