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The Effects of Omega-3 PUFA Infusions During Late Gestation on Developmental Pathologies in the Intrauterine Growth Restricted Fetus

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THE EFFECTS OF OMEGA-3 PUFA INFUSIONS DURING LATE GESTATION ON DEVELOPMENTAL PATHOLOGIES IN THE INTRAUTERINE GROWTH RESTRICTED FETUS

by

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THE EFFECTS OF OMEGA-3 PUFA INFUSIONS DURING LATE GESTATION ON DEVELOPMENTAL PATHOLOGIES IN THE INTRAUTERINE GROWTH RESTRICTED FETUS

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Low birthweight due to intrauterine growth restriction is associated with metabolic disorders after birth. Our 1st study assessed deficits in skeletal muscle glucose metabolism and pancreatic β cell function in IUGR fetal sheep. We aimed to evaluate the effectiveness of daily intravenous infusions of the anti-inflammatory ω-3 polyunsaturated fatty acid (**PUFA**), eicosapentaenoic acid (**EPA**), as a means of improving deficits previously observed in the IUGR fetus by targeting fetal systemic inflammation. The presence of systemic inflammation in IUGR fetuses was evident by increased total circulating populations of total leukocytes, lymphocytes, and monocytes. However, these were decreased by 5-day ω-3 PUFA infusions. Additional blood parameters including lactate and CO_2 were increased in IUGR fetuses, regardless of infusion of EPA. Poor β cell function in IUGR fetuses was improved by EPA infusion, as evident by improved circulating plasma insulin, O_2, CO_2, HCO_3, Na^+, K^+, and Cl^- during a hyperglycemic clamp. Hindlimb glucose uptake and oxidation rates, which were impaired by IUGR were improved by EPA infusion as well. These findings indicate that targeting systemic fetal inflammation by infusion of the anti-inflammatory ω-3 PUFA, EPA, many IUGR-associated deficits in glucose metabolism and β cell function in the IUGR fetal sheep were resolved.

Our 2nd study sought to determine the effectiveness of daily EPA infusions on fetal growth biometrics and skeletal muscle-specific deficits associated with IUGR. In IUGR fetuses, decrease in mass were observed for whole fetus, hindlimb, *semitendinosus*, *soleus*, *longissimus dorsi*, lungs, and kidneys, but 5-day EPA infusion improved most of these deficits. The fiber type ratios of skeletal muscle estimated by myosin heavy chain proportions were also altered by IUGR but were recovered by EPA infusion as well. *Ex vivo* glucose uptake and oxidation capacities were impaired in IUGR muscle but were improved after daily EPA infusions. Results from this study indicate that daily fetal ω-3 PUFA infusions of EPA were effective in improving fetal growth biometrics, body composition, and muscle-specific glucose metabolism.

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Chapter 1

Literature Review

Introduction

The physiological response to any external stimulus defines the result to that event. Whether to everyday life events or catastrophic insults, bodily responses to events define how life will move forward. Everitt (1968) discussed the extent to which future responses are altered by events that occur during fetal development. Subsequent epidemiological studies have defined the concept of fetal programming and the thrifty metabolic phenotype hypothesis was established in the early 1990's (Hales and Barker, 1992; Godfrey and Barker, 2000). The building literature in this field show that detrimental effects of maternal stress during critical windows of fetal development cannot be overstated. Intrauterine growth restriction (**IUGR**) is the phenotypic result of poor intrauterine conditions causing nutrient-sparing metabolic and growth deficits that predispose individuals to a multitude of metabolic disorders (Thorne et al., 2014; Brown et al., 2015). Fetal adaptations occur to aid fetal survival but the repartitioning of fetal nutrients toward vital organs and tissues occurs at the expense of fetal growth and glucose homeostasis (Yates et al., 2014; Yates et al., 2018). Using the well-characterized heat stress model for placental insufficiency induced IUGR (**PI-IUGR**) in the ovine, we have previously reported links between adrenergic and inflammatory signaling pathways, reduced fetal growth, and metabolic inefficacy (Cadaret et al., 2019a; Posont and Yates, 2019). We also found that post-natal supplementation yielded increased body performance in IUGR-born offspring. Although this provides a basis for treatment of pathologies once established, there is a gap in literature regarding interventions for

these mechanisms before they become pathogenic. Thus, the basis for prenatal treatment targeting fetal systemic inflammation has yet to be determined.

Intrauterine Growth Restriction

Prenatal Stress & Developmental Programming Mechanisms.

Fetal growth is contingent upon many factors, including environmental and maternal influences. However, alterations to these influences result in changes to the intrauterine environment, producing IUGR (Murphy et al., 2006; Scifres and Nelson, 2009). Poor intrauterine conditions in late gestation induce fetal survival mechanisms and adaptations that result in IUGR (Murphy et al., 2006). Individuals born IUGR are at an increased risk for perinatal morbidity and mortality. The largely muscle-centric adaptations that induce metabolic dysfunction result from chronic maternal stress that is almost always associated with placental insufficiency (**PI-IUGR**) (Limesand et al., 2007). IUGR-born individuals are predisposed to an increased risk of poor thermoregulation, hypoglycemia, cardiac dysfunction, and infection (Limesand et al., 2007; Gaccioli and Lager, 2016). However, consequences are not limited to the fetal stage as fetal programming mechanisms yield lifelong greater risk of metabolic and cardiovascular disorders and growth deficits (Barker et al., 2006).

Maternal Causes of IUGR. Although etiologies of placental insufficiency in human and livestock pregnancies are still being studied, previous literature indicates the impact of known risk factors such as pre-eclampsia, hypertensive disorders, maternal drug use, decreased interpregnancy intervals nutritional intake, and environmental stressors (Hendrix and Berghella, 2008; Limesand et al., 2018; Yates et al., 2018; Wardinger and Ambati, 2021). The result of these maternal stressors is compromised placental development that is characterized by decreased placental

vascularity, oxygen permeability, and transporters required for fatty acid and glucose transport to the fetus (Thorn et al., 2011; Limesand et al., 2018). Reduced maternal blood flow to the fetus and umbilical-placental vascular resistance further compound the functional deficits of the placenta and worsen hypoperfusion of glucose and fatty acid in particular (Wardinger and Ambati, 2021). A summary of the hallmark fetal pathologies created in late gestation by placental insufficiency is presented in **Table 1-1**.

Placental Insufficiency Induces IUGR. As the facilitator of nutrient and waste exchange between the dam and the fetus, the placenta is a crucial regulator of fetal growth and, specifically, the ability for the fetus to meet its growth potential (Murphy et al., 2006). The placenta is a metabolically-active tissue that utilizes nutrients and oxygen for its own independent growth as well as providing these substrates to the fetus by simple diffusion and transporter-mediated transfer (Barry et al., 2008). However, when these processes are impeded by placental stunting due to maternal stress, both placental growth and the maternofetal interface are compromised. In animal models, placental insufficiency is characterized by as much as 50% decreased total placental mass (Brown et al., 2015), as well as reductions in placental villous number, diameter, surface area, and degree of branching (Barry et al., 2008). In addition, impaired synthesis of Nitric Oxide and polyamines is observed in the stunted placenta (Wu et al., 2006). In a study by Limesand et al. (2007), IUGR placentas collected near term in sheep weighed 61% less than control placentas ($P < 0.01$). During peak fetal growth in late gestation, delivery of carbohydrates, amino acids, and oxygen by the placenta must meet metabolic demand for rapid fetal growth. However, when placental stunting occurs, the stunted placenta cannot deliver sufficient oxygen and nutrients to the developing fetus. Thus, the fetus is compromised by a limited supply of nutrients, particularly, amino acids, glucose, and oxygen (Limesand et al.,

2007; Brown et al., 2015; Pendleton et al., 2021). The deprivation of these nutrients due to placental insufficiency ultimately lead the fetus to respond with nutrient-sparing programming that includes insulin resistance, impaired growth, and altered metabolic processes (Dunlop et al., 2015).

Physiological Responses of the Fetus to Placental Insufficiency. The fetal response to intrauterine stress is necessary for prenatal survival but also ultimately creates postnatal consequences for these individuals. The fetal stress response is characterized by increased secretion of catecholamines, pro-inflammatory cytokines, hypoxia, hypoglycemia, and hyperlactatemia (Yates et al., 2014; Cadaret et al., 2019c). As an innate survival mechanism, nutrient-sparing mechanisms are enacted to provide the essential organs with necessary substrates (Limesand et al., 2007). However, these developmental mechanisms reduce growth potential as the fetus adapts to being deprived of nutrients for less essential mechanisms such as myogenesis (Yates et al., 2018). Because skeletal muscle is a crucial regulator of glucose homeostasis, impaired development impacts metabolic health. More direct adaptive programming mechanisms in muscle include changes in metabolic processes that limit capacity for oxidative metabolism and increased lactate production (Brown et al., 2015; Cadaret et al., 2019c).

Nutrient Repartitioning at the Expense of Skeletal Muscle. A key fetal adaptation is redistribution of cardiac output towards the body's most vital organs such as the brain and adrenal glands (Dunlop et al., 2015). This acute survival mechanism allows these organs to maintain adequate growth and development but at the expense of other tissues and organs such as the heart, kidneys, and skeletal muscle. Near term and after birth, the consequences are made apparent by asymmetric body composition (Hales and Barker, 1992). Although it only composes

approximately 40% of the fetal body mass, skeletal muscle is responsible for 85% of fetal insulin-stimulated glucose metabolism (Brown, 2014; Hicks and Yates, 2021). Thus, compromised skeletal muscle growth and metabolism is the primary cause for glucose metabolic deficits in the IUGR fetus. The metabolic shift that inherently yields such biometric deficits are characterized by decreased mitochondrial metabolic capacity which couples oxygen consumption with substrate utilization (Pendleton et al., 2021). Although this mechanism is a barrier for muscle growth and development, it increases lactate production as a mechanism for providing carbon substrates for hepatic glucose production, which helps maintain glucose for brain development (Brown et al., 2015).

Because these adaptations are programmed, metabolic impairment and growth deficits persist after parturition even though oxygen and nutrient supply is no longer limited. Individuals maintain this thrifty phenotype, and increased fat deposition ultimately results. This further compromises intracellular regulation of insulin secretion and responsiveness, resulting in dysregulation of blood glucose concentrations and insulin resistance in muscle and adipose tissue (Limesand et al., 2007; Gibbs et al., 2020).

Fetal Programming Mechanisms. Metabolic adaptations that limit the oxidative capacity of skeletal muscle occur at the molecular level (Pendleton et al., 2021). These changes can include increased PDK1. This in turn inhibits PDH activity and its conversion of pyruvate to acetyl coA, which is the entry point for glucose into the TCA cycle that feeds into the electron transport chain. This helps explain the suppression of glucose oxidation even when glucose uptake is not impaired (Brown et al., 2015). Furthermore, a 4-fold increase in PDK4 expression in IUGR fetal skeletal muscle observed by Brown et al. (Brown et al., 2015) was likewise associated with a 10-15% reduction in IUGR fetal glucose oxidation, suggesting dynamic molecular changes

underlying limited capacity for glucose oxidation. The combination of normal glucose uptake by reduced entry into the TCA cycle means that skeletal muscle lactate production is increased, as illustrated by **Figure 1-1**. Despite concurrent insulin insensitivity, this typically equates to a lesser fraction of glucose being oxidized under both basal and insulin-stimulated conditions (Limesand et al., 2007; Brown et al., 2015). Work by our lab demonstrates the persistence of impaired muscle glucose oxidation at the neonatal and juvenile stages, despite the stress being alleviated by birth (Limesand et al., 2007; Gibbs et al., 2020).

The Health Impact of IUGR Pathologies.

The maternal etiology of IUGR can be variable, with many different environmental, nutritional, or lifestyle conditions leading to the multifaceted reduced functional capacity of the placenta. The fetal and postnatal outcomes that result, however, are more consistent and hallmarks of impaired metabolic function in IUGR offspring include hypertension, obesity, diabetes, and increased risk of cardiovascular diseases (Dunlop et al., 2015). The same molecular pathologies occur when pregnant livestock face chronic stress, and thus IUGR-born animals also exhibit poor growth and metabolic inefficiency. Therefore, IUGR is a relevant issue that must be addressed in both humans and livestock.

Relevance of IUGR in Humans. Although several common causes of maternal stressors have been linked to IUGR in human pregnancies, nearly 50% of IUGR cases occur as the result of an unidentified cause (Al-Azemi et al., 2017). IUGR adaptations can effect metabolic function even when growth restriction is mild and does not qualify clinically as small for gestational age (SGA) birthweight, which means that it and can be misdiagnosed when only bodyweight is measured (Cyrkowicz and Czekański, 1998). In fact, SGA individuals are classified by healthy, well-

nourished infants with a smaller and shorter frame in the 10th percentile whereas IUGR is associated with asymmetric body metrics and ultrasound-diagnosed uteroplacental deficiencies (Cyrkowicz and Czekański, 1998; Boehmer et al., 2017). In other words, SGA can occur independent of IUGR and conversely, IUGR does not always fit the clinical parameters for SGA. Nevertheless, IUGR is reported to afflict up to 53 million infants globally each year (Sedgh et al., 2014). Because of challenges associated with global healthcare, only 4-8% of cases are diagnostically linked to placental insufficiency (Boehmer et al., 2017). Fleiss et al. (2019) estimated that the rate of IUGR in human populations without confounding conditions to be up to 9%. However, the rate is exacerbated in lower-resource populations to as high as 30% (Fleiss et al., 2019). In addition to greater perinatal morbidity and mortality, these individuals are at 18-fold greater risk for metabolic disorders that reduce the length and quality of life, including Type 2 diabetes, obesity, hypertension, and hyperlipidemia (Gatford et al., 2010; Yates et al., 2018; Hicks and Yates, 2021).

Relevance of IUGR in Livestock. IUGR is also a relevant issue in livestock production where low birthweight due to IUGR occurs in approximately 10% of all livestock but can effect entire herds during harsh environmental or nutritional conditions (Wu et al., 2006). This contributes to an estimated annual loss of $12.1 billion in revenue (USDA, 2017) for producers due to a combination of poor growth performance, carcass merit discounts, and death loss (Smith et al., 1995; Boleman et al., 1998; Posont and Yates, 2019). Although most low-birthweight offspring survive the perinatal stage, decreased vigor and health further reduce the economic feasibility of having these individuals in the herd. As the animal ages, decreased muscle growth coupled with increased visceral fat compromise carcass value at the slaughterhouse due to less yield and smaller high-value cuts (Greenwood et al., 2000). Although studies are beginning to identify

underlying mechanisms and the issue has long been recognized by the industry, IUGR continues to cost the livestock producers 8% of their annual product, which is equal to the loss of 3 million beef calves, 930,000 dairy calves, and 530,000 lambs each year (Wu et al., 2006; Yates et al., 2018).

Sheep as a Model for IUGR. With the need to understand and treat IUGR in both humans and animals, ovine models have become popular tools to study fetal and placental growth restriction. Using the pregnant sheep as a physiological model is ideal for a multitude of reasons, including similar developmental milestones as humans and cattle, tolerance for surgical and experimental manipulation during gestation, and the translation of IUGR outcomes that are highly comparable to humans and cattle (Regnault, 2003; Beede et al., 2019). The induction of IUGR in the sheep model can be accomplished using several methods, including chronic maternal hyperthermia, overnutrition of the dam at a young age, chronic maternofetal inflammation, nutrient restriction, and surgical placental reduction (Beede et al., 2019). The frequent use of these models in current studies show that sheep are an effective model for biomedical and agricultural research aimed at assessing fetal programming mechanisms.

Inflammatory Pathologies in IUGR

As a part of the stress response to placental insufficiency, IUGR fetuses present physiological indicators of systemic inflammation, including changes in white blood cell (**WBC**) populations and increased inflammatory cytokines which play a vital role in metabolic programming. These indicators are summarized in **Table 1-2**. Tumor necrosis factor α (**TNFα**) and Interleukin-6 (**IL-6**) are major inflammatory cytokines that are secreted from leukocytes during nutritional or metabolic stress, typically in response to increased reactive oxygen species (ROS) or other stress

signals (Renshaw et al., 2010). In a study by Al-Azemi et al. (2012), the increased relevance of inflammation in the PI-IUGR fetus in comparison to IUGR fetuses produced from other methods was abundant as increased IL-2 and IL-12 suggested a propensity towards Th1 expression and subsequent TNFα secretion in the PI-IUGR fetus. Moreover, decreased IL-4 and increased IL-10 concentrations in non-PI IUGR fetuses further supported the correlation between inflammation and placental insufficiency (Al-Azemi et al., 2017).

Stress-induced cytokine Production. Although most commonly associated with immune responses, cytokines and other inflammatory factors are parts of the general stress response and metabolic regulation. Cytokines are produced in large quantities by macrophages located in circulation and peripheral tissues. These proteins can be secreted locally or systemically and are elevated in the IUGR fetus (Irani et al., 2009; Cadaret et al., 2019c). The secretion of T helper 1 (**Th1**) and T helper 2 (**Th2**) cells regulate these mechanisms by their individual roles in the immune response (Al-Azemi et al., 2017). Th1 cells are responsible for the secretion of inflammatory cytokines such as TNFα and IL-2, which activate macrophages in cell-mediated immunologic responses, whereas Th2 cells secrete anti-inflammatory cytokines such as IL-4, IL-5, and IL-10 (Al-Azemi et al., 2017).

In adult animals, presence of oxidative stress is indicated by alterations in WBC profiles in circulation, including increases in leukocytes, granulocytes, lymphocytes, and monocytes as well as of inflammatory cytokines (Barnes et al., 2019; Swanson et al., 2020). Romero et al. (1998) cited the strong correlation of elevated cytokine concentrations in fetal blood and amniotic fluid and acknowledged that both of these were indicators of pre-term labor. However, maternal IL-6 was not correlated with fetal concentrations in that study. Therefore, increased cytokines in the

fetus imposed stress induced adaptations, and these conditions were not correlated with pre-term labor.

Impaired Skeletal Muscle Growth. Fetal hyperplasia determines postnatal muscle fiber number and thus is crucial in establishing muscle growth potential. In livestock, this can have a major impact on meat yield and quality (Hegarty and Allen, 1978; Greenwood et al., 2000). Adaptive changes in IUGR muscle impair myogenesis by disrupting cellular signaling for proliferation and differentiation of muscle stem cells called myoblasts (Yates et al., 2014; Posont and Yates, 2019). These cause long term impacts for total muscle mass and growth potential as the animal ages (Bell, 2006). Although acute stimulation by cytokines promotes growth and development of skeletal muscle, prolonged exposure is one mechanism whereby proliferation and differentiation of myoblasts are restricted in the IUGR fetus (Posont and Yates, 2019). Because myoblast function is a rate-limiting process for muscle growth, the functional impairment by persistent exposure to cytokines *in utero* can be considered a key mechanism for decreased hypertrophic growth capacity (Cadaret et al., 2019b).

Inflammatory Effects on Glucose Metabolism & Insulin Secretion. Activation of the Nuclear Factor κ-B (**NFκB**) pathway by TNFα and other cytokines contributes to the shift toward muscle-centric glucose metabolism by inhibiting insulin-stimulated Akt phosphorylation and subsequent decreased translocation of GLUT-4 transporters for glucose uptake (Lorenzo et al., 2008; Cadaret et al., 2017). This directly and indirectly helps explain deficient glucose oxidation capacity in IUGR skeletal muscle (Cadaret et al., 2019a; Posont et al., 2021b).

Pancreatic islet cells are also affected by inflammation as proper IL-6 exposure promotes microvasculature development of the islets (Limesand et al., 2007). However, chronic exposure

to systemic inflammation is detrimental to islet size, density, and pancreatic mass (Boehmer et al., 2017). These anatomical deficits in islets lead to alterations in function, as IUGR fetuses exhibit altered gene expression for PDX1 and other regulatory factors, thus contributing to defects in β-cell replication and decreased insulin content (Limesand et al., 2007; Green et al., 2010; Boehmer et al., 2017).

ω-3 Polyunsaturated Fatty Acids as Anti-Inflammitants

In pregnant women, common additives to prenatal vitamins include eicosapentaenoic acid (**EPA**) and docasohexanoic acid (**DHA**), as studies support the benefits of the ω-3 polyunsaturated fatty acids (**ω-3 PUFAs**) in fetal neurodevelopment, immune system development, and central nervous system health (Greenberg et al., 2008; Best et al., 2016). Although these ω-3 PUFAs can be synthesized from α-linoleic acid (**ALA**) in the adult, the developing fetus does not have this ability and thus the only derivatives come from placental transfer as excess after satisfying the metabolic needs of the mother (Larque et al., 2012). Studies have established the capacity for ω-3 PUFAs to mitigate inflammatory pathways and also promote the production and secretion of anti-inflammatory cytokines (Chen et al., 2017; Inoue et al., 2017; Gonzalez-Soto and Mutch, 2021). Epidemiological studies in the past have associated the consumption of long-chain ω-3 PUFAs with decreased risk of inflammatory syndromes such as arthritis and pulmonary vascular resistance (Sharma et al., 2017). The designation between ω-3 PUFAs, which are anti-inflammatory, and ω-6 PUFAs, which can increase inflammatory tone, is determined by whether a double bond is located at the 3rd or 6th carbon from the terminal methyl group, respectively (Kim et al., 2019). As illustrated in **Figure 1-2**, EPA is a carboxylic acid chain that is 20 carbons in length and contains five double bonds, the 1st of which is located at the 3rd carbon (designating it as an ω-3). DHA is a carboxylic acid that is 22 carbons in length with six cis double bonds, the

1st of which is located at the 6th carbon from the terminal methyl. Both EPA and DHA are synthesized from the essential fatty acid, ALA, although the process is energetically costly (Burdge and Calder, 2005). This stepwise process has several intermediate steps but begins with the desaturation of ALA to stearidonic acid by delta-6-desaturase. It is this 1st step that is rate limiting, however. Next, elongase converts stearidonic acid into eicosatetraenoic acid and finally to EPA by delta-5-de-saturase (Lenihan-Geels and Bishop, 2016). ALA is highly abundant in common foods such as flaxseed, soybean, and canola whereas EPA and DHA can be obtained directly from fish, seaweed, and algae without having to be synthesized by the body. In order to be metabolized for energy, these PUFAs enter the cellular matrix as an acyl-carnitine by way of a carnitine transporter before phosphorylation back to a fatty acid, which can then undergo β-oxidation (Burdge and Calder, 2005). As a nutraceutical, EPA induced a 25% decrease in pulmonary vascular resistance in fetal lambs, whereas DHA had no effect (Sharma et al., 2017). Literature provides comprehensive evidence that ω-3 PUFA inhibit many aspects of inflammation including decreased cytokine and eicosanoid production (Caughey et al., 1996; Velten et al., 2014; Allam-Ndoul et al., 2016). Moreover, the expression of transcription factors inhibits the activity of the NFκB pathway (Allam-Ndoul et al., 2016). Therefore, the capacity for ω-3 PUFA to mitigate inflammatory pathologies is established.

During late gestation, fatty acids are required for brain and adipose tissue development but may only be acquired from the maternal diet via placental transfer (Murphy et al., 2006). This transport can occur in several ways, including the use of fatty acid transport proteins (FATP)-1, 2, 3, 4, 5, 6 and fatty acid binding protein (FABP) -1, 3, 4, 5, 7 (Devarshi et al., 2019). As circulating maternal concentrations of EPA and DHA increase, placental mRNA expression of

these FATP is also upregulated, and subsequent increase of these PUFA in cord blood are observed, along with the FABP required for their placental transfer (Greenberg et al., 2008).

The cumulative alterations in white blood cells and cytokine profiles are hallmarks of systemic inflammation and likewise, hallmarks of IUGR. Thus, ω-3 PUFA provide promise to mitigate inflammatory signaling its programmed deficits in the IUGR fetus. As transporter mechanisms are upregulated during gestation, we know that these nutraceuticals have the ability to be transported across the placenta and therefore provide promise in future treatment through maternal ingestion (Connor et al., 1996; Greenberg et al., 2008).

Conclusion

Work by our lab and others continue to characterize the mechanistic deficits of underlying IUGR that contribute to lifelong metabolic and biometric deficits in humans and livestock. Although these mechanisms are beginning to be identified, little is known regarding how they may be mediated *in utero*. The objectives of the following studies were aimed at establishing a basis for how fetal systemic inflammation may be alleviated by nutraceutical intervention during late gestation. Specifically, the effectiveness of direct fetal infusion of ω-3 PUFA on IUGR fetal growth and metabolic outcomes was evaluated by measuring fetal biometrics, β-cell function, skeletal muscle glucose uptake, and skeletal muscle oxidative metabolism in a well-characterized sheep model for placental insufficiency and IUGR.

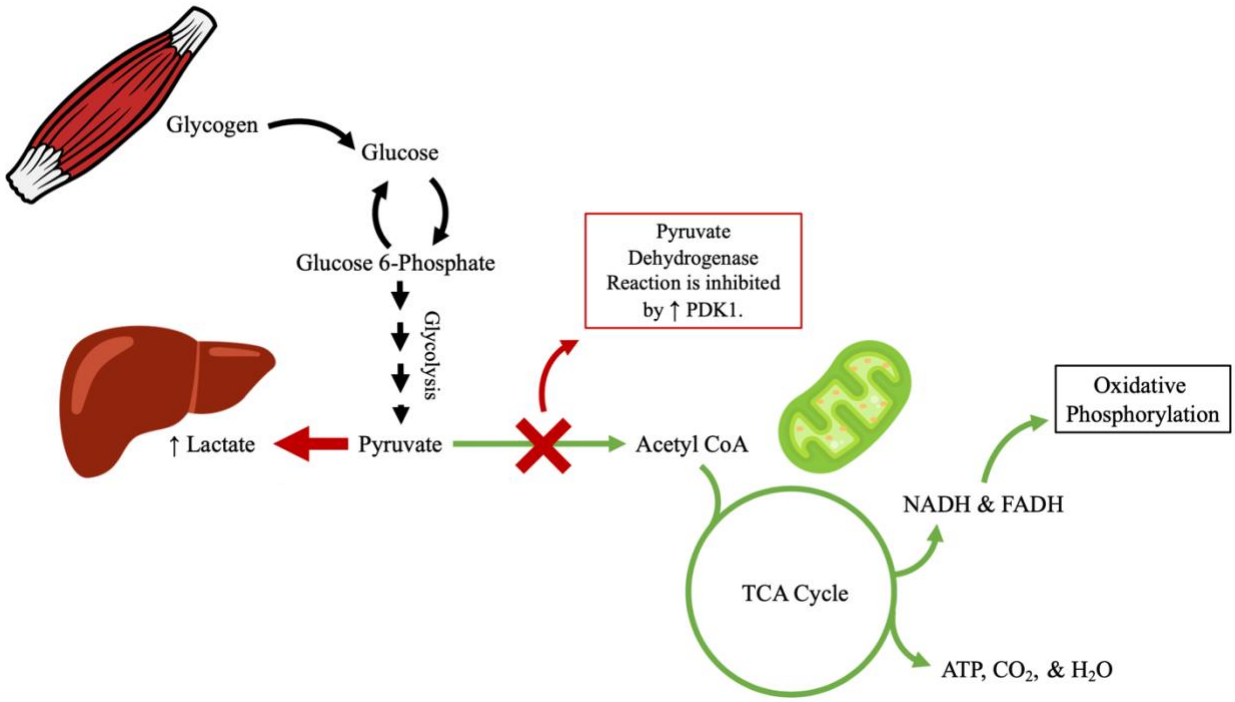

Figure 1-1. Simple Diagram of the part of glucose metabolism pathway which is being altered by IUGR

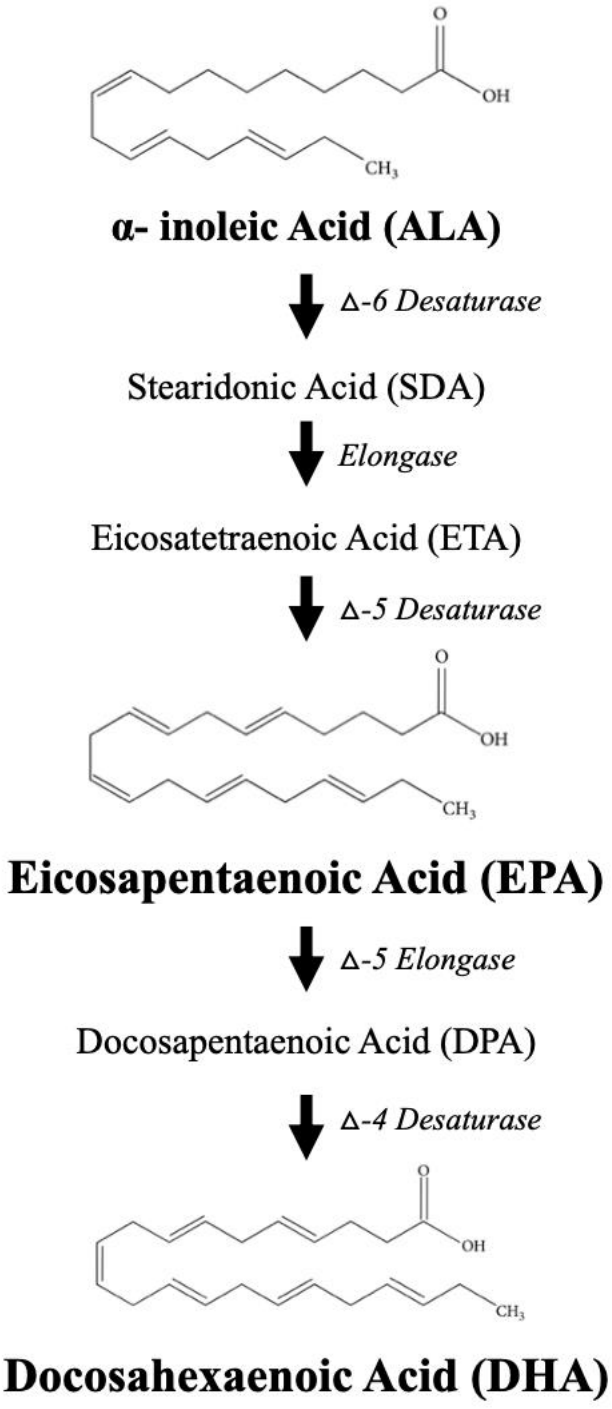

Figure 1-2. Biochemical Structures of α-linoleic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA).

Table 1-1. Summary of the physiological parameters which are altered in the IUGR fetus.

Physiological Parameter	Conditions in the IUGR Fetus
O_2	~60% ↓ arterial blood oxygen (Brown et al., 2015); ~50% ↓ fetal oxygen (Macko et al., 2016)
Lactate	4-fold ↑ lactate concentrations (Brown et al., 2015);
Insulin	~55% ↓ insulin concentrations (Brown et al., 2015); ~69% ↓ insulin concentrations (Limesand et al., 2007); ~56% ↓ β-cell mass (Leos et al., 2010)
Glucose	~50% ↓ glucose concentrations (Limesand et al., 2007); ~ 30% ↓ glucose concentrations (Brown et al., 2015); ~50% ↓ glucose oxidation rate (Limesand et al., 2007);
Catecholamines	7-fold ↑ Norepinephrine (Yates et al., 2016); 5-fold ↑ Epinephrine (Limesand et al., 2007)
IGF-1	~ 80% ↓ circulating fetal IGF-1 (Thorne et al., 2009); ~ 33% ↓ fetal arterial IGF-1 (Macko et al., 2016);
PDK4-1	4-fold ↑ PDK4 expression (Brown et al., 2015)
Myoblast Function	~13% ↓ proliferated and ~46% ↓ differentiated myoblasts (Yates et al., 2014); ~12% ↓ myoblast proliferation (Riley et al., 2016); ~50% ↓ cross-sectional hindlimb muscle fiber area(Posont et al., 2018)

Table 1-2. Summary of the indicators of inflammatory signaling in the IUGR fetus.

Physiological Parameter	Activity in the IUGR fetus
White blood cells (WBC)	~50% ↑ total WBC, ~35% ↑ monocytes, ~200% ↑ granulocytes (Cadaret et al., 2019); ↑(Armengaud, 2021; Jones et al., 2018)
TNFα	↑ (Cadaret et al., 2019; Bertucci et al., 2011)
IL-6	5-fold ↑(Bertucci et al., 2011)
IL-8	~72% ↑ (Raghupathy & Azizieh, 2012; Jones et al., 2018)
IL-13	~39% ↓ (Raghupathy & Azizieh, 2012)
Activin A	10-fold ↑ (Jones et al., 2018)

Chapter 2

Decreased skeletal muscle glucose metabolism and impaired β cell function in IUGR fetal sheep were improved by daily ω-3 PUFA infusions.

The findings presented in this chapter were published in the 2021 proceedings of the American Society of Animal Science, Western Section, which is peer-reviewed.

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ABSTRACT

Poor intrauterine conditions created by placental insufficiency result in intrauterine growth restriction (IUGR) of the fetus. Decreased fetal nutrients and O_2 coincide with fetal inflammation, which appears to have a role in thrifty programming mechanisms, including reduced skeletal muscle glucose oxidative metabolism. Thus, our objective was to target inflammation in IUGR fetal sheep by infusing an anti-inflammatory ω-3 polyunsaturated fatty acid (**PUFA**), eicosapentaenoic acid (**EPA**), to improve homeostatic blood parameters and metabolic outcomes. In this study, IUGR fetuses were produced by maternal hyperthermia-induced placental insufficiency. Surgical placement of indwelling fetal femoral catheters allowed arterial blood collection and daily intravenous infusion of IUGR fetuses with saline (i.e., IUGR; n=5) or eicosapentaenoic acid (i.e., IUGR+EPA; n=6) for 5 ± 1 days beginning at 119 ± 1 days of gestational age (dGA). Controls (n=12) were saline-infused fetuses from thermoneutral ewes. Daily arterial blood samples were collected to assess complete blood cell count (**CBC**). Daily

blood parameters were assessed by arterial blood collection for CBC, blood gases, and metabolites. Glucose-stimulated insulin secretion was assessed at 122 ± 1 dGA and hindlimb-specific glucose metabolism was determined at 123 ± 1 dGA. Daily circulating white blood cells (**WBC**) were elevated ($P < 0.05$) in IUGR compared to control and IUGR+EPA fetuses. In addition, platelets were elevated ($P < 0.05$) in IUGR and IUGR+EPA fetuses compared to controls. Blood lactate and pCO_2 were increased ($P < 0.05$) in IUGR fetuses compared to controls however did not differ from IUGR+EPA fetuses. Basal plasma insulin was less ($P < 0.05$) for IUGR but not for IUGR+EPA fetuses compared to controls. Glucose-stimulated insulin secretion was less ($P < 0.05$) for IUGR and IUGR+EPA fetuses compared to controls but was greater ($P < 0.05$) for IUGR+EPA than for IUGR fetuses. Blood pO_2, pCO_2, HCO_3, Na^+, K^+, and Cl^- were less ($P < 0.05$) for IUGR fetuses than controls but were greater ($P < 0.05$) for IUGR+EPA than for IUGR fetuses. BUN and NEFA concentrations were increased ($P < 0.05$) for IUGR but not IUGR+EPA fetuses compared to controls. HDLC did not differ between controls and IUGR fetuses but was increased ($P < 0.05$) in IUGR+EPA fetuses. Hindlimb glucose uptake rates were decreased ($P < 0.05$) for IUGR and IUGR+EPA fetuses and hindlimb insulin-stimulated glucose oxidation was decreased ($P < 0.05$) for IUGR and IUGR+EPA fetuses, compared to controls. These findings demonstrate that infusion of the anti-inflammatory ω-3 PUFA, EPA, improved some aspects of metabolic dysfunction in the IUGR fetus.

INTRODUCTION

Placental insufficiency-induced intrauterine growth restriction (IUGR) results in muscle-centric fetal programming that impairs glucose metabolism (Yates et al., 2019) and yields asymmetric growth that favors fat deposition (Gibbs et al., 2020). Decreased nutrients and O_2 impose fetal stress, initiating fetal adaptations for survival that also increase the risk for hypertension, obesity, and Type 2 diabetes after birth (Yates et al., 2011). Chronic exposure of skeletal muscle and pancreatic islets to inflammatory cytokines appears to be a key culprit for the programming mechanisms that yield IUGR and in turn life-long metabolic dysfunction (Yates et al., 2018; Posont et al., 2021b). Thus, the objective of this study was to evaluate the effects of manipulating stress-induced inflammatory activity in the IUGR fetus. Previous studies have reported that ω-3 polyunsaturated fatty acids (**PUFA**) have anti-inflammatory actions and also stimulate glucose metabolism (Kim et al., 2019). If administration of ω-3 PUFA can effectively improve glucose metabolism and insulin sensitivity, then glucose homeostasis, metabolic efficiency, and growth in the IUGR fetus may be recovered. We hypothesized that manipulation of inflammatory pathways in the skeletal muscle of IUGR fetuses via daily infusion with eicosapentaenoic acid (**EPA**) in late gestation would improve fetal blood indicators of metabolic health, glucose homeostasis, biometric growth, and body composition.

MATERIALS & METHODS

Animals and Experimental Design

All studies were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln, which is AAALAC International-accredited. Time-mated Polypay ewes were used to produce control and placental insufficiency-induced IUGR fetuses as previously described (Yates et al., 2016). Briefly, ewes were exposed to ambient conditions of

40°C and 35% relative humidity from the 40th to the 95th day of gestational age (dGA) before returning to thermoneutral conditions (25°C) for the duration of the study. Ewes carrying control fetuses were pair-fed to the average daily intake of the ewes carrying IUGR fetuses. At 118 dGA, partial cesarean surgeries were performed to place indwelling catheters in the femoral vein and artery of the fetus and an arterial blood flow probe as previously described (Cadaret et al., 2019c). On dGA 119 ± 1, IUGR fetuses were randomly assigned to receive daily 1-hour i.v. infusions of 0.25 mg/d EPA (i.e., IUGR+EPA; n=6) or ethanol-spiked (**EtOH**) saline placebo (ie. IUGR; n=5) for 5 ± 1 d. EPA concentrate was suspended in EtOH (Sigma-Aldrich). Control fetuses (n=12) also received daily EtOH-spiked saline infusions.

Fetal arterial blood samples were collected daily from 120 to 125 dGA. Complete blood cell counts (**CBC**) were determined using a HemaTrue Veterinary Chemistry Analyzer (Heska, Loveland, CO). An ABL90 FLEX blood gas analyzer (Radiometer America, Bera, CA) was used to measure blood gases and metabolites.

In Vivo Fetal Metabolism

Glucose-Stimulated Insulin Secretion. Pancreatic β cell function was assessed using a square-wave hyperglycemic clamp at 122 ± 1 dGA as previously described (Cadaret et al., 2019c). Three arterial samples were taken in 5-minute intervals at baseline (i.e., resting) levels then a 3 mL bolus of 33% dextrose solution was administered intravenously (**i.v.**) followed by a continuous variable-rate infusion in order to achieve steady-state hyperglycemia (i.e., 2.5X baseline). After steady-state was achieved and at least twenty minutes after the bolus, three additional arterial samples were taken at 5-minute intervals at hyperglycemia. Blood samples collected in heparinized syringes were analyzed to measure blood gases and metabolites via ABL90. Samples collected into EDTA syringes were centrifuged (14,000 x g, 2 minutes, 4°C)

and plasma was collected and stored at -80°C. Plasma insulin concentrations were then determined in duplicate using the Bovine Insulin ELISA (Alpco, Salem, NH), which has been validated for sheep. Plasma NEFA concentrations were calculated via commercial NEFA colorimetric assay (Sigma-Aldrich). Plasma BUN, and HDLc concentrations were measured by Vitros 250 Chemistry Analyzer (Biomedical and Obesity Research Core, Lincoln, NE). Inter- and intra- assay coefficients of variance were below 10%.

In Vivo Hindlimb Glucose Metabolism. Hindlimb-specific glucose metabolism was estimated by a hyperinsulinemic-euglycemic clamp (HEC) at 123 ± 1 dGA as previously described (Yates et al., 2019). For this study, fetuses were administered a radiolabeled glucose tracer (18.75μCi/ml, U-[14C]-glucose; Perkin-Elmer, Waltham, MA) infused at a constant rate of 1 mL/hour following a 1 mL i.v. bolus. Four pairs of simultaneous femoral arterial and venous samples were then collected at 5-minute intervals to establish a baseline before bolusing 1 mL of 33% dextrose (150 mg/kg, i.v.) and 2mL of insulin (250 mU/kg; Humulin-R, Eli Lilly, Indianapolis, IN). A variable-rate infusion of 33% dextrose was used to maintain euglycemia. Insulin was infused at a constant rate of 0.5 ml/kg/hour until steady state HEC was achieved (±10% baseline glucose). Four more pairs of simultaneous arterial and venous samples were then taken at 5-minute intervals. Samples were analyzed for blood gases and metabolites via ABL90 and plasma was isolated and collected to be stored at -80°C until insulin concentrations were determined via Bovine Insulin ELISA.

Additional arterial and venous samples (0.33 mL, respectively) were deposited in 0.5 mL micro-centrifuge tubes containing 2M HCl and fixed inside 20 mL scintillation vials containing 1M NaOH to estimate hindlimb glucose oxidation rates as previously described (Posont et al., 2021b). Vials were then sealed and incubated for 24 hours at room temperature. In vivo glucose

oxidative metabolism is estimated by the amount of $^{14}CO_2$ released from the blood by HCl and recaptured by the NaOH at the bottom of the scintillation vial. Micro-centrifuge tubes were then manually removed, and scintillation vials were filled with UltimaGold scintillation fluid (Perkin-Elmer). A liquid scintillation counter (Beckman-Counter 1900 TA, Brea, CA) was then used to quantify radiolabeled $^{14}CO_2$. Glucose oxidation by the hindlimb tissues was then calculated using the difference in $^{14}CO_2$ between arterial and venous samples at each given time of sample collection. The response of the hindlimb tissues to insulin stimulation was determined by calculating the fold-change in glucose oxidation between basal and HEC periods. Glucose uptake was estimated by the difference in arterial and venous glucose concentrations. Technical replicates at baseline and HEC levels were averaged.

Statistical Analysis

All data were analyzed with SAS 9.4 (SAS Institute, Cary, NC) using the mixed procedure specific for repeated measures with the fetus as the experimental unit. Repeated measures of blood parameters in the GSIS, HEC, and daily blood samples were analyzed for effects due to treatment, time (dGA or period), and the interaction. Variables for time were treated as repeated measures. Sex was treated as a random effect. Technical replicates were averaged, and data are presented as mean ± standard error. Significant differences were declared at $\alpha \leq 0.05$ and tendencies at $\alpha \leq 0.10$.

RESULTS

Daily Hematology

No group x dGA interactions were observed for any hematological measures. Total white blood cell (**WBC**) concentrations tended to be greater ($P = 0.09$) in IUGR fetuses compared to

controls and IUGR+EPA fetuses, regardless of dGA (**Figure 2-1**). Circulating lymphocytes were greater ($P < 0.05$) in IUGR and IUGR+EPA and monocytes tended to be greater ($P = 0.06$) in IUGR and IUGR+EPA fetuses compared to controls. Granulocytes, hematocrit (**Hct**), mean corpuscular volume (**MCV**), hemoglobin concentrations (**Hb**), mean platelet volume (**MPV**), red blood cells (**RBC**), and red blood cell width distribution (**RWD**) did not differ among experimental groups. Hemoglobin concentrations were less ($P < 0.05$) in IUGR and IUGR+EPA fetuses than controls **(Figure 2-1**). Platelet concentrations were greater ($P < 0.05$) in IUGR fetuses compared to controls and were greater ($P < 0.05$) in IUGR+EPA fetuses than in IUGR and control fetuses.

Daily Blood Gases and Metabolites

No group x dGA interactions were observed for blood glucose, lactate, pH, partial pressure CO_2 (**pCO_2**), or partial pressure O_2 (**pO_2**). Daily blood glucose concentrations were less ($P < 0.05$) in IUGR fetuses than controls regardless of day and was intermediate ($P < 0.05$) for IUGR+EPA fetuses between controls and IUGR fetuses (**Figure 2-3**). Lactate concentrations were greater ($P < 0.05$) in IUGR and IUGR+EPA fetuses compared to controls. Blood pH did not differ among experimental groups. Blood pCO_2 was greater ($P < 0.05$) and pO_2 was less ($P < 0.05$) in IUGR and IUGR+EPA fetuses compared to controls. A group x dGA interaction ($P < 0.05$) was observed for daily blood HCO_3 concentrations, which were greater ($P < 0.05$) in IUGR and IUGR+EPA fetuses on dGA 122, 123, and 124. HCO_3 was also greater ($P < 0.05$) in IUGR+EPA than controls at dGA 120.

Glucose-Stimulated Insulin Secretion

Experimental group x glycemic period interactions were observed ($P<0.05$) for plasma insulin and glucose-to-insulin ratios but not for blood glucose or lactate. Blood glucose was less

(P<0.05) for IUGR fetuses than for controls and IUGR+EPA, regardless of period. Glucose was also increased (P<0.05) during hyperglycemia compared to baseline for all groups (**Figure 2-4**). Plasma insulin was less (P < 0.05) for IUGR fetuses than for controls and IUGR+EPA fetuses at baseline. At hyperglycemia, plasma insulin was less (P < 0.05) in IUGR fetuses compared to controls and was intermediate (P < 0.05) for IUGR+EPA between controls and IUGR fetuses. Glucose-to-insulin ratios were less (P<0.05) for IUGR fetuses than for controls and IUGR+EPA fetuses at baseline. At hyperglycemia, glucose-to-insulin ratios were less (P < 0.05) for IUGR fetuses than controls and were intermediate (P < 0.05) for IUGR+EPA fetuses between controls and IUGR fetuses. Blood lactate did not differ among treatment groups, regardless of period but was greater (P<0.05) during hyperglycemia than baseline in all groups.

No group x period interactions were observed for blood pO_2, pCO_2, pH, hemoglobin, hematocrit, hemoglobin bound O_2 (**O_2HB**), hemoglobin bound CO (**COHB**) or HCO_3. Blood pO_2 was less (P < 0.05) for IUGR fetuses than controls and was intermediate (P < 0.05) for IUGR+EPA fetuses between IUGR fetuses and controls, regardless of period **(Figure 2-5)**. Blood pCO2 was greater (P < 0.05) for IUGR fetuses but not IUGR+EPA fetuses than for controls and was greater (P < 0.05) during hyperglycemia than baseline regardless of experimental group. Blood pH was less (P < 0.05) for IUGR fetuses and was intermediate (P < 0.05) for IUGR+EPA fetuses between IUGR fetuses and controls. It was also decreased (P < 0.05) during hyperglycemia compared to baseline regardless of experimental group. Blood hemoglobin and hematocrit did not differ among experimental groups, regardless of period. Blood O_2HB was less (P < 0.05) in IUGR fetuses but did not differ between controls and IUGR+EPA fetuses. It was also decreased (P < 0.05) during hyperglycemia in all experimental groups. Blood COHB was less (P < 0.05) in IUGR fetuses compared to controls but did not

differ from IUGR+EPA fetuses, regardless of period. Blood HCO3 was greater ($P < 0.05$) for IUGR fetuses but less ($P<0.05$) for IUGR+EPA fetuses compared to controls, regardless of period.

No group x period interactions were observed for Na^+, K^+, Cl^-, or Ca^{2+} concentrations. Blood Na^+ was greater ($P < 0.05$) for IUGR fetuses but not IUGR+EPA fetuses than for controls, regardless of period. Blood K^+ was greater ($P < 0.05$) in IUGR fetuses and was intermediate ($P < 0.05$) for IUGR+EPA between controls and IUGR fetuses (**Figure 2-6**). Blood K^+ decreased during hyperglycemia, regardless of experimental group. Blood Cl^- was greater ($P < 0.05$) for IUGR fetuses than controls and IUGR+EPA fetuses, regardless of period. Blood Ca^{2+} did not differ among experimental groups regardless of period.

No group x period interactions were observed for non-esterified fatty acids (**NEFA**), blood plasma urea nitrogen (**BUN**), and high-density lipoprotein cholesterol (**HDLc**). Total blood NEFA concentrations were greater ($P < 0.05$) in IUGR fetuses compared to controls and IUGR+EPA fetuses (**Figure 2-7**). Blood NEFA were also decreased ($P < 0.05$) during hyperglycemia compared to baseline in all groups. BUN were greater ($P < 0.05$) in IUGR fetuses than controls, and were intermediate ($P < 0.05$) for IUGR+EPA fetuses between controls and IUGR fetuses. HDLc did not differ between IUGR fetuses and controls but were greater ($P < 0.05$) in IUGR+EPA fetuses, regardless of period.

Hindlimb Glucose Metabolism

A group x insulinemic period interaction was observed ($P < 0.05$) for blood K^+ but not for any other blood parameters. Blood glucose was less ($P < 0.05$) in IUGR fetuses compared to controls and was intermediate ($P < 0.05$) for IUGR+EPA fetuses between IUGR fetuses and controls (**Figure 2-8**). Blood lactate tended to be greater ($P < 0.10$) in IUGR and IUGR+EPA

than for controls. Blood pH was greater ($P < 0.05$) in IUGR fetuses than controls and IUGR+EPA fetuses (**Figure 2-9**). Blood pCO_2 did not differ among experimental groups but blood pO_2 was tended to be less ($P < 0.10$) in IUGR and IUGR+EPA compared to controls. There was no difference among experimental groups for hemoglobin or hematocrit concentrations (**Figure-10**). Blood plasma NEFA concentrations were greater ($P < 0.05$) in IUGR fetuses and was intermediate ($P < 0.05$) for IUGR+EPA fetuses and controls. HCO_3 was less ($P < 0.05$) in IUGR fetuses than controls and IUGR+EPA fetuses.

Blood Na^+ was greater ($P < 0.05$) in IUGR fetuses compared to controls and further increased ($P < 0.05$) in IUGR+EPA fetuses compared to IUGR fetuses, regardless of insulinemic period (**Figure 2-11**). A group x period interaction was observed ($P < 0.05$) for K^+ which were greater ($P < 0.05$) in IUGR fetuses than controls and intermediate for IUGR+EPA fetuses in the baseline period. During HEC, blood K^+ was greater ($P < 0.05$) in IUGR fetuses compared to controls and less ($P < 0.05$) in IUGR+EPA fetuses compared to controls. Blood Cl^- was greater ($P < 0.05$) in IUGR fetuses than controls and greater ($P < 0.05$) in IUGR+EPA fetuses compared to IUGR fetuses and controls, regardless of period. Blood Ca^{2+} was not different among experimental groups but was increased ($P < 0.05$) during HEC.

Hindlimb glucose uptake rates were less ($P < 0.05$) for IUGR fetuses and IUGR+EPA fetuses than controls (**Figure-12**). Similarly, hindlimb insulin-stimulated glucose oxidation was less ($P < 0.05$) in IUGR and IUGR+EPA fetuses compared to controls.

DISCUSSION

In this study, we found that some but not all deficits caused by IUGR were mitigated by daily infusion of fetuses with the anti-inflammatory ω-3 PUFA, EPA. The presence of systemic inflammation was evident by elevated WBC in our IUGR fetuses. Total circulating populations

of WBC, lymphocyte, and monocytes were increased in the IUGR fetuses and total WBC concentrations were recovered by 5-day EPA infusion to IUGR fetuses. Previous studies by our lab (Barnes et al., 2019; Swanson et al., 2020) have demonstrated in adult animals that the presence of increased leukocytes, granulocytes, lymphocytes, and monocytes are associated with chronic heat stress even in the absence of direct immune challenges. The chronic heat stress used in these studies produced many of the same physiological deficits as IUGR and demonstrates a correlation between changes in immune profile and blood indicators of inflammation, such as oxidative cellular stress and the secretion of inflammatory cytokines. In particular, the increase in lymphocytes and monocytes in the IUGR fetuses of the present study support our hypothesis that systemic inflammation occurs in IUGR fetuses. However, although the elevation of total WBC was decreased by EPA infusions, lymphocytes and monocytes remained elevated in EPA-infused IUGR fetuses, suggesting that full mediation of the mechanisms stimulating immune cell changes was not achieved. Surprisingly, platelet concentrations were increased only marginally in saline-infused IUGR fetuses but were 122% greater than normal in EPA-infused IUGR fetuses. We know from previous studies that increased circulating platelets are typically associated with classical immune insults to the body (Koupenova et al., 2018), which was perhaps an indirect component of this IUGR fetal model. Moreover, the further exacerbation by ω-3 PUFA infusion was unexpected based on previous literature. In studies by Dyerberg and Bang (1979) and Yamada et al. (1998), blood platelets were decreased in humans and rats receiving ω-3 PUFA supplementation, respectively. It is unclear why our results differed, but we would suspect there are other confounding mechanisms at work that may be responsible for these alterations, such as transient hyperlipidemia from the hour-long ω-3 PUFA infusions.

Daily blood glucose concentrations show that IUGR fetuses were moderately hypoglycemic, which was an expected outcome of placental insufficiency. However, blood glucose was partially recovered by daily EPA infusions. In contrast, blood lactate was increased in IUGR fetuses and EPA-infused IUGR fetuses alike, suggesting that administration of EPA had little effect on tissue glycolysis rates. Although we did not measure antioxidant status in our fetuses, it is possible that increased oxidative stress and the associated stimulation of inflammatory cytokine secretion (i.e., increase in inflammatory tone) impaired metabolic function earlier and more profoundly than our late-term intervention could overcome (Renshaw et al., 2010). Comparable impairments in blood pO_2 and pCO_2 between IUGR fetuses infused with saline and those infused with EPA further support this possibility. Interestingly, improvements in blood HCO_3 due to EPA-infusion appeared to take time as HCO_3 was increased in IUGR fetuses and were not improved by EPA until the final day of the 5-day infusion period. Circulating HCO_3 plays a pivotal buffering role to protect against oxidative stress, and thus increased HCO_3 concentrations in our IUGR fetuses is likely a compensatory buffering response that is ultimately made less necessary by daily ω-3 PUFA infusions.

Basal and glucose-stimulated insulin secretion, which were impaired by over 40% in our IUGR fetuses, were each improved by 5-day ω-3 PUFA infusion. This indicates that deficits in IUGR β cell function are at least in part due to enhanced inflammatory tone and thus benefit from inflammatory mitigation. Despite complete recovery of basal insulin secretion, only partial recovery of insulin secretion occurred under hyperglycemic conditions, indicating that there are clearly factors other than inflammation also contribute to poor glucose-stimulus-secretion coupling in the IUGR β cells. Although full islet morphology assessments were not performed, our results may coincide with improved β cell mass, as previous studies showed that fetal β cell

mass reduced by placental insufficiency directly leads to poor insulin secretion (Gatford and Simmons, 2013). Moreover, fetal β cells require glucose for proper development, and EPA has been shown to improve glucose uptake in other cell types (Figueras et al., 2011). Similar improvements in β cells would help explain the better functionality observed here. In addition, glucose-to-insulin ratios were recovered in IUGR fetuses receiving ω-3 PUFA treatment at baseline and were partially recovered at hyperglycemia, and the hypoglycemia observed in IUGR fetuses was also fully recovered. These results build upon our earlier studies that demonstrated fetal inflammation in the IUGR fetus (Cadaret et al., 2019a; Posont and Yates, 2019) by showing that metabolic function in IUGR fetuses could be improved when fetal inflammation is controlled.

Although previous studies suggested a shift to greater relative glycolytic lactate production concomitant with a reduction in glucose the oxidation in the IUGR concentrations, blood lactate concentrations did not differ among any of our groups under basal or hyperglycemic conditions. However, this may have been due to a coincident increase in hepatic lactate uptake, which would be consistent with increased Cori Cycle activity in the IUGR fetus (Thorne et al., 2014). Nevertheless, increased lactate production can cause insulin resistance in IUGR individuals (Kim et al., 2019), and previous work by us and others shows that limited capacity for glucose oxidation in favor of lactate production provides carbon substrates for hepatic glucose production in the IUGR fetus (Brown et al., 2015; Cadaret et al., 2019a). As expected, blood pH was decreased in our IUGR fetuses, despite our observation of greater blood HCO_3. Unlike HCO_3, however, blood pH was partially recovered by EPA infusion. Controlling fetal inflammation with ω-3 PUFA also improved CO_2 and O_2, which were predictably increased and decreased by IUGR, respectively. Although a beneficial outcome, it is unclear from this

study whether these improvements were due to change in placental function or fetal metabolism, and thus follow-up studies are warranted. Together, these findings further support our hypothesis that skeletal muscle glucose metabolism in IUGR fetuses reflects a key nutrient-sparing mechanism.

In addition to the adaptive change in glucose metabolism, lipid homeostasis and protein cycling may also be altered in the IUGR fetus. NEFA concentrations are indicative of increased lipolytic activity and are consistent with increased fat mobilization and/or decreased fat deposition, which is a hallmark of IUGR (Ortega-Senovilla et al., 2010; Yates et al., 2018). In our present study, IUGR fetuses exhibited increased blood NEFA concentrations that were completely resolved in EPA-infused IUGR fetuses, demonstrating a beneficial effect of these long-chain fatty acids in lipid homeostasis. Moreover, BUN levels were increased in IUGR fetuses but were lower in EPA-infused IUGR fetuses than IUGR fetuses and controls. We postulate that this was indicative of less protein catabolism, although we cannot rule out the possibility of increased kidney function. Indeed, decreased renal blood flow has been observed previously in IUGR fetuses and suggested disturbance of renal function (Todros et al., 1999). Thus, increased BUN in the blood of IUGR fetuses could have been due at least in part to a lack of filtration of residual urea, causing it to accumulate in the bloodstream. Interestingly, HDLc was not affected by IUGR alone, but was increased by EPA infusions. Considering the function of HDLc molecules, we presume that there were being taken from peripheral tissue back to the liver at greater rates. This occurs for the attached cholesterol to be utilized as precursor for bile salts, after which and is the HDL is esterified and returned to the liver (Gupta and Rajagopal, 2007). Because of this, it would be reasonable to expect the increase in circulating PUFA due to EPA infusion would make additional bile salts to break down EPA.

Hyperinsulinemic Euglycemic Clamp

Similar to our results during daily and hyperglycemic clamp assessments, blood glucose was diminished by approximately 64% in IUGR fetuses during the basal and hyperinsulinemic periods of the HEC study. However, these concentrations were partially rescued by infusion of EPA. Conversely, blood lactate concentrations were increased in IUGR fetuses and were not improved by EPA infusions. Together with other stress factors, the impact of lactate levels on insulin resistance in IUGR tissues could be a contributor to and/or cause of the change in glucose metabolism (Kim et al., 2019). Regardless, lactate production provides substrates for hepatic glucose production in the IUGR fetus via the Cori Cycle, since glucose itself cannot leave skeletal muscle (Brown et al., 2015; Cadaret et al., 2019a).These results allow us to postulate that, although glycemia was partially recovered, the lack of differences for blood lactate concentrations may be explained by increased hepatic lactate utilization that offsets the presumed increase in secretion of lactate by muscle. This, more direct studies of lactate production rates are needed. In contrast to lactate, blood pH was increased in IUGR fetuses, but was recovered by daily EPA infusion. This would indicate that more mechanisms than just lactate were influencing blood pH. For example, although pCO_2, which was recovered in the daily blood samples as well as during the hyperglycemic clamp did not differ among groups during the HEC study, which might help explain the paradoxical rise in pH which had previously been less in out IUGR fetuses.

Poor skeletal muscle glucose uptake is a well-characterized hallmark of IUGR fetuses and offspring (Cadaret et al., 2019c; Yates et al., 2019; Posont et al., 2021b). Thus, it was not surprising that in the present study, hindlimb glucose uptake was reduced by 400% in the IUGR fetuses in the present study. It was somewhat unexpected, however, that EPA-infusion resulted in

no improvement. This suggests that EPA was not successful in recovering glucose uptake despite our other evidence that inflammation was the underlying mechanism for this deficit. This contrasted previous findings in the literature, specifically when evaluating the effect of the ω-3 PUFA, EPA, on GLUT4 translocation, which is the rate-limiting step for insulin-stimulated glucose uptake (Kim et al., 2019). However, preserved glucose uptake in IUGR fetuses has been observed previously (Limesand et al., 2007; Brown et al., 2015) and it is worth noting that only about 40% of the hindlimb tissues are skeletal muscle (Hicks and Yates, 2021). Nevertheless, the response of skeletal muscle glucose oxidation rates to insulin was markedly impaired in the IUGR fetuses, and this deficit was not improved by EPA-infusion. Indeed, the 8-fold change in glucose oxidation observed when uncompromised fetuses were infused with insulin only increased 3-fold in IUGR fetuses, whether infused with saline or EPA. This suggests that although several components of glucose homeostasis were improved by daily EPA infusion into IUGR fetuses, insulin-mediated metabolic processes in the hindlimb tissues were not.

Together, these findings allow us to conclude that multiple factors contributing to the dynamic dysregulation of glucose homeostasis in the late-term IUGR fetus were improved by controlling fetal systemic inflammation through EPA infusion. Improvements in insulin secretion indicated that pancreatic β cell function was recovered. However, other important indicators of metabolic function and health remained deficient. This provides direction to the next step in order to determine what additional programming mechanisms must be targeted for a broader improvement in metabolism. Nevertheless, results of the current study indicate that moderation of fetal inflammation via EPA supplementation can have a beneficial impact on developmental programming of IUGR.

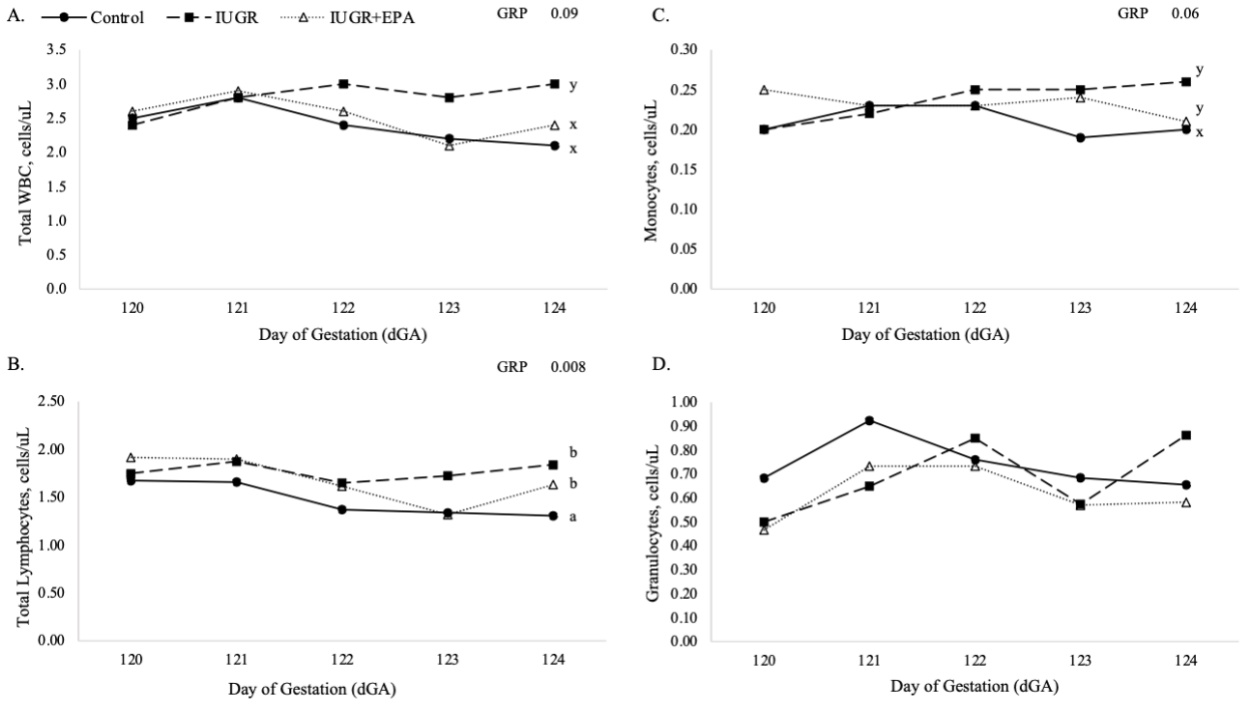

Figure 2-1. Daily total white blood cell (A), lymphocyte (B), monocyte (C), and granulocyte (D) concentrations from Control (n=12), IUGR (n=5), and IUGR+EPA (n=6) fetuses. a,b means with differing superscripts differ ($P<0.05$). x,y means with different superscripts tend to differ ($P < 0.10$).

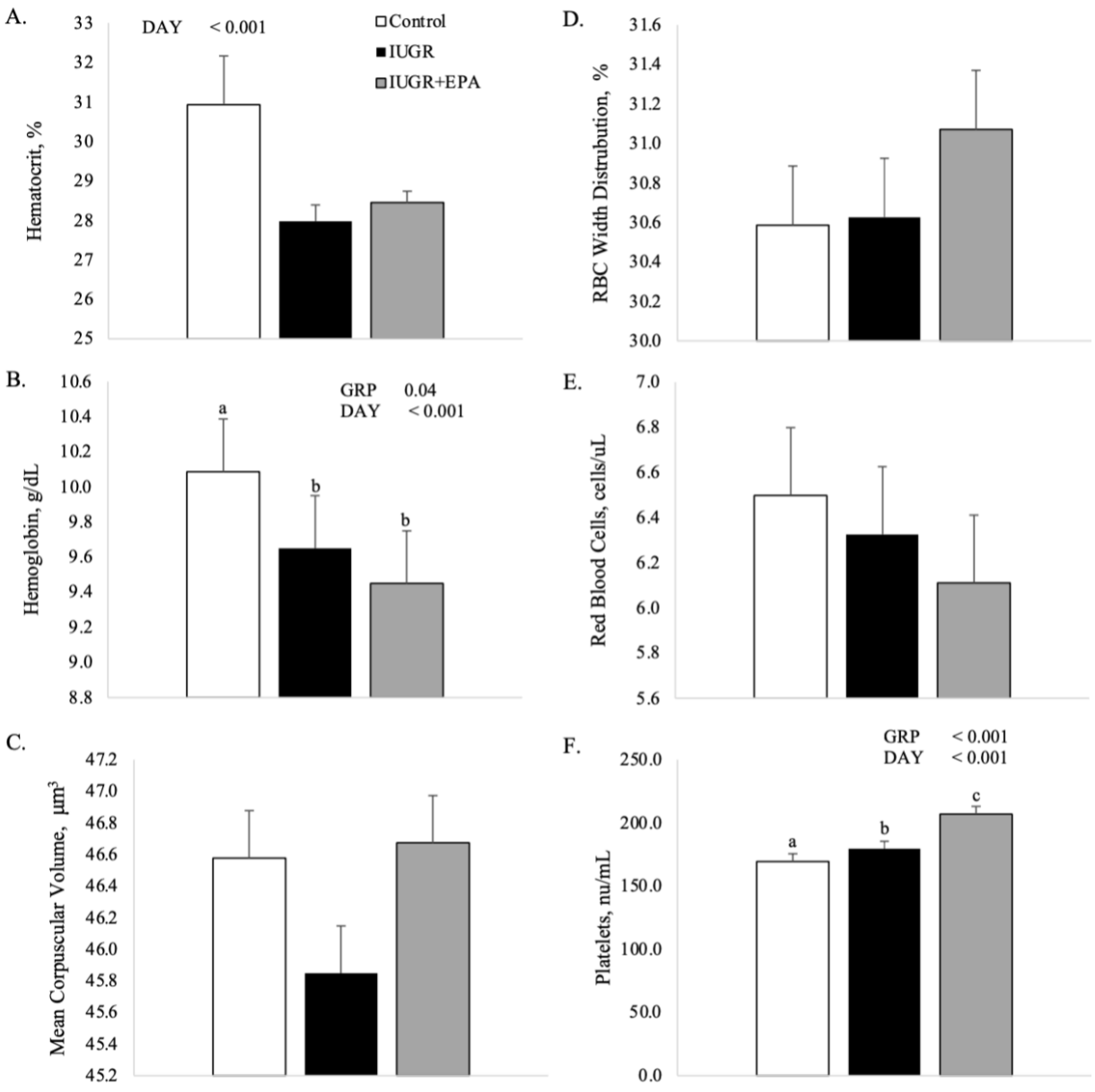

Figure 2-2. Data are shown for daily hematocrit percentage (A), hemoglobin (B), mean corpuscular volume (C), red blood cell width distribution (D), red blood cells (E) and platelet count (D) from Control (n=12), IUGR (n=5), and IUGR+EPA (n=6) fetuses. a,b means with differing superscripts differ ($P<0.05$).

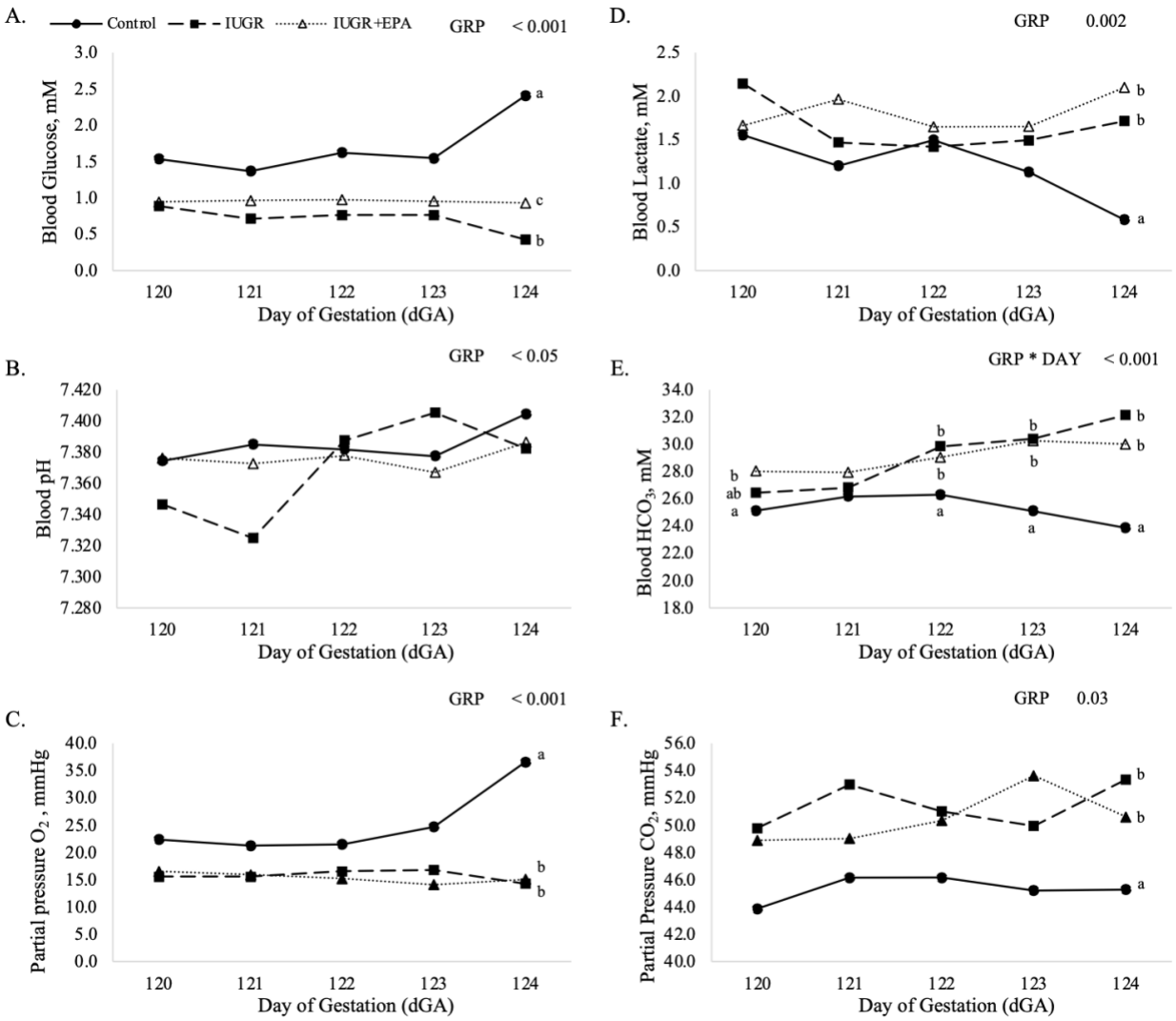

Figure 2-3. Daily blood glucose (A), pH (B), partial pressure O_2 (C), blood lactate (D), HCO_3 (E) and partial pressure CO_2 (D) from Control (n=12), IUGR (n=5), and IUGR+EPA (n=6) fetuses. a,b means with differing superscripts differ (P<0.05).

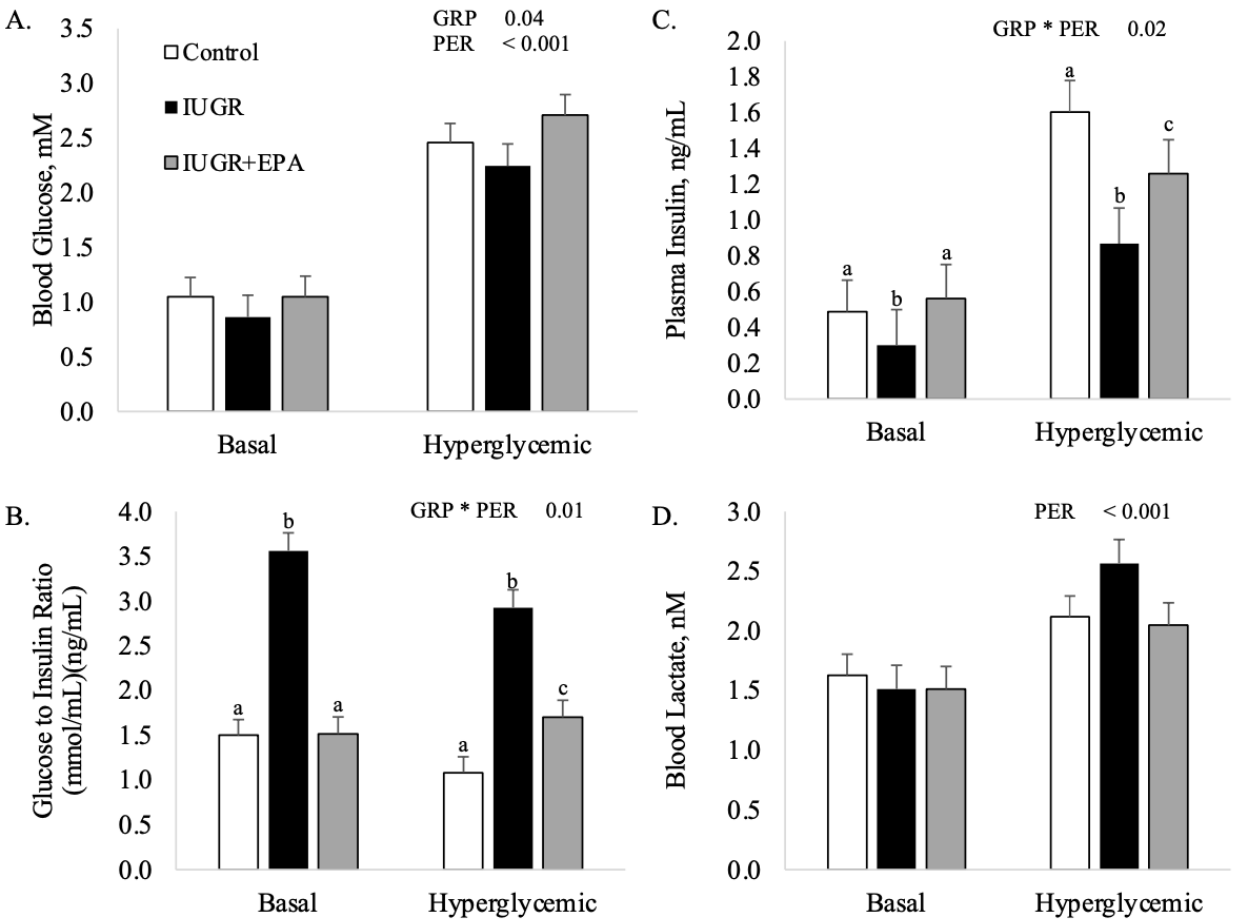

Figure 2-4. Blood glucose (A), glucose-to-insulin ratio (B), plasma insulin (C), and blood lactate (D) concentrations determined at basal and hyperglycemic conditions. Data are shown for Control (n=12), IUGR (n=3), and IUGR+EPA (n=4) fetuses. a,b means with differing superscripts differ (P<0.05).

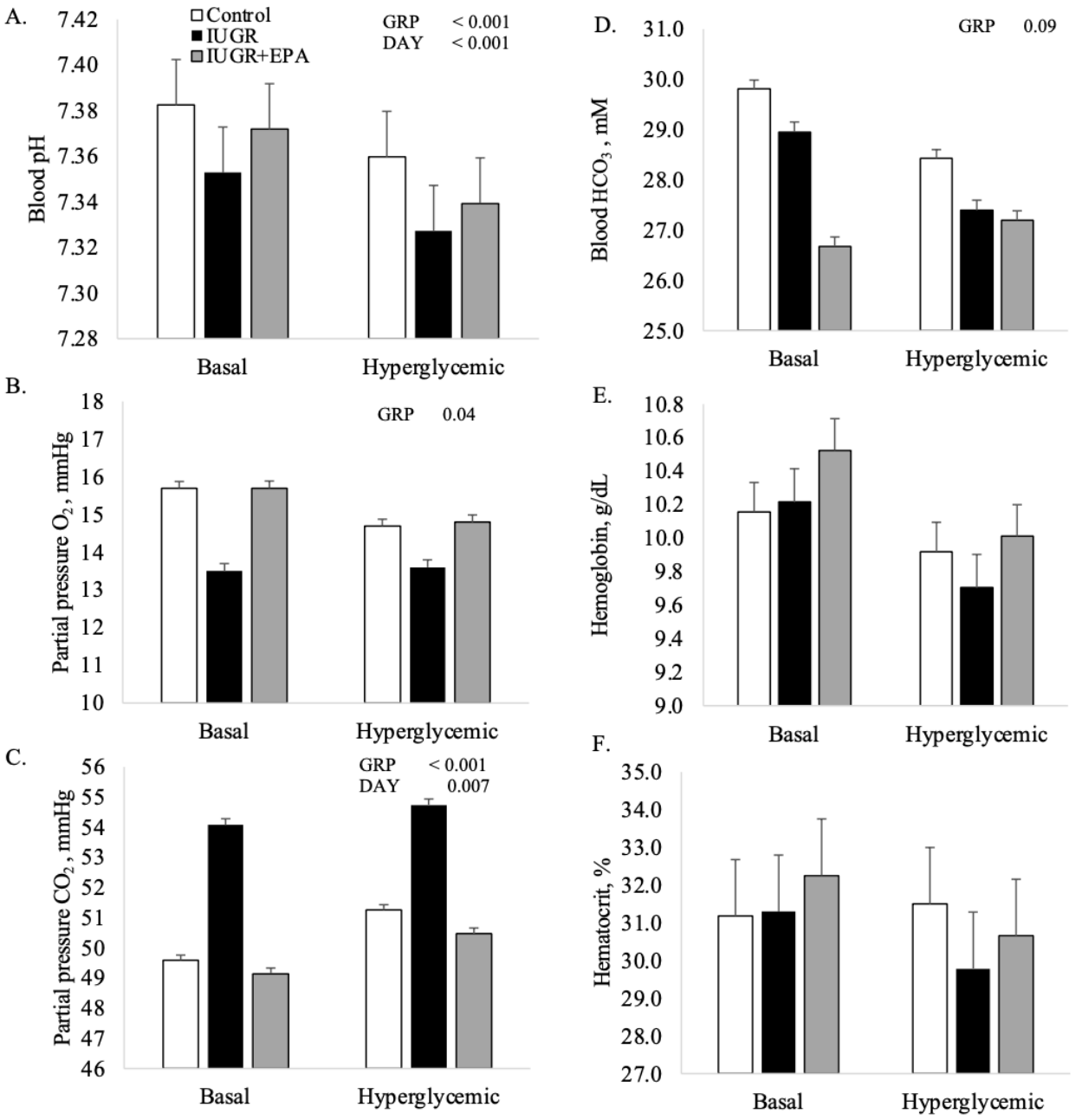

Figure 2-5. Data are shown for pH (A), partial pressure O_2 (B), partial pressure CO_2 (C), HCO_3 (D), hemoglobin (E), and hematocrit % (D) at basal and hyperglycemic conditions. Data are shown for Control (n=12), IUGR (n=3), and IUGR+EPA (n=4) fetuses.

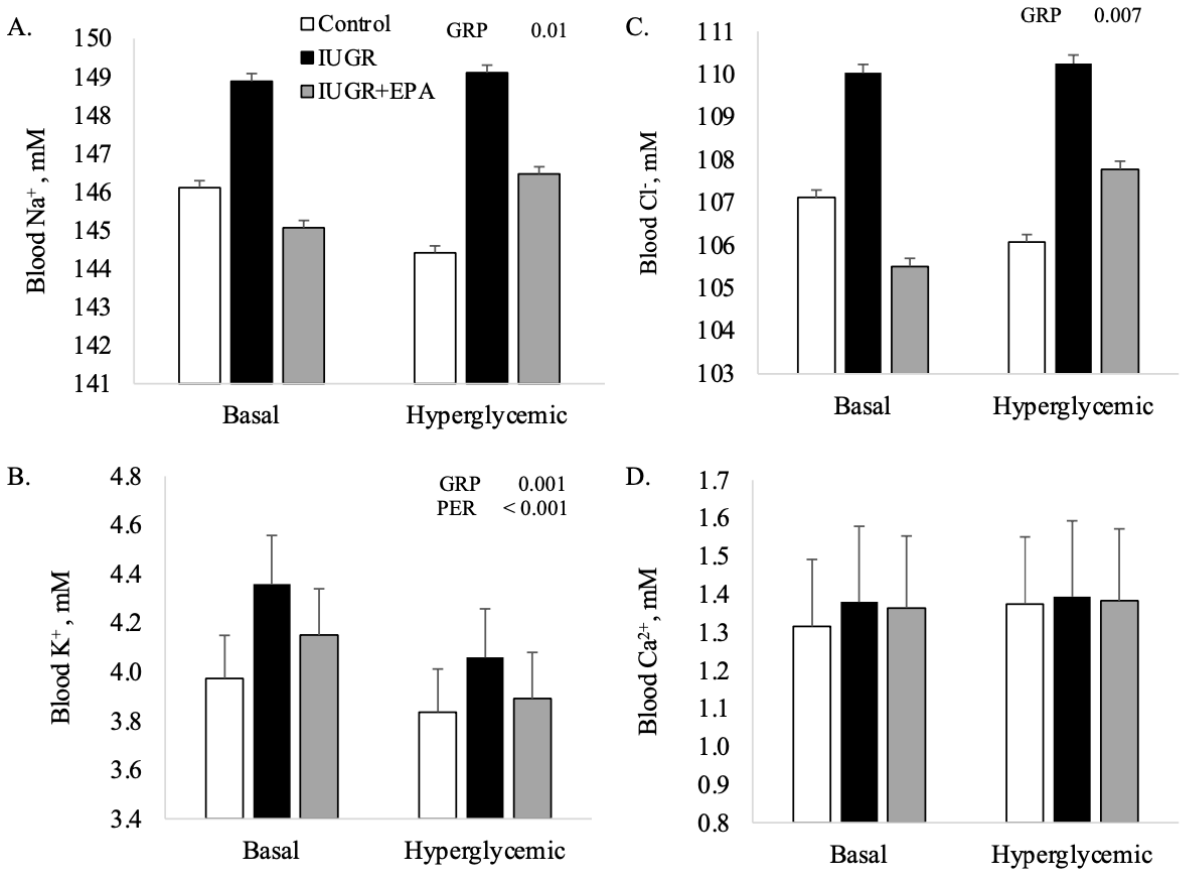

Figure 2-6. Na^+ (A), K^+ (B), Cl^- (C), and Ca^{2+} (D) measures are reported at basal and hyperglycemic conditions. Data are shown for Control (n=12), IUGR (n=3), and IUGR+EPA (n=4) fetuses.

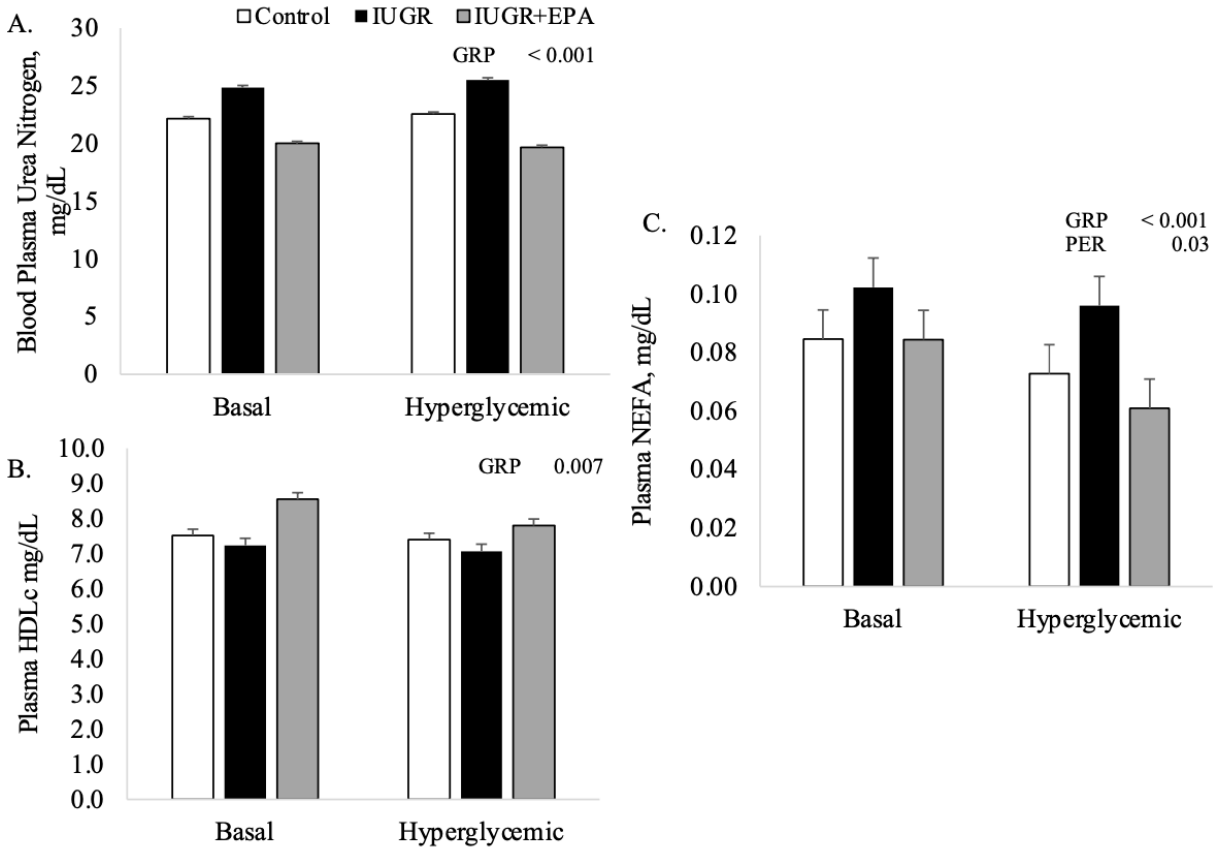

Figure 2-7. Data are shown for plasma blood urea nitrogen (A), plasma HDLC (B), and plasma NEFA concentrations (C) for Control (n=12), IUGR (n=3), and IUGR+EPA (n=4) fetuses. a,b means with differing superscripts differ (P<0.05).

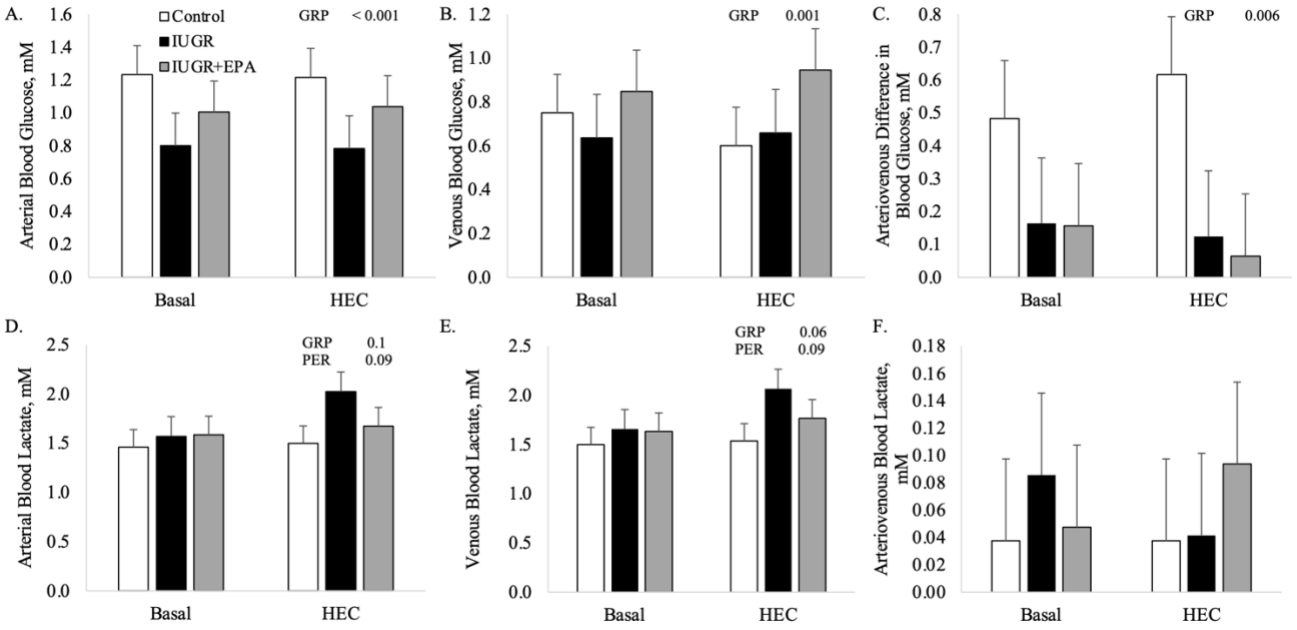

Figure 2-8. Blood glucose at arterial (A), venous (B), arteriovenous difference (C). Blood lactate at arterial (D), venous (E), and the arteriovenous difference (F). Data are shown at basal and HEC conditions for Control (n=3), IUGR (n=4), and IUGR+EPA (n=6) fetuses.

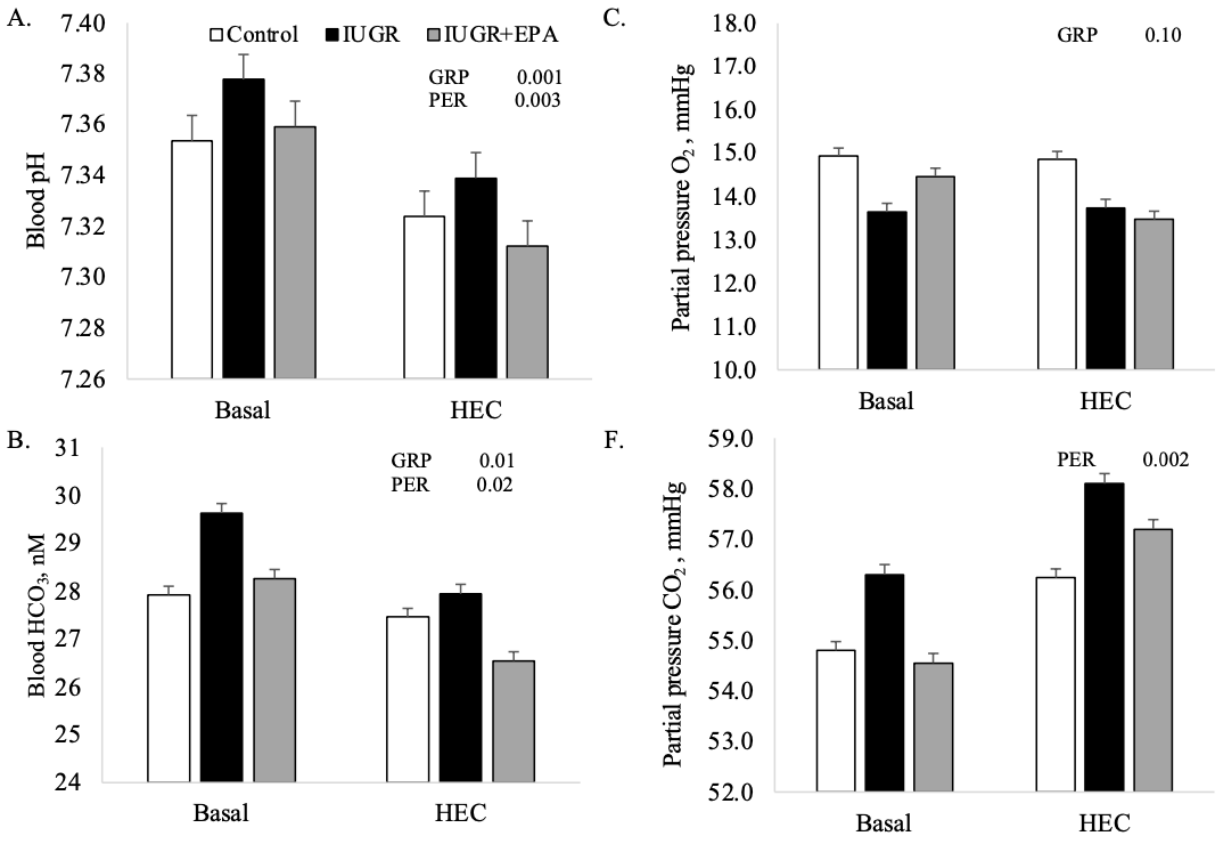

Figure 2-9. Blood pH (A), HCO_3 (B), partial pressure O_2 (C), and partial pressure CO_2 (D) at basal and HEC conditions. Data are shown for Control (n=3), IUGR (n=4), and IUGR+EPA (n=6) fetuses.

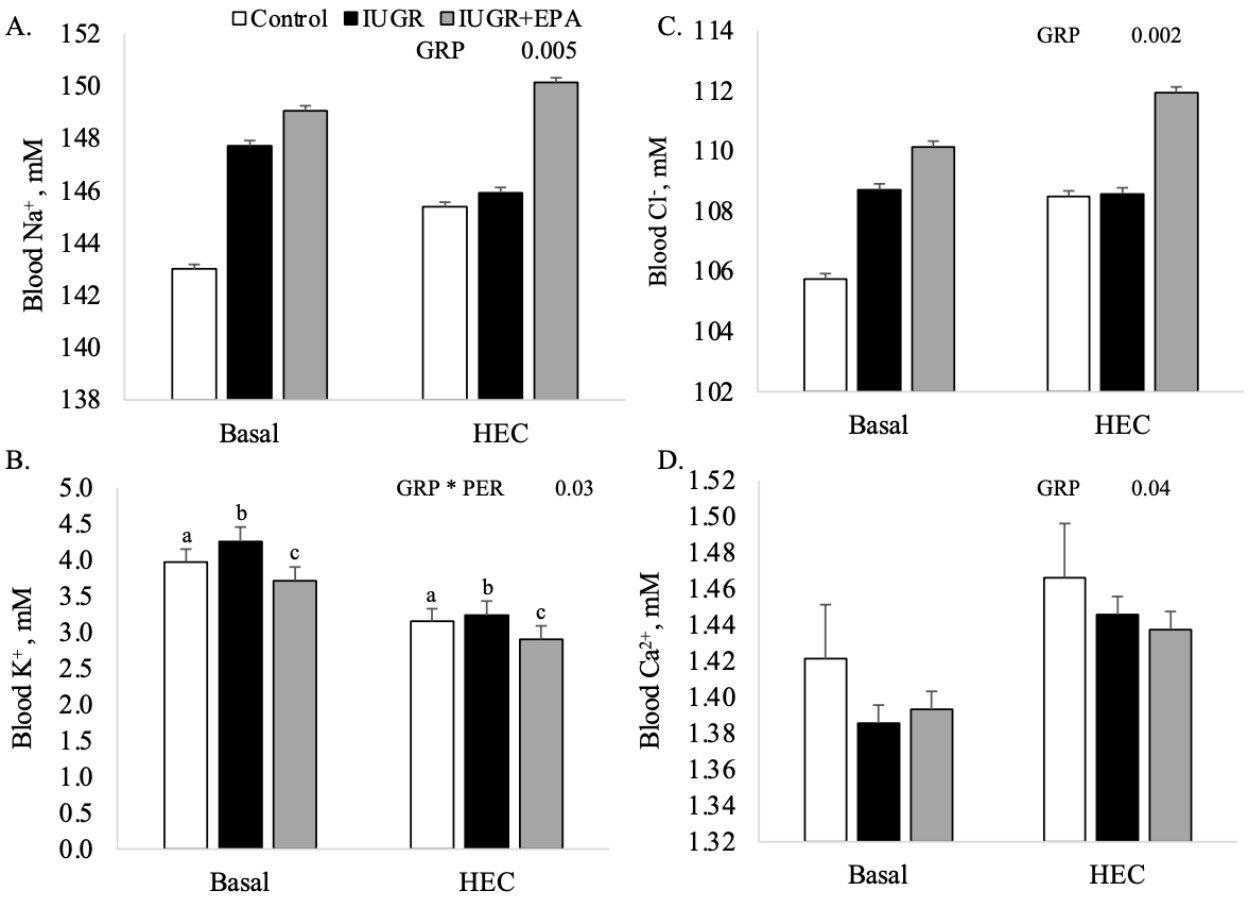

Figure 2-10. Data are shown for hemoglobin (A), hematocrit % (B), and plasma NEFA (C) are shown at basal and HEC for Control (n=3), IUGR (n=4), and IUGR+EPA (n=6) fetuses. a,b means with differing superscripts differ (P<0.05).

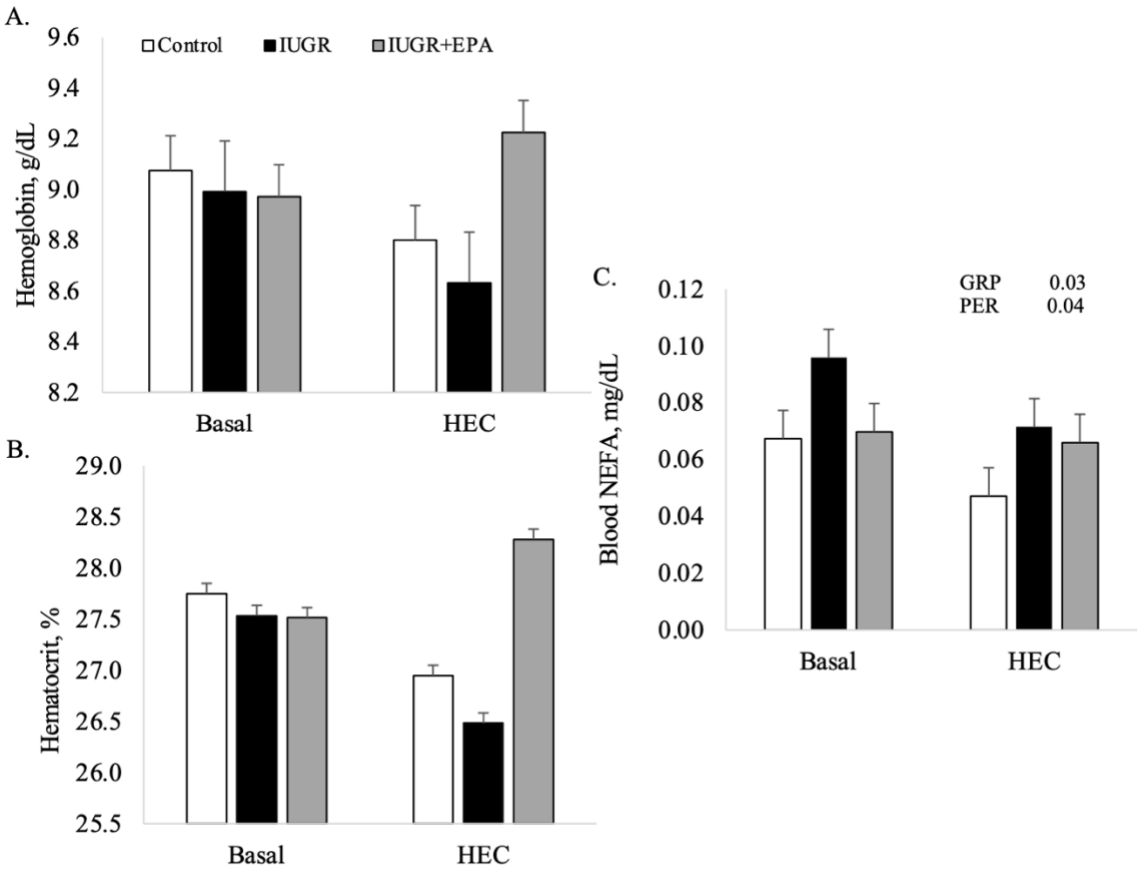

Figure 2-11. Basal and HEC concentrations for Na^+ (A), K^+ (B), Cl^- (C), and Ca^{2+} (D) are shown for Control (n=3), IUGR (n=4), and IUGR+EPA (n=6) fetuses.

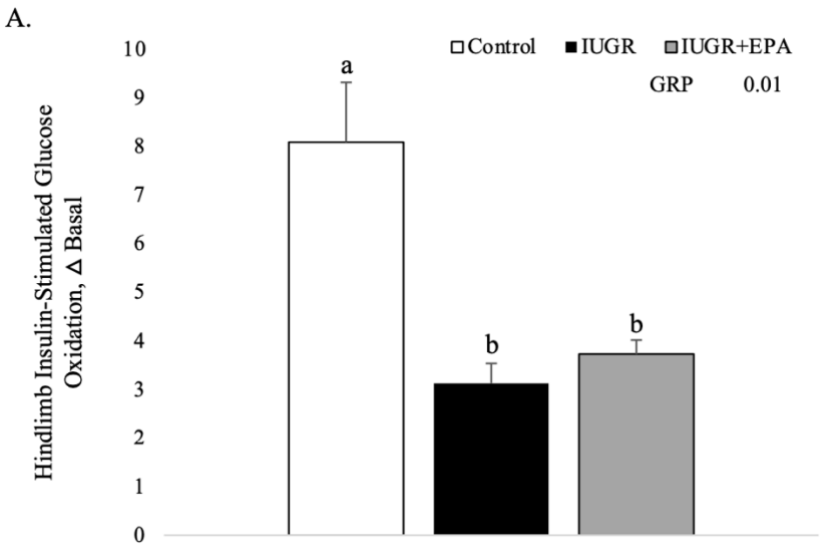

Figure 2-12. Insulin sensitivity for hindlimb skeletal muscle glucose oxidation during steady-state hyperinsulinemia measured by fold change from basal and hyper insulinemic-euglycemic conditions. Data are shown for Control (n=3), IUGR (n=4), and IUGR+EPA (n=6) fetuses. a,b means with differing superscripts differ ($P<0.05$).

Chapter 3

Fetal growth, body composition, and *ex vivo* glucose metabolism are impaired in the IUGR fetal sheep but are improved by daily ω-3 PUFA infusion.

ABSTRACT

Chronic maternal stress during peak placental development yields placental insufficiency-induced intrauterine growth restriction (**IUGR**) and causes biometric and metabolic deficits in the late-term fetus and offspring. Placental insufficiency limits nutrient transfer across the placenta, which forces the fetus to adapt its physiological processes for growth and metabolism in order to survive. These adaptations repartition limited nutrients to vital tissues and organs, and thus less vital tissues such as skeletal muscle are compromised by decreased growth potential and impaired metabolic capacity. In this study, IUGR fetal sheep were produced using the maternal hyperthermia model while controls were housed at thermoneutral conditions. Indwelling fetal femoral catheters were placed at 118 days of gestation (**dGA)** to allow for daily intravenous (**i.v.**) infusion of IUGR fetuses with eicosapentaenoic acid (**EPA**) (IUGR+EPA; n=6) or EtOH-spiked (**EtOH**) saline placebo (IUGR; n=5) and controls with an EtOH-saline placebo (n=12). Infusions were administered for 5 ±1 d beginning at 120 ±1 dGA. Necropsy was performed at 126 ±1 dGA and fetal biometrics were assessed. IUGR fetuses were lighter ($P < 0.05$) than controls but IUGR+EPA bodyweight was partially recovered. IUGR fetuses also exhibited reduced ($P < 0.05$) hindlimb, *semitendinosus* (**ST**), and *longissimus dorsi* (**LD**) masses and tended to exhibit reduced ($P < 0.10$) *soleus* mass compared to controls. EPA infusion increased ($P < 0.05$) hindlimb, ST, and LD mass and tended to increase ($P < 0.10$)

soleus and ST mass compared to saline-infused IUGR fetuses. *Flexor digitorum superficialis* (**FDS**) muscle mass did not differ among groups. IUGR reduced ($P < 0.05$) heart, lung, and kidney mass and tended to reduce ($P < 0.10$) liver mass. EPA infusion recovered ($P < 0.05$) lung and kidney mass and tended to recover ($P < 0.10$) liver mass but did not improve heart mass. Brain mass did not differ among groups. Fiber type proportions in the ST were estimated by myosin heavy chain ratios determined by electrophoresis. Myosin for Type I fibers did not differ among groups, but myosin for Type IIA fibers tended to be decreased ($P < 0.10$) and myosin for Type IIx fibers tended to be increased ($P < 0.10$) in IUGR fetuses compared to controls. EPA infusion tended ($P < 0.10$) to recover myosin ratios for two of these fiber types. ST glycogen content was not different among groups. *Ex vivo* analysis of skeletal muscle specific glucose metabolism indicated a decrease ($P < 0.05$) in glucose uptake for IUGR fetal muscle that was partially recovered ($P < 0.05$) by EPA infusion. Likewise, glucose oxidation rates were impaired ($P < 0.05$) for IUGR fetal muscle but were fully recovered by EPA infusions. From these findings, we can conclude that daily administration of ω-3 PUFA directly to the fetus was effective in improving several biometric and metabolic deficits in late-term IUGR fetuses.

INTRODUCTION

Acute stress is beneficial for a multitude of reasons, including its activation of growth factor synthesis and physiological alterations as part of the fight or flight response (Weissman, 1990). However, prolonged stress exposure in pregnant animals is detrimental to the life-long health of offspring (Hales and Barker, 1992). Placental insufficiency-induced intrauterine growth restriction (IUGR) occurs as a result of chronic maternal stress during peak placental development, which causes stunting of the placenta by re-routing uterine blood flow (Murphy et al., 2006; Brown et al., 2015). Placental stunting results in a limited supply of nutrients to the fetus and therefore prompts fetal stress responses that induce nutrient-sparing adaptive mechanisms (Limesand et al., 2007; Brown et al., 2015; Dunlop et al., 2015). Although these adaptations ensure intrauterine survival, they lead to programmed deficits such as impaired myogenesis, asymmetric growth, and dysregulation of glucose metabolism that persist after birth (Yates et al., 2014; Cadaret et al., 2019c; Gibbs et al., 2020). Such adaptive mechanisms appear to occur in part as a response to systemic inflammation and remain even when the systemic inflammation is alleviated after birth. Therefore, the objective of this study was to evaluate the effectiveness of daily ω-3 polyunsaturated fatty acids (PUFA) infusions on fetal IUGR sheep in order to mediate inflammation. Previous literature has established the effectiveness of ω-3 PUFA in decreasing inflammatory signaling (Caughey et al., 1996; Velten et al., 2014; Allam-Ndoul et al., 2016; Kim et al., 2019). Thus, use of this nutraceutical in mediating the fetal inflammatory response may improve metabolism and biometric deficits. We hypothesized that these deficits will be improved by daily infusion of IUGR fetuses with the ω-3 PUFA, eicosapentaenoic acid (EPA), in late gestation.

MATERIALS & METHODS

Animals and Experimental Design

These studies were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln, an AAALAC International-accredited institution. The well-characterized maternal hyperthermia model was used to generate placental insufficiency induced IUGR fetal sheep as previously described by Yates et al. (2016) and Cadaret et al. (2018). Briefly, gestating ewes were exposed to elevated ambient conditions of 40°C and 35% relative humidity during peak placental development (dGA 40-95) before returning to thermoneutral conditions (25°C) alongside their control counterparts. This study utilized time-mated Polypay ewes to carry control and IUGR fetuses. Indwelling catheters were placed at 118 dGA in the femoral vein and artery of the fetus as previously described (Cadaret et al., 2019a). Catheterizations allowed for the daily infusion of fetuses. IUGR fetuses were randomly assigned to receive daily infusions of 0.25 mg/d EPA (i.e., IUGR+EPA; n=6) or saline placebo (i.e., IUGR; n=5) from dGA 121 to 125 ± 1 d. Control fetuses (n=12) also received a saline infusion. Infusions were administered intravenously (i.v.) into the fetal femoral vein over a period of 1 hour. Animals were euthanized at dGA 125 ± 1 d by double barbiturate overdose and tissue and organ metrics were assessed. Fetal bodyweight and total hindlimb mass were recorded in addition to masses for *semitendinosus* (**ST**), *soleus*, *longissimus dorsi* (**LD**), and *flexor digitorum superficialis* (**FDS**) muscles. Organ metrics included heart, lung, liver, kidney, and brain masses.

Ex Vivo Glucose Metabolism

Flexor digitorum superficialis (**FDS**) and *soleus* muscles were collected at necropsy for *ex vivo* analysis of glucose metabolism. Muscle was washed in ice-cold Phosphate-buffered

saline solution (**PBS**), and strips were split from tendon-to-tendon, creating at 50 ± 5 mg strips. Strips were pre-incubated for 1 hour in Krebs-Heneslit Buffer (KHB) spiked with no additive (basal), or insulin (5 mU/ml Humulin-R). Muscle strips were then washed in respectively-spiked glucose-free KHB media for 20 min. Glucose oxidation was measured using sealed dual-well chambers as previously described (Cadaret et al., 2017). Briefly, muscle strips were incubated in KHB spiked with 5 mM [^{14}C-U] D-glucose for 2 hr. The adjacent well contained 1M NaOH. Chambers were then cooled on ice and wells containing muscle strips were injected with 2M HCl and allowed to incubate at 4°C for an additional 1 hr. During this final incubation, bicarbonate-bound $^{14}CO_2$ was liberated from the media and captured by the NaOH. The forementioned NaOH was collected and deposited in a 20-mL scintillation vial and filled with with UltimaGold scintillation fluid (Perkin-Elmer). Vials were assessed with an LC counter (Beckman-Counter 1900 TA, Brea, CA) to measure $^{14}CO_2$ concentrations. To measure glucose uptake, muscle strips were incubated in KHB media containing no additive (basal) or insulin (5 mU/ml Humulin-R) and 1 mM [^{3}H] 2-deoxy-d-glucose for 20 min. Following incubation, muscle strips were cooled and washed in PBS, and placed in 20-mL scintillation vials with 2M NaOH to be lysed at 37°C for 2 hours. Liquid scintillation was then used to determine the concentration of [^{3}H] 2-deoxy-d-glucose for each lysate.

Myosin Heavy Chain Electrophoresis

At necropsy, 100 mg of snap-frozen ST muscle was collected, homogenized, and sonified in 400 µl of RIPA buffer (Thermo Fisher). Samples were then centrifuged (14,000 x g, 2min, 4°C) and supernatant was collected. Pierce BCA Assay (Thermo Fisher) was used to measure total protein content. Samples were then combined with Bio-Rad 4× Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and dry-boiled for 10 minutes at 70°C. Samples were then equilibrated

to room temperature and 40 μg of protein was loaded into each well. Upper running buffer contained 100 mM Tris, 150 mM glycine, 0.1% SDS, and 0.07% 2β-mercaptoethanol in distilled water, and the lower running buffer consisted of 50 mM Tris, 75 mM glycine, and 0.05% SDS in distilled water. Mini-PROTEAN gradient gels (4-15%; Bio-RAD). Electrophoresis was performed at room temperature on a PowerPac Basic (Bio-RAD) at a constant voltage of 110V for 3 hours. Following electrophoresis, gels were stained overnight in Gel-Code Blue (Thermo Fischer) at room temperature, washed in distilled water, and imaged using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Quantification of MyHC-I, MyHC-IIA, and MyHC-IIx was completed by densitometry (Image Studio Lite Ver 5.0; LI-COR) in order to estimate fiber type ratios.

Glycogen Colorimetric Assay

Snap-frozen ST muscle samples (100 mg) were homogenized with 1 mL distilled water and sonicated. Homogenates were boiled (95°C x 5 minutes), centrifuged (13,000g x 5 minutes), and supernatant was collected. Samples were diluted in Hydrolysis buffer (Sigma-Aldrich) at 1:200. Glycogen content was then measured by a commercial Glycogen Assay Kit (Sigma-Aldrich, St. Louis, MO). Inter- and intra- assay coefficients of variance were less than 15%.

Statistical Analysis

All data were analyzed with SAS 9.4 (SAS Institute, Cary, NC) using the mixed procedure, with the fetus as the experimental unit. Sex was treated as a random effect. Technical replicates were averaged, and data are presented as mean ± standard error. Significant differences were declared at $\alpha \leq 0.05$ and tendencies at $\alpha \leq 0.10$.

RESULTS

Fetal Biometrics

At necropsy, fetal body weight was 23% less ($P < 0.05$) for IUGR fetuses than for controls and was intermediate ($P < 0.05$) for IUGR+EPA fetuses (**Table 3-1**). Fetal hindlimbs were lighter ($P < 0.05$) for IUGR fetuses but not for IUGR+EPA fetuses than for controls. ST muscle mass was less ($P < 0.05$), and soleus muscle mass tended to be less ($P < 0.10$) for IUGR fetuses than controls and were intermediate ($P < 0.05$) for IUGR+EPA fetuses. LD muscle mass was less ($P < 0.05$) for IUGR fetuses but not for IUGR+EPA fetuses than for controls. FDS muscle mass did not differ among groups. Heart mass was less ($P < 0.05$) and liver mass tended to be less ($P < 0.05$) for IUGR and IUGR+EPA fetuses than for controls. Lung mass and kidney mass were lighter ($P < 0.05$) for IUGR fetuses but not for IUGR+EPA fetuses than for controls. Brain mass did not differ among experimental groups.

Ex vivo Skeletal Muscle Glucose Metabolism

Glucose uptake rates in FDS and soleus muscle were 36% less ($P < 0.05$) for IUGR fetal muscle than controls but were only 14% less ($P < 0.05$) for IUGR+EPA fetal muscle, regardless of media conditions (**Figure 3-1**). Glucose oxidation likewise was less ($P < 0.05$) for IUGR fetal muscle compared to controls, but fully recovered in IUGR+EPA fetal muscle compared to controls, regardless of media conditions. Glucose oxidation was increased ($P < 0.05$) when muscle was incubated in insulin-spiked media compared to basal media, regardless of experimental group.

Skeletal Muscle Composition

Proportions of MyHC-I in ST protein isolates did not differ among groups. However, both MyHC-IIA and MyHC-IIx proportions tended to be less ($P < 0.10$) in the IUGR fetus

compared to IUGR+EPA fetuses and controls (**Figure 3-2**).. Semitendinosus glycogen content did not differ among groups.

DISCUSSION

In this study, we found that biometric deficits in the IUGR fetus were consistent with the decreased muscle mass and asymmetric growth as previously observed (Cadaret et al., 2019c; Yates et al., 2019; Posont et al., 2021b). However, targeting systemic inflammation in these fetuses provided promising results that indicate improvement in total body and muscle-specific growth. As expected, bodyweight was less in IUGR fetuses because of restricted growth *in utero*. Moreover, partial recovery of several growth and body symmetry metrics indicates that EPA administration is effective on potential intervention for biometric deficits. In fact, the mass of the hindlimb, two of the three hindlimb muscles (ST and *soleus)* and the LD, were fully recovered by this daily ω-3 PUFA regimen of EPA. The importance of skeletal muscle to metabolic health and glucose homeostasis cannot be overstated, as it clears 65% of total glucose from circulation (Yates et al., 2018). Thus, improved muscle mass almost assuredly contributed to improved glucose-to-insulin ratios reported in the previous chapter. Improved muscle mass presumably increased the capacity for glucose utilization and oxidation independent of any changes in metabolic function proper. Consistent with known nutrient repartitioning mechanisms, our IUGR fetuses developed smaller hearts, lungs, livers, and kidneys (Brown et al., 2015; Dunlop et al., 2015). Daily ω-3 PUFA administration of EPA was successful in recovering lung and kidney size but did not improve heart or liver mass. The later observations were somewhat unexpected, as both heart and liver appeared to be priority tissues for preserved growth in earlier studies (Cadaret et al., 2019a; Posont et al., 2021a). Nevertheless, comparable brain weights among the

three groups indicates that growth of the fetus' highest priority, neural tissue, was indeed maintained.

Deficits in skeletal muscle-specific glucose metabolism observed in IUGR fetuses was improved by ω-3 PUFA in our *ex vivo* analysis. Inflammation in the IUGR fetuses presumably decreased translocation of GLUT-4 transporters which is the rate-limiting mechanism for insulin-stimulated glucose uptake (Lorenzo et al., 2008). Although this study did not directly assess GLUT-4, the results of decreased glucose uptake was observed in muscle from IUGR fetuses. Kim et al. (2019) provided a description of the mechanism by which EPA increases AMP:ATP ratios, thus increasing phosphorylation of AMP-activated protein kinase (**AMPK**). This AMPK activation step is important in the general regulation of cellular growth and metabolism but acts in part by increasing translocation of GLUT-4 in muscle. Whether by this or other mechanisms, our study found a partial recovery of glucose uptake when IUGR fetuses were infused with EPA, suggesting that inflammatory mediation was at least partially inhibiting inflammatory action and, in turn, reducing glucose uptake. In addition to glucose uptake, we observed reduced glucose oxidation rates in muscle from IUGR fetuses. This was expected based on the findings of previous studies that reported reduced whole-body glucose oxidation in IUGR fetal sheep (Limesand et al., 2007; Brown et al., 2015). Brown et al. (2015) described this as being the result of a shift in metabolism to greater lactate production concurrent with a reduced conversion of pyruvate to acetyl coA. Ultimately, this resulted in downstream changes that decreased the capacity for glucose oxidation, regardless of insulin stimulation. In our study, glucose oxidation was reduced by 28% in the IUGR fetal muscles but was recovered in both basal and insulin-spiked media when the muscle strips were from IUGR fetuses that had received ω-3 PUFA infusions.

Decreased Type I and IIA muscle fibers in IUGR fetuses are correlated with insulin insensitivity and altered glycolytic enzyme activity (Jensen et al., 2007). As such a shift in relative numbers from Type I and IIA fibers, which are oxidative in nature, to Type IIx fibers, which are glycolytic, would have obvious detrimental effects on glucose oxidation (Yates et al., 2016). Although myosin heavy chain indicators assessed in this study indicated that Type I fibers were not decreased in numbers by IUGR, decreased number of Type IIA fibers and increased numbers of Type IIx fibers was consistent with reduced glucose oxidation found in this study. Moreover, although ω-3 PUFA infusion unexpectedly failed to improve glucose oxidation *in vivo*, the combined findings of improved Type IIA fiber ratios and improved *ex vivo* glucose oxidation, indicate that ω-3 PUFA infusion was indeed beneficial for muscle glucose metabolism. Furthermore, the recovery of glucose oxidation *ex vivo* was independent of insulin activity. This likely explains the lack of an effect *in vivo*, as hindlimb glucose oxidation was assessed as the responsiveness to insulin stimulation.

IMPLICATIONS

The growth and metabolic deficits imposed by IUGR have been widely established in literature. These deficits, which result from physiological adaptations made *in utero*, yield prenatal and postnatal consequences for offspring. Nutraceutical moderation of inflammatory signaling in these fetuses, however, appeared to pose a promising intervention strategy to improve the pathological outcomes of these programming deficits. This study demonstrates that ω-3 PUFA was dynamically effective at recovering biometric deficits and partially recovering skeletal muscle-specific glucose metabolism. Although a fundamental step in establishing their efficacy, i.v. infusions of ω-3 PUFA are not feasible for humans or livestock producers, and therefore

further research is warranted to evaluate the effectiveness of administering EPA maternally via the diet or otherwise to target the fetus through placental transfer.

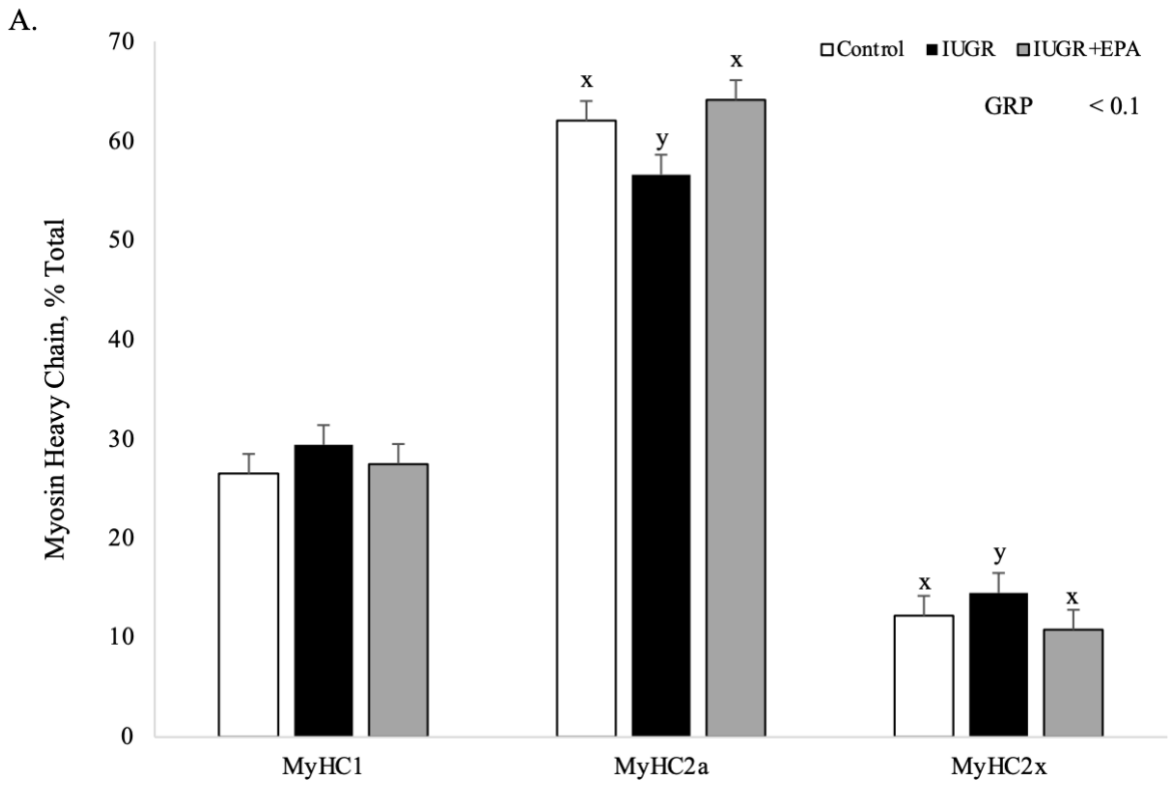

Figure 3-1. Proportion of myosin heavy chains, Type 1, 2A, and 2X, are shown for Control (n=12), IUGR (n=4), and IUGR+EPA (n=4) fetuses. x,y means with differing superscripts tend to differ (P<0.10).

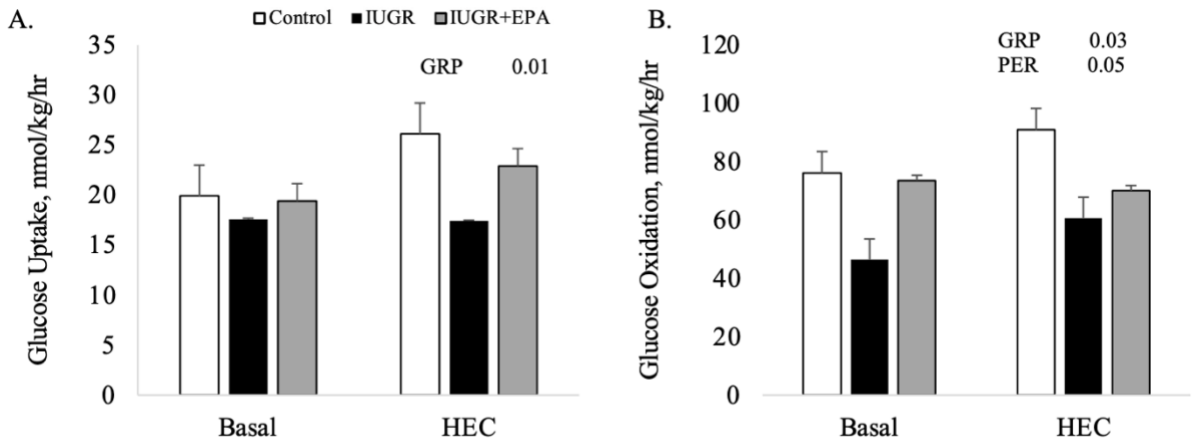

Figure 3-2. *Ex vivo* hindlimb specific glucose metabolism was assessed at necropsy by isolation of FDS and *soleus* muscle. Glucose uptake (A) and oxidation (B) are reported for Control (n=12), IUGR (n=5), and IUGR+EPA (n=6) fetuses.

Table 3-1. Fetal biometrics for ω-3 PUFA-infused IUGR fetal sheep.

Mass	Experimental Group			*P*-Value
	Control	IUGR	IUGR+EPA	
Fetal Weight, kg	3.1 ± 0.1^{a}	2.4 ± 0.2^{b}	2.9 ± 0.1^{c}	0.03
Hindlimb, g	308 ± 13^{a}	238 ± 20^{b}	294 ± 17^{a}	0.04
ST, g	6.5 ± 0.3^{a}	4.5 ± 0.5^{b}	5.5 ± 0.4^{c}	0.03
Soleus, g	1.03 ± 0.13^{x}	0.63 ± 0.11^{y}	0.89 ± 0.15^{z}	0.09
LD, g	61 ± 3^{a}	47 ± 2^{b}	60 ± 2^{a}	< 0.001
FDS, g	5.57 ± 0.39	3.83 ± 0.88	5.66 ± 0.45	NS
Heart, g	27.1 ± 1.3^{a}	21.5 ± 1.4^{b}	24.1 ± 1.4^{b}	0.02
Lungs, g	101 ± 4^{a}	88 ± 3^{b}	95 ± 4^{a}	0.02
Liver, g	121 ± 8^{x}	102 ± 8^{y}	104 ± 7^{y}	0.07
Kidneys, g	21.8 ± 1^{a}	13.9 ± 1.7^{b}	20.5 ± 1.3^{a}	<0.001
Brain, g	45.1 ± 1.3	41.5 ± 2.1	41.7 ± 1.6	NS

Data are presented as mean ± SE.

^{a,b,c} means with different superscripts differ ($P < 0.05$).

^{x,y,z} means with different superscripts tend to differ ($P < 0.10$).

NS, not significant.

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