Biochemical changes in animal models of fetal alcohol spectrum disorder

Christine E. M. Keller
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By Christine E.M. Keller

Entitled
Biochemical Changes in Animal Models of Fetal Alcohol Spectrum Disorder

For the degree of PhD

Is approved by the final examining committee:

Paul B. Brown
Chair
Maria S. Sepúlveda

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Charles R. Santerre

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Approved by Major Professor(s): Paul B. Brown

Approved by: Robert Swihart 22 July 2016

Head of the Departmental Graduate Program Date
BIOCHEMICAL CHANGES
IN ANIMAL MODELS
OF FETAL ALCOHOL SPECTRUM DISORDER

A Dissertation
Submitted to the Faculty
of
Purdue University
by
Christine E.M. Keller

In Partial Fulfillment of the
Requirements for the Degree
of
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</table>
4.8 Ethanol treatment resulted in one or more individuals per group with abnormal morphology, as qualitatively graded. Atypical tissue extending from the eye (blue arrows) was seen in individuals in several treatment groups (0.001, 0.01, 0.03, 0.3% lateral views). Abnormal tails were seen in many individuals across treatment groups (0.003, 0.3, and 1% in lateral views). Moderate pericardial edema was seen in 3/8 individuals in the control group as well as in individuals in all ethanol exposure groups except 3%. Moderate yolk sack edema at was observed in a few individuals across the treatment groups (0.003 and 0.1%). These individuals are shown in the lateral view. Individuals with moderately abnormal head morphology were present in several treatment groups, including 0.003 (2/6) and 0.3% (1/6), appearing to have a shortened forebrain segment. Moderate narrowing of the OOS was seen in a single individual in the control, 0.003, 0.03, and 0.1% groups.
Fetal alcohol spectrum disorder (FASD) is a completely preventable disease, that has profound effects on life-long health and function of the affected individual. Prevalence estimates of FASD in the United States indicate 33.5 per 1,000 live births are affected with this disorder [1]. FASD is caused by maternal ethanol intake during pregnancy. However, recommendations of the amounts of alcohol safe to drink during pregnancy are not established. Further, we lack a comprehensive understanding of the biochemical pathways modified in prenatal ethanol exposure. Biomarkers are also lacking. Our results demonstrate the vast array of biochemical pathways modified in the chronic ethanol exposure zebrafish model of FASD. Many of these pathways agree with existing literature, but the comprehensive nature of this study expands upon several of them, and links branches that were previously considered in isolation as mediators of FASD. In addition, examination of the non-polar excised embryonic mouse demonstrates a significant difference between a single binge dose of ethanol and a two binge doses. Similar metabolites to the zebrafish are identified in this model, though the degree and direction of change is not always consistent. These findings suggest that more ethanol does not necessarily result in an amplified, same-direction response, and that longer duration exposures do not always change metabolites the same way binge exposures do. In addition, a novel application of the probabilistic principal component and covariate analysis (PPCCA) is used to examine latent variables suggested by the combined models. Lastly, we review biomarkers suggested from the mouse and zebrafish model metabolomes, the PPCCA, and Ingenuity Pathway Analysis® (Chapter 4). We consider these biomarkers with a morphometric analysis of the zebrafish, where few significant differences are noted. Together, these observations suggest that in spite of a lack of morphologic changes, biochemistry of the prenatally exposed individual is significantly changed.
1. INTRODUCTION

1.1 Introduction

Fetal alcohol spectrum disorder (FASD) is a completely preventable disease, that has profound effects on life-long health and function of the affected individual. Prevalence estimates of FASD in the United States indicate 33.5 per 1,000 live births are affected with this disorder [1]. FASD is caused by maternal ethanol intake during pregnancy. It is a difficult disease to study, for many reasons. It is difficult to determine the relationship between ethanol dose, consumption frequency, and the effects on the offspring. These factors are altered by the woman’s health, fasted or non-fasted state, and individual metabolism differences. Further, many women consume alcohol in early pregnancy, before they know they are pregnant. The social stigma surrounding alcohol consumption during pregnancy may make self-reporting less accurate. Also, clinical definitions of FASD have been evolving in recent decades, which make interpretation of older data difficult.

Because of the need for early intervention to minimize the effects of alcohol on the offspring, especially until a consensus on recommended limits on alcohol are achieved, early detection is key. In spite of a variety of proposed biomarkers, rapid and robust clinical biomarkers are lacking. Further, ethanol’s polar protic nature makes it a powerful molecule. To advance our understanding and ability to intervene in FASD, a broader understanding of the mechanisms involved in this disease is needed. Additionally, differences in models of ethanol dose and duration of exposure need to be better characterized. This chapter summarizes the relevance of FASD investigation, difficulties in understanding FASD, the need for better biomarkers, and our metabolomics studies investigating these problems.

1.2 Fetal Alcohol Spectrum Disorder

1.2.1 An Overview of FASD

For centuries, man has identified a link between maternal alcohol consumption and abnormal offspring. The link between maternal alcohol consumption and fetal
defects has been cited as far back as Greek and Roman mythology \[2\]. As early as 1971, scientists had begun to infer the effect of ethanol on offspring. Using chick and rat embryos, researchers demonstrated the effect of ethanol on abnormal nervous system morphology, somites, and growth in these models \[3\]. Through examination of case-studies of the children of two alcoholic mother and one other child that appeared to have similar features, Jones made the association between maternal alcohol and infant malformations. In the two infants he studied, he noted that they had respiratory deficiency, and biochemical abnormalities, including hypoglycemia, hypocalcemia, and hyperbilirubinemia. Both infants had microphthalmia, cardiac abnormalities, and a cleft soft palate. In autopsy of one of the children who died at 5 days of age, neural, glial structures were disoriented and brain structures were incompletely formed \[3\]. Yet, we still lack a real understanding of this disease, preventing the identification of successful therapies.

Fetal alcohol spectrum disorder (FASD), previously only recognized in its most severe form, fetal alcohol syndrome (FAS), has been characterized for over 40 years by its defects in craniofacial morphology, limb morphology, and heart defects \[2\]. FASD is a spectral disease, with variations in clinical features and severity defined by the Institute of Medicine. The diagnosis of FASD severity is weighted heavily on the abnormal morphology of the offspring, as well as neurologic or other physiology deficits. Diagnostic morphologic features include short palpebral fissures, thin vermilion border of the upper lip, smooth philtrum, and growth retardation. Based on the morphology and mental faculties of a child, he or she may be classified as having FASD. These classifications include alcohol-related neurodevelopmental disorder, alcohol-related birth defects, partial FAS (pFAS) (with or without known maternal drinking history), and FAS (with or without known maternal drinking history) \[4\].

Cognitive deficits are also present in FASD, with varying severity. The cognitive-behavior phenotype has been described as “generalized deficit in processing complex information” \[5\]. Verbal and non-verbal skills both appear to be diminished in FASD \[5\]. Executive control function is also affected as well as complex task functioning \[5\]. In reports of FAS, some cite the average IQ to be as low as 68 \[6\].

Not included in the Institute of Medicine classification system is the contribution of maternal alcohol consumption to early death, be it through spontaneous abortion, pre-term labor, still births, or sudden infant death syndrome \[2, 7–11\]. In a study of the National Danish Birth Cohort, women consuming seven of more drinks a week had a relative very preterm delivery (fewer than 32 weeks) risk of 3.26 and 1.58 for
moderate preterm labor (32–36 weeks) \cite{7}. The association for drinkers for moderate preterm labor was much lower for mothers consuming 4–7 drinks per week at only 1.15 \cite{7}. This study controlled for variables including maternal history of preterm labor, occupation, hypertension, education, age, child’s sex, and coffee and tobacco intake \cite{7}.

The consideration of FASD as a *spectral* disorder in and of itself may skew the data regarding the historical prevalence of this disease. Now that more phenotypes are considered a part of FASD than those of FAS, it is possible that earlier FAS diagnoses underestimate the count of those affected \cite{12}.

Understanding the physiological changes that occur in FASD is complicated by the variation in maternal intake, timing of consumption, and the maternal and fetal individual biological differences. All of these factors make the collection of relevant human data and its interpretation difficult. For example, in a study of the association between “light” and “moderate” maternal alcohol consumption pattern showed no difference in the growth rate of children of some ethanol-consuming mothers. This study examined a population cohort of over 7,300 pregnant women who were surveyed in early, middle, and late pregnancy regarding the frequency and quantity of alcohol they consumed. (This study defined “light-to-moderate” as equal to or less than 1 alcoholic beverage per day \cite{13}.) One study even reported women who were “mild drinkers” (defined as 0.1–0.25 oz of absolute alcohol per day) statistically showed a “protective” effect on intrauterine growth retardation, but preterm labor during the seventh month of pregnancy was associated with nearly 3 times the odds for both “light” and “mild-to-moderate” drinkers \cite{10}.

Timing of ethanol consumption during pregnancy is another major hurdle in studying FASD. A study comparing quantities, patterns, and timing of alcohol consumed by mothers showed a significant association between higher prenatal exposure and a smooth philtrum. The thin vermilion border associated with exposure during the second part of the first three months of pregnancy. Reduced length at birth was associated with alcohol exposure at any time during pregnancy \cite{14}. Growth and development are rapidly changing in the developing offspring, and the response to the teratogen is variable during gestation. Thus, another complication is that the variability alcohol’s effects upon the constantly changing stage of development.

Differing consumption patterns contribute to the difficulty in finding consistent outcomes of alcohol exposure. Some women drinking routinely, a glass or two of wine nightly. Other women consume alcohol more in a weekend “binge” fashion.
Another drinking pattern would include the chronic alcoholic. So, determining relevant explanatory alcohol concentrations to study may be difficult in the laboratory. Even within the field of alcohol research the broad categorization of “low-moderate”, “moderate”, and “heavy”, for example, are not uniformly defined. Further, while many researchers attempt to have mothers estimate alcohol consumed, dietary studies routinely show that recall of food and beverage intake and consumer estimates of measurement are highly inaccurate. Further, mothers are often not willing or potentially ashamed of having consumed alcohol while they are pregnant, making accurate exposure history difficult to obtain.

Further, differences in the rates of metabolism differ from individual to individual, stomach contents, and weight may contribute to the variety of outcomes in FASD-related research. In addition, maternal tissues, fetal tissues, and the placenta all vary in their contributions to ethanol metabolism. Further, amniotic recirculation contributes to alcohol reentry in the fetus [15].

Socioeconomic status and lifestyle choices also influence fetal outcomes, making human research of this disease further complicated. In the past, researchers largely linked FASD to a lack of education among women. The result of this in the US was the addition of an alcohol consumption label on bottles [6]. However, cross-sectional study using the National Center or Health Statistics data, of the 45% of women who report having consumed alcohol during the last 3 months, included in the highest risk factors were being 25 years or older and college educated [16].

Genetics have also been implicated as a dispositional cause of FASD in some. Very early investigations in FASD revealed the likely implication of genetic alterations. In FAS, an irreversible growth deficit also occurs, even when children are placed in foster home environments [2]. Twin studies have shown that in the occurrence of monozygotic twins, both children are affected, whereas in dizygotic twins, one (or both) may be affected [17,18]. However, other twin studies have shown dizygotic twins have reported only a single pair where both children presented with FAS [9,19].

In animal studies, polymorphisms of alcohol dehydrogenase (ADH1B) have been identified that predisposes/protects individuals to FASD [18]. In a comparison of different mice breeds, c57BL/6J, DBA/2J, and A/J mouse strains resulted in differing levels of malformations and mortalities. These mice had been treated with the same concentration and by the same route of administration, yet ended up with different blood alcohol levels and different fetal outcomes [18,20,21]. Another study that used excised mouse embryos demonstrated these effect were related to the difference
in susceptibility of organs at different times to teratogenic ethanol in the different mouse strains [22].

Due to variations in factors such as diagnostic criteria, sampling bias, and descent (native or non-native), global estimates of the prevalence of FASD are limited [1]. A recent meta-analysis highlights the lack of global research of FASD, identifying publications of only 10 countries with available prevalence data [1]. Pooled prevalence statistics from general population studies vary widely between country and severity of disease. South Africa, for instance, has a fetal alcohol syndrome prevalence of 55.42 per 1,000, and a point estimate of 113.22 for FASD [1]. In the United States, FAS prevalence is estimated at 0.67 per 1,000 live births and FASD is estimated at 33.5 per 1,000 live births [1]. Survey of a nationally representative population of women ages 15–44 in the United States suggest the rate of alcohol-exposed pregnancy risk is 7.3% [23].

FAS has an estimated annual cost in the United States of $3.6 billion [24]. The mortality rate for fetal alcohol syndrome has been estimated to be 3.5 times that expected of the general population [25]. Individuals with FASD have higher rates of behavioral problems in school and with the law, inappropriate sexual behaviors, and alcohol or drug problems [26].

1.2.2 Proposed Mechanisms

Understanding the nature of the mechanisms involved in FASD is essential to prevention, diagnosis, and treatment of FASD. Because of the polar protic nature of ethanol, this teratogen can interact with a wide variety of substances. Many different pathways have been proposed as being key mediators in the abnormalities that characterize FASD. Altered energy and nutrient metabolism that results in decreased growth was an early proposed key mechanism [27–29]. Accumulation of FAEEs, formed from the esterification by fatty acid ethyl ester synthase of ethanol and fatty acids, has been hypothesized as a cause of FAS [30]. Other lipid and cholesterol modifications are also altered in FASD [31][32]. Sphingolipid modifications have been hypothesized as the cause of neuronal loss in FAS [33]. Another hypothesis is the modified nuclear transcription factors. Vitamin A has been widely investigated as a causative alteration and potential point of intervention in FASD [34–36]. Cell death, beyond normal apoptosis, has been associated with FASD [37][38]. Abnormal tryptophan metabolism, particularly in the serotonin branch, has been well studied as a
key mechanism altered in FASD. Alcohol deters the outgrowth, migration, and development of serotonergic neurons \[39\] \[42\]. Increased oxidative stress is also associated with FASD \[43\] \[47\]. Glycosylation, important for protein translocation, has been proposed as well \[48\]. However, in spite of all of this work, there is still not a unified theory of alcohol induced alterations in FASD.

1.2.3 Lack of Diagnostics

We currently lack sufficient tools to diagnose FASD, particularly ones that can be used early pregnancy. Significant improvements in 3-D facial photography has allowed diagnosis of children with a maternal history of drinking without classical morphological features. Prenatal ultrasounds provide some success as a diagnostic tool in identifying a “hook”-shaped structure in the corpus colossum that was strongly associated with ethanol exposure during pregnancy \[49\]. However, researchers were successful in identifying only 12 of 23 ethanol-exposed infants and one false positive among the 21 unexposed or “lightly” exposed controls \[49\].

Several biomarkers demonstrate a correlation with the maternal exposure to alcohol, including enzymes like cytochrome P450, \(\gamma\)-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase \[50\] \[51\]. Large protein biomarkers, such as carbohydrate deficient transferrine, have also been proposed for FASD biomarker use \[51\]. Assessment of mean corpuscular volume of erythrocytes, is another proposed FASD biomarker \[51\]. However, all of these are limited to chronic or long-term alcohol use \[51\].

Fatty acid ethyl esters (FAEEs) have been proposed for decades as a biomarker of prenatal ethanol exposure \[30\] \[52\]. These molecules are formed from the esterification of alcohol and fatty acids or acyl-CoA/fatty acids by fatty acid ethyl ester synthases, and can be identified in a variety of tissues after ethanol ingestion \[30\]. FAEEs can be detected in meconium, however it is unclear when meconium formation begins, making correlation with gestational ethanol intake difficult \[53\]. False negatives for some FAEE analytical methods are also a concern, as this could lead to sending a child home to an at risk environment \[53\]. Importantly, meconial FAEEs have been reported in children of non-ethanol consuming mothers, limiting their robustness as population screening biomarkers \[54\] \[56\]. FAEE detection in hair has also been proposed, as hair will reflect historical alcohol consumption \[57\]. But, FAEEs have
been shown to change based on the use of hair products, which is a tremendous limitation \[58][59].

With these limitations, a robust biomarker(s) is still needed. It needs to capture both acute and chronic alcohol exposure, as well as correlate with quantity of alcohol consumed and potentially the duration of exposure. Screening should be rapid and robust, which has been a problem with many of the existing biomarkers proposed for FASD. Ideally for interventions, screening for alcohol-exposed children should take place in the womb. This would allow for interventions of the mother, so as to prevent further alcohol-induced abnormalities in development. Further, an ideal biomarker would reflect the effectiveness of FASD interventions, be they pharmacological or otherwise.

1.3 Specific Aims

1.3.1 Specific Aim #1: Investigate Mechanisms

The first aim of this work is to investigate the mechanisms, as a whole, changed in an animal model of FASD, through the use of metabolomics. Because a unified theory of FASD is lacking, a generalized method to explore a variety of mechanistic changes in FASD is essential. Metabolomics is an excellent tool, that allows for a wide-spread scan of small molecule changes. This method removes the limits on the number of mechanisms explored in a single study. Because of its “unbiased” nature, metabolomics does not limit the researcher to the preconceived notions regarding the pathways hypothesized in FASD. We will utilize a common zebrafish model for this purpose, where zebrafish embryos are incubated in an ethanol solution from 2–24 hours post fertilization (hpf). We will utilize a wide range of ethanol concentrations to mimic a variety of chronic drinking levels. While we anticipate we will confirm observations of many of the proposed pathways modified in FASD, we also anticipate identifying novel metabolites that will expand or alter some of the existing theories of FASD.

1.3.2 Specific Aim #2: Compare Models

The second aim is to compare and contrast metabolite findings in two different, well-established models of FASD. As is the case with most areas of biomedical re-
search, FASD relies heavily on a few established animal models. More than ten years ago, collaborators on this research project developed a model of prenatal ethanol exposure that utilizes an excised mouse embryo (E8), which is then cultured \cite{22}. This offers the benefit of minimizing confounding maternal influence on the embryo, while still generating observable developmental dysmorphology \cite{22,60}. We will explore the similarities and differences within this model of the non-polar metabolites changed between control (untreated) animals and groups dosed with 400 mg/dL on one or two consecutive days for six hours. As in our first specific aim, we anticipate confirming many of the previously hypothesized pathways in FASD. But, we also anticipate identifying novel metabolites that extend or alter some of these theories. These findings will be compared with those of the zebrafish models. In addition, a novel application of a principal component analysis will be applied to examine the differences in the non-polar metabolomes generated from the zebrafish and mouse models. A significant overlap is expected between the metabolomes of these two models. This methodology will allow examination of the universality of the metabolomics approach and validate zebrafish model findings in a second model.

1.3.3 Specific Aim #3: Identify Biomarkers

The third specific aim is to identify and characterize potential biomarkers from these models of FASD. Ethanol is a master solvent capable of acid-base modification, ester formation, halogenation, haloform reactions, dehydration, oxidation, and chlorination, and a great number of small molecules are altered by alcohol. Alteration of these small metabolites may serve as biomarkers for the detection of alcohol exposure. These biomarkers should be dose-responsive to ethanol, highly reproducible, and ideally present in both of these established models of FASD. Based on the outcomes anticipated from the second specific aim of similar metabolites from the zebrafish and mouse models, we anticipate identifying several potential biomarkers relevant to FASD. Some of these are anticipated to overlap with currently proposed (but insufficiently robust) biomarkers. In addition, these biomarkers will be compared with the morphologic differences visibly identified in the zebrafish model.
1.4 Introduction to chapters

This dissertation is a compilation of manuscripts prepared for submission to various journals.

Chapter 2 examines mechanisms highlighted by the metabolome changes identified in the zebrafish model of chronic prenatal ethanol exposure. Unlike many other metabolomics analyses, this presentation of a zebrafish FASD model metabolome attempts to explore the actual mechanisms implicated by the whole of the metabolome changes. Rather than simply presenting data, this work attempts to contextualize that material within the proposed mechanisms of FASD. This analysis provides a platform for the generation of new hypotheses of FASD as well as expanding upon existing hypotheses.

Chapter 3 examines the non-polar metabolome changes in embryonic mice exposed once or twice with 400 mg/dL ethanol for 6 hour binges, and compares these findings relative their unexposed controls. We will also explore here the similarities and differences in the zebrafish and mouse as models of FASD in humans, including application of a novel probabilistic principal component and covariate analysis (PPCCA).

Chapter 4 examines the biomarkers suggested by the metabolomics output and PPCCA, including a secondary analysis with the biomarker filtering agent of Ingenuity Pathway Analysis®. It also examines our findings relative the existing, commonly proposed biomarkers. Results of a morphologic measurement of modifications of head circumference, eye size, eye-to-eye distance, total length, and survival of ethanol exposed zebrafish are presented, as are qualitative scoring of body shape, tail shape, pericardial edema, yolk sac edema, head (brain) shape, and the otic-optic space. Together, our biomarker findings in conjunction with our morphology results emphasize the effect of ethanol, in spite of statistically significant morphology changes.

Chapter 5 summarizes the major findings of this dissertation. The limitations of these studies are also briefly examined. This chapter also proposes future directions for further investigation.
2. DEVELOPING THE BIG PICTURE OF FETAL ALCOHOL SPECTRUM DISORDER: A PROPOSED MECHANISM OF METABOLITE CHANGES

Abstract

Although fetal alcohol spectrum disorder (FASD) has been clinically identifiable by the medical community for decades, a comprehensive profile metabolic changes has not been developed. To that end we characterized ethanol-induced metabolome changes in the zebrafish model of FASD. Zebrafish embryos were exposed to ethanol at concentrations of 0–3% from 2–24 hours post-fertilization. This was followed by washing and further incubation with media for an additional 24 hours. At 48 hours post fertilization whole-embryos were extracted and analyzed by liquid chromatography using a Thermo Fisher Orbitrap mass spectrometer (non-polar fraction) or liquid chromatography coupled with an Agilent Time of Flight mass spectrometer (polar fraction). Data were aligned, normalized, and filtered followed by ANOVA. Significant differences were detected in lipids, including short chain fatty acids, phosphatidic acid, phosphatidylycerines, phosphatidylycerols, ceramide, and sphingosine, which support our existing understanding of neural abnormalities in FASD. We also noted significant differences in metabolites related to essential enzymatic cofactors, such as nicotinamide adenine dinucleotide, coenzyme A, pyridoxal phosphate biocytin (biotin), and tetrahydrofolate. We also noted significant differences in metabolites related to nuclear transcription factors, oxidative stress, and cardiovascular function. Our work supports the work of many previous FASD mechanistic hypotheses, but more importantly identifies novel metabolome changes that may alter our perception of these previously proposed hypotheses.

2.1 Introduction

Though the relationship between maternal drinking and abnormal prenatal development has been recognized for over a millennia, we still do not have a clear picture of the widespread biochemical influences alcohol has on offspring. Fetal alcohol spectrum
disorder (FASD) prevalence estimates vary widely due to the complex nature of this disease. The nature of ethanol as a polar protic solvent means that it chemically modifies a vast array of molecular species, making the modeling of FASD a complicated problem. (Polar protic, or proton donating, solvents, such as ethanol, are characteristically capable of hydrogen bonding, are acidic (weakly, in the case of ethanol), and dissolve salts through hydrogen bonding (for anions) or unshared free electron pairs (for cations). They have a strong electric field strength in a vacuum, meaning a high permittivity.) To address this issue, we pursued non-targeted metabolomics in a widely used zebrafish model of FASD. Here we examine both established and new mechanisms of FASD.

Significant contributions have been made to the understanding of FASD by exploring changes in many teratogenic pathways (nitric oxide and other reactive oxygen species, glutamate, vitamin A, serotonin signaling, lipids, glucose regulation, and adhesion molecules), which are altered after embryonic ethanol exposure. This has resulted in a wide range of theoretical models as potential causative agents of FASD. Because of the technologies previously available, researchers have had to limit the range of biochemical changes explored at one time, which has inhibited our ability to create robust, reliable detection strategies, develop successful interventions, and understand this complicated disease.

Metabolomics has become an excellent tool to determine the biochemical changes occurring in a given condition. Through the use of liquid chromatography-mass spectrometry coupled with evolving metabolite databases, we were able to use a non-targeted metabolomics approach to investigate the well-established zebrafish model of FASD. This technique allows us to explore how the metabolome changes under changing ethanol concentrations (dynamically), isolating systemic influences to include only that of ethanol, the embryo itself, and the maternally derived yolk sack. It bolsters existing research as well as sheds light on possible new avenues for follow-up research (hypothesis generating). With this approach, we seek a better understanding of important biochemical changes that are occurring in the entire developing organism.

After exposing AB strain *Danio rerio* zebrafish to ethanol in concentrations from 0–3% from 2 - 24 hours after fertilization, whole embryo extracts were analyzed using liquid chromatography coupled with a mass spectrometer (MS) Agilent Orbitrap (LC-MS/MS) technology (non-polar fraction) or Agilent 6220 Time of Flight (TOF) LC-MS (polar fraction) detection. We compiled the significantly different metabo-
lites, which we present here. Our results demonstrate embryonic ethanol exposure often has a hormetic (J- or U-shaped) effect on the biochemical changes that occur. In addition, our findings bolster the of the works of others on the impact of ethanol on nervous system changes related to lipids; alterations in tryptophan-related metabolites affecting serotonin, kynurenine, and growth; metabolome changes which alter vitamin A and D regulatory functions; oxidative stress; PI3K changes which may impact secondary messenger signaling and cardiovascular function; metabolites able to alter the HPA axis; and hormone changes relevant to the sex differences noted in FASD research. In addition, we noted many enzymatic cofactor-related metabolites, which affect a vast array of biochemical processes.
2.2 Materials and Methods

2.2.1 Zebrafish Embryo Ethanol Exposure

Animal experiments were performed in accordance with Purdue Animal Care and Use policies at Purdue University, West Lafayette using approved protocol 1111000275. Healthy AB strain zebrafish were housed in 15-gallon glass aquaria with approximately twenty-four individuals, mixed male and female, per tank. Pyrex dishes with mesh tops were set in each tank at 0925 AM for spawning. Fertilized eggs were collected shortly after spawning from six aquaria.

Embryos were examined microscopically to confirm fertilization and transferred to E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl$_2$, and 0.33 mM MgSO$_4$ in distilled water) $^{[63]}$. At 2 hours post-fertilization (hpf), randomized embryos (n=8 per group) were exposed to ethanol at concentrations of 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% v/v. Embryos were incubated in covered 6-well plates at 28°C (18 hours light, 6 hours dark) until 24 hpf. After washing with E3 twice, zebrafish were further incubated until 48 hpf. At that time, the larvae were flash frozen in liquid nitrogen and stored at $-80^\circ$C.

2.2.2 Metabolomics

Sample Preparation

After thawing at 4°C and removal of any visible water with a pipette, embryos were extracted in 2:1 methanol:water, homogenized, extracted in ice-cold chloroform, centrifuged and concentrated. The non-polar extract was dissolved in 50% ddH$_2$O, 25% methanol, 25% acetonitrile and analyzed with LC-MS/MS using Orbitrap (see below). The polar extract was dissolved in 20% acetonitrile, 80% water with 0.1% formic acid. Polar samples were then analyzed by LC-MS (Time of Flight).

Chromatography

Non-Polar Extract Chromatography  A Thermo Fisher LTQ Orbitrap (Waltham, MA USA) instrument was used for the non-polar sample analysis, where each sample was analyzed once. It was coupled to an Agilent 1100 series Liquid Chromatography (LC) (Agilent Technologies, Santa Clara, CA) equipped with a micro well plate auto
sampler and binary pump. Reverse phase liquid chromatography was used to analyze the samples. An Eclipse XDB-C8 column (Agilent Technologies, Santa Clara, CA) with 2.1x150 mm, 3.5 \( \mu \text{m} \) dimensions was used for the separation. Solvent A consisted of water + 0.1% piperidine and Solvent B contained acetonitrile:methanol (50:50 \( \text{v/v} \)) + 0.1% piperidine. The flow rate was 300 \( \mu \text{L/minute} \). A volume of 10 \( \mu \text{L} \) was loaded onto the column. The gradient was as follows: 0 minutes, 50% B; 25 minutes, 95% B; 45 minutes, 95% B; 50 minutes, 50% B; and 60 minutes, 50% B.

The mass spectrometry (MS) analysis used negative polarity electrospray ionization with a source voltage of 3.0 kV, source current 100 \( \mu \text{A} \), capillary voltage –45.50 V, and tube lens voltage –102.0 V. The capillary temperature was 275 \( ^\circ \text{C} \), sheath gas flow was 15, auxiliary gas was 30, and sweep gas was set to 0. Data were acquired using data dependent scanning mode. Fourier transform mass spectrometry (FTMS) resolution of 60,000 with a mass range of 100–1200 atomic mass units (based on the limit of detection) was used for full scan analysis and the FTMS was used for MS/MS data acquisition with a resolution of 7500. The top three most intense ions were acquired with a minimum signal of 1000, isolation width of 2, normalized collision energy of 35, default charge state of 1, activation Q of 0.250, and an activation time of 30.0. The samples were evaluated with Thermo XCalibur software (v.2.1.0) and downstream alignment done with an in-house data processing package (see below). Standards for palmitic and stearic acids were analyzed using the same method.

**Polar Extract Chromatography** For the polar fraction, only the 0.01, 0.03, 0.1, 0.3, and 1% ethanol groups were analyzed, with each sample analyzed one time. An Agilent 1100 series liquid chromatography (LC) instrument (Agilent Technologies, Santa Clara, CA) equipped with a well plate auto sampler and binary pump coupled to an Agilent 6220 TOF LC-MS instrument was used for the analysis of polar fraction samples. Reverse phase liquid chromatography was used to separate the samples before MS analysis. A Waters Atlantis T3 column (Waters Corporation, Milford, MA) with 2.1 x 150 mm, 3.0 \( \mu \text{M} \) dimensions was used for the separation. Solvent A consisted of water + 0.1 % formic acid. Solvent B consisted of acetonitrile + 0.1 % formic acid. The flow rate was 300 \( \mu \text{L/minute} \). A sample volume of 20 \( \mu \text{L} \) was loaded onto the column. The linear gradient was as follows: time 0 minutes, 0 % B; time 1 minute, 0 % B; time 41 minutes, 95 % B; time 46 minutes, 95 % B; time 50 minutes, 0 % B; time 60 minutes, 0 % B.
The MS analysis used positive polarity electro-spray ionization (ESI). The source conditions were as follows: capillary voltage 3.5 kV, gas temperature 325 °C, drying gas 8 L/minute, nebulizer pressure 40 psi, fragmentor 120 V, and skimmer 65 V. Data were acquired from 50–1000 m/z (based on the limit of detection) and the acquisition rate was 0.99 spectra/second. An Agilent calibration mix (Agilent G1969–85001) was used as an external mass correction for all samples. It was continuously infused into the dual ESI source at a rate of 15 µL/minute throughout the run. The samples were evaluated and preprocessed with Agilent Masshunter Qualitative Analysis software (version B.03.01) followed by data filtering and analysis.

Data Filtering and Analysis

Using the Purdue Bindley Bioscience Center Omics Discovery Pipeline Software, retention time, m/z, and peak intensity data from each data set were separately deconvoluted with a m/z variation setting of 0.07, an LC peak width of 10, and a minimum noise-to-signal ratio of 0.5 [65]. Alignment across non-polar samples required an m/z variation of 0.015 and a retention time variation across samples of less than 0.7 minutes. For the polar metabolites, alignment was required to be within 0.075 m/z with a retention variation of no more than 0.7 minutes. Due to poor alignment, two samples from the 0.03 and one from the 0.1% treatment groups were excluded from the polar analysis. In the non-polar samples, normalization required the detected peak to occur in 60% of the samples of at least one treatment group and 35% experiment-wide. For the polar samples, 70% and 35% were used for these cut-offs, respectively.

Some metabolites with identical m/z appeared in the data. If the retention times were within 1 minute, the metabolites were combined. For those that had a larger difference in retention time, these molecules were retained as separate metabolites, and are reported with their retention time ± standard deviation (SD). Metabolites were identified and classified using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa Laboratories), the PubChem Substance and Compound database, LipidMaps, the Human Metabolome Database, or METLIN [66–72]. Identified non-polar metabolites were filtered based on being within 3 parts per million (ppm) difference of the exact mass of the metabolite, with ppm difference defined as

$$
ppm\,\text{difference} = \frac{(\text{observed mass} - \text{theoretical mass})}{(\text{theoretical mass})} \times 1,000,000
$$
Polar metabolites were filtered based on being within 10 ppm of the theoretical mass. Data was further filtered as noise if it failed to have a minimum signal intensity across samples of $e^{10}$ or greater or were likely misidentified (such as drug metabolites only appearing in one sample concentration). Data for samples that were fractionated on differently aligned peaks were manually combined for each species if entire groups were split between metabolites identified as the same entity. Treatment groups for each metabolite were compared using an ANOVA with metabolites considered significant at $P < 0.001$. The fold for each metabolite was calculated as follows:

$$fold = \log_2 \frac{\bar{i}}{\bar{c}}$$

where $\bar{i}$ is the average of the intensity values for the group of interest and $\bar{c}$ is the average of the intensity values for the control group.

**Ingenuity Pathway Analysis®**

Ingenuity Pathway Analysis® (IPA) (Ingenuity Systems, Redwood, CA) was used for additional metabolome network analysis. Metabolites were required to have at least one concentration with a 0.001-fold or greater to be considered in the subsequent IPA analysis. The P-value reported expresses the association between the identified metabolite and a given pathway, based on the number of measured molecules that are reported in the literature to participate in a function, and the number of all molecules associated with that function in the Ingenuity Knowledge Base. These categories of function can be prioritized by the IPA $P$ (or $P$ range for supersets) for each concentration tested. Reported here are the 0.03% ethanol treatment significantly associated pathways, as the values from all ethanol doses provided similar $P$. Non-polar and polar data sets were not combined for analysis due to the different chromatography methods used to generate them.
2.3 Results

2.3.1 Metabolite Clustering

A total of 647 non-polar fraction metabolites were identified in the databases queried. Of these, 94 were identified as molecules unique from each other with less than a 3 ppm difference from the theoretical mass of their associated identified metabolite. Eighty-two metabolites significantly changed as a function of the ethanol concentrations tested \((P < 0.001, \text{ANOVA})\). Metabolites clustered in three main categories: fatty acids (9), glycerophospholipids (18), or sphingolipids (7). The remainder of the identified metabolites were prenol lipids or exogenous/synthetics. Hierarchical clustering resulted in samples within a treatment group appearing more similar to each other than to samples of other treatment concentrations (Figure 2.1). However, the groups did not always cluster in the order of ethanol concentration (0.03 and 1% groups were reversed).

The polar metabolome consisted of metabolites 219 identified by the databases queried. The metabolites in each sample clustered more like the samples within the same ethanol treatment group than to samples of other ethanol groups (Figure 2.2). Fifty-one polar metabolites were identified as synthetic compounds or drugs. Other classes of molecules included ethanol metabolites (15), proteins/amino acids (48), carbohydrates (5), and lipids (57).

2.3.2 Ethanol Metabolites

Many metabolites related to ethanol catabolism were identified in the polar fractions (Table 2.1, Structure 3-1). These included acetamide, butanal, dihydroxyacetone, acetoacetic acid, \(S\)-methylthioglycolate, and \(N\)-cyclohexylformamide. Acetamide is an amidated form of acetate. It was significant higher at concentrations of 0.1, 0.3, and 1% ethanol at a retention time of 10.87 ± 0.08 minutes. It also occurred at a later retention time of 15.53 ± 0.10 minutes, where it was nearly two-fold higher than controls at concentrations of 0.3 and 1% ethanol. Butanal (Structure 3-3) occurred with several different elution times. At 5.36 ± 0.11 minutes, with was significantly elevated over controls at concentrations of 0.01, 0.03, and 0.1%. At the later retention times of 14.07 ± 0.17 and 16.56 ± 0.27 butanol was nearly 5.94-fold higher than controls. The metabolite dihydroxyacetone (Structure 3-4) was identified as only slightly higher than control for
Fig. 2.1. Clustering of non-polar metabolites from zebrafish embryos exposed to ethanol. Divisional clustering of the non-polar fraction samples demonstrates group members were more similar to other same group members than to the members of another group. Clustering analysis is a statistical tool used for grouping samples on the basis of similarity to other samples. Clustering of the metabolite groups occurred in the reverse order of what was expected for groups 0.03% and 1%, but samples within each group were more like each other than samples of another group.
Fig. 2.2. Clustering of polar metabolites from zebrafish embryos exposed to ethanol. Divisional clustering of the polar fraction samples demonstrates group members were more similar to other same group members than to the members of another group. Clustering analysis is a statistical tool used for grouping samples on the basis of similarity to other samples. Clustering of the metabolite groups occurred in the reverse order of what was expected for groups 0.1% and 0.3%, as well as control and 0.01%. But, samples within each group were more like each other than samples of another group.
treatment groups of 0.01–0.3% ethanol, but dropped to −5.45-fold in the highest concentration treatment group. Acetoacetic acid \(\text{(Structure 3-5)}\) was higher than controls at the lowest concentrations tested of 0.01 and 0.03% ethanol. The metabolite \(S\)-methylthioglycolate \(\text{(Structure 3-6)}\) was elevated at all concentrations of ethanol higher than 0.01%. Lastly, \(N\)-cyclohexylformamide \(\text{(Structure 3-7)}\) was approximately 6-fold higher than control in the 0.01 and 1% ethanol treatment groups, but was not different in the intermediate ethanol treatments.

### 2.3.3 Lipids

**Fatty Acids**

**Stearic and Palmitic Acids** Lipids were identified in both the non-polar and polar data sets, though were predominantly in the latter. The metabolites were broadly
Table 2.1.
Ethanol catabolism associated metabolites were identified at several retention times within 10 ppm of their theoretical mass/charge from the polar fractions.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention Time (min.)</td>
<td>ppm</td>
<td>0.01</td>
</tr>
<tr>
<td>Acetamide</td>
<td>CID178</td>
<td>10.87 ± 0.08</td>
</tr>
<tr>
<td>Butanal</td>
<td>CID261</td>
<td>5.36 ± 0.11</td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>CID670</td>
<td>2.83 ± 0.08</td>
</tr>
<tr>
<td>Acetoacetic acid</td>
<td>CID96</td>
<td>3.86 ± 0.17</td>
</tr>
<tr>
<td>S-Methylthioglycolate</td>
<td>CID5179950</td>
<td>3.57 ± 0.13</td>
</tr>
<tr>
<td>N-Cyclohexylformamide</td>
<td>CID13017</td>
<td>2.34 ± 0.23</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–1%. Metabolites were identified from polar extraction fractions using LC-MS (TOF). The average retention time±SD are listed along with the PubChem database ID (CID), followed by the ppm difference (ppm) between the theoretical mass and the observed mass. Folds, relative to 0% controls, are listed for each ethanol concentration. All metabolites listed are significantly changed from control, \( P < 0.001, \) ANOVA.

classified as fatty acids, phospholipids, sphingolipids, and sterol metabolites. Of the fatty acids identified, stearate and palmitate were predominant...
in the non-polar fraction. For both palmitic (HMDB00220, average molecular weight 256.4241 g/mol) and stearic acids (HMDB00827, average molecular weight 284.4772 g/mol), multiple metabolites were identified with similar mass/charge (m/z), but different analyte retention times (Tables 2.2 and 2.3). Palmitic and stearic acid standards were subsequently analyzed. Chromatograms and mass spectra are shown in panels A and C of Figure 2.4 for palmitic acid (m/z 256.2370 [M + H]^+ with a retention time of 3.5 minutes). Panels B and D show the results for stearic acids (m/z 284.2682 [M + H]^+ with a retention time of 6 minutes), respectively. The standards’ retention times suggest that the native palmitic acid eluted at 4.49±0.19 minutes. At this retention time, palmitic acid is over 6-fold higher in ethanol concentrations of 0.03, 1, and 3%. The later elution times of palmitic acid showed increases up to 7.32-fold over that of the control embryos. The earliest metabolite identified as stearic acid eluted at 14.20±0.17 minutes. Similar to the earliest eluting palmitic acid, stearic acid eluting at this time was significantly lower, being –5.45-fold with 0.01% ethanol or greater, compared to control concentrations. The other metabolites identified as stearic acid tended to be significantly lower as well, with the exception of the 26.57±0.13 minute elution time, which was up to 7.08-fold greater than control at 0.1% ethanol.

Other Fatty Acids Additional fatty acids were identified from both fractions. Non-polar fraction fatty acids included caprolylglycine, tetradecanoic acid (myristic acid), pentadecanoic acid, (9Z)-hexadecenoic acid, methyl hexadecanoate, 2-oxotetradecanoic acid, monoacylglycerol (MAG), and (2S,3S,4S,5R,6R)-6-(hexadecyloxy)-3,4,5-trihydroxyoxane-2-carboxylic acid (palmitoyl glucuronide) (Table 2.3.3). In the polar fraction, fatty acids identified were 2-decene-4,6,8-triyn-1-al, 2-hexanamidoacetic acid, (E)9-oxodec-2-enoic, trans-2-decenoyl-acyl-carrier protein (ACP), (4Z)-decenediolic acid, 3(R)-hydroxy-dodecanoyl-ACP, 12(S)-hydroxy-16-heptadecynoic acid, and 16-feruloxylxypalmitate.

The non-polar fraction fatty acid metabolite 2-octanamidoacetic acid (capryloylglycine) changed from control at all tested concentrations, however was not significantly change (p = 0.035). It was –5.98-fold below controls at 1 and 3% ethanol, but at the other tested concentrations changed little. Similarly, pentadecanoic acid was 6.09-fold below control in the non-polar fraction, but only in the 3% ethanol. Methyl hexadecanoate was significantly lower by 6.33-fold in ethanol.
Table 2.2.

Palmitic acid was identified at several retention times within 3 ppm of its theoretical mass/charge.

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th>0.001</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.29 ± 0.08†</td>
<td>0.411333</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>6.87</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>4.49 ± 0.19†</td>
<td>2.002808</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>6.16</td>
<td>0.00</td>
<td>0.00</td>
<td>6.41</td>
<td>6.11</td>
<td></td>
</tr>
<tr>
<td>5.33 ± 0.09†</td>
<td>0.689587</td>
<td></td>
<td>7.13</td>
<td>7.32</td>
<td>6.63</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>5.70 ± 0.20</td>
<td>1.725334</td>
<td></td>
<td>5.48</td>
<td>5.86</td>
<td>5.46</td>
<td>5.83</td>
<td>6.60</td>
<td>6.83</td>
<td>7.08</td>
<td>6.59</td>
</tr>
<tr>
<td>7.60 ± 0.04</td>
<td>1.639477</td>
<td></td>
<td>0.00</td>
<td>0.02</td>
<td>-0.03</td>
<td>-0.04</td>
<td>-0.23</td>
<td>-0.05</td>
<td>-0.01</td>
<td>-0.70</td>
</tr>
<tr>
<td>7.71 ± 0.18</td>
<td>-2.088275</td>
<td></td>
<td>-0.91</td>
<td>-1.03</td>
<td>-0.89</td>
<td>-0.54</td>
<td>-0.93</td>
<td>-0.30</td>
<td>-0.23</td>
<td>-0.29</td>
</tr>
<tr>
<td>8.73 ± 0.25†</td>
<td>1.995393</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>5.48</td>
<td>5.53</td>
<td>6.27</td>
<td>6.74</td>
<td>6.72</td>
</tr>
<tr>
<td>21.65 ± 0.09†</td>
<td>1.846314</td>
<td></td>
<td>5.83</td>
<td>0.00</td>
<td>5.46</td>
<td>5.84</td>
<td>6.10</td>
<td>0.00</td>
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<td>0.00</td>
</tr>
<tr>
<td>31.32 ± 0.24</td>
<td>0.807836</td>
<td></td>
<td>0.45</td>
<td>0.03</td>
<td>0.43</td>
<td>0.40</td>
<td>0.45</td>
<td>-5.36</td>
<td>0.72</td>
<td>0.36</td>
</tr>
<tr>
<td>44.64 ± 0.04†</td>
<td>1.817045</td>
<td></td>
<td>-6.64</td>
<td>-6.64</td>
<td>-6.64</td>
<td>-6.64</td>
<td>-1.36</td>
<td>-0.99</td>
<td>-1.40</td>
<td>-6.64</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–3%. Metabolites were identified from non-polar extraction fractions using LC-MS/MS. The average retention time ± SD is listed, followed by the ppm difference (ppm) between the theoretical mass and the observed mass. Folds, relative to 0% controls, are listed for each ethanol concentration. †: $P < 0.001$, ANOVA.

treatments of 0.03% or greater. MAG and (2S,3S,4S,5R,6R)-6-(hexadecyloxy)-3,4,5-trihydroxyoxane-2-carboxylic acid, also in the non-polar fraction, were significantly lower (approximately 7-fold) at 0.3% ethanol on greater.

Conversely, tetradecanoic acid, was significantly higher than control by as much as 7.19-fold in ethanol concentrations except 0.003%. 2-Oxooctadecanoic acid was 6.23-fold higher than control, but only with the 0.03% ethanol treatment. Lastly, (9Z)-hexadecenoic acid, also in the non-polar fraction, was nearly 6.12-fold above control in the 3% ethanol treatment.

In the polar fraction, the unsaturated fatty acid 2-decene-4,6,8-triyn-1-al was elevated more than 5.45-fold in the 0.03 and 0.1% ethanol treatment groups only. The metabolite 2-hexanamidoacetic acid (hexanoylglycine) was different from control only in the 0.01 and 0.03% ethanol treatment groups. It was 4.97- and 5.72-fold higher in those two groups. (E)9-oxodec-2-enoic, increased to 5.63-fold higher than controls in the 0.03% treatment group and 4.55-fold in the
Stearic acid was identified at several retention times within 3 ppm of its theoretical mass/charge. Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–3%. Metabolites were identified from non-polar extraction fractions using LC-MS/MS. The average retention times ± SD is listed, followed by the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. †: P < 0.001, ANOVA.

<table>
<thead>
<tr>
<th>Retention Time (min)</th>
<th>ppm</th>
<th>0.001</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.20 ± 0.17†</td>
<td>0.700387</td>
<td>0.02</td>
<td>0.02</td>
<td>-5.45</td>
<td>-5.45</td>
<td>-5.45</td>
<td>-5.45</td>
<td>-5.45</td>
<td>-5.45</td>
</tr>
<tr>
<td>19.10 ± 0.13†</td>
<td>1.223830</td>
<td>0.17</td>
<td>0.17</td>
<td>0.13</td>
<td>-0.03</td>
<td>0.14</td>
<td>0.18</td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>24.98 ± 0.06†</td>
<td>1.477812</td>
<td>0.19</td>
<td>0.38</td>
<td>-0.59</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
</tr>
<tr>
<td>25.28 ± 0.22</td>
<td>0.66943</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
<td>-6.21</td>
</tr>
<tr>
<td>26.57 ± 0.13</td>
<td>1.614302</td>
<td>0.00</td>
<td>0.00</td>
<td>5.77</td>
<td>7.02</td>
<td>7.08</td>
<td>7.05</td>
<td>6.81</td>
<td>6.51</td>
</tr>
<tr>
<td>29.43 ± 0.13†</td>
<td>0.490376</td>
<td>-0.08</td>
<td>-0.41</td>
<td>-6.28</td>
<td>-6.28</td>
<td>-6.28</td>
<td>-6.28</td>
<td>-6.28</td>
<td>-6.28</td>
</tr>
<tr>
<td>34.22 ± 0.09†</td>
<td>1.131313</td>
<td>0.28</td>
<td>0.64</td>
<td>-0.20</td>
<td>0.12</td>
<td>0.01</td>
<td>0.00</td>
<td>-5.55</td>
<td>0.66</td>
</tr>
</tbody>
</table>

0.3% group. At all other ethanol concentrations, there was no difference relative to controls. 2(E)-Decenoyl-ACP increased in the 0.01 and 0.1% ethanol treatments to 5.55- and 4.75-fold greater than controls, but at the other tested concentrations was not different than controls. The metabolite (4Z)-decenediio acid was consistently higher than controls at 0.1% ethanol or greater, being maximally different (5.94-fold) in the 0.1% treatment group. (3R)-Hydroxy-dodecanoyl-ACP, was different than controls for the 0.01, 0.03, and 1% ethanol treatments. It was as large as 5.72-fold greater than controls (0.03%). (12S)-Hydroxy-16-heptadecynoic acid in zebrafish embryos was unchanged from control at the lowest and highest concentrations tested, but as much as 5.68-fold higher in the 0.03–0.1% treatments. Similarly, 16-feruloyloxyhexadecanoic acid was higher than controls in all ethanol treatment groups except 0.01%. This metabolite was approximately 5.6-fold higher than controls until the 1% treatment group, where it was 4.63-fold higher than control.
Fig. 2.4. LC-MS/MS standards of stearic and palmitic acids. Chromatograms of palmitic (panel A) and stearic acids (panel B) standards generated using the same method as the metabolomics analysis. Mass spectrogram of palmitic (panel C, $m/z = 255.2363$, mass = 256.2370) and stearic acids (panel D, $m/z = 283.2375$, mass = 284.2382) for the major peak(s) in panels A and B.
Table 2.4.: Other fatty acids were identified in zebrafish embryos exposed from 2–24 hpf to 0–3% ethanol. Metabolites were within 3 ppm (non-polar) or 10 ppm (polar) of the theoretical m/z.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>0.001</td>
</tr>
<tr>
<td>2-Decene-4,6,8-triyln-1-al†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>LMFA06000063</td>
<td>-2.21544</td>
</tr>
<tr>
<td>2-Hexanamidoacetic acid†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>HMDB00701</td>
<td>-5.42447</td>
</tr>
<tr>
<td>(E)9-Oxodec-2-enedioic†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>CID1713086</td>
<td>-1.60990</td>
</tr>
<tr>
<td>2(E)-Decenoyl-ACP†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>LMFA07060012</td>
<td>2.066991</td>
</tr>
<tr>
<td>(4Z)-Decenediolic acid†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>HMDB000603</td>
<td>2.801531</td>
</tr>
<tr>
<td>2-Octanamidoacetic acid†&lt;sup&gt;q&lt;/sup&gt;</td>
<td>HMDB000832</td>
<td>2.850303</td>
</tr>
<tr>
<td>Tetradecanoic acid†&lt;sup&gt;n&lt;/sup&gt;</td>
<td>C06424</td>
<td>2.285625</td>
</tr>
<tr>
<td>3(R)-Hydroxy- dodecanoyl-ACP†&lt;sup&gt;p&lt;/sup&gt;</td>
<td>LMFA07060015</td>
<td>6.164180</td>
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</table>

*continued on next page*
Table 2.4.: continued

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>ID</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ppm</td>
<td>0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>Pentadecanoic acid†(^n)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C16537</td>
<td>1.808239</td>
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<td>0.71</td>
</tr>
<tr>
<td>(9Z)-Hexadecenoic acid†(^n)</td>
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<td></td>
<td></td>
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<tr>
<td>C08362</td>
<td>1.631235</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Methyl hexadecanoate†(^n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16995</td>
<td>0.980922</td>
<td>0.69</td>
<td>0.49</td>
</tr>
<tr>
<td>(12S)-Hydroxy-16-heptadecynoic acid†(^p)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LMFA01050146</td>
<td>5.017726</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>2-Oxooctadecanoic acid†(^n)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C00869</td>
<td>0.335288</td>
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<tr>
<td>MG(18:0/0:0/0:0)†(^n)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>HMDB111131</td>
<td>0.913459</td>
<td>-0.95</td>
<td>-0.96</td>
</tr>
<tr>
<td>(2S,3S,4S,5R,6R)-6-(hexadecyloxy)-3,4,5-trihydroxyoxane-2-carboxylic acid†(^n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMDB10331</td>
<td>-0.099930</td>
<td>-0.64</td>
<td>-1.41</td>
</tr>
</tbody>
</table>

continued on next page
Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–3%. Metabolites were identified from non-polar (n) or the polar (p) extraction fractions using LC-MS/MS (Orbitrap) or LC-MS (TOF), respectively. The average retention time±SD is listed along with the matching m/z database identifier (HMDB: Human Metabolome; C: KEGG; LMFA: LipidMaps), followed by the ppm difference (ppm) between the theoretical mass and the observed mass. Folds, relative to 0% controls, are listed for each ethanol concentration. ACP: Acyl Carrier Protein; MG: Monoacylglyceride. †: $P < 0.001$, ANOVA.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16-Feruloyloxy-hexadecanoic acid†p C18217</td>
<td>ppm</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.921450</td>
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</table>

Table 2.4: continued
Glycerophospholipids

**Phosphatidic Acid** Several glycerophospholipids were significantly different with ethanol exposure. These included phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylglucose (PG), and glycerophosphoglucone (GP). Except as noted, PAs were observed in the non-polar fractions (Table ??). The PA 14:1/22:2 was significantly lower, by 6.50-fold, in all concentrations of ethanol. The moiety 16:0/18:1 eluted at several retention times, and was always significantly different. The direction of change, however, varied with retention time. The less saturated 16:0/18:2 decreased nearly 6.50-fold, only at the lowest treatment concentration. However, 18:0/18:1 was significantly higher at moderate to high ethanol concentrations, including 0.03, 0.3, and 1%. The less saturated 18:0/18:2 also had multiple retention times, all of which changed significantly. Palmitoyl 3-carbacyclic PA was 6.36-fold lower in all ethanol treatment groups greater than 0.003%. In the polar fraction, PA(13:0/0:0) was elevated in ethanol concentrations of 0.03% and greater. It was 5.94-fold greater than control at 0.1% ethanol, but dropped to 4.65-fold in 1% ethanol. Pyrophosphatidic acid (PPA) 16:0/18:1 was higher by at least 5.75-fold greater than controls in all ethanol treatments at 0.03% ethanol and higher.
Table 2.5.: Many phosphatidic acids significantly changed in ethanol-exposed embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA(13:0/0:0)(^p)</td>
<td>-0.944605</td>
<td>0.00 5.65 5.94 5.43 4.65 n/a</td>
</tr>
<tr>
<td>LMGP10050001</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>PA(14:1/22:2)(^n)</td>
<td>-0.864438</td>
<td>-6.50 -6.50 -6.50 -6.50 -6.50 -6.50 -6.50 -6.50</td>
</tr>
<tr>
<td>LMGP10010134</td>
<td>-6.50</td>
<td>-6.50 -6.50 -6.50 -6.50 -6.50 -6.50 -6.50 -6.50</td>
</tr>
<tr>
<td>PA(16:0/18:1)(^n)</td>
<td>-0.680664</td>
<td>0.00 6.21 0.00 0.00 0.00 0.00 0.00 0.00</td>
</tr>
<tr>
<td>M40905</td>
<td>-0.51 -0.45 -6.66 -0.15 -0.16 -0.17 0.25 0.17</td>
<td></td>
</tr>
<tr>
<td>32.84 (\pm) 0.11</td>
<td>-0.587705</td>
<td>0.00 0.00 0.00 6.51 6.50 6.49 6.91 6.83</td>
</tr>
<tr>
<td>35.30 (\pm) 0.17</td>
<td>0.034396</td>
<td>0.00 6.07 0.00 0.00 0.00 0.00 0.00 0.00</td>
</tr>
<tr>
<td>36.51 (\pm) 0.10</td>
<td>0.00 0.00 0.00 6.00 0.00 5.29 5.24 0.00</td>
<td></td>
</tr>
<tr>
<td>PA(16:0/18:2)(^n)</td>
<td>0.000446</td>
<td>-6.58 -0.24 -0.84 -0.20 -0.43 -0.74 -0.18 -0.49</td>
</tr>
<tr>
<td>HMDB07860</td>
<td>0.000446</td>
<td>-6.58 -0.24 -0.84 -0.20 -0.43 -0.74 -0.18 -0.49</td>
</tr>
<tr>
<td>PA(18:0/18:1)(^n)</td>
<td>-0.191027</td>
<td>0.00 0.00 0.00 6.00 0.00 5.29 5.24 0.00</td>
</tr>
<tr>
<td>M40933</td>
<td>-1.14075</td>
<td>-6.01 0.06 -6.01 0.56 0.10 0.09 0.75 0.29</td>
</tr>
<tr>
<td>PA(18:0/18:2)(^n)</td>
<td>-0.880651</td>
<td>0.00 6.07 0.00 0.00 0.00 0.00 0.00 0.00</td>
</tr>
<tr>
<td>HMDB07861</td>
<td>-0.880651</td>
<td>0.00 6.07 0.00 0.00 0.00 0.00 0.00 0.00</td>
</tr>
<tr>
<td>30.74 (\pm) 0.12</td>
<td>-1.14075</td>
<td>-6.01 0.06 -6.01 0.56 0.10 0.09 0.75 0.29</td>
</tr>
<tr>
<td>33.68 (\pm) 0.13</td>
<td>-1.14075</td>
<td>-6.01 0.06 -6.01 0.56 0.10 0.09 0.75 0.29</td>
</tr>
</tbody>
</table>

*continued on next page*
Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0 to 1 or 3%. Metabolites were identified from the database as indicated by the ID below the name based on the matching the m/z (HMDB: Human Metabolome; LMGP: LipidMaps; M: Metlin). Next listed is sparkline as well as the parts per million difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. PA: Phosphatidic acid; PPA: Pyrophosphatidic Acid. 

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID</strong></td>
<td>ppm</td>
<td>0.001</td>
</tr>
<tr>
<td>Palmitoyl 3-carba-cyclic PA&lt;sup&gt;n&lt;/sup&gt;</td>
<td>-0.200855</td>
<td>0.00</td>
</tr>
<tr>
<td><em>M</em>44867</td>
<td>0.08866</td>
<td>-0.69</td>
</tr>
<tr>
<td>PPA(16:0/18:1)&lt;sup&gt;p&lt;/sup&gt;</td>
<td>1.028955</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<sup>n</sup>: Non-polar Fraction Metabolite; <sup>p</sup>: Polar Fraction Metabolite. All metabolites were significantly changed, †: $P < 0.001$, ANOVA.
**Phosphatidylserines**  Many PS were present in both the polar and non-polar fractions, reported here in order of length and saturation (Table ??). PS in the form of 15:0/22:0 was decreased (6.31-fold) at all but the 0.003 and 0.01% ethanol concentrations. In the polar fraction, O-16:0/14:0 in the polar fraction was increased only in the 0.1% ethanol treatment group. The metabolite O-16:0/17:1 was identified in the polar fraction and was approximately 5.8-fold control in the 0.1 and 0.3% treatment groups. The PS 16:0/18:0, also in the polar fraction, was 5.13- and 5.93-fold higher than controls in the 0.1 and 0.3% treatment groups. The non-polar 18:0/20:4 was lower than controls by 6.37 for all concentrations tested. Less saturated 18:1/22:4 changed little throughout the ethanol concentrations tested, while 18:2/22:2 was 6.25-fold lower than control at all but 0.001% ethanol. However, the longer, unsaturated 19:1/22:4, 20:0/22:4, and 20:2/22:4 were all dramatically lower approximately 6-fold in all but the lowest ethanol concentration groups. In all ethanol treatments, O-20:0/20:3) from the polar fraction was elevated at a minimum of 5.44-fold control. Conversely, 20:1/22:4 did not follow this trend, decreasing to -6.56-fold for only the 0.001% treatment group. Lastly, the longer, fully saturated 22:0/22:0 was present in the polar fractions of all ethanol treatment groups as high as 6.18-fold control.
Table 2.6.: Many phosphatidyserines were significantly changed in ethanol-exposed embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>15:0/22:0†n</td>
<td>LMGP03010156</td>
<td>1.357278</td>
</tr>
<tr>
<td>O-16:0/14:0†p</td>
<td>LMGP03020003</td>
<td>-4.166436</td>
</tr>
<tr>
<td>O-16:0/17:1†p</td>
<td>LMGP03020008</td>
<td>2.747279</td>
</tr>
<tr>
<td>16:0/18:0†p</td>
<td>HMDB12356</td>
<td>6.295313</td>
</tr>
<tr>
<td>18:1/22:4†n</td>
<td>LMGP03010339</td>
<td>-0.439376</td>
</tr>
<tr>
<td>18:2/22:2†n</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*continued on next page*
Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0 to 1 or 3%. Metabolites were identified from non-polar (\(n\)) extraction fractions using LC-MS/MS (Orbitrap) or the polar (\(p\)) fraction using LC-MS (TOF). The database ID below indicating the reference matching the m/z (HMDB: Human Metabolome; LMGP: LipidMaps) is listed, followed by the parts per million difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. \(\dagger: P < 0.001, ANOVA\).
Other Phospholipids

**Phosphatidylcholine**  In addition to the PA, both non- and polar metabolomes contained several glycerophospholipids. The only PE identified was \( P-16:0/0:0 \), which did not change more than 0.10-fold from the control group at any tested concentration (Table 2.3.3). Other identified phospholipids included PC, GP, PI, PS.
Table 2.7: Many glycerophospholipids significantly changed in ethanol-exposed embryos. Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–3%. Metabolites were identified from non-polar (\(n\)), using LC-MS/MS (Orbitrap), or polar (\(p\)), using LC-MS (TOF), extraction fractions, with the database ID below indicating the reference matching the m/z (HMDB: Human Metabolome; C: KEGG; LMGP: LipidMaps; M: Metlin). Next listed is the parts per million difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested, and are expressed relative to 0% controls. PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: Phosphatidylglucose; PI: phosphatidylinositol. \(\dagger\): \(P < 0.001\), ANOVA.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>PC(15:1/22:4)+p(\dagger)</td>
<td>-9.411992</td>
<td>n/a</td>
</tr>
<tr>
<td>LMGP01011460</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(P-16:0/20:5)+p(\dagger)</td>
<td>-8.396944</td>
<td>n/a</td>
</tr>
<tr>
<td>LMGP01030040</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(18:4/P-18:1)+p(\dagger)</td>
<td>0.903672</td>
<td>n/a</td>
</tr>
<tr>
<td>M59706</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LysoPC(10:0)+p(\dagger)</td>
<td>-10.42851764</td>
<td>n/a</td>
</tr>
<tr>
<td>HMDB03752</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE(P-16:0/0:0)+p(\dagger)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(continued on next page\)
<table>
<thead>
<tr>
<th>Metabolite ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID</strong></td>
</tr>
<tr>
<td><strong>ppm</strong></td>
</tr>
<tr>
<td><strong>Fold (Relative to 0% Ethanol)</strong></td>
</tr>
<tr>
<td>0.001</td>
</tr>
</tbody>
</table>
| 2-(α-D-Mannosyl)-3-PG\(^p\)
| C11516       | -0.089643 | 0.04 | 0.02 | 0.03 | 0.07 | 0.09 | 0.10 | 0.07 | 0.09 |
| PG(14:1/0:0)\(^p\)
| LMGP040500035 | 1.50756 | n/a | n/a | 4.81 | 6.24 | 6.28 | 5.59 | 6.00 | n/a |
| PG(16:0/16:1)\(^p\)
| HMDB10571    | 1.379908 | n/a | n/a | 4.83 | 5.59 | 0.00 | 0.00 | 0.00 | n/a |
| PG(0-18:0/22:6)\(^n\)
| LMGP04020083 | 4.281506 | n/a | n/a | 5.87 | 5.82 | 6.53 | 6.53 | 0.00 | n/a |
| PI(16:1/22:2)\(^n\)
| LMGP06010192 | 2.764662 | 0.00 | 6.35 | 5.75 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| PI(18:0/20:4)\(^n\)
| HMDB09815    | -2.181116 | -6.03 | -6.03 | -6.03 | -6.03 | -6.03 | -6.03 | -6.03 | -6.03 |
| PI(22:0/12:0)\(^n\)
| LMGP06010819 | -0.393944 | -0.97 | 0.22 | -0.65 | 0.15 | -1.03 | -0.63 | 0.15 | -0.39 |
| | | 0.00 | 0.00 | 6.20 | 5.48 | 6.18 | 6.19 | 5.44 | 0.00 | 0.00 |
PCs were identified in the polar fractions (Table 2.3.3). The glycerophosphocholine lysoPC(10:0) was elevated in the 0.1 and 1% treatment groups to 5.38- and 5.74-fold controls. Similarly, 15:1/22:4 was elevated in the 0.03, 0.1, 0.3, and 1% ethanol by 6.24-, 6.25-, 6.07-, and 5.23-fold higher than controls. The PC $P_{-16:0/20:5}$ in the polar fraction was significantly higher than controls when treated with ethanol at 0.03 to 0.3% ethanol. It was maximally elevated to 6.08-fold higher at 0.03% ethanol and fell to 4.96-fold at 0.3% ethanol. Lastly, 18:4/$P_{-18:1}$, also in the polar fraction, was inconsistently increased relative to the control treatments. It was 5.41-fold higher at 0.01%, 5.92-fold at 0.1% ethanol, and 6.39-fold at 0.3% ethanol. It was not different from control at 0.03 and 1% ethanol.

**Glycerophosphoglucose** Several GP moieties were present in the metabolome (Table 2.3.3). In the non-polar fraction, $O_{-18:0/22:6}$ was different from controls at only 0.003 and 0.01%. These were 6.35-fold and 5.75-fold, respectively, however were not significantly different. The remaining GPs identified were in the polar fraction. PG(14:1/0:0) was elevated for only the lowest two ethanol concentrations tested. At 0.01% the it was at 4.83-fold and at 0.03% ethanol, it was 5.59-fold elevated over controls. At all ethanol treatment concentrations except the highest 1%, PG(16:0/16:1) was about 6-fold control levels. At 0.1 and 0.3% ethanol, it was 6.53-fold higher than controls. However, at 1% ethanol, it was not different than controls. 2-(α-D-mannosyl)-3-phosphoglycerate was elevated in all ethanol treatments. It was maximally 6.53-fold higher than controls in the 0.1% ethanol treatment group.

**Phosphatidylinositol** PI, an important non-polar fraction secondary signaling molecule, was present in the non-polar fraction (Table 2.3.3). It increased in all ethanol concentrations greater than 0.01% for 22:0/12:0, but the shorter, less-saturated 16:1/22:2 was lower by 6.03-fold in all treatment groups except 0.003% ethanol. The PI $18:0/20:4$ varied little amongst the various ethanol treatment groups.
Sphingolipids

Sphingolipids were present in both the non-polar and polar extraction fractions. In general, ethanol exposure tended to increase sphingolipids in the polar fractions, but not in the non-polar fractions (Table 2.3.3). Ceramides, present in the non-polar fractions, had an increasing trend at ethanol concentrations of 0.03% ethanol and higher. Sulfatides in the non-polar fraction were found at lower levels than controls when treated with ethanol.
Table 2.8.: Many sphingolipids were also significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th>0.001</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
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<tbody>
<tr>
<td>C16 Sphinganine&lt;sup&gt;p&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>n/a</td>
<td>n/a</td>
<td>0.00</td>
<td>5.87</td>
<td>6.34</td>
<td>5.78</td>
<td>5.58</td>
<td>n/a</td>
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<td>-7.685164</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>C17 Sphinganine&lt;sup&gt;n&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.04</td>
<td>-0.73</td>
<td>0.47</td>
<td>-0.75</td>
<td>0.48</td>
<td>-0.05</td>
<td>-0.04</td>
<td>0.41</td>
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<td></td>
</tr>
<tr>
<td>C17 Sphinganine&lt;sup&gt;p&lt;/sup&gt;</td>
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<td></td>
<td>n/a</td>
<td>n/a</td>
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<td>6.68</td>
<td>7.02</td>
<td>6.82</td>
<td>6.80</td>
<td>n/a</td>
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<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>N-Acetyl-sphingosine 1-phosphate&lt;sup&gt;p&lt;/sup&gt;</td>
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<td>n/a</td>
<td>n/a</td>
<td>0.00</td>
<td>5.92</td>
<td>5.77</td>
<td>5.54</td>
<td>0.00</td>
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<tr>
<td>M53980</td>
<td>8.742596</td>
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<tr>
<td>(4E,8E,10E-d18:3)-Sphingosine†&lt;sup&gt;n&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.766805</td>
<td>5.44</td>
<td>5.86</td>
<td>6.72</td>
<td>6.74</td>
<td>6.96</td>
<td>6.50</td>
<td>5.91</td>
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<td>LMSP01080013</td>
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<td></td>
</tr>
<tr>
<td>Ceramide (d18:1/24:1)†&lt;sup&gt;n&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>0.388807</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>5.47</td>
<td>6.15</td>
</tr>
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<td></td>
</tr>
</tbody>
</table>

*continued on next page*
Table 2.8.: continued

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th>0.001</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lignoceric ceramide†</td>
<td>-0.349097</td>
<td>-1.23</td>
<td>-0.23</td>
<td>-0.75</td>
<td>-0.41</td>
<td>-1.12</td>
<td>-1.15</td>
<td>-0.74</td>
<td>-0.46</td>
<td></td>
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<tr>
<td>HMDB00831</td>
<td>0.16717</td>
<td>0.00</td>
<td>0.00</td>
<td>6.10</td>
<td>5.42</td>
<td>5.86</td>
<td>6.39</td>
<td>5.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ceramide (d18:1/25:0)†</td>
<td>0.005274</td>
<td>0.00</td>
<td>0.00</td>
<td>5.68</td>
<td>0.00</td>
<td>5.77</td>
<td>6.29</td>
<td>5.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20 Sulfatide†</td>
<td>-1.564932</td>
<td>-6.25</td>
<td>-6.25</td>
<td>-6.25</td>
<td>-0.57</td>
<td>-6.25</td>
<td>-0.48</td>
<td>0.04</td>
<td>-1.02</td>
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</tr>
<tr>
<td>LMSP06020010</td>
<td>-0.157014</td>
<td>-6.25</td>
<td>-6.25</td>
<td>-6.25</td>
<td>0.31</td>
<td>-0.15</td>
<td>-0.17</td>
<td>0.49</td>
<td>-0.22</td>
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</tr>
<tr>
<td>C22 Sulfatide</td>
<td>-1.699464</td>
<td>-6.46</td>
<td>0.01</td>
<td>-0.40</td>
<td>0.32</td>
<td>0.07</td>
<td>0.05</td>
<td>0.47</td>
<td>0.20</td>
<td></td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–3%. Metabolites were identified using LC-MS/MS (Orbitrap) for non-polar (n) or LC-MS (TOF) for polar (p). ID indicates the reference database entry used to identify the metabolite (HMDB: Human Metabolome; C: KEGG; M: Metlin). The ppm is the difference between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. †: $P < 0.001$, ANOVA; PI-cer: Inositol-1-phospho-ceramide.
C16 sphinganine was present in the polar fraction and elevated with ethanol treatments higher than 0.01%. It was in its highest concentration in the 0.1% treatment group at 6.34-fold above controls. C17 sphinganine was also present in the polar fraction. In all ethanol treatments of 0.03% and higher, this metabolite was approximately 7-fold that of controls. However, C17 sphinganine, from the non-polar fraction, did not change more than 0.75-fold in either direction for all treatments. Non-polar fraction sphingosine (d18:3) was increased by as much as 6.96-fold over the concentrations tested.

N-Acetyl-sphingosine-1-phosphate increased at 0.03–0.3% ethanol, maximally 5.92-fold at the low end of that range. Lignoceric ceramide increased up to 6.10-fold relative to controls in all treatment groups from 0.03% and higher. Ceramide (d18:1/24:1) was significantly higher at treatment concentrations of 0.3% and higher. Ceramide (d18:1/25:0), by comparison, increased at the 0.03% and at or above 0.3% ethanol, yet was not elevated at 0.1%. Inositol-1-phosphate-ceramide (PI-cer) (d18:1/22:0), a metabolite analogous to PI fluctuated, lowering to 6.46-fold below controls at the lowest ethanol concentrations, then returning to near control levels across all concentrations. C20 Sulfatide differed little from controls. But, C22 Sulfatide was 6.25-fold lower than controls at concentrations of 0.001, 0.01, and 0.1% ethanol. C20 sulfatide, in contrast, was lower than the control by 1.23-fold at most.

**Sterol Metabolites**

**Hormones** Many sterol metabolites, including hormones, were identified in our analysis (Table 2.9). These included hormones, such as estrogens, androgens, and prostaglandins, as well as vitamins and other sterol lipids. Several different estrogens were significantly changed in ethanol-exposed embryos. 1α-Hydroxyesterone was not different in the 1% ethanol treatment, but was increased in all lower ethanol treatments tested, with the highest at 5.68-fold increased in the 0.03% ethanol treatment. The steroid 16-dehydroprogesterone was increased as much as 6.16-fold that of controls in all ethanol treatment groups except 0.3%. 13-Ethyl-16,17-dihydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one was maximally increased in the 0.03% ethanol treatment only to 1.17-fold of control. In all other treatments, it was decreased to –4.54-fold of control. In addition to estrogens, an androgen, 13-hydroxy-3-oxo-13,17-secoandrost-4-en-17-oic acid, was sig-
nificantly different in the ethanol-exposed embryos, at 0.01 and 0.03% ethanol doses. 13-Hydroxy-3-oxo-13,17-secoandrostan-4-en-17-oic acid was not different from control at 0.1% ethanol and higher but was increased in the lowest two ethanol treatments of 0.01 and 0.03% ethanol to 5.42- and 4.84-fold higher than controls.
Table 2.9.
Many hormones, including several estrogens and an androgen, significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold Change (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α-Hydroxyestrone</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>-0.64545</td>
<td>4.55 5.68 5.18 5.27 0.00</td>
</tr>
<tr>
<td>16-Dehydroprogesterone</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>-2.836562</td>
<td>5.20 6.16 5.94 0.00 5.75</td>
</tr>
<tr>
<td>13-Ethyl-16,17-dihydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>5.903102</td>
<td>-4.54 1.17 -4.54 -4.54 -4.54</td>
</tr>
<tr>
<td>13-Hydroxy-3-oxo-13,17-seco-androst-4-en-17-oic acid</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>4.739243</td>
<td>5.42 4.84 0.00 0.0 0.00</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–1%. Metabolites were identified from the polar fraction using LC-MS (TOF). Database ID indicates the reference database used to identify the molecule using the m/z (HMDB: Human Metabolome; LMST: LipidMaps; M: Metlin), followed by the structure. Next listed is the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. All metabolites were significantly changed, $P < 0.001$, ANOVA.
A variety of icosanoids were significantly different in embryos exposed to ethanol. Anandamide was significantly lower (approximately 6.5-fold) at 0.01% ethanol or greater. The 6-keto-prostaglandin F$_{1\alpha}$-d$_4$ was not different from control in the 0.01% ethanol treatment group, but was elevated in all higher ethanol treatments tested. This metabolite is likely a misnomer, as we did not use deuterated compounds in our study, and further clarification is needed. The maximum level was 5.68-fold higher than controls. The leukotriene 12-oxo-10,11-dihydro-20-COOH-LTB$_4$ was increased in only the 0.01 and 0.03% ethanol treatment groups to 5.52- and 5.29-fold, respectively. Similarly, prostaglandin G$_1$ was increased to 5.55- and 4.93-fold higher than control at these concentrations. 9-Oxo-11α,(16R)-dihydroxy-17-cyclobutyl-5(Z),13(E)-dien-1-oic acid was somewhat elevated from controls in the 0.01 and 1% ethanol treatment groups, but decreased to –4.69-fold control levels in the intermediate ethanol treatments. 17-Phenyl trinor-13,14-dihydro prostaglandin A$_2$ was elevated in the lowest three ethanol treatments to 5.64-fold control levels, at most. At the two highest ethanol treatments, it was not different than control. Lastly, 17-phenyl trinor prostaglandinE$_2$ serinol amide was increased in the lowest ethanol treatments to 5.74- and 4.95-fold for the 0.01 and 0.03% ethanol treatment groups.
Table 2.10.: Many icosanoids significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ID</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolite ID</td>
<td>ppm</td>
<td>0.001</td>
</tr>
</tbody>
</table>
| Anandamide (20:1, n-9)
LMFA08040010 | 0.502930 | -0.01 | -1.03 | -6.53 | -6.53 | -6.53 | -6.53 | -6.53 | -6.53 |
| 12-Oxo-10,11-dihydro-20-COOH-LTB4
M45059 | 0.017299 | n/a | n/a | 5.52 | 5.29 | 0.00 | 0.00 | 0.00 | n/a |
| Prostaglandin G1
HMDB13039 | 1.253418 | n/a | n/a | 5.55 | 4.93 | 0.00 | 0.00 | 0.00 | n/a |
| 17-Phenyl trinor-13,14-dihydro prostaglandin A2
M45409 | 4.124907 | n/a | n/a | 5.48 | 5.64 | 4.70 | 0.00 | 0.00 | n/a |
| 6-Keto-prostaglandin F1α-d4
LMFA03010037 | 2.076897 | n/a | n/a | 0.00 | 5.59 | 5.41 | 5.49 | 5.68 | n/a |

continued on next page
Table 2.10.: continued

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ID</strong></td>
<td>ppm</td>
<td>0.001</td>
</tr>
<tr>
<td>9-Oxo-11α,16R-dihydroxy-17-cyclobutyl-(5Z,13E)-dien-1-oic acid&lt;sup&gt;p&lt;/sup&gt;</td>
<td>-8.485524</td>
<td>n/a</td>
</tr>
<tr>
<td>17-Phenyl trinor prostaglandin E&lt;sub&gt;2&lt;/sub&gt;serinol amide&lt;sup&gt;p&lt;/sup&gt;</td>
<td>7.974096</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–3%. Icosanoids were identified from the non-polar ("n") fraction using LC-MS/MS (Orbitrap) or the polar ("p") fraction using LC-MS (TOF). ID indicates the reference database used to identify the molecule using the m/z (C: KEGG; HMDB: Human Metabolome Database; LMFA: LipidMaps; M: Metlin; SID: PubChem). Next listed is the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. All metabolites were significantly changed, $P < 0.001$, ANOVA.
**Vitamins** A variety of vitamin-related metabolites were identified in our metabolome, often significantly changing with ethanol exposure, including A, D, E, and K. Three vitamin A metabolites were significantly different, including 9,13-\textit{cis}-retinoate, 1′-hydroxy-\textgamma-carotene glucoside, and 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Table 2.11). 9,13-\textit{cis}-Retinoate minimally altered from controls in the 0.03, 0.3, and 1% ethanol treatments. For the 0.01 and 0.1% treatments it dropped to −4.67-fold controls. 1′-Hydroxy-\textgamma-carotene glucoside at all ethanol treatment concentrations was increased, to as much as 6.34-fold higher than controls. 1−(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, an isoprenoid, eluted at four distinct times (17.19, 32.25, 33.32, and 34.49 minutes). At the early elution time, there was little difference from the control as a result of the ethanol exposure. For the later elution time, 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one was significantly lowered at all concentrations above 0.01%.
Table 2.11.
Several vitamin A related metabolites were significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th>ppm</th>
<th>0.001</th>
<th>0.003</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>9,13-cis-Retinoate†</td>
<td><img src="image1" alt="Structure" /></td>
<td>n/a</td>
<td>n/a</td>
<td>-4.67</td>
<td>0.41</td>
<td>4.67</td>
<td>1.21</td>
<td>0.67</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMDB12874</td>
<td></td>
<td>-9.837865</td>
<td>n/a</td>
<td>n/a</td>
<td>6.05</td>
<td>6.32</td>
<td>6.34</td>
<td>4.97</td>
<td>5.65</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1'-Hydroxy-γ-carotene†</td>
<td><img src="image2" alt="Structure" /></td>
<td>0.990089</td>
<td>n/a</td>
<td>n/a</td>
<td>6.05</td>
<td>6.32</td>
<td>6.34</td>
<td>4.97</td>
<td>5.65</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15861</td>
<td></td>
<td><img src="image3" alt="Structure" /></td>
<td>n/a</td>
<td>n/a</td>
<td>6.05</td>
<td>6.32</td>
<td>6.34</td>
<td>4.97</td>
<td>5.65</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one</td>
<td><img src="image4" alt="Structure" /></td>
<td>17.19 ± 0.12†</td>
<td>2.932932</td>
<td>0.02</td>
<td>0.02</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.01</td>
<td>-0.04</td>
<td>-0.03</td>
<td>-0.07</td>
<td></td>
</tr>
<tr>
<td>HMDB35245</td>
<td></td>
<td>32.25 ± 0.29†</td>
<td>3.092996</td>
<td>-0.71</td>
<td>0.11</td>
<td>0.40</td>
<td>-0.18</td>
<td>-6.20</td>
<td>0.34</td>
<td>0.33</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>33.32 ± 0.14†</td>
<td></td>
<td>34.49 ± 0.07†</td>
<td>2.559449</td>
<td>0.16</td>
<td>-0.24</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td>-6.89</td>
<td></td>
</tr>
<tr>
<td>17.19 ± 0.12†</td>
<td></td>
<td>1.793080</td>
<td>0.00</td>
<td>6.16</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–1%. Metabolites were identified from non-polar ("n") using LC-MS/MS (Orbitrap) and polar ("p") fractions using LC-MS (TOF). ID indicates the reference database used to identify the molecule using the m/z (HMDB: Human Metabolome; C: KEGG). Next listed is the structure and ppm difference (ppm) between the theoretical mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. †: $P < 0.001$, ANOVA.
Four forms of vitamin D were identified (Table 2.3.3). 1α-Hydroxy-24,25,26,27-tetranorvitamin D$_3$ 23-carboxylic acid, calcitroic acid, was not different than controls in 0.01, 0.03, and 0.3% ethanol treatments. It was elevated to 5.61- and 5.27-fold control levels in the 0.1 and 1% treatment groups. This contrasts with 1α-hydroxy-25,26,27-trinorvitamin D$_3$ 24-carboxylic acid, which, while significantly higher than controls with ethanol treatment, was only 1.13-fold at most. 1α-Hydroxy-22-(3-methyl-phenyl)-23,24,25,26,27-Pentanorvitamin D$_3$ increased with all ethanol treatments, peaking to 6.28-fold higher than control in the 0.3% ethanol group. 1α,25-Dihydroxy-26,27-dimethyl-20,21-didehydro-23-oxavitamin D$_3$ increased to 6.69-fold higher than controls with ethanol treatments. At 0.01 and 1% ethanol it was 5.71- and 5.58-fold higher than controls, respectively.
Table 2.12.: Many vitamin D metabolites were significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1α-Hydroxy-24,25,26,27-tetranor-vitamin D₃ 23-carboxylic acid</td>
<td><img src="image1" alt="Structure" /></td>
<td>-7.136755</td>
<td>0.00</td>
</tr>
<tr>
<td>1α-Hydroxy-25,26,27-trinor-vitamin D₃ 24-carboxylic acid</td>
<td><img src="image2" alt="Structure" /></td>
<td>-2.68685</td>
<td>1.05</td>
</tr>
</tbody>
</table>

*continued on next page*
Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–1%. Metabolites were identified from polar fraction using LC-MS (TOF). ID indicates the reference database used to identify the molecule using the m/z (LMST: LipidMaps). Next listed is the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. All metabolites were significantly changed. $P < 0.001$, ANOVA.
In addition, two vitamin E metabolites were observed (Table 2.13). The metabolite 7′-carboxy-α-tocotrienol did not differ from controls in the 0.01 and 1% ethanol treatments, but was increased by as much as 5.73-fold higher than controls in the intermediate ethanol concentrations. Contrastingly, 9′-carboxy-α-tocotrienol was only higher than controls in the 0.1 and 1% ethanol-treated embryos and ~4.43 fold-lower than controls in the other tested concentrations. Vitamin K₃ was irregularly increased with ethanol treatment. At the 0.1% ethanol levels, embryos had 5.14- and 5.86-fold higher than controls. At all other ethanol treatments, it was not different than controls. Dissimilarly, sulfated dihydromenaquinone-9 was significantly decreased at all ethanol doses. Lastly, another non-vitamin antioxidant was identified as significantly different. Ethanol treatment, at any of the tested doses, increased N,N′-diphenyl-p-phenylenediamine. It was 5.79-fold higher with 0.01% and 6.19-fold higher with more concentrated treatments.
Table 2.13.

Several other vitamins were significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Vit.</th>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>7'-Carboxy-α-tocotrienol&lt;sup&gt;p&lt;/sup&gt;</td>
<td>HMDB12849</td>
<td>n/a n/a 0.00 5.73 4.81 4.97 0.00 n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HMDB12867</td>
<td>0.37665</td>
<td>-4.43 -4.43 1.19 -4.43 0.45 n/a</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>Vitamin K&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;p&lt;/sup&gt;</td>
<td>HMDB01892</td>
<td>n/a n/a 0.00 0.00 5.14 0.00 5.86 n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sulfated Dihydromenaquinone-9&lt;sup&gt;n&lt;/sup&gt;</td>
<td>LMPR02010038</td>
<td>-2.56837 -0.05 -0.18 -0.24 -0.72 -0.45 -1.0 -0.43 -0.74</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>N,N'-Diphenyl-p-phenylenediamine&lt;sup&gt;p&lt;/sup&gt;</td>
<td>C14501</td>
<td>7.923689 n/a 5.79 6.19 6.17 6.19 6.01 n/a</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–1%. Metabolites were identified using LC-MS/MS (Orbitrap) for non-polar ("n") and LC-MS (TOF) for polar ("p") fractions. Database ID indicates the reference database used to identify the molecule using the m/z (HMDB: Human Metabolome; LMPR: LipidMapsC: KEGG). Next listed is the structure and ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. All metabolites were significantly changed. 

*P < 0.001, ANOVA.*
Other Steroid Lipids  Several other steroid lipids were identified as significantly different in our metabolome. These include 3α,7α,12α-trihydroxy-5α-cholan-24-yl sulfate, cortexolone, 6-hydroxymethasone, 3α,7α-dihydroxy-12-oxo-5β-cholanate, 3α-hydroxy-6-oxo-5β-cholan-24-oic acid, diginatigenin, and (25S)-3-oxo-12β-acetoxy-cholest-1,4-dien-26-oic acid. 3α,7α,12α-Trihydroxy-5α-cholan-24-yl sulfate was increased to 5.46- and 4.42-fold in the 0.1 and 1% ethanol treatment, but was not different from controls in the other ethanol concentrations (Table 2.3.3). Cortexolone was not different in the 0.01–0.1% ethanol treatment groups, but increased to 5.82- and 4.62-fold in the 0.3 and 1% ethanol treatments, respectively. For 6-hydroxydexamethasone, the 0.01 and 0.03% treatments were elevated to 5.56- and 5.35-fold controls, but was similar to controls in all of the higher ethanol treatments. 3α,7α-Dihydroxy-12-oxo-5β-cholanate at all ethanol concentrations was increased, by as much as 6.20-fold control levels. 3α,7α-Dihydroxy-12-oxo-5β-cholanate increased with all ethanol doses, approximately 6-fold. 3α-Hydroxy-6-oxo-5β-cholan-24-oic acid eluted at three different times. At 2.04±0.06 it was elevated to 5.59-, 5.38-, and 5.96-fold higher than controls for 0.1, 0.3, and 1% ethanol treatments, respectively. At 10.70±0.22 it was only 1.21-fold higher than control at these concentrations, but was decreased by -5.32 in the 0.01 and 0.03% ethanol treatments. When eluting at 13.81±0.11 it was not different from controls at 0.03 and 0.3% ethanol, but was 4.99-, 6.42-, and 5.19-fold higher than controls at 0.01, 0.1, and 1% ethanol. Diginatigenin was minimally altered from controls in most ethanol treatment groups, and dropped with the 0.3% ethanol to -4.46-fold of controls. Finally, (25S)-3-oxo-12β-acetoxy-cholest-1,4-dien-26-oic acid increased to as much as 6.44-fold control in the 0.03–0.3% ethanol treatment groups, but was not different in the highest and lowest treatment groups.
Table 2.14.: Many steroids significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortexolone</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>9.481696</td>
<td>0.00 0.00 0.00 5.82 4.62</td>
</tr>
<tr>
<td>HMDB00015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3α-Hydroxy-6-oxo-5β-cholan-24-oic acid†</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>-4.116307</td>
<td>0.00 0.00 5.59 5.38 5.96</td>
</tr>
<tr>
<td>LMST04010146</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2.04±0.06</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>10.70±0.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13.81±0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3α,7α-Dihydroxy-12-oxo-5β-cholanate</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>-3.133862</td>
<td>5.92 6.11 6.15 6.20 5.95</td>
</tr>
<tr>
<td>HMDB00490</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*continued on next page*
Table 2.14: continued

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Hydroxydexamethasone M613</td>
<td><img src="image1" alt="Structure" /></td>
<td>3.640174</td>
<td>5.56 5.35 0.00 0.00 0.00</td>
</tr>
<tr>
<td>(14α,17β,20S,22R)-14,20-Epoxy-17-hydroxy-1-oxo-3,5,24-trienolide HMDB32685</td>
<td><img src="image2" alt="Structure" /></td>
<td>-8.017420372</td>
<td>5.05 5.78 5.55 5.86 5.81</td>
</tr>
<tr>
<td>3α,7α,12α-Trihydroxy-5α-cholan-24-yl sulfate M57971</td>
<td><img src="image3" alt="Structure" /></td>
<td>3.315656</td>
<td>0.00 0.00 5.46 0.00 4.42</td>
</tr>
</tbody>
</table>

continued on next page
Table 2.14: continued

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Structure</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(25S)-3-Oxo-12β-acetoxy-cholest-1,4-dien-26-oic acid</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>7.022646</td>
<td>0.00 5.45 6.44 6.25 0.00</td>
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<tr>
<td>LMST04030214</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>1.967824</td>
<td>1.06 1.19 0.95 -4.46 1.35</td>
</tr>
<tr>
<td>Diginatigenin monodigitoxoside</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>9.299595</td>
<td>6.76 6.43 6.64 6.86 6.49</td>
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<td>M57772</td>
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<td></td>
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<tr>
<td>Goyaglycoside c</td>
<td><img src="image4.png" alt="Structure" /></td>
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<td>HMDB32685</td>
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</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations of 0–1%. Metabolites were identified from the polar fraction using LC-MS (TOF). ID indicates the reference database used to identify the molecule using the m/z (HMDB: Human Metabolome; C: KEGG; LMST: LipidMaps; M:Metlin). Next listed is the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. All metabolites were significantly changed, $P < 0.001$, ANOVA.
2.3.4 Protein-related Metabolites

Proteins were another major class of metabolites identified in the ethanol-treated zebrafish embryos. Several metabolites are associated with amino acid metabolism, including some related to histidine, lysine, and tryptophan. Numerous oligopeptides were identified as well, though by nature of our method, it is difficult to determine the order of the component amino acids or their identity. Other protein-related metabolites included urea, creatinine, crotono-betaine, aspidinol, ifosfamide, N-acetyl-leucyl-leucyl-methioninal, and laserpitin.

Amino Acids

Histidine Metabolism  Several metabolites associated with amino acid metabolism were altered with ethanol treatment. Broadly, these included metabolites related to histidine, lysine, and tryptophan metabolism, amongst others. Three metabolites associated with histidine metabolism included \( N-\omega \)acetylhistamine, imidazol-5-yl-pyruvate, and thiourocanic acid (Table 2.15). \( N-\omega \)-Acetylhistamine was not different than controls with the 0.01% ethanol treatment, but was increased at all higher ethanol concentrations. It peaked at 6.24-fold higher than control in the 0.1% ethanol group. Imidazol-5-yl-pyruvate was not different than control at most ethanol concentrations. However, at 0.03 and 1% ethanol it was 5.75 and 5.02-fold higher. Lastly, thiourocanic acid was not different than control with low ethanol doses, but was 5.64-fold higher in the 0.1% treatment group. By the 1% ethanol dose, it decreased to 5.42-fold control levels.

Tryptophan Metabolism  Several tryptophan catabolism products were identified in our analysis. The metabolite oxindole eluted with two separate retention times (Table 2.16). At 18.64±0.23 minutes it was irregularly elevated. With 0.03 and 0.3% ethanol treatments it was not different than control. With 0.01, 0.1, and 1% ethanol treatments, it was increased by 4.64-, 6.01-, and 5.52-fold control. The later eluting oxindole (24.64±0.10 minutes) it was unchanged from control in the lower ethanol concentrations, but then peaked to 5.73-fold higher than controls in the 0.1% dose. For higher concentrations, it decreased slightly to 5.49-fold higher than controls at 1%. Kynurenic acid was different than controls only at the 0.03% ethanol treatment, where it was 5.70-fold higher. (Note: this metabolite appeared
Table 2.15.
Histidine-related metabolites were identified from the polar fraction within 10 ppm of their theoretical m/z in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>0.01</td>
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<tr>
<td>N-ω-Acetylhistamine</td>
<td>6.60068</td>
<td>0.00</td>
</tr>
<tr>
<td>Imidazol-5-yl-pyruvate</td>
<td>-3.74064</td>
<td>0.00</td>
</tr>
<tr>
<td>Thiourocanic acid</td>
<td>-1.26930</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Histidine-related metabolites were identified from polar extraction fractions by LC-MS (TOF). ID indicates the database reference for the m/z (C: KEGG; M: METLIN). Next, is the ppm difference (ppm) between the theoretical and observed masses. Folds are expressed relative to 0% controls. All metabolites listed were significantly changed from control, \( P < 0.001 \), ANOVA.

only in this ethanol concentration, and therefore was filtered out by our protocol during data analysis. It is included here due to its potential mechanistic-relevance, as discussed below.) Cotinine methonium ion was 4.63-, 5.72-, and 4.82-fold higher than controls in 0.01–0.1% ethanol treatment and was not different from controls at higher concentrations. 2,6-Dihydroxypteridine was nearly –5.67-fold lower than controls at both 0.1 and 3% ethanol concentrations. The metabolite 7-chlorokynurenene increased only with the 0.03% ethanol to 1.23-fold. At all other ethanol concentrations, it decreased to –4.40-fold lower than controls. Nicotine glucuronide was increased by at least 5.23-fold with all ethanol treatment concentrations except 0.3%, where it was not different than controls. Uncaric acid A was only different than control in the 1% ethanol treatment, where it was 5.66-fold higher. Other significant tryptophan metabolites included oxindole, kynurenic acid, and 7-chlorokynurenic acid. Mugineic acid increased to 1.14-fold higher than controls in 0.03% ethanol. With all other ethanol treatments, it dropped to –5.13-fold below that of controls.
Table 2.16.
Tryptophan-related metabolites were identified in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
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<th>Metabolite</th>
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<th>Fold (Relative to 0% Ethanol)</th>
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<tr>
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<tr>
<td>Oxindolep</td>
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<td>M34541</td>
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<td>Kynurenic acidp</td>
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<td>n/a</td>
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<td>HMDB00715</td>
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<td>Cotinine methonium ionp</td>
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<td>n/a</td>
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<tr>
<td>HMDB01365</td>
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<td></td>
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<tr>
<td>2,6-Dihydroxypseudo-oxy nicotinep</td>
<td>2.285098</td>
<td>0.62</td>
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<td>M63883</td>
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<tr>
<td>7-Chlorokynurenic acidp</td>
<td>-5.924567</td>
<td>n/a</td>
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<td>M44301</td>
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<td>Nicotine glucuronide</td>
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<td>n/a</td>
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<tr>
<td>HMDB01272</td>
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<tr>
<td>Uncaric acidp</td>
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<tr>
<td>C17874</td>
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<tr>
<td>Mugineic acidp</td>
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<tr>
<td>C15500</td>
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</tbody>
</table>

Tryptophan-related metabolites were identified from non-polar (\(^n\)) extraction fractions using LC-MS/MS (Orbitrap) or polar (\(^p\)) fractions using LC-MS (TOF). ID indicates the database reference for the m/z (HMDB: Human Metabolome; C: KEGG; LMFA: Lipidmaps; M: METLIN). Next, is the ppm difference (ppm) between the theoretical and observed masses. Folds are expressed relative to 0% controls. All metabolites listed were significantly changed from control, \( P < 0.001, \text{ANOVA} \).
Other Amino Acid-Related Metabolites  Several additional amino acid-related metabolites were identified, including 2-hydroxymethylserine, β-aminopropionitrile, N-(3-o xo-octanyl)-homoserine lactone, purine, 2,6 dimethylheptanoyl carnitine, and hydroxybutyrylcarnitine. 2-Hydroxymethylserine was identified in the polar fractions (Table 2.17). At 0.1 and 0.3% ethanol treatments it was 6.44- and 5.44-fold higher than controls. With all other ethanol treatments, ethanol-treated embryos were not different than controls. β-Aminopropionitrile was not different than controls with low ethanol doses. However, at 0.01% and higher, it was 4.90-fold or more that of control levels. N-(3-Oxo-octanoyl)-homoserine lactone was similarly only increased in the 0.1% and higher ethanol treatments and was 5.52-fold higher than controls at most. Purine increased in almost all ethanol treatments by at most 6.34-fold controls. The exception the 1% concentration, where it was not different than controls. Finally, two carnitine metabolites in the polar analysis included 2,6 dimethylheptanoyl carnitine and hydroxybutyrylcarnitine. The metabolite 2,6 dimethylheptanoyl carnitine increased at all ethanol treatments of 0.03% ethanol and higher. It was at a minimum of 5.05-fold higher than controls, peaking at 5.81- in the 0.1% treatment group. Hydroxybutyrylcarnitine eluted at two different retention times. At 18.62±0.13, it increased to 5.01- and 5.71-fold higher than controls in the 0.01 and 0.03% ethanol treatments, respectively. For the later retention time, 29.29±0.21 it was increased in the 0.03, 0.1, and 1% ethanol treatments to 5.91-, 4.93-, and 5.44-fold higher than controls.

A lysine-associated molecule, L-pyrrolysine, was significantly higher in the ethanol-treated embryos. With all ethanol treatments, L-pyrrolysine increased by a minimum of 4.97-fold controls.

Peptides

Many peptides were identified in our analysis. However, due to the large number of alternative peptide sequences for each m/z, consideration of functional characteristics at all identified peptide masses is beyond the scope of this paper. α-Aspartyl Lys was maximally 5.80-fold at 0.03% ethanol (Table 2.3.4). The peptide His-Ile increased in all ethanol treatments to a maximum of 6.08-fold higher than controls in the 0.03% ethanol treatments, but was not increased in the 1% treatment group. Pro-Ala-Pro was increased to 6.27-fold control levels, only at 0.3% ethanol. Gly-Asp-Val similarly was increased with all
Amino acid-related metabolites were identified from the polar fraction within 10 ppm of their theoretical m/z in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Aminopropionitrile HMDB04101</td>
<td>4.68002</td>
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<td>0.00</td>
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<td>5.67</td>
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<td>Purine HMDB01366</td>
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<td>6.00</td>
<td>5.78</td>
<td>6.34</td>
<td>0.00</td>
</tr>
<tr>
<td>2-Hydroxymethylserine M65894</td>
<td>-1.06180</td>
<td>0.00</td>
<td>0.00</td>
<td>6.44</td>
<td>5.44</td>
<td>0.00</td>
</tr>
<tr>
<td>N-(3-Oxo-octanoyl)-homoserine lactone LMFA08030004</td>
<td>-9.57403</td>
<td>0.00</td>
<td>0.00</td>
<td>4.77</td>
<td>5.52</td>
<td>5.54</td>
</tr>
<tr>
<td>Hydroxybutyrylcarnitine HMDB13127</td>
<td>18.62±0.13</td>
<td>-1.41902</td>
<td>5.01</td>
<td>5.71</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>29.29±0.21</td>
<td>3.19007</td>
<td>0.00</td>
<td>5.91</td>
<td>4.93</td>
<td>0.00</td>
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<tr>
<td>L-Pyrrolysine C16138</td>
<td>5.26183</td>
<td>4.97</td>
<td>5.79</td>
<td>6.03</td>
<td>5.62</td>
<td>5.62</td>
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<tr>
<td>2,6 Dimethylheptanoyl carnitine HMDB06320</td>
<td>-3.76263</td>
<td>0.00</td>
<td>5.06</td>
<td>5.81</td>
<td>5.35</td>
<td>5.05</td>
</tr>
</tbody>
</table>

Amino acid-related metabolites were identified from polar extraction fractions by LC-MS/MS. ID indicates the database reference for the m/z (C: KEGG; HMDB: Human Metabolome; LMFA: Lipidmaps; M: Metlin). Next, is the ppm difference (ppm) between the theoretical and observed masses. Folds are expressed relative to 0% controls. All metabolites listed were significantly changed from control, \( P < 0.001 \), ANOVA.

ethanol treatments except 1% by a minimum of 4.87-higher than controls. Isoleucine-tyrosine eluted with two different retention times. With a retention time of 12.56±0.02 minutes it was increased to as much as 6.05-fold higher than control for 0.01–0.1% ethanol. For the later retention time 52.97±0.14 minutes it was increased in all ethanol treatment groups except at 1%, also peaking in the 0.1% treatment group to 5.75-fold higher than controls. Pro-Gln-Pro was inconsistently
elevated. It was not different in the 0.01, 0.1, and 0.3% treatments, but was 5.72- and 4.56-fold higher than controls in the 0.03 and 1% treatments. Asp-Lys-Pro was also only different from controls with 0.1 (5.40-fold higher) and 0.3% (4.91-fold higher) ethanol. Glu-Thr-Leu was increased by 5.71-fold, only at the lowest ethanol treatment concentration. (Ac)2-L-Lys-D-Ala-D-Ala was significantly different with all ethanol treatment levels, except 0.3% ethanol. It peaked with 0.03% ethanol treatment to 5.65-fold higher than controls. Leu-Phe-Pro was not different from controls at higher ethanol concentrations. At low ethanol concentrations (0.01–0.1%) it was 5.87-, 5.31-, and 5.09-fold higher than controls. Val-Pro-Tyr increased with 0.01 and 0.03% ethanol, but was not different from controls in higher ethanol treatments. Cys-Lys-Lys was not different in 0.01 and 1% ethanol, but in intermediate concentrations it was increased by a minimum to 5.27-fold higher than controls. The peptide Pro-His-Gln increased to 5.24- and 5.69-fold higher than controls with 0.01 and 0.03% ethanol. Otherwise it was similar to controls. Gly-Arg-Arg was not different from controls at 0.1 and 1% ethanol treatment and was increased to 4.58-, 5.86-, and 5.05-fold greater than controls for 0.01, 0.03, and 0.3% treatments, respectively. Lys-Ser-Arg was 5.99-fold control levels, only at the 0.01% ethanol treatment. The peptide Val-Lys-Phe was similarly inconsistently increased with ethanol treatment. Only at 0.01% (5.79-fold) and 0.3% (4.67-fold) was it different than control. Ile-Lys-His increased to 5.95- and 4.71-fold higher than controls with ethanol treatments of 0.01 and 0.3% and was otherwise not different than controls. The tripeptide Lys-Lys-Lys increased at 0.1% ethanol and higher to a maximum of 5.62-fold higher than controls. Pro-Arg-Arg increased at those concentrations as well, but peaked at 1% ethanol (5.79-fold higher than controls). Lys-Phe-Phe increased to 5.60- and 5.40-fold higher than controls at 0.01 and 0.03% ethanol. Otherwise it was not different than controls. Ile-Arg-Arg was increased in all ethanol treatments except 0.03%. It peaked to 5.88-fold higher than control with 0.3% ethanol. The tripeptide Trp-Ile-Lys was not different from controls at concentrations of 0.1% or higher. It was 5.59-fold increased at 0.01% and 5.43-fold at 0.03% ethanol. Tyr-Leu-Arg was higher than controls by 5.00-fold and 5.51-fold in only the 0.03 and 0.3% treatments. Lastly, Leu-Pro-Glu-Ile increased with all ethanol treatments up to 0.3%. It was highest at 0.01% ethanol, where it was 6.14-fold higher than controls.
Table 2.18.: Peptide polar metabolites were identified within 10 ppm of their theoretical m/z in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>ppm Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td>His Ile</td>
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</tr>
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<td>Gly Asp Val</td>
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<td>M23691</td>
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<tr>
<td>12.56 ± 0.02</td>
<td>-2.88145</td>
<td>5.57 5.97 6.05 0.00</td>
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<tr>
<td>52.97 ± 0.14</td>
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<tr>
<td>Gly Arg Arg</td>
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continued on next page
Table 2.18.: continued

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<th>Metabolite ID</th>
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<th>Fold (Relative to 0% Ethanol)</th>
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<tr>
<td>Tyr Leu Arg</td>
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<td>Leu Pro Glu Ile</td>
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<td>M180346</td>
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</table>

Peptide-related metabolites were identified from polar extraction fractions by LC-MS (TOF). ID indicates the database reference for the m/z (C: KEGG; HMDB: Human Metabolome; M: METLIN). Next, is the sparkline and the ppm difference (ppm) between the theoretical and observed masses. Folds are expressed relative to 0% controls. All metabolites listed were significantly changed from control, $P < 0.001$, ANOVA.
Other Proteins and Related Compounds

Several other proteins and related compounds were identified, including urea, carnosine, creatinine, crotono-betaine, aspidinol, ifosfamide, N-acetylleucyl-leucyl-methioninal, and laserptin. Urea increased in the 0.03 and 1% ethanol treatments to 5.25- and 5.84-fold greater than controls (Table 2.19). Carnosine increased in the 0.01, 0.1, and 0.3% ethanol treatments to a maximum of 1.13-fold controls. In the other two ethanol treatment doses, it decreased to −4.70-fold below controls. Creatinine was not different in all ethanol concentrations except 0.1 and 0.3% ethanol. At those doses it was 6.09- and 5.57-fold higher than controls, respectively. The polar metabolite crotono-betaine was slightly elevated with low ethanol doses (maximum of 1.49-fold controls in 0.03% ethanol). At higher ethanol concentrations, it dropped to −4.63-fold lower than control. Aspidinol was elevated slightly in treatment groups of 0.01, 0.03, and 0.1% ethanol. In the 1% treatment group, aspidinol dropped to −4.60-fold lower than controls. Ifosfamide was significantly higher in all ethanol treatment groups. It was maximally 1.40-fold higher than controls. N-Acetylleucyl-leucyl-methioninal was increased in all ethanol treatment groups except the highest. It was at the highest level of 5.69- that of controls. In the 0.3% ethanol group it was decreased to 5.07-fold of controls. Lastly, laserpitin was significantly decreased to −4.34 in all ethanol treatment groups except the highest (1%), where it rose to 1.14-fold higher than controls.

2.3.5 Carbohydrates

The polar metabolites included several carbohydrates. These included 3-hydroxyglutarate, 2-oxosuberate, 5-methylthio-D-ribose, (R)-(homo)2-citrate, N-acetyl-D-glucosamine, fructoselysine, bis-D-fructose 2′,1:2,1′-dianhydride, 8-oxo-deoxy guanosine monophosphate (dGMP), and minosaminomycin. 3-Hydroxyglutarate was present in the polar fraction (Table 2.20). It was elevated to 5.59- and 5.05-fold in the ethanol concentrations of 0.1 and 0.3% ethanol. The metabolite 2-oxosuberate was elevated in intermediate ethanol treatment groups at 0.03% or higher, not exceeding 5.58-fold above controls. 5-Methylthio-D-ribose was nearly the same as controls in the 0.01 and 0.03% ethanol treatments. At 0.1% and higher, it was −5.07-fold lower than controls. (R)-(Homo)2-citrate was
Table 2.19.
Other protein-related metabolites were identified within 10 ppm of their theoretical m/z in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>-6.12370</td>
<td>0.00</td>
</tr>
<tr>
<td>Creatinine</td>
<td>4.51092</td>
<td>0.00</td>
</tr>
<tr>
<td>Crotono-betaine</td>
<td>3.20426</td>
<td>0.80</td>
</tr>
<tr>
<td>Aspidinol</td>
<td>-3.97091</td>
<td>0.99</td>
</tr>
<tr>
<td>Carnosine</td>
<td>7.845857</td>
<td>0.63</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>-1.23219</td>
<td>5.75</td>
</tr>
<tr>
<td>N-Acetyl-leucyl-leucyl-methioninal</td>
<td>-5.72657</td>
<td>5.69</td>
</tr>
<tr>
<td>Laserpitin</td>
<td>5.01042</td>
<td>-4.34</td>
</tr>
</tbody>
</table>

Amino acid and related metabolites were identified from polar fractions by LC-MS (TOF). Metabolite ID indicates reference database for the m/z (C: KEGG; HMDB: Human Metabolome). Next, is the sparkline and the ppm difference (ppm) between the theoretical and observed masses. Fold changes are expressed relative to 0% controls. All metabolites listed were significantly changed from controls, \( P < 0.001, \text{ANOVA} \).

at a minimum 5.47-fold higher than controls with ethanol treatment, except at the highest ethanol concentration, where it was similar to controls. The metabolite \( N \)-acetyl-D-glucosamine was significantly higher by 5.84-fold at 0.1% and 5.32-fold at 0.3% ethanol concentrations, but was otherwise not different than controls. Fructoselysine was not different in the 0.03 and 1%, but increased in the 0.01, 0.1, and 0.3% ethanol treatments. It was 4.75-, 4.83-, and 5.78-fold higher for these treatments, respectively. At 0.03 and 0.1% ethanol treatment bis-D-fructose
2′,1′,2′,1′-dianhydride was 1.67- or 5.5-fold higher than controls, depending on the retention time. It was not different than controls at other ethanol treatments tested. 8-Oxo-dGMP was increased in all ethanol treatment concentrations, peaking at 6.42-fold or 6.14-fold with 0.1% ethanol. Lastly, the metabolite minosaminomycin was elevated only at the intermediate concentrations of 0.1 and 0.3% ethanol.

2.3.6 Other Metabolites

Several other metabolites were identified in both the non-polar and polar fractions (Table 2.3.6). In the non-polar fraction, dethiobiotin, a precursor to biotin and metabolite of caproic acid, eluted at two different retention times, increasing up to 6.16-fold higher in some ethanol treatment groups. A metabolite known to be a cereal constituent, identified as α-bergamotenol, was significantly lower, by nearly −6.55-fold, at 0.03, 0.3, 1, and 3% ethanol by nearly −6.55-fold. Epoxymurin-A, previously reported as a molecule from plant bark, was lower by −6.44-fold at 0.03, and 0.1, and 3% ethanol [75]. Several other metabolites identified that did not vary significantly from the control group, including DL-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (DL-DPPD).

In the polar fraction, sodium bicarbonate levels were elevated approximately 5.21-fold above controls at 0.1% ethanol and higher. Dimethyl trisulfide was increased to 1.03- and 0.23-fold higher than control with 0.01 and 0.1% ethanol treatments. At the other tested concentrations, however, it dropped to −4.60-fold below controls. Also, 4-sulfobenzoate was increased with 0.01–0.1%, but was not different with 0.3 and 1% ethanol treatments. 3-Amino-4,7-dihydroxy-8-chlorocoumarin increased with all ethanol treatment doses except 1%. It peaked with 0.03% ethanol, but did not drop below 4.85-fold in other doses. Trinitrotoluene was not different from controls for some ethanol treatments, but was 5.67- and 4.78-fold higher than controls with 0.1 and 1% ethanol. Metyrapol was elevated by 5.54- and 4.96-fold in the 0.01 and 0.3% ethanol treatments only. Thysanone, previously reported as a fungally derived antibiotic, was not different than controls at low ethanol doses, but was nearly two-fold higher than controls at 0.1% or greater ethanol concentrations [76]. Ethylenediaminetetraacetic acid tetrasodium salt (EDTA) was elevated with 0.1% (6.12-fold higher) and 1% (5.54-fold higher), but was not different than controls in the
Table 2.20.
Significantly changed carbohydrate-related metabolites were identified within 10 ppm of their theoretical m/z in zebrafish embryos exposed from 2–24 hpf to 0–1% ethanol.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
<th>0.01</th>
<th>0.03</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxyglutarate HMDB00426</td>
<td></td>
<td></td>
<td>-5.43985</td>
<td>0.00</td>
<td>5.59</td>
<td>5.05</td>
<td>0.00</td>
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<td>5-Methylthio-d-ribose C03089</td>
<td>-2.204442</td>
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<td>0.53</td>
<td>1.05</td>
<td>-5.07</td>
<td>-5.07</td>
<td>-5.07</td>
</tr>
<tr>
<td>2-Oxosuberate C16589</td>
<td>7.056471</td>
<td></td>
<td>0.00</td>
<td>4.97</td>
<td>5.58</td>
<td>5.06</td>
<td>0.00</td>
</tr>
<tr>
<td>(R)-(Homo)2-citrate M71244</td>
<td>4.987315</td>
<td></td>
<td>5.47</td>
<td>6.20</td>
<td>5.70</td>
<td>4.73</td>
<td>0.00</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine HMDB00215</td>
<td>7.943374</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
<td>5.84</td>
<td>5.32</td>
<td>0.00</td>
</tr>
<tr>
<td>Fructoselysine C16488</td>
<td>1.35320</td>
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<td>4.75</td>
<td>0.00</td>
<td>4.83</td>
<td>5.78</td>
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<tr>
<td>bis-d-Fructose 2', 1:2,1'-dianhydride C04333</td>
<td>3.46 ± 0.04</td>
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<td>3.727828</td>
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<td>5.61</td>
<td>0.00</td>
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<tr>
<td></td>
<td>6.76 ± 0.25</td>
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<td>-1.187884</td>
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<td>5.54</td>
<td>5.56</td>
<td>0.00</td>
</tr>
<tr>
<td>8-Oxo-dGMP M62410</td>
<td></td>
<td></td>
<td>-6.413025</td>
<td>4.96</td>
<td>6.38</td>
<td>6.42</td>
<td>5.73</td>
</tr>
<tr>
<td></td>
<td>8.10 ± 0.06</td>
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<td>-0.415912</td>
<td>5.07</td>
<td>5.84</td>
<td>6.14</td>
<td>5.11</td>
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<tr>
<td>Minosaminomycin M71965</td>
<td>-2.677195</td>
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<td>0.00</td>
<td>5.82</td>
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</table>

Carbohydrate-related metabolites were identified from polar fractions by LC-MS (TOF). ID indicates reference database for the m/z (C: KEGG; HMDB: Human Metabolome; M: METLIN). Next, is the ppm difference (ppm) between the theoretical and observed masses. Fold changes are expressed relative to 0% controls. All metabolites were significantly changed, \( P < 0.001 \), ANOVA.

Other treatment groups. Pyochelin was not different in the lowest ethanol treatment, and increased by as much as 6.37-fold higher than controls in the 0.1% treatments. Inosine 5’-monophosphate (IMP) was increased to 5.80- and
4.62-fold higher than controls with 0.1 and 0.3% ethanol treatment, but otherwise was not different from controls. Aspidospermine increased with 0.1% or greater ethanol treatment and was not different at lower doses. It peaked at 5.70-fold in the 0.3% treatment group. 3′-Hydroxy-5,6,7,8,4′-pentamethoxyflavone was not different than control for 0.01, 0.03, and 0.3% ethanol. It was increased to 5.55- and 5.39-fold higher than controls with 0.1 and 1% ethanol treatments. Also in the polar fraction, a flavonoid 8-prenylafzelechin 5-methyl ether was increased at ethanol concentrations of 0.03% and greater. The metabolite increased up to 5.66-fold of the control group. At only the 0.1 and 1% ethanol treatment was devapamil different than controls. It was 4.85- and 5.59-fold higher in these, respectively. Lastly, 3-geranyl-4,2′,4′,6′-tetrahydroxy-5-prenylidihydrochalcone increased only at the 0.1 and 0.3% ethanol treatment doses to 4.83- and 5.64-fold greater than controls.
Table 2.21.: Other metabolites were significantly changed in ethanol-exposed zebrafish embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate†</td>
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<tr>
<td>Dimethyl trisulfide†</td>
<td>HMDB13780</td>
<td>-6.195462</td>
<td>n/a</td>
</tr>
<tr>
<td>4-Sulfobenzoate†</td>
<td>C02236</td>
<td>5.799689</td>
<td>n/a</td>
</tr>
<tr>
<td>Dethiobiotin</td>
<td>C01909</td>
<td>14.49 ± 0.23 ‡</td>
<td>2.862257</td>
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<tr>
<td></td>
<td></td>
<td>18.03 ± 0.13 ‡</td>
<td>2.502198</td>
</tr>
<tr>
<td>α-Bergamotenol†</td>
<td>HMDB36402</td>
<td>-1.3520387</td>
<td>0.19</td>
</tr>
<tr>
<td>3-Amino-4,7-dihydroxy-8-chlorocoumarin†</td>
<td>C12469</td>
<td>1.515869</td>
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</tr>
<tr>
<td>Trinitrotoluene†</td>
<td>C16391</td>
<td>-6.512265</td>
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*continued on next page*
<table>
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<th>Metabolite</th>
<th>Sparkline</th>
<th>Fold (Relative to 0% Ethanol)</th>
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<td>Metyrapol†p</td>
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<td>0.00</td>
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<td>Thysanone†p</td>
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<td>0.00</td>
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<td>6.26</td>
<td>6.41</td>
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<td>Ethylenediaminetetraacetic acid†p</td>
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<tr>
<td>Pyochelin†p</td>
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<td>n/a</td>
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<tr>
<td>Inosine 5′-monophosphate†p</td>
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<td>Aspidospermine†p</td>
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<td>5.70</td>
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<td>8-Prenylafzelechin 5-methyl ether†p</td>
<td>LMPK12020136</td>
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<td>5.56</td>
<td>5.01</td>
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Table 2.21: continued on next page
<table>
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<th>Metabolite ID</th>
<th>Sparkline ppm</th>
<th>Fold (Relative to 0% Ethanol)</th>
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</thead>
<tbody>
<tr>
<td>3′-Hydroxy-5,6,7,8,4′-pentamethoxyflavone&lt;sup&gt;†&lt;/sup&gt;</td>
<td>3.361368</td>
<td>0.00 0.00 5.55 0.00 5.39 n/a</td>
</tr>
<tr>
<td>Devapamil&lt;sup&gt;†&lt;/sup&gt;</td>
<td>9.41918</td>
<td>0.00 0.00 4.85 0.00 5.59 n/a</td>
</tr>
<tr>
<td>3-Geranyl-4,2′,4′,6′-tetrahydroxy-5-prenyl dihydrochalcone&lt;sup&gt;†&lt;/sup&gt;</td>
<td>-5.225479</td>
<td>0.00 0.00 4.83 5.64 0.00 n/a</td>
</tr>
<tr>
<td>dl-PPMP&lt;sup&gt;n&lt;/sup&gt;</td>
<td>2.808495</td>
<td>0.21 -0.04 -0.22 -0.25 -0.05 -0.05 -0.29</td>
</tr>
<tr>
<td>Epoxymurin-A&lt;sup&gt;†&lt;/sup&gt;</td>
<td>0.498426</td>
<td>-0.06 0.44 -0.06 -6.44 -6.44 0.02 -0.72 -6.44</td>
</tr>
</tbody>
</table>

Zebrafish embryos were exposed from 2–24 hpf to ethanol at concentrations from 0–3%. Metabolites were identified from non-polar (<sup>n</sup>) extraction fractions using LC-MS/MS (Orbitrap) or the polar (<sup>p</sup>) fraction using LC-MS (TOF). ID indicates the reference database used to identify the molecule using the m/z (HMDB: Human Metabolome; C: KEGG; LMPK: LipidMaps; M: Metlin; CID: PubChem). Next listed are the sparkline and the ppm difference (ppm) between the theoretical mass and the observed mass. Folds are listed for each ethanol concentration tested and are expressed relative to 0% controls. dl-PPMP: dl-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol. † : $P < 0.001$, ANOVA.
2.3.7 Ingenuity Pathway Analysis®

Ingenuity Pathway Analysis® (IPA) was utilized to identify relevant pathways changed with the ethanol exposure. In the non-polar metabolome, due to the levels of the non-polar metabolites tetradecanoic acid, palmitic, and stearic acids, lipid metabolism was ranked highly in IPA. Relative to controls, the increases at 0.03% ethanol treatment of palmitic, stearic, and tetradecanoic acids are associated with the accumulation of diacylglycerol (DAG) \((P < 0.001)\), where P-value reflects the IPA generated association between the identified metabolite and a given pathway) and synthesis of lipids \((P < 0.001)\). The increases in palmitic and stearic acids are also associated with accumulation and synthesis of ceramide, the conversion of succinic and palmitic acids, the synthesis of d-erythro-C16-ceramide, localization of triacylglycerides, and fat oxidation. Changes in the concentration of palmitic acid were linked to toxicological functions, including renal and liver necrosis /cell death as well as cardiac dysfunction. Increases in palmitic acid were associated with apoptosis of renal podocytes \((P = 0.008)\) and proximal tubules \((P = 0.008)\), as well as dysfunction of cardiomyocytes \((P = 0.011)\). Additional identified fatty acids associated in IPA with other pathways. Increases in tetradecanoic, palmitic, pentadecanoic, and stearic acids were all associated significantly with the quantity of Ca\(^{+2}\) in cell signaling, vitamin and mineral metabolism, and molecular transport. Increased stearic acid as well as decreased palmitic acid have been associated with apoptosis of hepatocytes \((P = 0.003)\). Stearic and palmitic acids were also associated with canonical stearate biosynthesis I pathway changes. Tetradecanoic, palmitic, and stearic acids decreases were associated with the transport of d-glucose and the oxidation of glucose–6-phosphate \((P < 0.001)\); however tetradecanoic and palmitic acids molecules were generally increased in our analysis studies \((P < 0.001)\). Increases in these molecules are associated with transport of d-glucose \((P < 0.001)\). These pathways were associated predominantly by tetradecanoic acid, palmitic, and stearic acids.

Analysis of the polar metabolite data identified 221 metabolites, 45 of which were mapped in IPA. The highest rank pathways identified were cell signaling, molecular transport, and vitamin and mineral metabolism. This was due to the presence of carnosine, dihydroxyacetone, kynurenic acid, purine, tretinoin, and urea. Carbohydrate and lipid metabolism as well as molecular transport was indicated by the increases of dehydroxyacetone, kyurenenic acid, urea, tretinoin, and decreases in carno-
sine (P=0.029, 0.047, 0.047, respectively). The increases in metabolite kynurenic acid, tretinoin, and purine and the decrease in carnosine indicated cell-to-cell signaling (P=0.042), nervous system development and function (P=0.047) as an important pathway in the ethanol-exposed embryo. Due to the increases in urea, ifosamide, and tretinoin, dermatological disease was indicated (P=0.003). Tissue development was indicated with increases in tretinoin and kynurenic acid and the decrease in carnosine (P=0.033). Decreased carnosine and increased tretinoin indicated toxicologic abnormalities in renal cell death of kidney and tubular cells (P=0.038). Increases in dihydroxyacetone, urea, and tretinoin, and decreases in carnosine were associated with small molecule biochemistry (P=0.047), specifically synthesis of nitric oxide (P=0.008). Hematological system development and function was indicated by increases in N-Ac-leucyl-leucyl-methionial, tretinoin, and kynurenic acid as well as decreases in carnosine (P=0.038). Increases in tretinoin, urea, and ifosfamide combined with decreases in carnosine (P=0.043) resulted in inflammatory response being indicated by IPA. Lastly, Free radical scavenging was indicated by increases in butyraldehyde, tretinoin, and urea in addition to the decrease in carnosine.
2.4 Discussion

2.4.1 Introduction

Overview of Results

To compare the effects of different ethanol treatment concentrations on a zebrafish model of FASD, we analyzed the changes in metabolome for zebrafish embryos treated from 2–24 hpf with 0–3% ethanol. We used LC-MS/MS (Orbitrap) to measure non-polar changes and LC-MS (TOF) to measure polar changes. Metabolite classes that changed significantly in the non-polar zebrafish fractions included fatty acids, glycerophospholipids, sphingolipids, and other metabolites. These findings were anticipated, as our extraction method should isolate non-polar metabolites. Lipid metabolism pathways were ranked highly in IPA due to higher levels of tetradecanoic, palmitic, and stearic acids. As these are the predominant fatty acids of fish and other animal fats, these findings were not surprising [65,77]. In the polar fraction where 219 metabolites were identified, many were identified as synthetic compounds or drugs (51). The remaining metabolites were lipids (57), proteins or amino acids (48), ethanol metabolites (15), and carbohydrates (5). Forty-five metabolites were mapped in IPA, which highlighted pathways of cell signaling, molecular transport, and vitamin and mineral metabolism.

In our data set, many metabolites lacked consistent linear dose-response trends over the concentration curves. Often they were highly altered relative to controls at 0.03% ethanol, having a hormetic response (U- or J-shaped dose response curve). Similar results have been demonstrated in the morphologic profiling of several strains of ethanol-exposed zebrafish, as well as human embryonic stem cells differentiated to neural cells in culture [78,79]. This might be attributable to a shift from apoptosis to necrosis occurring at some intermediate (i.e. 0.03 to 0.1%) ethanol concentration. Capturing the physiological changes at that switch-point may be essential to determining the type of intervention required for ethanol-exposed children.

What mechanisms are demonstrated in our data?

Part of the devastating effect of ethanol is that it alters multiple tissue types and organ systems. Here we highlight changes supported by our metabolomics data in the neural, cardiovascular, endocrine, renal, and placental systems, as well as impacts
on cell function, enzymatic cofactors, and the general disease resistance in ethanol-exposed embryos.

Zebrafish as our model

Fig. 2.5. Developmental comparison of zebrafish FASD model versus humans. In our experiment, zebrafish were exposed from 2–24 hpf to 0–3% ethanol, indicated on the timeline as a blue horizontal bar. The blue arrow indicates the time of zebrafish sacrifice. Eye icon: lens vesicle complete; Open heart icon: beginning of heart development; Filled and checked heart icon: completion of heart development; A: initial development of arches; L: liver development begins; M: beginning of meconium production; N: neural tube closure; N*: neural rod cavitation completed; S: first somites appear

2.4.2 Ethanol Catabolism

Ethanol may be metabolized several ways that may result in small metabolites. These small metabolites are not expected to be present in our metabolome, however,
we did find several metabolites that are related to ethanol catabolism (Figure 2.6). In response to ethanol, alcohol is oxidized to acetaldehyde via alcohol dehydrogenase (ADH). This enzyme, mediated by the ethanol-inducible cytochrome P450 enzyme (Cyp) 2E1, oxidizes the alcohol to form acetaldehyde. Acetaldehyde is known to be a teratogen and has a much greater catalytic efficiency with acetaldehyde than for ethanol. Thus, production of acetaldehyde during the catabolism of ethanol has been proposed by many to cause FASD. In adults, hepatic ethanol breakdown by ADH occurs in the cytosol. This is followed by microsomal oxidation to acetate by aldehyde dehydrogenase. The acetate is converted to carbon dioxide and water.

Prenatally, Cyp is at a very low abundance, only 25–50% that of an adult. CYP2E1 mRNA expression has been detected on placenta, including that of non-drinkers. Liver microsomes can also eliminate alcohol, but they only function between 12 and 27% that of adults.

It is worth noting that there are differences in the ADH present in zebrafish and mammals. Both lineages contain ADH3, though it is hypothesized that the acquisition of ADH1 occurred independently in these two genetic lineages. Further, teleosts underwent a whole-genome duplication event, including the genes encoding ADH. The result of these changes created the ADH variants 1A1, 1A2, 3H, 3L, while humans have 1A, 1B, 1C, 2, 3, 4, 5. Mice and rats have H1, H2, H3, H4, H6A, H6B, with the variant H5ps being present in mice and H5 occurring in rats.

Several known inhibitors of ADH were identified in the polar metabolome. N-Cyclohexylformamide uncompetitively inhibits alcohol metabolism by the enzyme ADH II activity in mice. N-Cyclohexylformamide is also a competitive inhibitor against acetaldehyde. ADH2 requires NADH as a coenzyme, and interestingly was elevated at the lowest (0.01%) and highest (1%) ethanol concentrations tested, but did not appear different than controls in intermediate concentrations. (We noted several nicotine-related metabolites changed in our metabolome as well, discussed below.) ADH2 causes a reversible oxidation of ethanol, but has fairly low activity as compared to the other 5 endogenous classes of alcohol dehydrogenases. Like cyclohexylformamide, octanoic acid is an inhibitor of mammalian ADH2 activity, able to competitively inhibit octanol.

We identified acetamide, which increased significantly at mid- and higher-dose ethanol concentrations. Although N,N-dimethyl acetamide has been detected in exhalations from rat models of chronic renal failure, we have identified no reports of
Fig. 2.6. Ethanol catabolism of metabolites isolated from extraction fractions of whole zebrafish embryos exposed to ethanol from 2–24 in 0–3% ethanol. Metabolite changes are indicated by sparklines.
acetamide generated with ethanol exposure. However, given the polar nature of this metabolite ($\text{C}_2\text{H}_5\text{NO}$), and that of ethanol, in conjunction with the structural similarity of these two molecules, understanding acetamide formation may be valuable. This may be especially true in the context of the alterations noted in peptides and proteins.

Also identified was $S$-methylthioglycolate (significantly differed from controls in $0.3\%$ ethanol and higher), a derivative of acetate. The higher levels of this metabolite suggest increased reactant acetate, as commonly reported by others in investigations of FASD. In addition, our metabolome demonstrated significant differences in acetoacetate, which can react with ethanol to reversibly form both butyric and caproic acids. 2-Octanamidoacetic acid (capryloylglycine) is a fatty acid metabolite, which varied little until concentrations of $1$ and $3\%$ ethanol, where they were nearly $6$-fold lower than controls. While acyl glycines, such as 2-octanamidoacetic acid, are common metabolites of fatty acids, they are also associated with inborn errors of metabolism.

2.4.3 Nervous System

It is widely understood that ethanol alters the developing nervous system and subsequent function and behavior. Here we will explore metabolome changes related to this system. In particular we will address the relationship between glycerophospholipids, sphingolipids, and tryptophan-related metabolome changes and their association with FASD.

Glycerophospholipids

The membranes of all cells, including neurons, are complex structures with high phospholipid content, including glycerophospholipids. Some of these function as signaling molecules, such as PA. Glycerophospholipids, including PS, PA, PE, and PI, are modified with ethanol exposure and showed a variety of changes in our model. Previous investigators have demonstrated changes the concentrations of glycerophospholipids, indicating a shift in the degree of saturation as well as the length of the phospholipids. In the male Sprague Dawley® rat forebrain, the makeup of glycerophospholipids changed in response to chronic (three week) $5\%$ ethanol. These changes included a decrease in the degree of unsaturation of PS, an increased unsat-
uration of PC, but brain PI composition was not significantly changed [93]. These observations are consistent with much of our data.

Multiple abnormalities have been associated with alteration of PLD function, including changes in PA signaling targets, astrocyte mitogenesis, myelination, and astroglial migration (Figure 2.7). An overview of phospholipase D (PLD) in the brain during development is available [94]. Changes in downstream PA signaling have also been hypothesized as a result of the lack of normal PLD conversion of PC to PA.

We detected a variety of PA moieties that responded differently at differing ethanol concentrations. A shorter, unsaturated PA moiety was significantly lower with higher ethanol doses, and more saturated and longer PA moieties were higher than controls with ethanol exposure. The longer, mixed saturation PAs tended to be significantly higher at mid and high ethanol concentrations. The variety of PA responses denoted are likely from multiple ethanol-induced causes. These may include altered enzymatic activity, such as inducing PLD to generate more PA from PC or instead make phosphatidylethanol from PC. Changes in conversion of diacylglycerol to PA by diacylglycerol kinase may also contribute to the inconsistent PA response we observed [95]. It also may be due to changed needs structural components that can be accommodated by PA, increased dihydroxyacetone conversion via lysophosphatidic acid, or there may be changed requirements or utilization of PA as a second messenger.

Ethanol affects PLD in cells such as astrocytes and astroglia, and is altered in our zebrafish embryo model of FASD. Based on data from neonatal rat astrocytes, ethanol binds to PC, preventing the conversion to PA by membrane bound enzyme PLD [96]. In permeabilized astrocytes, this can cause a decrease in DNA synthesis [97]. PA can also cause an increase in DNA synthesis, which is not sensitive to ethanol [97]. Physiologically, PA deficit can cause significant developmental defects, as alteration of this signaling pathway can also prevent astroglial proliferation [95,97,98]. In whole larvae Drosophila melanogaster extract, dietary ingestion of ethanol has been shown to increase activity of PC-induced PLD [99]. These experiments provide support that changes in PA, PC, and PE in our model are induced by ethanol-induced alteration of PLD function.

PLD has both transferase and hydrolysis activities that require an activator. Of the fatty acids that have been shown to be strong activators of PLD, the strongest fatty acid activators appear to be lauric, palmitoleic, oleic, linoleic, and arachidonic acids [100]. In our analysis of ethanol-exposed embryos, only palmitoleic acid was
Fig. 2.7. Metabolites isolated from extraction fractions of whole zebrafish embryos exposed to ethanol from 2–24 in 0–3% ethanol that influence the nervous system. Metabolite changes are indicated by sparklines.
significantly changed. It was significantly higher than controls at the 3% ethanol concentration, where it was 6.12-fold higher. It is possible that it was consumed in PLD activation at lower ethanol concentrations, but by 3% ethanol normal enzymatic functioning had been interrupted, leaving additional palmitoleic acid present.

PA has numerous downstream functions such as membrane trafficking, signal transduction, intracellular signaling, and protein kinase C activation [97,101]. Others have demonstrated that ethanol also appears to increase triglyceride, diacylglycerol, and fatty acid ethyl esters generated from PC [99]. One difficulty in understanding the ramifications of changes in PA is that no clear binding site has been established for its targets. However, it is understood that PA is a lipase product in the cell membrane [102].

Another PA function of note is the regulation effects on sphingosine kinase 1(SK1) [102]. Upon formation of PA by PLD in the lipid bilayer, SK1 translocates from the cytosol to the membrane [103]. Then, SK1 phosphorylates sphingosine forming sphingosine 1-phosphate, a polar G-protein coupled receptor substrate. (These receptors are conserved in zebrafish [104].) We noted a significant increase in sphingosine with ethanol treatment. The product, sphingosine 1-phosphate was not identified as a significantly changed metabolite in our analysis, but is known to be an intracellular second messenger and extracellular mediator, important for functions such as cytoskeletal rearrangements, control of growth, calcium mobilization, and cell survival pathways [105–107].

In addition to the effects of PA via sphingolipids and cell signaling, PA appears to be an important player in neuronal development via cytoskeletal reorganization and neuronal surface enlargement via exocytosis of cytoplasmic vesicles [101]. Due to its small, negatively charged head group in the phospholipid membrane, PA provides membrane flexibility, to a greater degree than other phospholipids, and attracts positively charged molecules [101]. Conversely, ethanol has also been shown to cause increase membrane fluidity [96]. Thus, ethanol-induced interruptions in PA also alter physical structure of neurons.

Dihydroxyacetone is a monosaccharide that eventually can be converted to PA or interconvert to glyceraldehyde [108]. It was marginally decreased at all concentrations, but was 5.45-fold lower than controls at 1% ethanol. Dihydroxyacetone can be phosphorylated by dihydroxyacetone kinase, which can eventually become lysophosphatidic acid and then structural PA [101]. We found PA moieties to generally be significantly increased with high ethanol concentrations. In conjunction
with significant decreases in dihydroxyacetone, this could indicate that dihydroxyacetone available to support structural PA are depleted at the highest ethanol dose. In essence, ethanol’s attack of membrane structure can no longer be overcome with dihydroxyacetone conversion to PA.

In addition to its structural role, PA, via PLD signaling, is associated with neurite outgrowth, so alterations in PLD function discussed above also alter normal neurite outgrowth [101]. We also identified significant lowering of palmitoyl 3-carbacyclic PA (CPA) at 0.03% ethanol concentrations and higher. This cyclic PA has neurotrophin-like activity and can inhibit DNA polymerase, fibroblast cell proliferation, and Cdc25 phosphatase [109–111]. Further, CPA is neuroprotective, promotes neurite development, and has been shown to decrease cell death in the adult male Wistar rat hippocampus (CA1) after ischemic injury [109]. Thus, the large decrease observed at 0.03% ethanol or more might indicate a point of no return for the damaged neuron or possibly the the onset of neuronal cell death. CPA is discussed further below with respect to the cardiovascular system.

Bile acids have also been shown to activate PLD [96, 100]. The bile acid derivatives 3α-hydroxy-6-oxo-5β-cholan-24-oic acid, 3α,7α-dihydroxy-12-oxo-5β-cholanate, and (25S)-3-oxo-12β-acetoxy-cholest-1,4-dien-26-oic acid were present in our data set. (Addressing possible functional differences between these bile acids is beyond the scope of this article, rather they will be considered as a single class of molecule.) These metabolites generally were increased at mid-range ethanol concentrations relative to unexposed controls. However, at the highest ethanol treatment, levels had returned control levels. It is possible these bile salts are activating PLD, resulting in the increased PA present in our data set. However, the amount of PC substrate did not differ over the dose response curve of ethanol in our analysis. Thus, the substrate source is unclear.

Insulin-like growth factor–1 stimulates rat astroglial PLD activity and mitogenesis, which can be inhibited by ethanol or 1-butanol [112]. Others have demonstrated that 1-butanol is a substrate for PLD transphosphatidylation activities [113]. 1-Butanol decreases the amount of PA formed via transphosphatidylation [112]. 1-Butanol is the reduced form of butanal, a marker for oxidative damage to lipids, proteins, and DNA. We saw significantly higher butanal at all ethanol concentrations tested, which may indicate both oxidative damage to lipids, proteins, and DNA, as well as an influence on PA levels.
It is possible the increase we detected in PA is of a protective nature, in spite of ethanol-induced decreases in PLD activity. In the presence of alcohol, PA is increased, perhaps through increased DAG kinase activity or another mechanism besides PLD. Further investigation is needed to understand if there are alternative enzymatic source (beyond PLD) of PA increases when exposed to ethanol, such as via lysophosphatidic acid acylation or by DAG kinase. In addition, further characterization is needed regarding the changes in PA-related secondary signaling and cytoskeletal changes that are occurring in our model. Also, whether this bile acid-induced PLD stimulation is physiologically relevant or simply an artifact of using whole-embryo extracts is unclear.

We did not identify phosphatidylethanol in our analysis. Under normal conditions, PC is converted by PLD to PA. However, with ethanol exposure, PLD instead shunts PC towards phosphatidylethanol, which occurs in most organs \[96, 97, 99, 114–116\]. This was surprising as several have suggested phosphatidylethanol as a biomarker for FASD.

In addition to its role in the coagulation cascade discussed below, changes to plasminogen maintaining reactions may be relevant to altered neural structural development. Conversion of plasminogen to plasmin is activated by tissue-plasminogen activator (tPA). TPA is activated by plasminogen activator inhibitor–1 (PAI–1). In addition to mediating fibrinolysis, tPA also is important for neuronal structure mediation \[117\]. Ethanol exposure in neonatal tPA null mice appear to be protected from neuronal loss in the cortex and thalamus as well as cognitive deficits in adulthood. This appears to be mediated by preventing normal NMDA receptor subunit B2 induced apoptosis \[118\].

In rat primary cortical astrocytes, ethanol treatment dose dependently increases tPA mRNA, and increases cellular and released tPA \[119\]. Ethanol also decreases DNA methyltransferase and DNA methylation in the promotor region of tPA \[119\]. Ethanol has been shown \textit{in vitro} to dose dependently inhibit carbachol-induced (activates PLD in astrocytes) hippocampal neurite outgrowth, an effect attributed to inhibition of astrocyte PAI–1 \[120\]. The abnormal neurite extension effect has also been demonstrated in tPA knock-out mouse neuroprogenitor cells \[117\]. This may suggest that even if low levels of ethanol and acetate are converted to caproic acids, conversion to the epsilon amino form, this can be devastating in the neurite outgrowth in the neonate.
Saturated PI in our experiments also increased with ethanol treatment, while less saturated forms decreased. The formation of PI is derived from PA. Under ethanol exposure in neurons, this is known to be inhibited due to the formation of phosphatidylethanol instead. Also, PI (4,5)-bisphosphate and PI (3,4,5)-triphosphate levels decrease, which are both involved in cytoskeleton organization and intracellular trafficking [121]. In addition, PI changes may be relevant to activation of various protein kinase C isoforms, discussed below.

PE, while significantly different, was unremarkable in its change from the control group when compared to other glycerophospholipid changes. This is noteworthy because of PE’s structural role in the neuron cell body, axon, and myelin. PE composes as much as 43% of myelin in some species [122]. So, this could indicate triaging of PE levels in attempt to maintain neural structures.

Our results demonstrate a mixed response of PS moieties to increasing concentrations of ethanol. There were increases in longer, more saturated moieties, but decreases in in shorter and less saturated PSs. Alcohol decreases more unsaturated PS moieties in subcellular fractions of synaptosomes, mitochondria, and myelin, and to a lesser extent liver homogenate [93]. However, in hippocampi from Sprague Dawley® rat embryos exposed to maternally consumed ethanol (up to 35% energy from ethanol) from E11 to E18 ethanol exposure decreased 13 of 14 moieties of PS reported as well as total PS; only 18:0/22:5 PS was reported as increased with ethanol exposure [123]. This inhibition of PS accumulation is associated with neuronal apoptosis, potentially due to decreased caspase–3 inhibition [123].

**Sphingolipids**

Another important lipid class identified in our analysis was the sphingolipids, a group of molecules that have a variety of biological functions of cells, including as part of membranes, and regulating growth, differentiation, senescence, and apoptosis [124]. We will focus here on the relationship between sphingolipids and the nervous system, which impact astroglial migration, neurite outgrowth, myelin structure, and cell membranes. Depending on the sphingolipid moiety, metabolites in this class tended to be significantly higher at either mid-range ethanol doses or with all concentrations tested. These results are consistent with findings in neonatal (days 5, 15, 21, and 30) c57BL/6J mouse brains after a single 1.5, 3.0, and 6.0 g/kg (15.8, 31.6, and 63.2% ethanol) dose of ethanol was given to their mothers during the third trimester [33].
authors proposed that maternal ethanol increases offspring ceramide concentrations by increasing serine-palmitoyl transferase activity resulting in de novo synthesis of ceramide, rather than conversion from sphingomyelin via sphingomyelinase [33]. vitamin K3, which was significantly increased in our metabolome, has also been shown in bacteria to activate serine-palmitoyl transferase [125,126]. Others have shown in rat cerebellar granule cells that ethanol increased the rate of ceramide sphingosine recycling for use by gangliosides (composed of ceramide, oligosaccharide, and sialic acids that function in cell communication), as opposed to de novo ganglioside synthesis [127].

Likewise, we did not detect sphingomyelin as a significantly different metabolite, but our results show that at 0.01 or 0.03% ethanol and higher, ceramides, sphinganine, and sphingosine are elevated. Additionally, vitamin D3 has been shown to induce conversion of sphingomyelin to ceramide via sphingomyelinase activity. Providing promyelocytic HL–60 cells with vitamin D3 increases sphingomyelin turnover. Two hours after treatment, ceramide levels remain increased, but then return to baseline [128]. We demonstrated significant difference in four vitamin D metabolites. Whether the ceramide increase in our metabolome is a result of greater stearate supply, abnormal conversion from increased sphingosine, deficient sphingomyelinase resulting in less utilization of ceramide for sphingomyelin, abnormal vitamin D levels, or increased recycling of damaged neurons is unclear.

In addition to the transcription regulation functions of vitamin D discussed below, vitamin D is essential in many capacities in brain development and normal functioning related to neurite growth and regulation of nerve growth factor. Vitamin D has the capability to pass through the blood-brain barrier [129]. The brain possesses P450 enzymes capable of converting the active form 1,25-dihydroxy vitamin D3 and then the inactive 24,25-dihydroxy vitamin D3 (CYP27B1 and CYP24B1, respectively) [129]. In neural development, vitamin D3 also is involved in inducing neurite formation and upregulating nerve growth factor (NGF) [130]. Adding 1,25-dihydroxy vitamin D3 to cultured primary hippocampal cells results in increases neurite outgrowth [129]. Subsequent to the 1,25-dihydroxyvitamin D3 stimulation of NGF in the Sall2 transcription factor mediated promotion of hippocampal neurite outgrowth, ceramide formation is induced; then ceramide binds to the p75 neurotrophin receptor [131,133].

Increases in ceramide is of great importance as it can lead to an eventual increase in activation of sphingomyelinase causing additional ceramide accumulation. Ceramide has also been shown to inhibit PLD, which consequently may have contributed to
the decreased levels of PA observed relative to controls [116]. Ceramide accumulation with ceramidase activity, can lead to increases in sphingosine, as demonstrated in our results, and result in consequent toxicity to neurons [134]. Additionally, sphingosine can inhibit protein kinase C (PKC), as can ceramide when activated with the ubiquitously expressed caspase–3 substrate prostate apoptosis response 4 [33,135,136].

Accumulation of DAG is biphasic [137]. Initially PI, via PI–4,5-bisphosphate specific phospholipase C-mediated hydrolysis, can form polyunsaturated 1,2-DAG [137,138]. Then mono-unsaturated and saturated DAGs are then generated from PC, via PLD’s dephosphorylation of PA) [137,139]. In conjunction with DAG, PS can activate “novel” PKC isoforms present in the central nervous system (such as ε or θ) [?,140,141]. Signaling functionality of DAG may be dependent on its fatty acid composition. Polyunsaturated DAGs (those from PI) appear to activate PKC more efficiently than mono-unsaturated or saturated DAGs, perhaps due to its receptor affinity or possibly a physical alteration generated in the phospholipid bilayer that allows improved PKC access [138,139].

Saturated forms of PI increased, but polyunsaturated ones decreased, as a function of ethanol in our experiments. We did not detect mono-unsaturated PI. This may suggest decreased production of mono-saturated DAG, resulting in decreased efficiency of PKC activation. PI-Cer can result from a reaction between ceramide and PI. In spite of increases in other ceramides with increased ethanol concentrations, PI-Cer was significantly decreased at very low doses of ethanol. These results show that PKC inhibition is relevant in FASD, due to the accumulation of inhibitors sphingosine and ceramide and the increased in suboptimal saturated PI.

In a study of cultured cerebellar neurons from juvenile Sprague Dawley® rats, incubation with 50 or 100 mM of ethanol for 48 hours followed by culture medium resulted an apparent increase in the recycling of sphingosine [127]. This is inconsistent with our results. Our data show that with increased ethanol dose sphingosine was elevated, but we also saw its precursor/product ceramide increased. (Given that the ceramide precursor sphinganine shows similar changes with ethanol exposure as ceramide in our polar fraction samples, we are assuming the reaction between ceramide and sphingosine favors sphingosine.) Thus, if increased ceramide contributed to elevated sphingosine, the consistent, elevated levels of sphingosine would be a result of the increased ceramide. However, at lower doses, sphingosine is elevated when ceramide is not, which at that concentration may be consistent with increased recycling.
In SH-SY5Y neuroblastoma cell culture, neutral ceramidase, an enzyme critical for control of ceramide turnover, has been shown to be downregulated (mRNA, protein, and enzymatic activity) during all-trans retinoic acid induced neuron differentiation [142]. This occurs without a change in the level of sphingosine or sphingosine-1-phosphate [142]. The significant increase in sphingosine in conjunction with the biphasic dose response of retinoic acid may result in abnormal neuron differentiation.

Myelination

Sulfatides, a class of sulfoglycosphingolipid, play a role in a variety of immune system responses, insulin secretion, hemostasis, and thrombosis [143]. They compose ~4.4% of myelin lipids (Sprague Dawley®, dry weight) [143, 144], and function as a negative regulator in oligodendrocyte differentiation [143, 145], as well as influence myelin stability [143, 146]. Schwann cells appear to have myelin formation initiated in part by sulfatide [143, 147]. Sulfatide is a myelin-associated neurite outgrowth inhibitor, which may contribute to axon regenerative failure [143, 148]. C22 sulfatide was 6.25-fold lower than controls at both 0.001 and 0.003% ethanol levels, potentially indicating a breakdown in myelin components. In addition, C20 sulfatide was reduced from controls in some treatments by 1.23-fold. These may represent important alterations in metabolism, the nervous system, and cardiovascular system.

In addition to the effect of vitamin K on coagulation, discussed below, the elevation in vitamin K$_3$ has important neural implications that remain underexplored in FASD. Vitamin K plays essential roles in development, particularly in the nervous system [126]. A case study of individuals with the congenital skeletal dysplasia chondrodysplasia punctata were also associated fetal alcohol syndrome or related vitamin K abnormalities [149]. In addition, vitamin K has been suggested to provide an anti-oxidant benefit to Sprague Dawley® primary oligodendrocytes and embryonic cortical neurons [150].

Lignoceric acid is converted to $\alpha$-hydroxy fatty acids and ceramide in the brain via $\alpha$-hydroxylation, which is associated with myelination [151, 152]. The increases we noted in lignoceric ceramide may suggest an increased myelin generation, potentially as compensation for the decreased sulfatides. Our findings in sphingolipids and sphingolipid-mediating metabolite levels suggest novel pathways in FASD that require further investigation. These include the relationship between myelin and other
neural components, vitamins A and D, and newly associated sulfatides and lignoceric ceramides.

**Tryptophan**

Tryptophan, the least abundant essential amino acid, is an important component of proteins and normal functioning of cells, including neurons. It also is a precursor of serotonin and nicotinamide adenine dinucleotide. Several tryptophan metabolites significantly differed with ethanol treatment in our data set, including oxindole, kynurenic acid, 7-chlorokynurenate acid, and uncaric acid (Figure 2.8). Additionally, significant differences in vitamin D$_3$ and glucocorticoid metabolites may also influence the tryptophan related metabolites in ethanol-exposed individuals. Several studies have suggested the increased conversion of tryptophan to nicotinic acid ribonucleoside is related to the changes occurring in response to pregnancy-related estrogen and progesterone changes\[153\]. Therefore, related estrogen changes will be explored here as well.

**Serotonergic Changes** Serotonin (5-hydroxytryptamine, 5-HT) is a tryptophan metabolite recognized as being altered in FASD. Previously our colleagues have demonstrated that mouse embryos from alcohol-exposed dams have decreased neuronal migration or differentiation in 5-HT neurons originating in the midline raphe of the brainstem and projecting to the dorsal and median raphe\[41\]. These effects persist into adulthood of male offspring animals\[40\]. In addition, prenatal ethanol exposure leads to persistent decreases in the number of serotonin immunoreactive neurons in the brainstem of both males and females\[154\].

In a model of chronic, low dose ethanol exposure in pups of Wistar rat dams fed 6.6% ethanol in drinking water, tryptophan hydroxylase immunoreactivity was significantly increased in dorsal and median raphe nuclei. 5-HT was increased, but not significantly in both nuclei\[155\]. While our metabolomics data does not address specific alterations to serotonin synthesis or metabolism, our data does demonstrate an ethanol induced effect. Tryptophan-related pathways outside of 5-HT are altered in our metabolome, including indole and kynurenine metabolites. Increased synthesis of these metabolites could explain the decrease in 5-HT related pathways, signaling, and synthesis. In addition, changes noted in vitamin D related pathways also may influence normal 5-HT function in ethanol-exposed individuals.
Fig. 2.8. Tryptophan-related pathway changes with metabolites isolated from extraction fractions of whole zebrafish embryos exposed to ethanol from 2–24 in 0–3% ethanol. Metabolite changes are indicated by sparklines.
Kim and coauthors showed that maternal ethanol consumption in Sprague Dawley® rats leads to decreased 5-HT synthesis and expression of tryptophan hydroxylase in the dorsal raphe, where cell bodies of most serotonergic neurons are and then project to other parts of the brain. This result was more pronounced at 5 wks of age compared to 3 wks \[156\]. Schneider and coauthors examined the effects of carrying the gene (homozygous or heterozygous) for the short allele of 5-HT transporter promoter region in rhesus monkeys exposed to alcohol during gestation. They found that when mother rhesus monkeys were exposed to 0.6 g/kg/d during early (0–50 days) or middle-late (50 to 135 days) gestation, these allelic carriers had lower levels of the a 5-HT metabolite 5-hydroxyindoleacetic acid in cerebral spinal fluid when offspring were 30 months of age. Homovanillic acid was also decreased at baseline in these test groups as well \[157\].

Tryptophan hydroxylase gene transcription is activated by a vitamin D response element (VDRE) \[158\]. The VDREs “on two different tryptophan hydroxylase genes involved in serotonin synthesis ... are functionally opposite to one another: one of them induces transcriptional activation of tryptophan hydroxylase 2 (TPH2) by vitamin D in the brain and the other induces repression of tryptophan hydroxylase 1 (TPH1) in tissues outside the blood-brain barrier or peripheral to the brain” \[158\]. Our metabolome demonstrates increases in vitamin D$_3$ that occur with ethanol exposure. Given the well-established links between serotonin system abnormalities and behavioral and cognitive disruption, such as anxiety, aggression, impulsive behavior, and learning and memory impairment, it is logical to associate serotonin malfunction in FASD functional changes. “For example, polymorphisms in the serotonin transporter gene have been linked to social behavioral defects including aggression, impulsivity, anxiety, psychopathology, and personality disorder. Experimentally lowering brain serotonin levels in normal people has a wide range of behavioral consequences: impulsive behavior, impaired learning and memory, poor long-term planning, inability to resist short-term gratification, and social behavioral deficits characterized by impulse aggression or lack of altruism” \[159\].

Patrick and Ames proposed a mechanism for the development of autism, emphasizing the relationship with fetal-maternal interactions influenced by vitamin D status and how they impact changes associated with brain defects in autism spectrum disorder. While FASD and autism spectrum disorder are very different, both disorders share some common features in presentation, such as diminished social and behavioral function. The effect of a low vitamin D (<30 ng/mL) status in maternal blood
causes increased activation of placental tryptophan hydroxylase 1, resulting in higher concentrations of serotonin and lower amounts of indoleamine-derived kynurenine, causing fewer T_{\text{reg}} cells. The decreased T_{\text{reg}} cells results in an autoimmune response from maternal antibodies in the fetal brain and abnormal brain development \[158\].

In addition, Patrick and Ames hypothesize that diminished supplies of marine-derived fats such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA), in conjunction with low vitamin D, contribute to the abnormal regulation of serotonin and thus associated behavioral abnormalities \[159\]. The association of DHA inadequacies in FASD has also been proposed by others \[160\]. Given the elevation in vitamin D\textsubscript{3} isoforms occuring with ethanol exposure in our experiment, it is possible that the vitamin D synthesis from cholesterol has been upregulated in response to alcohol. This could be an attempt to correct serotonin imbalance caused by increased metabolism of tryptophan to the kynurenine pathway, rather than serotonin. This hypothesis, on the surface, is contradicted by literature in rodents demonstrating the vitamin D deficiency during gestation being causal for behavioral abnormalities in later life as well as Kim’s findings of decreased tryptophan hydroxylase activity. However, if one considers the Patrick and Ames hypothesis in the context of increased levels of vitamin D in order to regulate the decreased level of serotonin induced by ethanol, these ideas do not contradict each other, but rather suggests that there is a feedback loop between the serotonin insufficiency and vitamin D regulation. Vitamin D can activate tyrosine hydroxylase in the brain to stimulate the synthesis of serotonin. EPA aids in the release of serotonin from the presynaptic membrane, and DHA increases membrane permeability of the post-synaptic membrane of the serotonin receptor \[159\].

In a dietary recall survey of South African mothers of children with FASD, DHA and EPA consumption tended to be less than controls. Nearly all women consumed less vitamin D, vitamin E, dietary fat, and cholesterol than the estimated average requirement \[161\]. Additionally, zebrafish on a low vitamin E diet have reduced polyunsaturated fatty acids \[162\]. We did not directly measure the effects of ethanol on EPA or DHA in our zebrafish model, but that would be an important next step in the examination of this hypothesis. We did, however observe significant decreases in anandamide with increasing dosages of ethanol. DHA and EPA can be converted to docosahexanoyl-ethanolamide and eicosapentanoyl-ethanolamide, respectively, and these molecules as well as anandamide are endocannabinoids. Further details about the relationship of endocannabinoids and FASD are beyond the scope of this article.
Sex hormones also have an impact on 5-HT and ethanol’s effects. In studies by Sliwowska, et al. in female rats, prenatal alcohol exposure (PAE) causes a decrease in the 5-HT immunoreactive brainstem neurons in the dorsal raphe (p=0.060). Ovariectomization, which resulted in a significant decrease in the plasma estradiol and progesterone levels, caused an even greater decrease of these neurons (p<0.018) [154]. In our dataset, 1α-hydroxyesterone, a metabolite of estradiol, was significantly higher in mid-dose range levels followed by a return to baseline at the highest dose. Estradiol has relevant neuroprotective effects on 5-HT neurons, including promoting cell survival, preventing cell death, and decreasing DNA fragmentation [163]. Exogenous subcutaneous estrogen administration to ovariectomized control animals showed a non-significant increase in the number of 5-HT neurons. But, PAE ovariectomized animals that were estrogen-supplemented had a significant increase in dorsal (P<0.029) and median (P<0.004) raphe 5-HT immunoreactive neurons, an effect that was ablated by progesterone supplementation. However, alcohol exposed animals had no difference in estrogen or progesterone levels without ovariectomization. Our results contrast to Sliwowska’s findings in sham operated prenatal alcohol exposed rats. 16-Dehydroprogesterone was also higher than control at most ethanol concentrations tested as well. Our data showed similar responses, in that 16-dehydroprogesterone was significantly higher in both mid- and highest-ethanol doses tested.

Previously, we have documented the disruption of the neural tube midline and decreased brain size in mice whose mothers consumed alcohol. In addition, we have shown that 5-HT production is disrupted in these offspring, which may be mediated with activity-derived neurotrophic factor agonist peptide (Ser-Ala-Leu-Leu-Arg-Ser-Ile-Pro-Ala) and to a lesser extent brain-derived neurotrophic factor (Asn-Ala-Pro-Val-Ser-Ile-Pro-Gln) [164,165]. While our results in zebrafish embryos demonstrated many peptide chains, those identified through our filtering and analysis process did not align with fragments of these bioactive peptides. However, since we did not attempt to examine all possible peptide sequences, it is possible we did not align our peptide masses with correct amino acid sequences. It is possible that if these bioactive peptides were endogenously produced in attempts to repair ethanol-induced damage, the prolonged duration of our exposure would have caused the bioactive peptides to be a limiting reagent in any such repair. Further investigation is needed to examine if the peptide masses identified in our metabolome are important to the FASD etiology.
**Oxindole** Oxindole, elevated approximately 5-fold or higher at most ethanol treatment concentration, is a metabolite of tryptophan catabolism via indole production, and may be produced in several tissues, including the brain \[166, 167\]. This is proposed to occur in the intestine by bacterial tryptophanase and then oxidation of the indole in mammalian tissues \[167\].

Oxindole contributes to a sedative effect, demonstrated both in animal models or patients with acute or chronic hepatic impairment, correlating to declines in mental and neuromuscular function in patients with hepatic encephalopathy \[166, 167\]. It has been shown to accumulate in the brains of rats with hepatotoxin thioacetamide or galactosamine-induced liver failure, potentially relevant due to the associated neural abnormalities \[166\]. In addition, plasma levels of the precursor indole have been shown to be increased in the plasma of cirrhotic patients, especially those with associated altered mentation (hepatic encephaly) \[167\]. And, oxindole has been shown to modify the voltage-gated sodium channel function of neurons resulting in decreased excitation \[168\]. In low doses, this metabolite can cause vasodilation, loss of muscular tone, and decreased activity, but in higher doses it can cause coma or even death \[168\].

5-Hydroxyindole is also produced by tryptophanase’s catabolism of tryptophan found in bacteria \[169\]. In rat pyramidal hippocampal neurons, addition of 5-hydroxyindole causes modifications of both pre- and post-synaptic neurons’ potentials. On the presynaptic side, the time until the neuron fires after depolarization is reduced. At the post-synaptic side, there is increased the amplitude of excitation potential and slowed inhibition of the glutamate neuron \[170\]. The indole metabolite 5-hydroxyindole has also been shown to cause release of glutamate via the selective activation of presynaptic α7 nicotinic acetylcholine receptors (α7nAchR), a process that can be negatively modulated by kynurenine \[171\]. In addition, 5-hydroxyindole has an inhibitory effect on dopamine, noradrenaline, and acetylcholine receptors \[171\]. This change may be relevant to the etiology of FASD, as increased oxidase activity generating oxindole with ethanol treatment may indicate less indole available for 5-hydroxyindole functioning.

**Indoleacetylaldehyde** The tryptophan metabolite (via indole-pyruvate) indoleacetylaldehyde has been identified as a potential biomarker of prenatal alcohol exposure in vitro. Ethanol-induced increases in indoleacetylaldehyde decrease tryptophan hydroxylase activity, thus may be the origination of increased kynurenine in neural progenitor cells \[79\]. Palmer, et al. reported results of a metabolomics study using
human embryonic stem cells, including embryoid bodies, neuronal progenitors, and neurons at two ethanol doses (0.1 and 0.3% ethanol) [79]. Each cell type had a different metabolomic profile with “no significant features” common to the three cell types; fifteen metabolites appeared to have a “dose response” [79]. They were able to confirm four entities, including L-thyroxine, 5-methylthioadenosine, L-kynurenine, and indoleacetaldehyde. Surprisingly, there was little overlap in the significantly different metabolites from their data and ours. This may be attributed to our analysis covering such a wide range of alcohol doses as well as being whole-embryo extracts. Additionally, they also reported significant differences from controls at both ethanol treatments concentrations for methylated purine 7-methylinosine and succinyladenosine, neither of which were present in our ethanol-altered metabolome. Unlike Palmer, we did not identify a metabolite matching the tryptophan metabolite indoleacetaldehyde [79]. This is likely due to a limitation of the detection method used in the non-polar fraction analysis, for which a 200 amu cut-off value was utilized.

Kynurenine Pathway  Production of serotonin from ingested tryptophan accounts for only 1% of the total tryptophan catabolism, with more than 95% catabolized in the kynurenine pathway [153, 172, 173]. In our analysis, the kynurenine metabolite kynurenic acid was identified in the 0.03% ethanol group, where it was elevated to 5.70-fold over control, but in other ethanol concentrations was not different from controls. The significant difference in this metabolite with ethanol demonstrated in our analysis supports the relevance of kynurenine-pathway changes to FASD biochemistry. This increase in non-serotonin tryptophan catabolic products supports findings of previous studies that demonstrated in 19- and 35-day old Sprague Dawley® offspring of dams fed 6.6% (v/v) ethanol have decreased 5-HT motor neurons [174]. Similarly, offspring of female Sprague Dawley® dams gavaged with 5.1 g/kg ethanol on E20 had decreased 5-HT neurons relative to dams dosed from E1–E20 [175]. E13 c57BL/6 offspring of mice on a 4.49% (v/v) ethanol diet from E7 to E13 had significantly reduced serotonin levels [176].

Enzymatic conversion via IDO  Two enzymes are responsible for the rate-limiting conversion of tryptophan to kynurenine. Indoleamine 2,3-dioxygenase (IDO) converts indoles like tryptophan in the presence of oxide or superoxide to kynurenine, functioning to open the indole ring of tryptophan [177–180]. IDO is expressed by most organs, including the placenta [158, 179]. Tryptophan 2,3-dioxygenase (TDO)
converts tryptophan (specifically) and oxide to kynurenine \[181\]. It is expressed in the liver, but is absent or very low in the newborn \[153\].

IDO can be influenced by several factors, including nutrient levels and immune system activation. In normal conditions, the rate of tryptophan degradation increases due to immune-mediated activation of IDO with duration of pregnancy, with the highest kynurenine/tryptophan ratio at birth \[182\]. IDO is “highly expressed in the trophoblasts surrounding the developing fetus. This starves local immune cells, conferring fetal immunotolerance and preventing rejection of the foreign fetus by the immune system” \[183\]. This enzyme can also be stimulated by stress, high B \(_6\), insulin resistance or magnesium insufficiency \[153, 184\]. Lignans have been shown to limit IDO activity and limit tryptophan catabolism, which has potential relevance due to the lignans identified in our metabolome \[177\].

IDO plays an important role in the health of the cell. Outside of superoxide dismutase, IDO is the only enzyme known to use superoxide as a substrate \[180\]. This suggests that any alteration increasing the conversion of tryptophan to kynurenine via IDO is limiting capabilities of the cell contributed by IDO.

Steroids and inflammatory cytokines such as interleukin-1\(\beta\) and interferon-\(\gamma\) are also able to increase IDO, while TGF-\(\beta\) and IL-4 are able to decrease IDO in macrophage or glial cells, thereby altering kynurenine products \[180\]. Increases in immune activation markers that occur concurrently with interferon-\(\gamma\), tryptophan decreases, and kynurenine increases, indicate the function of IDO, as opposed to TDO \[182\]. This is considered by some to be an “anti-proliferative mechanism of monocyte-derived macrophage and dendritic cells” \[182\].

IDO-activated tryptophan catabolism is a marker of several diseases, including infection, malignant disease, autoimmune diseases, and neurodegenerative disease \[182\]. Diseases such as neurodegenerative diseases (Alzheimer’s, Huntington’s, and Parkinson’s), cardiovascular disease, and pregnancy can all cause tryptophan degradation with immune system activation \[182\]. In addition, IDO is a useful biomarker of several pregnancy or fetal problems, such as chorioamnionitis, spontaneous abortion, and inborn errors of metabolism. Chorioamnionitis, an infection of the fetal-maternal membrane, is associated with higher risk of preterm delivery, as is FASD. In an investigation of “sterile chorioamnionitis”, intra-amniotic interleukin–1 injection resulted in a significant increase in immunomodulatory enzyme IDO \[185\]. In addition, in a prospective study of healthy 5–9 week pregnant women undergoing elective abortions or with a history of recurrent spontaneous abortion, levels of IDO normally found at
the placenta and decidua were significantly lower in women with a history of recurrent spontaneous abortions [186]. Lastly, in the inborn error of metabolism glutaric aciduria type I, the deficiency of glutaryl-CoA dehydrogenase has been hypothesized to increase IDO in macrophages and monocytes, resulting in an eventual increase in quinolinic acid, resulting in antagonism of the NMDA glutamate receptors [187]. These examples of IDO as a biomarker support the importance of the kynurenine pathway abnormalities in our metabolome.

**Enzymatic conversion TDO** Tryptophan 2,3-dioxygenase (TDO) is the heme-containing enzyme that converts L-tryptophan to N-formyl-L-kynurenine. It exists in low concentrations in the newborn and can be influenced by a variety of factors, including several steroid structures [153,188].

TDO has been shown to be elevated in pregnant rats, but it is reportedly extremely low in newborn animals and humans [153]. Thus, any changes in the kynurenine pathway occurring via TDO would be maternally-derived alterations, initially, then offspring. This may explain the increase and then sudden decrease in kynurenic acid and 7-chlorokynurenic acid in our data, as our model system has finite ability to provide resources. Stress due to lower concentrations may be handled by maternally or the low amounts of embryonic enzyme. But, with higher enzymatic demands, existing concentrations of the enzyme is exceeded. Because of the limitations of TDO, there may be an increase in kynurenine 3-hydroxylase utilization, resulting in the potential for 3-hydroxykynurenine and the resultant neuronal toxicity [179].

Steroids with glucocorticoid activity increase TDO, and TDO can be increased by compounds containing the indole nucleus [153]. The opposite can be achieved with injection of nicotinamide in rats, metabolites of which were significantly altered in our model [153]. In assessment of human liver biopsy samples, hydrocortisone stimulation caused an increase in TDO, leading Altman and Greengard to conclude “any form of stress or treatment with a variety of drugs that act directly or indirectly to stimulate the pituitary-adrenal system may also increase the level of [TDO]” [189]. Hydrocortisone administered to normal male patients or benign prostatic hypertrophic subjects also showed a significant increase in urinary excretion of kynurenine, kynurenic acid, and other kynurenine pathway catabolites [153]. These studies support the notion that stress, such as that which alcohol exposed zebrafish are subjected to, causes additional catabolism of tryptophan via the kynurenine pathway.
Due to the presence of a glucocorticoid response element on TDO [179,190], the metabolite 6-hydroxydexamethasone, present in our analysis, may be of particular relevance. This metabolite was significantly higher at low concentrations of ethanol. 6-Hydroxydexamethasone is normally a metabolite of the synthetic glucocorticoid dexamethasone, shown to be significantly more potent than endogenous cortisol, so its presence in our analysis requires further investigation.

In addition to glucocorticoid-response changes, estrogens have been shown to induce TDO in vivo [153,191]. In addition, peri- and post-menopausal women administered estrogen in conjunction with tryptophan show no increase in urinary kynurenic acid, suggesting that estrogens inhibit TDO [192]. In our metabolome, 1α-hydroxyesterone was significantly different, elevated at all ethanol concentrations tested until the highest dose, suggesting TDO may be decreased by 1α-hydroxyesterone.

**Kynurenic acid** Once L-tryptophan has been converted by IDO or TDO to L-kynurenine, kynurenine aminotransferase enzymes are able to convert it to kynurenic acid. Kynurenic acid is an astrocyte-derived neuromodulator and neuroprotector [193,194]. Most brain kynurenine metabolism occurs in the glial cells [195]. It can be formed by oxidation of tryptophan residues in close proximity to heme or copper binding sites [196]. Kynurenic acid metabolism can also occur in astrocytes, converted by kynurenine aminotransferases and vitamin B_6 to kynurenic acid, which can then be converted to quinolinic acid [195]. Kynurenine is actively transported into the brain, but most kynurenine catabolites, such as kynurenic and quinolinic acids are poor at crossing the blood brain barrier [197,198]. Because of this, it is hypothesized that these catabolites must be made in the brain [198], and the regulation is largely enzymatic [195].

Before birth, kynurenic acid levels increase, and then on the day of birth decrease, changes which are suggested to reflect neuronal plasticity [172,199,200]. Fetal astrocytes contain most enzymes of kynurenic pathway, with the exception of kynurenine 3-hydroxylase, which is hypothesized to provide a protective mechanism in the developing brain from damaging quinolinic acid-induced NMDA synthesis [180].

Kynurenic acid acts as an antagonist of the nicotine cholinergic (α7nAchR), glutamate receptors, as well as the glycineB site on N-methyl-D-aspartate (NMDA) receptors [179,180,194,201]. Kynurenic acid has been shown to inhibit the permissive effect of 5-hydroxyindole on the α7 nicotinic acetylcholine receptors discussed above [171]. Kynurenine also attenuates NMDA- or ischemia-induced brain damage
in neonates [179], a protective effect that may be reduced with the ethanol-induced mid-range increase in kynurenic acid shown in our data.

In adult male Sprague Dawley® rats, kynurenic acid applied extracellularly reversibly decreases GABA concentrations in a dose-responsive manner. 7-Chlorokynurenic acid, which also acts at the glycineB site of the NMDA receptor, has no effect on GABA [193]. We demonstrated the kynurenic acid catabolite 7-chlorokynurenic acid, significantly decreased with ethanol treatment at all concentrations except 0.03%.

Additionally, we demonstrate that higher ethanol concentrations resulted in increased vitamin D, potentially suggesting activation of some sort of protective effect from alcohol. The increases in oxindole, another tryptophan catabolite (via indole), during ethanol treatment may indicate indole that would otherwise be used in the formation of kynurenine is not available with ethanol exposure. It also suggests that additional tryptophan utilization of for indole generation with ethanol exposure in the embryo likely limits its availability for protein and NAD and serotonin synthesis.

Others previously demonstrated that mid-range ethanol concentrations are associated with an increase in kynurenic acid, possibly through modification of kynurenine aminotransferase [202]. But, at higher ethanol concentrations, additional dysregulation occurs preventing the activation of the Treg cells that prevent fetal rejection [203].

Furthermore, stress alone affects this pathway. Notably, in tryptophan-infused sheep with late-term chronically diminished placental blood flow, kynurenine is increased in the fetal plasma to a lesser degree than demonstrated with tryptophan-infusion alone [204]. Fetal brain levels show higher levels of kynurenic and quinolinic acids with tryptophan infusion, but embolized placenta offspring had a greater amount of these metabolites [204]. In addition, explants of placental villi from normal mothers and mothers of small for gestational age babies, cultured in hypoxic conditions caused a significant decrease in kynurenine, relative normoxic samples. Likewise, culturing in hyperoxic conditions resulted in a significant increase in kynurenine [205]. These observations support the notion that kynurenine is in essence a marker of oxidation, emphasizing the importance of the lack of ROS protection in the fetus discussed above.

Another kynurenine, 7-chlorokynurenic acid, was present in our metabolome. Though this metabolite has only been described as a synthetic molecule, its changes mirror in a reduced fashion what is happening with kynurenic acid. 7-Chlorokynurenic acid has been shown in rat cortical slices to act as in antagonist to NMDA by binding at its glycine receptor site [206,207]. This inhibition of NMDA was not reversible by
the addition of additional NMDA, but was reversible by the addition of glycine [206]. Further, in 10-week old Sprague Dawley® rats, activation of the NMDA receptor glycine site by the addition of glycine in the dorsal vagal complex results in a decrease in glucose production in the liver [208]. The antagonist 7-chlorokynurenic acid, decreased production of glucose [208]. 7-Chlorokynurenic acid is more potent and selective to the glycine binding site on NMDA than kynurenic acid [197]. These studies support the importance of 7-chlorokynurenic acid in our metabolome, and support the need for further investigation.

Quinolinic acid, an indirect metabolite of kynurenine via kynurenic acid, can promote NMDA activation of select glutamate receptors that can eventually cause excitotoxic neuronal death [179,209]. Accumulation of quinolinic acid occurs in neuroinflammatory diseases such as Alzheimer’s, cerebral malaria, poliovirus brain infection, and ischemic brain disease [173,210,212]. This increased level of quinolic acid had a strong correlation with upregulation of IDO as well as the severity of injury [211]. The tryptophan catabolites 3-hydroxykynurenine, 3-hydroxyanthranilic acid, anthranilic acid, and quinolinic acid were not present in our data set, likely due to their small sizes being beyond the limits of detection in our assay. However, their potential impact should not be ignored in future investigations.

Vitamin B₆ in the form pyridoxal phosphate, is a limiting coenzyme for kynureninase and cofactor of kynurenine aminotransferases. It was not identified as a significantly different metabolite in our experiments [153]. However, chronic alcoholism is associated with low pyridoxal phosphate. Our model of chronic embryonic exposure may similarly be taxing the zebrafish embryo. In addition, pregnancy can often result in decreased levels of B₆. This can have a marked effect on kynureninase levels. Thus, diminished levels of B₆ can result in increased levels of 3-hydroxykynurenine as well as the neurotoxin xanthanurenic acid [153]. In addition, this potential increase in xanthanurenic acid has implications on energy metabolism. This compound forms a chelate complex with insulin and reduces receptor sensitivity of insulin receptors [213].

Interestingly, we also identified uncaric acid A elevated in the 1% ethanol treatment group. This alkaloid has been reported to act as a kynurenine-3-hydroxylase inhibitor, which if inhibited, could cause an excessive amount of kynurenine to be present or cause an increase of tryptophan conversion to oxindole [202,214,215]. Uncaric acid can inhibit IDO, potentially inhibiting the protective IDO-induced maternal T cell fetal tolerance [203].
In conclusion, at the highest ethanol concentration, levels of inhibitory uncaric acid are quite elevated, potentially causing the reduction IDO activity and a resulting decrease in kynurenic acid and 7-chlorokynurenic acid at the same concentration. This also might explain the increase in oxindole at the highest ethanol concentration, as tryptophan would be shunted away from kynurenine production. This could affect the activation of \( T_{\text{reg}} \) cells that prevent fetal rejection \[203\]. The decrease in kyurenine catabolites at the highest concentration, however, may be due to increased production of 3-hydroxykyurenine, which is known to cause neuronal cytotoxicity and oxidative stress \[179\]. Quinolinic acid, another metabolite of the kynurenine pathway, can cause NMDA activation of glutamate neuroreceptors as well as act as an endogenous excitotoxin \[179\]. Our evidence supports the work of others that demonstrate kynurenine pathway modifications are important in the pathways involved in FASD. Further work is needed to fully understand the ramifications and possible biomarkers of the kynurenine pathway.

**Oxidative Stress**

One hypothesis as to why alcohol is such a potent teratogen is that its catabolism results in the formation of reactive oxygen species (ROS) \[9\]. Denham Harman originally put the concept of oxidative stress forth in the 1950s. Advanced as a “free radical theory”, he hypothesized that endogenous oxygen radicals generated within cells caused a net result of damage to the tissues. This concept of oxidative stress is now believed to be largely associated with generation of oxygen radicals by mitochondrial metabolism within the cell. The presence of acetylaldehyde in elevated concentrations can lead to oxidative damage and the consumption of glutathione \[9\]. Further, it is the change in the cellular responses to the free radicals that is a major regulator in the aging process throughout the body \[216\]. The damage caused by free radicals can dramatically alter the composition and function of macromolecules in the cell. ROS have the capability to alter lipids, protein, and DNA by the very presence of the unpaired electron, which is relevant to brain-related changes in FASD. Recent evidence has shown that abnormalities in sphingosine kinase, discussed above, are associated with increased risk of spontaneous abortion. This appears to be due to an increase in neutrophils followed by oxidative damage to the decidua \[217\].

Free radical damage has been advocated by many as an important mechanism in FASD. ROS are generated during alcohol metabolism. Due to the early developmental
stage of the fetus, free radical scavengers that would normally mediate the effects of ROS are absent or insufficient to counteract threats \textsuperscript{[218]}. Changes in acetylaldehyde, butanal, carnosine, aspidospermine, anandamide, and tryptophan may support the oxidant stress hypothesis of FASD.

**Acetylaldehyde**  Butanal can inhibit PLD-induced mitogenesis, as discussed above. The lack of ROS protection makes butanal a greater threat to abnormal PA signaling. We show butanal is significantly different with ethanol exposure, which is likely related abnormalities noted in PA levels in the embryo, and therefore PA signaling functions. Other direct catabolites of ethanol have also been shown to influence astroglial cells. Acetylaldehyde in the presence of astroglial cells inhibits their growth, and in conjunction with ethanol can increase superoxide dismutase \textsuperscript{[218][219]}. While increased SOD would be protective, the oxidative stress noted in FASD suggests that such an increase would be sufficient enough to ameliorate the alcohol-induced distress demonstrated in our model.

**Carnosine**  Carnosine was identified in our polar metabolites with a biphasic response. Such a response is common amongst other members of the vitagene family, important to neuroprotective mechanisms \textsuperscript{[220]}. It is synthesized in the brain (glial and neuron) by carnosine synthetase, as demonstrated in Sprague Dawley\textsuperscript{\textregistered} rat olfactory glial cells in culture \textsuperscript{[220][221]}. Importantly in our model, carnosine is neuroprotective against nitrosative and oxidative stress \textsuperscript{[220][222][223]}, functioning as a “proton buffer, metal chelator, antioxidant, antiglycating, immunostimulant, antitumoral and wound-healing agent” \textsuperscript{[220]}, and alters neuronal excitability due to zinc and copper induced neurotoxicity \textsuperscript{[220][224]}. Carnosine has been proposed as a treatment for neurodegenerative disorders \textsuperscript{[220]}. But, to our knowledge, it has not been reported on for its role in FASD since 1981, when it was proposed to be relevant to a 50% decrease in fetal free plasma histidine demonstrated after maternal ethanol exposure \textsuperscript{[225]}. Given the significant decrease at mid- and high- ethanol concentrations, this metabolite requires further exploration in its role in FASD etiology as well as a potential intervention.

**Tryptophan and Aspidospermine**  Other metabolites possibly changed via FASD oxidative stress include tryptophan and aspidospermine. Oxidative stress may also
play a role in tryptophan catabolism, as tryptophan’s aromatic nature makes it susceptible to oxidation to \( \text{N}-\text{formylkynurenine} \)\textsuperscript{226}.

In spite of the increased aspidospermine \( \text{with mid-range and high ethanol doses, relevance of this metabolite in our system is unclear. Aspidospermine is an alkaloid known to be an inhibitor of \text{Trypanosoma cruzi} \text{trypanothione reductase} \textsuperscript{227}. Trypanothione (di(glutathionyl)spermidine)reductase is a protazoic “NADPH-dependent flavoenzyme that helps fight oxidative stress by maintaining adequate levels of trypanothione T[SH]_2”} \textsuperscript{227,228} It functions as a “glutathione-glutathione reductase system in these parasites and appears to be involved in defense against oxidants and some heavy metals” \textsuperscript{228}.

**Oxidative Stress Resistance**

**Vitamin E** In addition to alterations in metabolites associated with oxidative stress, we noted changes in metabolites associated with mediation of oxidative stress. Vitamin E is believed to function largely in an antioxidant capacity. Supplementation with \( \alpha \)- or \( \gamma \)-tocopherols to ethanol-exposed chick eggs prevents fatty acid changes of membrane composition, brain mass, and brain protein levels \textsuperscript{229}. 7′-Carboxy-\( \alpha \)-tocotrienol \( \text{in our metabolome, increased at mid-range ethanol doses, while 9′-carboxy-\( \alpha \)-tocotrienol generally decreased with ethanol. Because of the opposite effect of ethanol on these metabolites, it is possible that the data merely reflects a conversion between the two products, favoring the more saturated hydrocarbon chain with increased ethanol doses. The overall increase in vitamin E may suggest an increase in mechanisms which preserve fatty acid membranes and brain composition.} \textsuperscript{229}

\( \text{N,N′-Diphenyl-p-phenylenediamine} \) \( \text{N,N′-Diphenyl-p-phenylenediamine (DL-DPPD)} \) \( \text{has antioxidant and anti-inflammatory capabilities, and was elevated approximately 6.1-fold in all concentrations tested} \textsuperscript{230}. The endogenous origin of this metabolite is unclear. This metabolite has been shown to be well-tolerated in adults, but negatively impacts pregnant rats. Individuals suffered from hypothermia, decreased litter size, significantly prolonged gestation, dystocia, and even death \textsuperscript{231}. In neonatal rat cardiomyocytes \( \text{DL-DPPD} \) has also been shown to reduce lipid peroxidation caused by oxidative stress \textsuperscript{232}. In cultured, serum-starved rat cortical neurons, supplementation with \( \text{DL-DPPD} \) significantly decreased the production of
reactive oxygen species [233]. And, in cultured hepatocytes, supplementation with dl-DPPD prevents ferrous, vanadium, and copper ion-induced lipid peroxidation, protecting even more that α-tocopherol [234]. Similarly, dl-DPPD inhibited the lipid peroxidation of rat liver microsomes incubated with ascorbic acid and ferric sulfate, as well as protect enzymes (aldehyde dehydrogenase), from activation [235]. When administered to rats, dl-DPPD does not cause an increase in ubiquinone, but has been describe as having a protective effect on the levels of vitamin A, which is known to influence ubiquinone levels [236]. These functionalities of dl-DPPD may contribute to difficulties in elucidating FASD mechanisms.

8-Oxo-dGMP The mutagenic 8-oxo-dGMP was increased in all ethanol treatment concentrations. This metabolite can be generated through MutT Homolog 1 by binding to the nucleotide and hydrolyzing 8-oxo-dGTP to 8-oxo-dGMP [237]. This is a mechanism that protects DNA from damage from oxygen free radicals generated by mitochondrial oxidation. The significant increase in 8-oxo-dGMP supports the commonly held belief that ethanol exposure is increasing oxidative stress in FASD, and that the embryos is compensating for this.

Other

Anandamide Anandamide and PKC-related metabolites may also be relevant to FASD nervous system changes. Astroglial cells are important support neurons in the nervous system. Astroglial cells perform support functions to other neurons, including providing nutrients, supporting biochemical reactions and homeostasis, and repair of damaged neurons. They also direct synapse formation, maturation, and elimination by the secretion of chemicals that induce relevant changes [238]. Importantly for lipid alterations occurring in FASD, this process requires cholesterol, which has been shown to increase excitatory synapse formation in retinal ganglion cells as well as influence pre-synaptic neurotransmitter release [238, 239].

We demonstrated a significant decrease in anandamide. Others have demonstrated that under acute ethanol exposure (0.75, 2, or 4 g/kg), anandamide in Wistar male rats decreases (not statistically significantly) in the cerebellum, nucleus acumbens and hippocampus [240]. Anandamide has been demonstrated to have many relevant neurophysiological properties, including the ability to induce PLD in some cell types [139, 241], modulate ROS production [242], and modulates both L-
and T-type calcium channels \[243\]. Anandamide modulates intracellular calcium levels via interacting with the cytosolic domain of the transient vanilloid receptor type 1 (TRPV1) channel protein \[244\]. Additionally, at low concentrations anandamide prepares the mammalian blastocyst for implantation, however at high concentrations anandamide alters calcium concentrations \[245\]. Thus, ethanol’s alteration of anandamide, which must be narrowly controlled for successful implantation, may contribute to decreased rates of successful pregnancy in alcohol-consuming mothers.

In astroglial cells in particular, calcium signaling is important in signal transmission. However, compared to the dramatic changes detectable in neuronal action potentials, calcium signaling in astroglial cells appears to occur at a much slower and more difficult to detect (smaller potential difference) than in other neurons \[246\]. This implies it would take low levels of alteration of calcium signaling capabilities to disrupt normal astroglial function and therefore synaptogenesis. Therefore, calcium signaling interruptions caused by changes such as the significant decrease in anandamide we demonstrated, could be an important astroglial change in FASD.

**Protein Kinase C** Membrane bound PKC is present in neurons, and in conjunction with calcium may be activated by DAG and possibly PI-ceramide. PKC in turn phosphorylates other proteins in astrocytes and responds uniquely to alcohol, as compared to neuronal stem cells. In human neuronal stem cells, 10 mM ethanol activates membraneous, but not cytosolic, PKC \[247\]. Astrocytes, in contrast, do not show activation of membranous nor cytosolic PKC \[247\]. Upon stimulation with 25–200 mM of ethanol in LRM55 astroglial cells, PKC appears to translocate from the cytosol to the membrane \[248\]. This change may precede activation of apoptosis in response to the cellular damage \[247\]. However, PKC translocation acutely decreases in “neurons, astrocytes, keratinocytes, lymphocytes, and platelets” in response to ethanol as reviewed by Carter and Kane \[249\]. PKC and one of its substrates, growth and plasticity protein–43, expression have been shown to decrease in response to ethanol exposure in rat hippocampus. Further, it has been proposed that this is due to the deficiency in PKC\(_{\beta2}\) and PKC\(_{\epsilon}\) translocation from the membrane surface of hippocampal cells PKC, an effect that was statistically significant in animals that underwent contextual fear conditioning \[250\].

In addition, ethanol appears to alter activation of targets downstream from PKC. In cultured cortical astrocytes, “ethanol raises the set-point for basal [mitogen activated protein kinase (MAPK)] activity, thereby changing [Platelet-Derived Growth
Factor (PDGF)-mediated increases in MAPK activity into an antiproliferative signal” [251]. In the brains of ethanol-exposed adult zebrafish, MAPK-related changes in microRNA occur [252]. In lysates from cultured guinea pig airway smooth muscle cells, sphingosine, which was significantly increased in our metabolome, prevented activation of MAPK and PKC [253].

2.4.4 Cardiovascular System

Myocyte Contraction

PKC is activated by calcium in order to induce excitation-contraction in the L-type cardiomyocyte. (This type of channel is long-lasting, and passes calcium inward to the cardiomyocyte; it couples excitation-contraction coupling.) It may also be activated by DAG and PI-ceramide. The latter is present at decreased levels when zebrafish embryos are exposed to ethanol. This may be due to alcohol-associated shunting of ceramide to sphingosine in conjunction with decreased PI. Ceramide is a known inhibitor of PKC [136]. Perhaps as compensation, PKC activity is acutely increased by ethanol [249].

PKC activation of myocyte contraction is regulated by phosphoinositol-3 kinase (PI3K) signaling [254]. Ethanol can interfere with excitation-contraction coupling through calcium loading and apoptosis in neonatal myocytes [255,256]. Interruption of this pathway might be associated with persistent cardiac abnormalities in FASD. PI3K also plays an important role in the movement of cells during gastrulation. In response to chemoattractant gradient signals, PI3K localizes directionally with respect to migrating cell [257]. In the zebrafish, gastrulation has been established as 5.25–10 hpf, during the window of our model’s ethanol exposure [258].

In addition to the neural-related functions of anandamide discussed above, anandamide can inhibit L-type calcium ventricular myocyte signaling [259]. The significant decrease in anandamide at mid- to high-doses of ethanol suggest abnormal modulation as a result of these alterations.

Minosaminomycin, originally identified as a mycobacteria antibiotic, was elevated at 0.1 and 0.3% ethanol. A major component of this molecule is L-myoinosamine-1, which may be synthesized from D-inositol [260]. It is unclear what the relationship is to ethanol-induced changes in our model or how it might relate to the...
overall etiology of FASD. However, PI-cer also contains a similar structure and was significantly changed in our model.

In addition, pathways downstream of PKC are associated with FASD cardiovascular abnormalities. PKC activates sphingosine kinase, which phosphorylates sphingosine to form sphingosine-1 phosphate. Morpholinos generated with sphingosine-1 phosphate receptor 1 demonstrated a phenotype with similarities to FASD, including global and pericardial edema and vascular abnormalities. Knockdown of sphingosine-1 phosphate receptor 2 in conjunction generated even more severe vascular anomalies as well as earlier death.

Many changes noted in our model support the notion that in spite of alterations induced by ethanol, surrounding molecules are being modified to compensate for ethanol’s effects on PKC activity or more broadly bioactive lipid functions. Increases in palmitate metabolites may be to maintain PKC signaling induced by DAG, to maintain levels of sphingolipid *de novo* biosynthesis, or as a result from reduction of phosphatidylethanolamine to eventually generate palmitate. It may also be compensating for decreases in anandamide, which induces calcium signaling for PKC activation. DAG may also be contributing to the increases noted in PA via conversion by DAG kinase. Further, levels of these products are innately disproportional to each other, so small changes in one reactant may have a large impact, making it critical to understand precisely what metabolites are acting as signaling effectors in FASD. The complexity and variety of bioactive lipid changes are important to explore to understand cardiovascular etiology.

Lastly, diginatigenin is a cardiac glycoside previously identified in *Digitalis lantana*. Though it is unclear how this metabolite was generated endogenously, we identified a significant change with ethanol treatment. It was increased in many concentrations, but decreased greatly in the 0.3% ethanol treated embryos. Cardiac glycosides inhibit Na⁺/K⁺-ATPase increasing cardiac contractility, as demonstrated in human-induced pluripotent stem cells.

### Vasculature

Many metabolites identified are associated with the cardiovascular system. In addition to the neural-related effects ethanol has on PA discussed above, there is strong evidence that PA has a role in normal angiogenesis and cardiovascular function. In our model, at 0.03% or more ethanol we observed significant lowering of palmitoyl
3-carbacyclic PA (CPA). This cyclic PA, as discussed above with respects to its neural impacts, is a product of PLD and can also be produced via transphosphatidylation of lysophosphatidylcholine by autotaxin in human serum [111]. CPA is also produced by autotaxin in blood, which has also been linked to vascular development in zebrafish [111]. CPA has also been reported as a bioactive lipid bound to human serum albumin [110]. This suggests the significant lowering of CPA at 0.03% ethanol and higher may exacerbate cardiovascular and circulatory abnormalities [266].

We also noted a significant increase in β-aminopropionitrile, an irreversible inhibitor of lysyl oxidase (LOX). LOX is expressed in healthy coronary artery endothelium and in the nucleus of vascular smooth muscle cells [267]. In mice, LOX mRNA is highly expressed in the developing cardiovascular system [268, 269]. Low-density lipoprotein as well as β-aminopropionitrile inhibit this enzyme and alter normal membrane permeability [267]. The inhibition of collagen cross-linking by β-aminopropionitrile has also been linked to reduced aortic stiffness in young SPF Wistar rats [270]. LOX downregulation in the lab mimics the atherosclerotic process observed in endothelial dysfunction in cardiovascular disease [267].

Coagulation

Several metabolites related to coagulation were identified in our metabolome. Vitamin K₃ was elevated, inconsistently, with ethanol treatment. In 0.1% and 1% ethanol treatments, vitamin K was 5.14 and 5.86 fold higher than control embryos, respectively. Intake of vitamin K below adequate intake has been noted as a nutrition deficit in individuals with FASD. In Western Cape Provence, South Africa, as indicated in a 24-hour recall survey of mothers of children with FASD, as compared to mothers of children without FASD [271]. In an analysis of 31 children with FASD using parental 24-hour dietary recall, most children did not receive an adequate intake of vitamin K [272]. Similar findings were made in a 24-hour dietary recall of school-aged children with heavy FAE [273].

Vitamin K₃ is a synthetic chemical that can be converted to vitamin K₁ or vitamin K₂ (menaquinone) in the liver. In addition, vitamin K exists in the brain as meaquinone (MK–4), which is converted by a cellular, rather than bacterial process [126]. Reduced vitamin K hydroquinone functions as a cofactor for γ-glutamylcarboxylase. It posttranslationally modifies certain proteins, most of which are involved in coagulation, γ-carboxylating at specific glutamic acid residues [126, 274]. As summarized
by Tsaioun, “the highly negatively charged [product] appear[s] to be involved in Ca\(^{2+}\)-dependent phospholipid binding, necessary for the biologic activity of all the blood coagulation factor” \[274\]. Vitamin K is rarely used in treatment of deficiencies due to hemorrhage in infants resulting from difficulties in dosing (it has been linked to brain damage and even death, among other symptoms) \[130,149\].

Two coumarin compounds were identified in our metabolome. Coumarins are known to be vitamin K antagonists and anticoagulator \[275\]. Laserpitin, recognized as a coumarin extract from *Angelica keiskei* plants, thus it is unclear why it was present in our metabolome. It was significantly decreased in all ethanol doses, except the highest. Nonetheless, this metabolite change may reflect a causation for cardiovascular abnormalities induced in FASD. This metabolite has been shown to effect lipid metabolism. Six-week old spontaneous hypertensive rats fed a diet with 0.1% laserpitin for 7 weeks demonstrated significant increases in serum total cholesterol, phospholipid, and apolipoprotein E in low- and high-density lipoproteins. There was a significant increase in 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase mRNA and a significant decrease in H-triglycerides and peroxisome proliferation-activated receptor α mRNA \[276\]. In addition, 3-amino-4,7-dihydroxy-8-chlorocoumarin was increased with all ethanol doses. This metabolite is recognized in novobiocin synthesis, an aminocoumarin antibiotic. It is unclear why the directional change of these two metabolites appears in opposition.

However, coumarins have been shown to significantly reduced sulfatides in 16-day old male Swiss mice (ICR), an effect that was resuable with vitamin K treatment \[126,277\]. This occurs via inhibition of sulfotransferase activity \[278\]. We noted that C20 sulfatide changed little from controls, but C22 sulfatide was increased significantly with ethanol. Our findings suggest that ethanol may be inducing a compensatory effect on vitamin K, which in turn supports maintenance of sulfatide levels. However, the functional difference between sulfatides C20 and C22 in this model remain unclear, and should be further explored in the context of FASD—perhaps they are related to the opposing coumarin effects noted above.

The electron carrier sulfated dihydromenaquinone–9, was also identified as a significantly changed metabolite, decreased marginally with ethanol treatment. The inter-relationship between menaquinone and sulfated dihydromenaquinone–9 is unclear in this model \[279\].
2.4.5 Endocrine System

Sex Hormones

Several important components of the endocrine system were modified in our metabolome, including sex hormones, the hypothalamic-pituitary-adrenal (HPA) axis, and icosanoids. Numerous studies have documented differences in male and female offspring responses to prenatal alcohol exposure. While the design of our study does not allow for examination of direct effects between sexes, many of the changes noted in our metabolome are relevant to exploring the nature of these differences. Changes upon ethanol exposure in steroids related to sex-hormones were noted in our metabolome, including significant decreases in 13-ethyl-16,17-dihydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one as well as significant increases in 16-dehydroprogesterone, 1α-hydroxyesterone, and 13-hydroxy-3-oxo-13,17-secoandrost-4-en-17-0ic acid. These changes are consistent with previous studies reporting that pre-natal ethanol exposure alters sex hormones in offspring. In normal metabolism cholesterol can be converted in to pregnenolone, which is then converted to progesterone. This is then converted to downstream products such as cortisol, corticosterone (and then aldosterone), or testosterone (eventually converted to estradiol). Although the role in normal metabolism of 13-ethyl-16,17-dihydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one is unclear, the significant decrease in may suggest an increase in conversion of pregnenolone to progesterone. This is supported by the increase in 16-dehydroprogesterone which occurred with ethanol treatment. Previous findings of transient nuclear progesterone receptor expression in the brain of developing rodents suggest that progesterone plays a role in normal cortical development [280]. Adult zebrafish express progesterone receptors in all regions of the brain, and strongly in radial glial cells [281]. Further, ethanol alters the ability of cultured placenta cytotrophoblast cells to generate progesterone, potentially by preventing cholesterol from entering organelles, perhaps highlighting a compensatory mechanism of the increased 16-dehydroprogesterone, if also true in placentates [31]. In addition, 1α-hydroxyesterone is significantly increased, supporting the notion of increased conversion of pregnenolone to progesterone. Serum hormone analysis of young male rats fed a 35% isocalorically-controlled ethanol diet from postnatal days 35–99 show a significant increase of estradiol and leptin, decrease in IGF–1, and no change in testosterone levels. In females, progesterone, insulin, and IGF–1 are significantly reduced [282]. (In this study progesterone levels in males and testosterone levels in females were not reported.) Dihydrotestos-
terone is known to be significantly decreased in the brains of 1–2 postnatal day male Sprague Dawley® rats from dams given 35% isocallorically-controlled ethanol diet for gestational days ~16 and on 283. 13-Hydroxy-3-oxo-13,17-secoandrost-4-en-17-oic acid, common name testolate, is an androgen derivative that was significantly changed with ethanol exposure in our metabolome. The relevance of this metabolite is also unclear, but it can be derived from steroid-lactonase conversion of testololactone, a progesterone derivative and steroid estrogen synthase inhibitor. Testolactone can be synthesized from phytosterols 284. The significant increase in this metabolite may indicate decreased levels of estrogen synthase inhibitor, which may be related to the significant increases in estradiol noted in ethanol-exposed offspring. Further research is needed to understand the implications of the sex-hormone related metabolites demonstrated in this metabolome.

**HPA Axis**

The ethanol-induced alterations of the hypothalamic-pituitary-adrenal axis has been extensively examined 285. As reviewed by Smith, et. al, during normal metabolism, the periventricular nucleus of the hypothalamus releases corticotrophin-releasing factor (CRF), which signals to the pituitary. The pituitary gland responds with activation of cAMP signaling, which induces adrenocorticotropic hormone (ACTH) release into circulation. ACTH binds to a melanocortin receptor, stimulating glucocorticoid synthesis (generally cortisol and corticosterone) and release, which acts in a negative feedback loop 286. Glucocorticoids are produced in the adrenal cortex and largely regulate carbohydrate metabolism. Glucocorticoid receptor trafficking proteins are altered in prenatally alcohol exposed c57BL/6J mice, linked to frontal cortical learning deficits 287.

Adult animals with prenatal ethanol exposure are hyper-responsive to handling and chronic stress. Female Sprague Dawley® rats prenatally exposed to ethanol have increased plasma adrenocorticotropic when subjected to repeated handling restraint stress 288. This was a persistent effect in middle-aged adult animals as well, and appears to be associated with the increase in the basal levels of hypothalamic CRF, in spite of the actual levels of cortisol and adrenocorticotropic hormone having no difference between sexes, whether ethanol teated, pair fed, or controls 289 290. This work also demonstrated a sex-specific effect of cold stress on the HPA axis, showing the ethanol-exposed males had higher hormone responses to cold stress 289. Ad-
ditionally, prenatally ethanol exposed Sprague Dawley® rats also exhibit enhanced ACTH sensitivity to increased NO levels in the brain, particularly in males [291].

Corticosterone is known to be significantly increased in the brains and plasma of 1–2 postnatal day Sprague Dawley® rats from dams given 35% ethanol diet from gestational days ~16 and on [283]. 19-Hydroxydeoxycorticosterone, or the same m/z entity cortexolone, was 5.82-fold higher than controls at 0.3% ethanol, but was not different from controls at lower ethanol concentrations. Cortexolone is a glucoorticoid, which when oxygenated can form cortisol. In intact or regenerating rat adrenals, 19-hydroxydeoxycorticosterone can be produced from progesterone, which was significantly increased in our metabolome, or alternatively deoxycorticosterone [292 294]. Elevations in this metabolite may therefore suggest that in response to moderate or higher ethanol concentrations the adrenal gland function has been altered. In adrenal adenomas in humans and canines with Cushing’s disease, a decreased responsiveness to ACTH has been documented in the release of steroid hormones, such as progesterone. This has been suggested to be due to a blockade of 11β-hydroxylase, which hydroxylates steroids at the 18-position to generate aldosterone [295 296]. Interestingly, decreased cortisol levels in premature infants in response to illness is hypothesized to be due to an immature response of this enzyme [297]. Thus, further investigation may be useful to understand the role of 11β-hydroxylase in FASD.

It is worth noting that there is some maternally derived cortisol in the yolk of the zebrafish, and offspring cortisol does not increase significantly until after the embryo hatches [298]. Little, if any, maternally-derived steroid acute regulatory protein or 11β-hydroxylase are present, though the latter does significantly increase prior to hatching between 0–15 hpf [298]. Further, Alsop hypothesizes that the only after hatching is the corticosteroid axis fully developed [298]. As discussed by Tokarz and reviews referenced within, steroid synthesis in teleosts is largely controlled by the hypothalamus-pituitary-interrenal axis and hypothtlamic-pituitary-gonadal axis, taking place primarily in the gonads, brain, and interrenal gland (similar to the adrenal) [299].

In our work, very low levels of ethanol result in a significant increase in 6-hydroxydexamethasone, but it was not different at mid- or high-ethanol concentrations. Normally a metabolite of the synthetic compound dexamethasone, this metabolite functions like cortisol as an anti-inflammatory and an immunosuppressant [71 300]. Dexamethasone has been shown to act primarily on the glucocorticoid
receptors in the pituitary, resulting in suppression of adrenocorticotropin hormone, thus preventing subsequent production and release of adrenal cortisol [301]. Thus, 6-hydroxydexamethasone being elevated with low ethanol concentrations may indicate cortisol levels are lowered by ethanol. Dexamethasone suppresses both resting and stress-altered levels of the glucocorticoid corticosterone and ACTH in prenatal ethanol exposure [302]. Although the mechanism of endogenous formation of 6-hydroxydexamethasone is unclear, the changes in 6-hydroxydexamethasone, in conjunction with the increase in 19-hydroxydeoxycorticosterone may suggest that at low- to mid-range doses of ethanol an endogenous suppression of the glucocorticoid response element by 6-hydroxydexamethasone acts to prevent stimulation of the HPA. However, at mid- to high-range ethanol concentrations the system can no longer suppress pathological induction of ACTH-induced glucocorticoid release. Alternatively, the increase in cortexolone may indicate a failure of the negative feedback loop responsible for suppressing further CRF release from the pituitary.

Icosanoids

We identified several icosanoid metabolites, including anandamide (discussed further below), a leukotriene B$_4$ (LTB$_4$), and prostaglandins. Associations between alcohol induced pathogenesis, including FASD, has long been associated with altered essential fatty acid and prostaglandin metabolism, including increases in prostaglandin E [303, 304]. 12-Oxo-10,11-dihydro-20-COOH-LTB$_4$ was greatly increased in low concentrations of ethanol, but was not different from controls at moderate to high ethanol concentrations.

Consistent with previous reports in the literature, we found several prostaglandins significantly changed with ethanol exposure. These include prostaglandin G$_1$, 17-phenyl trinor-13,14-dihydro A$_2$, and 17-phenyl trinor E$_2$ serinol amide. We also identified 6-keto-prostaglandin F$_{1\alpha}$-d$_4$ was significantly elevated at mid-range ethanol concentrations and higher. It is unclear what the identity of this metabolite is, as we were not using deuterated chemicals. The presence of this metabolite, however, is consistent with findings that infants with fetal alcohol effects excrete higher levels of 6-keto-prostaglandin F$_{1\alpha}$ and its metabolite 2,3-dinor-6-keto-prostaglandin F$_{1\alpha}$ [305]. 6-Keto-prostaglandin F$_{1\alpha}$ was increased in fetal, but not significantly changed on maternal or fetal aspects of ethanol perfused human placental cotyledon [306]. Each of these metabolites demonstrated a profile similar to
the 12-oxo-10,11-dihydro20-COOH-LTB in response to ethanol treatment. The metabolite 17-phenyl trinor prostaglandin E serinol amide was significantly increased at low ethanol concentrations. Prostaglandin E has a variety of important functions, including inducing uterine concentrations, participating in platelet aggregation, maintaining fetal patent arteriosus, mediating portions of the stress response, and can act in both pro- and anti-inflammatory manners. It is known to be increased in uterine-embryo tissue of c57BL/6 mice after 5.8 g/kg ethanol treatment. Fetal sheep exposed to ethanol microdialysis have no change on preterm or near-term plasma PGE, but mixed effects on cerebral cortical PGE. It is believed that ethanol enhances conversion of dihommo-γ-linoleic acid to PGE and thromboxane B2.

(12S)-Hydroxy-16 heptadecynoic acid, elevated at intermediate ethanol concentrations, can inactivate pulmonary Cyp4A4, which catalyzes ω-hydroxylation of prostaglandin. This may explain prostaglandin increases with mid-range ethanol doses noted in our metabolome.

Melatonin, a tryptophan derivative, and its kynuramine metabolites inhibit formation of prostaglandins in a dose dependent manner. Kynuramines may also be formed from kynurenine or indoleamines. We noted ethanol-induced changes in kynurenine metabolites, as discussed above, which showed kynurenic acid increased at mid-range ethanol doses, but otherwise unchanged from controls. While at first glance these notions may contradict each other, the alterations may be due to altered pathway shunting prior to kynurenine’s conversion to kynurenic acid, resulting in a reduction in tryptophan-pathway related inhibition of prostaglandin synthesis.

### Renal System

(12S)-Hydroxy-16-heptadecynoic acid is a hydroxylated, saturated fatty acid that was elevated at intermediate ethanol concentrations. It has been reported to have the capability to inhibit renal vessel vasoconstriction by preventing arachidonic acid ω-hydroxylation.

In zebrafish, pronephros nephron morphogenesis is identifiable at 32–33 hpf, but the rudiments are present as early as the 3-somite stage and identifiable at the 8-somite stage. The primary function of the pronephros in zebrafish is osmoregulation, and early on are functionally limited. In spite of this, the pronephros is considered a relevant model for the human kidney. The pronephros duct is
apparent at the 14-somite stage [258]. Vascularization and glomerular filtration occurs as early as 36–48 hpf [314]. Thus, during the time of our ethanol exposure, increases in intermediate ethanol concentrations may inhibit vasoconstriction via an increase in (12S)-hydroxy-16-heptadecynoic acid.

2.4.7 Growth and Energy

Prenatal ethanol exposure has been associated with later-life changes in energy metabolism, including weight gain, insulin resistance, and body weight. FASD-related growth deficits are not recovered after pregnancy. In a follow-up study of mother-child pairs, the reported pregnancy alcohol consumption was a significant predictor of size [316]. This included levels less than one drink per day. Some have suggested this is due to ethanol-induced hypothalamic oxidative stress and altered melanocortin [317]. Studies have shown that 80% of individuals with in utero ethanol exposure have decreased subcutaneous fat, congruous with abnormalities in energy production [318]. Kaminen-Ahola, et al., have investigated ethanol-mediated postnatal growth reductions in FASD. Pregnant c57BL/6J dams were provided 10% ethanol solution until E8.5, and were then cross-fostered by control dams. (It is important to note that this study was not matched with an isocalorically-controlled group.) In the liver, they noted significant change in genes that encode hepcidin. Genome-wide association indicated enrichment of genes associated with lipid metabolism (Grb10) and growth (Vldlr, Pparg, Scp2, Nr5a2, Ehhadh, and Abcg5), amongst others. We noted changes relevant to many of these altered genes in our metabolome.

Glycolysis and Gluconeogenesis

Glucose regulation, glycolysis, and gluconeogenesis are important for normal growth, development, and metabolism. Abnormalities in glucose metabolism have been widely suggested as a causation for the reduced growth demonstrated in FASD. For example, glucose turnover is significantly reduced in term Sprague Dawley® rats fed a diet with 30% calories derived from ethanol [319]. The use of radioactive 2-deoxyglucose suggests that placental transport of glucose is likely inhibited [28]. In gluconeogenesis, bicarbonate combines with pyruvate and ATP to generate oxaloacetate, ADP, and inorganic phosphate. We noted significant increases in sodium bicarbonate at mid-range and higher ethanol doses, which may support increased utilization of ATP.
3-Hydroxyglutaric acid is known to accumulate in glutaryl-CoA dehydrogenase deficiency, which contributes to altered brain energy metabolism [320]. It is hypothesized to act as an “endogenous uncoupler of mitochondrial respiration” resulting in a blockade of lysine and tryptophan catabolism [321]. The increases in 3-hydroxyglutaric acid at mid- to high-doses of ethanol may account for a portion of the tryptophan- and lysine-related abnormalities in this metabolome.

In addition to the functions of CPA in neural and cardiovascular aspects discussed above, it is an antagonist of peroxisome proliferator-activated receptor-γ, which coordinates fatty acid storage and glucose regulation [322]. The significant decrease of CPA, even at low ethanol concentrations, may also have implications for energy regulation in FASD.

The disaccharide fructoselysine was elevated at several of the ethanol doses tested. Human gut bacteria can convert fructoselysine to butyrate [323]. 2-Oxosuberate is a precursor to coenzyme B in redox reactions in methanogens, so the relevance of this significantly changed methane metabolite is unclear in this model. However, like 2-oxosuberate, (R)-(homo)2-citrate also participates in coenzyme B synthesis, and was elevated at all ethanol concentrations tested except the highest dose.

bis-D-Fructose 2′,1:2,1′-dianhydride increased at intermediate ethanol concentrations. Because of the anhydrous nature of this metabolite, it may simply be a product of ethanol acting on fructose. It may also signify an inhibition in the ability to convert fructose in the liver to glycogen for storage or triglyceride synthesis, or disfunction in glycolysis, which could contribute to abnormal energy regulation.

Fatty Acid Metabolism

Fatty acid oxidation is altered in FASD. For example, consumption of ethanol by adult Sprague Dawley® intact male and female testosterone-treated offspring of dams who had consumed ethanol has been shown to result in a significant increase in fasting serum triglyceride levels as well as diaphyseal bone marrow triglycerides [?, 324]. Triglycerides are then hydrolyzed, forming free fatty acids that can eventually be oxidized in the mitochondria.

β-Oxidation The quaternary amino acid carnitine predominantly functions in transport of long-chain fatty acids into the mitochondria for β-oxidation. It is derived
from N-methylated lysine. Sbriccoli, et al., have stated that supplementation with acetyl-L-carnitine mediates fetal ethanol exposure in Wistar rats, however do not present data to support this. Acylcarnitine, the transport form of fatty acids, is generated by carnitine palmitoyltransferase-1, which conjugates the activated fatty acid with carnitine. This is then translocated across the mitochondrial membrane. In the adult ethanol-exposed Caenorhabditis elegans, carnitine palmitoyltransferases mRNA is slightly higher. We noted increases in hydroxybutyrylcarnitine and 2,6-dimethylheptanoyl carnitine, an acylcarnitine, were increased with ethanol treatment. In addition, crotono-betaine, which was significantly decreased in moderate to high ethanol doses, has been shown to undergo reversible hydrolysis to form L-carnitine in Escheria coli and Proteus sp.

Several metabolites identified in our metabolome are known to be associated with other types of β-oxidation abnormalities. The unsaturated dicarboxylic acid cis-4-decenedioic acid was elevated by as much as 5.94-fold relative to controls. This metabolite has been associated with abnormal fatty acid oxidation due to deficiencies in medium chain acyl-CoA dehydrogenase. In addition, excretion of acetoacetic acid can occur in instances of fatty acid catabolism errors. Acetoacetic acid was increased with low doses of ethanol. This metabolite is a product of acetyl-coenzyme A mitochondrial fatty acid β-oxidation in the liver. Although ethanol treatment did not statistically significantly alter it, 2-octanamidoacetic acid (capryloylglycine), at high ethanol concentrations it did fall below controls by 5.98-fold. 2-Octanamidoacetic acid is the acylated, glycine form of octanoic (caprylic) acid. While normally present as a metabolite of fatty acids, its presence in excretion also occurs in an inborn error of metabolism in the β-oxidation of fatty acids.

Fatty Acid Synthesis In fatty acid synthesis fatty acids are covalently bound to acyl-carrier protein (ACP). We noted significant change in two ACP-bound fatty acids, including trans-2-decenoyl-ACP and (3R)-hydroxy-dodecanoyl-ACP. However, the response was not consistent between doses. Our results suggest abnormal fatty acid synthesis and β-oxidation in FASD. Additionally, the fatty aldehyde 2-decene-4,6,8-triyn-1-al was increased in mid-range ethanol doses in our metabolome. Fatty aldehyde dehydrogenase functions to convert fatty aldehydes to fatty acids, suggesting abnormal fatty acid generation in FASD.
8-Prenylafzelechin 5-methyl ether increased with most ethanol doses. This metabolite is a member of the polyketide class, which are biosynthesized in a similar manner as fatty acids. However, polyketides are not produced by all organisms, so the relevance is unclear.

16-Feruloyloxyhexadecanoic acid, previously reported as a plant extract, increased in all but the lowest ethanol concentration. It is generated by feruloyl-CoA and 16-hydroxyhexadecanoate and belongs to the plant acyltransferase family. While it is unclear why this metabolite appears in this metabolome, it is possible that this is involved with the aberrant fatty acid metabolism noted in FASD.

**Energy and Bile Acids**  As noted above, several bile acids were increased in our experiment at mid-ethanol concentrations relative to unexposed controls, but not at the highest ethanol treatment. Bile acids are formed through the oxidation of cholesterol, so it is possible that increases in bile acids indicate that there has been an increase in the oxidation of cholesterol as the concentration of ethanol increases. At 42 hpf the liver primordium has begun development in the zebrafish embryo. Since there is a limited amount of liver functional capacity at that time, increased bile salts generated from cholesterol oxidation is sourced from the maternally-derived yolk sac. Secondary bile acids, such as cholanoic acids, are formed by bacterial metabolism. The key is the function of bile acids, which is in the digestion and absorption of fats. Thus, the increased levels of bile acids in the zebrafish embryo may be an attempt to increase lipid and energy supply of the compromised zebrafish embryo. Importantly, bile acids have been recognized to have important functional roles as signaling factors in regulating energy expenditure via G protein-coupled receptors, regulating farnesoid X receptor binding that controls cholesterol metabolism, and mediating glucose regulation via both types of receptor. Given the variety and significant changes in bile acids noted in our experiments, further consideration of bile acid modification in FASD is warranted.

**Other Cholesterol Derivatives**  Two other cholesterol metabolites were identified. (25S)-3-Oxo-12β-acetoxy-cholest-1,4-dien-26-oic acid was increased at intermediate ethanol does, and is known to have antihepatotoxic effects. Mid- and high-concentrations of ethanol moderately increased 3α,7α,12α-trihydroxy-5α-cholan-24-yl sulfate, a steroid sulfate oxoanion. The functional relevance of the latter is unclear.
4-Sulfobenzoate is present in some microorganisms, and is known to be generated in *Comamonas testosterone* T–2. *C. Testeosteroni* is capable of growing on steroids which are reversibly converted by α- or β-hydroxysteroid dehydrogenase to the ketosteroid. The enzymes responsible for conversion of sulfobenzyl-alcohol and sulfobenzaldehyde to 4-sulfobenzoate are classified as members of the short-chain dehydrogenases/reductases family of enzymes, of which ADH in some species is included. Our findings demonstrate a variety of cholesterol-derived steroids are modified. We must consider if there is a common lineage to these modifications, to determine if a common alteration exists among them. After cholesterol is synthesized, Cyp side chain cleavage enzyme acts on it to generate pregnenolone. The steroidogenic acute regulatory protein transfers it across the mitochondrial membrane. "Downstream of the synthesis pathway, several enzymes modify the steroid nucleus including side chain cleavage, D5/D4-isomerization, hydrogenation, and aromatization. Other enzymes add and modify functional groups by hydroxylation, reduction, or oxidation". In addition, we identified many metabolite previously recognized only in plants or microorganisms. However, several of them, such as mugineic acid and coumarin compounds, have structures reminiscent of cholesterol. It is possible that these unusual compounds reflect the remains of destroyed cholesterol units.

**Anandamide**

Anandamide, as well as other fatty acid ethanolamides (FAE), function in a variety of conserved signaling mechanisms regulating uterine blastocyst implantation, feeding behavior, energy metabolism, and immune function. Anandamide also modulates growth and lipid metabolism, as demonstrated in the zebrafish, via sterol regulatory element binding protein and insulin-like growth factors. We observed a significant change in the amount of anandamide present with even low concentrations of ethanol, which further decreased with higher ethanol doses. This is consistent with previous research demonstrating that acute intraparitoneal ethanol administration results in significant decreases in anandamide in the brain. Endogenous synthesis of anandamide has been demonstrated. PE is combined with sn-1 arachidonoyl phosphate by the calcium mediated N-acyltransferase. The resulting N-arachidonyl PE is subsequently converted to anandamide, with the loss of PA. And, acute ethanol administration had been shown to not inhibit either of these en-
zymes \(^{240}\). Phospholipase C and \(\alpha,\beta\)-hydrolase 4 can generate intermediates that generate anandamide \(^{344}\). Interestingly, we noted a general trend of increasing concentrations of PA with mid- to high-levels of ethanol, suggesting anandamide is not the source of the increased PA, or alternatively that anandamide is being consumed at a rate greater than it can be generated, contributing to the rise in PA. Also, fatty acid ethanolamine signaling is initiated by the high-affinity, reversible binding of bile acids to membranous \(N\)-acylphosphatidylethanolamine-hydrolyzing phospholipase D \(^{345}\). The erratic response of bile acids to differing ethanol concentrations demonstrated with our metabolome, may indicate alterations in this arm of anandamide signaling as well.

**Amino Acid Metabolism**

**Histidine Metabolism** Histidine is important in the metabolism of several amino acids, as well as the pentose phosphate pathway, and purine synthesis. \(N\-\omega\)-Acetylhistamine is an imidazole intermediate in the metabolism of histidine. Histamine has a potent impact on gastric secretions, bronchial smooth muscle cell constriction, vasodilation, and as a neurotransmitter \(^{346\text{–}350}\). This metabolite is known to be present at high concentrations only the brains of poikilothermic vertebrates, such as zebrafish \(^{351,352}\). However, it was only significantly changed at 0.03% ethanol and higher. Further investigation is needed to understand which this metabolite is significantly higher with higher doses of ethanol.

In rats, chronic maternal alcohol exposure during gestation is associated with increased levels of cerebral histamine and free histidine in fetal plasma in the offspring \(^{225,353,354}\). Histamine, a metabolite of histidine decarboxylation, was not present in our data set. But, using an \emph{in situ} hybridization of larval Turku strain zebrafish, Puttonen et al. showed that the histaminergic neurons were morphologically unaffected by short duration (10 or 30 minute) exposure to ethanol \(^{355}\). However, in 7-day old fish, a 10-minute ethanol treatment of 0.75% and higher resulted in a significant increase in the mRNA of histidine decarboxylase, but levels of the histamine neurotransmitter in whole larvae were unchanged with the ethanol treatment \(^{355}\). We did note a significant change in imidazol-5-yl-pyruvate \(\rightleftharpoons\rightleftharpoons\), which was increased at some ethanol concentrations, is a histidine metabolism intermediate.

\(L\)-Histidine may be converted to hercynine and then ergothioneine. Ergothioneine may then be enzymatically converted to thionuromicanic acid. In our experiments,
this metabolite was elevated at the 0.1% ethanol treatment level or higher. While not present in our data set, its predecessor, the amino acid ergothioneine, has been reported to have antioxidant effects, including prevention of copper-induced DNA and protein damage [356]. Ergothioneine is not produced by animals, but has been reported in marine organisms. However, the increase that occurs with ethanol may indicate increased enzymatic activity that is converting more ergothioneine to thiouroncanic acid.

Additionally, some labs have shown that ergothioneine can prevent the oxidation of hemoglobin and myoglobin, again in contrast to copper [357,358]. Real-time PCR has demonstrated high levels of the ergothioneine transporter in fetal liver and cord blood CD71+ cells [359,360]. Ergothioucanic acid has also been shown to provide an anti-inflammatory effect. In cultured C2C12 mouse myoblasts, palmitic acid reduced cell viability, p38 and JNK phosphorylation, and IL–6 mRNA expression were all significantly improved with ergothioneine treatment [361]. Neuroprotection has also been associated with ergothioucanic acid. Injection of ergothioucanic acid provided to has been shown to prevent toxic over-stimulation of NMDA receptors [359]. It has shown neuroprotective effects against the chemotherapeutic cisplatin in CBA mice [362]. A reduction in β-amyloid-induced apoptosis in rat pheochromocytoma (commonly used for neurobiological cell studies) by ergothioucanic acid has also been demonstrated [359].

The increase in the thiouroncanic acid may indicate that ergothioneine originally present in the embryo has been utilized with the ethanol exposure. As only the higher levels of ethanol exposure resulted in an increase in thiouroncanic acid, this may indicate that metabolism is being shunted away from other histidine metabolites. Less would then be available in the ergothioneine form to provide protection from the copper-related oxidative damage.

**Methionine Metabolism** 5-Methylthio-d-ribose, a metabolite in the methionine metabolism pathway, was significantly decreased with mid- or high-doses of ethanol. This is consistent with previous reports of methionine metabolism changes. Embryoid bodies derived from 0.1% or 0.3% ethanol-cultured human embryonic stem cells show significant decrease of the precursor of this metabolite, 5′-methylthioadenosine [79]. Both of these products are metabolites of S-adenosyl-methionine (SAM), and occur in preparation for methionine salvage. Additionally, response to methionine treatment
of ethanol-exposed mouse embryos has been mixed, showing rescue of only some non-neural malformations [363].

Pyrrolysine, Vitamin B$_{12}$, and Folate

Pyrrolysine, the 22nd amino acid, is genetically encoded in some methanogenic archaea and bacteria, including some strains present in the human gastrointestinal tract [364, 365]. In our experiments, pyrrolysine was increased at all ethanol concentrations tested. In a brief review on the subject, Krzycki discusses that pyrrolysine is an alternative amino acid and one of 300 non-canonical amino acids that often arise as a result of post-translational modification of standard amino acids [366]. Like selenocystein, pyrrolysine is introduced through what normally is a stop codon by a transfer RNA. Lysine, commonly a limiting amino acid of protein synthesis, is believed to be the source of pyrrolysine, formed from two lysines residues in a S-adenosylmethionine dependent process [367, 368]. Unique among amino acids due to its electrophilic imine bond, pyrrolysine may function in the activation, orientation, and transfer of methylamines to corrinoids, proteins that bind corrinoid cofactors containing cobalt [366, 368].

Cobalt toxicity is associated with hematological, endocrine, neurologic, reproductive, and cardiac effects in humans [369]. In zebrafish, previous studies have shown that elevations in cobalt concentrations are associated with increased apoptotic gene expression; concentrations of >100 µg/L copper or higher are associated with abnormalities including decreased rates of survival, increased morphologic abnormalities, bradycardia, and decreased growth [370]. Cobalt ions also “inhibit nucleotide excision and DNA repair by the inhibition of incision and polymerization” [370]. Thus the increase in L-pyrrolysine shown in our metabolome could relate to increased cobalt-bound corrinoids that cause copper-associated teratogenicity.

Vitamin B$_{12}$

Essential vitamin B$_{12}$ has the most complicated structure of all vitamins. It consists of three components, including the corrinoid structure, dimethylbenzimidazole, and associated amides. The complex structure is because of the corrin portion, which is maintained in all human cobalamin transporters [371]. While there are several different forms of B$_{12}$ that vary in charge, the main form in plasma is the protein bound methylcob(II)alamine [372]. Contained within the corrin structure is a cobalt atom. The carbon-cobalt bond is a central building block of complex vita-
min B<sub>12</sub> structure, also known as cyanocobalamin<sup>[371]</sup>. This carbon-metal bond is considered unique in nature.

B<sub>12</sub> is made only by bacteria and can be provided through dietary meat, dairy, and some seafoods<sup>[372]</sup>. A complicated uptake and metabolism has evolved for the handling of B<sub>12</sub>. First, it requires intrinsic factor (IF) from the stomach parietal cells to be bound to B<sub>12</sub> in the lumen of the distal ileum. Only then can the intrinsic factor receptor recognize it and transport it into the enterocyte. Eventually lysosomes target the IF-B<sub>12</sub> complex and break B<sub>12</sub> off. B<sub>12</sub> is then bound to transcobalamin II (TC-II), where it is released into the plasma<sup>[372]</sup>.

Tissue uptake of B<sub>12</sub> requires the TCII receptor for transport to the cytosol. There lysosomes reduce cobalamin to eventually form methionine synthase. The folate-dependent methionine synthase has several functions, notably the catalyzation of nitrous oxide to nitrogen, which can result in the destruction of the polypeptide back bone and inactivation of enzymes<sup>[372]</sup>. In the mitochondria, it is also reduced using cofactors. Eventually methylmalonyl-CoA mutase is formed, which is converted to succinyl-CoA. This is they utilized in amino acid carbons and propionyl CoA, both of which are consumed in gluconeogenesis.

Methyl B<sub>12</sub> (methylcobalamin) functions as a cofactor to aid methionine synthetase in the conversion of 5-methyltetrahydrofolate and homocysteine to tetrahydrofolate and methionine<sup>[373]</sup>. Pre- or co-treatment of hybrid motor neuron-neuroblastoma cells (NSC–34D) with methylcobalamin (but not methyltetrahydrofolate) provided neuroprotection or neurorescue to homocysteine induced caspase-3/7 mediated apoptosis<sup>[374]</sup>. Methyl vitamin B<sub>12</sub> has also been reported to inhibit the homocysteine-induced activation of caspases 3 and 7, thus preventing the associated apoptosis of motor neurons<sup>[374]</sup>. Thus the increased pyrrolysine may indicate an increase in this anti-apoptosis activity.

Amongst complications of low B<sub>12</sub>, spinal column demyelination, peripheral myopathy, memory loss, and altered moods such as depression and anxiety have all been reported<sup>[375]</sup>. Biochemically this deficit can also be associated with increased levels of serum methylmalonic acid and total homocysteine<sup>[375]</sup>. Due to the large size of the B<sub>12</sub> complex, it does not fall within the scope of our assay parameters.

**Folate and 2-Hydroxymethylserine**  Vitamin B<sub>12</sub> and folate function cooperatively. While it has long been understood that ethanol ingestion alters levels of folic acid, some effects of ethanol exposure have been shown to be ameliorated by the com-
combined treatment of folic acid and B12. Cultured Balb/c mouse embryos treated with both folic acid and B12 did not show growth and gross developmental deficiencies when treated with ethanol [376]. The “ethanol-resistant” CD–1 mouse strain is protected from developmental toxicity when treated with folic acid and B12 [377]. Also, folic acid supplementation suppresses changes in microRNA, target genes, and tearatogenesis that occurs in fetal mouse brains after prenatal ethanol exposure [378]. In addition to the well-associated neural tube defects, spontaneous abortions, and decreased weight and length of offspring have been reported [375]. Further, folate insufficiency limits methylation as well.

2-Hydroxymethylserine was significantly higher than controls at intermediate ethanol concentrations. This non-proteinaceous metabolite stabilizes oligopeptide complexes with nickel(II) and copper(II) ions [379]. 2-Hydroxymethylserine, when combined with tetrahydrofolate, can be reversibly converted to yield 5,10-methylenetetrahydrofolate and d-serine by α-methyl serine hydroxymethyltransferase [380]. (A complete review of folate metabolism is beyond the scope of this article, however folate deficiency is associated with neural tube defects in development, decreased DNA synthesis resulting in megaloblastic anemia, placental abruption, congenital heart defects, spontaneous abortion, stillbirths, and fetal growth restriction [381].) Since this reaction is reversible, it is unclear whether the increase in 2-hydroxymethylserine suggests increased conversion from 5,10-methylenetetrahydrofolate or an increased supply of 2-hydroxymethylserine for conversion to 5,10-methylenetetrahydrofolate.

This reaction is dependent on pyridoxal-5′-phosphate (vitamin B6). In conjunction with acetylaldehyde formed by ethanol metabolism, in alcoholic subjects there is a phosphatase-dependent degradation of phosphorylated vitamin B6 that occurs in erythrocytes, which may relate to altered α-methyl serine hydroxymethyltransferase [382]. Cytosolic α-methyl serine hydroxymethyltransferase acts as a metabolic control, preferentially regulating synthesis of deoxythymidine monophosphate over SAM, the primary methylating agent in all cells [383]. SAM is consumed by phosphatidylethanolamine N-methyltransferase, which converts PE to PC. PE was unchanged from controls, however many downstream metabolites of PC were significantly decreased (see above). This supports the notion that deoxythymidine is being preferentially produced to supply needs for DNA synthesis, since it is possible that SAM is unavailable for PE conversion to PC.
Hemizygosity for the cytosolic α-methyl serine hydroxymethyltransferase is associated with Smith-Magenis syndrome [384]. Smith-Magenis patients frequently display a broad flat midface, broad nasal bridge, diminished growth, as well as hyperactivity, and self-destructive behaviors [385]. In addition, in an analysis of human placental villus explants, hydroxymethylserine was significantly increased in tissues from small for gestational age individuals when hypoxic conditions were simulated by changing the oxygen from 6% to 1% (p=0.0010) [205].

Once converted to a coenzyme, folate functions to donate or accept one-carbon moieties reactions related to nucleotide metabolism, amino acid metabolism, or as a regulatory compound. Mitochondria provide most of the single carbons for these reactions. However, cytosolic serine (generated from serine hydroxymethyl transferase) as well as histidine and purines (from 5,10-methenyl-tetrahydrofolate) can provide these carbons as well [381]. Purine was significantly increased with most ethanol doses. The purines adenine and guanine function as nucleic acids in DNA and RNA. Inosine 5′-monophosphate (IMP) was increased at mid-range ethanol doses. It is a purine nucleotide involved in purine and histidine metabolism and secondary metabolite biosynthesis. It is unclear whether the metabolite changes noted here are due to an increased supply of one-carbon moieties for metabolism or decreased demand. However, if we assume that deoxythymidine is increasing, and we see that purines are increased, this may suggest a deficiency in the mechanisms of DNA synthesis.

Ngai and colleagues recently published a study of one-carbon metabolism in dams and fetuses of Sprague Dawley® rats. Their work attributes alterations in one-carbon metabolism and the (implied) long-term programming to genes in the hypothalamic-pituitary-adrenal axis. More work is needed in this area, however, as pair-fed fetuses were not significantly different from ethanol treated fetuses in serum methionine concentrations [386].

Folate interventions have been proposed to mediate FASD. Ethanol appears to interfere with the transport of N-5 methyltetrahydrofolic acid from storage [387]. In addition, measurements of serum folate in maternal blood and umbilical cord blood in chronic drinkers demonstrate decreased ratio of fetal:maternal folate, suggesting fetal supply may be shunted to the chronic alcohol-exposed mother [388]. However, conclusive studies still need to be performed. Our evidence supports folate abnormalities in FASD, and that these changes occur in absence of decreased nucleic acids for DNA and RNA synthesis.
**Mechanistic Relevance**  The changes noted in pyrrolysine levels in our metabolome could implicate several different alterations under the alcohol-exposed condition. It is interesting to note the significant change that occurs across the ethanol treatment concentrations. One possible source of the increased pyrrolysine is an increase in the amino acid lysine. This could be due to the degradation of protein tertiary structure present in the sample, which in turn allows for increased pyrrolysine production. But, given the importance of pyrrolysine in the orientation and transfer of the critical cobalt atom to corrinoid B\textsubscript{12} structure, it is also possible that there is an increased demand for the cobalt of vitamin B\textsubscript{12}. However, one might expect more of a dose response in this instance. An exception to this is if the demands of even our very low ethanol concentrations required increased pyrrolysine-supplied cobalt for B\textsubscript{12}. This theory is supported by existing literature that demonstrates amelioration of morphological abnormalities with folic acid and B\textsubscript{12} supplementation as described above. However, since we are unable to evaluate the quantity of folic acid or B\textsubscript{12} moieties with this protocol, we cannot currently determine whether there is an increased demand remaining at the ethanol concentrations utilized. Further work is needed in this area to determine the roles of each of these important players and to understand how pyrrolysine changes that have been shown here resolve with B\textsubscript{12} and the documented folic acid changes in FASD.

### 2.4.8 Morphology

Abnormal craniofacial morphology and neuronal cell migration occur in FASD\footnote{389}. At 0.01% ethanol and higher, β-aminopropionitrile \footnote{390} was 4.90-fold or more than of control levels. This metabolite is a naturally occurring, toxic amino acid derivative used in the manufacture of polyurethane foam, β-alanine and pantothenic acid. With maternal occupational exposure, it has reported in case studies as being associated with rare Cantrell-sequence birth defects. In addition to other severe morphological abnormalities, children with this genetic defect have “anterior thoraco-abdominal wall defects with ectopia cordis, and diaphragm, sternum, pericardium, and heart defects” \footnote{390}. In this case, the premature infant had aplasia of the right foot and femur, “aplasia of the right kidney, cerebellar hypoplasia, and aplasia of the cutis” \footnote{390}. Craniofacial abnormalities including short neck, low-set ears, and cleft palate were also present in this infant, who died at 2 days postnatally \footnote{390}. 
β-Aminopropionitrile irreversibly inhibits LOX, thereby preventing normal covalent collagen, elastin, and bone cross-linking of lysine and hydroxylserine [267, 268, 391]. LOX is “a copper-dependent amine oxidase that initiates the covalent cross-linking of collagen and elastin. LOX catalyses an oxidative de-amination of lysine and hydroxylysine residues to peptidyl α-aminoadipic-δ-semialdehydes” [267]. Some research has been published linking the copper-dependent enzyme LOX and changes as a result of alcohol consumption, but to date we have found no reports associating LOX and fetal alcohol syndrome.

Rats fed a low-protein ethanol diet showed increased collagen deposition in the liver, which correlated strongly with increased LOX [392]. While this initially may appear to contrast our findings, this could indicate that β-aminopropionitrile is present in the alcohol treated animals and as a result LOX is upregulated to compensate for β-aminopropionitrile’s inhibitory effects.

In zebrafish, LOX morpholinos show “mildly undulated notochord, truncated anterior-posterior axis, tail bending and smaller head”, “a complete disorganization of muscle somites and neural defects”, and “pigment defects and pharyngeal arch deformities consistent with neural crest dysfunction” [393]. LOX is detectable at 3 hpf in zebrafish embryos, with a peak expression at 90% epiboly stage, indicating it is maternally and zygotically expressed [393]. By 48 hpf, Reynaud and co-authors saw expression predominantly in the central nervous system, eyes, pectoral fin, and muscles [393]. They also noted frequent pericardial edema at 48 hpf and beyond in morpholinos [393].

2-Mercaptopyridine-N-oxide has demonstrable LOX inhibitory capabilities, which result in abnormal zebrafish notochord morphogenesis resulting in visible undulations in the embryo’s notochord [394]. These abnormal notochord result in zebrafish that look similar to copper deficient zebrafish, having a wavy notochord [395]. While review of our own morphological experiments with alcohol did not reveal similar patterns, this could be due to the concentration of 2-mercaptopyridine-N-oxide or β-aminopropionitrile used in these experiments, which result in an inhibition of LOX to a much greater extent than that in our experiments (Chapter 4). β-Aminopropionitrile can be formed by the reaction of 2-aminopropionitrile and ammonia at 70°C to 100°C in the laboratory [396]. However, how this compound is formed under biological conditions such as that in our experimental design is unclear and will need further investigation.
In addition, IPA identified dermatological disease as significant due to the presence of the significantly changed polar metabolites urea, ifosfamide, and tretinoin as well as tissue development due to increases in tretinoin and kynurenic acid and the decrease in carnosine. Soft tissue morphological abnormalities have are a key marker in the diagnosis of FASD. A flat mid face with smooth philtrum, abnormal nasolabial fold, abnormal genital formation, and reduced subcutaneous fat, and abnormal palmer creases. (These features are based in soft tissue disorganization rather than epidermal diseases.) In addition, a few laboratories have reported an association of ethanol exposure prenatally with different dermatoglyphics. These included a decrease in whorls, increased ulnar and radial loops, and ridges as well as increased asymmetry.

Dimethyl trisulfide, a volatile organic compound, was significantly decreased with ethanol exposure. This pungent, malodorous metabolite is commonly associated with tissue decay and fungating lesions. It is present in human feces and urine. However, it is unclear why this metabolite is present, but possibly may be due to tissue decay with the ethanol treatment.

### 2.4.9 Cellular and Molecular Mechanisms

**Glycosylation**

We noted changes in several metabolites related to glycosylation, including N-acetylglucosamine, isoprenoids, and 2-(α-D-mannosyl)-3-phosphoglycerate. With mid-range to high ethanol doses, N-acetylglucosamine was significantly increased. N-Acetylglucosamine is used in co-/post-translational glycosylation, marking a glycoprotein for transfer to a lysosome, a secretory granule, or the cell membrane. This metabolite binds to the nitrogen atom of asparagine in the rough endoplasmic reticulum. In addition to directing cellular proteins, glycosylation is essential for normal protein folding as well as normal protein secretion, which has been shown to be disrupted with alcohol exposure. N-Glycans affect the solubility and thermal stability (physiochemical) properties of proteins. Glycosylation can also be used as an indicator of zebrafish egg quality. α-1,3-mannosyltransferase ALG2 mRNA is differentially expressed with differences in fish egg quality.

Theory of glycosylation as mechanisms of FASD was specifically proposed in 2011 by Binkhorst, et al., and findings from previous reports support this notion.
Binkhorst, et al., noted a newborn with a history of maternal alcohol abuse and morphological features consistent with FASD, congenital disorders of glycosylation screening results were temporarily abnormal (assumed to be a false positive) [48]. Ethanol decreases the secretion of the sugar moieties used to glycosylate proteins [404]. The sugar moieties used in these structures vary from prenatal, postnatal, and adult ages [405]. Lastly, with ethanol exposure, tubulin secretion has been shown to increase as much as 33% in neonatal rat livers, while the polymerization of microtubules is diminished [404]. This has been attributed to abnormal glycosylation.

Like the decreased 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one we noted in our metabolome, other isoprenoids are known to decrease with prenatal ethanol exposure and lipoperoxidative ROS [48]. Dolichols, polyisoprenoids that occur in all eukaryotic cell membranes, are essential for \( N \)-glycosylation of proteins (asparagine residues) in the endoplasmic reticulum, which has been shown to be disrupted with ethanol [406]. Dolichol in blood and urine was proposed as an indirect marker for chronic or long-term alcohol use (in spite of referencing a study that marginalized its biomarker value) [51, 407]. Dolichol, while increased in urine after long-term and binge drinking in non-pregnant subjects, was not different in pregnant mothers of infants displaying FASD compared to normal infants [407,408]. However, its involvement in FASD may still be important, as sufficient dolichol is required for glycosylation to begin. In addition, in hepatocytes from rats treated with 6 mg/kg of ethanol for 12 hours, there was a “significant reduction of the de novo synthesis of both dolichyl phosphate and free dolichol” [406]. Measurement of malondialdehyde levels demonstrating the amount of lipoperoxidation, significantly decreased dolichyl phosphate and free dolichol [48,406].

After leaving the endoplasmic reticulum, proteins are transported to the \( cis \)-side of the Golgi apparatus in vesicles. When they emerge from the \( trans \)-side of the Golgi complex, proteins are sorted to lysosomes, for secretion, or to the plasma membrane. “Upon exposure to ethanol, developing L6 neurons manifest disruptions in Golgi apparatus [(compactions)] and cytoskeletal elements which may in turn trigger selective and significant perturbations to primary neurite formation and neuronal polarity” [409].

The metabolite 2-(\( \alpha \)-D-mannosyl)-3-phosphoglycerate was increased by at least 4.81-fold with any ethanol treatment. Upstream of this metabolite is mannose–6-phosphate (M6P). M6P is generated by the activation of \( N \)-acetyl-glucosamine-1-transferase by cathepsin D and is the major lysosomal targeting tag for
lysosomal enzymes, resulting the endocytosis of these enzymes. Dephosphorylation of this metabolite generates α-D-mannosylglycerate, which has demonstrated protective effects under extreme stresses, such as extreme heat or freezing. This metabolite also has protective effects in neuroprotective effects in *in vitro* assays against fibrils that form and are associated with Alzheimer’s disease [410].

Cathepsin D is an aspartic protease, involved in non-specific, acidic lysosomal degradation, apoptosis, and postnatal tissue maintenance [411]. Although cathepsin D is not required for embryonic development, developmental abnormalities have been associated with cathepsin D [411]. Cathepsin D deficient mice demonstrate atrophy of the intestinal mucosa, intestinal necrosis with thromboemboli, and loss of thymus and spleen function two to three weeks after birth [412]. However, these animals do appear to maintain lysosomal proteolysis, suggesting a redundancy of function [412]. As noted by Benes and references within, in *Drosophila*, a natural mutation in sheep, and the American bulldog, inactivation of cathepsin D is associated with accumulation of lipofuscinosis, which is associated with neuodegeneration and neural regression, amongst other symptoms [411].

Zebrafish appears to be missing a domain of cathepsin D to activate phospho-transferase; zebrafish appear to be N-glycosylated at Asn-134 only [413]. Zebrafish experiments that knocked-down cathepsin D have demonstrated a profound morphologic effect, not dissimilar to FASD. Normally present in eggs and developing embryos, knock-down of cathepsin D results in shorter body length, impairment of yolk sac absorption, microophthalmia, and other abnormal morphology [414]. Together, the changes demonstrated in N-acetylglucosamines, isoprenoids, and 2-(α-D-mannosyl)-3-phosphoglycerate suggest glycosylation is important in FASD.

**Transcription Factors**

**Vitamin A** Several transcription factors were noted to change in our metabolome, including vitamins A and D. Vitamin A participates in a wide variety of essential biological processes, including vision, nervous system development and patterning, and a variety of cellular functions. For placentates, maternal plasma is the main source of retinol, while in oviparous species it is supplied by β-carotene in the yolk [415]. In embryonic cells, retinol binds to retinol binding protein 4, which is then transferred to a receptor protein that has been stimulated by retinoic acid 6. Retinol binds to cellular retinol binding protein, and is then converted to retinaldehyde by retinaldehyde
dehydrogenase 10 or ADH. β-Carotene is converted to retinaldehyde by β-carotene oxygenase. Retinaldehyde is converted to retinoic acid by three retinaldehyde dehydrogenases. Retinoic acid can then participate in nuclear transcription regulation or exit the cell [41]. During embryogenesis vitamin A controls homeobox genes responsible for anterior-posterior patterning and tetrapod digit specification [415]. A gradient of morphogenic retinoic acid is established in conjunction with fibroblast growth factor, retinaldehyde, and Cyp26A1, which designates anterior and posterior patterning [416, 417]. In addition, vitamin A is required for normal cardiomyocyte differentiation and heart patterning in the mouse [415, 418].

Retinoid-related metabolism has long been studied as an importantly modified mechanism in FASD. Enzymes required for catabolism of ethanol functionally overlap with those required to convert retinol to retinal and retinoate. For example, during gastrulation in Xenopus embryos, ethanol competitively inhibits retinaldehyde dehydrogenase [419]. Among its several functions, ADH3, along with the cofactor NAD\\(^+\), converts retinol to retinal, which is then able to be converted to retinoic acid [84]. Due to mobilization of hepatic retinyl esters, some tissues are found to have increased all-trans-retinoic acid with ethanol exposure [420, 422]. Additionally, cerebella of Long Evans pups postnatally (P7) exposed to ethanol (~80 mM blood alcohol concentration) show decreased expression of retinoic acid receptors α/γ and increased expression of retinoic acid X receptors α/γ [423]. Interestingly, in genomic interactome analysis of brains of offspring of c57BL/6J mice exposed at various times to 2.5 g/kg ethanol, retinoate metabolism genes were underexpressed [130]. Because of the multiple points of regulation and the extensive array of vitamin A functions, pinpointing cause and effect of vitamin A abnormalities can be difficult [424].

Supplementation with retinoic acid during ethanol exposure has been shown to reduce some ethanol-induced changes that occur in early gastrulation and somitogenesis, including embryo length, ear circumference, and neural defects [423]. However, co-treatment with retinoic acid does not rescue microphthalmia in zebrafish, but does appear to reduce optic nerve hypoplasia and photoreceptor differentiation defects [425, 426]. Ocular developmental defects in FASD models may be due to independent inhibition of retinoic acid and sonic hedgehog pathways [427].

We identified very large decreases of retinoate with low and mid-high doses of ethanol, while 1′-hydroxy-γ-carotene glucoside increased at any ethanol dose (Figure 2.9 and Chapter 3). γ-Carotene can be formed from β-carotene [428]. γ-Carotene can also be converted in to retinol and subsequently
form retinoids, a process that is believed to be blocked directly by ethanol [428]. It has been proposed that 1’-hydroxy-γ-carotene glucoside is formed γ-carotene by the actions carotinoid 1,2-hydratase and glucosyl transferase [429]. The latter is hypothesized to have increased antioxidant capacity because of the addition of the hydroxyl group [429, 430]. Taken together, these may suggest that shunting of β-carotene to γ-carotene may decrease supply for retinoids, suggesting altered function of chembeta-carotene 15,15'-dioxygenase, the oxidoreductase which normally converts β-carotene to all-trans-retinal in animals.

In addition, 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one was present in our metabolome. This metabolite appears as a cleavage product, potentially derived from 11-cis-retinoate. This finding is significant, as it could be a causative mechanism of aberrant vitamin A in FASD.

Relevant to the high number of lipid metabolites that were significantly changed in our data set, retinoic acid does appear to influence lipid metabolism. De novo fatty acid synthesis generates palmitate, which then undergoes processes of elongation and desaturation to synthesize other fatty acids. Stearyl-CoA desaturase I (SCD1), which desaturates saturated fatty acids, and to our knowledge has not previously been associated with FASD, appears to be regulated by vitamin A [431]. In normal and vitamin A deficient BALB/c mice, feeding 0.01 and 0.1% retinyl palmitate lead to induction of SCD1 mRNA [432]. Δ-5-Desaturase, required for synthesizing polyunsaturated fatty acids like eicosapentaenoic and arachidonic acids, also appears to be regulated by vitamin A [433, 434]. As discussed above, anandamide, derived from arachidonate, was significantly decreased in our metabolome with all but the lowest ethanol dose tested. The aberrant vitamin A metabolism might be important to explore for this reason. Our data demonstrates a variety of responses of palmitate and the next larger fatty acid stearate to ethanol treatment. Considered in conjunction with our findings on retinoate and 1’-hydroxy-γ-carotene glucoside, vitamin A disruptions may explain some of the changes we noted in fatty acids.

In conclusion, while the debate about the role of vitamin A disregulation in FASD continues, we, not surprisingly, found significant changes in retinoate, 1’-hydroxy-γ-carotene glucoside, and 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one. However, when taken in conjunction with abnormalities in fatty acids in our ethanol model metabolome, SCD1 should be considered as a novel and interesting pathway modification that requires further investigation.
Fig. 2.9. Zebrafish embryos were treated from 2–24 hpf to 0–3% ethanol. Two vitamin A metabolites, 9,13-cis-Retinoate (Structure 4-8) and 1′-hydroxy-γ-carotene glucoside (Structure 4-10), were identified in the polar fraction. In the non-polar fraction, 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Structure 4-9) is potentially a cleavage product of retinoate. All metabolites were significantly changed, $P < 0.05$.

**Vitamin D**  We detected several changes in vitamin D metabolites that may influence FASD etiology. Classical vitamin D molecules, such as cholecalciferol ($D_3$), 25-hydroxyvitamin $D_2$, 25-hydroxycholecalciferol, 1,25-hydroxyvitamin $D$, 24,25-hydroxyvitamin $D_3$, 25,26-hydroxyvitamin $D_3$, or 7-dehydrocholesterol, were not present in our data set. However, our data showed several other vitamin D analogs that changed
with ethanol exposure. $1\alpha,25$-Dihydroxy-26,27-dimethyl-20,21-didehydro-23-oxavitamin D$_3$ and $1\alpha$-hydroxy-22-(3-methylphenyl)-23,24,25,26,27-pentanorvitamin D$_3$ were elevated up to 6.7-fold from controls at all tested ethanol concentrations. Calcitroic acid (1$\alpha$-hydroxy-24,25,26,27-tetranorvitamin D$_3$ 23-carboxylic acid) and $1\alpha$-hydroxy-25,26,27-trinorvitamin D$_3$ 24-carboxylic acid changed modestly and inconsistently from controls. Because of vitamin D’s ability to act as nuclear hormone receptor, alterations of vitamin D pathways are important. In addition to critical role in serotonin metabolism and neural development (discussed above), calcium homeostasis, bone building and maintenance, cell cycle regulation, immune function, insulin secretion, cardiovascular regulation, and muscle development [435].

Normal vitamin D metabolism begins via ingestion of vitamin D$_3$ from food sources or by generation of 7-dehydrocholesterol in the skin (derived from cholesterol) when it is exposed to UVB radiation and heat. These conditions result in its conversion to vitamin D$_3$, also in the skin. Vitamin D$_3$ then enters the blood, where it binds with vitamin D binding protein (DBP) and is transported to the liver. In the liver, it is converted by 25-hydroxylase and then stored in the form of 25-hydroxyvitamin D$_3$, where it can be transported to the kidney. Low levels of calcium in the blood stimulate the parathyroid gland to release parathyroid hormone, which acts on the kidney. This stimulation by parathyroid hormone or low blood calcium causes increases the conversion of 25-hydroxyvitamin D$_3$ by extra-renal 1$\alpha$-hydroxylase (Cyp27B1) into 1,25-dihydroxyvitamin D$_3$ [436]. The DBP can the be recycled for transport of other vitamin D molecules. From the kidney, the vitamin D once again travels through the blood as 1,25-dihydroxyvitamin D$_3$, bound to DBP in order to reach the target tissues. In addition, 25-hydroxy vitamin D$_3$ can be locally converted in some target tissues by 1$\alpha$-hydroxylase. In the target tissues, the 1,25-dihydroxyvitamin D functions as an active metabolite at a vitamin D receptor (VDR) site, altering transcription. Alternatively, it is converted to 1,24,25-trihydroxy vitamin D$_3$ where it is degraded by 24-hydroxylase (Cyp24) and then excreted [436]. $1\alpha,25$-Dihydroxyvitamin D$_2$ can also be oxidized by P450 into calcitroic acid.

Vitamin D$_3$ acting at the VDR regulates transcription via as a nuclear receptor. VDR plays an important role in intestinal calcium absorption, pancreatic $\beta$-cell secretion, and growth and differentiation of a variety of tissues, such as cartilage, smooth muscle, keratin, and bone [437]. It appears to also play an important role in
brain cell differentiation, regulating calcium in neurons and preventing excitotoxicity, protection from reactive oxygen species, and regulation of neurotrophic factors. VDR is initially expressed in the central nervous system in rodents at approximately embryonic day 12. During embryonic development, maternal vitamin D is the only source available.

Like humans, fish require vitamin D, having diminished growth and lowered calcium turnover under conditions of vitamin D deficient diet, as demonstrated in sea bream (Sparus auratus). Both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D₃ are present in the serum of fish. At both 48 and 96 hpf, immunostaining for VDR is positive in the ganglion cells of the developing eye, brain (diencephalon), and epithelial cells lining the otic vesicle. “VDR is widely expressed in tissues of the adult male and female zebrafish, Danio rerio, specifically in epithelial cells of gills, tubular cells of the kidney, and absorptive cells in the intestine,” as well as in “skin, the olfactory organ, the retina, brain, and spinal cord. Sertoli cells of the testis, oocytes, acinar cells of the pancreas, hepatocytes, and bile duct epithelial cells.” Quantities of VDR in intestinal, but not gill, epithelial cells are responsive to administration of exogenous 1,25-dihydroxyvitamin D₃.

VDR expression in rats appears increased during times of decreased proliferation and programmed cell death. Given this, the metabolites identified in our metabolome that can activate VDR may be inhibiting proliferation and increasing apoptosis in FASD. “Analogs eliciting VDR activity must contain a 1α-hydroxyl group and a side-chain that contains no aromatic substituents and most importantly a hydroxyl at C24 or C25.” 1α,25-Dihydroxy-26,27-dimethyl-20,21-didehydro-23-oxavitamin D₃ binds poorly to vitamin D binding protein and VDR, estimated to be 33% that of 1α,25-dihydroxy vitamin D₃ (as measured in pig intestine). However, 1α-hydroxy-22-(3-methylphenyl)-23,24,25,26,27-pentanorvitamin D₃, compared to 1α,25-dihydroxy D₃, can inhibit 96% of 1α-hydroxylase activity.

1α-Hydroxy-25,26,27-trinorvitamin D₃ 24-carboxylic acid is marginally higher with ethanol treatment, staying nearly identical at 1.1-fold control with all ethanol doses. Mizwicki noted that “to be effectively transported by DBP, [vitamin D analogs] must contain a side-chain that is, for the most part, rotationally free and contains a hydroxyl that is not sterically hindered by additional methyl groups at C25 and C26.” This suggests that while this metabolite may not react with VDR due to the lack of a hydroxyl group at C24 or C25, but does bind to DBP, where it can continue to be modified by the changed environment caused by ethanol exposure.
It is possible this carboxyl group is a result of transcarboxylation with ethanol in the system. The extent to which the carboxyl group substituted at C24 influences these actions or the relationship to the similar 1α-hydroxy-24,25,26,27-tetranorvitamin D₃ 23-carboxylic acid (calcitroic acid) in FASD is unclear and requires further investigation.

1α-Hydroxy-24,25,26,27-tetranorvitamin D₃ 23-carboxylic acid (calcitroic acid) is the renal catabolism product of 1,25-dihydroxy vitamin D₃ and can be formed through P450 oxidation. It mobilizes bone calcium and increases the transport of intestinal calcium. The inconsistent levels of calcitroic acid demonstrated in our metabolome may suggest that calcium levels are in a state of flux over the ethanol concentration curve tested.

In pregnancy, the maternal decidua of the placenta produces 1α-hydroxylase, which is especially high in the first trimester. This has been hypothesized to influence 1,25-dihydroxyvitamin D₃’s role in suppressing the cytokines that trigger maternal immune system, preventing induction of fetal rejection. In addition, vitamin D status plays an important role in the regulation of metabolism. When vitamin D levels are low, tryptophan 5-hydroxylase, also present in the placenta, is upregulated to increase the conversion of tryptophan to 5-HT. Tryptophan hydroxylase 2 on the vitamin D response element in the brain is also upregulated, allowing greater conversion of tryptophan to serotonin. Because of these alterations, there is less tryptophan available for protein synthesis, which may result in decreased growth, such as that demonstrated in FASD. In a comparison of offspring placenta of heterozygous c57BL/6 VDR ablated mice, microarray of VDR null showed significant differences in many genes, notably a 6.47-fold increase in Cyp24a1. In spite of the genetic differences, no functional differences in vitamin D were noted. The relevance of the above is questionable given that our model does not contain a placenta, and likely all of the maternal vitamin D present is in the yolk sac, however this information is important for understanding FASD.

Further, due to the positioning of the metabolite on the ligand binding domain, vitamin D analogs bound to DBP in circulation remain “highly exposed to the external environment”, making DBP different than many of the other albumin related proteins. This may be important, especially in the context of pharmacological models of FASD that utilize very high concentrations of ethanol, as these vitamin D analogs would remain susceptible to modification by circulating ethanol.
Previous works have demonstrated ethanol alters the vitamin D pathway. In one study, pups from ethanol-fed Sprague Dawley® dams had reduced skeletal ossification compared to pair-fed or control animals, in spite of there not being a difference in calcium content between groups [448]. However, this experiment also noted that the ethanol fed dams consumed less calcium and phosphorus, likely due to decreased intake in these groups. In another study, cholecalciferol, the prehormone synthesized from 7-dehydrocholesterol, given to Sprague Dawley® rats at 5 mg/kg significantly reduced the number of perseverative-type errors demonstrated in a serial spatial discrimination reversal task by 11.9% alcohol-exposed rats age postnatal day 49–52 [449]. However, cholecalciferol treatment did not improve the number of trials it took rats to find the escape ladder in the trial. Alcohol treatment did not appear to reduce vitamin D status, nor did vitamin D status appear to alter peak blood alcohol concentrations [449]. The authors hypothesize that cholecalciferol is providing a neuroprotective benefit to VDR containing regions of the brain, such as the cerebellum [449].

Pooled data from multiple ethanol exposure studies in developing c57Bl/6J showed underexpression of the gene Cyp2R1 [130]. This gene encodes a Cyp liver enzyme microsomal vitamin D₃-25-hydroxylase, which converts cholecalciferol to 25-hydroxy vitamin D₃ [450]. Similarly, Cyp27B1, the P450 1α-hydroxylase responsible for conversion of 25-hydroxy to 1α,25-dihydroxy vitamin D₃, is underexpressed in ethanol exposed pups, [130,450].

Other researchers investigating the liver metabolome of alcohol-dosed rodents have discounted their own reports of 3-deoxyvitamin D₃ ((5(Z),7(E))-9,10-seco-5,7,10-(19)cholestatriene (LMST03020618), instead determining the metabolite to be cholesterol ((3)-cholest-5-en-3-ol; m/z 369.3516 [M H₂O + H]+) to be more consistent with analysis of their standards of this metabolite [451]. This, in conjunction with our findings, suggest further investigation is warranted to explore these vitamin D metabolome changes in FASD.

**Nicotinamide**

2,6-Dihydroxypseudooxynicotine was significantly changed in several ethanol doses. In addition, cotinine methonium ion and nicotine glucuronide was increased with most ethanol doses. Both of these are generally recognized as a products of nicotine metabolism. Mugineic acid is known to be produced by plants. Importantly, this metabolite is very closely related
Fig. 2.10. Several vitamin D metabolites were identified in the polar fraction.

in structure to nicotinamide, and was decreased in all but one of the ethanol treatment groups \[452\]. Work in plants demonstrate mugineic acid antibodies also are cross reactive with nicotinamide antibodies in assays. These changes suggest these metabolites reflects changes in nicotinamide metabolism. Tryptophan is the precursor
of nicotinic acid mononucleotide, which can eventually be converted to nicotinamide adenine dinucleotide [183]. Tryptophan pathway abnormalities are well-established in FASD (see above). Because of its critical function as a coenzyme in redox reactions, changes in nicotinamide would have critical, far-reaching effects in FASD. This represents a novel finding, and should be explored further as to the generality of this effect.

2.4.10 Disease Resistance

\(N\)-(3-Oxo-octanoyl)-homoserine lactone was also elevated in the 0.1% or greater ethanol treatments. Acylated homoserine lactones are common in the environment. These are a type of quorum sensing molecule, which responds when a cell population proliferates in a given volume to activate transcription. These molecules influence a pathogen’s virulence, making them important for disease susceptibility, suggesting a protective response by the embryo in generating \(N\)-(3-oxo-octanoyl)-homoserine lactone [453,454].

2.4.11 Study Weaknesses

With all of the metabolites identified, several factors should be considered when examining the overall trends present in our data set. First, the fact that these were whole-embryo extracts must be considered. For some metabolites, it is likely that our method underestimates important metabolite changes in some tissues, due to the dilution from other tissues. A second concern is that, through the homogenization of the whole embryo, we created interactions that would not naturally occur. Biochemicals that may normally be compartmentalized from each other could be exposed to what otherwise would appear as exogenous substances and result in some degree of catabolism. Tissue compartmentalization and subcellular compartmentalization confer too great of a functional difference on molecules to ignore these physiological separations. Thus, it is a mistake to assume cells and zebrafish embryos always respond in the same manner.

Further, some have suggested that generalized extraction methods, such as those used here, can result in a loss of very polar lipids to the aqueous component during extraction [455]. Thus, our values may underestimate the actual lipid modifications taking place during fetal ethanol exposure. Further, since the polar and non-
polar metabolome were generated from separate experiments, it is possible that some metabolites which are borderline polar/non-polar may be over- or underestimated by being in both or neither fraction.

Hence, a follow-up investigation divided by tissue compartments for comparison to these results may be warranted. This will be a difficult task, in no small part due to the highly friable nature of the ethanol-cultured embryo. Conversely, metabolites identified here may therefore be important in a wider-array of organ types than previously anticipated by compartmentalized studies alone. Lastly, because of the developing nature of metabolomics databases and the relatively novel use of zebrafish in such experiments, it is likely that some of the metabolites could have alternate identities. Periodic re-examination of metabolome databases could modify the findings from work.

Why is FASD difficult to study?

Metabolomics offers a powerful method to gain insights in normal biochemistry or disease processes. Metabolome changes are dynamic, especially during the rapid evolution of development. Different developmental stages can be metabolomically characterized, which has been demonstrated in the zebrafish [456].

In many biological contexts, an assumption can be made that changes in the levels of a biochemical intermediate are due to changes in the rate of production or the rate of degradation [153,189]. But, due to the protic solvent properties of ethanol, alterations could occur at reaction precursors, intermediates, or products, in addition to alterations in enzymes that normally alter metabolites. An additional complication to describing mechanisms in FASD is that various enzymes have differential expression during development, indicating that the normal metabolic and catabolic functions are at less than maximal capacity.

Triage theory is the hypothesis that when nutrient availability is insufficient, evolutionarily-conserved mechanisms are preserved over non-essential mechanisms [457,459]. This theory has largely been applied to nutrient deficiencies that result in disease, but it is logical to extend it to abnormalities caused in frank disease of non-nutrient deficiency origin. By extension of triage theory, the developing embryo self-preserves critical nutrient-utilizing processes acutely, disfavoring non-critical evolutionary developmental processes. Thus, changes that occur in our model from ethanol-exposure as compared to control are attributable to both the direct ethanol-
induced biochemical changes as well as self-preservation triage of critical nutrient-related in the embryo.

An additional difficulty in modeling FASD is the variety of tissue influences occurring as a result of maternal, fetal, and placental metabolism of ethanol and its products. Similarly, dosing of zebrafish in culture is not 100% efficient. Finding relevant ethanol concentrations is important to creating useful models of FASD. Based on blood alcohol content reported in a variety of studies, relevant ethanol concentrations can range between 20 - 170 mM [460]. Ethanol is a teratogen, with an LC$_{50}$ in zebrafish of 338.5 mM after 48 hours or 280.5 mM after 24 hours of exposure [82]. Zebrafish embryos are even more sensitive to the metabolite acetylaldehyde (LC$_{50}$ 0.541 mM after 24 hours) [82].

The use of ethanol dosed in the water of zebrafish embryo medium is common. The applied waterborne concentration and the embryonic dose is different, and appears at least partially dependent on the proportion of time the embryo is exposed to ethanol during development. After 24 hours ethanol exposure for 100 mM waterborne dosage, embryos contained 17.10±6.27 mM of ethanol, as determined by enzymatic assay (alcohol dehydrogenase [ADH]), and with 200 mM dose waterborne dose resulted in a 57.36±5.29 mM embryonic ethanol level (n=3) [82]. Others have reported that ethanol applied at 167 mM between 24–27 hpf resulted in an embryonic ethanol concentration of 51.7±5.6 mM (n=8–30 embryos per plate 100 mm plate), a much higher embryonic exposure than previously reported [461]. But, it is clear that waterborne doses are much greater than the embryonic exposure.

Multiple retention times

Due to the multiple retention times observed for some of the fatty acids, standards of both palmitic and stearic acids were run (Appendix A). These standards eluted at approximately 2.8 and 6 minutes, respectively. Mass spectra of peaks empirically identified as palmitic and stearic acids were subjectively verified to ensure they were similar to the standard. We hypothesize the differences in retention times of these smaller fatty acids may be due to several factors. First, larger fatty acids and glycerophospholipids separate in the column due to differing polarity based on their tail lengths. Then, during MS, some of these larger chains may be fragmented, resulting in the appearance of multiple metabolites with the same m/z values. Additionally, given these were whole embryo extracts it is possible these fatty acids were associ-
ated with atypical matrix components that impacted the elution times. In the future, these could be examined by spiking standards into whole-embryo extracts to verify differences in retention times or studying the metabolites in their native tissues.

Zebrafish Concerns

The embryonic yolk in zebrafish (and other model species) is an important consideration when comparing human development. Prior to hatching, the entire supply of nutrition comes from the yolk, which is largely made of the maternally-originating lipoprotein vitellogenin [462]. Studies in multiple fish species have demonstrated that in estrogen-stimulated fish vitellogenin is composed of 16–18% lipids, including glycoproteins, polyunsaturated fatty acids, and the [462]. The yolk components are also the primary source of phosphorous (phosphovitin), calcium, protein, and lipids for the embryo. Provided by the lipovitellin component of vitellogenins, large protein droplets form in the yolk, which provide amino acids for development. During the maturation process, proteins become completely degraded, likely due to cathepsin-induced proteolysis. Additional components present in the yolk include major receptor class components, such as retinoids, retinoid binding protein, steroids, and thyroid hormones. Also during this process, innate and adaptive immune responses are also transferred to the egg [?].

At the early developmental stage of our experimental embryos, lipids would derive only from the yolk, and not from ingestion of cholesterol and unsaturated fatty acids obtained as adults from the diet [463]. On top of that, the gut is fairly undeveloped until 60 hpf, making intestinal absorption of lipids of little importance in this model [258]. Fish store lipids as triglycerides in visceral adipose, but visceral adipose does not begin to accumulate until they begin eating [463]. Accordingly, more emphasis may be needed on the role of yolk lipids (or maternal supply of lipids) with respect to abnormal lipogenesis and lipolysis that appears to be occurring in this model of FASD.

Oxygen Solubility

One possible problem in our experimental design, which immerses zebrafish embryos in ethanol solution, is that in incubating the embryos for development at normal laboratory protocol temperature of 28°C likely alters the dissolved oxygen of the wa-
Baek, et al., proposed that higher dissolved oxygen concentrations in alcoholic beverages decreased the time required for participant blood alcohol concentrations to return to baseline [464]. It has long been known that even while using repeated distilled ethanol, when placed in temperatures above 25°C (up to 50°C) results in a decrease in oxygen gas solubility [465]. This is important in the interpretation of changes that might involve ROS mechanisms, as our system would possibly minimize potential ROS effects. But, normal oxygen-requiring metabolism may also be altered, especially in higher experiment ethanol concentrations. We did not examine the effects of dissolved oxygen in our system, and that might be advisable in future experiments. However, with the relatively small differences between alcohol concentrations we utilized in a normal laboratory incubator, the experiment must be well designed to capture comparatively small differences experienced by the embryo. This may also be important in the comparison of zebrafish data across laboratories, as different laboratories may have different protocols regarding incubation environment, which would greatly be affected by the use of recirculation of water as compared to the standing water of many culture incubators.

Multiple assay methods

Two different detection modalities were used in these experiments, having different levels of expert-established accuracies. However, the data presented in this work does not weight these differences in this interpretation of the FASD model metabolome. In the future, this should be addressed through development of a statistical or algorithmic penalty to provide more emphasis on data derived from the Orbitrap. As presented in this article, the differences in detection modalities are denoted by the ppm difference, included in the associated tables, as well as indications to demonstrate which fraction (and thus which detection modality is used).

2.4.12 Future Directions

Weinberg, et al. astutely point out that all of our understanding about ethanol-related mechanisms can never be separated from absorption, dispersion, metabolism, and excretion of nutrients [285]. For example, maternal nutritional status may be impacted if she is a chronic drinker. In heavy drinkers alcohol interferes with thiamine absorption, a nutrient essential for normal energy metabolism and synthesis of
lipids. Gaining a better understanding of the metabolic changes is essential to diagnosing and intervening in FASD.

Many studies targeting mechanisms of FASD in the brain focus specifically on the hippocampus. This may be due to the behavioral changes noted in FASD in conjunction with our current understanding of hippocampal functional modalities. However, reactional changes induced with ethanol would be unlikely to specifically target this single structure in the brain. The effects of ethanol are systemically profound during development. Thus, in the future, we should either 1) work toward an understanding of what makes hippocampal structure more susceptible to change than other structures, or 2) refocus our interpretation of the FASD phenotype (including behavior) to be more relevant to the global changes induced by ethanol.

Many of the abnormalities we have identified center at cholesterol metabolism. From cholesterol is derived pregnenolone, progesterone, testosterone, estradiol, glucocorticoids (like cortisol), vitamin D, and bile acids/salts. All of these metabolites had significant changes in our analysis when exposed to ethanol. The question must therefore be raised if 1) cholesterol regulation might be targeted and 2) our analysis indirectly targets cholesterol-derived products biasing our findings.

In addition to differences noted in steroids related to sex hormones, we noted metabolome differences relevant to metabolites reported by other scholars as having a differential outcome in prenatally ethanol-exposed males and females. Females display elevated ACTH stress responses, while males display higher levels of β-endorphins. In addition, “normal testicular influences on HPA function are markedly reduced in PAE males.” Further, male and female Sprague Dawley® rats exposed to prenatal alcohol exhibit differential regulation of hypothalamic-pituitary-adrenal (HPA), dopaminergic-adrenal (DA), and likely HPA-DA. Due to the sex-specific differences noted in the response of the brain to alcohol exposure, it will be important to follow-up with work examining the correlation between sex of the individual and metabolome differences in steroid hormones.

2.5 Conclusions

In conclusion, we found a vast array of significantly changed metabolites in our zebrafish model of FASD. Pathways related to phospholipid structure and function, cardiovascular development and functioning, oxidative stress, and vitamin A are some of the interesting findings. Many were metabolites associated with established FASD
mechanistic hypotheses. However, the novel metabolites we have identified may shift our interpretation of many of these theories.

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3. COMPARATIVE MODELING OF FETAL ALCOHOL SPECTRUM DISORDER: MORE IS NOT JUST MORE

3.1 Abstract

A comprehensive mechanistic profile of metabolite changes occurring in fetal alcohol spectrum disorder has yet to be developed. We used our well-established *Mus musculus* (mouse) model to compare 0, 1, or 2 six-hour 400 mg/dL binge-type ethanol exposures, and analyzed the whole-embryos at E10 extracts using a Thermo Fisher LTQ Orbitrap coupled to an Agilent 1100 for liquid chromatography. From this data, we generated a profile of significantly different metabolites resulting from ethanol treatment, which included fatty acids, monoacylglycerides, glycerophospholipids, and prenols. Many of the metabolite changes related to protein kinase C, as well as lipid metabolism and lipid droplets. As a post hoc analysis, we also compared these mouse model findings with our previously reported chronic ethanol exposure *Danio rerio* (zebrafish) analysis, where embryos are exposed to 0—3% ethanol from 2—24 hours post-fertilization. Six significantly different, commonly-identified metabolites were present, including fatty acids, phosphatidic acid, and sulfatide, which were analyzed using a probabilistic principal components and covariates analysis. Interestingly, metabolite responses in the two models diverged, suggesting that the chronic ethanol exposure is not the same as more of the binge response. We also contextualize these findings with a novel relativistic inter-specific timeline. These metabolite profiles provide a more comprehensive understanding of ethanol-induced changes during development and may lead to better biomarker(s) identification or novel therapies for FASD.

3.2 Introduction

3.2.1 FASD affects many, but alcohol is non-specific

Though it has been widely studied for decades, a comprehensive model for fetal alcohol spectrum disorder (FASD) has yet to be determined. Ethanol acts as a solvent, causing tissues to break apart. In addition to the alcohol itself, its constituent
hydroxyl group, make excellent nucleophiles. In the laboratory, such reactions have been shown to convert fatty acids into their ethyl esters via lipase catalyization, with a preference for short chain fatty acids in ethanol:fatty acid, especially in the presence of CO$_2$ [468]. With the evolution of our understanding about FASD, development of new models, and improvement in technologies, many hypotheses have been advanced regarding the primary drivers of this disease. These have included nitric oxide and other reactive oxygen species, glutamate, vitamin A, serotonin signaling, lipids, glucose regulation, adhesion molecules, and more [61, 62]. Yet, it is unclear how the most important pathway changes caused by this far-reaching molecule of ethanol can be discerned, targeted, and tracked as a biomarker(s). To that end, we conducted a non-targeted, non-polar metabolomics analysis of our well-established *Mus musculus* binge alcohol model. We also compare and contrast findings of this experiment with metabolomics results from previously reported experiments using a *Danio rerio* chronic ethanol exposure model. A novel application of probabilistic principal component and covariate analysis (PPCCA), applied interspecifically, was also utilized to determine predominant underlying biological drivers in these differing FASD models. Lastly, we contextualize the findings of these models by presenting a cross-species relative timeline of these models.

### 3.2.2 Animal Modeling Considerations

Fetal alcohol spectrum disorder has been modeled in a variety of species and modalities, ranging from rodents, to fish, to guinea pigs. Rodents dominate the field of FASD research, due to their availability and well-published understanding of their physiology and volume of work in the literature. Because of this, it is easy to put on blinders with respect to the selection of the best model for a research question. But it is important, in spite of the complexities of the process, that we prioritize models that best captures the physiology or symptoms at hand [469]. Because we have relied so heavily on rodent models, there remains a significant phylogenetic separation between knowledge of the effects of alcohol in humans and alternative models.

When selecting an animal model, several important considerations should be made, but ultimately the goal is to mimic the human condition. Many factors influence the applicability of animals to understanding human dietary- or consumption-induced toxicity and terratogenicity. These factors include genetics and physiology, environment, diet and nutritional status, behavior, bioaccessibility, and bioavailabil-
Judgement of the success of an animal model can only be made by understanding the overall context of the research question, physiology, environment, and experimental design.

Various techniques have been used to classify relationships between model organisms and humans (or target species). Some use parsimony, trying to determine relationship with fewest possible point of evolutionary divergence and convergence [469]. Others prefer to rely on quantifiable differences (i.e. genetics) to construct basis to create “maximum likelihood estimates” [469]. When no longer considering a single genetic modification from wild-type to knock-out, such as those used here for FASD, genetics comparisons are based on ancestry [469]. Another consideration in comparative modeling is cladistic relationships, wherein some species are necessarily nested within each other [469].

The environment, behavior, and diet of a model organism should be considered as well. The environment is an important driver of genetic, behavioral, and physiologic differences. Also, animal models often perform insufficiently in modeling human behavior. (Human behavior is difficult for humans to predict, much less for an animal model to predict.) While single behaviors may be mimicked in the laboratory, the variety of human habits will likely always exceed that of what animals can model. Additionally, diet is another extremely important factor. Humans in more developed countries have a greatly varied diet. This is compared to a laboratory animal that is fed routinely a single “complete” chow diet. Laboratory animals, while creating a reductionist model of human nutrition, greatly simplify the impact of diet variety (including sources) and nutritional status.

Finally, bioaccessibility and bioavailability are tremendously important, especially in consideration of teratogenic studies. Bioaccessibility is defined as “the amount of a chemical that is released from food into gastrointestinal tract fluid following a simulated digestion and, as a result, is available for absorption by the intestinal mucosa” [470]. This value is likely to vary between species, as the physiology of the gastrointestinal tract changes. This factor makes comparative modeling more difficult, since the maturity and functioning of the digestive tract varies during development, especially when compared between species like mice and zebrafish. Bioavailability is “the extent to which the active ingredient of a drug dosage form becomes available at the site of drug action or in a biological medium believed to reflect accessibility to a site of action” [471]. This would be highly affected by any changes in the metabolism of a substance between species, whether due to changes in development or just physio-
logical differences. Bioaccessibility, bioavailability, genetics, physiology, environment, and behavior must all be considered in establishing the merit and appropriate scope of an animal model.

3.2.3 Utility of Zebrafish for FASD

Toxicology and developmental research has already demonstrated the usefulness of fish, specifically zebrafish, as a model. This work is now being extended for research-friendly intermediate scale screens of drugs at a stage between cell culture models and mammalian models. The small size of the zebrafish may make sample collection more difficult, but the ability to carry out larger experiments provides increased statistical power. In general, zebrafish are much less expensive to maintain than other animal models (however, do require vigilant monitoring of water quality). They reproduce readily (typically laying 75–120 eggs) and rapidly in captivity [472]. Transparent embryos make transgenesis easy. Their small size requires little space and husbandry costs are much less than in rodent models [473]. Inducing the FAS zebrafish model is also very simple compared to dosing a stressed pregnant rodent [472].

Though zebrafish seem to lack construct homology (e.g. living in water, fins instead of arms), these vertebrates are highly conserved, providing genetic homology. As such, a clear disadvantage of the zebrafish if that they require water for oxygen and not air. As a result, any lung abnormalities that might appear in other animal models may not be seen with the zebrafish. Use of the lower level phylogenic vertebrate supports the reduce, refine, and replace objectives in animal research [472]. Relative to other lower metazoans, zebrafish contain greater face validity, including more anatomic similarities than others (Drosophila, Caenohabditis elegans, etc.), have comparable early development, and general patterning similar to humans [389, 474, 475]. Importantly, zebrafish demonstrate one-to-one face validity for human FASD. Zebrafish serve as a predictive construct, as they develop decreased growth, craniofacial abnormalities, spinal malformations, and damage to CNS, like humans [63, 472]. Other researchers have demonstrated homology of heart malformations in the zebrafish FASD model similar to humans [476]. In addition, zebrafish respond dose-dependently to alcohol [389]. The model is very fast, as the time required for alcohol exposure is limited (24 hours at 2 hours post fertilization [hpf] or even just 2 hours at 24 hpf has been reported) [477]. Furthermore, untreated zebrafish possess key mammalian (human, rat, and mouse) liver regulatory factors for dyslipidemia. In terms of lipid metabolism,
Zebrafish show many similarities to humans. For example, zebrafish conserve the ability to store cytoplasmic lipid droplets. These factors support the construct validity of the zebrafish in FASD research.

Zebrafish have provided an important model for FASD, in no small part due to the tremendous amount of work that has been done in zebrafish developmental biology. Developmental norms are well-established and compare well with early human development, which is important as “fetal cellular mechanisms often differ from adult processes.” However, it “fetal development is activity dependent” requiring previous stages to be successful prior to next. This has important implications on the long duration of ethanol exposure we use in the zebrafish model, since early exposure effects surly compound later ones.

Zebrafish and humans share considerable genetic homology. Over 70% of human genes contain at least one zebrafish ortholog. There are a variety of genes for alcohol dehydrogenase (ADH), the enzyme that catabolizes ethanol to acetylaldehyde. There are differences in the alcohol dehydrogenases present in zebrafish and mammals. Both lineages contain ADH3, though it is hypothesized that the acquisition of ADH1 occurred independently in these two genetic lineages. Further, the teleosts whole-genome duplication event includes the genes encoding ADH. The result of these changes created the ADH variants 1A1, 1A2, 3H, 3L, while humans have 1A, 1B, 1C, 2, 3, 4, 5. Mice and rats have H1, H2, H3, H4, H6A, H6B, with the variant H5ps being present in mice and H5 occurring in rats. ADH8A in zebrafish metabolizes ethanol, and is not yet expressed at 24 hpf, but is at 36 hpf. This has important implications on our model, as it suggests the effects noted in our model are likely impacted by this diminished ethanol-catabolizing capacity.

Beyond these advantages to using the zebrafish in FASD, there are some important considerations including the lack of placenta, nutrient supply, the and tank environment. Zebrafish cannot support investigations in to the role of the placenta in FASD. It is clear that the placenta is important in fetal programming. But, the functional similarities of the chorion membrane to the placenta is not. Furthermore, maternal interaction in zebrafish is completed at time of egg release; ethanol exposure may likely not be a single event in humans, and the zebrafish model (as currently implemented) does not mimic that very well. But, zebrafish and the excised mouse embryos both provide useful information as a negative and partial-negative animal models, respectively.
Zebrafish embryo nutrients are parentally derived, supplied in the yolk, which isolates ongoing fetal-maternal nutrient interactions otherwise presented in embryonic development. The composition of the zebrafish yolk is important in examining FASD metabolic alterations. Vitellogenesis is the process of producing and accumulating egg yolk proteins (vitellogens) from circulating precursors in the oocytes of non-mammalian. Vitellogenins are largely composed of phospholipids and glycoproteins. In teleosts, vitellogenins are composed of approximately 16–18% lipid, including the polyunsaturated fatty acids phosphatidylcholine (PC), phosphatidylethanolamine, and phosphatidylinositol (PI) \[462,481\]. Additional fat is required for the oil droplets that provide buoyancy for the egg \[481\]. Energy reserves for embryonic life come from lipoproteins that are hydrolyzed \[481\]. Phosvitins, composed largely of phosphorous bound to amino acid polyserine, provide vitellogenins much of the minerals required for embryonic development \[481\]. Additionally, proteins are another important component that are provided by the lipovitellin component of vitellogenins. They form large protein droplets in the yolk and provide amino acids for development.

The watery environment makes the zebrafish an easily induced FASD model, as embryos take up the alcohol through immersion. This difference may influence the uptake of alcohol, since the phenotype occurs at much higher concentrations than what human effects occur. However, there is a significant \((P < 0.0001)\) correlation between alcohol concentrations in the media relative to alcohol inside of the egg \[477\]. The FASD phenotype response may be strain dependent, as some strains respond with cyclopia only at higher ethanol concentrations (2.4%) relative to others (vs. 1.5%) \[63\]. Given the method of ethanol exposure used in zebrafish, the use of circulating water or still water has important implications on the dissolved oxygen of the water. While this may be less of a concern for incubating embryos that have less exposure to circulating water due to being at the bottom of a tank, it is certainly a factor for hatched individuals. Further, it has been demonstrated that higher dissolved oxygen concentrations in alcoholic beverages decreased the time required for participant blood alcohol concentrations to return to 0.000% \[464\].

As research shifts to a better understanding of the genetic diversity in our world, our potential for animal models also greatly expands. With the advancements in bioinformatics, researchers are beginning to appreciate what genotypic, epigenetic, protein, and metabolic changes between species means phenotypically and physiologically. The zebrafish model of FASD offers many benefits to researchers. These benefits, must be weighed against the models downfalls to determine the hypothe-
ses for which this model is most relevant. But, taken together, our well-established c57BL/6J model of binge alcohol and our chrnoic zebrafish exposure can be beneficial.

3.2.4 Comparative Modeling

A potential method of assessing the strengths and weakness of an animal model is through interspecific comparative modeling. Through the use of comparative study, researchers can perform “the analysis of trait variation across rather than within species, with the aim of testing hypotheses or generating new ones” [482]. Consideration of phylogeny is important in comparative animal modeling, as understanding relationships between disease related traits may alter the utility of a given animal model. Some other important bases for model comparison include anatomy, physiology, genetic variation, biomarkers, or evolutionarily (dendrologically). However, interspecific comparisons of development are complicated by differences in the sequence of development events as well as variations in the rates of these events. Further, overall development rates in general may vary between species as well as at different stages during development.

In the field, the degree of genetic conservation and chromosomal synteny has long been identified as a method to compare species [483]. Historically, we have identified models through characterization of the human disease, then finding a suitable natural model or inducing one through genetic manipulation [484]. However, even our workhouse lab rodents are known to have undergone a large amount of chromosomal rearrangement, making this method difficult to use to align species maps, much less draw conclusions [483]. Further, the strong reliance in the industry upon highly inbred strains also removes a large degree of individual variation, which may in turn be contribution to the reduction in statistical findings in trials in later non-inbred species, including humans [485]. Two genetic comparison methods have been used traditionally, including quantitative genetics and segregation and linkage analysis [485].

Using the best animal models possible can reduce the number of animals in research, refine our experiments to best answer the correct questions, replace the use of phylogenically higher level animals with lower ones. However, we still lack quality quantitative methods to compare developmental staging interspecifically. To that end, we present here a tool to compare development of multiple species across one timeline.
3.2.5 Comparison of PCA

An enormous limitation to our capabilities in translational medicine is the lack of ability to quantitate the inter-specific differences observed in the laboratory. Principal component analysis (PCA) is a method to take assumingly linearly-related observations and transform them into a data set with reduced dimensionality. “PCA asks: Is there another basis, which is a linear combination of the original basis, that best reexpresses our data set?”[486] First a linear function is identified to account for as much of the variance as possible, and then they are orthogonally transformed. It is commonly used in the analysis of complex data sets, such as -omics data, since it puts the data “into lower dimensional space, revealing inherent data structure, and providing a reduced dimensional representation of the original data”[487]. It has several limitations, however, including the lack of a probability component, does not address other important underlying characteristics of the sample, and fails to address missing data[487].

Since its inception decades ago, many iterations have been developed to address these limitations, including the probabilistic PCA (PPCA), the probabilistic principal components and covariate analysis (PPCCA), and the mixtures of PPCA models (MPPCA). “In contrast to the more conventional view of PCA which is a mapping from the high dimensional data space to a low dimensional latent space, the PPCA framework is based on a mapping from a latent space to the data space[487].” The PPCCA extends upon the PPCA, incorporating covaritaes into the PPCA model. These methods used the Expectation Maximization model, which uses an iterative approach to find the Maximum Likelihood Estimates (selecting a parameter based on maximizing the likelihood of obtaining the values observed) of a parameter[488]. Further, probabilistic models also offer the ability to determine the optimal number of principal components, in MPPCA through the incorporation of a modified Bayesian Information Criterion[487]. “This modified version of the BIC evaluates the likelihood at the maximum a posteriori (MAP) estimator instead of the MLE”[487]. Jackknife resampling refits the PPCA model with random observations removed, allowing the “loadings of any probabilistic principal components based model [can] be used to identify variables responsible for the structure in the data” and the associated jackknife standard error can be used to compute confidence intervals for each of the loadings[487].
Notably, one weakness in selecting PCA, PPCA, or PPCCA for metabolomics analysis is the underlying assumption of linearity in the latent variables. We demonstrate many, but not all, metabolites having horneretic responses to ethanol, which intuitively suggests non-linearity of latent variables, making validity of these methods questionable. MPPCA generates a collection PPCA models, which “can be used to cluster subjects whilst facilitating dimensionality reduction of metabolomic data” [487]. “Under the MPPCA model, in addition to the latent location variable, the unobserved group membership of each observation is also viewed as a latent variable [487].” However, MPPCA does not incorporate a covariate analysis. Further work is needed in this area to explore better models for addressing this important weakness. Many machine learning models have been developed to address such limitations, the use of kernel PCA, which utilizes a nonlinearity to transform the data [486, 489]. It may be possible to apply such tools to metabolomics data to improve the interpretation of such large and complex data sets.

In comparing only the metabolites present in both mouse and zebrafish metabolomes, we lose important species differences in the reaction to ethanol-exposure. To examine if species-specific differences can be identified posteriori in the MPPCA, in spite of not being specified as a covariate, an additional analysis was run. It was anticipated that this would also provided a statistical measure of the validity of this inter-specific comparison. Using the MPPCA algorithm to analyze interestingly did not provide an obvious identification of either the species differences nor the different ethanol treatment concentrations (data not shown). Further research is needed to identify weaknesses in the MPPCA method, or to explore optimization of the data entered into the model which would reveal the anticipated differences.

### 3.2.6 Findings

We used an un-biased metabolomics, to examine the non-polar small molecule changes that occur in a binge model of FASD. We used liquid chromatography (LC) with a Thermo Fisher LTQ Orbitrap mass spectrometer (MS/MS), which provides a highly accurate identification of metabolites. We anticipated finding tryptophan metabolites, as we have demonstrated decreased neuronal migration or differentiation in 5-HT neurons originating in the midline raphe of the brainstem and projecting to the dorsal and median raphe in the mouse embryos from alcohol-exposed dams have [41]. These effects persist into adulthood of male offspring animals [40]. We also
anticipated protein kinase C (PKC)-related metabolites to be significantly different, based on similar findings in zebrafish. While we did not find the former, we indeed identified significant differences in glycerophospholipids and sphingolipid changes that are relevant to PKC. In addition, we identified metabolite related to abnormal lipid metabolism and lipid droplets.

However, some have criticized rats and mice as models of prenatal ethanol exposure, due to the majority of brain development occurring postnatally. Because of this limitation, it is important to consider our findings in the context of our work in zebrafish as well. Previously we reported our work in zebrafish from a similar metabolomics study, where we saw nervous system related metabolite changes, including myelination, tryptophan metabolism, and oxidative stress. In addition, we saw cardiovascular related abnormalities, including significant differences in metabolite related to myocyte contraction, vasculature, and coagulation. In addition, we noted many changes in metabolites related to the endocrine system, including sex hormones, and alterations in cellular and molecular function. Through comparing and contrasting our non-polar mouse and zebrafish metabolomes, we have identified many pathways that are differently altered in binge and chronic ethanol exposure modalities. We further compare these findings through the use of a PPCCA and contextualize them with a inter-specific development modeling tool.

### 3.2.7 Specific Aim and Study

The aim of this study is to develop an un-biased metabolome of ethanol-induced changes in our binge-type mouse model. In addition, we compare similarities and differences obtained from non-polar metabolomics analysis of mice and zebrafish using PPCCA. In this work we will explore benefits and deficits of these model systems and examine the translational impact when they are considered together in the context of relativistic development. With this technique, we demonstrated both expected and unexpected results of metabolite divergence between the two animal models, a finding that suggests “more is not just more”.

### 3.3 Results

We utilized our well-established method of exposing excised c57BL/6J mouse embryos to ethanol in a petri dish to examine the effects increased number of ethanol
exposures on non-polar, whole-embryo metabolome changes. We identified a total of 32 metabolites that changed with ethanol exposure, including significant differences in several fatty acids, glycerolipids, glycerophospholipids, ceramides, and retinoate. A histogram of the one-way ANOVA P-value is included in Figure 3.9. Below we will discuss in detail the metabolites we identified.

### 3.3.1 Fatty Acids

A total of ten fatty acids were identified in this metabolome (Table 3.3.1). Similar to our previously published zebrafish analysis, many of the fatty acid metabolites eluted at multiple retention times. Dodecanoic acid eluted at three different retention times, the earliest of which significantly differed from controls (Chapter 2). For mice exposed to multiple ethanol doses, dodecanoic (lauric) acid was 6.423-fold higher than untreated animals, significantly increased over the single-ethanol animals. Tetradeconoic (myristic) acid eluted at two retention times, the latter of which was significantly different. Hexadecanoic (palmitic) acid eluted at three separate retention times, decreasing to –0.2-fold compared to controls with a single ethanol dose, then increasing statistically significantly when in animals given two doses. Methyl hexadecanoic acid changed significantly at the earlier of two elution times, decreasing to –0.021-fold and then significantly increasing to as much as 0.027-fold controls with two ethanol exposures. At the later elution time, a larger decrease to –0.535-fold control was observed with a single dose, which then increased to –0.148-fold in mice exposed to ethanol twice. (9Z)-Octadec-9-enoic acid differed significantly with two ethanol exposures to 0.062-fold control level, but was not different with a signal ethanol exposure. Similarly, the earlier eluting octadecanoic (stearic) acid differed significantly from controls in animals with two ethanol exposures 0.021-fold, while the later eluting moiety significantly differed as well, being 0.043-fold greater than controls with two ethanol exposures. This was statistically different from the –0.043-fold noted in animals with a single ethanol dose. (5Z,8Z,11Z,14Z)-Icosa-5,8,11,14-tetraenoic (arachidonic) acid eluted at four retention times, changing statistically significantly in two of them. Most retention times were associated with a significant increase in the levels from singly and multiply dosed animals. 18-Methyl-(8Z,11Z,14Z)-nonadecatrienoic acid differed significantly with repeated ethanol doses, including relative to singly dosed animals. Lastly, (8Z,11Z,14Z)-icosatrienoic acid did not vary signifi-
cantly at its earlier retention time, but at the later retention time showed significant increase in multiply dosed animals to as 0.327-fold.
Table 3.1.: Fatty acid metabolites identified in the non-polar fraction of ethanol-exposed mouse embryos.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>P</th>
<th>Fold 6 h</th>
<th>Fold 6+6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dodecanoic acid</td>
<td>1.17 ± 0.01*</td>
<td>1.784915</td>
<td>&lt;0.01</td>
<td>0.000</td>
<td>6.423</td>
</tr>
<tr>
<td>HMDB00638</td>
<td>3.06 ± 0.12</td>
<td>1.779919</td>
<td>0.87</td>
<td>0.025</td>
<td>-0.184</td>
</tr>
<tr>
<td></td>
<td>5.10 ± 0.18</td>
<td>1.982239</td>
<td>0.38</td>
<td>-0.251</td>
<td>-0.030</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>12.51 ± 0.29</td>
<td>0.955265</td>
<td>0.37</td>
<td>0.212</td>
<td>0.275</td>
</tr>
<tr>
<td>HMDB00806</td>
<td>22.70 ± 0.05*</td>
<td>1.209856</td>
<td>0.04</td>
<td>-0.682</td>
<td>0.617</td>
</tr>
<tr>
<td>Hexadecanoic acid</td>
<td>15.88 ± 0.22</td>
<td>0.845691</td>
<td>0.17</td>
<td>-0.348</td>
<td>-0.037</td>
</tr>
<tr>
<td>HMDB00220</td>
<td>16.60 ± 0.24§</td>
<td>0.423041</td>
<td>0.37</td>
<td>-0.207</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>24.93 ± 0.12‡§</td>
<td>0.384015</td>
<td>0.27</td>
<td>-0.236</td>
<td>0.057</td>
</tr>
<tr>
<td>Methyl hexadecanoic acid</td>
<td>18.00 ± 0.23*</td>
<td>0.657895</td>
<td>&lt;0.01</td>
<td>-0.021</td>
<td>0.027</td>
</tr>
<tr>
<td>HMDB61859</td>
<td>25.83 ± 0.12‡§</td>
<td>0.432183</td>
<td>0.62</td>
<td>-0.535</td>
<td>-0.148</td>
</tr>
<tr>
<td>(9Z)-Octadec-9-enoic acid</td>
<td>17.69 ± 0.25‡§</td>
<td>0.073338</td>
<td>&lt;0.01</td>
<td>-0.001</td>
<td>0.062</td>
</tr>
<tr>
<td>HMDB00207</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>19.48 ± 0.17*</td>
<td>-0.00985</td>
<td>0.02</td>
<td>-0.005</td>
<td>0.021</td>
</tr>
<tr>
<td>HMDB00827</td>
<td>26.62 ± 0.16*</td>
<td>0.468214</td>
<td>0.02</td>
<td>-0.043</td>
<td>0.043</td>
</tr>
<tr>
<td>(5Z,8Z,11Z,14Z)-Icosa-</td>
<td>16.09 ± 0.26‡§</td>
<td>-0.021693</td>
<td>0.02</td>
<td>0.023</td>
<td>0.137</td>
</tr>
</tbody>
</table>

continued on next page
<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Elution Time (pm)</th>
<th>ppm Diff</th>
<th>One-way ANOVA $P$-value</th>
<th>Fold 6 h vs. control</th>
<th>Fold 6+6 h vs. control</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,8,11,14-tetraenoic acid, HMDB01043</td>
<td>24.02 ± 0.14§</td>
<td>0.000000</td>
<td>0.07</td>
<td>-0.003</td>
<td>0.043</td>
</tr>
<tr>
<td>18-Methyl-(8Z,11Z,14Z-)nonadeca-trienoic acid, LMFA01020221</td>
<td>31.84 ± 0.40*‡§</td>
<td>-0.061465</td>
<td>0.04</td>
<td>0.001</td>
<td>0.036</td>
</tr>
<tr>
<td>18-Methyl-(8Z,11Z,14Z-)nonadeca-trienoic acid, LMFA01020221</td>
<td>36.08 ± 0.45†</td>
<td>-0.157113</td>
<td>0.13</td>
<td>-0.522</td>
<td>0.065</td>
</tr>
<tr>
<td>(8Z,11Z,14Z)-Icosatrienoic acid, LMFA01030158</td>
<td>37.57 ± 0.45†§</td>
<td>-0.295831</td>
<td>0.69</td>
<td>-0.233</td>
<td>-0.182</td>
</tr>
</tbody>
</table>

Fatty acid metabolites were identified in the non-polar fraction of ethanol-exposed mouse embryos. The database identity of the metabolite is listed, followed by the elution time, ppm difference, one-way ANOVA $P$-value, and fold, relative to controls. *: $P < 0.05$ using ANOVA; †: $P < 0.05$ using t-test, 6 h vs. control; ‡: $P < 0.05$ using t-test, 6+6 h vs. control; §: $P < 0.05$ using t-test, 6 h vs. 6+6 h. Database ID abbreviations include LMFA: LipidMaps and HMDB: Human Metabolome Database.
3.3.2 Glycerolipids

Two glycerolipids were identified in the mouse metabolome (Table 3.2). Monoacylglycerol (16:0/0:0/0:0) (MG) was significantly decreased from controls with a single ethanol dose to −0.693, as well as significantly increased from controls with a second ethanol dose to 0.395-fold (significantly different from singly dosed animals). MG(18:0/0:0/0:0) differed similarly to −0.238-fold with a single ethanol dose and 0.060-fold with twice-dosed animals.

Table 3.2.
Two glycerolipid metabolites were identified in the ethanol-treated mouse non-polar fraction, neither of which were significantly changed by one-way ANOVA. The elution time of each metabolite is given, followed the database identity of the metabolite, the ppm difference, ANOVA P-value, and fold, relative to controls. ‡: P < 0.05 using t-test, 6+6 h vs. control; §: P < 0.05 using t-test, 6 h vs. 6+6 h. MG: monoglyceride; HMDB: Human Metabolome Database.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>P Value</th>
<th>Fold 6 h</th>
<th>Fold 6+6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG(16:0/0:0/0:0)</td>
<td>24.88 ± 0.12 ‡‡ § §</td>
<td>0.096888</td>
<td>0.21</td>
<td>-0.693</td>
<td>0.395</td>
</tr>
<tr>
<td>HMDB11564</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MG(18:0/0:0/0:0)</td>
<td>26.60 ± 0.16 ‡‡ § §</td>
<td>-0.958393</td>
<td>0.26</td>
<td>-0.238</td>
<td>0.060</td>
</tr>
<tr>
<td>HMDB11131</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

3.3.3 Glycerophospholipids

Five glycerophospholipid metabolites were identified in the mouse FASD metabolome (Table 3.3). Phosphatidic acid(18:0/18:2(9Z,12Z)) (PA) was not different from control with a single ethanol dose, but after two doses was 6.213-fold higher than controls. PC (O-22:2(13Z,16Z)/22:3(10Z,13Z,16Z)) similarly significantly increased to 6.371-fold higher than control animals in the 6+6 h group. Phosphatidylglucose (12:0/19:0) (PG) was not significantly different with ethanol treatment. PG (P-20:0/22:4(7Z,10Z,13Z,16Z)) was significantly different, decreasing to −5.809-fold with a single ethanol dose, then increasing to 1.011-fold in those dosed twice. Lastly,
PS (20:1(11Z)/22:2(13Z,16Z)) was significantly different with multiple ethanol exposures, increasing to 6.291-fold.
Glycerophospholipid metabolites were identified in the non-polar fraction of ethanol-exposed mouse embryos. The database identity of each metabolite, elution time, ppm difference, one-way ANOVA P-value, and fold, relative to controls are given. *: $P < 0.05$ using ANOVA; †: $P < 0.05$ using t-test, 6 h vs. control; ‡: $P < 0.05$ using t-test, 6+6 h vs. control; §: $P < 0.05$ using t-test, 6 h vs. 6+6 h. Metabolite abbreviations include PA: phosphatidic acid; PC: phosphatidylcholine; PG: phosphoglycerol; PS: phosphatidylserine. Database ID abbreviations include LMGP: LipidMaps; HMDB: Human Metabolome Database.

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>$P$</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA(18:0/18:2(9Z,12Z))</td>
<td>32.44 ± 0.18</td>
<td>-1.13561</td>
<td>&lt;0.01</td>
<td>6.213</td>
</tr>
<tr>
<td>HMDB07861</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC(O-22:2(13Z,16Z)/22:3(10Z,13Z,16Z))</td>
<td>38.73 ± 0.28</td>
<td>2.340455</td>
<td>&lt;0.01</td>
<td>6.371</td>
</tr>
<tr>
<td>HMDB13456</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG(12:0/19:0)</td>
<td>36.09 ± 0.45</td>
<td>1.537769</td>
<td>0.18</td>
<td>0.037</td>
</tr>
<tr>
<td>LMGP04010057</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PG(P-20:0/22:4(7Z,10Z,13Z,16Z))-</td>
<td>33.73 ± 0.35</td>
<td>2.6191</td>
<td>&lt;0.01</td>
<td>-5.809</td>
</tr>
<tr>
<td>LMGP04030085</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS-(20:1(11Z)/22:2(13Z,16Z))-</td>
<td>32.03 ± 0.25</td>
<td>-1.7663</td>
<td>&lt;0.01</td>
<td>6.291</td>
</tr>
<tr>
<td>LMGP03010555</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.4 Prenol Lipids

Three prenol lipids were identified (Table 3.4). α-Bergamotenol was significantly increased in the repeatedly dosed animals to 0.021-fold. Retinoate eluted at two retention times. At the earlier retention time it was not significantly different, but at the later retention time was, increasing to 0.032-fold control. 30-(2-(O-2Hydroxy-ethane)-3-hydroxy-propane)-hopane was significantly increased to 6.339-fold in the 6+6 h treatment group.
Table 3.4.
Prenol lipid metabolites identified in the non-polar fraction of ethanol-exposed mouse embryos. Three different prenol metabolites were identified in the mouse non-polar fraction after ethanol treatment. The elution time of each metabolite is given, followed by the calculated mass and the database identity of the metabolite, the ppm difference, one-way ANOVA P-value, and fold, relative to controls. *: $P < 0.05$ using ANOVA; †: $P < 0.05$ using t-test, 6+6 h vs. control; §: $P < 0.05$ using t-test, 6 h vs. 6+6 h. Database ID abbreviations include C: KEGG; LMPR: LipidMaps; M: Metlin

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>$P$</th>
<th>Fold 6 h</th>
<th>Fold 6+6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Bergamotenol M91544</td>
<td>22.65 ± 0.04†</td>
<td>-0.482781</td>
<td>0.43</td>
<td>0.006</td>
<td>0.021</td>
</tr>
<tr>
<td>Retinoate C00777</td>
<td>29.31 ± 0.20</td>
<td>0.151561</td>
<td>0.16</td>
<td>0.002</td>
<td>0.050</td>
</tr>
<tr>
<td>30-(2-(O-2-Hydroxy-ethane)-3-hydroxy-propane)-hopane LMPR04000024</td>
<td>31.86 ± 0.39‡§</td>
<td>0.032644</td>
<td>0.04</td>
<td>-0.002</td>
<td>0.032</td>
</tr>
<tr>
<td>33.90 ± 0.23‡§</td>
<td>-0.775539</td>
<td>&lt;0.01</td>
<td>0.000</td>
<td>6.339</td>
<td></td>
</tr>
</tbody>
</table>
3.3.5 Sphingolipids

Six sphingolipids were identified in mouse treated once or twice with ethanol (Table 3.5). Ceramide (d18:1/16:0) (Cer) significantly differed, decreasing to –0.354-fold with a single ethanol-exposure and increasing significantly to 0.992-fold in multiply-exposed animals. Similarly, Cer(d18:1/22:0) decreased to –5.664-fold that of control animals with a single ethanol dose, then increasing to 1.009-fold in multiply dosed animals. Cer(d18:1/23:0) was significantly different, increasing to 6.358-fold only after the second ethanol dose. Nervonic ceramide differed significantly as well, decreasing to –0.626-fold with a single ethanol exposure, then increasing to 0.648-fold with two exposures. N-Ligno-ceroyl-sphingosine increased in both treatment modalities. PI-ceramide (d18:1/22:0) decreased to –0.238-fold in the 6 h group, but was significantly increased from controls to 0.628-fold in the 6+6 h group. Lastly, C22 sulfatide was significantly different because of the 6.381-fold levels in the twice-dosed group.
Table 3.5.
Seven sphingolipid metabolites were identified in the ethanol-treated mouse non-polar fraction, many of which changed significantly from untreated control animals. C22 Sulfatide, a glycerosphingolipid, is also included. The database identity of each metabolite, elution time, the ppm difference, one-way ANOVA P value, and fold relative to controls are given. *: $P < 0.05$; †: $P < 0.05$ using t-test, 6 h vs. control; ‡: $P < 0.05$ using t-test, 6+6 h vs. control; §§: $P < 0.05$ using t-test, 6 h vs. 6+6 h. Metabolite abbreviations include Cer: ceramide. Database ID abbreviations include LMSP: LipidMaps; HMDB: Human Metabolome Database

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>$P$</th>
<th>Fold 6 h</th>
<th>Fold 6+6 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cer(d18:1/16:0)</td>
<td>32.37 ± 0.31*†§</td>
<td>-0.50157</td>
<td>0.03</td>
<td>-0.354</td>
<td>0.992</td>
</tr>
<tr>
<td>HMDB04949</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer(d18:1/22:0)</td>
<td>39.62 ± 0.30*†§</td>
<td>-1.391557</td>
<td>&lt;0.01</td>
<td>-5.664</td>
<td>1.009</td>
</tr>
<tr>
<td>HMDB04952</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cer(d18:1/23:0)</td>
<td>41.74 ± 0.28*†§</td>
<td>-1.592142</td>
<td>&lt;0.01</td>
<td>0.000</td>
<td>6.358</td>
</tr>
<tr>
<td>HMDB00950</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nervonic ceramide</td>
<td>39.78 ± 0.32*‡</td>
<td>-1.539479</td>
<td>0.04</td>
<td>-0.626</td>
<td>0.648</td>
</tr>
<tr>
<td>HMDB04953</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Ligno-ceroyl-sphingosine</td>
<td>44.11 ± 0.34§</td>
<td>-1.671702</td>
<td>0.26</td>
<td>0.177</td>
<td>0.889</td>
</tr>
<tr>
<td>HMDB00831</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylinositol-ceramide (d18:1/22:0)</td>
<td>33.97 ± 0.40‡</td>
<td>-2.466695</td>
<td>0.12</td>
<td>-0.238</td>
<td>0.628</td>
</tr>
<tr>
<td>LMSP03030001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C22 Sulfatide</td>
<td>32.53 ± 0.25*†§</td>
<td>-1.940678</td>
<td>&lt;0.01</td>
<td>0.000</td>
<td>6.381</td>
</tr>
<tr>
<td>LMSP06020009</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.6 Other Metabolites

Four other metabolites were also identified in the mouse metabolome (Table ??). 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one increased significantly with either modality of ethanol-treatment. Dimethisterone was significantly different from controls in the twice-dosed animals, decreasing to −0.199-fold. The peptide proline-serine-argining decreased with singly-exposed animals, and increased in multiple-exposed animals. Lysine-histidine-methionine eluted with two elution times, with variable results.
Table 3.6.
Other metabolites identified in the non-polar fraction of ethanol-exposed mouse embryos. Five metabolites of other types were identified in the mouse non-polar fraction. The database identity of each metabolite, elution time, the ppm difference, ANOVA P-value, and fold relative to controls are given. ∗: $P < 0.05$ using one-way ANOVA; †: $P < 0.05$ using t-test, 6 h vs. control; ‡: $P < 0.05$ using t-test, 6+6 h vs. control; §: $P < 0.05$ using t-test, 6 h vs. 6+6 h. Database ID abbreviations include C: KEGG; HMDB: Human Metabolome Database; M: Metlin

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Metabolite Name</th>
<th>Elution Time</th>
<th>ppm Diff</th>
<th>P</th>
<th>Fold 6 H</th>
<th>Fold 6+6 H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Cleaved A) HMDB35245</td>
<td></td>
<td>21.00 ± 0.07*</td>
<td>-1.864391</td>
<td>0.01</td>
<td>0.005</td>
<td>0.041</td>
</tr>
<tr>
<td>Dimethi-sterone C07628</td>
<td></td>
<td>22.60 ± 0.06†</td>
<td>-0.220726</td>
<td>0.95</td>
<td>-0.182</td>
<td>-0.199</td>
</tr>
<tr>
<td>Proline-serine-arginine M15698</td>
<td></td>
<td>21.58 ± 0.04</td>
<td>0.597437</td>
<td>0.36</td>
<td>-0.200</td>
<td>0.276</td>
</tr>
<tr>
<td>Lysine-histidine-methionine M17238</td>
<td></td>
<td>15.58 ± 0.21</td>
<td>-2.250094</td>
<td>0.46</td>
<td>0.044</td>
<td>-0.517</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16.86 ± 0.17§</td>
<td>-1.533058</td>
<td>0.11</td>
<td>-0.504</td>
<td>0.017</td>
</tr>
</tbody>
</table>
3.3.7 Common Metabolites

Six significantly different metabolites common to both mouse and zebrafish FASD models (Table 3.7). The m/z values were identified as 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, tetradecanoic acid, methyl hexadecanoic acid, octadecanoic acid, PA(18:0/18:2(9Z,12Z)), and C22 sulfatide. In general the magnitude of change was much greater for zebrafish than in mice. Changes with a single dose of ethanol in the mouse model never exceeded 1-fold. However, with a second 6 h ethanol exposure in the mouse model, PA(18:0/18:2(9Z,12Z)) and C22 sulfatide were increased by more than 6.2-fold control levels. In the zebrafish, these metabolites showed more than a 6-fold decrease with low ethanol concentrations, then increased to levels similar to controls at moderate-ethanol doses. 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one increased marginally in the repeated mouse exposure model, but was decreased at most ethanol doses in the zebrafish model, falling to as low as –7.059 with 0.1% ethanol. Tetradecanoic acid changed slightly in the mouse model, but was increased more than 6-fold with all ethanol doses in the zebrafish model, except at 0.003% ethanol, where it was not different from controls. Methyl hexadecanoic acid and octadecanoic acid both varied little from controls in the mouse model. In the zebrafish model methyl hexadecanoic acid increased slightly with 0.001% ethanol, then decreased to –6.326-fold control levels at 0.3% ethanol and higher. Octadecanoic acid similarly increased with 0.003% ethanol to 0.190-fold control levels, and then fell to –0.825-fold control levels at 3% ethanol.
Table 3.7.
Few significantly changed (one-way ANOVA within-species) metabolites were common to both the *Danio rerio* and *Mus musculus* non-polar metabolomes. The database identity of each metabolite, as well as the fold, relative to the same species controls are given. For the mouse treatments, embryos were excised and exposed to one \((n=7)\) or two \((n=7)\) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice \((n=6)\) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos \((n=8\) per group) were exposed from 2 –24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). Database ID abbreviations include C: KEGG; M: Metlin

<table>
<thead>
<tr>
<th>Metabolite ID</th>
<th>Fold <em>Mus musculus</em></th>
<th>Fold <em>Danio rerio</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>6 h</td>
<td>6+6 h</td>
</tr>
<tr>
<td>1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Cleaved A)</td>
<td>C09690</td>
<td>0.005</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>HMDB00806</td>
<td>-0.682</td>
</tr>
<tr>
<td>Methyl hexadecanoic acid</td>
<td>C16995</td>
<td>-0.021</td>
</tr>
<tr>
<td>Octadecanoic acid</td>
<td>C01530</td>
<td>-0.043</td>
</tr>
<tr>
<td>PA(18:0/18:2(9(Z),12(Z)))</td>
<td>LMGP10010036</td>
<td>0.000</td>
</tr>
<tr>
<td>C22 Sulfatide</td>
<td>M41627</td>
<td>0.000</td>
</tr>
</tbody>
</table>
3.3.8 Probabilistic Principal Component and Covariates Analysis

Because we were comparing two different species with different modalities of ethanol exposure, we pursued a PPCCA. This model is an extension on the probabilistic principal component analysis, and allows for the incorporation of observed covariates into the model. The MetabolAnalyze package was selected due to the need to include the covariates (species and treatment) in the analysis. Standard principal component analysis (PCA) does not accommodate this \[487\]. Further PPCCA also accommodates missing variables using the “Expectation Maximum” algorithm.

The PPCCA was generated from the five common metabolites present in both the mouse and zebrafish models that were significantly different. The Bayesian Information Criterion plot indicated that the maximum number of principal components (PrC), \( q \), should be 4, and so subsequent models were generated using a maxq = 4 (Figure 3.10). Loadings plots were generated to examine the relationship between each metabolite on the latent variable (Figure 3.2). The loadings plot demonstrated the PrC1 was positively effected by the response of 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (the metabolite we believe to be a cleaved vitamin A moiety, Cleaved A, based on their structural similarity [Figure 3.1, Structure ]), methyl hexadecanoic acid, and to a lesser extent octadecanoic acid to ethanol. Tetradecanoic acid and octadecanoic acid were the only metabolites that were negatively loaded in PrC2. For PrC3, tetradecanoic acid, octadecanoic acid, PA(18:0/18:2), and C22 sulfatide were positively loaded, while 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one and methyl hexadecanoic acid were negative. Lastly, all metabolites except octadecanoic acid loaded negatively on PrC4. PA(18:0/18:2) and C22 sulfatide always had the same directional loading on the latent variables. Scores plots demonstrate the estimated score of each individual within the reduced PrC space (Figure 3.3). The increasing size of the circles, the 95% posterior set, indicate that PrC3 and PrC4 are less explanatory of our data than PrC1 and PrC2. The model was refit using jackknife sampling to examine the uncertainty of the loadings estimates (Figure ??). The loadings plot, which includes 95% confidence intervals calculated from the standard error generated by recomputing the loadings, indicating which loadings are significantly different from zero (Figure ??) \[487\]. 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, methyl hexadecanoic acid, and octadecanoic acid loaded positively on PrC1, however octadecanoic acid was not significant. Tetrade-
Fig. 3.1. Zebrafish embryos were treated from 2–24 hpf to 0–3% ethanol. Two of the vitamin A-related metabolites observed, included 9,13-cis-Retinoate (Structure 3-1) and 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Structure 3-2), potentially a cleavage product of retinoate degradation.

canoic acid, PA(18:0/18:2), and C22 sulfatide loaded significantly negatively on PrC1.

3.4 Discussion

FASD is caused by ethanol exposure during development. Because of the complex nature of this syndrome, it has been difficult to determine biochemical effects common to both binge and chronic alcohol exposure modalities. We have utilized our well-established mouse model to generate an “unbiased” non-polar metabolome of FASD. For mice, excised c57BL/6J E8.2 embryos were exposed to 400 mg/dL of ethanol bursts on consecutive days for zero (control, n=6), one (6 h, n=7) or two 6 h (6+6 h, n=7). This model represents a binge ethanol effect. We compared these results to our chronic ethanol exposure model, where zebrafish embryos are exposed from 2–24 hours post fertilization (hpf) to ethanol at concentrations from 0–3%.
In the mouse metabolome, we noted differences in fatty acids (9), two glycerolipids, several glycerophospholipids (5), three prenols, sphingolipids (7), and other metabolites (4). These results echo similar findings in our chronic zebrafish FASD model, however the magnitude of changes in the binge mouse metabolites was greatly diminished (Chapter 2). In addition, our results demonstrate statistically significant differences in the mouse metabolome between singly and doubly dosed animals, suggesting a compounding effect of repeated binge exposure.

When we compared our previously reported zebrafish metabolome with the results of the mouse metabolome, six common, significantly different metabolites were identified. These included 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one (Cleaved A), tetradecanoic acid, methyl hexadecanoic acid, octadecanoic acid, PA(18:0/18:2), and C22 sulfatide. We conducted a PPCCA of these models, to determine latent similarities and differences of significantly different metabolites in these contrasting exposure modalities and species. The PrC loadings of these models underscore the importance of alterations of chemicals involved in neural structure formation, cell death, and secondary cellular signaling in the etiology and pathogenesis of FASD. The biochemical changes occurring in the non-polar FASD metabolome common to both models, may represent a unifying approach to the dose and/or timing debate in FASD research, and show that more is not just more.

3.4.1 Growth and Energy

Growth and energy metabolism changes are important in understanding FASD. Animals consuming alcohol, generally reduce their intake of food \(^{285}\). This is generally addressed through the use of pair-fed dams. This might convey an overt advantage to the zebrafish model, as they are not ingesting nutrients. However, we have not examined whether or not there is a change in the uptake of nutrients sourced from maternal yolk with ethanol. Also, Weinberg et al. perceptively point out that mothers pair-fed to match the animals on the alcohol-based diet are therefore in a restricted intake state, as the ethanol-based diets are formulated to be complete. In their experiments, pair-fed animals appeared to be chronically hungry, consuming their food much more rapidly than \textit{ad libitum} fed animals. Therefore, the pair fed animals are under an induced stressed by this limited diet \(^{285}\). These findings were confirmed with post-adrenalectomy pair-fed animals showed increases in adrenocorticotropic and corticotrophin-releasing hormones, above that of control animals and
similar to alcohol fed animals. However, even beyond these energy differences, we noted changes that may influence lipid droplets and lipid uptake, which are likely to alter growth and energy metabolism.

**Hepatic Stellate Cells May Mobilize Lipid Droplets in Response to Retinoid Metabolism Changes**

Similar to our findings in zebrafish, we found many binge mouse metabolome changes that impact the growth and energy. These included differences in fatty acids, glycerophospholipids, and sphingolipids. PA, PC, and PS are all important in the nervous system, but all three of these metabolites are also important in the structure and function of lipid droplets [490]. Abnormal lipid droplets are associated with ethanol consumption or prenatal ethanol exposure. Female UCh ethanol-prefering rats fed ad libitum ethanol demonstrate unusual cytoplasmic lipid droplet accumulation in the lining of the endometrium [491]. In addition, in prenatally ethanol exposed male Wistar rats fed a high-fat diet after weaning, lipid droplets were abnormally accumulated in the hypothalamus, pituitary, and liver [492]. In newborn offspring of Hartley guinea pig dams fed 6 g ethanol/kg body weight for the second half of gestation, electron micrographs of the gastrocnemius show enlarged lipid droplets [493].

In normal conditions, cytosolic lipid droplets in rat hepatic stellate cells (HSC) contain “retinyl ester, triglyceride, cholesteryl ester, cholesterol, phospholipids, and free fatty acids”, with retinyl esters and triglycerides making up the largest portion of total lipids [494]. Lecithin:retinol acyltransferase is required for formation of these lipid droplets [494]. In a distressed, diseased, or chronic alcohol-exposed state, HSC become activated and lose these droplets, including the retinoid [494]. Recent work in isolated HSC from Sprague Dawley® rats has demonstrated that vitamin A and insulin are required to maintain HSC in their inactivated form [495]. Due to mobilization of hepatic retinyl esters, some tissues are found to have an excess of all-trans-retinoic acid with ethanol treatment [420–422].

Vitamin A has been well studies for its relationship to FASD. There is important mechanistic overlap between alcohol catabolism and vitamin A metabolism. ADH3, along with the cofactor NAD⁺, converts retinol to retinal, which is then able to be converted to retinoic acid [84]. During gastrulation of *Xenopus* embryos, retinaldehyde dehydrogenase is competitively inhibited by ethanol [419]. There appears to be a diminished effectiveness of vitamin A, as Long Evans pups postnatally exposed to
ethanol (∼80 mM blood alcohol concentration) have decreased cerebellar expression of retinoic acid receptors α/γ, but increased expression of retinoic acid X receptors α/γ [423]. Further, genomic interactome analysis of the brains of c57BL/6J prenatally ethanol exposed mice (exposed at various times to 2.5 g/kg ethanol) showed retinoate metabolism genes were underexpressed [130].

Vitamin A, significantly increased with ethanol treatment in our mouse model and our previously reported zebrafish polar metabolome (Chapter 2). In addition, 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one significantly increased in the binge mouse model, but was significantly decreased in the zebrafish. We hypothesize that this metabolite, based on its structural similarity, is actually a cleaved form of vitamin A. Vitamin A is an isoprenoid, a class of molecules noted to be perturbed in FASD [48]. Similarly, we found two other isoprenoids that differed in our model, one of which increased 6.339-fold after two alcohol exposures.

Since we are examining whole-embryo extracts, simply mobilizing the lipid droplets from the HSC would not result in a change in the metabolite levels detected. However, as retinyl esters and retinoate differ in mass, the increased vitamin A we detect could be a result of increased mobilization and conversion to retinoate. Ethanol mobilizes HSC lipid droplets in response to decreased function in vitamin A metabolism, decreased retinoic acid receptors, and underexpressed genes, all caused by ethanol. In addition, ethanol induces a cleavage of vitamin A, which likely further disrupts function. With a single burst of ethanol, vitamin A metabolism is not different and seemingly able to manage the insult. But, with an additional binge of ethanol, HSC mobilize lipid droplets in compensation for the effects of ethanol. With the chronic ethanol exposure of the zebrafish, the effects of ethanol result in vitamin A being completely consumed, in spite of the lipid mobilization, and the cleavage product is also broken down.

**Lipid Metabolism**

The non-polar extract metabolome of the ethanol-exposed embryo shows significant differences in fatty acids. We noted significant alterations in dodecanoic, tetradecanoic, hexadecanoic, octadecanoic, (9Z)-octadec-9-enoic acid, amongst others. In the zebrafish, we noted increased, decreased, and hormetic responses to ethanol treatment in the significantly different fatty acids, which we believe are associated with abnormal growth and development caused in part by aberrant fatty acid metabolism.
(Chapter 2). In the mouse model, significantly different metabolites were similar to or marginally increased with a single ethanol dose, while a repeated dose increased the fatty acid variation. From our data, it is unclear whether these increases are due to decreased fatty acid utilization or increased synthesis.

MG can be generated via enzymatic alcoholysis. Several lipases have aversion to polyunsaturated fatty acids, resulting in a bias toward the reaction of saturated and monounsaturated fatty acids being converted to ethyl esters. 2-Monoacylglycerol (2-MG) has also been shown to be produced in vitro by similar lipase activities. Particularly lipase D has been shown in vitro to result in high levels of 2-MG, and can be deactivated by contact with ethanol, making a chronic drinking mother exacerbate this lipase malfunction. This ethanol-induced deactivation of lipases could explain the decrease in MG noted in singly-dosed animals. Levels of MG increased significantly between singly- and doubly-dosed animals, which may suggest increased levels or function of lipase with repeated exposure.

Additionally, monoacylglycerol-acyltransferases (MGAT) and diacylglycerol (DAG) acyltransferases are essential building blocks for triacylglycerol synthesis. MGAT is localized in the endoplasmic reticulum, absorbing dietary lipids in the enterocyte. It also mediates intracellular concentrations of MG and participates in energy regulation and mediation of insulin sensitivity. Monoacylglycerol-O-acyltransferase 1 gene expression has been shown to increase 1.20-fold in Danio rerio exposed to $e^{-7} M$ 17$\beta$-estradiol for three weeks. In analysis of our polar fraction metabolome in zebrafish, we noted that even with low ethanol doses 1a-hydroxyesterone increased significantly (Chapter 2). In addition, we found a significant increase in 16-dehydroprogesterone and a significant decrease in a precursor-related product 13-ethyl-16,17-dihydroxy-18,19-dinorpregna-4,9,11-trien-20-yn-3-one. Interestingly, others have reported no difference in 17$\beta$-estradiol in ethanol treated alcohol-preferring or Wistar female rats. Taken together, these findings may suggest that with ethanol exposure, increases in estrogen-related metabolites upregulate MGAT, which in conjunction with inactivated lipase, causes changes in MG.

**PPCCA and Lipids**

Many researchers use PCA in order to analyze their data, but fewer go beyond and look for the biological meaning behind them—the driver of the principal components. Without a more in-depth exploration of interpretation of PCA, we are left in the realm
of interesting coincidence rather than a meaningful research driver. Given the limited amount of coinciding, significantly different metabolites in these species, it is difficult to make the leap to interpretation. However, based on the PPCCA PrC1 loading being positive for cleaved A, methyl hexadecanoic acid, and octadecanoic acid, the latent variable may be representing the lipid metabolism changes noted above.

3.4.2 Nervous System

Glycerophospholipids

As in our previous work, we found many binge mouse metabolome changes that impact the nervous system. Glycerophospholipid changes that occur with ethanol exposure have important implications in the nervous system. With one exception, the glycerophospholipids we identified were not different from control after a single ethanol dose, but increased significantly after a second ethanol dose. PA, PS, and PC increased the most, to more than 6.2-fold controls. These metabolites play an important role in the nervous system. PC is converted by phospholipase D (PLD) to PA. PA has a variety of functions, including in astroglial proliferation, neurite outgrowth, function as a signaling molecule, or contribute to the phospholipid bilayer of all cells [101, 490].

Ethanol can shunt PLD to generate phosphatidylethanol instead of PA from PC [99, 115]. In addition, ceramide, which was generally decreased with one ethanol dose and increased with two, can inhibit PLD [116]. With chronic ethanol exposure in the zebrafish, there were a variety of differences in PA moieties, but PC moieties were all significantly increased with ethanol treatment. In the binge mouse model, the PA and PC detected significantly increased, but only after the second ethanol dose. It is unclear whether these differences are due to decreased conversion of PC to PA by PLD and/or decreased utilization of PA.

Sphingolipids

Ceramides have a variety of functions, including as precursor of the myelin component sphingomyelin, as an activator of phosphatases and thereby cell death, and also as a precursor to sphingosine [33]. It has been hypothesized that prenatal ethanol exposure causes an increase of ceramide by increasing serine-palmitoyl trans-
ferase activity. This results in *de novo* synthesis of ceramide, rather than conversion from sphingomyelin via sphingomyelinase [33]. Others have proposed ethanol increases ceramide sphingosine recycling in rat cerebellar granule cells [127]. During all-trans-retinoic acid-induced neuron differentiation, SH-SY5Y neuroblastoma cells have downregulated (mRNA, protein, and enzymatic activity) neutral ceramidase, an enzyme critical for control of ceramide turnover [142]. This occurs without an alteration in the level of sphingosine or sphingosine–1-phosphate [142]. In conjunction with the significant increase in retinoate and the established abnormalities in retinoid metabolism that occurs with ethanol, our results may suggest that neutral ceramidase is downregulated in FASD. However, we did note twice-dosed mice had significantly increased *N*-ligno-ceroyl-sphingosine and ceramides than controls, so this notion requires further investigation.

Many ceramides decreased significantly with a single ethanol dose, while others increased significantly with two ethanol doses. The ceramide precursor octadecanoic acid similarly decreased with one dose, but then significantly increased with two doses. These differences are important as ceramide accumulation with ceramidase activity, can lead to increases in sphingosine, and result in consequent toxicity to neurons [134].

PI-ceramide was present in both the zebrafish and mouse metabolomes. With a single dose in the mouse or at the lowest chronic zebrafish dose, this metabolite decreased. However, with higher zebrafish ethanol doses it was marginally increased, and with a second ethanol dose in the mouse it was significantly increased from controls. PI, which can be converted to the PKC activator DAG. Sphingosine and ceramide accumulation are known to inhibit PKC [33, 135, 136]. Contrary to the significant increase in PA and PC in twice-dosed animals, ceramides have been shown to inhibit phospholipase D, preventing the conversion of PC to PA [116]. So, while the decreases in PI in conjunction with increases in ceramides suggests decreased PKC activation, the increases in PA and PC are confounding and require further investigation.

**Sulfatides**

Sulfatides are a class of sulfoglycosphingolipid that participate in important physiological functions such as the immune system responses, insulin secretion, hemostasis, and thrombosis [143]. Studies of Sprague Dawley® show they compose ∼4.4% of myelin [143, 144]. Sulfatides function as a negative regulator in oligodendrocyte
differentiation, influence myelin stability, and a myelin-associated neurite outgrowth inhibitor \[143,145,146,148\].

In the chronic zebrafish model, sulfatides were significantly decreased. However, in the binge mouse model, a single ethanol dose did not modify sulfatide, but a second dose increased it to 6.381-fold. It is possible that with minimal ethanol exposure, sulfatides are unaffected. But, with repeated dosing, sulfatides are increased for the purpose of myelin preservation. However, when alcohol exposure is chronic, the decreased sulfatides represent destabilized myelin. Further study through examination of labelled sphingolipids may help clarify the exact relationship between ethanol modalities and sulfatides.

**PPCCA and the Nervous System**

Based on the loadings of PrC2 and PrC3, the underlying latent variable of these components is likely neural related. PPCCA PrC2 was positively loaded by cleaved A, PA\(18:0/18:2\), and C22 sulfatide. PA\(18:0/18:2\) and C22 sulfatide. PPCCA PrC3 was positive for ocatdecanoic acid, PA\(18:0/18:2\), and C22 sulfatide, and to a lesser extent tetradecanoic acid. It is clear that these metabolites are important in the development and functioning of the nervous system. However, the exact mechanism generating PrC2 and PrC3 requires further investigation.

### 3.4.3 Relativistic Inter-specific Development Model

An enormous limitation to our capabilities in translational medicine is the lack of ability to quantitate the inter-specific differences seen in the laboratory. To be effective as translational scientists we must develop methods to measure differences in species to account for these not-so-nuanced differences. We have mainly focused as a community on the use of biomarkers, but working toward quantitating model differences will lead to better predictive models of human disease. To that end, we have developed a novel application of the PPCCA, as discussed above. In addition, to contextualize our metabolome findings, we have developed a tool for intra-specific development comparison.

For over 100 years, even as early as Haekel’s artistic rendering of developmental stages in the chick, the debate as to the similarity between species has continued. While some find homology in the pattering of development stages, others find nu-
ances that indicate clearly different developmental order, compartmentalization, and modularity. Relative to mice and humans, many developmental systems are conserved in zebrafish, such as the presence of somites, body axis patterning, and central nervous system. However, there are several important considerations. Zebrafish demonstrate phylotypic variability, supporting the notion that “there is no highly conserved embryonic stage in vertebrate embryos but rather a period of general similarity.” Further, vital to many experiments is breeding of fish. Individual aquarium tanks require the manual insertion of breeding media (marbles or mesh with plants over a collection vessel) into the tank for breeding. This results in asynchronous breeding which has a great potential to influence embryonic or larval temporal experiments.

There remains, however, appreciable utility in comparing and contrasting findings from these exposure modalities, in spite of the species difference. An extremely important consideration in the evaluation of any research model of development is the asynchronous order or progression rate that occurs between species. For perspective, an approximate timeline comparing human, mouse, and zebrafish development is provided (Figure 3.6). The x-axis is $ln$ proportion of days until reproductive maturity.
Fig. 3.2. Principal component loadings barplots for the PPCCA model generated from metabolites significantly changed in both the mouse and zebrafish models of FASD, using species and ethanol exposure as covariates. For the mouse treatments, embryos were excised and exposed to one ($n=7$) or two ($n=7$) for 6 h exposures of 400 mg/dL alcohol on consecutive days. Control mice ($n=6$) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos ($n=8$ per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). The PPCCA model incorporates significantly changed (one-way within-species ANOVA, $P < 0.05$) metabolites appearing in both species. Cleaved A: 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one; PA: Phosphatiic Acid.
Fig. 3.3. PPCCA scores plot of the individuals in each treatment group with respect to the PC loading, demonstrating the estimated score of each individual within the reduced PrC space. The increasing size of the circles, the 95% posterior set, indicate that PrC3 and PrC4 are less explanatory of our data than PrC1 and PrC2. The x- and y-axes represent the relationship of one principal component to another. The filled circles indicate mice and triangles indicate zebrafish. The color represents a spectrum of ethanol exposure from zero (red) through 805.91 μ M (blue). The grey circles represent 95% posterior sets, which are large, indicating some degree of uncertainty. For the mouse treatments, embryos were excised and exposed to one (n=7) or two (n=7) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice (n=6) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos (n=8 per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). The PPCCA model incorporates significantly changed (one-way within-species ANOVA, $P < 0.05$) metabolites appearing in both species.
Fig. 3.4. Scatterplot of the PPCCA model loadings generated from metabolites significantly changed in both the mouse and zebrafish models of FASD, using species as a covariate.

The relationship of each metabolite in the principal components space is plotted. For the mouse treatments, embryos were excised and exposed to one (n=7) or two (n=7) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice (n=6) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos (n=8 per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). The PPCCA model incorporates significantly changed (one-way within-species ANOVA, P < 0.05) metabolites appearing in both species. Cleaved A: 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one; PA: Phosphatiic Acid.
Fig. 3.5. Barplot of the PPCCA model after Jackknife resampling, generated from metabolites significantly changed in both the mouse and zebrafish models of FASD, using species and ethanol exposure as covariates. Cleaved A, methyl hexadecanoic acid, and octadecanoic acid loaded positively on Principal Component 1, while tetradecanoic acid, PA(18:0/18:2), and C22 sulfatide loaded negatively. Error bars representing 95% confidence interval show stearic acid is not significantly loaded on PC1. For the mouse treatments, embryos were excised and exposed to one (n=7) or two (n=7) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice (n=6) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos (n=8 per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). The PPCCA model incorporates significantly changed (one-way within-species ANOVA, $P < 0.05$) metabolites appearing in both species. Cleaved A: 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one; PA: Phosphatiic Acid.
Based on this timeline, we note the magnitude of difference between the ethanol exposures of the chronic and binge models is quite large. In addition, the ethanol exposure for the binge mouse model occurs within the relative time-frame of the zebrafish chronic exposure. At the time of sacrifice of the mice, they are approximately equivalent to Carnegie stage 15 humans. The time of zebrafish sacrifice would approximate to early to mid 2nd trimester human development.

Due to the differences in rates of reproductive maturity in these species, the timeline shows variation in key stages between species (i.e., each species has a different point for fertilization). While there are limitations to this approach as a result, it can still provide useful information regarding cross-species developmental comparisons in animal modeling. For example, with this comparison of development, human and zebrafish enter gastrulation relatively closely to one another, but mouse gastrulation is later than these two. Zebrafish also differ in development during the early cleavage stage in that it has meroblastic discoidal cleavage as opposed to mammals that have equal holoblastic cleavage [503]. Also, development of the pharyngeal arches in the zebrafish begins slightly after the human, but well before the mouse. (These relative delays in mice are counterintuitive, as they are more precocial at birth than humans.) Gastrulation and pharyngeal arch development are during the time of the ethanol exposure in the zebrafish model. However, in the mouse, this process occurs after the time of sacrifice in our experiments.

Another potentially important difference is that heart development in the mouse begins sooner than the zebrafish and later in the human, relatively. Heart development completes in all three at a relatively similar time. This may have particular relevance in the cardiovascular abnormalities that develop in FASD. In particular,
this may include the effects surrounding PKC, as PI-3 kinase activation regulates excitation-contraction in myocytes [254].

Zebrafish liver development begins after that of the human and before the mouse, relatively. This is important in understanding how our models function in catabolism of alcohol. However, based on this depiction of our model, liver development does not begin until after our mouse binge exposures. This could explain why so many metabolites relevant to the lipid droplet mobilization hypothesis discussed above are significantly differed only after the second ethanol exposure. In the future, this could be better controlled by not exposing the single-dose group to ethanol until the time of the second dose of the twice-dosed group.

In terms of relative neural tube closure, humans begin much before mice. In zebrafish the completion of neural cavitation falls between these two, and is near the end of the ethanol exposure window in this model. The implication of this is important, as this suggests that nervous system-related changes noted in the mouse impact neural development, more so than neural function. This distinction is important, as it can help separate some of the complexities of development that inhibit our understanding of neural defects in FASD.

While this method of relativistic developmental comparison is not without limits, this information is still an important consideration. With such an approach, selecting the most appropriate animal model for a development or aging-related disease or pharmacology can be made easier. Further, for complicated diseases, such as FASD, it can help explain differences in metabolism observed between different species, such as what we have shown here with metabolomics. While no single animal model can provide a comprehensive substitute for human research, intra-specific comparisons between models such as zebrafish and mice provides tremendous power as FASD research tool.

3.5 Conclusion

We have identified many mouse metabolites that appear to be significantly different only after a second dose of alcohol. We noted several changes that support the hypothesis of abnormal lipid droplets in FASD, based on significant differences in glycerophospholipids and vitamin A. In addition, we noted several changes in lipid-related metabolites, that support the notion that FASD alters lipid metabolism, potentially via ethanol’s inhibition of lipase and increased estrogen, resulting in MGAT
inhibition. We also found ethanol-induced alterations that occurred with repeated binges that may decrease PKC activation. These included changes in glycerophospholipids, ceramides, and sphingolipids. Though we utilized post hoc analysis in the comparison and contrasting of zebrafish and mouse models under differing exposure modalities, we have observed interesting differences between our models of binge and chronic alcohol-induced FASD. In addition, we have contextualize the metabolome differences between these models with respect to their relative development.

3.6 Materials and Methods

3.6.1 Animal Husbandry and Ethanol Exposure

Animal experiments using *Danio rerio* were performed in accord with Purdue Animal Care and Use policies at Purdue University (West Lafayette, Indiana, USA) using approved protocol 1111000275. Extensive details of the *Danio rerio* ethanol exposure are available in our previous publication (Chapter 2). In brief, healthy adult AB strain zebrafish, were housed in 15-gallon glass aquaria with approximately twenty-four mixed male and female. Light timing was used to induce spawning. To collect fertilized eggs, mesh topped pyrex dishes were placed in aquaria at 0925 AM. After confirming eggs were fertilized via visual microscope examination, they were incubated in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, and 0.33 mM MgSO₄ in distilled water) [63]. At 2 hours post-fertilization (hpf), embryos were randomized into ethanol treatment groups (*n*=8 per group) and transferred in to 6-well plates with the appropriate ethanol concentration in each well. Ethanol treatment groups included 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% v/v. Embryos were incubated in covered plates at 28°C (18 hours (h) light, 6 h dark) until 24 hpf. Embryos were washed with E3 media twice and further incubated until 48 hpf, at which time they were flash frozen in liquid nitrogen and stored at −80°C until time of sample processing.

Animal experiments for *Mus musculus* were performed in accord with Indiana University School of Medicine (Indianapolis, Indiana, USA) Animal Care and Use policies using approved protocol 000000 ethanol exposure was achieved using our previously reported protocol. In brief, c57BL/6J mouse embryos were excised from the dam at noon on E8.2. Culture of the embryos began an hour later in petri dishes with medium (control embryos, *n*=6). For ethanol exposed animals in the 6 h (n=7)
Mouse embryos were excised from the dam at noon on E8.2, with culture of the embryos beginning an hour later in petri dishes with medium (control embryos, \(n=6\)). Ethanol-treated animals were exposed once (6 hour, \(n=7\)) or twice (6+6 hour, \(n=7\)) to ethanol 400 mg/dL for six hour increments. Alcohol was added from 1:00–7:00 PM, and then the media was changed. On the second day, the media was again changed at 3:00 PM. For the 6+6 hour group, alcohol was again added from 9:00 AM–3:00 PM. All embryos were harvested the following day at 10:00 AM, at which time the embryos were dissected with the yolk sac removed and snap frozen. Embryos were stored at \(-80^\circ\)C until shipment to the processing lab at Purdue University (West Lafayette, Indiana, USA).

Fig. 3.7. Experimental design for c57BL/6J *Mus musculus* embryos cultured in ethanol.
3.6.2 Metabolomics

Sample preparation was conducted as previously reported (Chapter 2). In brief, samples were thawed at 4 °C, extracted in 2:1 methanol:water, homogenized, extracted in cold chloroform, centrifuged and concentrated. The non-polar fraction was dissolved in 50% double-distilled water, 25% methanol, 25% acetonitrile.

The samples were analyzed using Thermo Fisher LTQ Orbitrap (Waltham, MA USA) mass spectrometer (MS) coupled to an Agilent 1100 series Liquid Chromatography (LC) (Agilent Technologies, Santa Clara, CA, USA), using an Eclipse XDB-C8 column (Agilent Technologies, Santa Clara, CA, USA). Solvent A was water + 0.1% piperidine and solvent B was acetonitrile:methanol (50:50 v/v) + 0.1% piperidine [64]. Extensive details on the separation settings have also been previously reported (Chapter 2). The mass spectrometry (MS) analysis used negative polarity electrospray ionization. A mass range of 100–1200 atomic mass units was used for full scan analysis and XCalibur software (v.2.1.0) was used for analysis.

3.6.3 Data Processing

Results for the non-polar metabolites from each species were processed separately. Using the Purdue Bindley Bioscience Center Omics Discovery Pipeline Software, retention time, m/z, and peak intensity data from each data set were separately deconvoluted with a m/z variation setting of 0.07, an LC peak width of 10, and a minimum noise-to-signal ratio of 0.5 [65]. Alignment across non-polar samples required an m/z variation of 0.015 and a retention time variation across samples of less than 0.7 minutes. The peak frequency cutoff was 60. Normalization required the detected peak to occur in 60% of the samples of at least one treatment group, and 35% species-wide.

As previously reported, metabolites with identical m/z appeared in the data and when the retention times were within 1 minute, metabolites were combined, but those with a larger difference in retention time were retained as separate metabolites. Metabolites were identified and classified using the Human Metabolome Database (HMDB), the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa Laboratories), LipidMaps, METLIN, or the PubChem Substance and Compound database [66–72]. Identified metabolites from both species were filtered based on being within
3 parts per million (ppm) difference of the exact mass of the metabolite, with ppm difference defined as

\[
\text{ppm difference} = \frac{(\text{observed mass} - \text{theoretical mass})}{(\text{theoretical mass})} \times 1,000,000
\]

Data were excluded as noise if it failed to have a minimum signal intensity across a species’s samples of $e10$ or greater or were likely misidentified (such as drug metabolites only appearing in one sample concentration). Data for samples that were fractionated on differently aligned peaks were manually combined for each species if entire groups were split between metabolites identified as the same entity. Treatment groups for each metabolite were compared using a within-species one-way ANOVA with metabolites considered significant at $P < 0.05$. The differences between repeated exposures of ethanol in the mouse were also compared using a t-test. The fold for each metabolite was calculated as follows:

\[
\text{fold} = \log_2 \frac{\bar{i}}{\bar{c}}
\]

where $\bar{i}$ is the average of the intensity values for the group of interest and $\bar{c}$ is the average of the intensity values for the control group.

### 3.6.4 Statistical Analysis

After the data processing, significantly different ($P < 0.05$) metabolites identified in both species were assembled. Since very few of the metabolites were identified in both data sets and having similar retention times, lots of data appears “missing” data in the combined set are treated as “0.1”, since some analytical tools cannot handle null values. PPCCA is designed to handle missing values, but for internal consistency the choice was made to maintain them as “0.1”. In addition, since the PPCCA we are using is a post hoc analysis and the ethanol exposure concentrations and modalities differed between species, ethanol exposure values, multiplying the ethanol concentration and the proportion of development time the embryos were exposed to ethanol.

We used the MeabolAnalyze package for R to generate a PPCCA [487]. R Studio (R Studio, Boston, Massachusetts, USA), Version 0.99.902, and R (The R Foundation for Statistical Computing, Vienna, Austria), Version 3.2.2, were used for this analysis. Spectral data for metabolites common to both species and having one-way
ANOVA $P < 0.05$ were entered in the model in addition to the species and relative ethanol exposure values. (Note: this is a different P-value limit than used for our previously published zebrafish analysis.) Where metabolites with multiple retention times existed, the maximum signal intensity for a defined metabolite was used for each sample. Functions for plots provided in the package were modified to generate color plots with appropriate axes labels and to run on Diagrid.org (Purdue University, West Lafayette, Indiana, USA) cluster computers unmonitored.

In order to compare the two species, an estimation of the average ethanol exposure both the zebrafish and mouse models (Table 3.8). The ethanol concentrations used to treat the zebrafish and mice were converted to mol/L. To compare the models, given their different ethanol treatment concentrations, durations, and stages of development, a modified ethanol dose was calculated as follows:

$$\text{RelativeExposure}(nM) = \frac{[(t_{out1} - t_{in1}) \text{Ethanol}_1(nM)] + [(t_{out2} - t_{in2}) \text{Ethanol}_2(nM)]}{t_{ReproductiveMaturity}}$$

where $t_{out_n} - t_{in_n}$ is the duration of each exposure, $\text{Ethanol}_n$ is the ethanol concentration given in nM. $t_{ReproductiveMaturity}$ was approximated from c57BL/6J mice for “Weeks at first mating” as 7.7 weeks, and for zebrafish as 57 dpf [504,505].

Fig. 3.8. This adjustment accounted for the development age during the ethanol exposure(s) and the proportion of development captured during the exposure. For the mouse treatments, embryos were excised and exposed to one ($n=7$) or two ($n=7$) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice ($n=6$) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos ($n=8$ per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol (v/v).
<table>
<thead>
<tr>
<th>Concentration Given (mM)</th>
<th>Species</th>
<th>Exposure Time (h)</th>
<th>Relative Ethanol Exposure (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 Mouse, Fish (control)</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.17 Fish</td>
<td>22</td>
<td>2.76</td>
<td></td>
</tr>
<tr>
<td>0.51 Fish</td>
<td>22</td>
<td>8.27</td>
<td></td>
</tr>
<tr>
<td>1.71 Fish</td>
<td>22</td>
<td>27.56</td>
<td></td>
</tr>
<tr>
<td>5.14 Fish</td>
<td>22</td>
<td>82.68</td>
<td></td>
</tr>
<tr>
<td>8.68 Mouse</td>
<td>6</td>
<td>403.04</td>
<td></td>
</tr>
<tr>
<td>8.68 Mouse</td>
<td>6 + 6</td>
<td>805.91</td>
<td></td>
</tr>
<tr>
<td>17.1 Fish</td>
<td>22</td>
<td>275.59</td>
<td></td>
</tr>
<tr>
<td>51.41 Fish</td>
<td>22</td>
<td>826.76</td>
<td></td>
</tr>
<tr>
<td>171.37 Fish</td>
<td>22</td>
<td>2755.88</td>
<td></td>
</tr>
<tr>
<td>514.10 Fish</td>
<td>22</td>
<td>8267.65</td>
<td></td>
</tr>
</tbody>
</table>

The developmental landmarks for calculations and on the timeline were derived from publications describing the typical time of these events for zebrafish, mice (c57BL/6J, when available), and humans \cite{258, 506-515}. Additional reference images used for drawing the human figures \cite{516}.

### 3.6.5 Data Management Plan

Raw data sets from the metabolite analysis are available for viewing at https://purr.purdue.edu/publications/1398 or doi:10.xxxxxxxx.

### 3.7 Acknowledgements

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3.8 Competing Interests Statement

The authors have no competing interests to declare.

3.9 Funding

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3.10 Supplementary Material
Fig. 3.9. Histogram of the p-values obtained from the ANOVA for the FASD mouse model non-polar metabolome
Fig. 3.10. Bayesian Information Criterion plot for the PPCCA model. The plot indicates that the optimal number of principal components, q, for our PPCCA model is four. For the mouse treatments, embryos were excised and exposed to one \((n=7)\) or two \((n=7)\) consecutive days for 6 h exposures of 400 mg/dL alcohol. Control mice \((n=6)\) were not exposed to ethanol, but were incubated in culture medium. Zebrafish embryos \((n=8\) per group) were exposed from 2–24 hpf to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol \((v/v)\). Samples were then extracted and the non-polar fraction was analyzed by LC-MS/MS (Orbitrap). The PPCCA model incorporates metabolites that appeared in the metabolomes of both species and were significantly changed, one-way within-species ANOVA, \(P < 0.05\).
4. BIOMARKERS OF FETAL ALCOHOL SPECTRUM DISORDER: THE DIFFERENCE YOU CAN’T SEE

Although fetal alcohol spectrum disorder (FASD) has been clinically identifiable after birth by the medical community for decades, a comprehensive and robust biomarker(s) has yet to be established to allow for early diagnosis and intervention. To that end we previously characterized the ethanol-induced changes in the zebrafish model of chronic ethanol and the binge mouse model exposures. We examined the morphologic features of zebrafish embryos exposed to the same treatments, finding little evidence of significant difference as a whole. We were able to replicate radical morphologic abnormalities of other laboratories by modifying ethanol dose and timing.) In spite of this lack of statistical difference in morphologic measurements, our previous work has demonstrated there are statistically significant metabolite differences, demonstrating that ethanol alters embryonic physiology even at concentrations of ethanol where statistically significant morphologic differences are absent. Here we exam many of the commonly proposed biomarkers for FASD, many of which were absent in our metabolomes. We also examine the possibility of biomarkers suggested from statistically significantly different metabolites common to our mouse and zebrafish models. This striking contrast between significantly different metabolites in the absence of morphologic of statistically significant visible changes reiterates the need for more robust biomarkers to diagnose and characterize FASD.
4.1 Introduction

Several factors contribute to the missed diagnosis of FASD, including subtle or absent facial dysmorphology, unreliable reports of maternal drinking history, and insufficiently robust biomarkers. Thus, intervention is difficult, whether through behavioral therapy, or potential pharmacological treatment [51,517]. A wide variety of embryonic metabolites are altered by alcohol exposure, which offer the possibility of novel biomarker(s) identification. However, broad metabolite screens have not been fully assessed for their biomarker(s) potential in whole vertebrate embryos exposed to ethanol.

Several teratogenic pathways (nitric oxide and other reactive oxygen species, glutamate, vitamin A, serotonin signaling, lipids, glucose regulation, and adhesion molecules) are altered after embryonic ethanol exposure. This has resulted in a wide range of theoretical models as potential causative agents of FASD [61,62], and many metabolites have been proposed as biomarkers of ethanol exposure. Sampling of meconium has shown the presence of fatty acid ethyl esters (FAEEs) correlating with maternal alcohol consumption [518,519]. While offering the benefit of not being invasive, meconial FAEE biomarkers have been reported in children of non-ethanol consuming mothers, potentially limiting their robustness as population screening biomarkers [54,55]. FAEEs have also been reported in hair, which, while also a useful medium for noninvasive biomarker testing, has shown changes in some FAEEs corresponding with use of hair cosmetics [59]. Further, animal studies of dizygotic twins have shown differing FAEE levels in the offspring, indicating that equivalent maternal ethanol doses do not result in equivalent FAEE levels or possibly even equivalent ethanol exposures [520]. Thus, while widely studied, FAEEs still face limitations in clinical diagnostic use. Therefore, we sought to measure FAEEs via metabolomics and also to identify novel biomarkers of FASD.

Zebrafish have become a widely utilized species in the laboratory, especially for FASD and craniofacial abnormality studies [521,522]. In our previous publications, we examined the metabolome generated from zebrafish embryos to 0–3% ethanol from 2–24 hours post fertilization (hpf). In addition, we have examined the non-polar extracts from a binge ethanol exposures to embryonic mice. After extraction of the whole embryo from each species, we used metabolomics via liquid chromatography with mass spectrometry detection (Orbitrap or Time of Flight). We found changes in metabolites associated with lipid metabolism, molecular transport,
and small molecule biochemistry. Significant differences occurred in glycerophospholipids and sphingolipids, which are consistent with previously proposed mechanisms in FASD neuronal and cardiac abnormalities (Chapter 3).

Here, we examined the morphologic differences occurring with these treatments in zebrafish. Surprisingly, in spite of many individuals with abnormal features, we found few morphologic measurements that were significantly different from controls. This highlights the startling impact ethanol has on embryonic metabolites, even in the absence statistical differences in morphology.

The aims of this study are to examine the utility of biomarkers suggested in our previous work, as discovered through hypotheses supported by our metabolomics studies, comparative species Probabilistic Principal Component and Covariate Analysis (PPCCA), and Ingenuity Pathway Analysis® (IPA) biomarker suggestions. We will do this in the context of biomarkers already suggested in the literature, as well as suggest new metabolites that may be useful in the identification, assessment, or treatment of FASD. Our evidence underscores the need for robust biomarkers, as biochemical changes are occurring even in the absence of clinical features used to diagnose FASD. From this work we conclude that a biomarker panel may be a more reliable strategy for characterizing risk to offspring of maternal drinking, exposure patterns, and severity of FASD.

4.2 Results

4.2.1 Mortality and Morphology

To examine the correlation between shortened lifespans and metabolites, we measured the mortality/teratology and morphometry of embryos at each ethanol concentration tested. We treated zebrafish embryos from 2–24 hpf with ethanol concentrations of 0 (n = 10), 0.001 (n = 8), 0.003 (n = 8), 0.01 (n = 9), 0.03 (n = 7), 0.1 (n = 8), 0.3 (n = 8), 1 (n = 7), and 3% (n = 7) (vol/vol). As shown in Figure 4.1, 49 of 72 total embryos (eight of the ten controls) survived to hatch. For several ethanol concentrations, the Kaplan-Meier estimates of the median survival could not be generated because the mortalities at the end of the observation period did not exceed 50%. Thus, the reported bound of the upper confidence intervals are listed as not applicable (NA). These concentrations included the control, 0.001%, 0.003%, 0.01%, and 0.3%. The median survival time for the 0.03% and 0.1% treatment group
(n = 8) was 40.0 hpf (95% CI = 38.5 to NA) and 40.1 hpf (95% CI = 40.1 to NA), respectively. For the 1% (n = 7) and 3% (n = 7) treatment groups the median survival times were 40.2 (95% CI = 39.1 to NA) and 39.0 (95% CI = 33 to NA) hpf. The log-rank test did not demonstrate a statistically significant difference between the survival rates ($\chi^2 = 14.9$, df = 8, $P = 0.061$). Nevertheless, the log-rank test for trend showed a significant trend for mortalities with increasing concentrations of ethanol ($\chi^2 = 11.0$, df = 1, $P < 0.001$). The Cox Proportional Hazards model

$$h(t, X) = h_0(t)e^{\sum_{i=1}^{p} \beta_i X_i}$$

for the ethanol concentration was

$$h(\text{time}, [\text{Ethanol}]) = (\text{time})e^{\sum_{i=1}^{p} 0.506 \times [\text{Ethanol}]} + 0.164$$

where $z = 3.08$ and $p = 0.002$. The likelihood ratio was 7.59 (df = 1, $n = 72$, number of events = 23, $P = 0.006$).

Zebrafish larvae were measured to assess ethanol’s effects on morphology, as shown in boxplots of the morphological measurements (Figure 4.2). Measurements included the total length (Figure 4.2A), head circumference (Figure 4.2B), distance from the outer boarders of each eye (Figure 4.2C), and the area of the eye (Figure 4.2D), as illustrated in Figure 4.2L. Q-Q plots and Shapiro-Wilk test ($P < 0.05$) values indicated most measurements within each group were normally distributed (Figures 4.3, 4.4, 4.5, 4.6, 4.7, and Table 4.1). At the 3% concentration only one individual survived, prohibiting inclusion of this treatment group. By ANOVA, ethanol treatment failed to result in a significant differences between treatment groups and controls for length ($F_{1,29} = 0.590$, $P = 0.449$), head circumference ($F_{1,29} = 2.753$, $P = 0.108$), eye to eye distance ($F_{1,29} = 2.693$, $P = 0.112$), or eye area ($F_{1,29} = 2.693$, $P = 0.112$). Similar results were obtained through MANOVA, treating ethanol as a factor for the combined dependent variables of length, head circumference, eye to eye distance, and eye area ($\text{Wilks - Lambda } F_{8,36} = 1.281$, $P = 0.170$).

Qualitative evaluations of zebrafish larvae were made according to the procedure described by Panzica-Kelly, et al., utilizing a detailed morphological scoring system. This system uses a scale from 0–5, with 5 indicating the “Anatomical structure is entirely normal for developmental stage”. Of the qualitative evaluations, body shape ($\chi^2 = 17.7$, df = 8, $P = 0.023$) and yolk sack edema ($\chi^2 = 15.8$, df = 8, $P = 0.046$) were the only categories that significantly differed, as shown in Figures 4.2F
Fig. 4.1. Kaplan-Meier survival curve demonstrating zebrafish embryos exposed to increasing concentrations of ethanol were less likely to survive to hatch. Only 1/8 of those exposed to 3.0% ethanol hatched at 48 hpf as opposed to untreated embryos, where 8/10 survived. The Cox Proportional Hazards model for the ethanol concentration was
\[ h(t, \text{Ethanol}) = \text{time} e^{\sum_{i=1}^{z} 0.506 \times \text{Ethanol} + 0.164} \], where \( z = 3.08 \), Ethanol = Ethanol Concentration, and \( p = 0.002 \). The likelihood ratio was 7.59 (df = 1, n = 72, number of events = 23, \( P = 0.006 \)).
Fig. 4.2. Boxplots of qualitative and quantitative morphometric evaluations. Ethanol exposed zebrafish larvae were imaged at time of hatching and subsequently digitally measured. Significant differences were seen only in the qualitative measure of body shape and yolk sack edema. Distances are shown in µm. (*: P < 0.05, ANOVA) (A) Average Length, (B) Head Circumference, (C) Distance Between Exterior Boarders of the Eyes, (D) Eye Area, (E) Eye Area/Average Length, (F) Body Shape, (G) Tail Shape, (H) Pericardial Edema, (I) Yolk Sack Edema, (J) Head (Brain) Space, (K) Otic-Optic Space, (L) Morphometrics Guide. hb: hindbrain, mb: midbrain, fb: forebrain, OOS: Otic-Optic Space.
Table 4.1.
Shapiro-Wilk normality test of quantitative morphological values. *: $P < 0.05$, indicating the data are not normally distributed.

<table>
<thead>
<tr>
<th>Group (n)</th>
<th>Eye-to-Eye Distance W</th>
<th>P</th>
<th>Eye Area W</th>
<th>P</th>
<th>Head Circumference W</th>
<th>P</th>
<th>Length W</th>
<th>P</th>
<th>Eye Area:Length W</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (8)</td>
<td>0.924</td>
<td>0.459</td>
<td>0.916</td>
<td>0.398</td>
<td>0.898</td>
<td>0.318</td>
<td>0.958</td>
<td>0.793</td>
<td>0.981</td>
<td>0.968</td>
</tr>
<tr>
<td>0.001% (6)</td>
<td>0.846</td>
<td>0.147</td>
<td>0.794</td>
<td>0.052</td>
<td>0.925</td>
<td>0.544</td>
<td>0.720</td>
<td>0.010*</td>
<td>0.841</td>
<td>0.132</td>
</tr>
<tr>
<td>0.003% (5)</td>
<td>0.793</td>
<td>0.051</td>
<td>0.780</td>
<td>0.039*</td>
<td>0.928</td>
<td>0.564</td>
<td>0.856</td>
<td>0.215</td>
<td>0.819</td>
<td>0.116</td>
</tr>
<tr>
<td>0.01% (7)</td>
<td>0.774</td>
<td>0.022*</td>
<td>0.679</td>
<td>0.004*</td>
<td>0.877</td>
<td>0.212</td>
<td>0.611</td>
<td>0.0004*</td>
<td>0.745</td>
<td>0.018*</td>
</tr>
<tr>
<td>0.03% (4)</td>
<td>0.766</td>
<td>0.054</td>
<td>0.799</td>
<td>0.100</td>
<td>0.954</td>
<td>0.744</td>
<td>0.672</td>
<td>0.005*</td>
<td>0.869</td>
<td>0.293</td>
</tr>
<tr>
<td>0.10% (6)</td>
<td>0.870</td>
<td>0.225</td>
<td>0.955</td>
<td>0.776</td>
<td>0.833</td>
<td>0.113</td>
<td>0.841</td>
<td>0.132</td>
<td>0.962</td>
<td>0.837</td>
</tr>
<tr>
<td>0.30% (6)</td>
<td>0.968</td>
<td>0.877</td>
<td>0.768</td>
<td>0.030*</td>
<td>0.837</td>
<td>0.123</td>
<td>0.986</td>
<td>0.977</td>
<td>0.968</td>
<td>0.878</td>
</tr>
<tr>
<td>1% (4)</td>
<td>0.922</td>
<td>0.550</td>
<td>0.929</td>
<td>0.585</td>
<td>0.908</td>
<td>0.472</td>
<td>0.758</td>
<td>0.046*</td>
<td>0.940</td>
<td>0.654</td>
</tr>
<tr>
<td>3% (1)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
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</tr>
</tbody>
</table>
Fig. 4.3. Q-Q plots of quantitative morphometric length to examine for normal distribution. Measurements were generally normally distributed. Each plot represents the distribution of the zebrafish for a given ethanol concentration from 0 – 3% ethanol.

and 4.2, respectively. Both measures were significantly different from control with the 0.003% treatment ($P < 0.05$). The mean score for body shape was 4, indicating “Slight variation in morphology suggestive of a developmental delay or anomaly that is potentially recoverable.” Yolk sack edema was also significantly different from
controls in the 0.01% and 0.3% treatments, with the means of these groups being
4. Tail shape ($\chi^2 = 12.0$, $df = 8$, $P = 0.153$, Figure 4.2G), pericardial edema
($\chi^2 = 9.1$, $df = 8$, $P = 0.334$, Figure 4.2H), head (brain) shape ($\chi^2 = 10.7$, $df =$
Fig. 4.5. Q-Q plots of the distance between external portions of the eyes to examine for normal distribution. Measurements were generally normally distributed. Each plot represents the distribution of the zebrafish for a given ethanol concentration from 0 – 3% ethanol.

8, $P = 0.218$, Figure 4.2J), and otic-optic space (OOS, $\chi^2 = 9.9$, $df = 8$, $P = 0.282$, Figure 4.2K) were not different from control.

Ethanol treatment resulted in one or more individuals per group with abnormal morphology, as qualitatively graded (Figure 4.8). Atypical tissue extending from the
Fig. 4.6. Q-Q plots of eye area to examine for normal distribution. Measurements were generally normally distributed. Each plot represents the distribution of the zebrafish for a given ethanol concentration from 0 – 3% ethanol.

eye (blue arrows) was seen in individuals in several treatment groups (0.001, 0.01, 0.03, 0.3% lateral views). Abnormal tails were seen in many individuals across treatment groups (0.003, 0.3, and 1% in lateral views). Moderate pericardial edema was seen in 3/8 individuals in the control group as well as in individuals in all ethanol exposure
Fig. 4.7. Q-Q plots of the ratio of eye area to length in order to examine for normal distribution. Measurements were generally normally distributed. Each plot represents the distribution of the zebrafish for a given ethanol concentration from 0–3% ethanol.

Groups except 3%. Moderate yolk sack edema at was observed in a few individuals across the treatment groups (0.003 and 0.1%). These individuals are shown in Figure 4.8 in the lateral view. Individuals with moderately abnormal head morphology were present in several treatment groups, including 0.003 (2/6) and 0.3% (1/6), appearing
to have a shortened forebrain segment. Moderate narrowing of the OOS was observed in a single individual in the control, 0.003, 0.03, and 0.1% groups.
Fig. 4.8. Ethanol treatment resulted in one or more individuals per group with abnormal morphology, as qualitatively graded. Atypical tissue extending from the eye (blue arrows) was seen in individuals in several treatment groups (0.001, 0.01, 0.03, 0.3% lateral views). Abnormal tails were seen in many individuals across treatment groups (0.003, 0.3, and 1% in lateral views). Moderate pericardial edema was seen in 3/8 individuals in the control group as well as in individuals in all ethanol exposure groups except 3%. Moderate yolk sack edema at was observed in a few individuals across the treatment groups (0.003 and 0.1%). These individuals are shown in the lateral view. Individuals with moderately abnormal head morphology were present in several treatment groups, including 0.003 (2/6) and 0.3% (1/6), appearing to have a shortened forebrain segment. Moderate narrowing of the OOS was seen in a single individual in the control, 0.003, 0.03, and 0.1% groups.

To confirm the lack of statistical difference in morphology was not part of a systemic protocol problem, a subsequent study included 1.5 and 2.4% ethanol in the concentration curve. Also, a time variable of when the alcohol was added was included, having a set at 2 hpf and a set at 4 hpf. Embryos from the 1.5 and 2.4% exposures at both time points elicited several aberrant morphological changes and resembled those reported from other laboratories [63,521,525]. Abnormalities included individuals missing tails and/or faces in the 2 hpf group, and narrowing of the eye to eye distance, missing faces, microphthalmia, cyclopia, bent tails, and generalized apparent disruption of cellular patterning in the 4 hpf group (Figure 4.8). Pericardial edema, yolk sack edema, and tail and craniofacial abnormalities were also present in individuals in the 1.5 and 2.4% ethanol treatments.

4.2.2 IPA Biomarkers

IPA was utilized to identify relevant molecules pathways modified with the ethanol exposure. Using the biomarker analysis tool within IPA, biomarkers for each ethanol concentration tested in the zebrafish were identified. The greatest number of identified biomarkers occurred with the 0.03% ethanol data set. A few of these metabolites were excluded as mis-identifications (drug-related metabolites) by the metabolite databases, including butabarbitur, ecgonine, fasoracetam, and harmaline. The metabolites identified with the biomarker filter for the non-polar data set included
1-(1(Z)-hexadecenyl)-sn-glycero-3-phosphoethanolamine, 2-oxostearic acid, 3-acetyl-
tropine, cyclic 2,3-bisphospho-D-glycerate, d-desthiobiotin, methyl palmitate, tetradecanoic acid, palmitic acid, pentadecanoic acid, and stearic acid. The metabolites identified with the biomarker filter for the non-polar data set included creatinine and urea, which were both significantly different in the zebrafish metabolome. Creatinine applications included diagnosis, efficacy, prognosis, safety and unspecified application. Urea applications included diagnosis, efficacy, prognosis, and safety.

In addition to the work conducted in zebrafish, we examined the IPA biomarkers suggested from our established mouse model of binge prenatal ethanol exposure. For this dataset, only the metabolite arachidonic acid was identified for both the single and doubly exposed mouse embryos with a biomarker application of efficacy.

4.3 Discussion

4.3.1 The Need for Biomarkers

Several factors contribute to the missed diagnosis of fetal alcohol spectrum disorders (FASD), including subtle or absent facial dysmorphology, unreliable reports of maternal drinking history, and insufficiently robust biomarkers. Thus, intervention is difficult, whether through behavioral therapy, or potential pharmacological treatment. A wide variety of embryonic metabolites are altered by alcohol exposure, offering the possibility of identifying novel biomarkers. However, broad metabolite screens have not been pursued in whole vertebrate embryos exposed to ethanol for the purpose of biomarker(s) identification.

Diagnostic techniques such as ultrasound and 3-D facial analysis have been proposed as FASD identification tools. In prenatal ultrasounds of the midline shape of the corpus callosum, a “hook”-shaped structure (splenium angel greater than 90 degrees) was strongly associated with ethanol exposure during pregnancy, even with confounders such as gestation al age, birth weight, sex, ultrasound age. Analysts were able to successfully identify this structure 12 of 23 ethanol-exposed infants and a one false positive among the 21 unexposed or “lightly”-exposed controls.

A newer tool has been developed to help diagnose children lacking classical dysmorphismology but did have a history of heavy prenatal ethanol exposure. Using a 3-D facial photograph, this tool agreed significantly agreed with classical criteria (0.90–.92, depending on view), even correctly identifying those classified as having
partial FASD [526]. However, as we demonstrate here, offspring biochemistry is altered even in the absence of dysmorphology. Further, based on the specific metabolites identified as significantly different in the chronic zebrafish and binge mouse ethanol exposure models, biochemical changes are of physiological importance with long-term implications.

Based on the 2001 Biomarker Definitions Working Group, a biomarker is “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. [527] For FASD, biomarkers predominantly are aimed at serving as an indicator of the disease, for the “prediction and monitoring of clinical response to an intervention”, as a clinical endpoint that “reflects how a patient feels, functions, or survives”, or a surrogate endpoint, which is “a biomarker that is intended to substitute for a clinical endpoint expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence”. [527]

Many factors effect biomarker utility, including physical factors such as tissue accessibility and biomarker stability and reliability over time [528]. Sampling of biomarkers for FASD requires consideration of developmental stage, ease of tissue access, be it maternal or offspring, or sampling after birth or during childhood. Meconium has often been proposed as a tissue for assessment for FASD, in part for its ease of sampling. However, this matrix does not allow for prenatal intervention. In this discussion, we will not focus on biological matrices appropriate for biomarkers, as our metabolites reflect whole embryos and therefore cannot address these differences.

4.3.2 Mortality and Morphology

The log-rank test for trend was significantly different between groups ordinated by ethanol concentration, but the Kaplan-Meier log rank test failed to show a significant difference in survival between ethanol concentration groups. This indicates the survival observations should be extended, which would also allow generation of the median survival statistics. Further, the morphological differences may be attributed to the natural variation that occurs in hatching of a clutch of eggs [502]. Nevertheless, the lack of Kaplan-Meier statistical difference further supports the notion that physiologically important metabolite changes are occurring, even in the absence of survival effects.
Morphological differences were not significant by ANOVA, but zebrafish embryos exposed to ethanol responded with alterations in normal development. Morphological abnormalities were noted with ethanol treatments, as well as qualitative differences in body shape. The lack of statistical findings may be due to the small number of survivors in the high dose groups or that the original concentrations tested missed the critical 1.5 and 2.4% ethanol. These doses have been shown to elicit significant abnormalities in morphology in zebrafish [63, 524, 525]. In non-survivors, tissue degraded rapidly and therefore did not provide reliable samples for imaging. In spite of the lack of statistically significant morphologic outcomes, changes did occur in our original concentration curve, underscoring the relevance of the metabolite changes at low ethanol doses.

Another potential reason for the lack of significance in morphologic findings may be related to the developmental modularity of the zebrafish. Others have reported that during the phenotypic stage, only during less than optimal conditions (temperature, oxygen, and salinity) does modularity become important [529]. That is to say that intra-group developmental consistency is favored over individual variation in morphology as a result of the strain induced by suboptimal environmental conditions. Thus, in our experiments, we would expect the variation of the untreated groups to be larger than the variation in ethanol treated groups. As shown in Q-Q plots of the morphometric values (Figures 4.3, 4.4, 4.4, 4.5, and 4.7), there is a tendency for the trend lines to become flatter in slope as the ethanol concentration increases.

4.3.3 Existing Biomarkers

A variety of biomarkers have been proposed for FASD, and have been extensively reviewed [51, 53, 530]. The objective of this discussion is to review commonly proposed metabolite biomarkers for FASD that are detectable with our metabolomics methods. This includes reviewing common biomarkers that were absent in our metabolome as well as proposing additional biomarker strategies.

Direct Biomarkers

Oxidative Ethanol Metabolism Several FASD biomarkers reported in the literature of FASD were not identified in our analyses. For instance, products of alcohol catabolism were not identified in the metabolome, as our Orbitrap assay method
accurately detects molecules with a m/z of 100–1200. Ethanol (46.0421866 g/mol), acetaldehyde (44.05300 g/mol), and acetate (60.05200 g/mol) are beyond the limit of detection.

Acetaldehyde, a metabolite of ethanol via Cyp450, has been explored as a direct biomarker particularly for moderate to high levels of drinking \[51\]. But, it is detectable in blood for only a very short time after ingestion \[530\]. We did not detect acetaldehyde in our zebrafish non-polar or polar metabolomes, however did detect similar metabolites, such as butanal and acetamimde (Chapter 2). Acetylaldehyde protein adducts have also been considered as potential biomarkers, however these products are much too large for our metabolite detection strategy \[51\].

Increased excretion of acetoacetic acid has been detected with proton nuclear magnetic resonance spectroscopy in a zebrafish disease model of alcoholic fatty liver \[531\]. We found a significant decrease in acetoacetic acid at mid-range and higher ethanol doses. In addition, betaine excretion decreased in the alcoholic fatty liver model, and crotono-betaine was similarly decreased at mid-range and higher ethanol doses in our experiments \[531\].

Non-oxidative Ethanol Metabolism

**Fatty Acid Ethyl Esters** Based on the literature, we examined our metabolome for FAEEs. Formed from the esterification of alcohol and fatty acids or acyl-CoA/fatty acids, FAEEs have been proposed as a biomarker of in utero ethanol exposure, and can be measured in meconium where it correlates with maternal alcohol consumption \[30, 51, 518, 532, 538\]. While offering the benefit of not being invasive, meconial FAEEs have been reported in children of non-ethanol consuming mothers, potentially limiting their robustness as population screening biomarkers \[54–56\]. FAEEs have also been reported in hair, which, while also a useful medium for noninvasive biomarker testing, has shown changes in some FAEEs corresponding with use of hair cosmetics \[59\]. Further, animal studies of dizygotic twins have shown differing FAEEs levels in the offspring, indicating that equivalent maternal ethanol doses do not result in equivalent FAEE levels or possibly even equivalent ethanol exposures \[520\]. FAEEs have been demonstrated in serum as well \[539\]. Thus, while widely studied, FAEEs still face limitations in clinical diagnostic use. These metabolites are believed to be produced by the ethanol-exposed fetus, making it a direct biomarker for ethanol expo-
Ethyl linoleate, a FAEE, has been proposed as a negative biomarker from meconium, indicating “infants not exposed in utero to high levels of alcohol”. 532

Given the emphasis placed in the literature on FAEEs as FASD biomarkers, we compared metabolites identified in our analysis with those FAEEs reported by Burd and Hofer in their meta-analysis of FAEEs as biomarkers for fetal alcohol exposures 53. In this analysis, ethyl esters of palmitate, stearate, oleate, linoleate, and arachidonate were identified as potential biomarkers 53, 54, 518, 532, 535, 537, 541. None of these nine FAEEs were identified in our study, including when the peak scan width was narrowed to 5, rather than the 10 used to identify the other metabolites. This is in contrast to the findings of by Loftus et al., who detected several FAEEs using LC-MS in the liver of rats and mice exposed to ethanol 451. We believe this is due to their use of acetonitrile to stabilize of the FAEEs, which we did not do.

Ethyl arachidonate has been proposed as a FAEE biomarker 542. Gas chromatography of umbilical cord vessel fatty acids from self-reported alcohol-consuming black women demonstrated that alcohol consumption positively correlated with arterial vessel wall docosahexaenoic acid and arachidonic acid 543. Similar findings are associated with periconceptional ethanol intake and neonatal cord blood serum 32. In the mouse metabolome, arachidonic acid ((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic) was significantly increased, especially after the second ethanol binge-dose. In our zebrafish metabolome, its precursor, anandamide, significantly decreased at all but the lowest ethanol dose. This suggests either an increase in enzymatic function producing ((5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoic), shift in the demand of it, or decrease in its utilization. However, it is important to note this is not the only route to anandamide, as it can also be a product of multiple pathways 344, 544.

**Phosphatidylethanol** Phosphatidylethanol (PEth) is another proposed direct biomarker of ethanol 51, 545. It can be measured in dried blood spots, with high specificity, relative other FASD biomarkers 546. We anticipated finding PEth in our metabolome, as it is generated by phospholipase D conversion from phosphatidylcholine 547. We found many PLD-related metabolites altered in the zebrafish metabolome, including phosphatic acids, phosphatidylcholines, and phosphatidylethanolamine, which were all significantly different. However, we did not identify metabolites with m/z corresponding to PEth.
Ethyl glucuronide and ethyl sulfate  Ethyl sulfate and ethyl glucuronide are direct non-oxidized, minor metabolites. Sulfotransferase can form ethyl sulfate from ethanol. Ethyl glucuronide is generated by glucuronidation by uridine diphosphate-glucuronosyltransferase, for the purpose of excreting toxic substances. Ethyl glucuronide and ethyl sulfate show correlation with specific but not total FAEEs in meconium [51,548]. However several weaknesses have been presented for these metabolites. For ethyl glucuronide non-consumers and those consuming small amounts of alcohol may present this biomarker at levels below the limit of quantification [51]. We did not identify metabolites with masses corresponding to ethyl glucuronide and ethyl sulfate in our whole embryo extracts. However, we did note palmitoyl glucuronide was significantly decreased and nicotine glucuronide was significantly increased.

Indirect Biomarkers

Many of the biomarkers proposed for FASD are enzymes, such as cytochrome P450, γ-glutamyltransferase, aspartate aminotransferase, and alanine aminotransferase [50, 51]. These are too large to be detected by our method. In addition, large protein biomarkers, such as carbohydrate deficient transferrine, are also too large for our detection method [51]. Assessment of mean corpuscular volume of erythrocytes, also a proposed biomarker for FASD, is not directly detectable via our metabolomics method [51]. However, glycosylation- and tryptophan-related metabolites may have utility as biomarkers.

Glycosylation Biomarkers  Dolichols and sialic acid have been proposed as biomarkers relevant to glycosylation. Based on a case study of a newborn with maternal history of alcohol abuse in comorbid with morphological abnormalities similar to FASD, some have suggested changes in glycosylation as an important factor in FASD [48]. In this individual, glycosylation screening was transiently abnormal. In addition, ethanol is known to decrease secretion of the sugars use to glycosylate proteins [404].

Excretion of urinary dolichols has been demonstrated in newborns of alcoholic mothers, however has also been described by Halmesmki as “a poor indicator of abusive drinking” in pregnant alcohol abusers [407,530,549]. Ethanol is also known to disrupt dolichols, polyisoprenoids that occur in all eukaryotic cell membranes, which are essential for N-glyosylation of proteins (asparagine residues) in the endoplasmic reticulum [406]. In our whole embryo extracts, none of our metabolites were identified
as dolichol, but we did detect other isoprenoids, such as 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, which decreased significantly with ethanol exposure in the chronic zebrafish model and increased significantly in the binge mouse model.

Sialic acid, “the name for a series of acyl-derivatives of neuraminic acids that occur as nonreducing terminal residues of glycoproteins or glycolipids in biological fluids and cell membranes”, has been proposed as a biomarker for alcohol abuse \[530\,\,550\]. Like most of the other proposed biomarkers, we did not identify this in the zebrafish or mouse metabolomes. But we did identify other indicators of glycosylation malfunction in the zebrafish metabolome. \(N\)-Acetylglucosamine, which marks a glycoprotein for transfer to the lysosome, was significantly increased at mid-range ethanol doses and higher.

We did find 2-(\(\alpha\)-d-mannosyl)-3-phosphoglycerate, which was significantly increased at any ethanol dose tested in our zebrafish model. This metabolite is downstream of mannose-6-phosphate, a tag for lysosomal degradation that is generated via activation of \(N\)-acetyl-glucosamine-1-transferase by cathepsin D. Zebrafish knockouts for cathepsin D demonstrate abnormal morphology, including shorter body length and microophthalmia, similar to reports by others of FASD, although inconsistent with our morphologic findings \[414\]. Based on this evidence, in spite of not identifying sialic acid metabolites in our datasets, glycosylation-based biomarkers are likely a useful tool in profiling FASD.

**Tryptophan Metabolites** 5-Hydroxytryptophol and 5-hydroxyindolylacetic acid are both serotonin metabolites, a pathway known to be modified with prenatal ethanol exposure \[551\]. 5-Hydroxytryptophol and the ratio of 5-hydroxytryptophol/5-hydroxyindolylacetic acid (5-HTOL/5-HIAA) have been proposed as indirect biomarkers of recent moderate to high levels of drinking \[51,\,552\]. However, there remain no published studies in pregnant women.

In addition, embryonic stem cell-derived embryoid bodies and neural cells cultured with 0.1 or 0.3% ethanol for four days have demonstrated features suggestive of other potential biomarkers \[79\]. Four confirmed \(\text{L-thyroxine}, \,5\)-methylthioadenosine, and the tryptophan metabolites, \(\text{L-kynurenine}\) and indoleacetaldehyde. While we did not identify any of these exact metabolites in the zebrafish or mouse metabolomes, we did identify many tryptophan-related metabolites, including oxindole, kynurenic acid, 7-chlorokynurenic acid, and uncaric acid. Taken together, further investigation
of tryptophan-related metabolites may result in identifying robust biomarkers for FASD.

4.3.4 Biomarkers Suggested from Metabolomics

Biomarkers Suggested the Zebrafish Metabolome

Previously we have reported on a comprehensive metabolome from whole-embryo extracts taken from zebrafish exposed to 0–3% ethanol from 2–24 hpf. In that publication, we cite numerous pathways that are significantly different. These pathways provide an interesting framework from which to find multi-metabolite profiles, which could be used as a biomarker for FASD. Such pathways include changes related to the nervous system, cardiovascular system, and growth and energy. Nervous system-related metabolite changes included molecules involved in myelination, tryptophan metabolism, and oxidative stress. Metabolites related to vascularization, myocyte contraction, and coagulation were modified, which affect the cardiovascular system. Growth and energy are modified in part through altered fatty acid and amino acid metabolism. Together, these profiles provide a characteristic dynamic profile of changes occurring in FASD.

Non-polar Comparative Animal PPCCA

We have previously reported a cross-species Probabilistic Principal Component and Covariate Analysis (PPCCA), which highlighted several changes in metabolites potentially relevant as FASD biomarkers (Chapter 3). Six metabolites were common to the chronic zebrafish and binge mouse ethanol exposure models. These included 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, which we believe could be a cleaved molecule of vitamin A, tetradecanoic acid, methyl hexadecanoic acid, octadecanoic acid, PA(18:0/18:2(9(Z),12(Z))), and C22 sulfatide (Table 3.7). Several of these metabolites had responses to ethanol that diverged between species, where the metabolite varied positively in the mouse binge exposure model but negatively in the chronic zebrafish model. It is unclear if these differences are rooted in differences in species’ responses to the ethanol treatment, an effect of exposure duration, or likely a combination of both. Further, several of the metabolites decreased in the binge mouse model with a single dose, but increased with a second dose. These in-
cluded tetradecanoic acid, methyl hexadecanoic acid, and octadecanoic acid. Due to the simultaneous treatment of all test mice with the first dose of ethanol, it is unclear whether the change noted after the second dose reflects direct effects of alcohol, remnants of a compensating response to the first ethanol dose, or a biological variation in the response that occurs after a second ethanol exposure.

Many of the relationships of these metabolites to alcohol in the zebrafish did not appear dose responsive. This may indicate the need for examination of intermediary doses within the curve. However, C22 sulfatide was decreased in zebrafish with low ethanol concentrations and trended to slightly increase with the highest ethanol dose. Singly dosed mice did not differ in C22 sulfatide, but doubly-dosed mice significantly increased. PA(18:0/18:2(9(\text{Z}),12(\text{Z}))) similarly responded. Therefore, these may be relevant to investigate as potential biomarkers. They are both important to neural changes occurring in FASD (Chapter 3).

Lastly, tetradecanoic acid increased significantly in doubly dosed mice and in nearly all ethanol doses in the zebrafish, which may suggest another potential biomarker. While none of the metabolites that were significantly different and common to both FASD models appear to be presumptive biomarkers of FASD, several of these metabolites may provide an important tool for characterizing the type of ethanol exposure (binge or chronic) and the severity of FASD.

IPA

The biomarkers identified via the Biomarker Filter in IPA demonstrate some weaknesses, and would likely not be robust for use in FASD. The biomarkers IPA identified were vague and not specific for development, zebrafish, or alcohol metabolism, and were disproportionally for drugs or exogenous chemicals that were unlikely to be in our system. For example, fatty acid differences on their own are too vague of a change to pinpoint a biological abnormality. Further development of metabolite databases and pathway analysis tools is needed before such automated strategies can suggest biologically relevant and robust biomarkers.

An alternative to the single compound biomarkers that

An alternative to the single compound biomarkers that have often been proposed is the use of a metabolome profile, where differences of several metabolites that would
individually be insufficient as a biomarker are considered together to generate a dis-
ease profile. We propose this as a method to capture the vast biochemical changes
that are occurring in FASD. Metabolomics analysis can be performed for a relatively
low-cost. However, further examination and optimization, possibly through artificial
intelligence strategies, is required.

To determine the exact composition of this profile would need to be conducted. But, it
would likely involve some of the cardiovascular and nervous system related
metabolites we have previously identified. It may also involve vitamin A related
metabolites, which were identified previously in our binge mouse and chronic zebrafish
exposure models.

4.4 Conclusion

Our findings of significantly different metabolites in zebrafish model conjunction
with a lack of statistical difference in most morphologic measures suggest current rec-
ommended limits for ethanol consumption during pregnancy need to be reexamined.
With our previous reliance on dysmorphology for diagnoses, it is likely there has been
a gross underestimation of the effects of ethanol on the fetus. Based on that variety
of changes in many different classes of metabolites, we observe a significant difference
even with low levels of chronic ethanol exposure. Repeated exposure to ethanol under
binge-type conditions in mice shows different effects than singly-dosed, which is an
important consideration in the allowable dose recommended by health professionals.
Further work is needed in these models to determine metabolome changes in even
lower doses and shorter durations in the zebrafish model show similar modifications.
However, we can effectively determine that in spite of unseen morphologic statisti-
cal differences, the metabolome is changed with embryonic ethanol exposure in the
zebrafish.

4.5 Materials and Methods

4.5.1 Study Design

Animal experiments using Danio rerio were performed in accord with Purdue
Animal Care and Use policies at Purdue University (West Lafayette, Indiana, USA)
using approved protocol 1111000275. Details of our study design for the zebrafish and
mouse have been reported previously. In brief, healthy AB zebrafish embryos (n = 8) were exposed from 2–to 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, or 3% ethanol from 2–24 hpf. At 48 hpf, whole embryo extracts were snap frozen and stored at −80°C. The same experimental design was used for morphometric and survival analysis, except the embryos remained in E3 media until hatching (~48 hours) and then were imaged. A follow-up experiment was also conducted using the above protocol with the addition of 1.5 and 2.4% ethanol concentrations. Additionally, ethanol treatments beginning at 4 hpf in addition to the 2 hpf were added.

Animal experiments for the binge *Mus musculus* model were performed in accord with Indiana University School of Medicine (Indianapolis, Indiana, USA) Animal Care and Use policies using approved protocol 000000. Excised E8.2 c57BL/6J mouse embryos were cultured in a dish with medium (controls, n=6). Treated animals were exposed on one (n=7) or two (n=7) days to 400 mg/dL of ethanol for six hours, being washed and incubated with medium between exposures. The embryos were harvested the next day and dissected, removing the yolk sac, snap frozen, and stored at −80°C until shipment to the processing lab at Purdue University (West Lafayette, Indiana, USA).

Samples for metabolomics from both species were thawed at 4°C, extracted in 2:1 methanol:water, homogenized, extracted in cold chloroform, centrifuged and concentrated. The non-polar fraction was dissolved in 50% double-distilled water, 25% methanol, 25% acetonitrile. The samples were analyzed using Thermo Fisher LTQ Orbitrap (Waltham, MA USA) mass spectrometer (MS) coupled to an Agilent 1100 series Liquid Chromatography (LC) (Agilent Technologies, Santa Clara, CA, USA), using an Eclipse XDB-C8 column (Agilent Technologies, Santa Clara, CA, USA). Solvent A was water + 0.1% piperidine and solvent B was acetonitrile:methanol (50:50 v/v) + 0.1% piperidine. Extensive details on the separation settings have also been previously reported (Chapter 2). The mass spectrometry (MS) analysis used negative polarity electrospray ionization. A mass range of 100–1200 atomic mass units was used for full scan analysis and XCalibur software (v.2.1.0) was used for analysis.

The zebrafish polar fraction was dissolved in 20% acetonitrile, 80% water with 0.1% formic acid. Polar samples were then analyzed by LC-MS (Time of Flight). Data processing was conducted as previously reported (Chapters 2 and 3).
4.5.2 Imaging and Morphometry

Using a Nikon NIS Elements System, ice-water sedated larvae were imaged twice—first with the right pectoral, then dorsal fin upwards. Individuals without a visible heartbeat were designated as mortalities. Subsequently, larval images were measured digitally for total length (averaged from both views), head circumference (dorsal view), area of the eye (right pectoral view), and distance between outside portions of the eyes (usually from dorsal view), as shown in Figure 3L. White-balance adjusted images (Photoshop v.11.0.2, 2010) were subjectively evaluated for morphologic abnormalities in body shape, tail shape, pericardial edema, yolk sack edema, head (brain) shape, and the otic-optic space (OOS) according to guidelines published by Panzica-Kelly et al. [523]. The evaluators were blinded to the treatment groups.

4.5.3 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis® (IPA) (Ingenuity Systems, Redwood, CA) was used for additional metabolome network analysis, as previously reported. Metabolites were required to have at least one concentration with a 0.001-fold or greater to be considered in the subsequent IPA analysis. The Biomarker Filter was applied using the default settings in IPA, selecting all for Species, Tissue and Cell Lines, Node Types, Diseases, Biofluids, and Biomarker Applications and Diseases. Separate filters were generated for the polar and non-polar zebrafish data sets and the mouse data set.

4.5.4 Statistical Analysis

Survival proportions were calculated using the Kaplan-Meier method in R Studio version 3© 2007. Individuals surviving until the time of hatching were censored. The survdiff function of the Survival CRAN package was used, which weighted deaths using the Peto-Peto modification of Gehan-Wilcoxon to detect early differences in survival (Version 2.37–4). Additionally, a logrank test for trend was used to compare the Kaplan-Meier survival curves over all concentrations as well as Cox proportional hazard ratio model (coxph) [553].

Analysis of morphometry measures began with analysis of the distributions using Q-Q plots and Shapiro Wilk testing by ethanol concentration groups. Non-parametric
analysis of the morphological variables was conducted on each measurement type using the a one-way ANOVA. Individuals that were dead prior to hatching were not included in these analyses.
5. CONCLUSION

5.1 Summary

FASD is resultant of a wide-array of biochemical pathway modifications, in no small part due to the polar protic nature of ethanol. Ethanol is a solvent and can function in acid-base modification, ester formation, halogenation, haloform reactions, dehydration, oxidation, and chlorination. These reactions can have a devastating effect on offspring of ethanol-consuming mothers, generating life-long disease. The estimated prevalence of FASD in the United States is 33.5 per 1,000 live births [1]. Currently, there are no robust biomarkers or effective interventions for FASD, due to the lack of comprehensive understanding of the mechanisms of FASD.

This dissertation explores the mechanisms involved in FASD. The first aim of this work is to investigate the mechanisms, as a whole, that are changed in an animal model (zebrafish) of FASD, with the use of metabolomics (Chapter 2). The second aim is to compare and contrast metabolite findings in two different, well-established models of FASD, the zebrafish and excised embryonic mouse (Chapter 3). Finally, the third specific aim is to identify and characterize potential biomarkers from these models of FASD (Chapter 4).

Our results demonstrated that a vast array of biochemical pathways are modified in the chronic ethanol exposure zebrafish model of FASD (Chapter 2). Many of these pathways confirmed earlier literature, but the comprehensive nature of this study expands upon many of these pathways, and links several that were previously considered in isolation as mediators of FASD. In addition, examination of the non-polar excised embryonic mouse demonstrates a significant difference between a single binge dose of ethanol and a two binge doses (Chapter 3). Similar metabolites to the zebrafish are identified in this model, though the degree and direction of change is not always consistent. These findings suggest that more ethanol does not necessarily result in an amplified, same-direction response, and that longer duration exposures do not always change metabolites the same way binge exposures do. In addition, a novel application of the probabilistic principal component and covariate analysis (PPCCA) to examine latent variables suggested by the combined models. Lastly, we examine
biomarkers suggested from the mouse and zebrafish model metabolomes, the PPCCA, and Ingenuity Pathway Analysis® (Chapter 4). We consider these biomarkers with a morphometric analysis of the zebrafish, where few significant differences are noted. Together, these observations suggest that in spite of a lack of morphologic changes, biochemistry of the prenatally exposed individual is significantly changed.

This dissertation contributes to the understanding of FASD, by examining mechanistic changes in a non-specific manner. Rather than simply presenting classes of metabolites significantly differed with the chronic ethanol exposure model, it contextualizes the metabolites within the altered biochemical pathways. This reduces publication bias, allows for the expansion of suggested mechanisms, and identifies novel pathways. This dissertation also examines differences in models of chronic and binge-type ethanol consumption. It contributes to comparative animal modeling by exploring mathematical means to compare interspecific data. Lastly, it advances the field of FASD biomarkers, suggesting that a panel of biomarkers may be more suitable for FASD than a single entity.

5.2 Major Findings

5.2.1 Developing the Big Picture of Fetal Alcohol Spectrum Disorder: A Proposed Mechanism of Metabolite Changes (Chapter 2)

1. A total of 647 non-polar fraction metabolites were identified. Of these, 94 were unique from each other with less than a 3 ppm difference from the theoretical mass of their associated identified metabolite. Eighty-two metabolites significantly differed as a function of the ethanol concentrations tested \( (P < 0.001, \text{ANOVA}) \).

2. Several metabolites that are related to ethanol catabolism were identified.

3. Many metabolites that significantly differed are implicated in the development, structure, and function of the nervous system.

(a) Glycerophospholipids, including phosphatidylserine, phosphatidic acid, phosphatidylethanol, and phosphatidylinositol, are modified with ethanol exposure and showed a variety of changes in our model. Many of these metabolites are associated with abnormalities in phospholipase D functioning.
(b) Sphingolipid moieties tended to be significantly higher at either mid-range ethanol doses or with all concentrations tested. These metabolites have important roles in the nervous system, which impact astroglial migration, neurite outgrowth, myelin structure, and cell membranes.

(c) Metabolites associated with myelin structure and function were significantly different, including sulfatides, lignoceric ceramide, and metabolites related to vitamins A, D, and K.

(d) Many metabolites identified were related to tryptophan metabolism. These included differences in the serotonergic, oxindole, and kynurenine arms of tryptophan pathways.

(e) Oxidative stress abnormalities were associated based on the metabolites acetylaldehyde, butanal, carnosine, aspidospermine, anandamide, and tryptophan. Some antioxidants were also significantly different, indicating an altered capacity to manage oxidative insult.

(f) Alterations in metabolites related to anandamide and protein kinase C were also identified.

4. Metabolite changes suggested alteration of the cardiovascular system, including metabolites related to myocyte contraction, vasculature, and coagulation.

5. Several important components of the endocrine system were modified in our metabolome, including sex hormones, the hypothalamic-pituitary-adrenal (HPA) axis, and icosanoids.

6. Renal system functioning via (12S)-hydroxy-16-heptadecynoic acid was identified.

7. Many participants in pathways related to growth and energy were modified.

(a) Metabolites related to glycolysis and gluconeogenesis were modified.

(b) Fatty acid metabolism appears highly altered in this model of FASD. Metabolites related to β-oxidation, fatty acid synthesis, energy and bile acids, and other cholesterol derivatives were identified.

(c) Modified amino acid metabolism was indicated by differences in histidine metabolites and methionine.
(d) Metabolites associated with pyrrolysine (functions in the activation, orientation, and transfer of methylamines to proteins that bind corrinoid cofactors containing cobalt), vitamin B$_{12}$, and folate were identified.
(e) Metabolites associated with abnormal morphology were significantly different in the metabolome.

8. Metabolites known to participate in normal cellular and molecular mechanisms were significantly different, including the glycosylation pathway and the transcription factors vitamins A and D.

9. The cofactor nicotinamide, a tryptophan derivative, is related to several significantly different metabolites. This may suggest a novel FASD mechanism.

5.2.2 Comparative Modeling of Fetal Alcohol Spectrum Disorder: More is Not Just More (Chapter 3)

1. We identified a total of 32 metabolites that changed with ethanol exposure, including significant differences in several fatty acids, glycerolipids, glycerophospholipids, ceramides, and retinoate.

2. Several metabolites were related to FASD-associated changes in growth and energy.
   (a) Based on our findings of phosphatidic acid, phosphatidylcholine, phosphatidylserine, and vitamin A, hepatic stellate cells may mobilize lipid droplets in response to retinoid metabolism changes induced by ethanol.
   (b) The non-polar extract metabolome of the ethanol-exposed embryo shows significant differences in fatty acids. In conjunction with results from the zebrafish model, these findings may suggest that with ethanol exposure, increases in estrogen-related metabolites upregulate monoacylglycerol transferase, which in conjunction with inactivated lipase, causes changes in monoacylglycerol.
   (c) Based on the PPCCA Principal Component (PrC) 1 loading being positive for a possible vitamin A cleavage product, methyl hexadecanoic acid, and octadecanoic acid, the latent variable may be representing the lipid metabolism changes noted above.
3. Other metabolites were related to nervous system changes.

(a) Several glycerophospholipid changes support the theory of abnormal function of phospholipase D.

(b) Ceramide alterations have implications in the nervous system, as this metabolite functions as a precursor of the myelin component sphingomyelin, as an activator of phosphatases and thereby cell death, and also as a precursor to sphingosine.

(c) Sulfatide differences were also noted in the mouse binge model.

(d) Based on the loadings of PrC2 and PrC3, the underlying latent variable of these components are likely neural related. PPCCA PrC2 was positively loaded by cleaved A, PA(18:0/18:2), and C22 sulfatide. PA(18:0/18:2) and C22 sulfatide.

5.2.3 Biomarkers of Fetal Alcohol Spectrum Disorder: The Difference You Can’t See (Chapter 4)

1. In examining the mortality of ethanol treated embryos, the log-rank test for trend was significantly different between groups ordinated by ethanol concentration, but the Kaplan-Meier log rank test failed to show a significant difference in survival between ethanol concentration groups.

2. Morphological differences were not significant by ANOVA for measurements of head circumference, eye size, eye-to-eye distance, total length, but zebrafish embryos exposed to ethanol did responded with alterations in normal development.

3. A single metabolite biomarker was not identified.

(a) None of the highly cited proposed biomarkers were present in metabolome. Many biomarkers are too large for detection with our method (proteins and enzymes). For fatty acid ethyl esters, our method was likely insufficient to stabilize these metabolites.

(b) Six significantly different metabolites were common to the chronic zebrafish and binge mouse ethanol exposure models. These included 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, which we believe could be a
cleaved molecule of vitamin A, tetradecanoic acid, methyl hexadecanoic acid, octadecanoic acid, PA(18:0/18:2(9(Z),12(Z))), and C22 sulfatide. Several of these metabolites had responses to ethanol that diverged between species, where the metabolite differed positively in the mouse binge exposure model but negatively in the chronic zebrafish model. It is unclear if these differences are rooted in differences in species’ responses to the ethanol treatment, an effect of exposure duration, or likely a combination of both.

(c) The biomarkers IPA identified were vague and not specific for development, zebrafish, or alcohol metabolism, and were disproportionally for drugs or exogenous chemicals that were unlikely to be in our system.

5.3 Limitations

This dissertation provides a comprehensive model of metabolites changed in FASD. With this information, hypotheses of several novel mechanisms have been generated, including nicotinamide metabolism changes and altered vitamin A. Several products of nicotine metabolism were significantly different, which may suggest a changes in nicotinamide metabolism are involved in FASD. Nicotinamide is the amide of vitamin B<sub>3</sub>. Tryptophan is the precursor of nicotinic acid mononucleotide, which can eventually be converted to nicotinamide adenine dinucleotide. This represents a novel finding, and should be explored further as to the generality of this effect. Modifications to this pathway have implications for redox reactions and post-translational modification of proteins. 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)-1-penten-3-one was present in both our mouse and zebrafish metabolomes. This metabolite is structurally similar to vitamin A, and could potentially be a cleavage product from 11-cisretinoate. This finding is significant, as it could be a causative mechanism of aberrant vitamin A in FASD. This dissertation also provides additional evidence about some of the hypothesized mechanisms of FASD, such as alterations of several branches of tryptophan metabolism.

5.3.1 Morphology

There are several limitations of this work. The morphologic analysis revealed little difference in the zebrafish. As a result, an additional study was conducted at ethanol
concentrations (1.5 and 2.4%) published by other laboratories that demonstrated a difference in morphology. These additional concentrations confirmed instances of cyclopia in the offspring, demonstrating the anticipated effects on craniofacial morphology. We conclude that these are critical doses for these phenotypes, although at unrealistically high doses for relevance in humans. Our concentration curve, which extends from 0–3% ethanol is sufficient to capture metabolomic change, including a higher dose than the often reported 1.5 and 2.4%.

5.3.2 Metabolomics

Metabolomics provides, at best, a snapshot of a system under a given condition. The difficulties in understanding FASD lie not only in the solvent nature of ethanol, but also in the capturing ethanol’s modifications to the unique conditions of development. Measuring the metabolome of a system can be a good monitor of the homeostatic state of that system. However, development cannot be considered as an extension of the normal homeostatic condition. It must be considered a unique process. Add to this the solvent nature of ethanol, and the disturbing nature of FASD become even more difficult to understand. So, while the metabolomics method does give us an unbiased estimation of changes in our system under ethanol exposure, it cannot capture the dynamic changes occurring in the system.

Because of the previous knowledge gained through centuries of biochemical exploration of enzymatic reactions and their substrates and products, we have a basis for understanding dynamic changes that occur in normal metabolism and homeostasis. The dynamics of development make understanding these changes even more difficult. While life sciences has gone far in understanding gene expression, protein regulation, etc., its considerations of these processes is still largely static. When we use a metabolomics, we are creating a static image of changes in the system, but because of the dynamic nature of development and homeostatic processes, these snapshots are still only a best guess at understanding changes in a system. We must work toward understanding these changes in a dynamic (think three-dimensional) environment. Changes to a system in disease cannot be considered singularly, but rather must be examined as part of that system as a whole. Thus our quantitative explanations of a disease must also.

The field of metabolomics is still a very young one. In such, often during the analysis of m/z values from the MS, metabolites that were quite illogical would appear
in the metabolome. This may reflect the immature nature of the database content. Secondly, this may also be a bias of the investment that pharmaceutical research has already put into developing rich metabolomics databases of their research. Over time, the database quality limitation will likely become less of an issue. Further, our output data can be archived and reanalyzed as these resources become further developed. New findings may come from this. Further, as more is known about the relationship between different genes, proteins, and metabolites, the vast research literature on FASD can be incorporate in to the comprehensive model of the ethanol-induced changes occurring in FASD. Likely, this global modeling of FASD mechanisms will rely heavily on bioinformatics tools, which are being developed at a rapid pace.

In the metabolomics study, the metabolite detection method used is not sensitive for metabolites which are very small (below 200 Daltons). Therefore, we were unable to examine changes in alcohol metabolism itself, such as the changing concentrations of acetylaldehyde. Similarly, many of the commonly proposed biomarkers are very large (proteins or enzymes) and therefore are not detectable with our methodology.

Secondary analysis tools also have limitation. Data entered in the IPA software is limited by the identification of metabolites by the software designers. IPA does not include metabolites listed in databases that more comprehensively catalog lipids, such as LipidMaps and Metlin. Further, many of the metabolites identified as relevant to the metabolome changes were exogenous compounds, more likely misidentified by the existing databases.

5.3.3 Mouse Study

One weakness in the mouse study is that the singly dosed animals were dosed at the same time of as the first ethanol dose in the doubly dosed animals. This makes the interpretation of the data more difficult, because it is unclear whether the differences in response to ethanol are a compounding effect of the second dose or an effect of a longer wash-out time before metabolomics. In the future, this could easily be corrected by dosing the singly-dosed animals only when the doubly-dosed animals were given their second dose.
5.3.4 Comparative Study

Finally, another limitation of this work is in the comparative modeling approach. This study examines the effects of chronic and binge ethanol exposures in two different species. Since it was a post-hoc analysis, study design could not be altered to address the compound nature of the study design differences, ethanol exposure type and species. In the future, a binge-type ethanol exposure could be explored in the zebrafish to examine the similarities and differences to the mouse response.

5.4 Future Directions

Several follow-up studies are suggested by these findings. In our morphologic analysis, we noted few statistically significant differences in common developmental parameters. This was surprising due to the significant difference in many metabolites observed in the zebrafish model. In the future, it would be important to measure the long-term effects of these ethanol doses, to determine the degree to which the individual could compensate metabolically.

Another strategy would be to use a radiolabeled ethanol and then examine the metabolome changes [554, 555]. Repeating the experiment would show where the metabolites are created through destruction of the embryo versus incorporation of ethanol into the embryo. However, analysis of metabolites may be difficult based on the current content of metabolite databases.

To further unique contributions, work is needed to confirm the identify and formation of 1-(2,6,6-trimethyl-2-cyclohexen-1-yl)-1-penten-3-one, as well as whether it is truly a cleaved molecule of vitamin A. Similarly, based on the importance of nicotinamide in redox reactions, post-translational modification of proteins, and as an enzymatic co-factor, further investigation is warranted of metabolites related to these pathways, including 2,6-dihydroxypseudoxyoxynicotine, cotinine methonium ion, and mugineic acid.

Lastly, because of the established limitations in existing proposed biomarkers for FASD, it is important to use new, unbiased methodologies like metabolomics to identify potential novel biomarkers. Most of the existing proposed biomarkers for FASD were not present in our metabolomes or were not appropriate to assess with metabolomics. While we did identify many metabolites that were significantly different in the zebrafish metabolome and a handful that were significantly different
in both the zebrafish and mouse models, a clear biomarker was still not identified. However, with the use of artificial intelligence computing strategies, in the future it may be possible to identify a panel of biomarkers that produces a characteristic FASD profile. To achieve this, further comparative modeling work is needed, to tease out modality-specific and species-specific metabolite changes that can create this profile.
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VITA
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EMPLOYMENT

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2001-2004 Associate Biochemist, Lilly Research Laboratories,
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PUBLICATIONS

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Book Chapters


Conference Proceedings


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AWARDS & HONORS


2014 Finalist for Most Outstanding Interdisciplinary Project Award, Office of Interdisciplinary Graduate Programs, Purdue University

2012 Outstanding Graduate Student Teaching Assistant in Nutrition Science, Committee for the Education of Teaching Assistants Teaching Award, Purdue University

2012 First Prize Ph.D. Student Poster, Forestry and Natural Resources Research Symposium, Purdue University

2005 Friends of Safety Award, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

GRANTS & FELLOWSHIPS

2012 School of Agriculture Graduate School Summer Research Grant, Graduate School, Purdue University

2009-2010 Lynn Fellowship, Graduate School, Veterinary Clinical Sciences, Purdue University

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TEACHING EXPERIENCE

Principles of Food Preparation and Nutrition, Instructor of Record for Nutrition (Fall 2012, Spring 2013)

Food Chemistry Laboratory (Fall 2011, Fall 2012)

Essentials of Nutrition (Spring 2012)

Food Science Laboratory (Spring 2010, Spring 2011, Spring 2012)

Applications and Integrations for 1st Year Veterinary Students (Fall 2010, Spring 2011)

RELATED PROFESSIONAL SKILLS

Animal Handling

Anesthesiology
Animal health monitoring of patient vital signs
Animal restraint
Aseptic surgery techniques
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Data entry of veterinary patient files
Drug administration
Laboratory assays and analysis
Necropsy
Specimen collection
Vaccine administration
Wellness exams
Cell and Tissue Culture

Adenovirus amplification and purification
Cardiomyocyte harvesting and culture
Cell-based assays
Tissue culture with primary cells and cell lines

Databases, Programming, Software, and Statistical Packages

Adobe Acrobat, DreamWeaver, Illustrator, InDesign, and Photoshop
ACTOR
Blackboard
Cluster computing
EndNote
Google-Docs, -Sheets, and -Slides
Ingenuity Pathway Analysis®
iWork
Keynote
LabGuru
LaTeX
Microsoft Office
NIS Elements System
Numbers
Omni-Focus, -Graffle, and -Plan
Pages
Papers
Prism
Qualtrics
R
Scrivener
SAS/JMP
SPSS
Zotero

Food and Nutrient Analysis

Bomb calorimetry
Brookfield Texture Analyzer
Brookfield Viscometer
Consistometer
Dry matter/ash content
Fat and fiber content
Hunter Colorimeter
Hydrometer
Linespread apparatus
Penetrometer/Compressometer
Rapid ViscoAnalyzer
Reflectance meter
Refractometer
Seed volume apparatus
Shear press
Shortometer
Stable MicroSystems Texture Analyzer
Water activity system

**Separation Biochemistry**

Antibody affinity chromatography
ELISA
Enzyme extraction, purification, and isolation
Fast protein liquid chromatography (FPLC)
Fluorescence-activated cell sorting
Gas chromatograph
High performance liquid chromatography (HPLC)
In-gel kinase
In-cell Western
LI-Cor Odyssey system
Metabolomics (Orbitrap mass spectrometer coupled to Agilent 1100 LC-MS)
Preparative chromatography
Thin-layer chromatography
Ultracentrifugation
Western blots

**Other General Laboratory**

Endotoxin analysis
Endotoxin removal
Hemoglobin analysis
pH meter
RT-PCR
Scintillation proximity assay
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Titration assays
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SERVICE TO UNIVERSITY

2011-2013  Assistant editor, Compass, Forestry and Natural Resources, Purdue University, West Lafayette.

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2011-2013  President, Forestry and Natural Resources Graduate Student Council, Purdue University

2006  Host to Veterinary Exchange Students from the Czech Republic
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2012 Supervisor of Masters Student, Forestry and Natural Resources, Purdue University; Student: Angela Goodman-Chan, Animal Sciences

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Biology of Aging
Food Chemistry
Food Science
Principles of Food Preparation and Nutrition
Current Topics in Ingestive Behavior

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German Reading: Can read and write with dictionary
German Speaking: Good
German Writing: Can write with dictionary

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