Maternal DHA supplementation influences sex-specific disruption of placental gene expression following early prenatal stress

Eldin Jašarević  
University of Maryland School of Medicine

Patrick M. Hecht  
University of Missouri

Kevin L. Fritsche  
University of Missouri

David C. Geary  
University of Missouri

Rocío M. Rivera  
University of Missouri

David Beversdorf  
beversdorfd@health.missouri.edu  
University of Missouri Columbia School of Medicine  
https://orcid.org/0000-0002-0298-0634

Research

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Abstract

**Background:** Early life adversity is a risk factor for early developmental perturbations and contributes to the presentation of neuropsychiatric disorders in adulthood. Neurodevelopmental disorders exhibit a strong sex-bias in susceptibility, presentation, onset and severity, although mechanisms conferring vulnerability are not well understood. Environmental perturbations during pregnancy, such as malnutrition or stress, have been associated with sex-specific reprogramming that contribute to increased disease risk in adulthood, whereby stress and nutritional insufficiency may be additive and further exacerbate poor offspring outcomes.

**Methods:** To determine whether maternal docosahexenoic acid (DHA) supplementation effects offspring outcome following exposure to early prenatal stress (EPS), dams were fed nutritionally complete semi-purified diets that either contained adequate essential omega-6 (n-6) and omega-3 (n-3) fatty acids from corn and soy oils (control diet, CTL) or an experimental diet that was nutritionally-equivalent to the CTL diet, but contained 1% by weight DHA, a long-chain omega-3 fatty acid (22:6n-3). Dams were administered chronic variable stress during the first week of pregnancy (Embryonic day, E0.5 – 7.5). Developmental milestones were assessed at E 12.5.

**Results:** Exposure to early prenatal stress (EPS) decreased placenta and embryo weight in males, but not females, exposed to CTL diet. DHA-enrichment reversed the sex-specific decrease in placenta and embryo weight following EPS. Early prenatal exposure upregulated expression of genes associated with oxygen and nutrient transport, including hypoxia inducible factor 3α (HIF3α), peroxisome proliferator-activated receptor alpha (PPARα), and insulin like growth binding factor 1 (IGFBP1), in placenta of CTL diet males exposed to EPS. DHA-enrichment in EPS-exposed animals abrogated male-specific upregulation of PPARα, HIF3α, and IGFBP1.

**Conclusions:** This suggests that maternal dietary DHA enrichment may buffer against maternal stress programming of sex-specific outcomes during early development.

Introduction

Environmental perturbations during pregnancy, such stress and malnutrition, are key risk factors for neurodevelopmental and neuropsychiatric disorders (Bale et al., 2010; Sapolsky, 2000). Epidemiological studies have shown that *in utero* exposure to infections, hypoxia, stress, and malnutrition during the first trimester predicted increased risk for schizophrenia in males, whereas chronic stress during the second and third trimester predicted a higher incidence of autism spectrum disorders in boys (Beversdorf et al., 2005; Khashan et al., 2008; Malaspina et al., 2008; Os and Selten, 1998). Indeed, nearly all neurodevelopmental disorders exhibit sex differences in the presentation, age of onset, and treatment outcome (Ober et al., 2008; Polyak et al., 2015). Parallel to epidemiological findings, exposure to chronic stress during the first week of pregnancy produced lasting effects in male offspring in rodent models of maternal stress experience (Bale et al., 2010; Dunn et al., 2011). While the exact mechanisms by which
environmental perturbations contribute to sex-specific vulnerability are not clear, mounting evidence suggests disruptions at the maternal-fetal interface (Bale, 2015; Nugent and Bale, 2015). As the metabolic and immune orchestrator between mother and offspring, the developing placenta is highly sensitive to availability of circulating nutrients and metabolites (Burton and Fowden, 2012; Fowden et al., 2006; Jansson and Powell, 2007; Nugent and Bale, 2015). Alterations to the maternal milieu during this critical window may fundamentally alter the structure and function of the placenta, resulting in downstream bottlenecks in placental nutrient and oxygen transport that ultimately contribute to elevated vulnerability and risk (Nugent and Bale, 2015). Sexually dimorphic patterns in energy substrate utilization have been observed during this window of early prenatal stress exposure, suggesting that the heightened male vulnerability to prenatal insults may be related to basal sex differences in metabolic requirements of male and female embryos (Bredbacka and Bredbacka, 1996; P et al., 2011). Further, the dynamic sex differences in placental transcriptomes may suggest that male and female placentas may respond to the same environmental insult through distinct pathways (Howerton et al., 2013; Howerton and Bale, 2014; Mao et al., 2010). Indeed, exposure to chronic variable stressors during the first week of gestation resulted in dysregulation of placental genes involved in fatty acid transport and glucose metabolic processes in male, but not female, placentas in a mouse model of early prenatal stress (EPS) (Bronson and Bale, 2014; Howerton et al., 2013; Mueller and Bale, 2008).

More recent work has demonstrated that maternal stress may influence the availability of nutrients essential for normal fetal neurodevelopment (Chiu et al., 2003; Coletta et al., 2010). Indeed, stress during pregnancy depletes maternal stores of polyunsaturated fatty acids (PUFAs) and decreases the amount of available PUFAs to the offspring (Chiu et al., 2003; Coletta et al., 2010). Moreover, maternal dietary deficiency of the omega-3 PUFAs, docosahexaenoic acid (DHA), is associated with reduced placental weight, increased inflammation and oxidative damage in the placenta, and long-term dysregulation of stress neurocircuity and behavior in offspring (Bhatia et al., 2011; Chiu et al., 2003; Coletta et al., 2010; D’Souza et al., 2013; Jones et al., 2013, 2014). Conversely, supplementation of maternal dietary DHA stimulates angiogenesis, resolves placental inflammation and oxidative stress, decreases pregnancy-associated risk, and improves behavioral outcomes in offspring (Fontani et al., 2005; Jones et al., 2014; Khaire et al., 2015; Le-Niculescu et al., 2011; Richardson, 2006). Taken together, maternal supplementation of dietary DHA may influence key pathways that are vulnerable to disruption by maternal stress experience during pregnancy.

Therefore, to examine the potential interaction between maternal stress and diet on sex-specific expression of genes involved in placental metabolic pathways, we used a mouse model of early prenatal stress (EPS), in which male, but not female, offspring demonstrate significant placental reprogramming, as well as increased stress sensitivity, cognitive dysfunction, and metabolic status in adulthood (Mueller and Bale, 2008). As maternal stress experience during pregnancy is associated with depleted maternal DHA stores, increased placental inflammation, and decreased DHA accumulation in the fetal brain, supplementation of dietary DHA to the dam may resolve stress effects on the placenta in a sex-specific manner. To examine this hypothesis, candidate genes involved in nutrient signaling and altered by EPS in a sex-specific manner were assessed in embryonic day (E) 12.5 placentas, with and without DHA.
supplementation (Mueller and Bale, 2008). In addition, to determine whether EPS and maternal dietary DHA supplementation exhibits an effect on early brain development, expression of the major neuroplasticity factors, brain derived neurotrophic factor (BDNF) and cyclic AMP response element-binding (CREB) protein were assessed in embryonic day 12.5 fetal heads, a period of rapid neurodevelopment that is particularly sensitive to maternal stress and nutrient availability (Bouret, 2010).

Materials And Methods

2.1. Animals

Sixty 6–8 week old C57BL/6J females (P0 females) were purchased from Jackson Laboratories (Bar Harbor, Maine) and fed the control diet (detailed below) for at least two weeks while the mice habituated to the vivarium. Following habituation, animals were randomly placed on one of two diets: (i) the control diet (CTL, n = 20) or (ii) the experimental diet that contained 1.0% by weight DHA (1.0% DHA, n = 20). Additional detail on the experimental diets are provided below. For all experiments, the litter was the experimental unit such that only one male and one female were used for subsequent analysis.

Animals were housed in clear polycarbonate cages (32 cm x 18 cm x 24 cm) provided with aspen bedding and a nestlet, and maintained under standard conditions (25 ± 2 °C and 50% ± 10% humidity), with ad libitum access to water provided in glass bottles and diet specific to each treatment group, and on 12:12 hr light cycle with lights on at 0600 CST. All experiments were approved by University of Missouri Animal Care and Use Committee and performed in accordance with National Institutes of Health Animal Care and Use Guidelines.

2.3. Diet composition

Our rodent diets started with the AIN-93G purified-diet (Dyets Inc., #110700) as the base. This base diet was modified slightly by substituting some soybean oil with corn oil in order to match the average n-6:n-3 ratio of 10:1 for individuals consuming a Western-type diet (Simopoulos, 2011; Blasbalg et al., 2011; Daniel et al., 2009; Sun et al., 2007). This modified AIN93G diet (Dyets Inc., #103619) served as the control (CTL) and as the base for the experimental DHA diets. The CTL diet contained no preformed DHA but did contain sufficient amounts of alpha-linolenic acid (ALA,18:3n-3) to meet normal brain DHA requirements (Domenichiello et al., 2015). An experimental diet was formulated to contain ~1% by weight DHA, while keeping caloric density constant as well as the concentrations of macro- and micro-nutrients. To accomplish this, we substituted a mixture of a DHA-enriched algal oil (i.e., life’sDHA™, a generous donation from DSM, Nutritional Products LLC Columbia, MD, USA) and safflower oil (i.e., a 25:45 ratio) for the corn oil and soy oil mixture used in the control diet. Our primary objectives of selecting the mixture of safflower oil and algal oil in the “experimental” diet was to maintain similar levels of the omega-6 essential fatty acid, linoleic acid (18:2n-6), while introducing DHA at a nutritionally-relevant level. Traditional diets of Greenland Inuits and some Japanese provide levels of long-chain omega-3 fatty acids (e.g., DHA) at levels comparable to that in our 1% DHA mouse diet (Meyer, 2011). Table 1 provides a detailed fatty acid profile of the two diets. Both diets were stabilized against auto-oxidation by the
addition of a synthetic antioxidant (i.e., 0.02 g tertiary-butylhydroquinone/100 g fat) (Irons and Fritsche, 2005).

2.3. Diet exposure protocol

Grain-based chow diets that are often utilized as gold-standard laboratory chow diets show significant batch-to-batch variability and formulations differ between manufacturers (Pellizzon and Ricci, 2018; Ricci, 2013). Given that central nervous system and systemic DHA levels are largely driven by dietary PUFA availability, variability in the source, amount and composition of dietary fatty acids in these chow diet formulations may represent a potential confounder of study outcomes, particularly those involving central nervous system development and function (Lardinois et al., 1989). To control for potential differences in the composition of dietary fatty acids that may result in physiologically relevant differences in omega-3 PUFA status, a multigenerational diet exposure protocol was applied to stabilize omega-3 PUFA levels and maintain the same levels across generations (Jašarević et al., 2014). The founding population of females (i.e., P0) transitioned from a chow diet to either CTL or DHA-enriched diet. P0 females were mated with males consuming the CTL diet and females were single housed upon detecting a mating plug. Females remained on the assigned diet through gestation and lactation, and F1 offspring remained on the same maternal diet. Similar to the P0 breeding scheme, F1 females were mated with CTL-fed males, remained on the assigned diet during gestation and lactation, and F2 offspring were maintained the same diet as their mothers. To generate the F3 embryonic day (E)12.5 offspring used in the present study, F2 dams were mated to males consuming the CTL diet.

2.4. Early prenatal stress

A chronic variable stress procedure was administered during gestational day 0.5–7.5 to F2 dams consuming either the CTL or DHA-enriched diets (early prenatal stress, EPS; n = 4 CTL diet, n = 4 1.0% DHA diet) for comparison to a control non-stressed (n = 3 CTL diet, n = 3 1.0% DHA diet) group. On detection of mating plug (e.g., E 0.5), pregnant mice assigned to the EPS group experienced each of the following stressors on a different day of the EPS period, as previously described (Mueller and Bale, 2008): 60 min (beginning at 11:00 AM) of fox odor exposure (1:5,000 2,4,5-trimethylthiazole; Acros Organics), 15 min of restraint (beginning at 11:00 AM) in a mouse restraint tube, 36 h of constant light, novel noise (White Noise/Nature Sound-Sleep Machine; Brookstone) overnight, three cage changes (at 7:00 AM, 1:00 PM, and 5:00 PM) throughout the light cycle, overnight exposure to a novel object (twelve marbles of similar size), and saturated bedding (700 mL, 23 °C water) overnight. These stressors were previously shown to be non-habituating and did not influence maternal food intake (Mueller and Bale, 2008). Together with the diet groups, there were four different groups in this study: 1) No EPS offspring exposed to the CTL diet (CTL-CTL); 2) EPS offspring exposed to the CTL diet (EPS-CTL); 3) No EPS offspring exposed to the 1.0% DHA diet (CTL-DHA); and 3) EPS offspring exposed the 1.0% DHA diet (EPS-DHA).

2.5. Mouse tissue dissection

On gestational day 12.5 pregnant dams were weighed and rapidly decapitated by cervical dislocation. Litter characteristics such as intrauterine position, number of offspring, sex ratio, and resorption sites
were noted. Corpora lutea from the left and right ovaries were collected and placed into ice-cold buffer, and total number of corpora lutea were counted. Fetal loss was calculated as the difference between number of corpora lutea and number of offspring.

The gravid uterus was transferred to a plastic culture dish containing ice cold 1 x PBS and the dish was placed on ice. Embryo sites were dissected to separate individual embryos, which were placed in ice-cold 1x PBS filled wells to preserve RNA, DNA, and protein integrity. The amnion sac was removed, and placenta was removed from the fetus. Tail snips from embryos were collected and used to identify sex of individual embryos by RT-PCR, using primers specific for Sry (5' -GAGTACAGGTGTCGAGCTCTA-3' and 5' -CAGCCCTACAGGCCATGAT-3'), as previously reported (Howerton and Bale, 2014). Thermal cycling conditions were 98 °C for 30 sec; 54 °C for 40 sec; 72 °C for 50 sec for 30 cycles. Embryo and placental samples were rapidly frozen in liquid nitrogen and maintained in -80°C until RNA isolation. In order to control for the significant contribution of uterine horn laterality (e.g., weight differences due to implantation in the left vs. right uterine horn) and intrauterine position, an adjacent pair of male and female conceptuses with no overt signs of developmental delay (e.g., small size relative to litters and not neighboring a resorption site) in the left uterine horn were selected for subsequent analyses.

2.6. Embryonic day 12.5 placental and fetal head RNA isolation and Quantitative real time PCR

Following sex determination of the placentas, RNA was isolated exactly as previously described by us (Chen et al., 2013). Candidate genes were chosen based on previous reports of sex-specific dysregulation of these placental genes following EPS (Howerton et al., 2013; Mueller and Bale, 2008). Changes in hypoxia inducible factor 3a (Mm00469375_m1; NM_001162950.1), O-GlcNAc transferase (Mm00507317_m1; NM_139144.3), vascular endothelial growth factor A (Mm01281449_m1; NM_001025250.3), insulin-like growth factor binding protein 1 (Mm00515154_m1; NM_008341.4), glucose transporter 4 (Mm01245502_m1; NM_009294.2), and peroxisome proliferator-activated receptor alpha (Mm00440939_m1; NM_001113418.1). Tissue samples were analyzed in at least triplicates with a critical threshold standard deviation of 0.5 within each triplicate. The threshold cycle was normalized to the housekeeping gene β-actin (Actb; NM_007393.3; Mm00607939_s1) using an ABI Real-time 7500 system (Applied Biosystems, Waltham, MA). The expression level for each gene in each tissue was calculated using the comparative CT method. In brief, the cycle number at threshold was used for calculations of relative amount of mRNA molecules. The CT value of each target gene was normalized by subtraction of the CT value from β-actin. This value is defined as the ΔCT. While it is likely that EPS and control animals within a diet are likely to differ, the central hypothesis of the present study is that maternal DHA-enrichment will buffer EPS-related programing relative to EPS animals exposed to a control diet. As a result, relative quantitative change was calculated using the formula $2^{-\left(\Delta CT_{EPSCTL} - \Delta CT_{EPSDHA}\right)}$ (n = 3–5 mice/sex/diet/stress).

2.7. Statistical analysis
Litter characteristics data is presented as (± SD), while data on placenta and fetal weight are compared across conditions and qPCR data is presented as mean (± SEM) and analyzed within the R environment using nlme and lattice packages (Pinheiro, 2009; R Core Team, 2018; Sarkar, 2008). Litter characteristics, placenta and embryo weights, and qPCR results from the placenta and embryonic tissue samples were analyzed with a 2 (stress) x 2 (sex) x 2 (diet) ANOVA. Tukey’s HSD post-hoc comparison was used for all group-level contrasts.

Results

3.1. Effect of EPS and maternal diet on litter characteristics

To determine whether prenatal stress, DHA enrichment, or its interaction alter litter characteristics, we assessed for the combined treatment effects on litter size, fetal loss, and sex ratio. Table 2 shows litter characteristics. An independent t-test revealed no difference in fetal loss (e.g., difference between corpora lutea and number of offspring present at time of collection) between EPS (M = 4, SD = 3.08) and non-EPS dams (M = 1, SD = 1) consuming the CTL diet; t(6) = -1.59, p = 0.16. Similarly, there was no difference in fetal loss between EPS (M = 0.50, SD = 1.0) and non-EPS dams (M = 0, SD = 0) consuming the DHA-enriched diet; t(6) = -0.845, p = 0.44. There was, however, a main effect of diet on fetal loss (F[1,11] = 6.40, p = 0.028), with CTL diet females exhibiting higher fetal loss than DHA-enriched diet females (p = 0.041), but the main effect of EPS and the diet x EPS interaction were not significant (ps = 0.11 and 0.26, respectively). No other litter characteristics were different across diet or stress treatment.

The effect of maternal diet and stress on placenta and embryo weight was then determined at E12.5. While there was no main effect of sex on placental weight (p = 0.11), there was a main effect of diet (F[1,22] = 7.56, p = 0.012) and a sex x EPS interaction (F[1,22] = 3.95, p = 0.0505); neither the diet x EPS (p = 0.28) nor sex x diet x EPS interaction was significant (p = 0.14). The DHA-enriched diet increased placental weight compared with CTL diet placentas (p = 0.012), and male placentas exposed to early prenatal stress weighed less than stress-exposed female placentas (p = 0.0526). Given the latter results and the diet effect, we examined the diet x EPS pairwise comparisons for males, which revealed one significant effect; with EPS, DHA-enriched diet increased placental weight relative to CTL (p = 0.0493). No other pairwise comparisons were significant.

There was a main effect of diet (F[1,22] = 5.26, p = 0.032) and EPS (F[1,22] = 8.03, p = 0.009) on embryo weight, but there was no main effect of sex (p = 0.51) or any significant interactions; diet x EPS (p = 0.16), sex x EPS (p = 0.78), diet x sex x EPS (p = 0.42). DHA-enriched embryos weighed more than CTL diet embryos (p = 0.032), and EPS decreased embryo weight (p = 0.009). Although the diet x EPS interaction was not significant, post-hoc contrasts revealed that exposure to EPS decreased embryo weight in animals exposed to the CTL diet relative to non-stressed animals consuming the same diet (p = 0.051), whereas no such difference emerged in the DHA diet animals (p = 0.926). No other pairwise comparisons were significant.
3.1. Effect of EPS and maternal diet on placental gene expression

To determine whether DHA-enrichment impacts EPS sex-specific disruption, control and EPS E12.5 placentas exposed to the diets were examined using a series of candidate genes; specifically, genes that have been previously shown to exhibit male-specific disruption in placental gene expression and are associated with increase disease risk in male in adulthood.

There was a diet x sex interaction of peroxisome proliferator-activated receptor alpha (PPARA) expression ($F_{1,10} = 12.34, p = 0.006$) (Fig. 1A). PPARA expression was upregulated in EPS-CTL diet male placentas relative to EPS-CTL female placentas ($p = 0.042$), but there was no sex difference in PPARA transcription in EPS-DHA diet placentas ($p = 0.36$). PPARA expression did not differ across EPS female groups ($p = 0.35$). Surprisingly, PPARA was downregulated in EPS-DHA diet male placentas relative to EPS-CTL diet male placentas ($p = 0.038$), which accounts for the lack of a difference comparing EPS-DHA diet male and female placentas.

There was a diet x sex interaction in expression of Insulin-like growth factor-binding protein1 (IGFBP1) in the placenta ($F_{1,10} = 6.17, p = 0.032$) (Fig. 1B). The interaction emerged because IGFBP1 was upregulated in EPS-DHA females and upregulated in EPS-CLT males; no pairwise contrasts were significant. There was a diet x sex interaction on hypoxia inducible factor 3a (HIF3α) expression ($F_{2,14} = 10.93, p = 0.008$) (Fig. 1C). HIF3α expression was upregulated in EPS-CTL diet male placentas relative to EPS-CTL diet female placentas ($p = 0.05$), but there was no sex difference in HIF3α expression in EPS placentas exposed to a DHA-enriched diet ($p = 0.41$). HIF3α expression did not differ across the EPS female groups ($p = 0.34$), but there was a trend for upregulation of HIF3α expression in EPS-CTL male placenta relative to EPS-DHA male placenta ($p = 0.062$).

Expression of O-linked N-acetylglucosamine transferase (OGT) was not related to sex ($p = 0.16$) or diet ($p = 0.76$) or the interaction ($p = 0.36$) (Fig. 1D). Expression of glucose transporter 4 (GLUT4) in the placenta was not related to sex ($p = 0.71$), diet ($p = 0.22$), or the interaction ($p = 0.13$) (Fig. 1E). There was a main effect of sex on expression of vascular endothelial growth factor A (VEGFA) ($F_{2,14} = 4.34, p = 0.053$), whereby females exhibited upregulation compared with males (Fig. 1F). Neither the main effect of diet ($p = 0.58$) nor the sex x diet interaction ($p = 0.68$) was significant.

Analysis of BDNF and CREB in E12.5 fetal heads

Expression of brain derived neurotrophic factor (BDNF) was not related to sex ($p = 0.16$) or EPS ($p = 0.73$) or its interaction ($p = 0.48$). Expression of cAMP response-binding element protein (CREB) was not related to sex ($p = 0.30$) or EPS ($p = 0.36$) or its interaction ($p = 0.31$). Based on the lack of differences between EPS and non-EPS groups exposed to the CTL diet, we did not measure BDNF and CREB expression in DHA diet cohorts.
Discussion

This study yielded two important results regarding the interaction between maternal stress and dietary DHA enrichment in embryonic day 12.5 conceptuses. First, maternal stress during the first week of gestation showed a marked influence on the composition of the litter and gene expression patterns in the placenta, with offspring sex largely determining the magnitude of disruption. Second, a maternal diet enriched with preformed DHA during periods of high stress shows partial rescue of stress-dependent dysregulation of gene expression in the placenta.

The observation that the placenta responds to maternal diet is not surprising given its specialized metabolic niche that is particularly sensitive to maternal resource availability. Maternal malnutrition during pregnancy, presumably through constrained exchange of maternal nutrients across the placenta, exerts long-term changes in morbidity and mortality, growth trajectory, and increased disease risk in adulthood (Waterland and Garza, 1999). Maternal diet also appears to exhibit contrasting programing on the placental transcriptome based on offspring sex, consistent with maternal diet influencing resource exchange through the placenta in a sex-specific manner (Mao et al., 2010).

In the present study, maternal diet was associated with some aspects of growth and development. Mothers consuming the DHA-enriched diet exhibited lower fetal loss relative to offspring of CTL-diet mothers, suggesting that the availability of DHA in maternal diet may be critical to fetal development at this development window. Although there were no sex differences in placenta weight within the same maternal diet, DHA-enrichment increased male placenta weight relative to that of males in the CTL-diet group. Previous reports have shown that placenta weight is associated with offspring size, which may be attributed to differential resource demand for growth-related nutrients and downstream consequences on newborn size (Godfrey et al., 1996). Indeed, DHA-enrichment increased embryo weight at E12.5 relative to offspring exposed to CTL diet, and this effect was independent number of offspring given no diet-related differences in litter size. These results must be interpreted with caution, however, as high rates of infanticide have been reported for the C57Bl/6J strain used in this study, and, as a result, increased offspring size at E12.5 may not reflect litter size or survival rates following birth (Brown et al., 1999).

Similar to maternal nutrition status, maternal stress during pregnancy has direct consequences on offspring growth and development in humans, and later phenotypic outcomes in adulthood across multiple species, including increased risk for neurodevelopmental and neuropsychiatric disorders in humans (Bale, 2015). Consistent with previous results, we observed that maternal stress trended toward reduction in male placental weight, with no effect of prenatal stress on female placenta weight (Mairesse et al., 2007). To assess the potential factors involved in the sex differences in placenta and embryo size, we measured the expression of placental genes previously reported to be sensitive to sex-specific disruption following EPS. Similar to previous reports, EPS resulted in male-specific upregulation of placental HIF3α and PPARα, with no effect on females (Mueller and Bale, 2008). However, there was no sex or EPS-related difference in expression of OGT, IGFBP1 or GLUT4 (Howerton et al., 2013; Mueller and Bale, 2008). The discrepancy between previous and current study is likely related to the differences in
genetic background of mouse strains (C57Bl/6J vs. mixed background of C57Bl/6J*129), which exhibit differential sensitivity to stress (Chan et al., 2017). DHA-enrichment exhibited no effect on placental expression in either EPS-exposed or control females, further suggesting male-specific vulnerability to early prenatal stress may be buffered by maternal diet.

Based on the observation that maternal diet and maternal stress result in similar sex-specific changes to placenta and embryo size, we examined whether maternal diet and maternal stress converge upon similar transcriptional pathways. DHA-enrichment reversed the EPS induced male-specific upregulation of placental HIF3α and PPARA back to comparable levels of males that were not exposed to early prenatal stress. Low oxygen conditions activate a cascade of physiological response that includes the upregulation of large class of hypoxia inducible factor (Forristal et al., 2010; Lee et al., 2004; Semenza, 2000). HIF proteins control a broad family of genes, including VEGFA, a canonical regulator of angiogenesis that is highly sensitive to hypoxic conditions (Forristal et al., 2010; Lee et al., 2004; Semenza, 2000). EPS increased expression of HIF3α but did not result in a parallel EPS-dependent upregulation of VEGFA. The inability of HIF3α to increase expression of VEGFA may be related to the unique structural properties of HIF3α. In contrast to family protein members HIF1 and HIF2, HIF3α lacks a C-terminal activation domain required for co-activator binding, and, as a result, is unable to recruit co-transcriptional regulators and basal transcriptional machinery to gene targets, including VEGFA (Forristal et al., 2010; Lee et al., 2004; Semenza, 2000).

An alternative interpretation to the finding that EPS induces male-specific reprogramming of candidate genes in the placenta would be that this represents a sex-specific adaptation to increase resources transfer during periods of stress, and, as a result, DHA-enrichment is hampering this adaptation (Burton and Fowden, 2012; Díaz et al., 2014). Such an alternative hypothesis would predict either no difference in placenta and embryo weight for EPS and non-stress animals exposed to the CTL diet or a negative effect of DHA diet on placenta and embryo weight. The present results show the opposite trend: DHA-enrichment increases placenta and embryo weight while concomitantly decreasing expression of genes that are normally expressed in low oxygen or constrained nutrient conditions.

Nevertheless, the present results can be readily understood within maternal life history trade-offs (Stearns, 1989). During pregnancy, mothers require resources to meet both maternal and offspring requirements. However, environmental cues, such as malnutrition and stress, may decrease the optimal resource transfer from mothers to offspring in a way that maximizes maternal reproductive success at the detriment to offspring. Sex-specific reductions in male, but not female, placenta and embryo in EPS exposed animals are consistent with reduction of maternal investment in male relative to female offspring. From an evolutionary perspective, the shift in maternal resource allocation may be related to sex differences in reproductive payoffs for mothers; specifically, smaller, less fit males are less likely to reproduce as adults than their sisters (Trivers, 1974). Indeed, rescue of this sex-specific vulnerability with dietary DHA-enrichment may suggests that EPS-dependent deficiency of nutrient and oxygen transfer may trigger this shift in maternal investment. The goal of future studies should focus on whether DHA-enrichment buffers from prenatal-stress dependent deficits in adulthood and on identifying sex- and diet-
dependent broad programmatic pathways following exposure to prenatal stress by leveraging whole transcriptome profiling approaches. Moreover, determining the possibility that DHA-enrichment may buffer from stress that extend across pregnancy and later developmental windows is a key avenue for future research.

**Declarations**

**Ethical Approval and Consent to Participate.**

All experiments were approved by the University of Missouri – Columbia Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Animal Care and Use Guidelines.

**Consent for publication**

Not applicable.

**Availability of data and materials**

No custom code or software was used for the analysis discussed in this manuscript. All data is available upon request of the senior author.

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**Contributions**

EJ, DCG and DQB conceived and designed the study. EJ conducted all experiments. PMH assisted with sample isolation. RMR carried out the ovarian structure dissection and quantification, assisted with the training, isolation, analysis and interpretation of RNA data. EJ and RMR performed analysis. KLF provided diet formulations and developed custom diets. EJ, DCG and DQB wrote the manuscript, with input from RMR and PMH, and with KLF providing the details regarding the custom diet and the rationale. All authors read and approved the final version of the manuscript.

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Competing interests

The authors declare that they have no competing interests.

Abbreviations

EPS = Early Prenatal Stress

CTL = Control

DHA = docosahexaenoic acid

HIF3α = Hypoxic Factor 3α

PPARA = Peroxisome Proliferator Activated Receptor Alpha

VEGFA = Vascular Endothelial Growth Factor A

GLUT4 = Glucose Transporter Type 4

BDNF = Brain Derived Neurotrophic Factor

CREB = cyclic AMP response element-binding protein

SRY = Testis Determining Factor

ACTB = Actin B

E12.5 = embryonic day 12.5

CT = Cycle Threshold

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**Tables**

**Table 1.** Fatty acid composition of diets
| Fatty acid | Standard diet | DHA-enriched diet |
|------------|---------------|-------------------|
| 16:0       | 0.77          | 0.39              |
| 18:0       | 0.24          | 0.16              |
| 18:1       | 1.76          | 1.00              |
| 18:2n6     | 3.86          | 4.01              |
| 18:3n3     | 0.37          | 0.02              |
| 20:5n3     | nd            | 0.11              |
| 22:5n3     | nd            | 0.26              |
| 22:6n3     | nd            | 1.01              |
| Ratio n-6/n-3 | 10.4  | 2.9               |

Note: nd = not detectable.

**Table 2.** Maternal and Litter characteristics by Diet and Early Prenatal Stress

| Dams       | Litter Size | Males       | Females     | Corpora Lutea | Fetal* Loss | Resorptions | Sex Ratio |
|------------|-------------|-------------|-------------|---------------|-------------|-------------|-----------|
| CTL – No EPS | 7 ± 1.63 | 4.66 ± 1.25 | 2.33 ± 0.47 | 8 ± 1.41 | 1 ± 0.82 | 1 ± 1.414 | 0.66      |
| CTL – EPS  | 5 ± 2.53 | 2.8 ± 1.33  | 2.2 ± 1.72  | 9 ± 2.76 | 4 ± 2.76 | 1.6 ± 0.8  | 0.64      |
| DHA – No EPS | 7.66 ± 0.47 | 4.33 ± 1.25 | 3.33 ± 0.94 | 7.66 ± 0.47 | 0 | 0.333 ± 0.47 | 0.56      |
| DHA – EPS  | 7.5 ± 0.5  | 5.25 ± 0.43 | 2.25 ± 0.83 | 8 ± 0.71 | 0.5 ± 0.87 | 0.5 ± 0.87 | 0.71      |

Data presented as Mean ±SD. Fetal loss was quantified as the difference between litter size and number of corpus lutea. * denotes a significant main effect of diet on fetal loss ($p = 0.041$), but diet*EPS interaction was not significant. Please refer to *Effect of EPS and maternal diet on litter characteristics in main text.*
Figure 1

Effects of DHA enrichment and EPS on expression of genes critical nutrient transport in male (■) and female (□) placentas. Relative expression with DHA enrichment is illustrated as a ratio to expression in CTL, and sex effects are compared. A sex-dependent dysregulation was observed with CTL diet male placentas exposed to EPS exhibiting upregulation in HIF3a and PPARα, but not females. Neither EPS, sex nor diet exhibited an effect on placental gene expression of IGFBP1, GLUT4, OGT, VEGFα, (see Results for further details). n = 3 – 5 animals/sex/diet/stress treatment (** = p<0.05 for male vs female, * = p<0.05 for DHA enriched vs control diet, # = p<0.10 for DHA enriched vs control diet).