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Toxicant Exposure during Pregnancy Increases Protective Proteins in the Dam and a Sexually Dimorphic Response in the Fetus

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Abstract

Endocrine disrupting compounds (EDCs) are ubiquitous environmental pollutants that alter endocrine system function. EDCs induce birth defects and a myriad of other negative health outcomes. Although the mechanism of toxicity of many EDCs have been studied in detail, little work has focused on understanding the mechanisms through which pregnant dams and fetuses protect themselves from EDCs or if those protective mechanisms are sexually dimorphic in fetuses. In this study, we examined proteomic alterations in the livers of mouse dams and their male and female fetuses induced by vinclozolin, a model antiandrogenic EDC. Dam livers upregulated nine phase I and phase II detoxification pathways and pathway analysis revealed that more pathways are significantly enriched in dam livers than in fetal livers. Phase I and II detoxification proteins are also involved in steroid and steroid hormone biosynthesis and vinclozolin likely alters steroid levels in both the dam and the fetus. The response of the fetal liver proteome to vinclozolin exposure is sexually dimorphic. Female fetal livers upregulated proteins in xenobiotic metabolism pathways, whereas male fetal livers upregulated proteins in oxidative phosphorylation pathways. These results suggest that female fetuses increase protective mechanisms, whereas male fetuses increase ATP production and upregulate several disease pathways that are indicative of oxidative damage. Females fetuses upregulate proteins and protective pathways that were more similar to the dams whereas males did not. If this sexually dimorphic pattern is typical, then males might generally be more sensitive to EDCs.

Keywords

Vinclozolin; Endocrine Disrupting Compounds; Proteomics; Detoxification; Liver

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Author Contributions

K. McCoy developed the study with assistance from Dr.s Nash and Bereman. Dr. Amato conducted the animal experiment and collected tissues. Dr. Nash ran the proteomics with guidance from Dr. Bereman. Dr.s Rister, K. McCoy, and M. McCoy worked together to analyze and interpret the data and write the results. Dr. Rister wrote the manuscript and incorporated the edits from all authors.

Introduction

Endocrine disrupting compounds (EDCs) are widespread environmental contaminants that alter the function of the endocrine system leading to a variety of negative health outcomes (Eertmans *et al.*, 2003; Swan *et al.*, 2003; Balabanič *et al.*, 2011; Giulivo *et al.*, 2016; Mariana *et al.*, 2016; Sidorkiewicz *et al.*, 2017). Embryonic exposure to EDCs is especially damaging as fetal development is mediated by endocrine regulation (Fowden, 1995; Costa, 2016). Exposure to EDCs during development can cause birth defects and induce a variety of disorders later in life (Skakkebæk *et al.*, 2001; Vaiserman, 2014).

Some EDCs have been so well studied that they are used as models to study specific mechanisms of toxicity. For example, vinclozolin, a currently used fungicide, is a model antiandrogenic EDC (Kelce *et al.*, 1994; Uzumcu *et al.*, 2004; Wickerham *et al.*, 2012; Jabali *et al.*, 2020) and also induces transgenerational effects likely via epigenetic alterations in the male germ line (Nilsson *et al.*, 2018). The anti-androgenic effects induced by vinclozolin have been well studied, and in males include hypospadias, infertility, cryptorchidism, decreased sperm quality, and virilization of female genitalia (Gray *et al.*, 1994; Monosson *et al.*, 1999; Shono *et al.*, 2004; Elzeinova *et al.*, 2008; Cowin *et al.*, 2010; Amato *et al.*, 2018). Transgenerational effects of vinclozolin exposure include a broad array of adult onset diseases in both males and females including prostate and kidney disease, immune and reproductive system abnormalities, and altered stress responses (Anway *et al.*, 2006a; Anway *et al.*, 2006b; Cruz-Hurtado *et al.*, 2018; Nilsson *et al.*, 2018).

Although the mechanism of toxicity of vinclozolin and other environmental toxicants have been studied in detail, less work has focused on determining the mechanisms through which individuals protect themselves and their fetuses from EDCs, or if those protective mechanisms are sexually dimorphic in fetuses. Indeed, understanding the mechanisms that decrease the effects of environmental chemicals is an important first step in determining how to develop protective therapies.

Fortunately, there are endogenous detoxification pathways in the liver that can metabolize EDCs, such as vinclozolin, into non-toxic compounds that can be excreted out of the body. There are two main phases of liver detoxification: phase I and phase II. In phase I, cytochrome P450 enzymes (CYPs) and other enzymes metabolize xenobiotics, or exogenous chemicals, to make them more water soluble. Phase II, enzymes conjugate polar groups onto the intermediary metabolites making them less toxic (less reactive) and allowing them to be excreted in urine and feces (Liska, 1998). Vinclozolin has been shown, in the rat, to increase the phase I CYPs in the liver during pregnancy, showing that it affects liver detoxification processes (de Oca *et al.*, 2015; Aquilino *et al.*, 2016). Indeed, CYPs are also involved in steroidogenesis, so increasing protective detoxification enzymes might alter steroid metabolism. Changes in steroid metabolism likely have different consequences for pregnant dams and their female vs. male fetuses. There is also evidence that expression or quantity of certain CYPs is influenced by steroid signaling (Honkakoski and Negishi, 2000; Tsuchiya *et al.*, 2005), which suggests that sex hormone feedback systems could affect the metabolism of vinclozolin.

Vinclozolin and its metabolites can also increase oxidative stress and influence the function of oxidant sensitive protective transcription factors (Radice *et al.*, 1998). There are multiple transcription factors that recognize increases in reactive oxygen species that result from toxin exposure (Baldwin, 2019). These transcription factors, such as AhR, PXR, Nrf2, and NF-κB, can regulate phase I and II detoxification enzymes and phase III transporters along with other proteins to protect the body from xenobiotic exposure. Therefore, there are multiple mechanisms through which vinclozolin can work to alter abundances of protective proteins in both the dam and fetal livers.

Although we have a basic understanding of how vinclozolin is detoxified in pregnant rodents, the way in which fetal livers respond to vinclozolin, whether this response is sexually dimorphic, and the relative roles that the dam and fetal livers play in detoxification are unknown. The current study uses proteomic analyses to investigate the broad mechanisms through which the dam liver upregulates detoxification and responds to the presence of the xenobiotic, vinclozolin. We also determine the pathways through which fetal livers respond to vinclozolin and test whether these responses are sexually dimorphic. Finally, we compare the strength and nature of the response between the dam and fetal livers. The results from this study increase our understanding of how vinclozolin affects detoxification within the liver of pregnant dams, illustrates how male and female fetal livers respond to exposure differently, and contrasts maternal and fetal responses to exposure. Understanding the endogenous protective mechanisms that are upregulated in toxic environments in pregnant mothers and their fetuses will help identify pathways that we can augment (e.g. through prenatal supplements) to protect the developing fetus from environmental contaminants.

Methods

Animal Handling and Treatment.

All studies were carried out under an approved animal use protocol established by East Carolina University (ECU) Institutional Animal Care and Use Committee (AUP D-297). Eight-week-old CD-1 mice were purchased (Charles River Breeding Laboratories Raleigh, NC) and acclimated for at least 7 days to 21–22 °C on a 12 h light-dark cycle with free access to food and water (Purina ISOCHOW).

CD-1 mice were time mated. Males and virgin females were placed together, and females were checked for vaginal plugs each morning. A vaginal plug indicated that a mating had occurred that night and the embryos were designated as embryonic day (E) 0.5 at noon the day the plug was identified. Pregnant dams (N=4) were treated with 125 mg/kg vinclozolin (cat# 45705, Sigma-Aldrich, St. Louis, MO), dissolved in tocopherol stripped corn oil (cat# 901415, Millipore, Burlington, MA) (VCZ), or tocopherol stripped corn oil alone (Solvent control, CO) once a day from E 13.5–16.5 (Figure 1a). Dosages were increased daily based on each dam's weight. This concentration of vinclozolin was selected because it consistently induces hypospadias in male mice and is therefore known to reach the fetus at toxic concentrations (Amato and McCoy, 2016; Amato *et al.*, 2018). All doses were given via gavage.

Discovery Proteomics.

Dams were sacrificed one hour after final treatment dose on day E16.5. Dams were dissected, and embryos' developmental stage (of E16.5) was verified by morphological evaluation using the Theiler staging system (stage 26) (Theiler, 2013). Embryos were then dissected in chilled, 1X Phosphate Buffered Saline (PBS). The sex of the pups was determined by examining gonad morphology, which is not morphologically affected by vinclozolin during this specific exposure time window (Amato *et al.*, 2018). Intact livers were collected individually, weighed, and snap frozen in liquid nitrogen. Frozen livers were stored at −80°C until protein extraction was conducted.

Dam and fetal livers (~25 mg) were washed in cold 1x PBS. Then, two volumes of cold lysis buffer (1% sodium deoxycholate surfactant (SDS)/50 mM ammonium bicarbonate) was added to each sample and the sample was fully homogenized and sonicated. The homogenate was then centrifuged at 16,000 rcf for 10 minutes at 4 °C. The supernatant was removed and prepared for tryptic digestion according to previously established protocols through the FASP method (Zhang *et al.*, 2012; Yang *et al.*, 2016). Briefly, protein solution was adjusted to a final concentration of 50 μg in 100 μL. MDL-Dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO) was added to each sample at 5 mM and incubated at 60 °C for 30 min. Iodoacetamide (IAM, Sigma Aldrich, St. Louis, MO) was then added at 15 mM and the solution was incubated in the dark for 20 min. The protein samples then underwent filter-aided sample preparation using a Vivacon 30 kDa molecular weight cutoff filters and reconstituted with 100 μL of 50 mM ammonium bicarbonate solution. The protein samples then underwent tryptic digestion by adding trypsin to the solution at 1:50 ratio and incubated at 37 °C for 4 hours. The peptides were then diluted and acidified with HCl to a final concentration of 250 mM (pH ≤ 3).

The samples were then analyzed using an Easy nanoLC 1000 coupled to a QExactive + mass spectrometer (Thermo Fisher, Bremen, Germany). Peptides (1μg) were loaded onto a 35cm C18 column (reverse phase ReproSilPur 120C-18-AQ 3μm particles, (Cat# H354, New Objective, Woburn, MA) and eluted using a 240-minute gradient of mobile phase A (98% water, 2% acetonitrile, and 0.1% formic acid) and mobile phase B (100% acetonitrile, 0.1% formic acid). The 270min method included an LC gradient with a linear increase from 2% B to 40% B across minutes 2 to 242. This was followed by an increase and wash at 80% B from 242–254 minutes, followed by equilibration of the column at 0% B. Tandem mass spectrometry was performed in a data-dependent manner with Bovine serum albumin run after every three injections to confirm reproducibility. The raw data were searched against the Uniprot *mus musculus* protein database (2017) using MaxQuant. Label free quantitation intensities were used for relative quantitation of proteins.

Statistical analyses.

To understand the effects of vinclozolin on the dam liver proteome we contrasted vinclozolin and corn oil treated dam livers. To investigate natural sexual dimorphisms in the fetal liver proteome we compare control males *vs.* control females. Comparisons between vinclozolin males *vs.* vinclozolin females were used to evaluate how vinclozolin affected the natural sexual dimorphisms relative to those found in the corn oil control contrast. To investigate

how each sex was affected by vinclozolin we compared control males *vs.* vinclozolin males, and control females *vs.* vinclozolin females.

We used t-tests to determine if there were differences in the log transformed abundances of proteins between corn oil and vinclozolin exposed livers, or between the sexes. To control for elevated risk of type I error rates associated with conducting multiple pairwise t-tests we used false discovery rate (FDR) adjusted p-values and a relaxed critical value ($q < 0.1$. We evaluated the protein-protein interaction networks formed by the FDR corrected differentially expressed proteins using STRING (https://string-db.org/) (Szklarczyk *et al.*, 2017).

To investigate the general functions and pathways that were affected by vinclozolin exposure, or that differed between fetal sexes we conducted functional enrichment analysis using DAVID 6.8 (https://david.ncifcrf.gov/summary.jsp) (Sherman and Lempicki, 2009). Note that for each focal contrast functional enrichment analysis was performed on proteins that were deemed to be differentially expressed based on the less conservative non-FDR corrected p-values that were compared to a background list of all the identified proteins in our data set (Jiao *et al.*, 2012). This allowed for a broader investigation of functional differences and is appropriate since we are not making conclusions about significant effects on specific proteins. Enrichment analyses were conducted separately for proteins that were up and downregulated for each focal contrast (Reimand *et al.*, 2016).

We illustrate how the total number of proteins used in the functional analyses varied across treatments and between dam and fetuses using Venn diagrams, which display the data in three ways: (1) only proteins downregulated in all comparisons, (2) only proteins upregulated in all comparisons, and (3) all differentially expressed (DE) proteins (this analysis includes proteins that did not change in the same direction among sexes or treatment, *e.g.* male fetuses may have upregulating levels of a protein and female fetuses have the same protein downregulated). As a result, the third contrast (all DE proteins) can include additional proteins that are not recorded in the first two contrasts. Additionally, we performed pathway visualization to evaluate how vinclozolin affected proteins involved in steroid and steroid hormone biosynthesis in the dam and fetuses using Pathview, which are shown in Figure S1–4 (Luo and Brouwer, 2013; Luo *et al.*, 2017).

Finally, to explore patterns of protein co-expression between sexes and vinclozolin treatments we conducted a principal components analysis on the correlation matrix of differentially expressed proteins using the prcomp function in the R programing environment version 4.0.0 (Team, 2013). Graphical summaries of PCA analyses were generated using the R library ggplot2 (Wickham, 2016).

Results

Dam Liver Proteomics.

In the dam, there were 356 proteins that were significantly differentially (a=0.05) expressed between corn oil and vinclozolin exposed livers, and 13 proteins that remained different after FDR correction (Figure 2a; Table S1). Network analysis illustrates a highly connected

group of nine of the 13 proteins: CYP2C54, CYP2C50, CYP2B19, CYP2C29, CYP2C37, CYP1A2, GSTM3, CYP2B10, and GSTM1, which were all increased in vinclozolin exposed dams (Figure 2b and 2c). These nine proteins have multiple interactions among each other and are involved in phase I (CYPs) and phase II (GSTM) detoxification.

Pathway enrichment analysis was conducted on the 356 non-FDR corrected differentially expressed proteins ($p < 0.05$). Dams exposed to vinclozolin had 14 significantly enriched pathways from proteins that were downregulated relative to corn oil which included multiple signaling pathways, as well as pathways associated with the immune system, nervous system, and cell proliferation. Vinclozolin exposure resulted in 14 significantly enriched pathways from proteins that were upregulated relative to corn oil including chemical carcinogenesis, retinol metabolism, steroid hormone biosynthesis, fatty acid synthesis, inflammation, and drug and xenobiotic metabolism (Figure 3; Table S2–S3). Many of these pathways are related to the metabolism of both endogenous and exogenous compounds. Therefore, to directly compare the broadest group of detoxification and metabolism proteins, the chemical carcinogenesis pathway was evaluated in more detail. The first step in the carcinogenesis pathway for each compound consists of detoxification/metabolic enzymes, which allows us to evaluate a large range of proteins including both phase I and phase II enzymes. The proteins that resulted in chemical carcinogenesis becoming significantly enriched in dam livers included 10 CYP enzymes, 1 epoxide hydrolase, 4 glutathione-s-transferases, 1 arylamine N-acetyltransferase, and 2 UDP-glucuronosyltransferases. These proteins also influence steroid metabolism. To evaluate this, we investigated how vinclozolin exposure affected steroid biosynthesis and steroid hormone synthesis in the pregnant dams using Pathview (Figure S1–S2). Interestingly, vinclozolin exposed females overwhelmingly upregulated proteins in the steroid biosynthesis pathway (Figure S1). However, they down regulated enzymes that drive steroid hormone synthesis (i.e. production of sex hormones), and upregulated enzymes leading to steroid hormone degradation (Figure S2), suggesting that vinclozolin induces strong negative effects on steroidogenesis in pregnant dams.

Fetal Liver Proteomics.

We compared female and male control animals to evaluate the natural sexual dimorphisms within the developing fetal liver, and female and male vinclozolin exposed animals to evaluate how these sexual dimorphisms were changed by toxicant exposure. We also evaluated the effects of vinclozolin on fetal liver proteomics by comparing controls and vinclozolin exposed individuals for each sex separately. Each focal contrast was characterized by different numbers of differentially expressed proteins. Prior to FDR correction there were 170 differentially expressed proteins between control females and males, 107 between vinclozolin females and males, 156 between control males and vinclozolin males, and 143 between control females and vinclozolin females. However, none of these proteins were significant after FDR correction primarily due to lower fold changes (Figure 4). Enrichment analyses showed that proteins were enriched for different functional pathways across the comparisons (Table S4–S7).

Control females upregulated proteins that were significantly enriched for pancreatic secretion and protein digestion and absorption pathways compared to control males

(Figure 5a). However, these pathways were no longer significantly enriched between vinclozolin females and males. Instead, vinclozolin exposed females downregulated proteins significantly enriched for synaptic vesicle cycle, endocrine regulated calcium reabsorption, tuberculosis, and lysosome pathways compared to vinclozolin exposed males (Figure 5b).

Relative to control males the vinclozolin exposed males had downregulated proteins that were significantly enriched for RNA transport, and upregulated proteins were significantly enriched for multiple pathways associated with oxidative stress including alcoholism, systemic lupus erythematosus, viral carcinogenesis, oxidative phosphorylation, Parkinson's disease, Alzheimer's disease, Huntington's disease, and non-alcoholic fatty liver disease relative to control males (Figure 5c) (Georgakilas *et al.*, 2010; Ambade and Mandrekar, 2012; Shah *et al.*, 2014; Foppoli *et al.*, 2015).

Female fetuses exposed to vinclozolin had downregulated proteins that were significantly enriched for pathways associated with calcium reabsorption and tight junctions and upregulated chemical carcinogenesis (which includes chemical degradation), steroid hormone biosynthesis, arachidonic acid metabolism, and metabolism of xenobiotics pathways relative to corn oil exposed females (Figure 5d). For chemical carcinogenesis, the proteins that resulted in its significant enrichment included glutathione-s-transferase mu 1, corticosteroid 11-beta dehydrogenase, cytochrome p450 2c40, and carbonyl reductase 1. These proteins are involved in drug metabolism, xenobiotic metabolism, and steroid metabolism pathways and could have effects on metabolic and endocrine processes. We evaluated how vinclozolin exposure affected steroid biosynthesis and steroid hormone biosynthesis in females and males using Pathview shown in Figure S1–S4 (https://pathview.uncc.edu/). Many of the enzymes involved in the early stages of steroid biosynthesis were affected by vinclozolin in opposite patterns in males and females, whereas in later stages both sexes are affected similarly (Figure S3). However, female fetal livers exposed to vinclozolin overwhelmingly upregulate enzymes involved in later stages of steroid hormone synthesis (Figure S4) relative to males. Many of these enzymes are associated with metabolizing the steroids into forms that are less potent or more easily excreted (Figure S4). One important exception is that females exposed to vinclozolin upregulate 5ß-reductase, the enzyme that converts testosterone into the more potent androgen dihydrotestosterone, and vinclozolin exposed males downregulate this enzyme. Taken together these data show that vinclozolin caused a complex, sexually dimorphic response where females upregulate proteins in xenobiotic metabolism pathways and steroid catabolism, whereas males upregulate proteins in oxidative stress pathways (Figure 5c & d) and down regulate enzymes that catabolize sex hormones.

To understand how vinclozolin affected natural sexually dimorphic protein expression, we first contrasted corn oil exposed females and males with females and males exposed to vinclozolin. Only 4 proteins were found to be differentially expressed in both contrasts (Figure 6a; Table S8), indicating that vinclozolin exposure almost completely changed the suite of proteins that are normally sexually dimorphic in expression. In addition to changing the identity of the proteins that are sexually dimorphic, the magnitude of the sexual dimorphism was reduced in vinclozolin exposed mice as they had fewer differentially expressed proteins between the sexes compared to control animals (Figure 6a). Reductions

in sexual dimorphism can occur because males become feminized by vinclozolin (an anti-androgen) or because females become masculinized, or both.

To further evaluate vinclozolin's influence on sexually dimorphic patterns of protein expression, we compared the number of differentially expressed proteins in the vinclozolin vs. corn oil contrast between males and females. Males exposed to vinclozolin responded differently than females as only 7 differentially expressed proteins were shared between sexes, out of the 7 differentially expressed proteins 6 were differentially expressed in the same direction (Figure 6b; Table S8).

To evaluate whether vinclozolin males were feminized, we contrasted the proteins that were differentially expressed between corn oil females and males (the natural sexual dimorphism) compared to those differentially expressed in vinclozolin males and corn oil males (proteins affected by vinclozolin). Therefore, corn oil males were held constant in this comparison. While the majority of proteins remained uniquely different, 25 proteins were different from corn oil males in both corn oil females and vinclozolin males (8 proteins decreased and 17 increased) (Figure 6c; Table S9). This suggests that the patterns of expression of these 25 proteins are feminized in males exposed to vinclozolin. We then conducted a similar comparison to evaluate if vinclozolin females had masculinized patterns of expression. We compared the differentially expressed proteins between corn oil females and males compared to the vinclozolin female and corn oil female contrast (corn oil females were held constant). We found that vinclozolin females shared 23 proteins with corn oil males that were different from corn oil females (Figure 6d; Table S10). Control males and vinclozolin females increased 5 proteins and decrease 17 proteins compared to CO females which suggests that 22 out of the 23 shared proteins are masculinized in females exposed to vinclozolin.

Given the complex way that vinclozolin affected sexual dimorphism, we decided to determine the effects of vinclozolin on overall protein co-expression between sexes. We performed principal component analysis on male and female liver proteomes from corn oil and vinclozolin exposures (Figure 7). Principal component 1 explained 19% of the variation in the data, and principal component 2 explained 16% of the variation (Figure 7a). The expression profiles were consistent with the patterns observed from our analyses of differential expression with profiles being separated along PC1 (the major axis of variation) according to vinclozolin exposure and along the PC2 axis according to sex. Specifically, the expression profiles of males and females exposed to corn oil fell out in distinct clusters from each other and were distinct from females exposed to vinclozolin. However, the expression profiles of males exposed to vinclozolin did not form a tightly clustered or distinct grouping along either PC axes, but tended to be more similar to females along the PC2 axis suggesting that the proteomic profiles of vinclozolin male livers were less well organized, and were feminized (Figure 7b,c). Indeed, there is clear sexual dimorphism in the proteomic profiles of corn oil females and males, and corn oil males and vinclozolin females, but vinclozolin males did not show the same clear pattern of sexual dimorphism (Figure 7b). The more dispersed proteomic profiles of males exposed to vinclozolin (Figure 7c) highlights that the response to vinclozolin is quite variable among males.

Dam and Fetal Proteomics Comparisons.

We compared the number of differentially expressed proteins between control *vs.* vinclozolin dam, and fetal livers (Figure 8a). The total number of proteins that responded to vinclozolin was more than two times higher in dam livers than in fetal livers. There was a higher number of the same proteins altered by vinclozolin between the dam and the fetuses of each sex relative to the number fetuses shared with each other. However, the direction in which vinclozolin affected these proteins was sexually dimorphic (Figure 8a). The dams shared 12 differentially expressed proteins with females and 10 differentially expressed proteins with males. However, while the majority (8 of 12, or 67%) of the shared proteins with female fetuses were altered in the same direction as the dam, less than half (4 of 10 or 40%) of the shared proteins between the dam and male fetuses were altered in the same direction. Only seven proteins responded to vinclozolin exposure in both female and male fetuses. There was one protein increased between all comparisons, pyridoxine-5′-phosphate oxidase (PNPO), which is involved in cofactor metabolism. This data suggests that the dam's response to vinclozolin stronger than the fetus' and confirms our earlier data showing that male and female fetuses respond to vinclozolin very differently from one another.

While there were not many differentially expressed proteins that were shared between dam and fetal livers, we wanted to investigate whether the proteins that were affected by vinclozolin were associated with shared significantly enriched pathways. Six significantly enriched pathways were shared between dam and fetal livers (Figure 8b). All of the significantly enriched pathways from upregulated proteins in the female fetuses and one of the two significantly enriched pathways from downregulated proteins (tight junction) was shared with the dam (Figure 5d). Interestingly, chemical carcinogenesis pathway was significantly enriched from upregulated proteins in both dam and female fetal livers (Figure 3 and 5). However, there was only one protein in that pathway, glutathione-s-transferase mu 1, that was similarly differentially expressed between the two groups. The female fetal liver also only upregulated 4 proteins whereas the dam liver upregulating 18 proteins in the chemical carcinogenesis pathway.

On the other hand, there was only one significantly enriched pathway between dam and male fetus which is the general metabolic pathway. Female and male fetuses did not share a single similar pathway in response to vinclozolin exposure. Males had more pathways that were significantly enriched (affected) than females, most of which are associated with oxidative phosphorylation (Figure 8b). These results indicate that female fetal livers are affected by vinclozolin exposure in a similar way but with fewer proteins than dam livers, and that the pathways affected in males in response to vinclozolin are different than dam or female fetal livers and are likely associated with cellular damage rather than protection.

Discussion

Characterizing the endogenous mechanisms that protect us from toxicants will facilitate research into therapies that can be used to augment those natural processes and strengthen our resilience to toxicants (Sargis *et al.*, 2019). As a first step to describe such mechanisms of resilience that occur within the liver during pregnancy and development, we use discovery proteomics to characterize the differences induced by vinclozolin exposure in both pregnant

and fetal mice. Discovery proteomics facilitates investigations that describe diverse effects generated by specific treatments or tests of hypotheses focused on determining broad effects or affected pathways. For example, previous studies have shown that the livers of pregnant mice exposed to vinclozolin upregulate phase I detoxifying enzymes (de Oca *et al.*, 2015), but our proteomic approach allowed us to document that vinclozolin also upregulates phase II enzymes in the livers of pregnant mice. In fact, GSTM enzymes were in higher abundance than five of the CYP enzymes, and only CYP1A2 and CYP2C29 had abundances similar to the GSTM abundances (Figure 2c). Furthermore, where de Oca *et al.* (2015) used a higher dosage of vinclozolin over a longer time, our results show a lower dosage over a shorter window still induces similar detoxification enzymes. Understanding the diverse detoxification mechanisms that are elicited by pregnant individuals (and their fetuses) when exposed to environmental toxicants is important for characterizing the natural mechanisms of resilience that protect individuals from toxicants.

To our knowledge, no studies have investigated how fetal livers respond to vinclozolin and whether these effects are sexually dimorphic. We show that fetuses have less drastic changes in their liver proteome compared to the dams. For example, no proteins were found to be significantly affected after FDR correction in fetal livers, and proteins significantly different in the fetal t-test comparisons (α=0.05) had lower fold changes than dams. In addition, fewer pathways were altered between corn oil and vinclozolin males (11), or similarly exposed females (7) relative to the dams (28), which generally also had higher p-values (Figure 8B). This lack of a strong response likely stems from the fetal liver not being sufficiently differentiated to function in an adaptive fashion (Hart *et al.*, 2009). For example, only 8 CYP proteins were measured in the fetuses compared to 32 CYP proteins measured in dams. These findings are consistent with the work of Hart *et al.* (2009) which showed that gene expression of many cytochrome P450 enzymes are not naturally upregulated until after birth. As these are the primary type I detoxification proteins and are known to metabolize vinclozolin (Sierra-Santoyo *et al.*, 2012), the reduced number in the fetuses suggests that they were either expressed at very low levels and were undetectable with our method, or that fetuses at E16.5 are not yet competent to upregulate all of these protective enzymes. In addition, there could be selective pressures or metabolic tradeoffs that reduce the probability that these proteins become upregulated in the fetus even in the context toxicant exposure (especially in males see below). Importantly, if fetuses have a reduced ability to detoxify environmental toxicants then research into protective therapies might need to focus on augmenting maternal or possibly placental detoxification.

Furthermore, while increased CYPs can aid in detoxification of vinclozolin, they can also alter the synthesis and degradation of steroids (Figure S1–S4). Therefore, the vinclozolin-induced increases in CYP enzymes in the dam and female fetal livers likely affect steroid balance, including feedback mechanisms, which are important during development. Our data suggests that vinclozolin induces the dams to upregulate steroid biosynthesis, but decrease enzymes involved in the production of steroid (sex) hormones and increase those involved in steroid hormone degradation (e.g., increasing glucuronidation, Figure S1–S2). This supports the hypothesis that detoxification processes affect steroid hormone production and that tradeoffs exist between detoxification and sex hormone synthesis. In fact, it appears that female fetuses do increase detoxification which is associated with increased metabolism of

steroid hormones whereas males take the opposite approach. Males exposed to vinclozolin do not increase detoxification and decrease the enzymes that degrade steroid hormones (Figure S4). This suggests that the tradeoff between detoxification and steroid (sex) hormone metabolism induces different physiological choices or outcomes in female and male fetuses. Interestingly, vinclozolin might increase dihydrotestosterone production in female fetal livers (Figure S4), which could explain why females exposed to vinclozolin have been reported to have masculinized genitalia (Moller and Backstrom, 2016). We are currently investigating how vinclozolin exposure affects steroid hormone balance in the dams and fetuses.

While fetal livers had less drastic changes, they still showed interesting effects. Females exposed to vinclozolin were better able to launch protective mechanisms and upregulated fewer pathways associated with cytotoxic effects than males (Figure 5c–d). Although not to the same extent as dam livers, the livers of female fetuses exposed to vinclozolin increased metabolism of xenobiotics whereas males did not. This suggests that female livers are able to increase the metabolism of vinclozolin and decrease exposure relative to male fetuses. Males had upregulated proteins that significantly enriched oxidative phosphorylation pathway (Figure 5c–d) including proteins in complex I, III, and V. If the protein abundance increases result in activity increases, then there will be an increase in ATP production and increased reactive oxygen species that could lead to higher oxidative stress in the liver (Cadenas, 2018). Males, therefore, are likely exposed to more vinclozolin, as detoxification is reduced, and could be experiencing more oxidative damage during development. If males are generally less able to detoxify environmental pollutants and protect themselves from associated cytotoxicity, they might suffer stronger effects of toxicant exposure than females.

The changes in sexually dimorphic proteins recorded here are likely resulting from both direct (antiandrogenic) and indirect (oxidative stress) actions of vinclozolin. Vinclozolin and its metabolites, including DTMBA, competitively inhibit the androgen receptor decreasing its function, but can also reduce androgen receptor concentrations (Gray *et al.*, 1994; Kelce *et al.*, 1994; Wang and Baskin, 2008; Galli *et al.*, 2014; Amato and McCoy, 2016; Amato *et al.*, 2018). These antiandrogenic effects are known to decrease masculinization of male genitalia during development (Gray *et al.*, 1994; Amato *et al.*, 2018). Androgen signaling is critical for masculinization so developing males might require fewer and lower concentrations of CYP enzymes that increase androgen catabolism (Figure S4). These reductions might make males less able to upregulate CYP enzymes that detoxify xenobiotics so that they ultimately suffer stronger negative effects than females. Alterations in androgen signaling can also affect CYP enzyme expression, therefore increased androgens in females could be driving the changes in their CYP enzymes (Hudson *et al.*, 2001; Moilanen *et al.*, 2007; Maksymchuk and Kashuba, 2020). Future work should formally evaluate these hypotheses. Furthermore, vinclozolin, M1, and M2 have also been shown to have antagonist or agonist activity against other steroid receptors including progesterone receptor, glucocorticoid receptor, or estrogen receptors (Molina-Molina *et al.*, 2006). Therefore, vinclozolin and its metabolites can interrupt the signaling of several sex hormones, and likely alters the steroidogenesis, as well as oxidative phosphorylation all of which could lead to the detrimental health effects discussed above (Wong *et al.*, 1995; Molina-Molina *et al.*, 2006). Such feedback mechanisms typically work to regulate hormone concentrations

to maintain homeostasis in the adult. However, during development, androgen signaling is critical for masculinization so developing males might require reduced CYP enzyme concentrations so that androgen catabolism remains low (Figure S4). These reductions might make males less able to upregulate CYP enzymes that detoxify xenobiotics so that they ultimately suffer stronger negative effects than females. Future work should formally evaluate these hypotheses.

There is also evidence that androgen signaling within the mitochondria reduces the expression oxidative phosphorylation proteins (Bajpai *et al.*, 2019). Therefore, reductions to androgen receptor function, could increase oxidative phosphorylation proteins and drive the sexual dimorphism. In addition, transcription factors that regulate the expression of detoxification and antioxidant genes have sexual dimorphic activity. Specifically, Nrf2, which induces the transcription of detoxifying and antioxidant proteins, was shown to have increased expression among with its target genes in females compared to males (Rooney *et al.*, 2018). Furthermore, vinclozolin and its metabolites have been shown to cause epigenetic alterations, which could contribute to the changes seen in this study (Anway *et al.*, 2006a; Cruz-Hurtado *et al.*, 2018). Therefore, there are multiple mechanisms through which vinclozolin induces sexually dimorphic effects and these likely interact with natural mechanisms of resilience that protect of the fetus and fetal liver from damage caused by xenobiotic exposure (i.e., Nrf2).

Vinclozolin induces toxicant-associated fatty liver disease, a form of non-alcoholic fatty liver disease (NAFLD) (Al-Eryani *et al.*, 2015). NAFLD presents in many ways, but generally results in liver damage and can lead to cirrhosis and hepatocellular carcinoma. Oxidative stress very likely plays a primary role in the beginning stages of NAFLD (Masarone *et al.*, 2018). Interestingly, NAFLD is a sexually dimorphic disease, where females at reproductive age have lower incidence of NAFLD. This distinction does not hold true after menopause, suggesting that sex hormones such as estrogens play a role in protecting against NAFLD (Ballestri *et al.*, 2017). Our data also shows that male fetuses exposed to vinclozolin are likely at a higher risk for developing liver disease since their upregulated proteins were significantly enriched for nonalcoholic fatty liver disease, oxidative phosphorylation pathway, and several disease pathways that are associated with oxidative stress.

While vinclozolin is known to feminize male genitalia and masculinize female genitalia, this is the first work showing that this same pattern can be identified for the liver proteome (Figure 6c–d) (Monosson *et al.*, 1999; Buckley *et al.*, 2006; Amato and McCoy, 2016; Amato *et al.*, 2018) . In fact, vinclozolin decreases overall sexual dimorphism of the liver proteome, and especially affects the male proteome (Figure 7). Only 16% of the sexually dimorphic proteins in vinclozolin treated animals are shared with those that are naturally different between males and females as see in the corn oil treatment (Figure 6c–d). The implications of this shift on fetal programing and the long-term consequences of this altered liver proteome are unknown and should be a topic of future work.

Overall, this study constitutes an important first step in understanding the proteomic responses of dam and fetal livers after exposure to a model antiandrogenic EDC. Dam

and female fetal livers increase detoxification enzymes in response to vinclozolin exposure, whereas male fetal livers upregulate proteins significantly enriched for the oxidative stress pathways. As a result, this is the first work illustrating a sexually dimorphic response from fetal livers, where females are protective, and males are more likely negatively affected. These differences might help explain why males can be at higher risk of developing diseases that have developmental origins associated with exposure to pollutants such as neurodevelopmental, and obesity disorders (Bolton *et al.*, 2012; Bolton *et al.*, 2014; Sobolewski *et al.*, 2018). Finally, the detoxification and antioxidant systems that were altered by vinclozolin should be studied further as possible mechanisms of resilience and therapeutic targets to decrease the effect of EDC exposure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

CO	Control/Corn Oil
CYP	Cytochrome P450 enzymes
E	Embryonic Day
EDC	Endocrine disrupting compounds
FDR	False discovery rate
GSTM	Glutathione-S-transferase mu
NAFLD	Non-alcoholic fatty liver disease
PBS	Phosphate buffered saline
PCA	Principal component analysis
PNPO	Pyridoxine-5′-phosphate oxidase
VCZ	Vinclozolin

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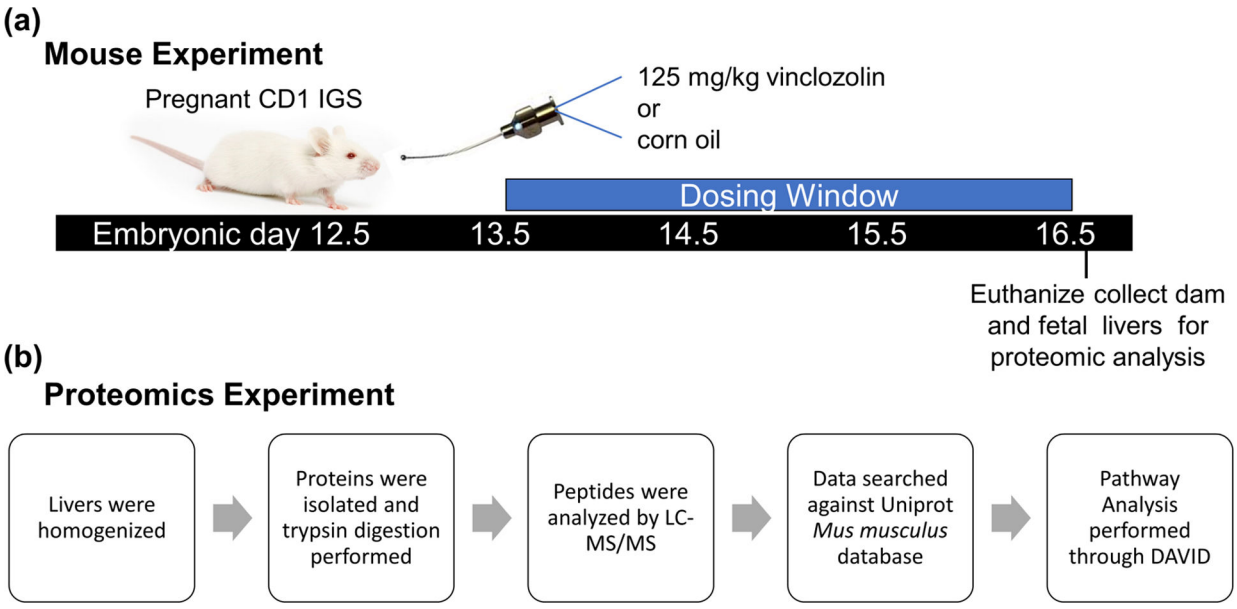

Figure 1.
Schematic for the animal experiment (a) and the proteomics experimental design (b).

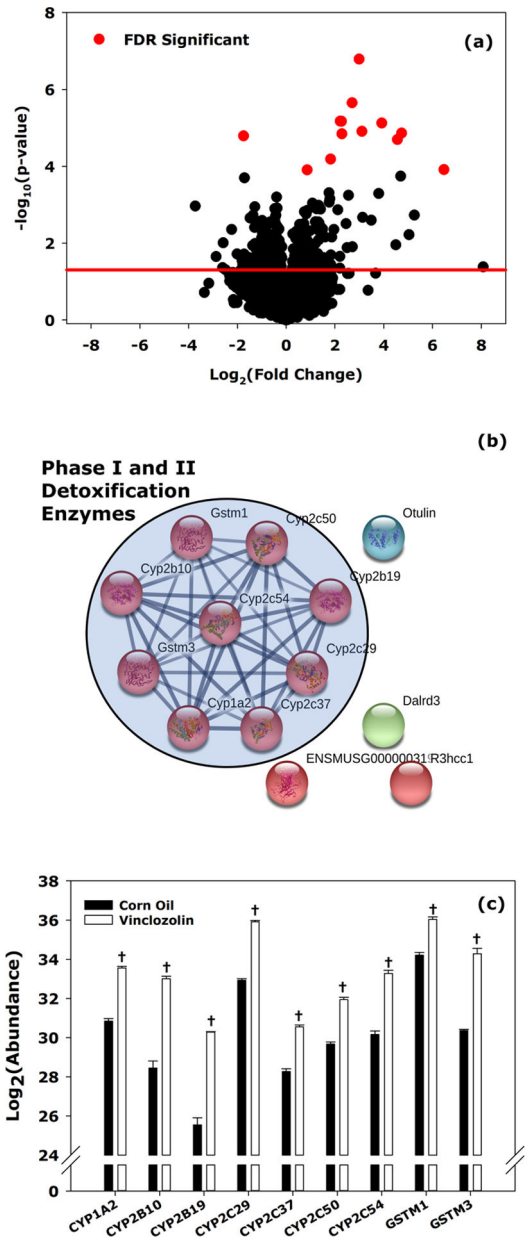

Figure 2.

Volcano plot of proteins from vinclozolin *vs.* control dam livers, where the red line represents a $p < 0.05$ and red circles represent false discovery rate significance (a). Network analysis of FDR significant proteins (b). Bar graph of the FDR significant proteins and their abundances with the error bars representing the standard error of the mean (c).

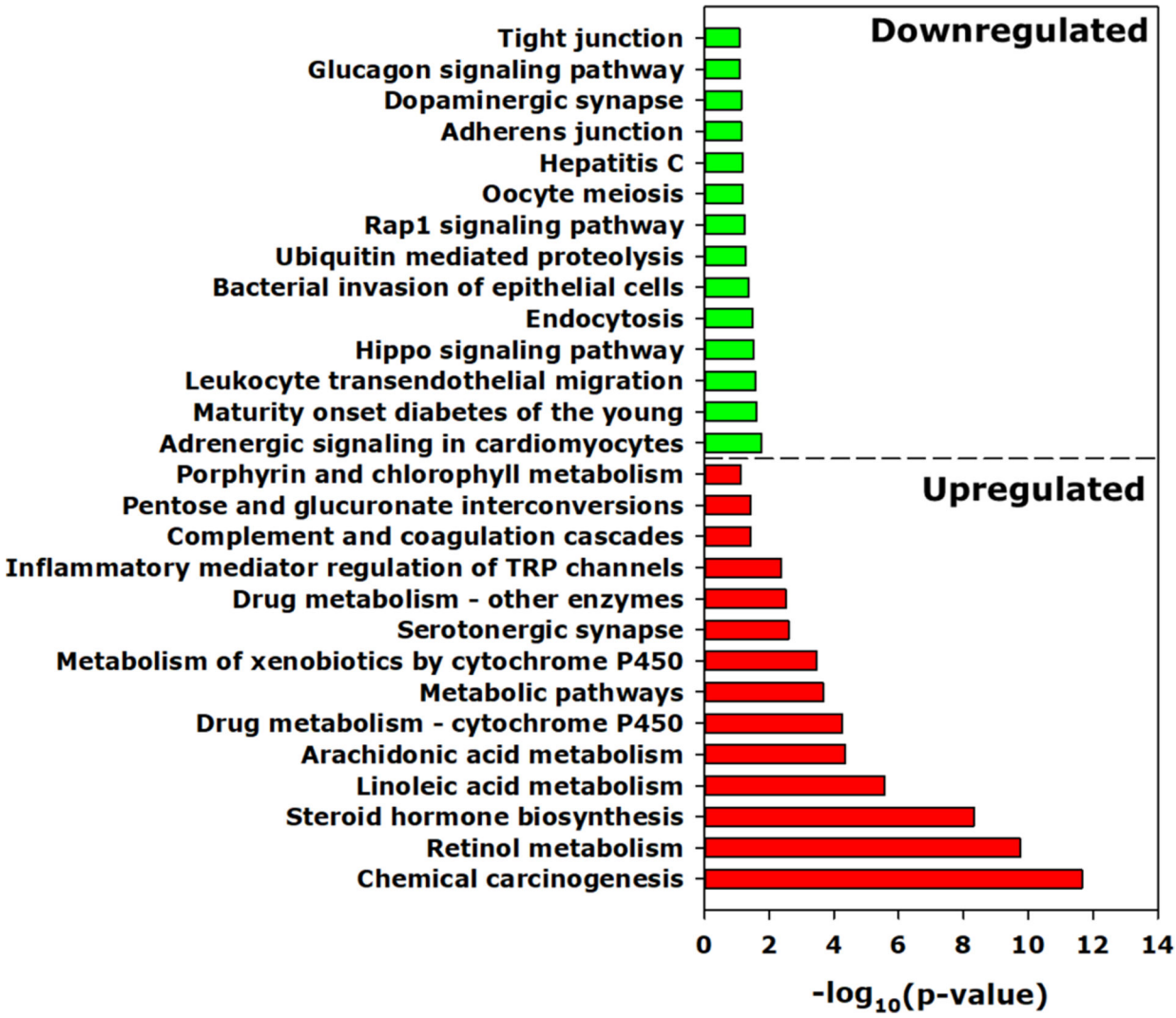

Figure 3.

Bar graph of the significantly enriched pathways from differentially expressed proteins between VCZ dam liver and CO dam liver, where downregulated (green) and upregulated (red) proteins in VCZ compared to CO are denoted.

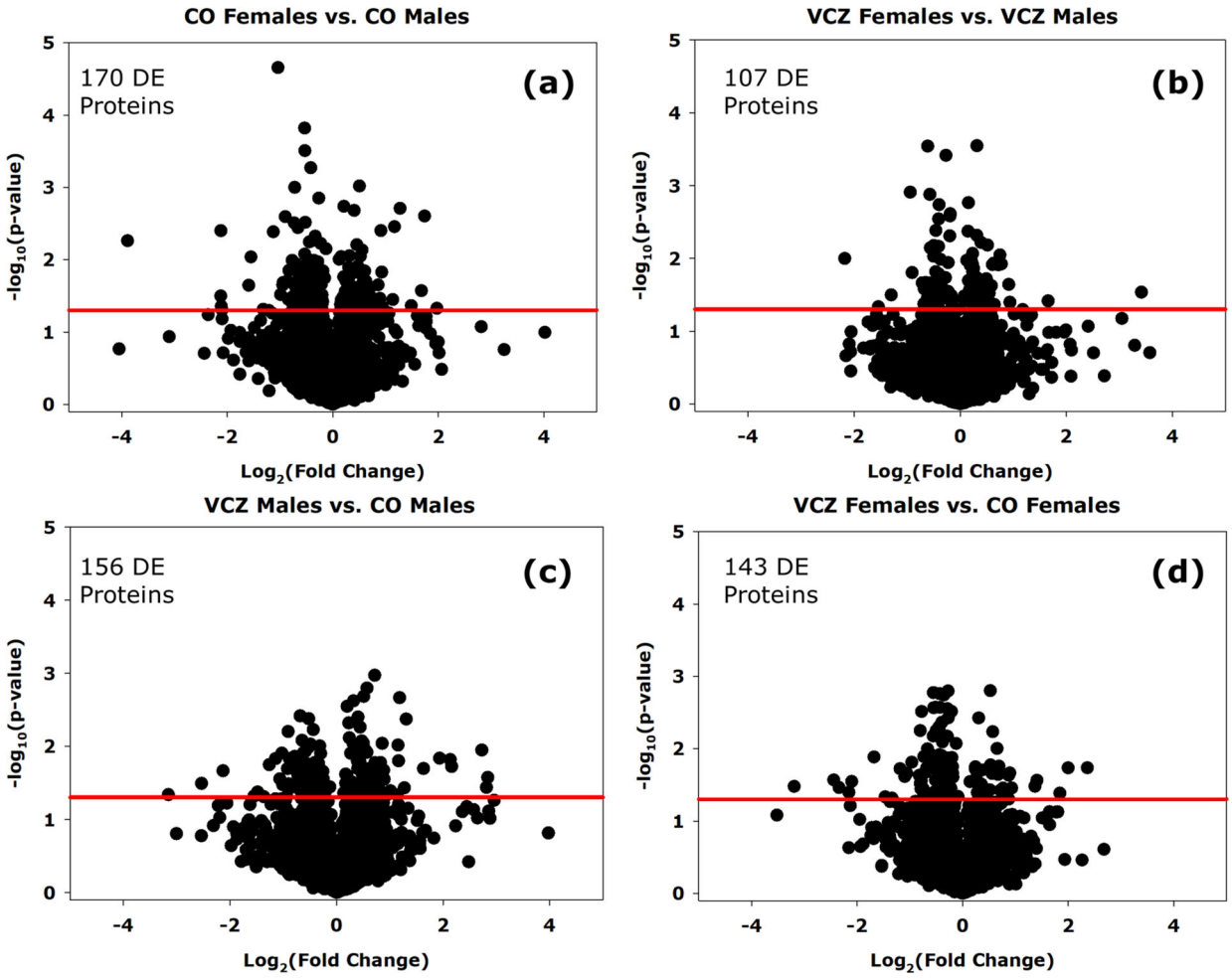

Figure 4.

Volcano plot of fetal liver proteins from control females *vs.* control males (a), vinclozolin females *vs.* vinclozolin males (b), vinclozolin males *vs.* control males (c), and vinclozolin females *vs.* control females (d), where the red line represents a $p < 0.05$. The total number of differentially expressed (DE) proteins with a p-value below 0.05.

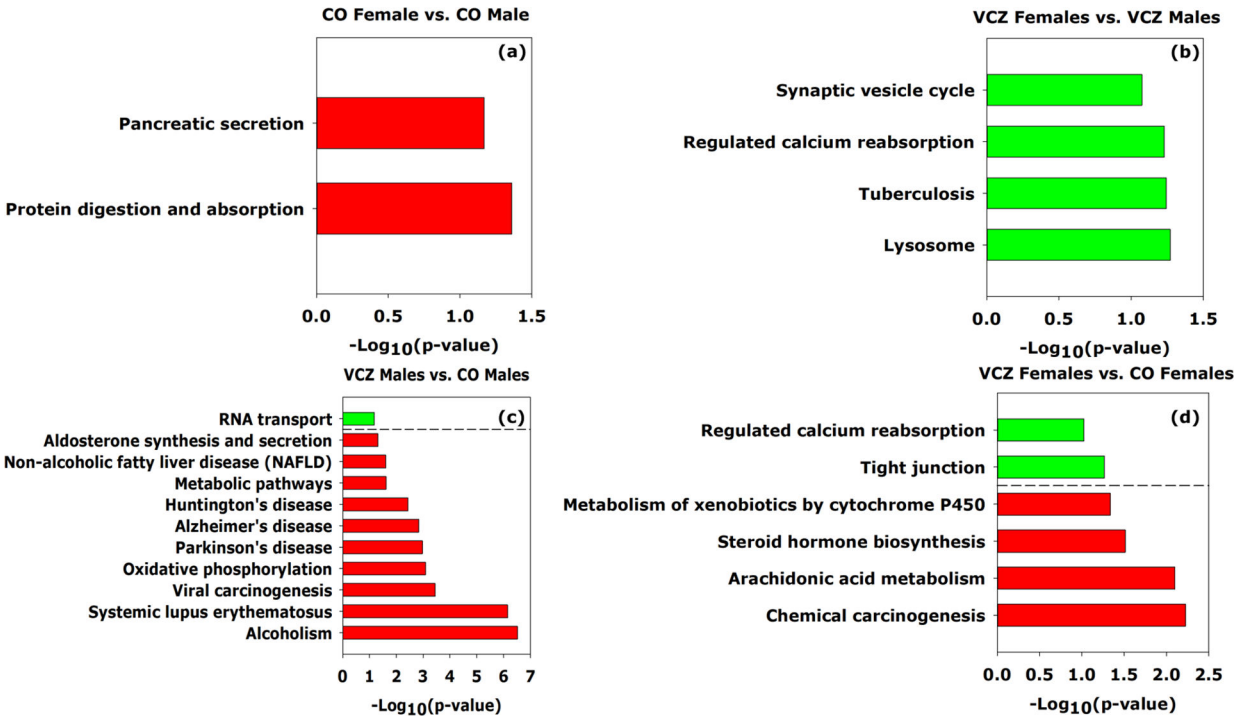

Figure 5.

Bar graph of the top 15 significantly enriched pathways and their p-values for control females *vs.* control males (a), vinclozolin females *vs.* vinclozolin males (b), vinclozolin males *vs.* control males (c), and vinclozolin females *vs.* control females (d). Significantly enriched proteins from upregulated proteins (red) and downregulated proteins (green) are shown separately with a dashed line between them if both are included on the same graph (c,d).

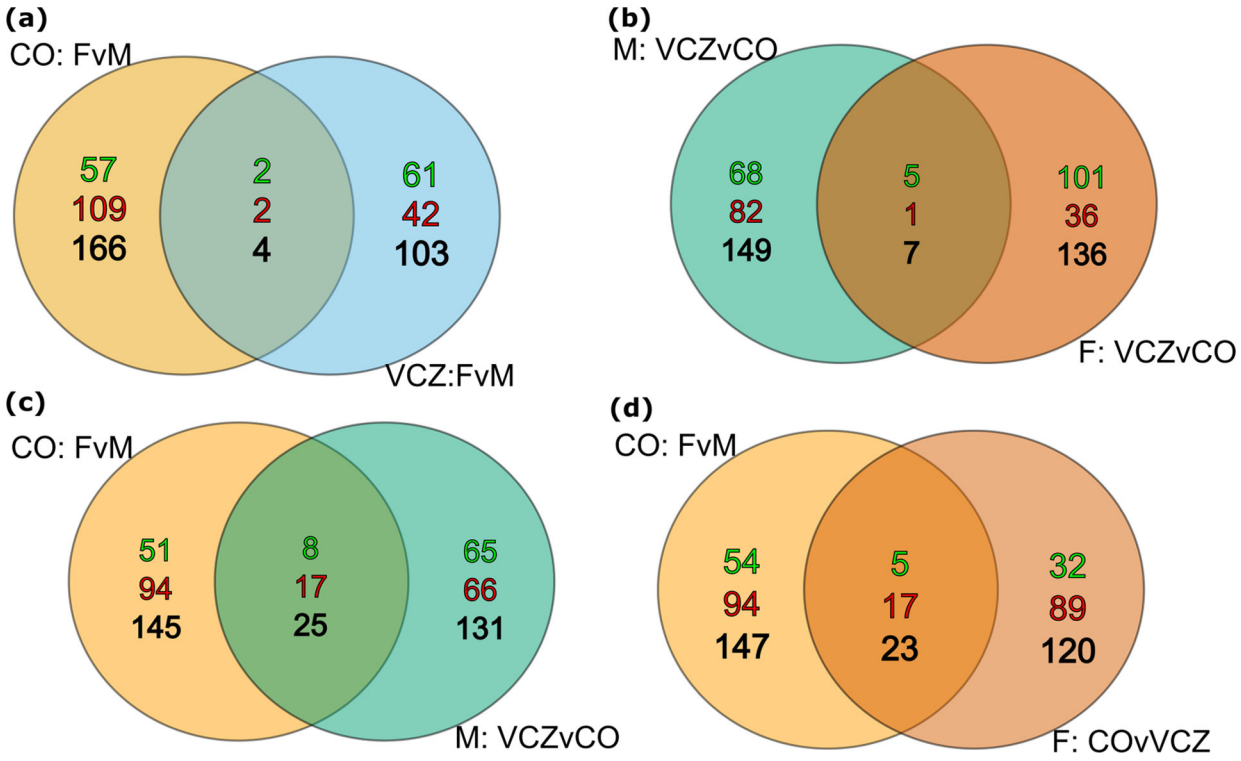

Figure 6.

Venn diagrams of proteins shared between different fetal liver comparisons: CO females *vs.* CO males/VCZ females *vs.* VCZ males (a), VCZ males *vs.* CO males/VCZ females *vs.* CO females (b), CO females *vs.* CO males/ VCZ males *vs.* CO males (c), and CO females *vs.* CO males/ CO females *vs.* VCZ females (d). The first number (green) represents proteins that are downregulated, the second number (red) represents proteins that are upregulated, and the third number (black) represents a comparison of a combined list of all proteins including those that were not affected by vinclozolin in the same direction.

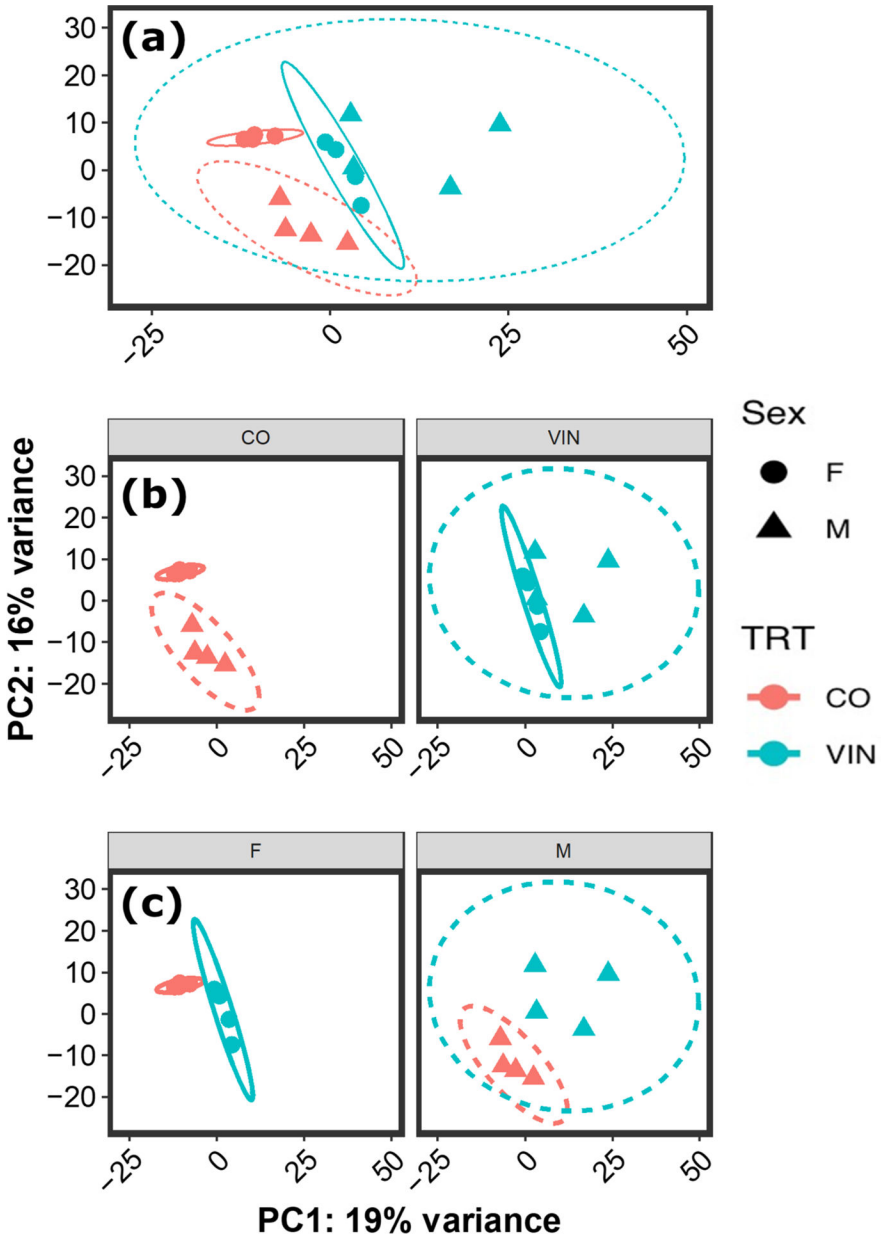

Figure 7.
Principal component analysis (PCA) score plots for fetal livers (a), comparison of sex (b), and comparison of treatment alone (c).

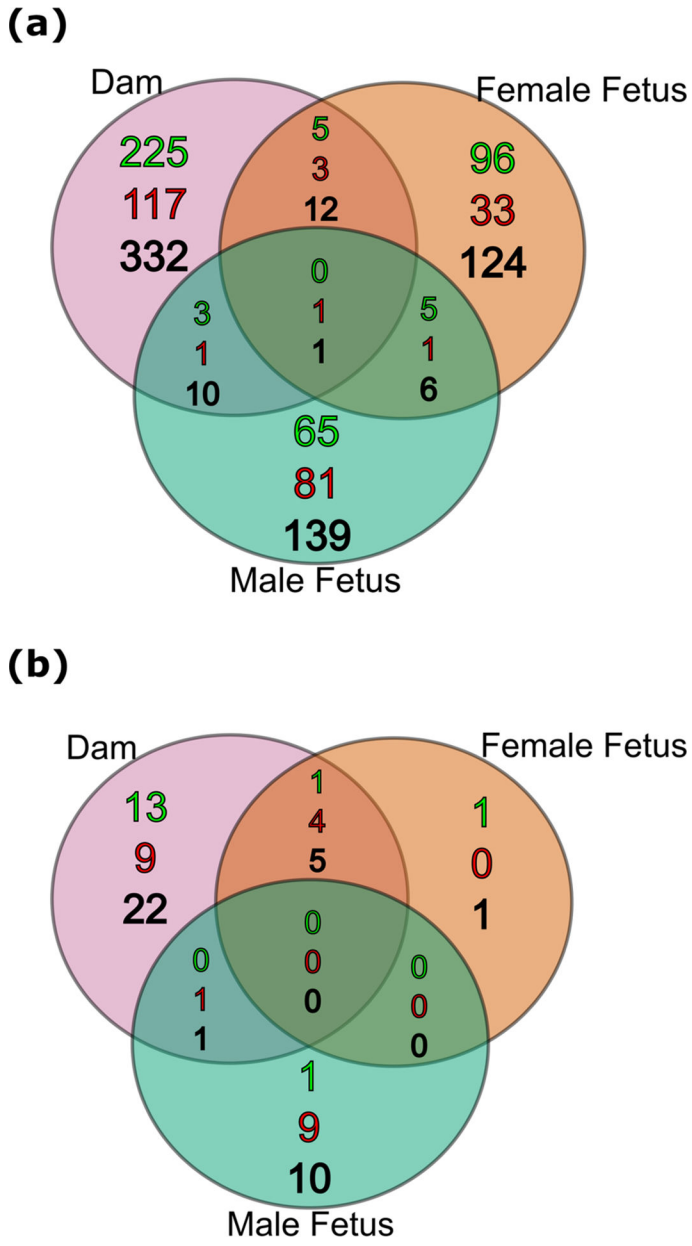

Figure 8.

Venn diagrams of proteins (a) and pathways (b) shared in the comparison of VCZ *vs.* CO between dam, female fetal, and male fetal livers. The first number (green) represents proteins that are downregulated, the second number (red) represents proteins that are upregulated, and the third number (black) represents a comparison of a combined list of all proteins including those that were not affected by vinclozolin in the same direction.