Early Life Exposures and Milk Composition Affect Offspring Health

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Nutritional Sciences)
in the University of Michigan
2022

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Dedication

This dissertation is dedicated to my husband who has always supported me and pushed me to achieve great things while pursuing his own doctorate degree, to my parents who provided me with many opportunities to succeed and always believed in me, to my siblings who were always there when I needed them, and to my friends who supported me throughout the doctoral program. It takes a village to get through the Ph.D., and I am so lucky to have had you all throughout this journey.

Acknowledgements

I would like to acknowledge and thank my mentors who have made this work possible. Thank you to Dr. Dave Bridges for believing in my potential and teaching me so much. Thank you to Dr. Brigid Gregg for her guidance and support, for teaching me everything she knows about lactation, and for allowing me to discover a new passion for human milk research. Thank you to Dr. Carol F. Elias for being supportive and empathetic throughout this journey and for sharing invaluable life experiences that helped me achieve my goals. Thank you to Dr. Kelly M. Bakulski for her constant encouragement and for being tirelessly supportive of women in STEM and consistently available to offer guidance. Thank you to Dr. Lindsay Ellsworth who provided invaluable knowledge on lactation. I would also like to acknowledge Dr. Rita Loch-Caruso and her lab for sharing their knowledge of the placenta. I would like to thank the current and former members of the Bridges and Gregg labs, especially JeAnna R. Redd, Molly C. Mulcahy, Allison C. Meyer, Detrick Snyder, Innocence C. Harvey, Laura Gunder, Hannah Hafner, and Zach Carlson for their contributions to the research reported here and for being a great team to work with.

Table of Contents

Dedication ii

Acknowledgements iii

List of Tables ix

List of Figures x

Abstract xii

Chapter 1 : Introduction 1

1.1 Developmental Origins of Health and Disease 1

1.2 Dexamethasone as a Model of Stress 2

1.3 Elevated Glucocorticoids and Placental Role 3

1.4 Murine Placental Development and Physiology 4

1.5 Cortisol/Corticosterone Levels in Pregnancy 5

1.6 Fetal HPA Axis Development 6

1.7 Glucocorticoid Treatments in Pregnancy 6

1.8 Effects of Glucocorticoid Exposure on Placental and Fetal Development 7

1.9 Effect of Glucocorticoid Exposure on Placental Endocrine Function 8

1.10 Effect of In Utero Glucocorticoid Exposure on Offspring 9

1.11 mTORC1 Hyperactivation as a Model of Excess Nutrient Sensing 11

1.12 Maternal Obesity Prevalence and Risk 11

1.13 Effect of Obesity on Offspring 12

1.14 Effects of Nutritional Excess on Lactation 13

1.15 Milk Macronutrient Synthesis 14

1.16 Mammary Adipocytes and Mammary Gland Function 15

1.17 mTORC1 Activity in Obesity 16

1.18 Role of mTORC1 in Mammary Gland Function 17

1.19 Maternal Obesity and Offspring Health 18

1.20 Obesity and Lactation 19

1.21 Dissertation Summary 20

Chapter 2 : Trophoblast-Specific Glucocorticoid-Receptor Knockout Reduces Fetal Resorptions but not Fetal Weight in a Sex-Specific Manner 21

2.1 Abstract 21

2.2 Introduction 22

2.3 Materials and Methods 25

2.3.1 Animal Husbandry 25

2.3.2 Dexamethasone Exposure 31

2.3.3 Body Composition 32

2.3.4 Euthanasia and Tissue Collection 32

2.3.5 Statistical Analyses 33

2.4 Results 34

2.4.1 Dexamethasone Treatment Reduces Dam Body Weight and Lean Mass Gain During Gestation 34

2.4.2 Dexamethasone significantly reduces litter size and offspring birthweight 37

2.4.3 Dexamethasone Causes Offspring Lethality by Postnatal Day 1.5, and Offspring Rescue Attempts Failed 38

2.4.4 Dexamethasone-Treated Placental Glucocorticoid-Receptor Wild-Type Dams had Similar Body Weights to Controls 40

2.4.5 Dexamethasone Increases Fetal Resorption Percentage at E14.5 Despite Similar Average Fetal Implantations 40

2.4.6 Higher Percent Implantation for Females on E14.5, but with Increased Percent Resorption in Wild-Type Females Rescued by the Knockout 41

2.4.7 Dexamethasone Reduces Male Implantations but Rescues Male Resorptions 42

2.4.8 Dexamethasone Reduces Placental Weight in Males, and this Effect is not Rescued by Knockout 43

2.4.9 Dexamethasone Reduces Fetal Weight in Males, and this Effect is not Rescued by Knockout 45

2.4.10 Placental and Fetal Weights are Significantly Correlated 46

2.5 Discussion 47

Chapter 3 : Activation of Adipocyte mTORC1 Increases Milk Lipids in a Mouse Model of Lactation 53

3.1 Abstract 53

3.2 Introduction 54

3.3 Materials and Methods 55

3.3.1 Animal Husbandry 55

3.3.2 Body Composition 57

3.3.3 Determining Milk Output Volume 57

3.3.4 Euthanasia and Tissue Collection 58

3.3.5 Milk Collection 59

3.3.6 Milk Composition Assessment 59

3.3.7 Transcriptomic Analyses 60

3.3.8 Mammary Gland Histology and Adipocyte Assessment 61

3.3.9 Statistical Analyses 62

3.4 Results 62

3.4.1 Fat Mass Gain in Adipocyte Tsc1Knockout Mice During Lactation 62

3.4.2 Adipocyte Tsc1 Knockout Mice Have Smaller Mammary Glands with More and Larger Adipocytes 65

3.4.3 Offspring Born to Adipocyte Tsc1 Knockout Dams are Heavier During Peak Lactation 68

3.4.4 Adipocyte Tsc1 Knockout Dams Produce Similar Volumes of Milk but with a Higher Milk Fat Percentage and Increased Desaturation of Milk Fatty Acids 70

3.4.5 Suppressed Expression of Adaptive Immune Markers and Increased Expression of Muscle Biosynthesis Genes 75

3.5 Discussion 78

Chapter 4 : Exploratory Analysis of the Associations Between Breast Milk Lipids and Infant Adiposity 82

4.1 Abstract 82

4.2 Introduction 83

4.3 Methods 85

4.3.1 Study Overview 85

4.3.2 Participants 86

4.3.3 Milk Collection 86

4.3.4 MIRIS analysis 87

4.3.5 Fatty Acid Lipidomic Analysis 88

4.3.6 Infant Growth Measurements 88

4.3.7 Statistical Analysis 89

4.4 Results 90

4.4.1 Participant Demographics and Characteristics 90

4.4.2 Milk Macronutrient and Energy Composition by MIRIS Analysis 91

4.4.3 Fatty Acid Composition at 2 Weeks and 2 Months 92

4.4.4 Seven Fatty Acid Levels Significantly Decreased from 2 Weeks to 2 Months 96

4.4.5 Multiple Fatty Acids were Significantly Associated with Infant Weight-for-Length z-Scores 96

4.4.6 Various Trends for the Association between Fatty Acid Percentages at 2 Weeks and 2 Months and Weight-for-Length z-Scores 102

4.5 Discussion 106

Chapter 5 : Conclusion..... 119

Bibliography 125

List of Tables

Table 1: Participant demographics and characteristics from the IMAGE study. 90

Table 2: Breast milk energy, macronutrient, and fatty acid composition at 2 weeks and 2 months. 92

Table 3: Fatty acid percentages at 2 weeks and 2 months and percent change between measurement periods using linear mixed models. Fatty acids are described using common and IUPAC nomenclatures. 93

Table 4: Significant fatty acid percentages and weight-for-length z-score nominal associations for all infants using Spearman and Pearson correlations (nominal $p<0.05$). 98

Table 5: Significant fatty acid percentages and weight-for-length z-score nominal associations for exclusively breastfed infants using Pearson correlation (nominal $p<0.05$). 100

Supplementary Table 1: Nominal and FDR-adjusted associations between milk components at 2 weeks and 2 months and weight-for-length z-scores (Wt/L Zscore) at 2, 6, 12, and 24 months of age. ……..110

List of Figures

Figure 1: Breeding schematic to generate Nr3c1$^{fl/fl}$;Cre$^{+/+}$ (wild-type) and Nr3c1$^{fl/fl}$;Cre$^{Tg/+}$ (knockout) mice. 28

Figure 2: Number of offspring born per genotype and sex from cross 1 in control, water-treated dams generating an expected 3:1 wild-type:knockout ratio. 29

Figure 3: Number of offspring born per genotype and sex from cross 2 in control, water-treated dams generating an expected 1:1 wildtype:knockout ratio. 30

Figure 4: Schematic showing the placental and fetal expression of the Cyp19a1-Cre in the F1 offspring born to wild-type dams at an expected 1:1 wild-type:knockout ratio. 31

Figure 5: Dam body composition, water intake, and gestational age for water (n=13 dams) and dexamethasone (n=14 dams) groups. 36

Figure 6: Litter size and offspring weight at delivery. 37

Figure 7: Offspring survival probability by treatment from birth (PND0.5) until postnatal day 21.5 using log-rank test. 39

Figure 8: Dam body composition prior to dexamethasone (DexE14.5) treatment and throughout gestation until E14.5 (n=7 water and n=7 dexamethasone dams). 40

Figure 9: Average implanted offspring and offspring percent resorption by treatment group on E14.5 (n=10 water and n=7 dexamethasone pGR-WT dams). 41

Figure 10: Fetal implantations and resorptions by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water and dexamethasone). 43

Figure 11: Placental weights by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water n-7 dams, and dexamethasone n=7 dams). 44

Figure 12: Fetal weights by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water n=7 dams, and dexamethasone n=7 dams). 46

Figure 13: Significant correlation between placental and fetal weights by sex. 47

Figure 14: Experimental timeline and dam body composition. 64

Figure 15: Mammary glands histology. 68

Figure 16: Litter size and offspring weight. 69

Figure 17: Offspring weight. 69

Figure 18: Milk volume measurements. 71

Figure 19: Milk fat analysis. 73

Figure 20: ω-3 and ω-6 fatty acid pathways. 74

Figure 21: Transcriptomic analysis of mammary glands. 78

Figure 22: Heatmap showing correlations between milk components at 2 weeks (2w) and 2 months (2m) and weight-for-length z-scores (displayed as Wt/L Zscore) for all infants clustered by similarity of association. 99

Figure 23: Heatmap showing correlations between milk components at 2 weeks (2w) and 2 months (2m) and weight-for-length z-scores (displayed as Wt/L Zscore) for exclusively breastfed infants clustered by similarity of associations. 102

Figure 24: Summary of saturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for saturated fatty acids only using Pearson correlation. 103

Figure 25: Summary of monounsaturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for monounsaturated fatty acids only. 104

Figure 26: Summary of n-3 polyunsaturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for omega-3 polyunsaturated fatty acids only. 105

Figure 27: Summary of n-6 fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for omega-6 polyunsaturated fatty acids only. 106

Abstract

The Developmental Origins of Health and Disease (DOHaD) theory describes how early life exposures impact offspring disease risk and health outcomes. Common early life exposures include maternal stress and obesity. Additionally, early life nutrition during lactation can affect offspring health. This work focuses on understanding the mechanisms that impact offspring health by examining fetal and lactational physiology using mouse models and clinical data.

Women who are subjected to chronic psychosocial stress and institutional racism have an increased risk of preterm delivery and infant morbidity and mortality. This is due to increased circulating levels of glucocorticoids, stress-induced steroid hormones. To understand the role of maternal elevated glucocorticoid levels on fetal and infant health, mice were exposed to the synthetic glucocorticoid, dexamethasone, during pregnancy. Mice treated with dexamethasone during gestation delivered reduced litter size and smaller offspring that were not viable postnatally. Hence, further studies were conducted to assess the role of the placental glucocorticoid receptor in mediating fetal growth restriction. A novel Cre-driven tissue-specific knockout of the placental glucocorticoid receptor was developed. Dexamethasone exposure during pregnancy caused fetal resorptions on embryonic day 14 in females only, but this was reversed by the placental glucocorticoid-receptor knockout. Furthermore, maternal dexamethasone exposure significantly reduced male placental and fetal weights on embryonic day 14, and this effect was not rescued by the placental glucocorticoid-receptor knockout. These

findings provide new information about the role of placental glucocorticoid receptor in fetal development in a sex-specific manner.

As maternal obesity is on the rise, it was important to assess the role of excess nutrient-sensing on lactational ability and milk composition. To better understand the effects of maternal obesity on early life nutrition, a mouse model of maternal adipose-specific *Tsc1* knockout predicted to hyperactivate the mechanistic target of rapamycin 1 (mTORC1) was used. This model revealed that the knockout dams had higher milk fat percentage compared to the wild-type dams. This contributed to higher milk caloric concentration and heavier offspring weight during lactation. Additionally, milk of knockout dams had a lower percentage of saturated fatty acids and a higher percentage of monounsaturated fatty acids and the omega-3 fatty acid, docosahexaenoic acid. Together, these results highlight a novel role of adipocyte mTORC1 in controlling lipid secretion during lactation and support the importance of adipose function physiology on milk composition.

To better understand the role of milk lipids on infant adiposity using clinical data, an exploratory analysis was performed to assess the relationship between the lipidomic profile of milk samples at two weeks and two months postpartum and infant adiposity. These results showed that several fatty acids were significantly correlated with infant weight-for-length z-scores, including positive correlations with vaccenic acid and negative correlations with docosahexaenoic acid. Dynamic changes in human milk lipids can have an impact on infant growth and allow us to better assess adiposity risk and develop interventions to reduce the risk of offspring morbidity and mortality.

This work shows that elevated maternal glucocorticoids and nutrient excess affect early life development and offspring health outcomes. Additionally, clinical examination of human breast milk fatty acids highlights the role of milk composition in affecting infant adiposity. Future work is warranted to fully elucidate the mechanisms by which maternal stress, excess nutrient sensing, and milk composition affect offspring health. These findings can be used to improve interventions to improve offspring health.

Chapter 1 : Introduction

1.1 Developmental Origins of Health and Disease

Early life encompasses critical windows of development that can have long lasting effects on the health of the offspring. Stress during early life can critically impact development. Specifically, maternal psychosocial and nutritional stressors lead to suboptimal offspring health (S. S. Kim et al., 2016; Moisiadis & Matthews, 2014; Reynolds, 2013). Psychological stress during pregnancy can impact fetal growth. Women with higher than average corticotropin-releasing hormone (>90pM) at midgestation, were 7.5 fold more likely to deliver preterm (Inder et al., 2001). Additionally, maternal over nutritional increased the relative risk of neonatal death by two fold for women with severe obesity (Aune, Saugstad, Henriksen, & Tonstad, 2014).

The placenta and mammary glands are primary sites of nutrient delivery to the developing offspring. The placenta and mammary glands are highly affected by maternal psychological and nutritional stressors and play a crucial role in development. Studies assessing the mechanisms by which maternal stressors alter placental and lactational development and function remain limited. This hypothesis is supported by data showing that maternal psychological stress can lead to reduced birthweight, altered offspring hypothalamic-pituitary-adrenal axis activity, reduced milk immune components, and earlier termination of lactation (Edwards, Benediktsson, Lindsay, & Seckl, 1993; Levine, 1967; J. Li et al., 2008; Matthews, 2000; Thibeau et al., 2016). Furthermore, maternal nutritional stress is associated with increased risk of fetal macrosomia,

increased likelihood of earlier weaning, and altered milk lipid composition (Castillo, Santos, & Matijasevich, 2016; Owens et al., 2010; Panagos et al., 2016).

1.2 Dexamethasone as a Model of Stress

There are two types of stress, acute and chronic. Acute stress, in response to an immediate threat, is processed through the sympathetic nervous system to trigger a fight-or-flight response. The acute stressor triggers the hypothalamic-pituitary-adrenal axis which releases the glucocorticoids cortisol/corticosterone. If acute stress persists, this leads to chronic stress whereby cortisol/corticosterone levels remain elevated (Stephens & Wand, 2012).

The majority of research has focused on glucocorticoid exposure during midgestation in animal models, long after placental development is complete. Lifestyle stressors that pregnant women may encounter like low socioeconomic status, institutional racism, shootings, and other traumatizing events that can affect health of the mother and child are common but not well studied. For example, institutional racism and the events of 9/11 in New York City caused reductions in birthweight (Collins et al., 2004; Eskenazi, Marks, Catalano, Bruckner, & Toniolo, 2007). These chronic stressful events are not yet well explored in animal models throughout pregnancy, and the mechanisms of action by which stress influences placental development are not understood.

Our model of chronic stress is providing a constant supply of the glucocorticoid dexamethasone. Dexamethasone is a potent synthetic glucocorticoid that mimics endogenous cortisol/corticosterone, and is a common therapeutic. When given at our dose of 1mg/kg/day,

dexamethasone causes a state of elevated glucocorticoids and mimics a state of stress (De Souza, F Adlard, & Souza, 1973).

1.3 Elevated Glucocorticoids and Placental Role

The placenta is the direct and only site of communication between mother and fetus during *in utero* development (Brett, Ferraro, Yockell-Lelievre, Gruslin, & Adamo, 2014). It is the rate-limiting step for fetal nutrient and gas acquisition (Brett et al., 2014). Additionally, the placenta plays an important endocrine role to promote fetal growth and nutrient supply (Malassine, Frendo, & Evain-Brion, 2003), and it is highly regulated to ensure adequate growth of the fetus in normal pregnancies (Napso, Yong, Lopez-Tello, & Sferruzzi-Perri, 2018). In cases of maternal psychological or glucocorticoid-induced stress, placental nutrient transport and endocrine function are compromised leading to potentially suboptimal fetal growth (Eskenazi et al., 2007; Kipmen-Korgun et al., 2012; Waffarn & Davis, 2012). In Denmark, 20% of women reported use of prescribed corticosteroids from 4 weeks prior to delivery until delivery between 1996-2008 for various conditions (Hviid & Mølgaard-Nielsen, 2011). Glucocorticoids such as betamethasone, dexamethasone, prednisolone, corticosteroids, or cortisol are prescribed to women who have acute asthma or asthma, hyperemesis gravidarum, depression, stress, or are at risk of delivering preterm babies (Singh, Cuffe, & Moritz, 2012). The mechanisms by which maternal corticosteroids influence fetal health and placental function are understudied (Kemp, Newnham, Challis, Jobe, & Stock, 2015). Some side effects like reduced birthweight, offspring hypertension, mental illness and higher childhood HPA axis activity remain controversial (Alexander et al., 2012; Braun, Challis, Newnham, & Sloboda, 2013; Duthie & Reynolds, 2013; Moisiadis & Matthews, 2014; Reynolds, 2013; Waffarn & Davis, 2012).

1.4 Murine Placental Development and Physiology

The definitive structure of the mouse placenta is achieved during midgestation (Malassine et al., 2003). The placenta encompasses two sides, an arc-shaped surface facing the maternal side and another flat surface facing the fetal side (Georgiades, Ferguson-Smith, & Burton, 2002). The mouse placenta has three distinct compartments, a decidual maternal zone which is the outermost compartment, a fetal-derived junctional zone that mediates placental endocrine function, and a fetal-derived labyrinth zone that comprises the majority of the placenta and is the main site for nutrient and gas exchange (Woods, Perez-Garcia, & Hemberger, 2018). Three exchange barriers exist moving inwards from the decidua to the fetal compartment including two syncytiotrophoblast layers (in the labyrinth layer) and one fetal endothelial cell layer (Georgiades et al., 2002). The two syncytiotrophoblast barriers comprise the microvillous membrane facing the maternal circulation and the basal membrane facing fetal circulation (Brett et al., 2014).

At midgestation, placental invasion of the maternal uterine cavity occurs to allow maternal blood flow into the placental cavity (Malassine et al., 2003; Woods et al., 2018). This invasion permits direct nutrient uptake from the maternal circulation to the fetus through the placenta. Prior to this invasion, the embryo acquires nutrients from the yolk sac, the initial placental structure that absorbs nutrients from maternal circulation (Malassine et al., 2003; Woods et al., 2018).

1.5 Cortisol/Corticosterone Levels in Pregnancy

During human pregnancy, mean cortisol rises gradually as pregnancy progresses (Carr *et al.*, 1981). Mean cortisol levels increase in humans during the first, second and third trimester by 1.6, 2.4 and 2.9 folds, respectively (Jung et al., 2011). The increased cortisol levels may be explained by placental secretions of estrogen stimulating maternal cortisol production and mitigating maternal negative feedback (Duthie & Reynolds, 2013; Lindsay & Nieman, 2005) and/or by placental production of corticotropin-releasing hormone (CRH) into the maternal circulation in mid- and late gestation (Duthie & Reynolds, 2013). Maternal cortisol promotes placental CRH production, which in turn promotes maternal HPA axis activity thus acting as a feed-forward positive mechanism.

However, in mouse pregnancy, corticosterone levels do not increase as much as humans near term although there are still increases. In pregnant control mice, corticosterone levels were not significantly different at mid to late gestation on E11 and E17 despite slightly higher levels at E17 (Jafari, Mehla, Afrashteh, Kolb, & Mohajerani, 2017). Other studies showed an increase in corticosterone levels at E19 compared to E16 in control unstressed mice (Owen R Vaughan, Sferruzzi-Perri, & Fowden, 2012). Unstressed pregnant mice had higher corticosterone levels with peak levels at E16 being 60 times higher than non-pregnant mice due to placental and maternal hormonal secretion changes during pregnancy (Barlow, Morrison, & Sullivan, 1973). The levels then dropped after E16 until delivery at E19 (Barlow et al., 1973).

1.6 Fetal HPA Axis Development

The human fetal hypothalamic-pituitary axis activity is detected as early as 8-12 weeks of gestation (Ng, 2000) and is fully developed in the second trimester of pregnancy (Moisiadis & Matthews, 2014). In early pregnancy, fetal cortisol is thought to primarily be attained from maternal cortisol, as the fetus is believed to sufficiently produce cortisol at 22 weeks of gestation (Buss et al., 2012). Given the critical developmental window by which fetal organs and HPA axis are developing, it is possible that increased maternal cortisol levels in early pregnancy compared to late pregnancy may have more deleterious effects on fetal development (Barker, 2007; Braun et al., 2013). In mice, the offspring HPA develops postnatally in two phases. On postnatal day (PND) 1 through 12, the mouse HPA is considered hypo-responsive, and after PND 12 the HPA system matures (Schmidt et al., 2003).

1.7 Glucocorticoid Treatments in Pregnancy

In addition to the naturally increasing cortisol levels in pregnancy, glucocorticoid (GC) treatments are also prescribed during pregnancy for multiple reasons. A single course of synthetic corticosteroid treatment is prescribed to women who are at risk of delivering premature babies. The treatment is proven to increase offspring chances of survival post-delivery (Baisden, Sonne, Joshi, Ganapathy, & Shekhawat, 2007; Doyle et al., 2000). Glucocorticoid treatments are prescribed for pregnancies at risk of preterm delivery as they enhance fetal growth, specifically fetal lung maturation to prevent respiratory distress syndrome (RDS), and aid in overall embryogenesis to prevent perinatal death due to hemorrhages, heart failure and other underlying causes associated with preterm birth (Lunghi et al., 2010; Singh et al., 2012). Specifically, betamethasone, dexamethasone, prednisolone, corticosteroids, or cortisol are prescribed to

women who have acute asthma or asthma, hyperemesis gravidarum, depression, stress, or are at risk of delivering preterm babies (Singh et al., 2012). The use of corticosteroids is widespread, but there is a scarcity of reports showing prevalence of glucocorticoid use during pregnancy. In a Danish cohort study encompassing all births in Denmark from 1996-2008, about 20% of women reported use of corticosteroids from 4 weeks prior to delivery until delivery (Hviid & Mølgaard-Nielsen, 2011). In an American cohort study including 152,531 pregnancies between 1996-2000, 3.5% of pregnant women who had a documented diagnosis associated with preterm birth used corticosteroids, while 1.7% of pregnant women who did not have a documented diagnosis used corticosteroid for non-pregnancy related conditions (Andrade et al., 2004). As maternal corticosteroid levels increase during pregnancy, the placenta aims to protect the fetus from the naturally high levels of maternal corticosteroid by deactivating nearly 85% of the maternal cortisol to cortisone, but this ability is compromised in cases of severe stress (Murphy, Clark, Donald, Pinsky, & Vedady, 1974). Placental 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) deactivates the majority of circulating maternal cortisol into inactive cortisone which passes through the umbilical cord to the fetus (Dahlerup et al., 2018; Duthie & Reynolds, 2013; Green et al., 2015). However, synthetic corticosteroids used in preterm treatments can readily cross the placenta bypassing inactivation by 11β-HSD2 (J.S.M. Cuffe, Dickinson, Simmons, & Moritz, 2011; Singh et al., 2012).

1.8 Effects of Glucocorticoid Exposure on Placental and Fetal Development

Pregnant rats treated with dexamethasone at mid and late pregnancy at E13 until E20 showed reduced placental and fetal weights (Ain, Canham, & Soares, 2005). Despite the evident placental and fetal growth restriction, dexamethasone did not affect litter size or fetal viability

(Ain et al., 2005). Rats exposed to the glucocorticoid triamcinolone (0.38 mg/kg dose) once at E16 had 53% reduction in placental weight and 73% reduction in fetal weights (Hahn et al., 1999). Mice exposed to a sound stressor on E10.5, E12.5, and E14.5 showed reduced fetal body weight and had growth restriction that was more evident in female fetuses (Wieczorek et al., 2019). Pregnant mice exposed to dexamethasone on E15, E16, and E17 had reduced placental and fetal weights and trophoblast swelling in the junctional and labyrinth zones (Baisden et al., 2007). Furthermore, mice given dexamethasone at E11-E16 had reduced fetal and placental weights at E16, but the volume of the placental junctional and labyrinth zone was unchanged despite less fetal capillaries in the labyrinth zone (Owen R Vaughan et al., 2012). Contrary to the evident reduction in placental and fetal weights seen in some studies, mice treated with the glucocorticoid dexamethasone (0.1 mg/kg dose) on E13.5 and E14.5 showed no effect on fetal weight at E15.5, E18.5, or at birth (Audette, Challis, Jones, Sibley, & Matthews, 2011). There was also no effect on placental weights at E15.5, E17.5 in male or female placentas, but at E18.5 female placentas had reduced weight while male placentas were not different (Audette et al., 2011). Placental junctional and labyrinth zone proportions with respect to the total placental area were unchanged (Audette et al., 2011).

1.9 Effect of Glucocorticoid Exposure on Placental Endocrine Function

The placental insulin-like growth factor 2 (*Igf2*) promotes the growth of the fetus during pregnancy (Sibley et al., 2004). Pregnant rats treated with dexamethasone at E13 had reduced *Igf2* mRNA expression in the junctional zone but unaltered expression in the labyrinth zone (Ain et al., 2005). Conversely, pregnant mice exposed to glucocorticoids at midgestation showed no

change in placental *Igf2* gene expression (Baisden et al., 2007; J.S.M. Cuffe et al., 2011; O. R. Vaughan et al., 2015).

Growth differentiation factor 15 (GDF15) is produced in the placenta, and changes are associated with a variety of complications including miscarriage, nausea and hypertension (Q. Chen et al., 2016; Petry et al., 2018; Tong et al., 2004). There are no studies assessing placental GDF15 activity in response to GC or psychological stress exposures. Placental GDF15 levels are positively correlated with maternal and fetal GDF15 levels, suggesting that the placenta is the primary source of this hormone during pregnancy (Sugulle et al., 2009). Based on our results, other placental hormones may be assessed in the future.

1.10 Effect of In Utero Glucocorticoid Exposure on Offspring

Women with higher corticotropin-releasing hormone (CRH) at midgestation, were 7.5 fold more likely to deliver preterm (Inder et al., 2001). However, the results on offspring outcomes remain conflicted. In humans, antenatal corticosteroid exposure was associated with higher systolic and diastolic blood pressure in children ages 14 years (Doyle et al., 2000). Looking at long-term effects of antenatal glucocorticoid treatments, 30-year old offspring of mothers who had received antenatal betamethasone showed higher insulin levels 30 minutes after a glucose tolerance test and lower glucose concentrations at 120 minutes, but they did not have altered cortisol levels, lipid profile or blood pressure (Dalziel et al., 2005). This suggests an impaired pattern of insulin secretion (Dalziel et al., 2005). Additionally, body composition of five year-old children of mothers who had higher cortisol levels during pregnancy showed increased fat mass index in girls but a lower fat mass index in boys indicating a sex-difference (Van Dijk, Van Eijsden,

Stronks, Gemke, & Vrijkotte, 2012). Higher maternal third trimester cortisol levels were associated with increased infant body fat percent from ages 1-6 months, suggesting programmed adiposity that can contribute to childhood obesity (Entringer et al., 2016). Antenatal GC treatment showed a blunted HPA axis activity in infants ages 3-6 days after a stressful exposure (Elysia Poggi Davis et al., 2004). Newborns exposed to antenatal glucocorticoids showed reduced cortisol levels, though long-term effects vary. Children exposed to antenatal glucocorticoids had higher cortisol levels following a standardized stressful test at ages 6-11 years, and this difference was mainly influenced by higher salivary cortisol in females, indicating a potential sex-dependent elevation in HPA axis activity effect (Alexander et al., 2012). Studies have shown multiple offspring outcomes including increased risk of preeclampsia, impaired cognitive development in infants, increased infant cortisol, reduced fetal weight, and other symptoms each varying with timing, dosage and type of corticosteroid treatment during pregnancy (Singh et al., 2012).

In mice, male offspring exposed to dexamethasone for 60 hours starting at E12.5 had lower fetal weights at E14.5 but not at E17.5 (O'Sullivan et al., 2013). The male offspring from similarly treated dams had similar weights at 2 weeks, 4 weeks, and 3 and 6 months of age (O'Sullivan et al., 2013). Twenty-one-day-old rats exposed to antenatal dexamethasone at E15 till delivery had 66% lower body weights than controls, lower corticotropin releasing hormone content and concentrations, lower adrenal and plasma corticosterone levels and severe adrenal atrophy (Dupouy, Chatelain, Boudouresque, Conte-Devolx, & Oliver, 1987). Conversely, rats exposed to antenatal glucocorticoids showed unaltered ACTH and corticosterone plasma levels at PND1,7,9, and 20 but had a suggested increased HPA axis activity when stress was induced

during adulthood (Bakker et al., 1995). Offspring of physically stressed rats during gestation showed higher plasma glucose levels at 24 months of age despite similar insulin secretion when challenged with an oral glucose tolerance test (Lesage et al., 2004).

1.11 mTORC1 Hyperactivation as a Model of Excess Nutrient Sensing

Obesity is a major public health crisis affecting 37.7% of Americans (Flegal, Kruszon-Moran, Carroll, Fryar, & Ogden, 2016). There are many molecular and physiological changes associated with obesity, but this work will focus on the nutrient sensing kinase, the mechanistic target of rapamycin 1 (mTORC1). In most cells, mTORC1 is activated by anabolic signals and an excess of nutrients. mTORC1 is a nutrient sensor and a main regulator of protein and lipid synthesis (Cai, Dong, & Liu, 2016; X. Wang & Proud, 2006). Obesity, identified as having excess fat mass, promotes mTORC1 activity (Catania, Binder, & Cota, 2011). In obese subjects, gene expression of mTORC1 was upregulated in the visceral fat compartments (Catalán et al., 2015). My model of adipocyte mTORC1 hyperactivation mimics the obesogenic environment and better allows us to understand the mechanisms by which milk composition and volume are altered in a nutrient-excess state of mTORC1 hyperactivation (Chapter 3). It is worth noting that our model has mTORC1 hyperactivation in all adipocytes and is not specific to the mammary adipocytes, as no mammary-gland-specific adipocyte driver has been identified yet.

1.12 Maternal Obesity Prevalence and Risk

Obesity in pregnancy is on the rise in the Unites States with an 11.3% increase from 2005 to 2014 in pregnant women, accounting for 7.3% of the global burden of obesity for 2014 (C. Chen,

Xu, & Yan, 2018). In a large cohort study of 287,213 participants, 24.3% of pregnant women were overweight (BMI 25-<30 kg/m^2) and 9.6% had obesity (BMI >30 kg/m^2) at the time of their first antenatal appointment booking (Sebire et al., 2001). Higher maternal BMI is associated with higher odds of having gestational diabetes, prolonged hospital postnatal stay, emergency cesarean section, offspring birthweight higher than the 90% percentile, stillbirth, and other antenatal, maternal and fetal complications (Sebire et al., 2001). In another cohort study of 96,801 participants, 18.2% of women were overweight and 10.1% were classified as obese pre-pregnancy (Baeten, Bukusi, & Lambe, 2001). Pre-pregnancy overweight and obesity increased the risk of preeclampsia and eclampsia, gestational diabetes, and delivering preterm (Baeten et al., 2001). Women with pre-pregnancy obesity had a significantly increased risk of infant death within the first year of life (Baeten et al., 2001). Furthermore, pre-pregnancy obesity is positively associated with large-for-gestational-age deliveries (Ehrenberg, Mercer, & Catalano, 2004).

1.13 Effect of Obesity on Offspring

Maternal obesity is associated with higher childhood risk of developing metabolic syndrome in large-for-gestational-age babies (Boney, Verma, Tucker, & Vohr, 2005). Indeed, maternal pre-pregnancy obesity was the strongest determinant of childhood obesity at 6-11 years of age (Patrick M Catalano et al., 2009). Furthermore, maternal obesity was correlated with higher fetal body fat percentage and higher fetal insulin resistance, whereby maternal obesity was associated significant increase in neonatal body fat percentage, neonatal fat mass, placental weight, and umbilical cord insulin levels (P. M. Catalano, Presley, Minium, & Hauguel-de Mouzon, 2009). Fetuses of mothers who had obesity were more insulin resistant than fetuses of lean mothers using umbilical cord blood at delivery (P. M. Catalano et al., 2009). Additionally, higher

maternal pre-pregnancy BMI was associated with preschool childhood obesity (Whitaker, 2004). Children born to obese mothers were two times more likely to be large for gestational age (LGA), and LGA was further predictive of early childhood obesity (Whitaker, 2004). In humans, maternal obesity was associated with early adulthood development of obesity and insulin resistance, even if the offspring had a normal birthweight (Mingrone et al., 2008). Finally, children of obese mothers had 3.84 times higher odds of being overweight at age six and 3.0 times higher odds of an adverse cardiometabolic disease profile (Gaillard et al., 2014), causing them to be at higher risk of developing non-communicable diseases like hypertension (Leddy, Power, & Schulkin, 2008), insulin resistance (Mingrone et al., 2008) and diabetes later in life (Stubert, Reister, Hartmann, & Janni, 2018).

1.14 Effects of Nutritional Excess on Lactation

Milk composition is important to provide essential nutrients for optimal offspring growth (Eriksen, Christensen, Lind, & Michaelsen, 2018). Given the links between maternal obesity and offspring health, it is plausible that maternal obesity or overnutrition may alter lactation, with important effects for the offspring. The mechanisms by which some micro and macronutrients are metabolized, transported, and incorporated into the secreted milk during lactation are not well understood, nor is their regulation by nutrient sensing pathways. In humans, maternal obesity affects lactation whereby lactation initiation is delayed, weaning occurs earlier, and milk composition is altered with a higher fat content (Castillo et al., 2016; Leghi et al., 2020; Panagos et al., 2016; Rasmussen & Kjolhede, 2004a).

1.15 Milk Macronutrient Synthesis

Lactation requires successful milk secretion, a process referred to as lactogenesis. To achieve that, lactogenesis occurs in two stages. Lactogenesis I encompasses the differentiation of mammary glands and is evident mid-gestation through term in humans. Lactogenesis II, the phase where milk production is initiated occurs prior to delivery in most animals, but in humans, lactogenesis II is initiated post-delivery due to placental removal and a gradual drop in progesterone levels (Ben-Jonathan, LaPensee, & LaPensee, 2008; Napso et al., 2018; Neville, Mcfadden, & Forsyth, 2002; Neville, Morton, & Umemura, 2001; Pillay & Davis, 2019; Soares, 2004). The critical macronutrients in mammalian milk are fat, protein and lactose. Mouse milk showed the highest fat and protein content on PND14 with 12.5% crude protein, 29.8% crude fat, and 1.58% lactose (Görs, Kucia, Langhammer, Junghans, & Metges, 2009). The highest lactose content of 2.41% was evident on PND18 (Görs et al., 2009). Proteins are synthesized in the rough endoplasmic reticulum of the alveolar epithelial cells (Anderson, Rudolph, McManaman, & Neville, 2007; Rezaei, Wu, Hou, Bazer, & Wu, 2016). Lipids, almost exclusively in the form of triglycerides, are synthesized in the smooth endoplasmic reticulum by de novo synthesis from available glucose, or they are derived from maternal diet or fatty acids from adipose tissue stores (Anderson et al., 2007; McManaman, 2009; Rezaei et al., 2016). The mechanisms by which lipids are packaged and transported into the milk remain elusive (McManaman, 2009). Lactose is synthesized in the Golgi of the alveolar epithelial cells (Anderson et al., 2007; Rezaei et al., 2016).

1.16 Mammary Adipocytes and Mammary Gland Function

Adipocytes form a major proportion of the mammary gland and are necessary for proper gland development and proliferation (Landskroner-Eiger, Park, Israel, Pollard, & Scherer, 2010; Machino, 1976). At puberty, alveolar ducts expand at the expense of the fat pad in the mammary gland (Hovey & Aimo, 2010; Macias & Hinck, 2012). A case study of a female with progressive lipodystrophy showed suboptimal lactation and early cessation of lactation due to ceased milk production 3 weeks postpartum (Russell, 1958). Two females with familial lipodystrophy had reduced mammary adipocytes despite normal mammary tissue size (Garg, Peshock, & Fleckenstein, 1999). A mouse model of lipodystrophy with underdeveloped fat tissues was developed to determine its effects on mammary gland development (R. Li, Zowalaty, Chen, Dudley, & Ye, 2015). The knockout mice had smaller mammary adipocytes, accelerated ductal growth, and potential sloughing of the ductal epithelial cells into the lumen indicating suboptimal mammary gland function and growth compared to controls (R. Li et al., 2015). A proliferator-activated receptor γ (PPARγ) knockout mouse model of impaired adipocyte function showed reduced expansion of the ducts at the expense of the fat pad along with prepubertal cessation of ductal growth (F. Wang, Mullican, DiSpirito, Peed, & Lazar, 2013). During pregnancy and lactation, adipocytes have a unique supportive function. Recently, it has been determined that mammary adipocytes de-differentiate gradually during gestation and almost disappear entirely during lactation allowing more space for milk production by the mammary alveolar epithelial cells (Q. A. Wang, Song, Gupta, Deplancke, & Scherer, 2018; Zwick et al., 2018). Adipocytes closest to the mammary epithelial cells de-differentiate quicker than those farther away in the cleared fat pad (Hovey & Aimo, 2010; Lawson, Werb, Zong, & Goldstein, 2015). The alveoli expand at the expense of the fat pad almost entirely covering its area (Richert, Schwertfeger,

Ryder, & Anderson, 2000). It is hypothesized that the adipocytes in the body mobilize their fat stores and provide for the mammary epithelial milk lipid production, which explains the reduction in size of the adipocytes during lactation (Cinti, 2018; D. Flint & Vernon, 1998; Richert et al., 2000). The exact fate of adipocytes during the de-differentiation phase of lactation remains unknown (Q. A. Wang et al., 2018). It is shown that the adipocytes do not transdifferentiate into epithelial cells unlike what was previously proposed (Morroni et al., 2004; Prokesch et al., 2014), indicating that the adipocytes do not contribute directly to the milk production function of the epithelial cells during lactation (Q. A. Wang et al., 2018; Zwick et al., 2018). As milk production gradually decreases at weaning, adipocytes later grow rapidly in size by taking up excess milk lipids from the alveolar lumen and alveolar epithelial cells (Zwick et al., 2018). This is referred to as a "refilling" process for the mammary gland adipocytes and it simultaneously occurs along with mammary epithelial cell regression (Zwick et al., 2018). The role of the adipocytes and the mechanisms regulating their regression and fate warrant further studies. Our model will focus on mTORC1 activation in differentiated adipocytes after a first pregnancy, not during the process of adipogenesis. Little is known about the role of mTORC1 in macronutrient synthesis and mobilization in the mammary gland (Rezaei et al., 2016).

1.17 mTORC1 Activity in Obesity

mTORC1 is a main regulator of protein and lipid synthesis (Cai et al., 2016; X. Wang & Proud, 2006). In the presence of insulin, an anabolic signal, mTORC1 function is upregulated via the Akt pathway (Catania et al., 2011). mTORC1 promotes lipogenesis via sterol-regulatory element-binding protein 1 (SREBP1) and promotes adipogenesis while inhibiting lipolysis (Cai et al., 2016; Laplante & Sabatini, 2009). Obesity, identified by having excess fat mass, promotes

mTORC1 activity (Catania et al., 2011). In obese subjects, gene expression of mTORC1 and its downstream substrate, the ribosomal protein S6 kinase 1 (S6K1), was upregulated in the visceral fat compartments (Catalán et al., 2015). Mice deficient in S6K1 are resistant to obesity and have a higher lipolytic rate and lower fat mass (Dann, Selvaraj, & Thomas, 2007; Um et al., 2004). This suggests the important and active role of mTORC1 in promoting an obese phenotype.

1.18 Role of mTORC1 in Mammary Gland Function

mTORC1 is a nutrient sensor and is crucial for proliferation and growth. Mice treated with rapamycin for 12 days starting at gestational day 19 had reduced mammary gland size and reduced epithelial tissue (Jankiewicz, Groner, & Desrivières, 2006). Furthermore, milk beta-casein protein composition was reduced by half in the rapamycin treated group (Jankiewicz et al., 2006). This indicates the important role of mTORC1 in mammary gland proliferation and protein synthesis. In bovine mammary epithelial cells, mTORC1 signaling was upregulated in response to the lactogenic stimuli insulin and prolactin (H. Li et al., 2017). The mechanisms by which mTORC1 promotes protein synthesis has been linked to downregulation of Menin protein, an inhibitor of AKT activity upstream of mTORC1 (H. Li et al., 2017). Transgenic pregnant mice with activated AKT in the mammary epithelial cells had comparable mammary gland development during pregnancy, but showed distended alveoli during lactation and a higher lipid droplet composition and size in the mammary epithelial during gestation and lactation (Schwertfeger, McManaman, Palmer, Neville, & Anderson, 2003). Milk composition from the transgenic mice revealed higher fat percentage and a higher protein concentration compared to controls (Schwertfeger et al., 2003). AKT, upstream of mTORC1, may play a significant role in regulating mammary gland differentiation and lipid and protein synthesis (Schwertfeger et al.,

2003). Furthermore, Th-inducing POK, a transcription factor, was found to be correlated with mTORC1 and a potential feed-forward regulator of insulin signaling via IRS1/Akt/mTORC1 pathway in the mammary gland (Zhang et al., 2018). Mice lacking Th-POK had lower pup survival rate that was attributed to lactation. Knockout mice further had reduced milk triglycerides and increased milk non-esterified fatty acids. This was due to large lipid droplet accumulation in the mammary alveolar cells. Th-POK knockout mice also showed signs of precocious mammary epithelial involution (Zhang et al., 2018). This implies the important role of mTORC1 in modulating lipid synthesis in the mammary alveolar cells.

1.19 Maternal Obesity and Offspring Health

Maternal obesity can influence the offspring health via pre-gestational, gestational and lactational exposures. Children of mothers with class III obesity (BMI >40 kg/m^2) are at 2.3 times higher risk of being large for gestational age (S. S. Kim et al., 2016). Children of overweight or obese mothers had increased weight gain at age 0-4 years and a higher BMI z-score compared to children of lean mothers (Hu et al., 2019). Another study found no effect on offspring weight. Pre-pregnancy obesity was associated with higher weight gain and higher obesity risk in early childhood. This association was unaltered when breastfeeding was accounted for (Hu et al., 2019). A systematic review revealed benefits of breastfeeding that were attenuated when accounting for maternal BMI, suggesting an interplay between maternal weight and benefits of lactation (Bider-Canfield et al., 2017). Furthermore, breastfeeding was associated with higher odds of childhood obesity in mothers who had a higher-than-expected gestational weight gain, suggesting that maternal pre-pregnancy weight and gestational weight gain are the main predictors of childhood obesity risk (Ohlendorf, Robinson, & Garnier-Villarreal, 2019).

This implies the effects of maternal weight on reducing benefits of lactation (Ohlendorf et al., 2019). Data collected in the United States showed that 50% of women had a pre-pregnancy BMI classified as overweight or obese in 2014 (Branum *et al.*, 2014). The exact mechanisms by which the offspring health is affected in response to early life exposures remain elusive due to the multiple critical developmental windows that can be influenced. This aim will focus on the developmental window of lactation in maternal obesity, as a lot of evidence points to the importance of lactation on offspring health (Neri & Edlow, 2015).

1.20 Obesity and Lactation

Maternal obesity can influence early postnatal development through its impact on mammary gland function. Maternal weight has been positively correlated with milk protein content and caloric value (kilocalories from protein, lipids and carbohydrates per 100 ml milk) in the third month of lactation postpartum (Bzikowska-Jura et al., 2018). Higher milk fat content was correlated with higher maternal weight at six months postpartum (Bzikowska-Jura et al., 2018). An altered milk lipid composition was found in milk of obese mothers with a higher omega 6:omega3 ratio (Panagos et al., 2016). Initiation of lactation was also affected by maternal weight, by which pre-pregnancy obesity or overweight reduced the suckling-induced prolactin secretion at 48 hours postpartum (Rasmussen & Kjolhede, 2004a). Furthermore, breastfeeding duration for 6 months or more was lower in mothers who were overweight or obese (Bider-Canfield et al., 2017). The probability of early weaning at 3 months postpartum was highest for infants of obese mothers (Castillo et al., 2016).

In rats, obesity induced by high-energy diet during pregnancy and lactation doubled fat content in milk (Rolls & Rowe, 1982). Mice fed a high fat diet had delayed lactogenesis which was evident by reduced litter weight gain on the first day of lactation which later normalized (D. J. Flint, Travers, Barber, Binart, & Kelly, 2005). The mice further had impaired alveolar development with abnormal reduced branching at gestational day 14 (D. J. Flint et al., 2005). However, the mechanisms by which the mammary gland senses nutritional excess remain unclear and the interplay between mammary adipocytes and alveolar cells requires further investigation. Our work (Chapter 3) will aim to assess the role of adipocyte excess nutritional sensing on milk composition, mammary gland physiology, and offspring outcomes.

1.21 Dissertation Summary

This dissertation describes my work that aimed to unravel underlying mechanisms by which early life exposures affect placental and mammary gland morphology and development using mouse models in addition to understanding how milk composition impacts infant adiposity using clinical data. Specifically, Chapter 2 highlights the detrimental effects of elevated glucocorticoids during pregnancy on mouse infant survival and the role of placental glucocorticoid receptor in mediating fetal growth in a sex-specific manner. Chapter 3 emphasizes the important role of adipocyte mTORC1 and excess nutrient sensing in altering mouse mammary gland physiology, milk composition, and offspring weight during lactation. Lastly, Chapter 4 explores associations between human breast milk fatty acids and infant adiposity during the first two years of life. These data provide novel insights into the important role early life exposures during gestation and lactation play in mediating offspring health and shed light on some of the mechanisms at play during these critical developmental windows.

Chapter 2 : Trophoblast-Specific Glucocorticoid-Receptor Knockout Reduces Fetal Resorptions but not Fetal Weight in a Sex-Specific Manner

2.1 Abstract

Glucocorticoid use during pregnancy is a common treatment for asthma, allergies, and COVID-19. Several studies have reported adverse effects including intrauterine growth restriction as a result of glucocorticoid exposure, yet little is known about the mechanisms by which short and long-term maternal glucocorticoid exposures affect placental biology and fetal development. To better understand the role of glucocorticoids on placental and fetal outcomes, we used a mouse model exposed to the synthetic glucocorticoid, dexamethasone, prior to and throughout gestation. We conducted a randomized controlled trial in mice with a treatment arm of dexamethasone exposure and water exposure as control. Dams treated with dexamethasone lost significant lean mass after one week of treatment. At PND0.5, dexamethasone-treated dams had reduced litter size and smaller offspring. Dexamethasone caused offspring lethality by PND1.5 and rescue attempts failed. These results demonstrate a novel finding regarding the chronic use of glucocorticoids before and during conception on offspring health.

To assess the role of placental glucocorticoid receptor in mediating the effects of dexamethasone, a novel Cre-driven tissue-specific knockout of the placental glucocorticoid receptor was developed. Dexamethasone exposure during pregnancy caused fetal resorptions on embryonic

day 14 in a females offspring only, but this was reversed by the placental glucocorticoid-receptor knockout. Furthermore, maternal dexamethasone exposure significantly reduced male placental and fetal weights on embryonic day 14, and this effect was not rescued by the placental glucocorticoid-receptor knockout. These findings provide new information about the role of placental glucocorticoid receptor in regulating fetal survival and growth in a sex-specific manner.

2.2 Introduction

Nearly 70% of pregnant women experienced a stressful life event, defined as unpredictable events that affect well-being such as traumatic experiences or relationship stressors, during pregnancy (Booth, Kitsantas, Min, & Pollack, 2021; Burns, Farr, & Howards, 2015). Maternal psychosocial stress during pregnancy is associated with adverse offspring health outcomes including preterm birth and low birthweight (Loomans et al., 2013; Whitehead, Hill, Brogan, & Blackmore-Prince, 2002). Premature birth is a leading cause of infant mortality, and it is estimated that around 10% of pregnant women are at risk of preterm delivery (Callaghan, MacDorman, Rasmussen, Qin, & Lackritz, 2006; Czamara et al., 2021; Torchin & Ancel, 2016). During the course of pregnancy, the hormone cortisol, which is a glucocorticoid, naturally increases by 3 fold as the pregnancy progresses (Jung et al., 2011; Mastorakos & Ilias, 2003). The increased cortisol levels are important for fetal development and lung maturation especially during the third trimester (Austin & Leader, 2000; Elysia P Davis & Sandman, 2010). However, the fetus needs to be protected from the excess circulating maternal glucocorticoids. The placenta is the primary organ responsible for deactivating maternal glucocorticoids and reducing fetal exposure. Placental 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) deactivates the

majority of circulating maternal cortisol into inactive cortisone which passes through the umbilical cord to the fetus (Dahlerup et al., 2018; Duthie & Reynolds, 2013; Green et al., 2015). However, acute maternal psychosocial stress reduces 11β-HSD2 expression leading to increased fetal cortisol exposure (Jahnke, Terán, Murgueitio, Cabrera, & Thompson, 2021). Additionally, some synthetic glucocorticoids prescribed during pregnancy, such as betamethasone and dexamethasone, can readily cross the placenta bypassing inactivation by HSD11B2 (J.S.M. Cuffe et al., 2011; Czamara et al., 2021; Singh et al., 2012). Synthetic glucocorticoids are commonly prescribed for pregnancies that are at risk of preterm delivery or during pregnancy as treatment for autoimmune diseases, allergies, and most recently, COVID-19 (Alangari, 2014; Saad, Chappell, Saade, & Pacheco, 2020; Singh et al., 2012). In a Danish cohort, 20% of women reported use of corticosteroids at least once within a month prior to pregnancy and until delivery (Hviid & Mølgaard-Nielsen, 2011).

In humans, the effects of elevated maternal glucocorticoids or synthetic glucocorticoid use on offspring health warrant further investigation to determine long-term health outcomes. Maternal third trimester cortisol levels are positively associated with infant body fat percent increase from 1-6 months postnatally suggesting programmed adiposity that may contribute to childhood obesity (Entringer et al., 2016). Antenatal glucocorticoid treatment caused a blunted hypothalamic-pituitary-adrenal (HPA) axis activity in 3-6 day old infant after a stressful exposure (Elysia Poggi Davis et al., 2004). A review of glucocorticoid use during pregnancy showed an increased risk of cleft palate (Bandoli, Palmsten, Forbess Smith, & Chambers, 2017). Another review showed that antenatal glucocorticoid use decreased neonatal mortality but may have unknown adverse long-term effects on offspring health if more than two courses of

glucocorticoid treatments are administered (George, Abramson, & Walker, 2012). Observational cohort studies have shown multiple altered offspring outcomes after antenatal use of glucocorticoids including a two-fold increased risk of preeclampsia, a 1.5 times increase in the odds of preterm delivery, and a 1.8 times increase in the odds of delivering low birth weight infants, but symptoms are associated with timing, dosage and type of glucocorticoid treatment during pregnancy (Schatz et al., 2004, 1997; Singh et al., 2012).

Several studies have reported adverse offspring effects as a result of glucocorticoid exposure, yet little is known about the long-term effects of antenatal glucocorticoid use and the mechanisms by which short and long-term maternal glucocorticoid exposures affect placental physiology and fetal development. The mechanisms underlying fetal programming are still being explored including several physiological pathways, but sex-specific placental and fetal physiological differences *in utero* need to also be considered (Douros et al., 2017; Solano & Arck, 2020; Sutherland & Brunwasser, 2018). There is a paucity of research in animal models that examine prenatal glucocorticoid exposure over the entire period of gestation to mimic prolonged elevated maternal glucocorticoid levels. To better understand the role of elevated maternal glucocorticoid levels on placental, fetal, and offspring outcomes, we used mouse models exposed to the synthetic glucocorticoid, dexamethasone, prior to and throughout gestation. We show that dexamethasone exposure throughout gestation reduces litter size, offspring weight, and offspring survival. To isolate the role of the placental glucocorticoid signaling on fetal development, we used a trophoblast-specific glucocorticoid receptor (GR, encoded by *Nr3c1*) knockout model that revealed a sex-specific reduction in female fetal loss and a sex-specific dexamethasone-induced effect on male fetal and placental weights.

2.3 Materials and Methods

2.3.1 Animal Husbandry

C57BL/6J mice were purchased from The Jackson Laboratory. Mice were fed a normal chow diet (Lab Rodent Diet; 5L0D) with *ad libitum* access to food and water (control groups) or dexamethasone water (treatment groups) one week prior to pregnancy and throughout pregnancy and lactation. C57BL/6J female mice were single-housed at 11 weeks of age, and water-soluble dexamethasone was introduced in the drinking water as a 1mg/kg/day dose. This dose has been found to mimic the systemic effects of the overproduction of cortisol as previously shown (Hochberg et al., 2015). This dose is equivalent to therapeutic doses administered to human patients (Fleseriu et al., 2012; Tyrrell et al., 1986) and comparable to the doses used in other rodent studies (Beaudry, D'souza, Teich, Tsushima, & Riddell, 2013). After one week of treatment, mice were bred with age-matched virgin males. Dam body composition and water intake were monitored weekly. C57BL/6J mice were used to determine dam fertility and postnatal offspring outcomes. All C57BL/6J mice were allowed to deliver at term. After 16 days of mating, male breeders (F0) were removed from the cage to avoid the occurrence of a second pregnancy. We checked for litters on a daily basis after 2.5 weeks of mating. Gestational age, litter size, and pup survival rate and birth weight at postnatal day (PND) 0.5 were also assessed. The number of offspring born (F1) was recorded to determine maternal fertility and offspring viability. After delivery (delivery day denoted as PND0.5), the dams continued to have *ad libitum* access to food and water.

To better understand the role of placental glucocorticoid receptor in mediating the effects of dexamethasone at midgestation, a novel Cre-driven tissue-specific knockout of the placental

glucocorticoid receptor was developed as shown in the breeding schematic (Figure 1). To generate these mice, we first crossed *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ male and female mice with *Nr3c1*$^{+/+}$; Cre$^{Tg/Tg}$ female and male mice, respectively, to generate *Nr3c1*$^{fl/+}$; Cre$^{Tg/+}$ offspring. This is denoted as cross 0 (C0). We then crossed *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ females with *Nr3c1*$^{fl/+}$; Cre$^{Tg/+}$ males generating an expected 1:1:1:1 ratio of *Nr3c1*$^{fl/fl}$; Cre$^{Tg/+}$ (knockout): *Nr3c1*$^{fl/+}$; Cre$^{Tg/+}$ (heterozygous flox-GR/Cre, considered wild-type): *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ (homozygous flox-GR/no Cre, considered wild-type): *Nr3c1*$^{fl/+}$; Cre$^{+/+}$ (heterozygous flox-GR/no Cre, considered wild-type). We monitored offspring births, and out of the 254 offspring born from this cross, referred to as cross 1 (C1), we had 29% less knockout offspring born compared to wild-type when accounting for the expected 3:1 ratio of wild-type: knockout offspring using chi-squared testing ($p=0.036$, Figure 2). However, when looking at genotype ratios by sex, there were no significant differences (Figure 2). The knockout and homozygous flox-GR/no Cre offspring were further used to generate our parental strains.

To determine viability of offspring prior to our experiment, we further crossed female *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ with *Nr3c1*$^{fl/fl}$; Cre$^{Tg/+}$ males. The expected offspring ratio was a 1:1 *Nr3c1*$^{fl/fl}$; Cre$^{Tg/+}$ (knockout): *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ (wild-type). Of the 287 offspring that were born from this cross, referred to as cross 2 (C2), there were no significant differences in the number of offspring born per genotype and between genotypes by offspring sex using chi-squared testing (Figure 3). The offspring born from this cross were used as our experimental parental strains (F0) as described below.

The parental strains (F0) for this experiment were 6-8-week-old male *Nr3c1*$^{fl/fl}$; Cre$^{Tg/+}$ (referred to here as knockout; pGR-KO) crossed with 6-8-week-old female *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ (referred to here as wild-type; pGR-WT). Note that in this case the dams are always phenotypically wild-type, reducing the risk of off-target effects to maternal physiology. The non-decidual region of the placenta, however, is predicted to be a knockout, as it is derived from fetal tissues as shown in Figure 4. The offspring (F1) were thus also a combination of knockout and wild-type mice. We consider knockout offspring to be genotypically knockout but phenotypically wild-type since the Cre driver was a *Cyp19a1* promoter-Cre, which is expressed in trophoblast cells in the placenta. Mice were fed a normal chow diet (Lab Rodent Diet; 5L0D) with *ad libitum* access to food and water (control groups) or dexamethasone water (treatment groups) with weekly body weight assessments. Similar to the C57BL/6J mice, all wild-type dams were single-housed at 11 weeks of age, and dexamethasone was introduced in the drinking water as a 1mg/kg/day dose. After one week of treatment, mice were bred with age-matched virgin knockout males. All trophoblast-specific glucocorticoid-receptor mice were checked daily for a vaginal plug formation to denote embryonic day 0.5. All dams were euthanized at embryonic day 14.5 at midgestation for fetal and placental collection. A breeding schematic below (Figure 1) details the generation of the placental glucocorticoid-receptor strain. All animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the University of Michigan Institutional Animal Care and Use Committee prior to the work being performed.

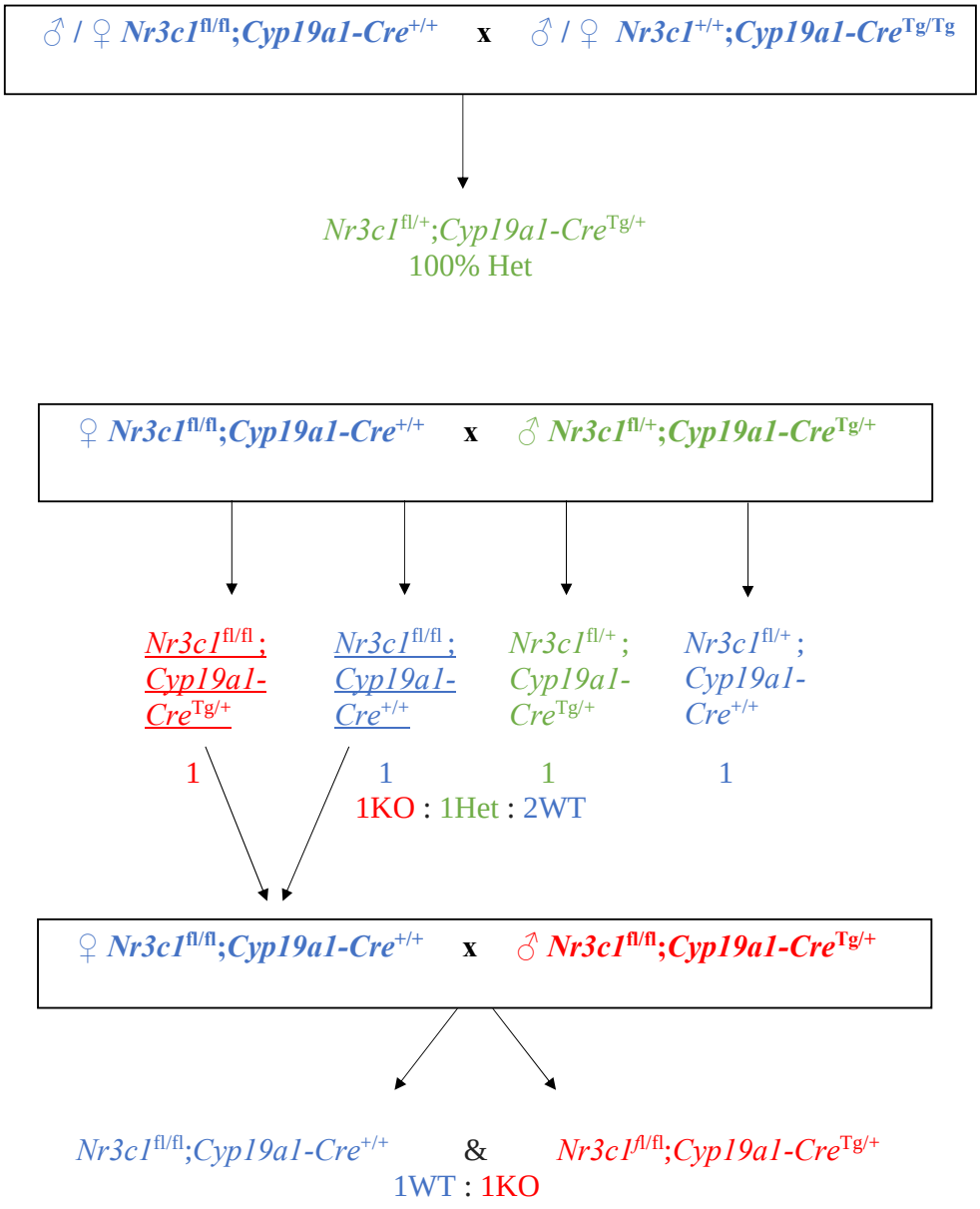


Figure 1: Breeding schematic to generate *Nr3c1*$^{fl/fl}$;*Cre*$^{+/+}$ (wild-type) and *Nr3c1*$^{fl/fl}$;*Cre*$^{Tg/+}$ (knockout) mice.

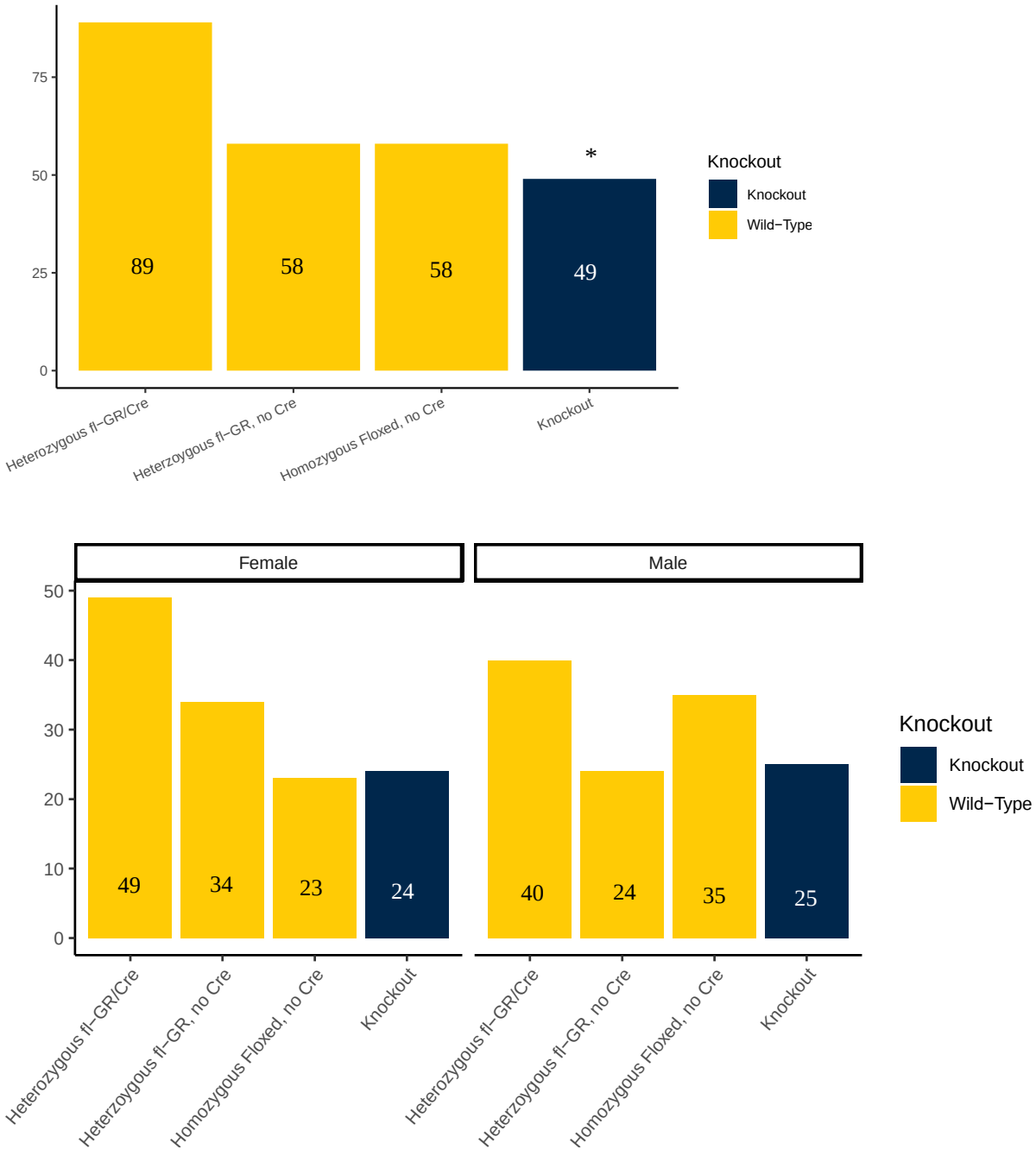
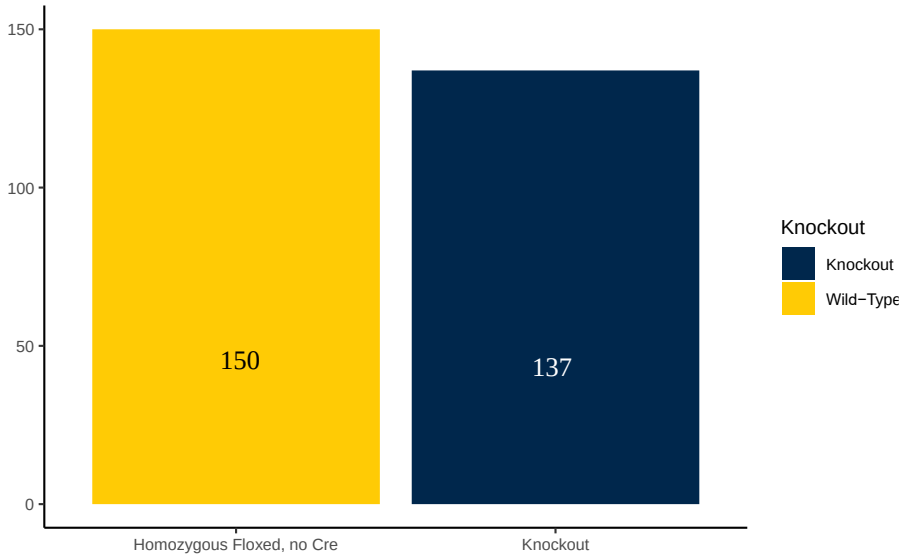


Figure 2: Number of offspring born per genotype and sex from cross 1 in control, water-treated dams generating an expected 3:1 wild-type:knockout ratio.

The numbers indicate number of offspring per genotype. Asterisks indicate a significant difference ($p<0.05$) between genotypes from a chi-squared test testing differences from the expected 3:1 wild-type:knockout ratio



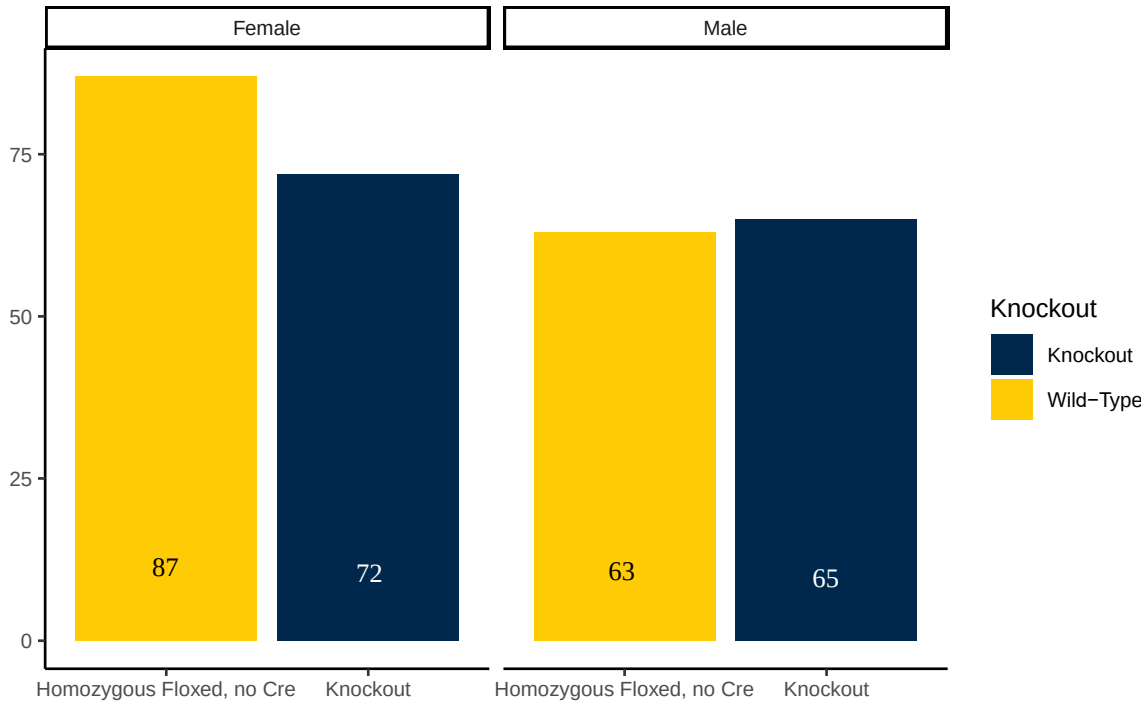


Figure 3: Number of offspring born per genotype and sex from cross 2 in control, water-treated dams generating an expected 1:1 wildtype:knockout ratio.

The numbers indicate the number of offspring per genotype.

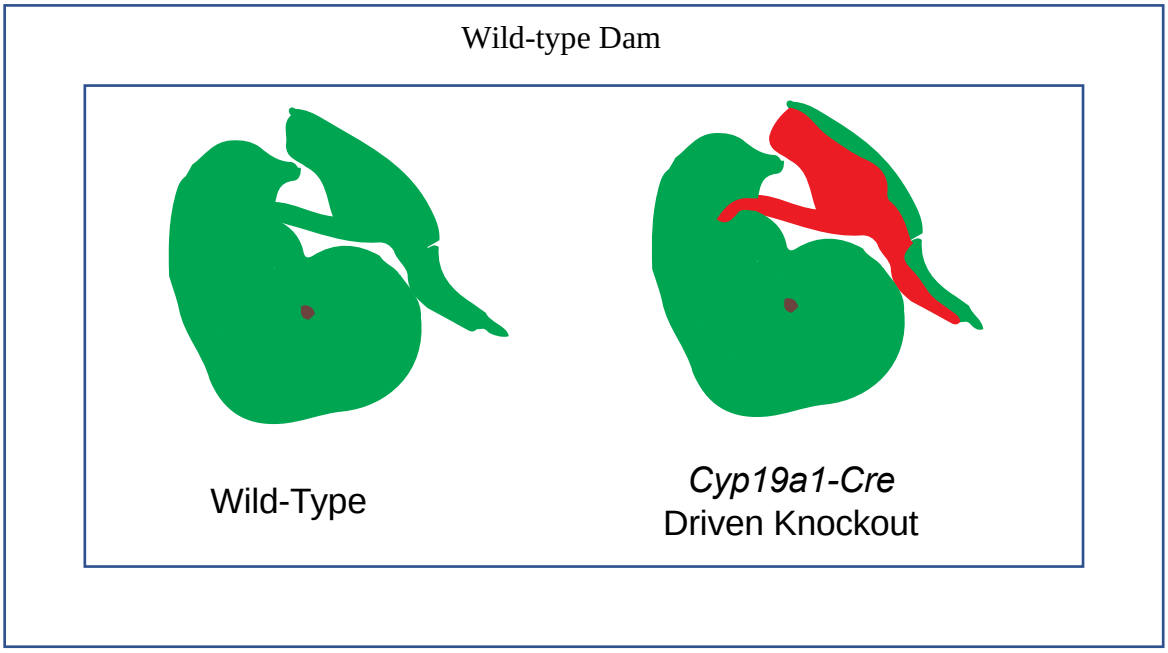


Figure 4: Schematic showing the placental and fetal expression of the Cyp19a1-Cre in the F1 offspring born to wild-type dams at an expected 1:1 wild-type:knockout ratio.

The wild-type placenta and fetus are expected to be genotypically and phenotypically wild-type (shown in green on the left). The knockout is shown on the right with the fetal-derived portion of the placenta being genotypically and phenotypically knockout (shown in red), whereas the maternal-derived portion of the placenta is wild-type (shown in green). The fetus with the knockout placenta is genotypically knockout but phenotypically wild-type (shown in green).

2.3.2 Dexamethasone Exposure

Water-soluble dexamethasone (Sigma) was prepared at a concentration of 53 mg/L. Based on normal water intake of mice, this corresponds to a dose of approximately 1 mg/kg/day. This is provided in the drinking water. Dexamethasone treatment was provided as the only water source for dams assigned to the dexamethasone treatment. Dexamethasone exposure began one week prior to mating to allow time for acclimatization to treatment prior to pregnancy. Dams assigned

to the dexamethasone treatment arm received freshly prepared dexamethasone water weekly. Water and dexamethasone intake were measured weekly.

2.3.3 Body Composition

Mice were weighed by dynamic weighing to capture accurate measurements using a digital scale (Mettler Toledo, ML6001T). For body composition assessment, mice were placed in the magnetic resonance imaging (MRI) tube and restrained during the magnetic resonance imaging measurement (EchoMRI, EchoMRI 1100). Fat, lean, free water, and total water mass (g) were recorded for each animal. Dams (F0) in all groups were weighed and underwent body composition assessment via MRI once a week during pregnancy and lactation. On the day of delivery, all C57BL/6J dams were weighed and their body composition was assessed via MRI. The offspring (F1) were weighed at PND0.5 and at embryonic day 14.5 (E14.5) for the placental glucocorticoid receptor knockout strain.

2.3.4 Euthanasia and Tissue Collection

All C57BL/6J dams were sacrificed using anesthetic gas inhalation (5% isoflurane via drop jar) after completion of the experiment without tissue collection. For the placental glucocorticoid-receptor strain, placental and fetal samples were extracted at E14.5. Briefly, dams were anesthetized using an isoflurane vaporizer. Toe punches were performed to ensure that the mouse was under anesthesia. After exposing the uterine horn, placental and fetal excision were conducted. The number of viable and resorbed placental-fetal pairs was recorded. The placenta for each offspring was detached from the maternal tissue and the umbilical cord then weighed

and collected in 2mL Eppendorf tubes, snap frozen in liquid nitrogen, and later stored at -80°C for future molecular assays with the number of samples being represented by the number of dams with a total n of: *Water groups:* n=5 male wild-type, 9 male knockout, 9 female wild-type, 8 female knockout; *Dexamethasone groups:* n=5 male wild-type, 6 male knockout, 7 female wild-type, 6 female knockout. A portion of each placenta was fixed in 10% formalin, dehydrated in 70% ethanol, and later embedded in paraffin for future histological analysis. For resorbed fetuses, the remaining placenta was taken for genotyping to determine placental sex and knockout or wild-type genotype. Fetuses were individually weighed after removal from the amniotic sac. Then fetuses were immediately sacrificed by decapitation and a tail clip was taken for genotyping (*Sry* and *Cre*). Fetuses were collected in 2mL Eppendorf tubes, snap frozen in liquid nitrogen, and later stored at -80°C for molecular assays. After the complete extraction of placental and fetal tissue, dams were euthanized while under anesthesia by cardiac exsanguination.

2.3.5 Statistical Analyses

Statistical significance was designated at $p<0.05$ for this study. All statistical analyses were performed using R v4.0.2 ("R: The R Project for Statistical Computing," n.d.). Data are presented graphically as mean +/- standard error of the mean. For longitudinal measurements, we looked at body composition changes and food and water intake over time using mixed linear models using lme4 v1.1-25 accounting for the random effect of the dam due to multiple recurrent measurements and measuring fixed effects of time (Bates, Mächler, Bolker, & Walker, 2014). For data pertinent to offspring genotypic ratios and resorptions, chi-square testing was used. Correlations between placental and fetal weights were determined using Pearson correlation tests

when distributions were normally distributed and Spearman correlation when distributions were non-normally distributed. Correlation coefficients reported here use Pearson correlation tests unless otherwise specified. We tested for sex-modification of all offspring outcomes involving both sexes using mixed linear models testing for interaction between genotype and fetal sex and report these when significant. For numerical variables, normality was assessed using Shapiro-Wilk tests followed by testing for homoscedasticity using Levene's test. Pending these results, appropriate parametric or non-parametric tests were done as noted in the figure legends.

2.4 Results

C57BL/6J Mouse Experiments

C57BL/6J mice were randomized into a control group receiving water or a treatment group receiving Dexamethasone at a dose of 1mg/kg/day. All dams were single housed and received Dexamethasone a week prior to mating (denoted here as week 1). Mating with a C57BL/6J male began on week 2. Dams were allowed to carry their pregnancies to term to determine the effect of *in utero* Dexamethasone exposure on postnatal offspring outcomes.

2.4.1 Dexamethasone Treatment Reduces Dam Body Weight and Lean Mass Gain During Gestation

Dam body weight and composition were assessed two weeks prior to mating and throughout gestation. While control dams gained weight during gestation, dexamethasone-treated dams maintained similar body weight, but gained fat mass similar to control dams (Figure 5A-B). Dams treated with dexamethasone were approximately 3 grams lighter than control counterparts

(Figure 5A, $p<0.001$). This was mainly driven by reductions in lean mass where dexamethasone caused reduced lean mass weight gain throughout pregnancy by 2.6 grams (Figure 5C, $p<0.001$). Fat mass, however, was unaltered throughout gestation in dams treated with dexamethasone (Figure 5B). Water and dexamethasone intake was recorded weekly, and dams consumed similar amounts a week prior to mating and throughout gestation (Figure 5D). Copulatory vaginal plugs were checked daily after mating started to determine gestational age. There were no significant differences in gestational age between dexamethasone and water dams (Figure 5E).

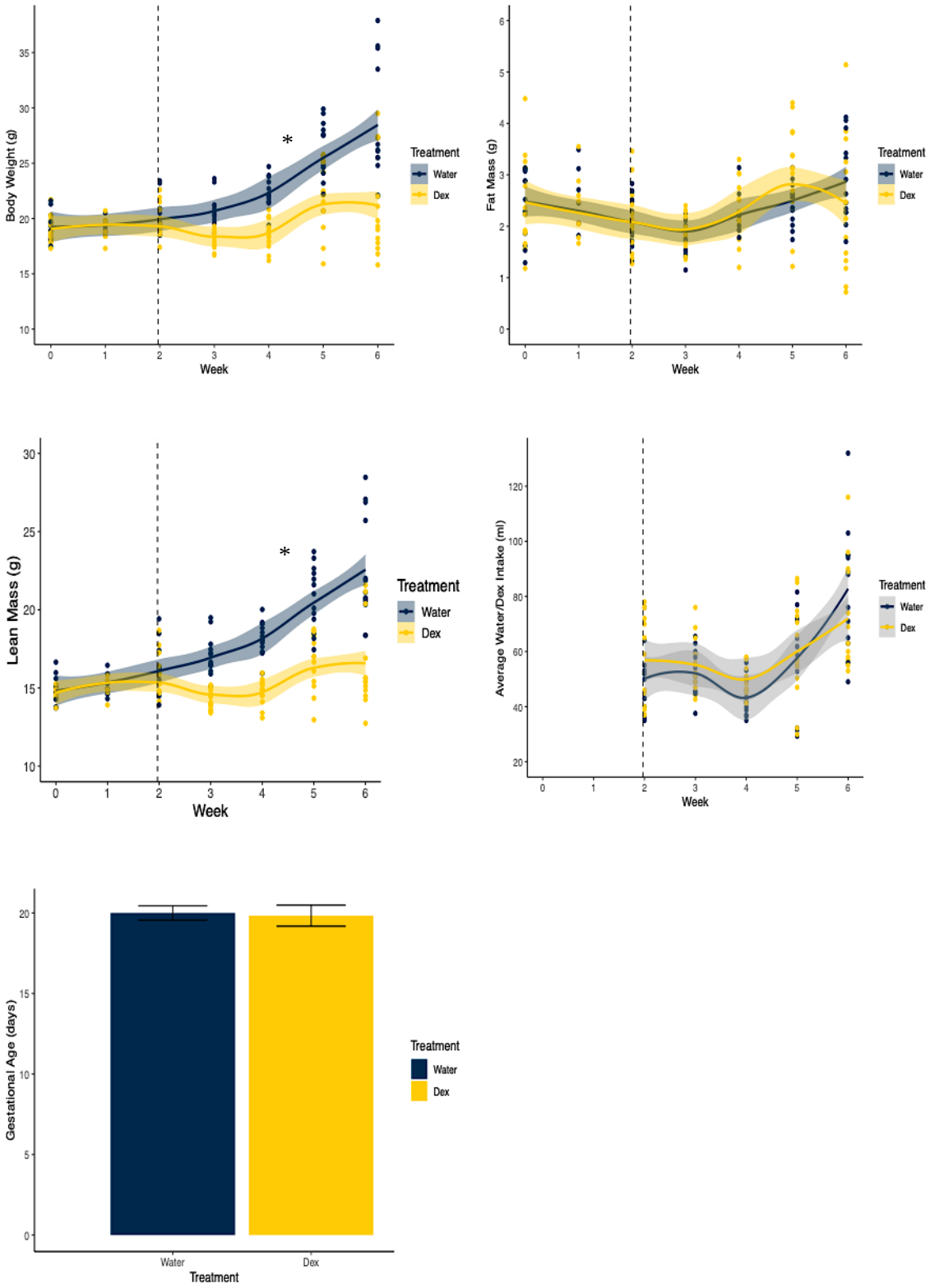


Figure 5: Dam body composition, water intake, and gestational age for water (n=13 dams) and dexamethasone (n=14 dams) groups.

(A) Dam body weight (g) before dexamethasone exposure (denoted as week 2) and throughout pregnancy (starting at week 3). (B) Dam fat mass (g). (C) Dam lean mass (g). (D) Average weekly dam intake of water or dexamethasone (ml). (E) Gestational age of dams (days) measured by determining the period between vaginal copulatory plug formation and delivery date. Asterisks indicate significant differences between control, water-treated dams and dexamethasone-treated dams using linear mixed model and pair-wise testing.

2.4.2 Dexamethasone significantly reduces litter size and offspring birthweight

All cages were checked daily for litters after 2.5 weeks of mating. The number of pups born to each dam was recorded. Dexamethasone dams delivered significantly less offspring with the litter size being reduced by 34% (Figure 6A, p=0.01). All offspring were weighed individually at delivery, denoted as postnatal day 0.5. Offspring of dexamethasone-treated dams were smaller by 37% compared to their water counterparts (Figure 6B, p<0.001).

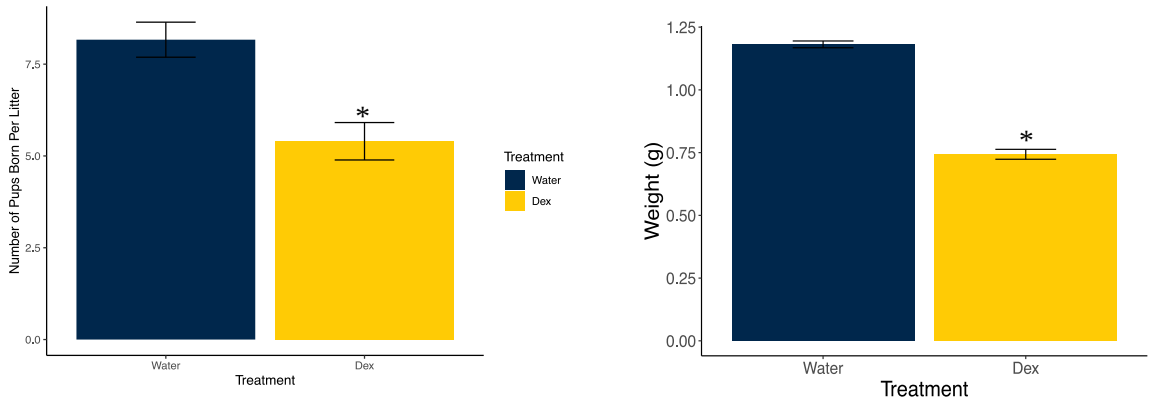


Figure 6: Litter size and offspring weight at delivery.

(A) Average number of offspring born per treatment. (B) Average weight of offspring after delivery on postnatal day 0.5 per treatment. Asterisks indicate significant differences between treatment groups using pair-wise testing (p<0.05). n=13 water dams and 14 dexamethasone dams.

2.4.3 Dexamethasone Causes Offspring Lethality by Postnatal Day 1.5, and Offspring Rescue Attempts Failed

Of the 82 offspring born to water-treated control dams, only 4 were not viable by postnatal day 3.5. All pups born to dams treated with dexamethasone were not viable by postnatal day 1.5 (Figure 7). Despite being viable at delivery, all 46 offspring born to dexamethasone dams did not survive past the first 24 hours of life. Using Cox proportional hazard modeling to determine survival probability, we find a 319-fold increase in offspring lethality probability with dexamethasone ($p<0.001$). We hypothesized that the reason the offspring were not viable could be due to lack of successful lactation or milk production by the dam or due to offspring adrenal insufficiency and hypoglycemia. To address these issues, we cross-fostered 7 offspring born to dexamethasone dams with control dams that were successfully lactating offspring of comparable age. To address concerns over hypoglycemia, we administered 10% glucose orally to 9 offspring born to dexamethasone dams once right after weighing the pups at delivery. Both rescue attempts failed and none of the dexamethasone offspring were viable.

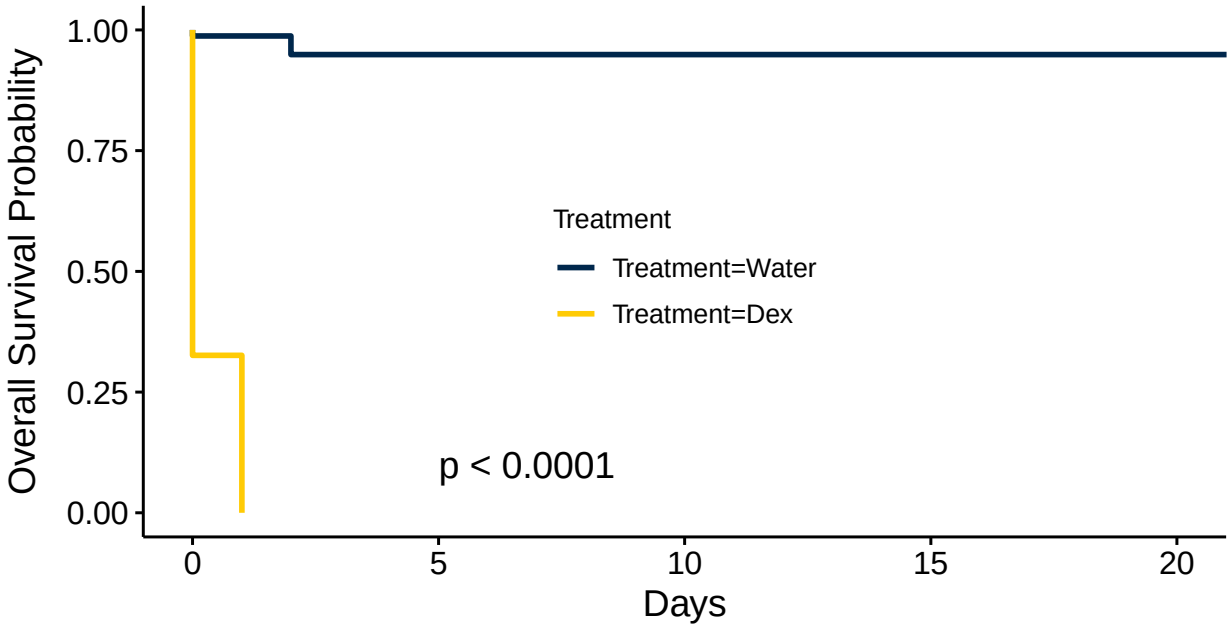


Figure 7: Offspring survival probability by treatment from birth (PND0.5) until postnatal day 21.5 using log-rank test.

Offspring n=82 from water-treated dams and n=46 from dexamethasone-treated dams.

Trophoblast-Specific Glucocorticoid Receptor Knockout Experiments

Our previous data demonstrated offspring lethality postnatally with dexamethasone exposure. As rescue attempts failed, we wanted to better understand the effects of dexamethasone on offspring development *in utero* and to determine if dexamethasone caused fetal or placental insufficiency. To isolate the role of placental glucocorticoid signaling on fetal development, we used a placental *Cre* driver *Cyp19a1-Cre* to generate a placental glucocorticoid-receptor knockout model (pGR-KO) solely in trophoblast cells. All dams were *Nr3c1*$^{fl/fl}$; Cre$^{+/+}$ wild-type to reduce the risk of off-target effects to maternal physiology. Only the males used in this experiment had the Cre transgene *Nr3c1*$^{fl/fl}$; Cre$^{Tg/+}$ knockout. This ensures that there is no effect of maternal genotype on offspring development and allows us to isolate the role of the placental glucocorticoid receptor.

2.4.4 Dexamethasone-Treated Placental Glucocorticoid-Receptor Wild-Type Dams had Similar Body Weights to Controls

Dam body weight was measured at 7-11 days prior to dexamethasone treatment initiation and throughout gestation until embryonic day (E) 14.5 when dams were euthanized. Body composition was not assessed for these mice. The dams, that were all wild-type, had similar body weight with no significant differences by treatment (Figure 8).

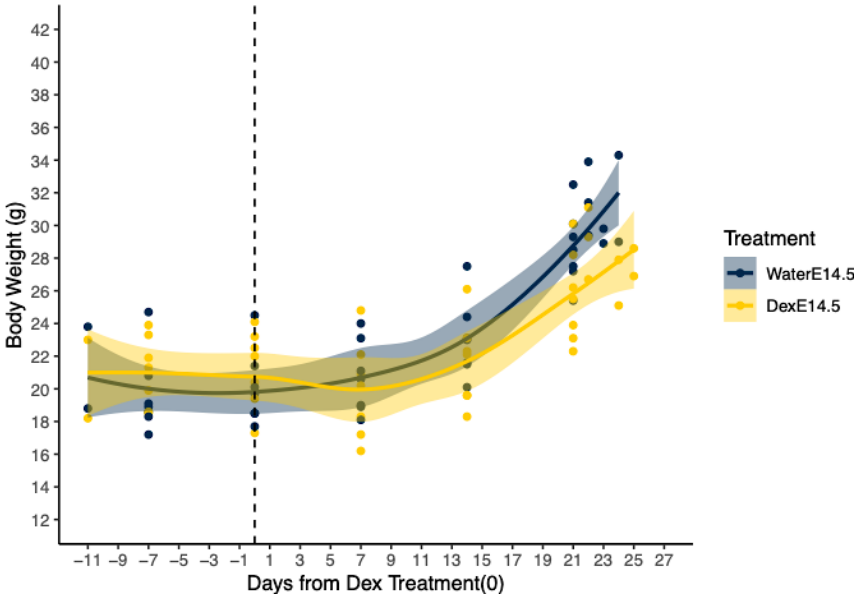


Figure 8: Dam body composition prior to dexamethasone (DexE14.5) treatment and throughout gestation until E14.5 (n=7 water and n=7 dexamethasone dams).

2.4.5 Dexamethasone Increases Fetal Resorption Percentage at E14.5 Despite Similar Average Fetal Implantations

On embryonic day 14.5, dams were euthanized for placental and fetal collection. All implantations were recorded despite resorption status. Resorptions were noted if the placenta was

a small dark circle or if no fetus was attached to the placental entity, however, resorbed placentas were also analyzed to determine fetal sex and genotype. The average number of fetal implantations was not significantly different between water and dexamethasone dams (Figure 9A, p=0.165). In spite of the similar average implantations, the percentage of resorptions was nearly two-fold higher for the dexamethasone-treated offspring although this did not reach statistical significance (Figure 9B, p=0.117).

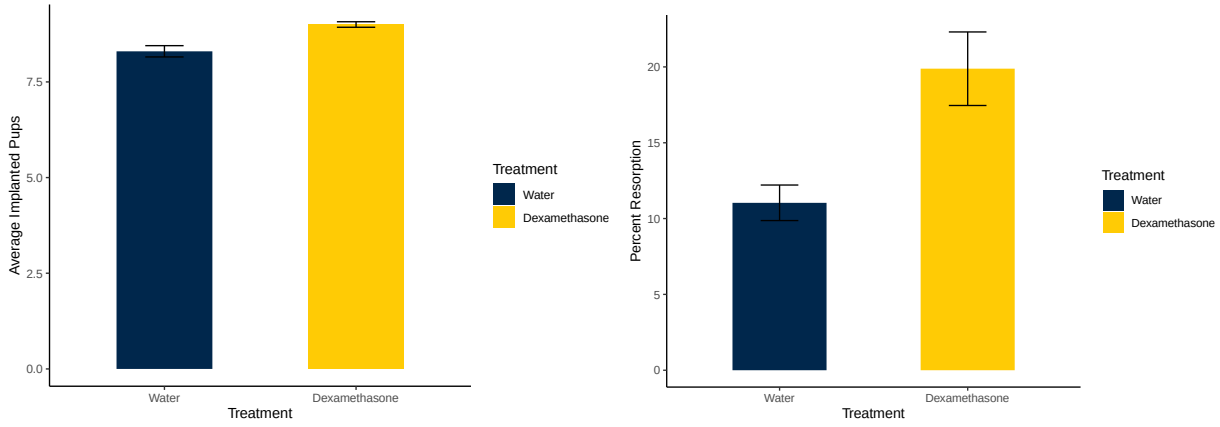


Figure 9: Average implanted offspring and offspring percent resorption by treatment group on E14.5 (n=10 water and n=7 dexamethasone pGR-WT dams).

2.4.6 Higher Percent Implantation for Females on E14.5, but with Increased Percent Resorption in Wild-Type Females Rescued by the Knockout

The expected genotypic ratio of the F1 offspring was a 1:1 wild-type:knockout with an expected 1:1 ratio of male and female implantations. Fetal genotypic analyses showed that on embryonic day 14.5, 60% of the implanted fetuses across treatments were females. Female implantations were 50% higher than male implantations when accounting for total implanted fetuses from water- and dexamethasone-treated dams (Figure 10, p=0.046). This significant difference was

mainly driven by the implantations from the dexamethasone-treated dams, where 68% of the implanted fetuses were females, nearly a two-fold increase compared to the male fetuses in the dexamethasone-treated dams (Figure 10, $p<0.001$). In the water-treated dams, female and male fetal implantations were comparable at 53% and 47%, respectively (Figure 10).

Resorption percentage by sex was 24% and 3% for females and males, respectively, demonstrating a significant 7-fold increase in female resorption percentage compared to males across treatment groups (Figure 10, $p<0.001$). Interestingly, for female offspring, 95% of the resorptions occurred in the wild-type while only 5% occurred in the knockout ($p<0.001$). In the males, 100% of the resorptions were in the wild-type offspring with 0 resorptions in the knockout (Figure 10, $p<0.001$). This shows that the knockout may reduce resorptions in a female-specific manner.

2.4.7 Dexamethasone Reduces Male Implantations but Rescues Male Resorptions

There were no significant differences in the percentage of fetal implantations by sex between water- and dexamethasone-treated dams. In female fetuses, percent resorption was higher in the dexamethasone group at 30% compared to 18% for the water control group, but this did not reach statistical significance (Figure 10, $p=0.083$). However, of the total implanted males at E14.5, 34% of the fetuses were from dexamethasone-treated dams which was lower than the 66% implanted fetuses from the water control dams (Figure 10, $p=0.001$) suggesting a sex-specific effect of dexamethasone on fetal implantations. The resorptions for male fetuses were only seen in the water-treated dams. Hence, 100% of the male resorptions were in the water group, and 0% of resorptions were seen in the dexamethasone group (Figure 10, $p<0.001$). This

suggests a potential protective effect of dexamethasone on male resorptions, but given the small number of male resorptions (n=2), it is hard to have confidence in the role of dexamethasone in male-specific resorptions.

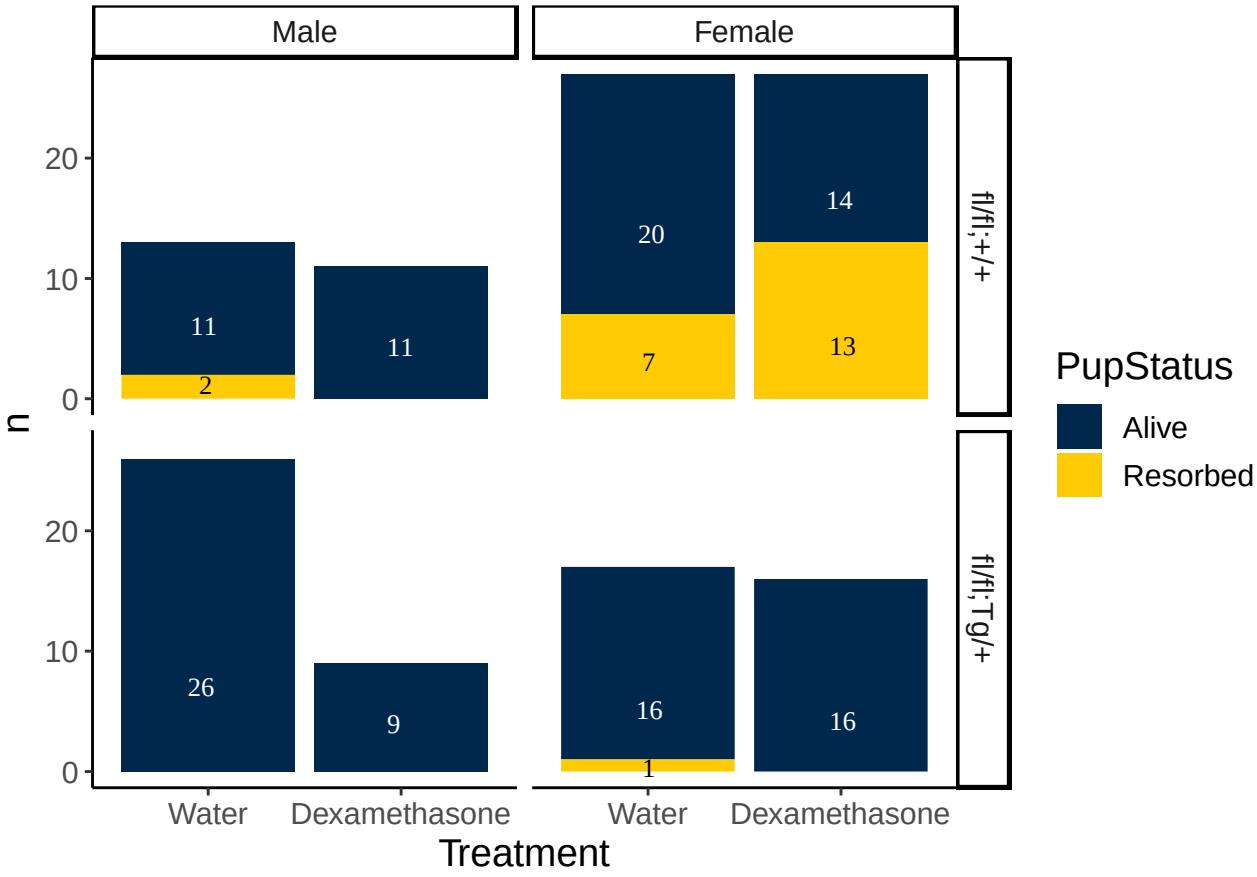


Figure 10: Fetal implantations and resorptions by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water and dexamethasone).

Each bar represents total fetal implantations per group. Resorbed fetuses are shown in yellow and their respective numbers are denoted on the bar. Alive fetuses are shown in blue with their respective number denoted on the bar.

2.4.8 Dexamethasone Reduces Placental Weight in Males, and this Effect is not Rescued by Knockout

To determine the role of dexamethasone and knockout on placental development, we individually weighed each placenta extracted at E14.5. There was no significant effect of dexamethasone treatment on placental weights (Figure 11, p=0.106) and no significant effect of the knockout genotype on pup weight (p=0.36). A sex specific analysis showed that for male fetuses, dexamethasone significantly reduced placental weights by 19 milligrams (Figure 11, p=0.038) with no significant effect of genotype. In female fetuses, there was no effect of dexamethasone treatment on placental weights, however, the knockout placentas were heavier than the wild-type by 9 milligrams although this did not reach statistical significance (p=0.134).

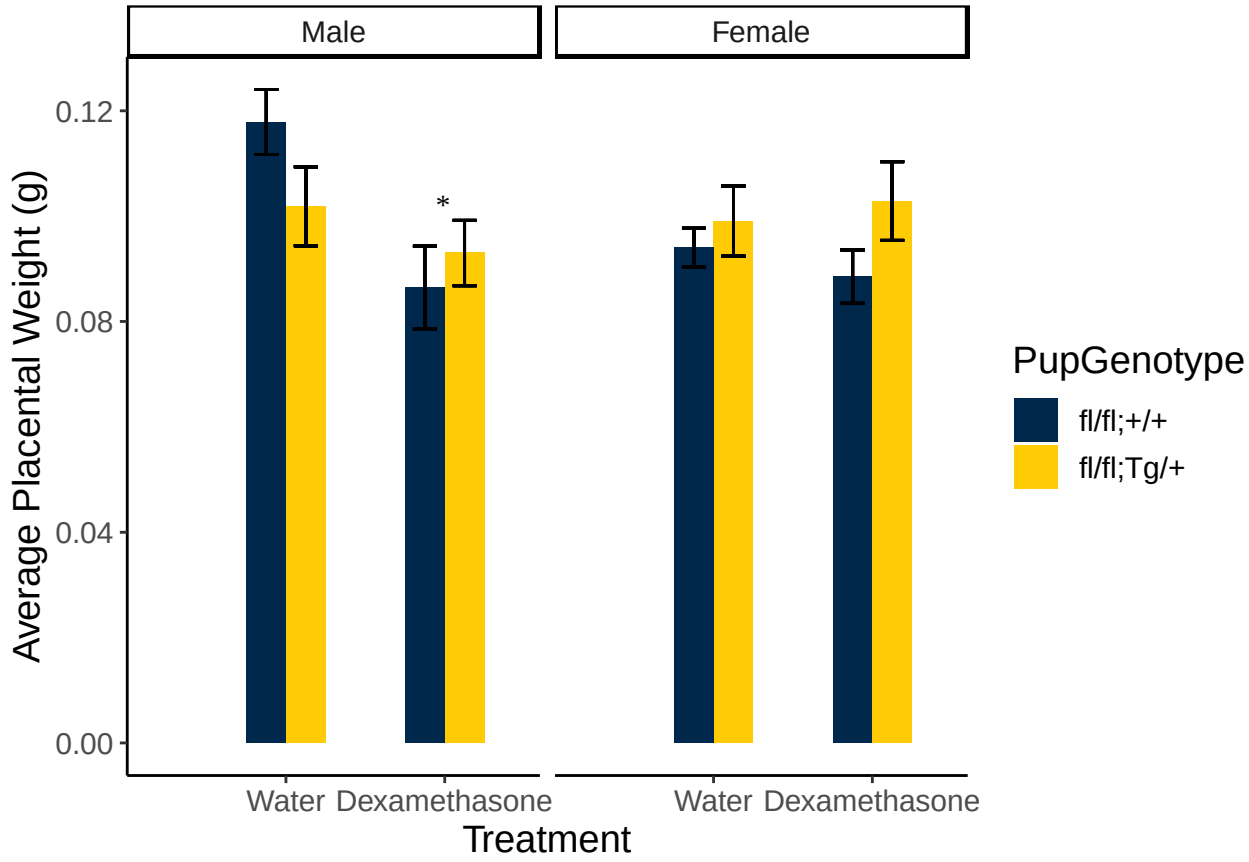


Figure 11: Placental weights by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water n-7 dams, and dexamethasone n=7 dams).

Asterisks indicate significant differences between treatment groups by sex from mixed linear model ($p<0.05$).

2.4.9 Dexamethasone Reduces Fetal Weight in Males, and this Effect is not Rescued by Knockout

Similar to placental weight, fetal weight weighed individually at E14.5 showed no significant difference between knockout and wild-type. However, there was a significant effect of dexamethasone on fetal weights where fetuses were 40 milligrams lighter than water groups (Figure 12, p=0.003). This effect was mainly driven by the weight of male fetuses where in male fetuses, dexamethasone significantly reduced fetal weights by 76 milligrams (p=0.001) with no significant effect of genotype. In female fetuses, there was no effect of dexamethasone treatment or genotype on fetal weights. This finding is consistent with the reductions in placental weights of males with dexamethasone treatment.

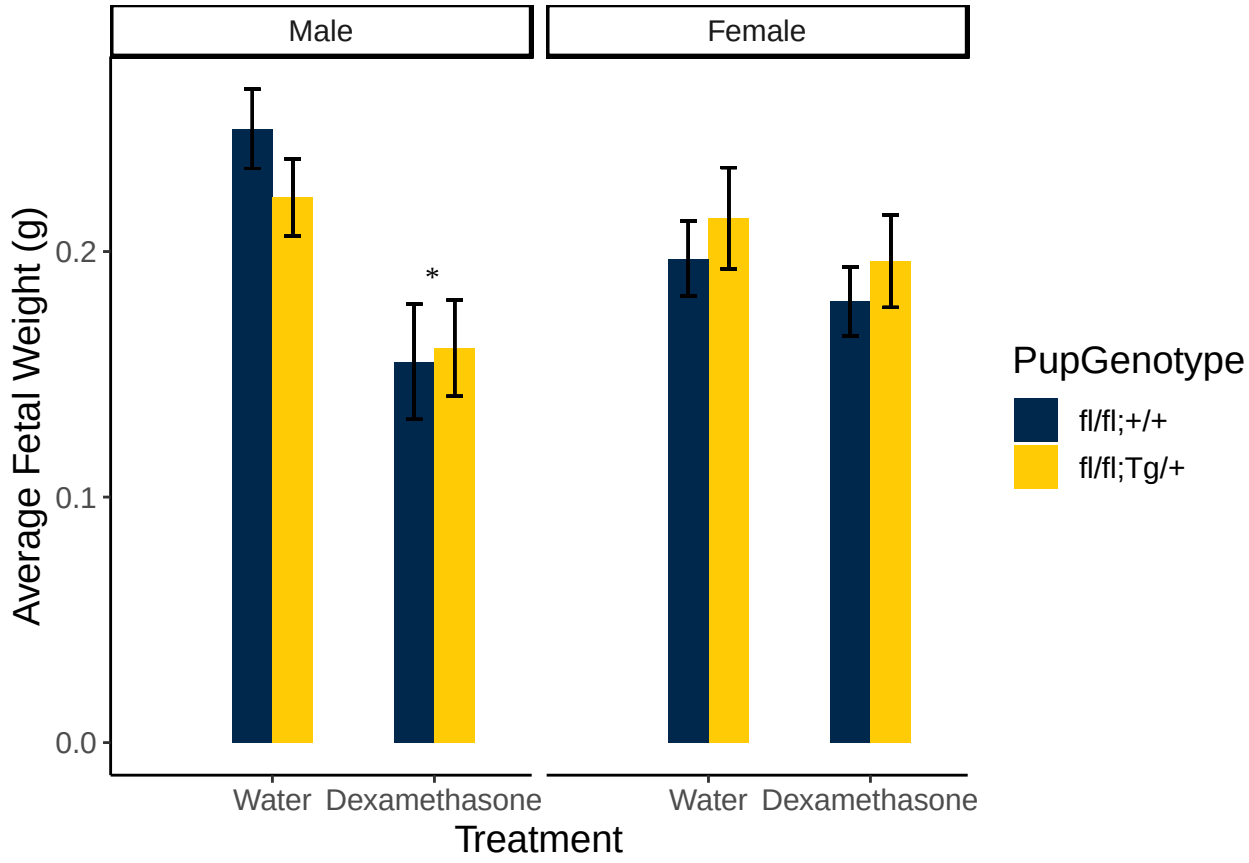


Figure 12: Fetal weights by sex (male and female), genotype (wild-type as fl/fl;+/+ and knockout as fl/fl;Tg/+), and treatment (water n=7 dams, and dexamethasone n=7 dams).

Asterisks indicate significant differences between treatment groups by sex from mixed linear model ($p<0.05$).

2.4.10 Placental and Fetal Weights are Significantly Correlated

As the placental and fetal weights were similarly altered by dexamethasone treatments especially in male fetuses, we wanted to test the correlation between fetal and placental weights to determine if they were proportionately affected by the glucocorticoid treatment. The overall placental-fetal weight correlation was significant (Figure 13; $r=0.65$, $p<0.001$). Male fetal and placental weights were significantly positively correlated (Figure 13; $r=0.72$, $p<0.001$).

Similarly, female fetal and placental weights were significantly correlated (Figure 13; r=0.54, p<0.001).

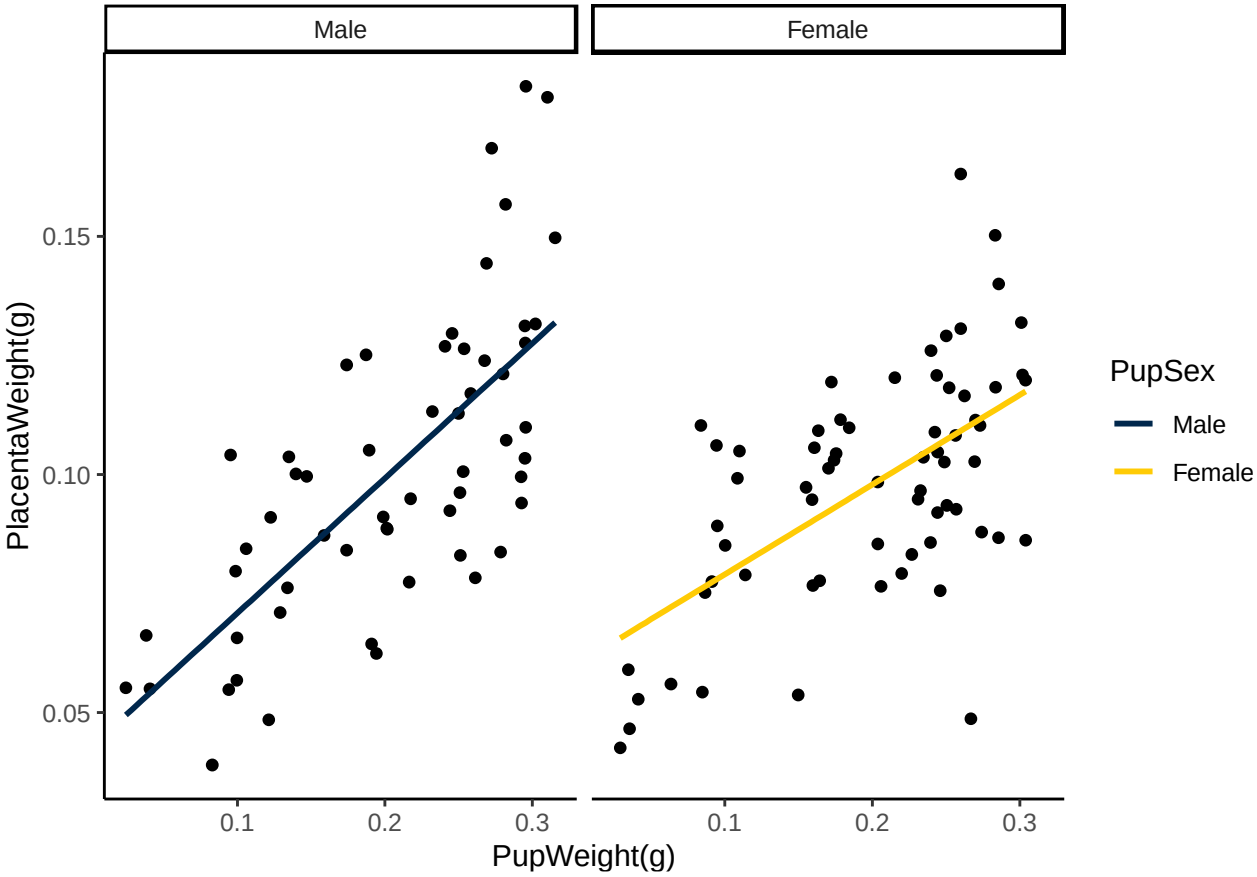


Figure 13: Significant correlation between placental and fetal weights by sex.

2.5 Discussion

In this study, we show that maternal dexamethasone treatment throughout gestation in mice did not affect female ability to conceive or gestational age. However, we show reduced litter size and offspring weight. Similar rodent glucocorticoid exposure experiments showed no effect on litter size or offspring viability when the dams were treated with glucocorticoids at mid or late gestation (Ain et al., 2005; Owen R Vaughan et al., 2012), but the timing and administration

route of glucocorticoids was not comparable to our study since we exposed the mice to dexamethasone a week prior to conception and all throughout gestation at a dose of 1mg/kg/day in their drinking water. Future studies using a series of lower doses may provide insights into the toxicological mechanisms of dexamethasone in antenatal mice. This is the first study to explore the effects of dexamethasone treatment throughout the entire gestational period. We also show that dexamethasone exposure was lethal to all born offspring within 24 hours postnatally. To our knowledge, there has not been any study that looked at offspring postnatal survival after a prolonged exposure of glucocorticoids throughout pregnancy. These findings showing reductions in viability and offspring size provide insight on how prolonged glucocorticoid exposure can affect offspring health. The mechanisms underlying offspring lethality have not been explored in this study. We can, however, eliminate the possibility that offspring are non-viable postnatally due to lack of maternal nursing ability, as cross-fostered offspring with water control dams were non-viable. In spite of this, impaired fetal suckling ability, adrenal insufficiency, and developmental anomalies could still be underlying issues.

In the placental glucocorticoid-receptor knockout strain (pGR-KO), dams exposed to dexamethasone had comparable weights during pregnancy to control dams given water. It is worth noting that the pGR-KO dams exposed to dexamethasone had similar fetal implantations compared to water control, which could contribute to weights of the dams being comparable. In the C57BL/6J mouse strain, the average number of offspring born was lower for the dexamethasone treatment, but the number of fetal implantations was not evaluated in the C57BL/6J experiments. It is possible that dexamethasone-treated C57BL/6J dams had lower weights compared to their water counterparts due to a reduced number of fetal implantations,

however, fetal implantations were not assessed in C57BL/6J mice, which is a limitation to our study.

In the placental glucocorticoid-receptor model, we further show sex-specific differences by treatment and genotype where female wild-type offspring were more prone to resorptions but all female offspring were resistant to dexamethasone treatments. The dams had similar fetal implantations across treatment groups although increased number of resorptions was demonstrated in the dexamethasone groups. Similar to our findings, in one study where mice were exposed to glucocorticoids a month prior to gestation and throughout gestation, increased fetal necrosis was shown at mid-gestation (Q. N. Li et al., 2018). Additionally, a short-term exposure to dexamethasone at mid-gestation reduced male offspring fetal weight at mid-pregnancy (O'Sullivan et al., 2013). Another study showed reductions in fetal weights in both sexes but reduced placental weights in female offspring only at mid-gestation after a brief glucocorticoid exposure (J.S.M. Cuffe et al., 2011).

Our study is the first that we are aware of to address resorption rates by offspring sex after a prolonged antenatal dexamethasone exposure. It would be interesting to examine litter sizes after delivery and offspring postnatal viability and birth weight by sex and genotype in the knockout model, but a limitation to our study is that postnatal data for dexamethasone-treated pGR-KO dams was not examined. Future work can better understand if postnatal effects of dexamethasone can possibly be mitigated by the knockout, as our current findings are promising showing female offspring resistance to dexamethasone at E14.5 but reduced fetal and placental weights and intrauterine growth restriction in males. This finding showing that antenatal dexamethasone

exposure did not significantly change female placental and fetal weights but reduced male placental and fetal weights matches the significant positive correlation between placental and fetal weights highlighting proportional effects of dexamethasone on placental and fetal weights. It has been hypothesized that female offspring are more resistant to *in utero* exposures (DiPietro & Voegtline, 2017), which is consistent with our findings with respect to fetal and placental weights as female weights were not altered by dexamethasone exposure, but inconsistent with our findings with respect to resorption rates that were higher in females. In our study, female fetuses had a higher percent implantation but also higher resorption percentage compared to males. This argues that it is possible that the female offspring that are born are the most resistant to *in utero* exposures, hence biasing our interpretation of risk susceptibility by offspring sex. Further work is warranted to better understand sex-specific risks, development, and viability to ascertain if females are less susceptible to exposures. We further show that resorptions with dexamethasone exposure occurred only in females, but males were resistant to dexamethasone-induced resorptions. Our findings that implantations and fetal and placental weights were reduced in male offspring upon antenatal dexamethasone exposure while female weights were unchanged is consistent with the literature showing an increased male susceptibility to early life exposures. However, another limitation to our study includes potential false positive data when genotyping for *Sry* and *Cre* which can affect our interpretation of the data (Isaksson, 2017; Marchini, 2011).

Our knockout model is a novel trophoblast-specific model that ablates placental glucocorticoid receptor, but this dissertation was not able to determine the effectiveness of GR knockout in placental tissue either at the level of GR, or in terms of GR responsive genes. In our initial

crosses to generate the parental strains for this study, we found a reduction in knockout offspring being born from cross 1. However, for cross 2, which generated our F0 parental strains, there were no differences in the wild-type to knockout ratio in males or females. Interestingly, in female fetuses assessed at E14.5, the knockout model rescued fetal resorptions at E14.5 regardless of treatment, and this rescue from resorptions could explain the similar number of viable female offspring we see from cross 2. It is difficult to assess if the knockout rescues male resorptions as we only had two male resorptions, both of which occurred in water and wild-type offspring. These differences could be driven by sex-specific differences in placental glucocorticoid receptor localization and activity in placenta of mice exposed to dexamethasone. In the mouse placenta, 8 isoforms of the glucocorticoid receptor were identified and differentially expressed by offspring sex and dexamethasone treatment (James S.M. Cuffe, Saif, Perkins, Moritz, & Clifton, 2017). A study conducted by Cuffe et al. showed that males exposed to dexamethasone have 55% increase in *Hspa1b* expression, increasing placental ability to inactivate glucocorticoid receptor signaling and show protective effects against dexamethasone in males (James S.M. Cuffe et al., 2017). Indeed, in our study, we did not see a significant effect of dexamethasone on male implantations in the wild-type group, potentially due to reduced glucocorticoid receptor activity. However, upon ablating the placental glucocorticoid receptor, dexamethasone reduced male fetal implantations in the knockout compared to water controls, highlighting a yet uncovered mechanism by which glucocorticoids can affect male fetal outcomes bypassing the glucocorticoid receptor altogether. This is not the case in female offspring, where the knockout rescued female resorptions across groups demonstrating a more direct mechanism by which maternal glucocorticoids can affect female placental glucocorticoid activity and lead to fetal death, which is consistent with an increased nuclear expression of

glucocorticoid receptors in female placentas when exposed to dexamethasone (James S.M. Cuffe et al., 2017). Interestingly, Cuff et al. found an increase in apoptosis in female placenta exposed to dexamethasone which could explain the higher number of resorptions seen in the wild-type females exposed to dexamethasone than the water group, although this difference was not statistically significant.

There are several strengths to our approach, including the use of matched diets, single parity, exposure to dexamethasone for a prolonged time-period, fetal sex assessment, and use of a novel trophoblast-specific *Cre* to isolate the role of placental glucocorticoid receptor in a wild-type pregnant dam. However, there are several limitations to this approach including the inability to exclude *in utero* effects on offspring postnatal viability in the C57BL/6J strain, the inability to determine the role of maternal systemic glucocorticoid exposure on offspring outcome, and the lack of a clear mechanism by which antenatal glucocorticoid exposure affects offspring. Future work can further examine these outcomes and mechanisms. This study demonstrated for the first time the role of placental glucocorticoid receptor in fetal viability by sex and the role of prolonged antenatal dexamethasone exposure on offspring postnatal outcomes.

Chapter 3 : Activation of Adipocyte mTORC1 Increases Milk Lipids in a Mouse Model of Lactation

3.1 Abstract

Human milk is the recommended nutrient source for newborns. The mammary gland comprises multiple cell types including epithelial cells and adipocytes. The contributions of mammary adipocytes to breast milk composition and the intersections between mammary nutrient sensing and milk lipids are not fully understood. A major nutrient sensor in most tissues is the mechanistic target of rapamycin 1 (mTORC1). To assess the role of excess nutrient sensing on mammary gland structure, function, milk composition, and offspring weights, we used an Adiponectin-Cre driven *Tsc1* knockout model of adipocyte mTORC1 hyperactivation. Our results show that the knockout dams have higher milk fat contributing to higher milk caloric density and heavier offspring weight during lactation. Additionally, milk of knockout dams displayed a lower percentage of saturated fatty acids, higher percentage of monounsaturated fatty acids, and a lower milk ω6: ω3 ratio driven by increases in docosahexaenoic acid (DHA). Mammary gland gene expression analyses identified changes in eicosanoid metabolism, adaptive immune function, and contractile gene expression. Together, these results suggest a novel role of adipocyte mTORC1 in mammary gland function and morphology, milk composition, and offspring growth.

3.2 Introduction

Human milk is considered the optimal source of nutrition for infants, and exclusive breastfeeding is recommended during the first 6 months of life (Lessen & Kavanagh, 2015). Successful lactation requires the development and differentiation of the mammary glands in preparation for milk production and secretion (Hartmann, Owens, Cox, & Kent, 1996; Macias & Hinck, 2012). The mammary gland is composed of several cell types including adipocytes, contractile muscles, and alveolar cells. Mammary adipocytes are necessary for proper gland development and structure (Landskroner-Eiger et al., 2010; Machino, 1976). The mammary adipocytes in close proximity to the alveolar epithelial cells are thought to provide primary lipids for milk production (Zwick et al., 2018). Given their role in maturation, development, and function of the mammary gland, adipocytes are crucial for successful lactation.

Maternal obesity has increased from around 26% in 2016 to 29% in 2019 ("Products - Data Briefs - Number 392 - November 2020," n.d.). The health of the offspring is highly influenced by intrauterine and early postnatal exposures (Barker, 2007). During early postnatal life, which is a critical developmental window, maternal obesity can alter breastfeeding capacity and milk composition (Ramji, Quinlan, Murphy, & Crane, 2016). Maternal obesity can delay the initiation of lactation (Rasmussen & Kjolhede, 2004b) and reduce the average duration of breastfeeding (Bider-Canfield et al., 2017). The probability of early termination of lactation at three months postpartum was 1.5 times higher for infants of obese mothers compared to lean mothers (Castillo et al., 2016). A meta-analysis of nine studies showed that maternal obesity increased mature milk fat composition but did not alter milk lactose or protein (Leghi et al., 2020). The fatty acid

composition of milk collected from obese women also showed a higher ω6:ω3 ratio compared to milk collected from non-obese counterparts (De La Garza Puentes et al., 2019).

Obese subjects have increased activity of the mechanistic target of rapamycin complex 1 (mTORC1) in the visceral fat compartment (Catalán et al., 2015). mTORC1 is a critical nutrient sensor and a major regulator of protein and lipid synthesis (Cai et al., 2016; X. Wang & Proud, 2006). mTORC1 promotes lipogenesis and adipogenesis and inhibits lipolysis (Cai et al., 2016; Laplante & Sabatini, 2009). In the presence of anabolic signals like insulin, energy abundance, and amino acid availability, mTORC1 function is upregulated (Catania et al., 2011). Developmental hyperactivation of mTORC1 in the mammary epithelium impairs the development of non-lactating mammary glands (Qin et al., 2016). However, little is known about the role of adipocyte mTORC1 with respect to macronutrient synthesis and secretion during lactation (Rezaei et al., 2016).

To elucidate the effects of excess nutrient sensor signaling on lactation, we used a genetic adipocyte *Tsc1* knockout model in mice. We show that chronic mTORC1 activation in maternal adipocytes increases adipocyte number and volume in mammary glands, changes milk fat levels and milk fatty acid composition, reduces gene expression of adaptive immune cell markers in the mammary glands, and increases the weight of suckling offspring.

3.3 Materials and Methods

3.3.1 Animal Husbandry

All mice were purchased from The Jackson Laboratory. Mice were fed a normal chow diet (Lab Rodent Diet; 5L0D) with *ad libitum* access to food and water. To hyperactivate adipocyte mTORC1 and generate an adipose-specific *Tsc1* knockout, *Tsc1*$^{fl/fl}$ mice (JAX stock #005680, RRID: IMSR_JAX:005680; (Kwiatkowski et al., 2002)) were crossed with *Adipoq*-Cre mice expressing the adipocyte-specific constitutive Cre recombinase controlled by adiponectin gene promoter (JAX Stock #010803, RRID: IMSR_JAX:010803; (Eguchi et al., 2011)).

The parental strains (F0) for this experiment were 6-8-week-old male *Tsc1*$^{fl/fl}$; Cre$^{Tg/+}$ (referred to here as knockout) and *Tsc1*$^{fl/fl}$; Cre$^{+/+}$ (referred to here as wild-type) crossed with 6-8-week-old female *Tsc1*$^{fl/fl}$; Cre$^{+/+}$ and *Tsc1*$^{fl/fl}$; Cre$^{Tg/+}$, respectively. The offspring (F1) were thus also a combination of knockout and wild-type mice. The Cre driver was Adiponectin-Cre, which is expressed in all adipocyte lineages (brown, white, and mammary adipocytes; (F. Wang et al., 2013; Z. V. Wang, Deng, Wang, Sun, & Scherer, 2010)). There is currently no known Cre driver that is specific to mammary adipocytes. All animal procedures were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and was approved by the University of Michigan Institutional Animal Care and Use Committee prior to the work being performed.

After 16 days of mating, male breeders (F0) were removed from the cage to avoid the occurrence of a second pregnancy. We checked for litters on a daily basis after 2.5 weeks of mating. The number of offspring born (F1) was recorded to determine maternal fertility and offspring viability. After delivery (delivery day denoted as postnatal day 0.5, PND0.5), the dams continued to have *ad libitum* access to food and water. At PND2.5, offspring were sexed by anogenital

distance assessment, and litters were culled to four animals (2 females and 2 males, when possible) to normalize milk supply.

3.3.2 Body Composition

Mice were weighed by dynamic weighing to capture accurate measurements using a digital scale (Mettler Toledo, ML6001T). For body composition assessment, mice were placed in the magnetic resonance imaging (MRI) tube and restrained during the magnetic resonance imaging measurement (EchoMRI, EchoMRI 1100). Fat, lean, free water, and total water mass (g) were recorded for each animal. Dams (F0) in all groups were weighed and underwent body composition assessment via MRI three times a week during pregnancy and lactation. On the day of delivery, dams were weighed and their body composition was assessed via MRI. At the end of our experiment, on PND16.5, all dams were weighed and underwent MRI prior to milk collection then were immediately euthanized. The offspring (F1) were weighed at PND0.5, 7.5, and 14.5. On PND16.5, the offspring were weighed and underwent body composition assessment via MRI then were immediately euthanized.

3.3.3 Determining Milk Output Volume

At peak lactation (PND10.5; (Q. A. Wang et al., 2018), we determined milk output volume for wild-type and knockout dams. To determine milk volume, we used the weigh-suckle-weigh technique (Boston, Bleck, Conroy, Wheeler, & Miller, 2001). Briefly, we weighed the dam separately and the offspring in aggregate. The dam and offspring were then separated for two hours to allow the milk reserves of the dam to build. During the two-hour separation, the

offspring were placed in a new cage and were kept warm using a heating pad under the cage. In the meantime, the dam remained in its initial cage with *ad libitum* access to normal chow diet and water. After the two-hour separation period, the dam was weighed and the aggregate weight of the offspring was measured, each for a second time. The offspring were then returned to the home cage and were allowed to nurse for one hour undisturbed. At the end of the nursing period, the dam was weighed again, and the aggregate weight of the offspring was measured for a third time. Milk volume was approximated as the weight change of the offspring and the dam after the one hour nursing period.

3.3.4 Euthanasia and Tissue Collection

All dams were sacrificed using anesthetic gas inhalation (5% isoflurane via drop jar method) at PND16.5 after milk collection. We extracted thoracic, inguinal, and abdominal mammary glands. Briefly, the peritoneum was pulled away from the skin, and the inguinal and abdominal 4^{th} and 5^{th} mammary glands (referred to here as lower mammary glands) were excised completely and weighed. The tissue from the left and right lower mammary glands were collected in 2mL Eppendorf tubes, snap frozen in liquid nitrogen, and later stored at -80°C for molecular assays. Portions of the thoracic mammary glands (referred to here as upper mammary glands) were fixed in 10% formalin, dehydrated in 70% ethanol, and later embedded in paraffin for histology and adipocyte assessment. Offspring of dams were sacrificed without tissue extraction at PND16.5 after body weight and composition measurements.

3.3.5 Milk Collection

On PND16.5, we collected milk samples (~0.5mL) from the nursing dams. To do this, after two hours of separation from the offspring, we anesthetized the dam by intramuscular injection of Ketamine/Xylazine (0.1275g/kg body weight) into the hindlimb muscle. Once the dam was fully anesthetized, we then injected oxytocin intramuscularly into the forelimb (2U/dam) to induce milk let-down. The dam's nipples were manually squeezed to promote milk secretion, and the milk was collected into a 1.5 mL tube via suction using a 50mL conical vacuum apparatus. After milking was complete, the dam was immediately euthanized using isoflurane and the mammary glands were extracted.

3.3.6 Milk Composition Assessment

Milk samples collected from wild-type and knockout dams on PND16.5 were assessed for fat content by the creamatocrit method using a microhematocrit centrifuge (Collares, Gonçalves, & Ferreira, 1997). Briefly, whole mouse milk was diluted four-fold with 1X PBS-EDTA solution. Milk samples were transferred into plain microhematocrit glass capillary tubes. The tubes were sealed from one end using Critoseal. The tubes were later placed in a microhematocrit centrifuge (Iris Sample Processing, StatSpin CritSpin M961-22). Samples were centrifuged for 120 seconds per cycle for a total of 8 cycles and a total spin time of 16 minutes. The capillary formed layers of non-fat milk and white fat. The length of the white fat layer was measured using a 150 mm dial caliper (General Tools, 6" Dial Caliper). The total volume of milk (non-fat and fat milk layers) was also measured in mm. Percentage of fat was determined with respect to the total milk volume.

Lipidomic analyses were done by the Michigan Regional Comprehensive Metabolomics Core. Milk samples were frozen at -80°C until analysis to prevent lipid hydrolysis and peroxidation. Samples were quickly thawed once for lipidomic analysis without undergoing multiple freeze-thaw cycles. Long chain fatty acid concentrations were determined following sample extraction, semi-purification and derivatization followed by fatty acid measurement by gas chromatography (GC) using an Agilent GC model 6890N equipped with flame ionization detector. Results were reported on 33 lipid classes from C14:0 to C24:1.

3.3.7 Transcriptomic Analyses

Using the lower right mammary gland tissues collected from the dams on PND16.5, we assessed whole-transcriptome RNA expression using five wild-type and six knockout samples. RNA samples were prepared from the mouse tissues using the PureLink RNA Mini Kit (Invitrogen by ThermoFisher Scientific, catalog #12183025). Briefly, tissues were cut on dry ice to ~50mg then homogenized and treated to collect purified RNA. The RNA was quantified using a nanodrop, and purity was verified by an Agilent Bioanalyzer. All samples had an RNA integrity number (RIN) higher than 7. Library preparation and next generation sequencing was conducted by the Advanced Genomics Core at the University of Michigan. Paired-end poly-A mRNA libraries were generated and sequenced to an average depth of 57M (range 46M-69M) reads per sample on Illumina NovaSeq 6000 (S4). Reads were aligned to the mouse reference genome GRCm38.p6 using Salmon v 1.3.0 (RRID: SCR _017036; (29)) with the gc-bias and validateMappings flags. Mapping efficiency was 54.8% (sample range 53-56.6%). Transcript-level data was reduced to gene-level data via tximeta v1.8.4 (Love et al., 2020) and txiimport v1.18.0 (Soneson, Love, & Robinson, 2016) prior to analysis by DESeq2 v1.30.1 (Love, Huber,

& Anders, 2014). To determine differentially expressed genes (DEGs), we evaluated 14242 genes, excluding those with low or no read counts. For gene set enrichment analyses, we used ClusterProfiler v3.16 (RRID: SCR_016884; (33)) after ranking genes by fold change and analyzing relative to Gene Ontologies. Similarities between enriched gene sets were calculated by Jaccard distances. Data are available from GEO at accession number GSE175620.

3.3.8 Mammary Gland Histology and Adipocyte Assessment

Upper right mammary glands collected from wild-type and knockout dams were embedded in paraffin and Hematoxylin and Eosin (H&E) stained at the Rogel Cancer Center's Tissue and Molecular Pathology Core. Slides were blindly assessed for adipocyte size and number using one slide per mouse. Using an EVOS XL Imaging System inverted fluorescent microscope (Invitrogen by ThermoFisher Scientific, catalog # AME3300), eight representative pictures per slide were taken at 10x magnification and covered the entire tissue area. Mammary gland adipocytes were quantified using ImageJ with Adipocyte Tools Macros Plugin (MontpellierRessourcesImagerie, 2020). In analyzing our images, the parameter filters for adipocytes using the processing options were set at minimum of 40 pixels, maximum of 1000 pixels, and 30 dilates. The parameters for segmentation options were set at minimum of 600 pixels and maximum of 1500 pixels. Potential adipocytes that were blurry, cut off, or below the threshold were excluded from the assessment to maintain accurate measurements. Once these two parameters were set on the image, manual additions and deletions were performed to ensure adipocytes were properly identified. After accounting for all adipocytes, they were further analyzed using the ImageJ software to obtain area measurements. The calculated adipocyte numbers were normalized to the total mammary gland area that was imaged.

3.3.9 Statistical Analyses

Statistical significance was designated at $p<0.05$ for this study. All statistical analyses were performed using R v4.0.2 ("R: The R Project for Statistical Computing," n.d.). Data are presented graphically as mean +/- standard error of the mean. For longitudinal measurements, body composition, food intake, and offspring weight gain data were analyzed over time using mixed linear models using lme4 v1.1-25 (Bates et al., 2014). We tested for sex-modification of all offspring outcomes involving both sexes using mixed linear models accounting for interaction between weight and fetal sex and report these when significant. For the mixed linear models, we accounted for the random effects of the offspring due to multiple recurring measurements and time was used as a fixed effect. For pairwise testing, normality was assessed using Shapiro-Wilk tests followed by homoscedasticity using Levene's test. Pending these results, appropriate parametric or non-parametric tests were done as noted in the figure legends.

3.4 Results

To understand how activation of mTORC1 in adipocytes affects lactation, we evaluated pregnant mice that were either wild-type or adipocyte *Tsc1* knockout. Virgin dams were mated with a male having the opposite genotype. The experimental timeline and mouse models are shown in Figure 14A-B.

3.4.1 Fat Mass Gain in Adipocyte Tsc1Knockout Mice During Lactation

Dam body weight and composition were assessed during pregnancy and lactation. Body weights were comparable between dams throughout the study (Figure 14C). Lean mass was also similar

between adipocyte *Tsc1* knockout and wild-type dams (Figure 14D). Knockout dams had a slightly lower fat mass during pregnancy and during lactation (Figure 14E). While wild-type dams lost a small amount fat mass during lactation, knockout dams gained fat mass (Figure 14F, $p<0.001$). This was not explained by differences in food intake, as wild-type and knockout dams had similar food intake throughout pregnancy and lactation (Figure 14G).

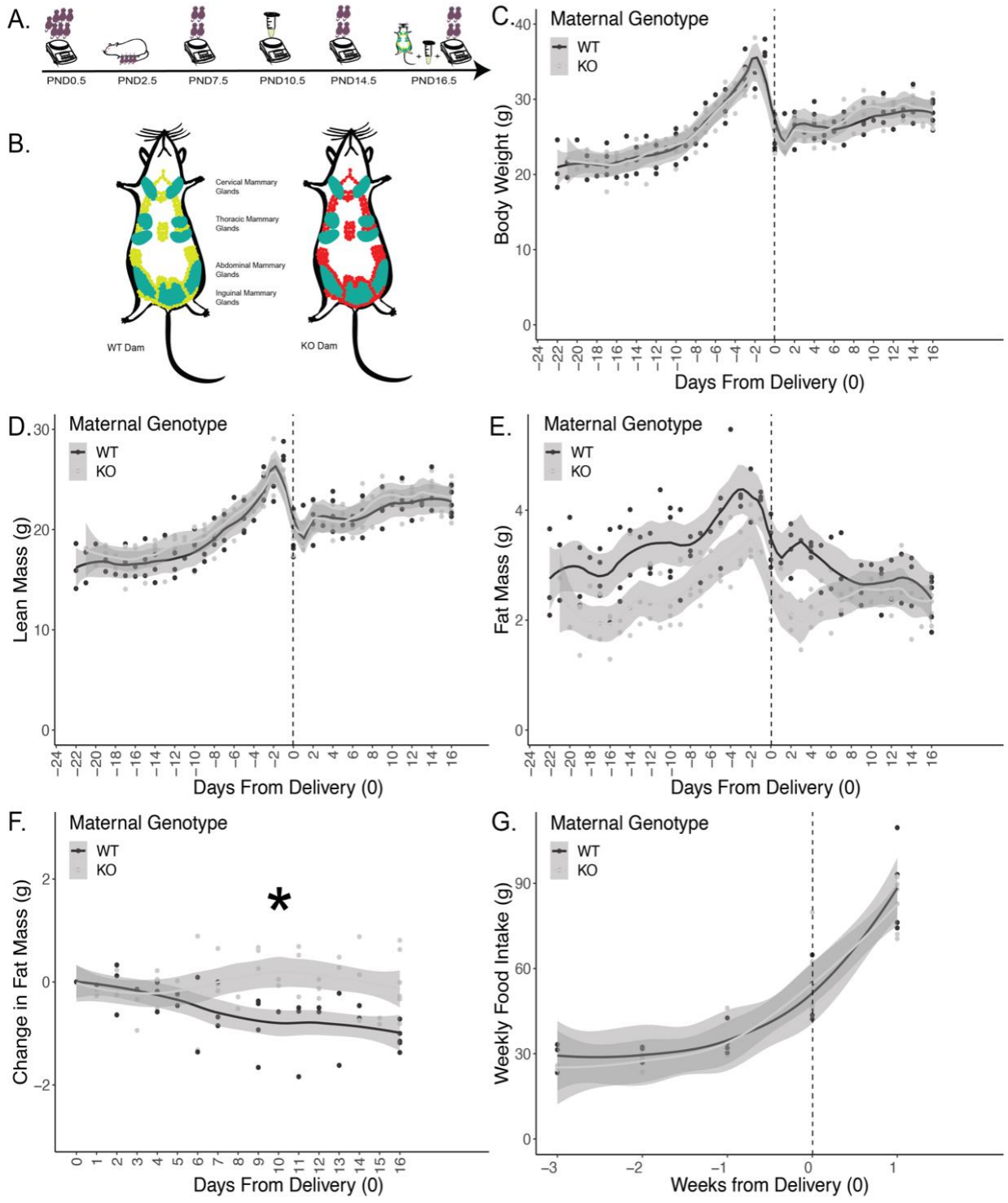


Figure 14: Experimental timeline and dam body composition.

(A) Experimental timeline. Dams and offspring were monitored throughout lactation. Offspring were born and weighed at PND0.5. Litters were culled to 4 offspring (2 males and 2 females) on PND2.5. Milk volume measurements were conducted on PND10.5. Offspring were weighed on PND7.5 and PND14.5. PND16.5 marks the end of the experiment where the dam and offspring were weighed then euthanized,

milk was collected from mammary glands of the dam, and mammary glands were excised for histological and molecular studies. Maternal body composition was measured on PND0.5 after delivery and three times a week thereafter until and including PND16.5. (B) Schematic showing mammary glands (in teal) and whole body adipocytes for wild-type (WT; in green) and knockout (KO; in red) dams with Tsc1 deletion. (C) Maternal body weights. (D) Maternal lean mass. (E) Maternal fat mass. (F) Maternal change in fat mass postnatally from the day of delivery until PND16.5 reveals more fat mass gain during lactation in knockouts. (G) Weekly food intake. Asterisk indicates $p<0.05$ from mixed linear model. (n=11 dams; 5 wild-type and 6 knockout).

3.4.2 Adipocyte Tsc1 Knockout Mice Have Smaller Mammary Glands with More and Larger Adipocytes

At sacrifice on PND16.5, we examined the inguinal and abdominal mammary glands. As shown in Figure 15A, knockout dams had a 21% reduction in mass of the right mammary glands ($p=0.042$) and a 29% reduction in mass of the left mammary glands ($p=0.001$) compared to the wild-type counterparts.

Right thoracic mammary glands were fixed and stained for histological analyses to assess the number and area of adipocytes in the dams (Figure 15G-H). Adipocyte *Tsc1* knockout mammary glands had 63% more adipocytes compared to the wild-type dams (Figure 15B, $p=0.057$). We then assessed the adipocyte area as a fraction of the total mammary gland and found that in the knockout, adipocytes occupied three times the mammary gland area compared to the wild-type mammary adipocytes (Figure 15C, $p=0.051$).

The mean area for mammary adipocytes was not significantly different (Figure 15D, $p=0.36$), however, the distribution of adipocyte sizes was different. Adipocyte *Tsc1* knockout mammary adipocytes had a significantly wider variation in the distribution of adipocyte area (Figure 15E, $p<0.001$). Knockout mammary glands had 52% more larger sized adipocytes (200-300 μm^2) compared to wild-type (Figure 15F, $p=0.039$). Similarly, *Tsc1* knockout mammary glands had 46% fewer adipocytes in the smallest range (0-100 μm^2) compared to wild-type adipocytes

(Figure 15F, p=0.060). Our results show histological differences where adipocyte *Tsc1* knockout dams have more adipocytes, more larger adipocytes, and a higher percentage of total mammary gland area comprised of adipocytes, despite having smaller mammary glands overall.

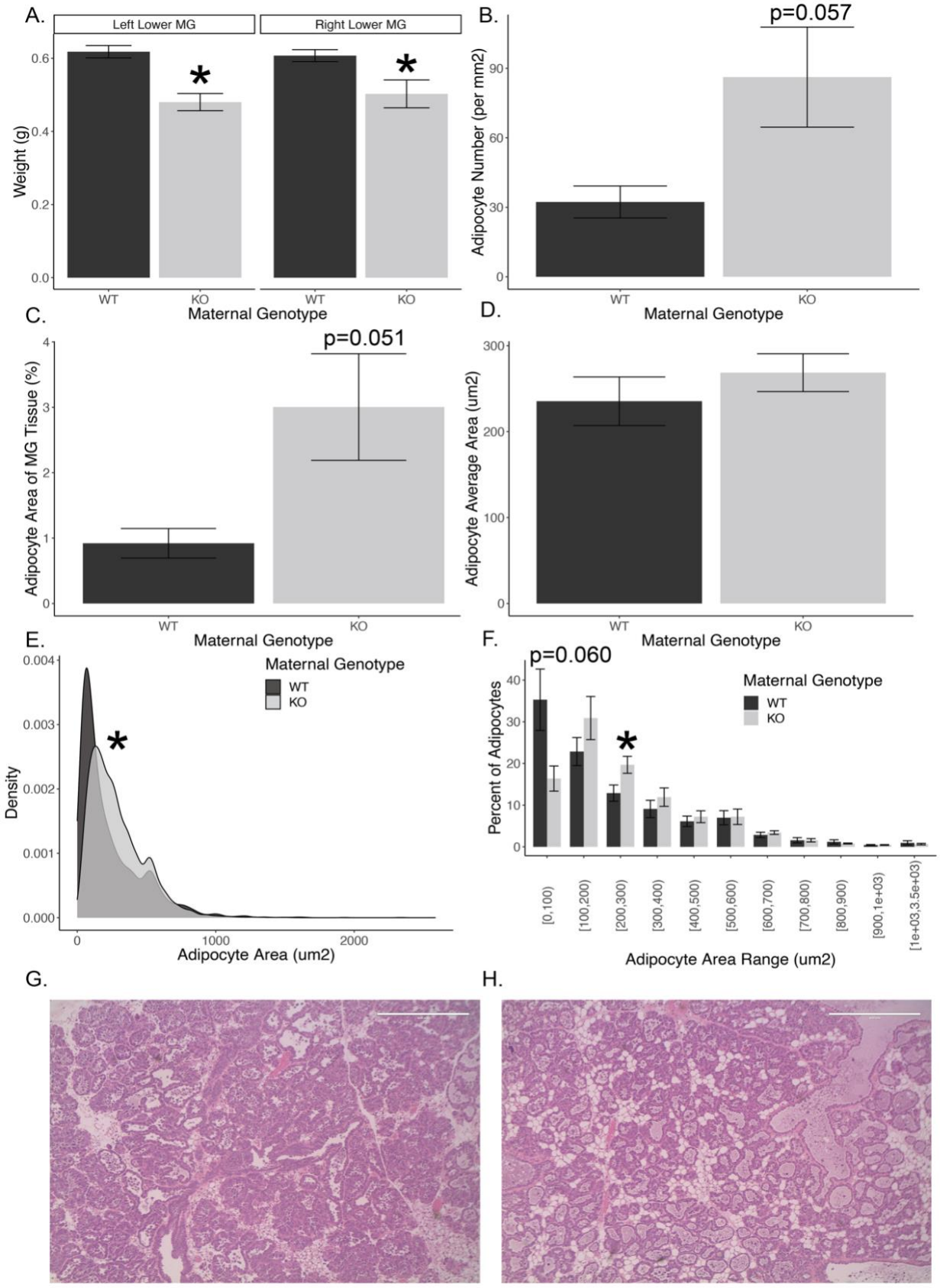

A.
Left Lower MG
Right Lower MG
Weight (g)
0.0
0.2
0.4
0.6
*
*
WT
KO
WT
KO
Maternal Genotype
B.
p=0.057
Adipocyte Number (per mm2)
0
30
60
90
WT
KO
Maternal Genotype
C.
p=0.051
Adipocyte Area of MG Tissue (%)
0
1
2
3
4
WT
KO
Maternal Genotype
D.
Adipocyte Average Area (um2)
0
100
200
300
WT
KO
Maternal Genotype
E.
Maternal Genotype
WT
KO
Density
0.000
0.001
0.002
0.003
0.004
*
0
1000
2000
Adipocyte Area (um2)
F.
p=0.060
Maternal Genotype
WT
KO
Percent of Adipocytes
0
10
20
30
40
*
[0,100]
[100,200]
[200,300]
[300,400]
[400,500]
[500,600]
[600,700]
[700,800]
[800,900]
[900,1e+03]
[1e+03,3.5e+03]
Adipocyte Area Range (um2)
G.
H.

Figure 15: Mammary glands histology.

(A) Inguinal and abdominal (lower) mammary gland (MG) weights. (B) Histological analysis showing increased number of adipocytes in adipocyte *Tsc1* knockout thoracic (upper) mammary glands. (C) Average area of the mammary gland comprised of adipocytes. (D) Mean adipocyte area. (E) Distribution of adipocyte area, cumulative over all images. (F) Percent of adipocytes by genotype and area range. For E, the asterisk indicates a significantly different distribution of adipocyte sizes by a Kolmogrov-Smirnoff test. (G) Representative images of a wild-type (G) or knockout (H) thoracic mammary gland sections. For B and C, mixed linear models were used with images as the random effect and genotype as the fixed effect. (n=11 dams, 8 images per dam, 174-3199 adipocytes per slide, 10533 total adipocytes for knockout dams and 3286 total adipocytes for wild-type dams).

3.4.3 Offspring Born to Adipocyte Tsc1 Knockout Dams are Heavier During Peak Lactation

The average litter size at birth across genotypes was similar (Figure 16A). Litters were culled to four offspring per dam at PND2.5 to normalize milk supply. There was no significant difference in offspring weight at birth (PND0.5; Figure 17A). At PND7.5, after adjusting for sex, offspring born to knockout dams were 7% heavier than offspring born to wild-type dams (Figure 16B, p=0.010 from a 2x2 ANOVA). Female offspring born to knockout dams were 9% heavier than females born to wild-type dams (Figure 16B, p=0.044). Weights of male offspring born to knockout dams were 5% heavier than males born to wild-type dams although this did not reach statistical significance (Figure 16B, p=0.14). At PND14.5 and PND16.5, there were no weight differences between groups or sexes (Figure 17B). Our results show that the offspring of knockout dams are heavier during the first week of life when they are solely reliant on lactation for nutrient acquisition. At later time points, we hypothesize that the weights converge between genotypes because the offspring may be eating more chow-based food and rely less on maternal lactation.

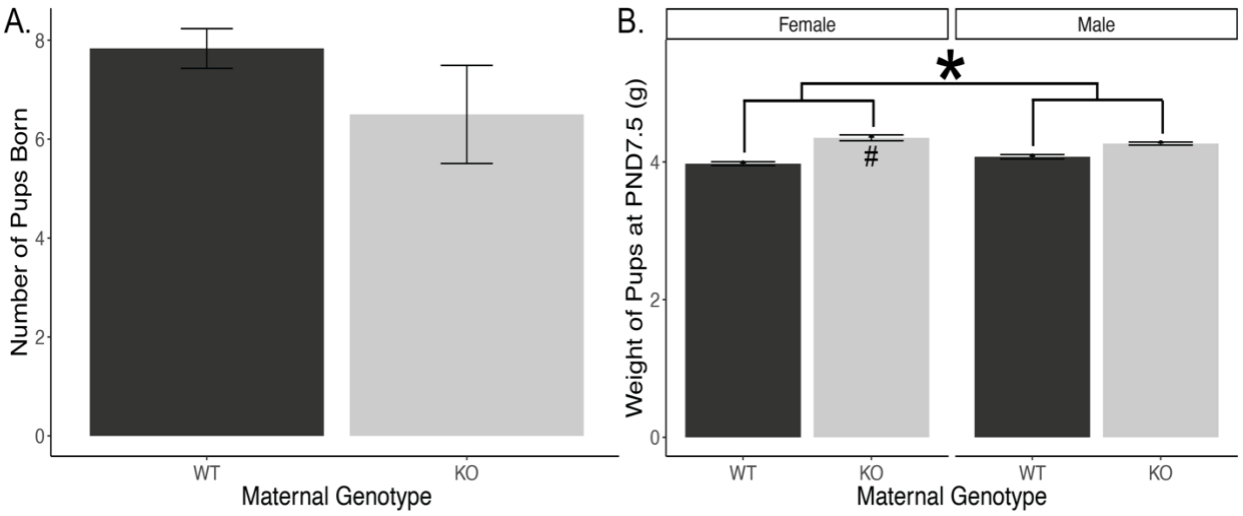


Figure 16: Litter size and offspring weight.

(A) Average number of offspring born to wild-type and knockout dams per their single litters. (B) Weights of male and female offspring of wild-type and knockout dams at PND7.5. Asterisk indicates a significant effect of maternal genotype from 2x2 ANOVA; number sign indicates significance within female offspring from a pairwise t-test. (n=44 offspring from 11 dams).

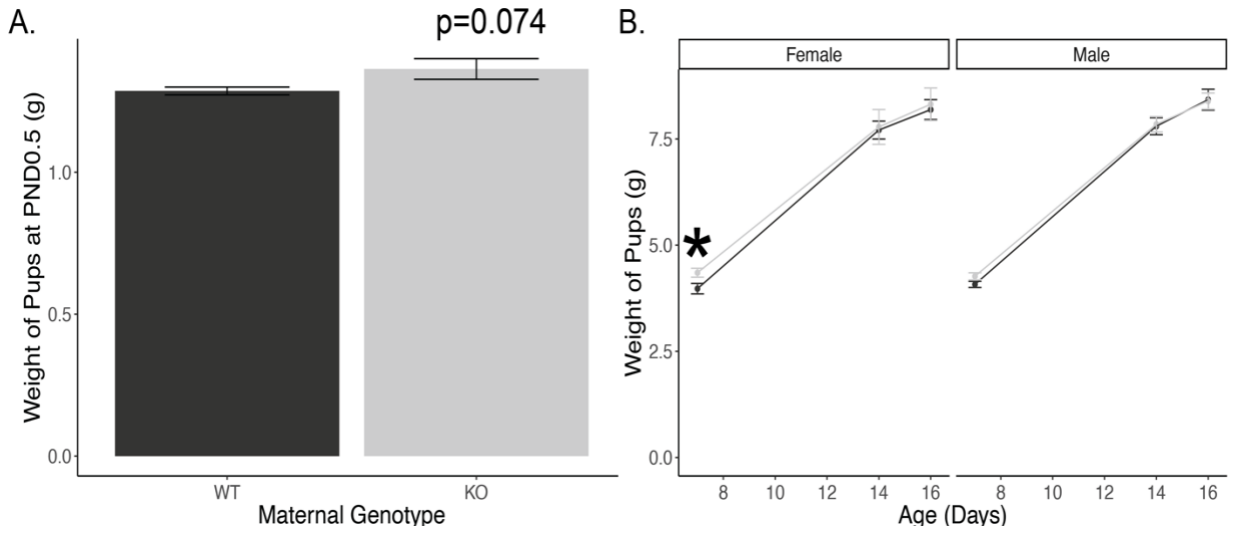


Figure 17: Offspring weight.

(A) Weight of offspring at birth. (n=84 offspring from 11 dams). (B) Weight of offspring at PND7.5 through PND16.5. (n=43 offspring from 11 dams).

3.4.4 Adipocyte Tsc1 Knockout Dams Produce Similar Volumes of Milk but with a Higher Milk Fat Percentage and Increased Desaturation of Milk Fatty Acids

Due to the differences in offspring weight and mammary gland size and histology, we calculated the mass of milk produced per dam at PND10.5. Milk output of the dams was not significantly different between groups (Figure 18A-B). This suggests that the differences we saw in offspring weight were not driven by increased milk output in the knockout dams. This prompted us to further evaluate the milk fat composition. Milk was collected from dams at PND16.5. Total fat analysis using the creamatocrit technique revealed that milk of adipocyte *Tsc1* knockout dams had a 34% higher fat percentage than milk of wild-type dams (Figure 19A, p=0.024). This finding shows that the increased fat content in the milk of knockout dams could be the main driver in increasing offspring weight due to increased milk caloric density.

Based on this finding, we assessed the specific fatty acid components of the milk fat using gas chromatography. These analyses showed a more desaturated and DHA-rich milk in the knockout (Figure 20). At an aggregate level, knockout dams produced milk with 11% lower saturated fatty acids (SFA, Figure 19B, p=0.008), 12% higher monounsaturated fatty acids (MUFA, Figure 19C, p=0.009), but similar percentages of polyunsaturated fatty acids (PUFA, Figure 19E). The MUFA/SFA ratio showed that the knockout had a 24% higher level of desaturation (Figure 19D, p=0.004).

While PUFAs overall were similar between groups, knockout milk had 28% higher levels of ω-3 fatty acids (Figure 19F, p=0.013), driven primarily by a 42% increase in the ω-3 fatty acid docosahexaenoic acid (DHA; Figure 20, p=0.031). There was a similar percentage of ω-6 fatty

acids (Figure 19G), resulting in a 31% lower ω-6:ω-3 ratio (Figure 19H, p=0.008) in the milk of knockout dams. Individual ω-6 fatty acids did not significantly differ between groups (Figure 20). Interestingly, the upstream precursors of DHA including alpha-linolenic acid (ALA) and eicosapentaenoic acid (EPA) were similar, suggesting that either the conversion from precursors into DHA may be increased or there is selective sparing of DHA from catabolism.

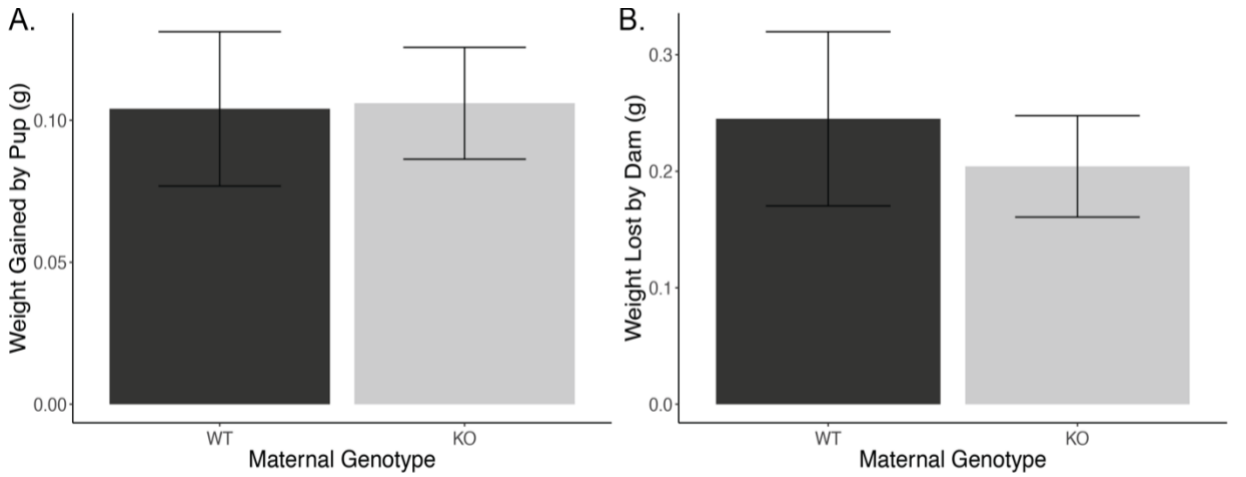


Figure 18: Milk volume measurements.

(A) Weight gained by the offspring during 1h of nursing. (B) Weight lost by dam during 1h of nursing. (n=11 dams).

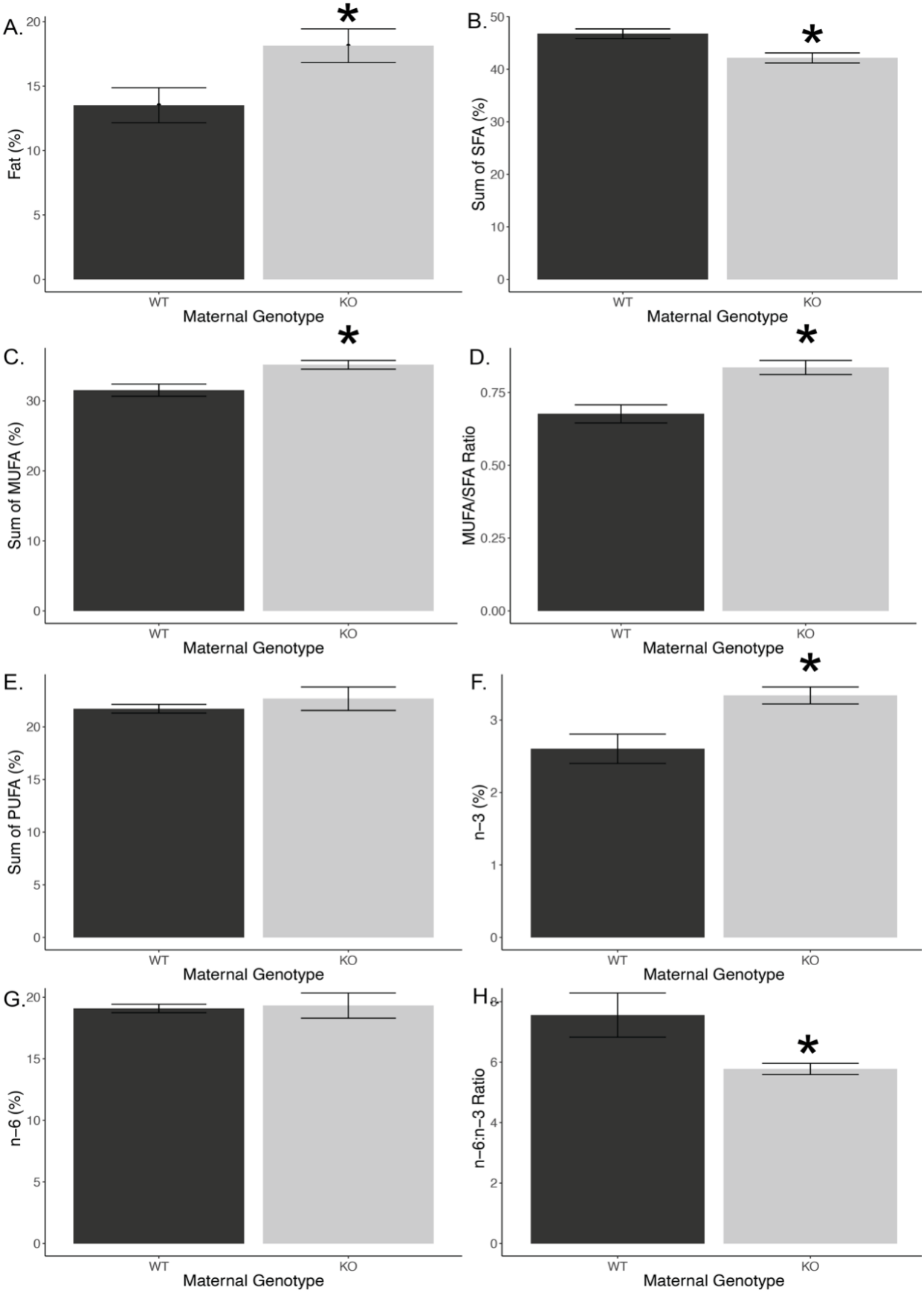

A.
Fat (%)
0
5
10
15
20
WT
KO
Maternal Genotype
*
B.
Sum of SFA (%)
0
10
20
30
40
50
WT
KO
Maternal Genotype
*
C.
Sum of MUFA (%)
0
10
20
30
WT
KO
Maternal Genotype
*
D.
MUFA/SFA Ratio
0.00
0.25
0.50
0.75
WT
KO
Maternal Genotype
*
E.
Sum of PUFA (%)
0
5
10
15
20
WT
KO
Maternal Genotype
F.
n−3 (%)
0
1
2
3
WT
KO
Maternal Genotype
*
G.
n−6 (%)
0
5
10
15
20
WT
KO
Maternal Genotype
H.
n−6:n−3 Ratio
0
2
4
6
8
WT
KO
Maternal Genotype
*

Figure 19: Milk fat analysis.

(A) Average percent fat composition of milk from knockout and wild-type dams. (n=21 dams). (B) Percentage of saturated fatty acids (SFA) in milk. (C) Percentage of monounsaturated fatty acids (MUFA) in milk. (D) MUFA/SFA ratio in milk. (E) Percentage of polyunsaturated fatty acids (PUFA) in milk. (E) Percentage of ω-3 fatty acids in milk. (F) Percentage of ω-6 fatty acids in milk. (G) ω-6:ω-3 ratio in milk. (n=10 dams).

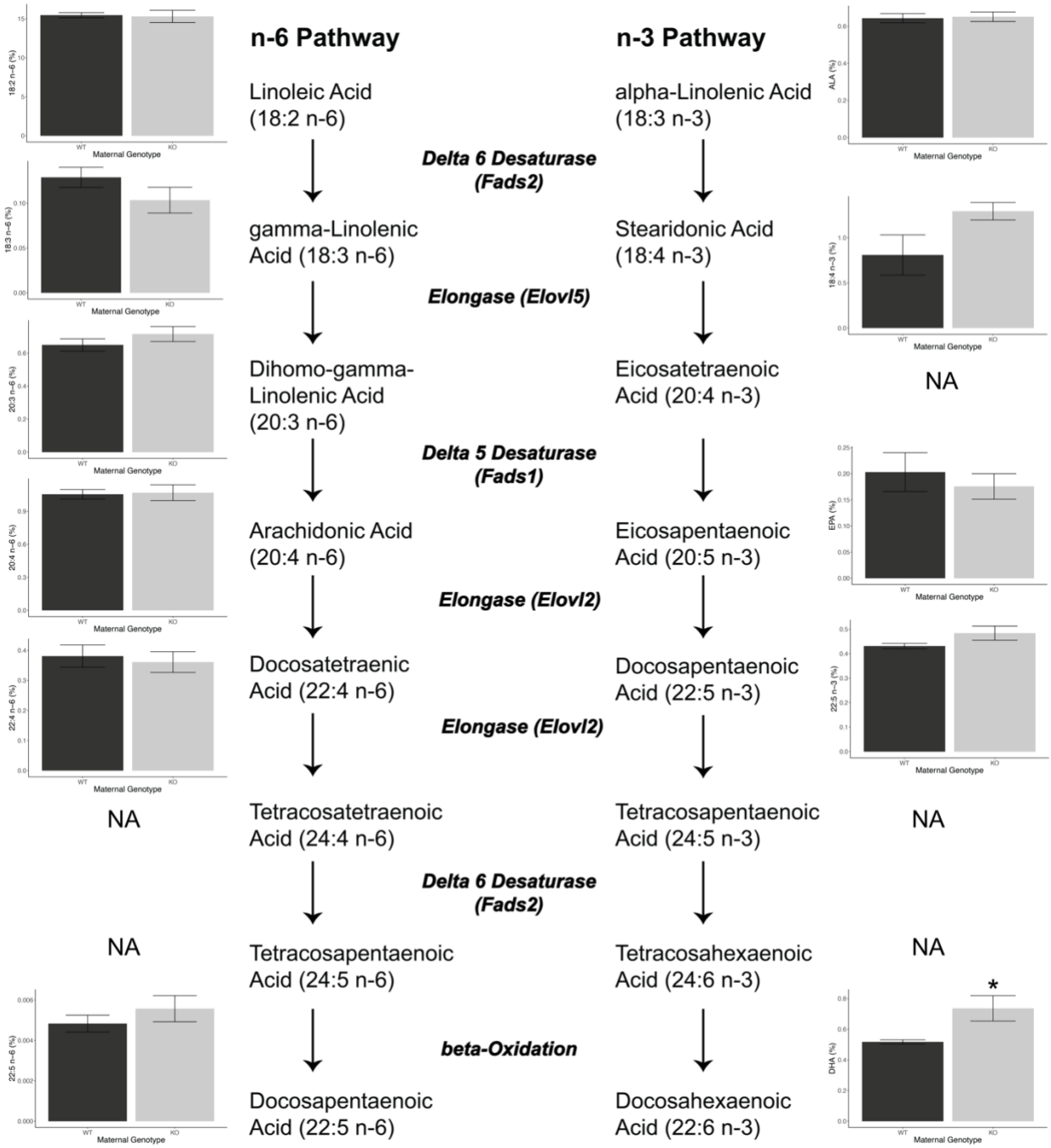


Figure 20: ω-3 and ω-6 fatty acid pathways.

ω-3 and ω-6 metabolic pathways, enzymes, and detected ω-3 and ω-6 fatty acids. Fatty acids that were not detected are noted with NA. (n=10 dams).

3.4.5 Suppressed Expression of Adaptive Immune Markers and Increased Expression of Muscle Biosynthesis Genes

To understand the mechanisms by which adipocyte mTORC1 activation affects mammary gland biology, we performed bulk RNAseq on whole mammary gland explants from lactating wild-type and knockout dams. We identified 139 significantly differentially expressed genes between these groups (Figure 21A-B). In spite of the observed differences in milk fat and milk fatty acid composition, we were surprised that most fatty acid and triglyceride synthesis enzymes were similar between groups (Figure 21C). Several markers of adipogenesis and PPARγ were upregulated in the knockout mammary glands including *Plin4, Adipoq, Cav2,* and *Fabp4* (Figure 21D), consistent with the observed increase in mammary adipocyte numbers (Figure 15B). There were no detectable changes in PPARγ transcripts. We also identified several upregulated genes involved in ω-3 eicosanoid metabolism, including the enzymes *Cyp2e1*, *Gpx3*, *Ephx2*, and *Pla2g4a* (Figure 21E). Conversely, COX1 (*Ptgs1*), was significantly downregulated (Figure 21E).

Despite the modest numbers of significantly differentially expressed genes, gene set enrichment analyses identified 220 significantly differentially expressed biological pathways (180 downregulated and 40 upregulated). By identifying genes in these pathways, they largely fell into two clusters of significantly differentially expressed pathways, one set related to the downregulation of adaptive immune differentiation and function, and another related to upregulation of striated muscle function (Figure 21F). To further explore the potential effects on adaptive immune cell function, we examined the expression of the T-cell marker genes encoding for CD3 and the B-cell marker genes encoding for CD45 and CD19. Each of these markers were

reduced 20-92% suggesting a potential reduction in adaptive immune cells in these mammary glands (Figure 21G). This could be primarily related to the increased DHA levels in the milk of knockout dams which could promote an anti-inflammatory state and reduce adaptive immune cell levels.

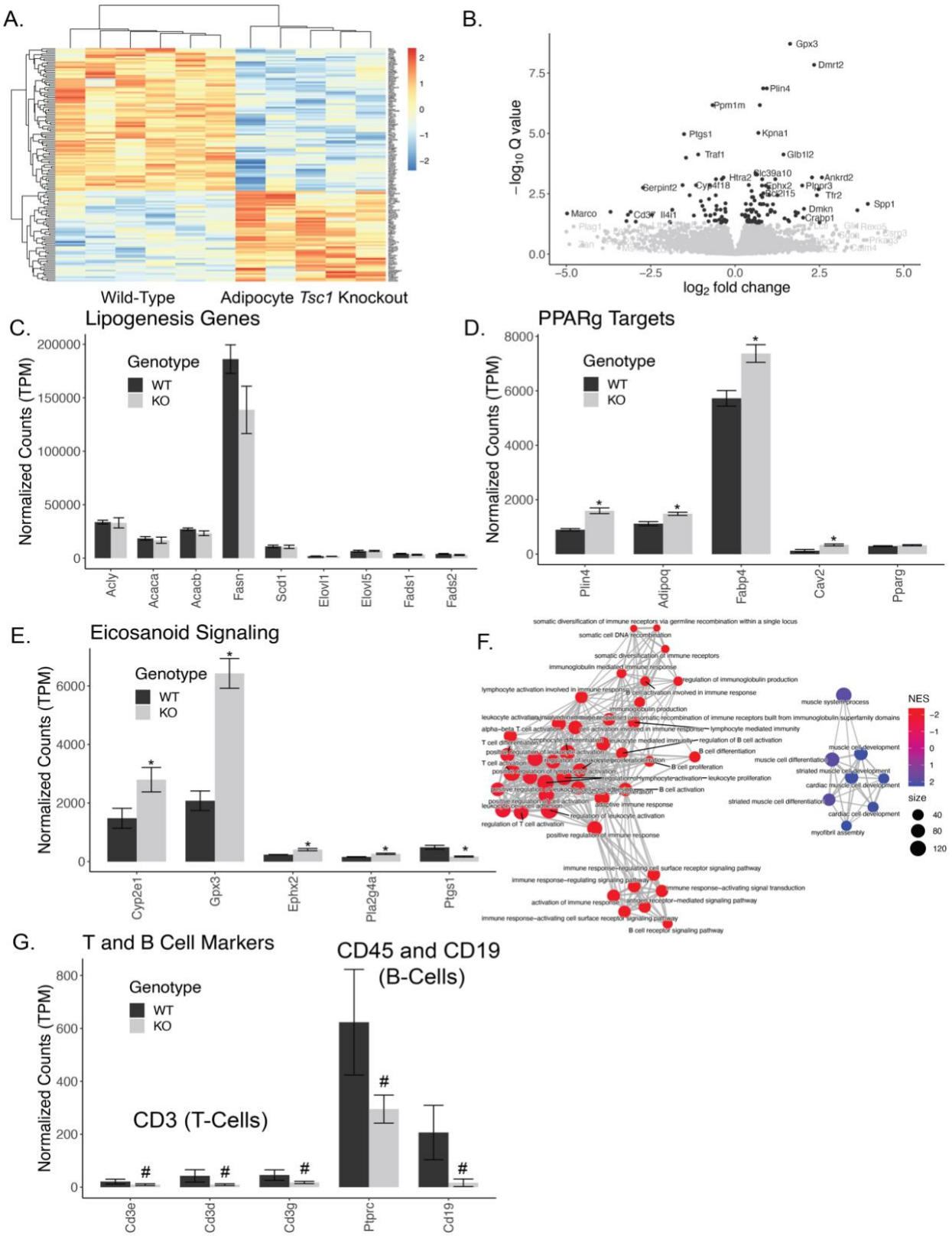

A.
Wild-Type
Adipocyte Tsc1 Knockout
B.
Gpx3
Dmrt2
Plin4
Ppm1m
Ptgs1
Kpna1
Traf1
Glb1l2
Htra2
Slc39a10
Ankrd2
Serpinf2
Cyp4f18
Ephx2
Ptprr3
Tfr2
Spp1
Marco
Cd36
Il4i1
Dmkn
Crabp1
– log10 Q value
log2 fold change
C.
Lipogenesis Genes
Genotype
WT
KO
Normalized Counts (TPM)
Acly
Acaca
Acacb
Fasn
Scd1
Elovl1
Elovl5
Fads1
Fads2
D.
PPARg Targets
Plin4
Adipoq
Fabp4
Cav2
Pparg
E.
Eicosanoid Signaling
Cyp2e1
Gpx3
Ephx2
Pla2g4a
Ptgs1
F.
somatic diversification of immune receptors via germline recombination within a single locus
somatic cell DNA recombination
somatic diversification of immune receptors
immunoglobulin mediated immune response
regulation of immunoglobulin production
B cell activation involved in immune response
immunoglobulin production
lymphocyte mediated immunity
regulation of B cell activation
B cell differentiation
B cell proliferation
leukocyte proliferation
B cell activation
regulation of leukocyte activation
positive immune response
regulation of T cell activation
positive regulation of immune response
immune response–regulating cell surface receptor signaling pathway
immune response–regulating signaling pathway
immune response–activating signal transduction
activation of immune response
immune response–activating cell surface receptor signaling pathway
B cell receptor signaling pathway
muscle system process
muscle cell development
muscle cell differentiation
striated muscle cell development
cardiac muscle cell development
striated muscle cell differentiation
cardiac cell development
myofibril assembly
NES
size
G.
T and B Cell Markers
CD45 and CD19
(B-Cells)
CD3 (T-Cells)
Cd3e
Cd3d
Cd3g
Ptprc
Cd19

Figure 21: Transcriptomic analysis of mammary glands.

(A) Heatmap of significantly differentially expressed genes between wild-type and knockout mammary glands. (B) Volcano plot comparing fold change to significance of specific genes. (C) Selected lipogenic gene expression. (D) Selected PPARγ target genes. (E) Selected genes involved in eicosanoid metabolism and signaling. (F) Network map of the top differentially expressed pathways from gene ontology – biological process. Jaccard distances were used to calculate similarity between pathways based on overlapping genes, and those distances are indicated as the edges. Net enrichment score (NES) indicate up or downregulation, while node size indicates the number of genes in that pathway. (G) T- and B-cell gene expression markers. Asterisks indicate $q<0.05$; number signs indicate $p<0.05$. (n=11 dams).

3.5 Discussion

Milk fat is the most variable macronutrient in human milk and contributes the most to differences in energy content of milk (Ballard & Morrow, 2013). In this study, we show that hyperactivation of adipocyte mTORC1 via adipocyte-specific deletion of *Tsc1* alters milk fat composition and mammary gland adipocyte histology. Importantly, our approach is expected to activate mTORC1 in all adiponectin-expressing cells, including both peripheral and mammary adipocyte depots (F. Wang et al., 2013; Z. V. Wang et al., 2010). The positive role of mTORC1 in adipocyte biology has been well established. mTORC1 is necessary for adipocyte differentiation in peripheral adipose depots (Cho, Park, Lee, Lee, & Kim, 2004; J. E. Kim & Chen, 2004; Yeh, Bierert, & Mcknightt, 1995) but less is known about its role in mammary adipocyte differentiation and growth during lactation. It is interesting that we observed increased adipocyte numbers and elevated markers of adipocyte differentiation in adipocyte *Tsc1* knockout mammary glands, as the Adiponectin-Cre is not expected to be activated until late in differentiation. It is also worth noting that there is evidence of de- and re-differentiation of post-involution mammary adipocytes, but to avoid this concern, our study focused on mice in their first pregnancy. The increased adipocyte hyperplasia could suggest a signal promoting mammary adipogenesis derived from peripheral adipocytes.

mTORC1 is important for lipogenesis, and the chronic absence of mTORC1 activity in adipocytes (by *Raptor* ablation) results in lipodystrophic mice (Lee, Tang, Li, & Guertin, 2016; Polak et al., 2008). Gain of function studies (via tissue-specific *Tsc1* knockout) of mTORC1 in adipocytes resulted in increased *in vitro* palmitate esterification in inguinal adipose tissue (Magdalon et al., 2016). In our adipocyte *Tsc1* knockout model, we were surprised that there were no obvious increases in lipogenic enzymes in the mammary gland in spite of increased milk fat composition. This is consistent with data from *Raptor* knockout adipocytes, which have elevated (not decreased) ACC, ACLY, and FASN protein levels (Lee et al., 2016). We propose that in the knockouts there is increased peripheral synthesis of lipids, which are then transported to the mammary gland adipocytes for storage and secretion into the milk, though we cannot rule out non-transcriptional upregulation of lipogenesis in mammary glands. Our hypothesis of increased trafficking of lipids is consistent with elevated expression of the fatty acid transporter *Fabp4* (Figure 21D). Further studies using depot-specific activation of mammary adipocytes will be important to separate the roles of peripheral adipocytes from mammary adipocytes with respect to lactation.

Transgenic activation of Akt (an upstream activator of mTORC1) in mammary epithelial cells resulted in higher milk fat percentage during lactation and larger milk lipid droplet size compared to the control mice (Schwertfeger et al., 2003). In a separate study, supplementation of the mTORC1 activating branched chain amino acid, valine, increased mammary gland lipogenic activity during lactation in a way that was reversible by the mTORC1 inhibitor rapamycin (Che et al., 2019). Together, these data suggest that mTORC1 activation may positively regulate milk

lipids through multiple cell-types. We found that the secreted milk volume measured at PND10.5 was similar across genotypes. This suggests that the main driver of the modestly increased offspring weight could be the increase in milk fat percentage.

In addition to total lipids, we show an increase in both the relative desaturation of lipids, and the levels of DHA in milk of adipocyte *Tsc1* knockout mice. DHA is an essential ω-3 fatty acid important for infant growth and development and has been linked to improved cognitive performance, psychomotor development, and visual acuity (Birch et al., 2010; Helland et al., 2008; Jensen et al., 2005). DHA and EPA levels are highly variable in human milk, and a better understanding of the physiological signals that control DHA levels in milk is important to optimize the delivery of essential lipids to the infant. We examined the expression of the phosphatidylcholine-DHA transporter *Mfsd2a* (Nguyen et al., 2014) but did not detect any differences in our mammary gland expression data. The increase in DHA levels may also be linked to our observation of reduced gene expression of markers of adaptive immune cells, as DHA has been shown to suppress the adaptive immune response (Gutiérrez, Svahn, & Johansson, 2019). We show that several enzymes that convert DHA into bioactive lipids are upregulated in our lysates (Figure 21E). DHA-derived eicosanoids, such as D-series resolvins and protectins, could serve as negative signals to reduce the number of B and T cells in the mammary gland. This in turn could affect both mammary gland morphology and the secretion of antibodies into the milk.

This is the first report that adipocyte mTORC1 activation alters the lipids in milk of lactating mice and provides important new data towards our understanding of lipid metabolism during a

critical developmental window. There are several strengths to our approach, including the use of matched diets, single parity (to avoid mammary adipocyte de- and re-differentiation concerns), and normalized litter sizes to comprehensively evaluate milk lipids and mammary gene expression. However, there are several limitations to this approach including the inability to exclude *in utero* effects on offspring growth, the inability to separate the roles of peripheral and mammary adipocyte depots, and the lack of a clear mechanism by which mammary (or peripheral) adipocytes result in increased milk lipids, milk fat saturation, and milk DHA levels.

Our data show that elevations in mTORC1 activity in adipocytes of pregnant and lactating dams can impact milk composition, offspring weight, and mammary gland gene expression and morphology. These findings are crucial to better understand the effects of nutrient sensing in the mammary gland on milk production and offspring health. These data support our hypothesis that mTORC1 activation in adipocytes increases the mammary gland capacity to produce fat and secrete it into the milk. Future work will focus on the mechanisms by which mTORC1 could be influencing mammary gland function and milk secretion to address the effects of maternal excess nutrient sensing on lactation and infant health.

Chapter 4 : Exploratory Analysis of the Associations Between Breast Milk Lipids and Infant Adiposity

4.1 Abstract

Breastfeeding is the recommended method of feeding for infants during the first six months of life. Milk composition changes dynamically throughout the lactation period to meet the nutritional needs of the infant and is thought to contribute to programming of infant adiposity. It is unclear, however, how each of the milk fatty acids, in the form of triglyceride, affect infant development and risk of adiposity. To better understand the role of milk lipids on infant adiposity, we performed an exploratory assessing the association between the 33 fatty acid percentages in milk samples collected from breastfeeding mothers at two weeks and two months postpartum and infant adiposity. Our results showed that several fatty acids were significantly correlated with infant weight-for-length z-scores, including positive correlations with vaccenic acid and negative correlations with docosahexaenoic acid. Dynamic changes in human milk lipids can have impacts on infant growth and allow us to better assess adiposity risk and develop interventions to reduce risk of offspring morbidity and mortality. Together, these results suggest the importance of assessing milk fatty acid composition at different timepoints throughout lactation and their role in infant lactational programming of adiposity.

4.2 Introduction

Eight percent of infants under two years of age have weight-for-length scores above the 95th percentile for sex (Ogden, Carroll, Kit, & Flegal, 2014), and a rapid weight gain during the first months of life has been associated with higher odds of childhood obesity (Stettler, Zemel, Kumanyika, & Stallings, 2002). Additionally, children who have obesity are more likely to become obese adults, putting them at a higher risk for morbidity and mortality (Geserick et al., 2018; Simmonds, Llewellyn, Owen, & Woolacott, 2016; Umer et al., 2017; Whitaker, 2004). Evidence from animal and human studies suggests that the lactational window plays an important role in programming infant and adult health (Ellsworth, Harman, Padmanabhan, & Gregg, 2018; Lanigan & Singhal, 2009). Exclusively breastfed infants within the first six months are less likely to develop obesity compared to non-exclusively breastfed infants, but the mechanisms at play remain elusive (Uwaezuoke, Eneh, & Ndu, 2017; L. Wang, Collins, Ratliff, Xie, & Wang, 2017). Specifically, breast milk short chain fatty acids have been found to play a role in infant adipose tissue development (Prentice et al., 2019; Yu et al., 2019), and thus studying the association between milk fatty acids and infant adiposity is crucial to better understand the role of lactational programming in infant health.

Breastfeeding is the recommended method of feeding for infants during the first six months of life (Kramer & Kakuma, 2012). Milk composition changes dynamically throughout the lactation period to meet the nutritional needs of the infant. This gradual progression involves production of colostrum for a couple of days after birth, transitional milk during the first two weeks postpartum, and mature milk after two weeks. The unique composition of the milk during each phase provides optimal nutrition for the infant. The milk produced after birth is called colostrum

and has a yellow color, thick texture, and is rich in antibodies and triglycerides. Within two weeks after birth, milk production capacity increases and the milk undergoes compositional changes from colostrum to transitional milk before becoming mature milk. Mature milk is comprised of 88% water, around 7% carbohydrates primarily in the form of lactose and non-caloric human milk oligosaccharides, around 4% fat primarily in the form of triglycerides, and around 1% protein primarily in the form of whey and casein (Ballard & Morrow, 2013; Emmett & Rogers, 1997). A main contributor to the caloric density of milk is fat. Milk fat, almost exclusively in the form of triglycerides, is synthesized in the smooth endoplasmic reticulum by *de novo* synthesis from available glucose or derived from maternal diet or fatty acids from adipose tissue stores (Anderson et al., 2007; Bravi et al., 2016; Garwolińska, Namieśnik, Kot-Wasik, & Hewelt-Belka, 2018; McManaman, 2009; Rezaei et al., 2016). Despite the levels of milk fat being fairly conserved throughout lactation, fat content varies by the time of day and within feed (Demmelmair & Koletzko, 2018). Given that lipids are the major source of calories for infants, it is important to assess the role of human milk lipids in infant growth and adiposity.

The associations between milk fatty acids and infant adiposity are inconsistent. Higher total milk fat percentage at 4-8 weeks postpartum was associated with lower infant weight and lower adiposity at one year of age (Prentice et al., 2016). Milk omega-3 fatty acids have been shown to be associated with infant growth. Breast milk α-linolenic acid (ALA) content at 4 weeks and 3 months postpartum was associated with higher infant body mass index at 7 years (Helland et al., 2008), and total ω-3 long-chain fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in breast milk at 6 weeks postpartum were associated with higher infant body composition assessed by skinfold thickness at 1 year of age (Much et al., 2013). In contrast, in

one study found a negative association between breast milk DHA levels at 14-30 days postpartum and body mass index at 7 years of age in addition to a negative association with fat percentage at 6-9 years of age (Pedersen, Lauritzen, Brasholt, Buhl, & Bisgaard, 2012). A higher omega-6 to omega-3 ratio corresponded to increased infant fat and total mass and was predictive of infant fat mass accumulation within 4 months postpartum (Rudolph et al., 2017). In addition, another study found positive associations between breast milk omega-6 to omega-3 ratio and infant weight-for-length z-score at 2 months of age (Ellsworth et al., 2020; Panagos et al., 2016). Data on the associations between human milk fatty acids and infant adiposity remain inconsistent and scarce, and therefore, our study aimed to add to the body of literature by looking at the role of 33 medium and long-chain fatty acids in offspring adiposity. To better understand the role of human milk lipidomic profile on infant health, we aimed to determine the association between breast milk fatty acids at two weeks and two months and infant adiposity using weight-for-length z-scores at 2, 6, 12, and 24 months of age.

4.3 Methods

4.3.1 Study Overview

The recruitment of mother-infant dyads was conducted after obtaining a written consent from the mothers as part of the IMAGE (Infant Metabolism And Gestational Endocrinopathy) study (Ellsworth et al., 2020). The mothers also provided consent on behalf of their infants. Mothers were provided with instructions regarding breastmilk collection to ensure consistency between participants. Maternal demographics, characteristics, and health history were obtained via surveys and medical record review. Infant feeding methods (breastfed, breastfed and formula, or formula fed) were obtained at 2 weeks and 2 months. Infant characteristics and weight-for-length

z-scores were obtained at 2 weeks, 2 months, 6 months, 12 months, and 24 months postpartum. The study protocol was approved by The Institutional Review Board of the University of Michigan (HUM00107801) and Saint Joseph Mercy Hospital (HSR -17-1686).

4.3.2 Participants

Mother-infant dyads were enrolled while admitted for birth at two participating hospitals in Ann Arbor, Michigan, USA. The inclusion criteria included being older than 18 years, having a healthy singleton pregnancy, no maternal pre-pregnancy diabetes, and gestational age of 35 weeks or more. The initial total number of recruited dyads was 114. After accounting for attrition (n=10 dyads) and availability of samples, 64 mothers provided milk samples at 2 weeks and 55 mothers provided milk at 2 months. 37 mothers consistently provided milk at both time points, and 11 mothers provided milk only at the two-month time period. Milk samples that contained a volume less than 5mL were not analyzed for milk composition (n=12). Of the available milk samples, 60 samples were run for lipidomic analyses at two weeks and 48 samples were run for lipidomic analyses at 2 months. Mothers recruited in this study intended to breastfeed their infants, but formula supplementation was not considered an exclusion criteria. If mothers did not breastfeed and infants received exclusive formula feeding, their data was excluded from this analysis (n=28).

4.3.3 Milk Collection

The milk collection protocol is further described in previous work (Ellsworth et al., 2020). A written protocol for milk collection was provided to participants. Additionally, the research team

members provided oral instructions to mothers on milk collection. Samples were collected at 2 weeks and 2 months postpartum around the times when the infants had their scheduled pediatrician well visits. Mothers were instructed to collect non-fasting milk in the morning between 8:00-10:00 a.m. at least two hours after the last feed using hand expression or pumping and emptying an entire single breast. Milk was expressed into containers then inverted to mix. Then 25 mL were aliquoted into 5 mL glass vials. Mothers were instructed to store the milk at home in the freezer prior to the physician visit. At the physician visit, milk samples were stored at -20°C for a few days prior to storage at -80°C. All milk sample analysis was conducted after thawing milk on ice.

4.3.4 MIRIS analysis

Whole milk samples were analyzed using a mid-infrared spectroscopy macronutrient analyzer (MIRIS HMA™, Uppsala, Sweden) to determine carbohydrate, protein, fat, and energy composition. All samples were run in duplicates and an average value was used. Detailed methods are explained elsewhere (Ellsworth et al., 2020). Briefly, 5mL of milk samples were warmed in a bead bath prior to undergoing ultrasonic homogenizations and analysis using MIRIS manufacturer protocol. All samples were run in duplicates, and macronutrient levels were verified against the range provided by the manufacturer. Milk samples that contained a volume less than 5mL were not analyzed for macronutrient composition.

4.3.5 Fatty Acid Lipidomic Analysis

Lipidomic analyses were done by the Michigan Regional Comprehensive Metabolomics Core. Detailed methods are discussed elsewhere (Ellsworth et al., 2020). Briefly, milk samples were frozen at -80°C until analysis to prevent lipid hydrolysis and peroxidation. Samples were quickly thawed on ice once for lipidomic analysis without undergoing multiple freeze-thaw cycles. Long chain fatty acid concentrations were determined following sample extraction, semi-purification and derivatization followed by fatty acid measurement by gas chromatography (GC) using an Agilent GC model 6890N equipped with flame ionization detector. Individual fatty acid concentrations were measured in nmol on 33 lipid classes from C14:0 to C24:1 and reported here as a percentage of total long chain fatty acids measured. Omega-6:omega:3 ratio is calculated from the total reported omega-6 and omega-3 concentrations, using the average omega-6:omega-3 ratio of each participant.

4.3.6 Infant Growth Measurements

Infant anthropometric measures were obtained during the scheduled physician visits at 2 weeks and 2, 6, 12, and 24 months postpartum. Weight-for-length z-scores (WFL) were obtained using the World Health Organization (WHO) growth charts for infants ages 0-2 years old ("WHO Child Growth Standards based on length/height, weight and age," 2006). Other measurements including infant length-for-age, head circumference-for-age, weight-for-length, and BMI were also assessed.

4.3.7 Statistical Analysis

Statistical significance was designated at $p<0.05$ for this study. All statistical analyses were performed using R v4.0.2 ("R: The R Project for Statistical Computing," n.d.). Data are presented graphically as mean +/- standard error of the mean. For longitudinal measurements, macronutrient changes from the MIRIS and lipidomic analyses were analyzed over time from two weeks to two months using mixed linear models with the fixed effect of time (2 weeks and 2 months) and milk macronutrients as the outcomes, but we did not account for any covariates. For the mixed linear models, our outcome was infant weight-for-length z-score at each time point and the exposure variables included fatty acid percentages at 2 weeks and 2 months. We tested categorical variables including delivery method, infant sex, ethnicity, and health condition using chi-squared testing. For continuous variables including maternal age, BMI, gestational age, and parity, normality was assessed using Shapiro-Wilk tests followed by testing for homoscedasticity using Levene's test. Pending these results, appropriate parametric or non-parametric tests were done. We explored nominal associations of milk fatty acid percentages from 2 weeks to 2 months. Correlations were calculated for the levels of 33 fatty acids between 2 weeks and 2 months. For correlation testing, normality was assessed to determine use of Spearman or Pearson correlation coefficients. determine use of Spearman or Pearson correlation coefficients. All nominal and FDR adjusted associations are reported in Supplementary Table 1. Adjustments for multiple comparisons were made using the method of Benjamini and Hochberg (Benjamini & Hochberg, 1995). Additionally, we explored nominal associations between fatty acid percentages at 2 weeks and 2 months and infant weight-for-length z-score at 2, 6, 12, and 24 months of age using mixed linear modeling with WFLz as our outcome, milk fatty acid percentages as the fixed effect per time point (2 weeks and 2 months) and random effect of the mother. In these models,

we did not account for any covariables, but a sensitivity analysis was conducted testing only exclusive breastfeeding at 2 weeks versus weight-for-length z-scores.

4.4 Results

4.4.1 Participant Demographics and Characteristics

Breast milk samples were collected from n=64 at 2 weeks and n=55 at 2 months. Lipidomic analyses were conducted on a subsample of n=60 samples at 2 weeks and n=48 at 2 months. Maternal age, ethnicity, health condition during pregnancy, body mass index (BMI), and parity are reported in the table below at 2 weeks and 2 months. Gestational age, delivery method, and infant sex are reported in the table below for participants at 2 weeks and 2 months. There were no significant differences between the two participant groups at 2 weeks and 2 months.

Table 1: Participant demographics and characteristics from the IMAGE study.
No significant differences were noted between the characteristics at 2 weeks and 2 months.

Variable	**Participant characteristics with 2 week human milk lipidomic analyses (n=60)**	**Participant characteristics with 2 month human milk lipidomic analyses (n=48)**
Maternal Age (years) mean (SD)	31.2 (0.5)	31.4 (0.5)
Ethnicity n (%)		
Non-Hispanic White	41 (68%)	33 (69%)
African American	3 (5%)	4 (8%)
Hispanic	6 (10%)	4 (8%)
Asian	6 (10%)	4 (8%)

Other/Not Available	4 (7%)	3 (6%)
Health Condition n (%)		
Healthy	32 (53%)	22 (46%)
Gestational Diabetes Mellitus	7 (12%)	8 (17%)
Obesity	14 (23%)	13 (27%)
Polycystic Ovary Syndrome	7 (12%)	5 (10%)
Parity mean (SD)	1.75 (0.1)	1.78 (0.1)
Maternal Pre-Pregnancy BMI (kg/m^2) mean (SD)	26.9 (0.8)	27.6 (0.9)
Gestational Age at Delivery(weeks) mean (SD)	39.7 (0.1)	39.5 (0.2)
Delivery Method n (%)		
Vaginal	40 (67%)	31 (65%)
Cesarean	20 (33%)	17 (35%)
Infant Sex n (%)		
Male	27 (45%)	24 (50%)
Female	33 (55%)	24 (50%)

4.4.2 Milk Macronutrient and Energy Composition by MIRIS Analysis

A total of n=63 samples and n=55 samples were analyzed using MIRIS at 2 weeks and 2 months, respectively. Lipidomic analysis via gas chromatography of a subsample of n=60 at 2 weeks and n=48 at 2 months identified fatty acid categories as reported in the table below in nmol. Protein content significantly decreased by 23% from 2 weeks to 2 months ($p<0.001$). However, other macronutrient levels were not significantly different between 2 weeks and 2 months.

Table 2: Breast milk energy, macronutrient, and fatty acid composition at 2 weeks and 2 months.

All milk macronutrients were not significantly changed from 2 weeks to 2 months except for protein, which significantly decreased by 23% from 2 weeks to 2 months.

MIRIS Component	**2 weeks n=60**	**2 months n=48**
	Mean (SD)	
Energy (kcal/100mL)	69.9 (1.3)	68.4 (1.8)
Carbohydrate (g/100mL)	7.0 (0.0)	6.9 (0.1)
True Protein (g/100mL)	1.2 (0.0)	0.9 (0.1)
Fat (g/100mL)	3.8 (0.1)	3.9 (0.2)
Sum Total Long Chain Fatty Acids (nmol)	1554.2 (61.2)	1522.1 (85.6)
Fatty Acid Categories (nmol)		
Saturated Fatty Acids	541.2 (22.3)	521.7 (32.3)
Monounsaturated Fatty Acids	689.7 (28.7)	659.5 (38.9)
Polyunsaturated Fatty Acids	323.4 (16.2)	340.9 (20.5)
Omega-3 Fatty Acids	23.5 (1.6)	23.7 (1.6)
Omega-6 Fatty Acids	294.2 (14.7)	312.7 (19.0)
Omega6:Omega:3 Ratio	13.7 (0.6)	14.8 (1.0)

4.4.3 Fatty Acid Composition at 2 Weeks and 2 Months

Fatty acid percentages at 2 weeks, 2 months, and the percentage change in their levels are reported in Table 3, including univariate analyses of changes in these lipids. Fatty acid levels are reported as percentage nmol of total long chain fatty acids (LCFA) measured.

Table 3: Fatty acid percentages at 2 weeks and 2 months and percent change between measurement periods using linear mixed models. Fatty acids are described using common and IUPAC nomenclatures.

Fatty Acid Component	**2 week %nmol of total LCFA**	**2 month %nmol of total LCFA**	**Percent change from 2 weeks to 2 months**	**p-value**
Mean (SD)				
%14:0 Myristic acid	3.71 (1.65)	3.03 (1.44)	-18.30	0.08
%14:1 (n-5) Myristoleic acid	0.19 (0.07)	0.20 (0.07)	1.22	0.70
%15:0 Pentadecyclic acid	0.26 (0.08)	0.24 (0.08)	-6.29	0.34
%16:0 Palmitic acid	22.68 (2.79)	22.23 (2.83)	-1.94	0.57
%16:1 (n-7)c Palmitoleic acid (cis)	2.08 (0.70)	1.93 (0.74)	-7.20	0.25
%16:1 (n-7)t Palmitoleic acid (trans)	0.24 (0.06)	0.21 (0.05)	-13.56	**0.005**
%18:0 Stearic acid	7.62 (1.49)	7.83 (1.73)	2.76	0.34
%18:1 (n-7) Vaccenic acid	3.35 (1.05)	3.29 (0.85)	-1.98	0.66
%18:1 (n-9) Oleic acid	37.94 (4.21)	37.43 (4.25)	-1.34	0.52
%18:2 (n-6)cc	17.02 (3.93)	18.99 (4.20)	11.58	0.06

Linoleic acid (cis)				
%18:2 (n-6)tt Linoleic acid (trans)	0.09 (0.06)	0.08 (0.05)	-10.53	0.97
%18:2 (n-7,9) Conjugate Conjugated linoleic acid	0.35 (0.18)	0.29 (0.18)	-15.37	0.44
%18:3 (n-3) Alpha-linolenic acid	1.03 (0.45)	1.16 (0.50)	12.58	0.83
%18:3 (n-6) Gamma-linolenic acid	0.10 (0.04)	0.11 (0.04)	19.08	0.058
%18:4 (n-3) Stearidonic acid	0.06 (0.04)	0.06 (0.06)	-11.31	0.78
%19:0 Nonadecyclic acid	0.15 (0.10)	0.16 (0.09)	4.36	0.18
%20:0 Arachidic acid	0.22 (0.07)	0.22 (0.07)	0.054	0.45
%20:1 Gadoleic acid	0.25 (0.23)	0.25 (0.17)	1.51	0.79
%20:2 (n-6) Eicosadienoic acid	0.44 (0.14)	0.35 (0.12)	-20.01	**<0.001**
%20:3 (n-6) Dihomo-gamma-linolenic acid	0.49 (0.14)	0.43 (0.11)	-13.54	**0.006**
%20:4 (n-6) Arachidonic acid	0.57 (0.16)	0.51 (0.12)	-9.34	**0.01**
%20:5 (n-3) Eicosapentaenoic acid	0.08 (0.04)	0.07 (0.04)	-11.4	0.29

%20:5 (n-6) Bosseopentaenoic acid	0.06 (0.03)	0.06 (0.02)	4.58	0.84
%21:0 Heneicosylic acid	0.08 (0.03)	0.09 (0.02)	11.05	0.21
%22:0 Behenic acid	0.08 (0.03)	0.09 (0.03)	6.19	0.18
%22:1 Brassidic acid	0.09 (0.02)	0.06 (0.02)	-31.28	<**0.001**
%22:2 (n-6) Docosadienoic acid	0.04 (0.04)	0.03 (0.03)	-33.06	0.21
%22:4 (n-6) Adrenic acid	0.11 (0.04)	0.08 (0.03)	-29.94	<**0.001**
%22:5 (n-3) Docosapentaenoic acid	0.13 (0.06)	0.12 (0.04)	-9.89	0.26
%22:5 (n-6) Osbond acid	0.008 (0.01)	0.01 (0.01)	23.67	0.381
%22:6 (n-3) Docosahexaenoic acid	0.17 (0.11)	0.13 (0.08)	-20.60	0.30
%24:0 Lignoceric acid	0.08 (0.02)	0.07 (0.02)	-13.26	0.09
%24:1 Nervonic acid	0.09 (0.03)	0.05 (0.03)	-37.47	<**0.001**

4.4.4 Seven Fatty Acid Levels Significantly Decreased from 2 Weeks to 2 Months

Of the 33 lipids measured, the percentages of 7 fatty acid significantly decreased from 2 weeks to 2 months (Table 3). The percent change and p-values are reported in Table 3 for %16:1 (n-7)t (palmitoleic acid, trans), %20:2 (n-6) (eicosadienoic acid), %20:3 (n-6) (dihomo-gamma-linolenic acid), %20:4 (n-6) (arachidonic acid), %22:1 (brassidic acid), %22:4 (n-6) (adrenic acid), and %24:1 (nervonic acid). There was a modest increase in %18:2 (n-6)cc (linoleic acid) and %18:3 (n-6) (gamma-linolenic acid) from two weeks to 2 months, but this did not reach statistical significance (Table 3, p=0.06, p=0.058, respectively). There were no significant changes in the percentages of other fatty acids between 2 weeks and 2 months.

4.4.5 Multiple Fatty Acids were Significantly Associated with Infant Weight-for-Length z-Scores

We then evaluated the nominal association between milk macronutrients, energy, and fatty acids at 2 weeks and 2 months with infant adiposity using the World Health Organization weight-for-length z-scores at 2, 6, 12, and 24 months of age. The 2 week weight-for-length z-score data was not included in our analysis since this study aimed to look at the role of milk components on infant growth, and we considered the 2 week time point to be the baseline.

First, we assessed the relationship between all milk components and weight-for-length z-scores for infants that were breastfed, formula fed, or mixed fed at 2 weeks and 2 months. However, it is worth noting that all infants were breastfed at 2 weeks. This analysis showed 15 significant nominal associations with 9 negative associations and 6 positive associations as shown in Table 4. Levels of 18:1 (n-7) (vaccenic acid) were positively associated with multiple weight-for-

length z-scores while 22:6 (n-3) (docosahexaenoic acid, DHA) was negatively associated with multiple weight-for-length z-scores. All nominal associations are shown in the heatmap below (Figure 22) and in Table 4 with the respective correlation coefficients.

Table 4: Significant fatty acid percentages and weight-for-length z-score nominal associations for all infants using Spearman and Pearson correlations (nominal $p<0.05$).

Fatty Acid (%)	Collection Time	Weight-for-Length (WFL) z-Score Measurement Period	Correlation Coefficient (r)	p-value
18:1 (n-7)	2 weeks	12 months	0.431	0.001
20:0	2 weeks	12 months	-0.367	0.006
22:6 (n-3)	2 weeks	6 months	-0.368	0.007
18:1 (n-7)	2 weeks	24 months	0.359	0.011
20:3 (n-6)	2 months	24 months	-0.409	0.016
20:0	2 weeks	6 months	-0.328	0.017
18:1 (n-7)	2 months	2 months	0.328	0.024
20:5 (n-3)	2 months	6 months	0.342	0.027
18:3 (n-3)	2 months	2 months	-0.319	0.029
22:5 (n-3)	2 weeks	6 months	-0.298	0.030
22:6 (n-3)	2 weeks	2 months	-0.277	0.034
20:2 (n-6)	2 weeks	12 months	-0.283	0.038
20:1	2 weeks	24 months	0.295	0.040
14:0	2 months	2 months	-0.299	0.041
18:3 (n-6)	2 weeks	2 months	0.266	0.042

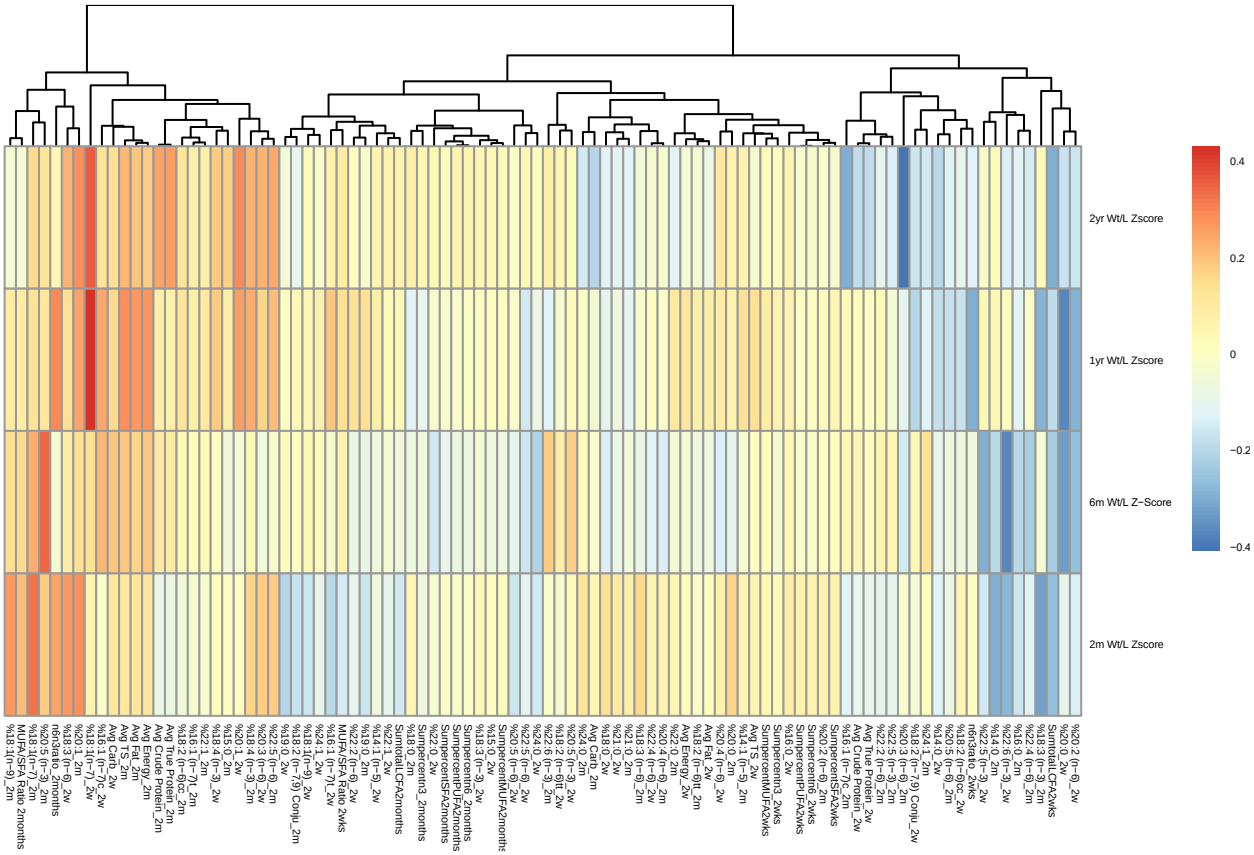


Figure 22: Heatmap showing correlations between milk components at 2 weeks (2w) and 2 months (2m) and weight-for-length z-scores (displayed as Wt/L Zscore) for all infants clustered by similarity of association.

Each row represents a time-period when weight-for-length z scores were measured. Positive correlations are represented in red, and negative correlations are represented in blue with the color intensity representing the strength of the correlation.

To better understand the role of human milk lipids on growth, we then conducted a sensitivity analysis to see if these associations remained true for exclusively breastfed infants. We assessed the nominal association between milk components and weight-for-length z-scores in a subset of infants who were exclusively breastfed at 2 weeks and 2 months (n=43 milk samples at 2 weeks from exclusively breastfed infants, n=37 milk samples at 2 months from exclusively breastfed infants). The nominal associations are shown in the heatmap below (Figure 24) and in Table 5

with the respective correlation coefficients. Interestingly, more significant nominal associations were detected when we excluded infants who were mixed fed or formula fed at either timepoint. There were 22 significant nominal associations with 9 positive associations and 13 negative associations. Of the total 22 significant associations, 17 were with fatty acid percentages and 5 with other milk components including milk energy, fat, and omega-6:omega-3 ratio.

Table 5: Significant fatty acid percentages and weight-for-length z-score nominal associations for exclusively breastfed infants using Pearson correlation (nominal $p<0.05$).

Fatty Acid (%) and Milk Components	**Collection Time**	**Weight-for-Length (WFL) z-Score Measurement Period**	**Correlation Coefficient (r)**	**p-value**
20:0	2 weeks	12 months	-0.468	0.004
20:1	2 months	2 months	0.458	0.006
20:0	2 weeks	6 months	-0.437	0.008
16:1 (n-7)c	2 weeks	12 months	0.405	0.014
18:0	2 months	6 months	-0.445	0.016
22:5 (n-6)	2 weeks	6 months	-0.399	0.016
Average Energy (kcal/100mL)	2 months	12 months	0.422	0.020
18:3 (n-3)	2 months	12 months	-0.424	0.022
18:1 (n-7)	2 weeks	12 months	0.375	0.024
20:2 (n-6)	2 weeks	6 months	-0.366	0.028
18:2 (n-7,9) Conjugate	2 months	2 months	-0.369	0.029

22:5 (n-3)	2 weeks	6 months	-0.361	0.030
24:1	2 months	24 months	-0.462	0.031
Average Fat (g/100mL)	2 months	12 months	0.395	0.031
22:0	2 months	24 months	-0.453	0.034
22:2 (n-6)	2 weeks	24 months	-0.372	0.036
Average Energy (kcal/100mL)	2 months	2 months	0.350	0.037
Omega6:Omega3 Ratio	2 months	12 months	0.385	0.039
Average Fat (g/100mL)	2 months	2 months	0.344	0.040
22:1	2 weeks	2 months	0.597	0.041
22:5 (n-6)	2 weeks	12 months	-0.331	0.049
24:0	2 weeks	6 months	-0.331	0.049

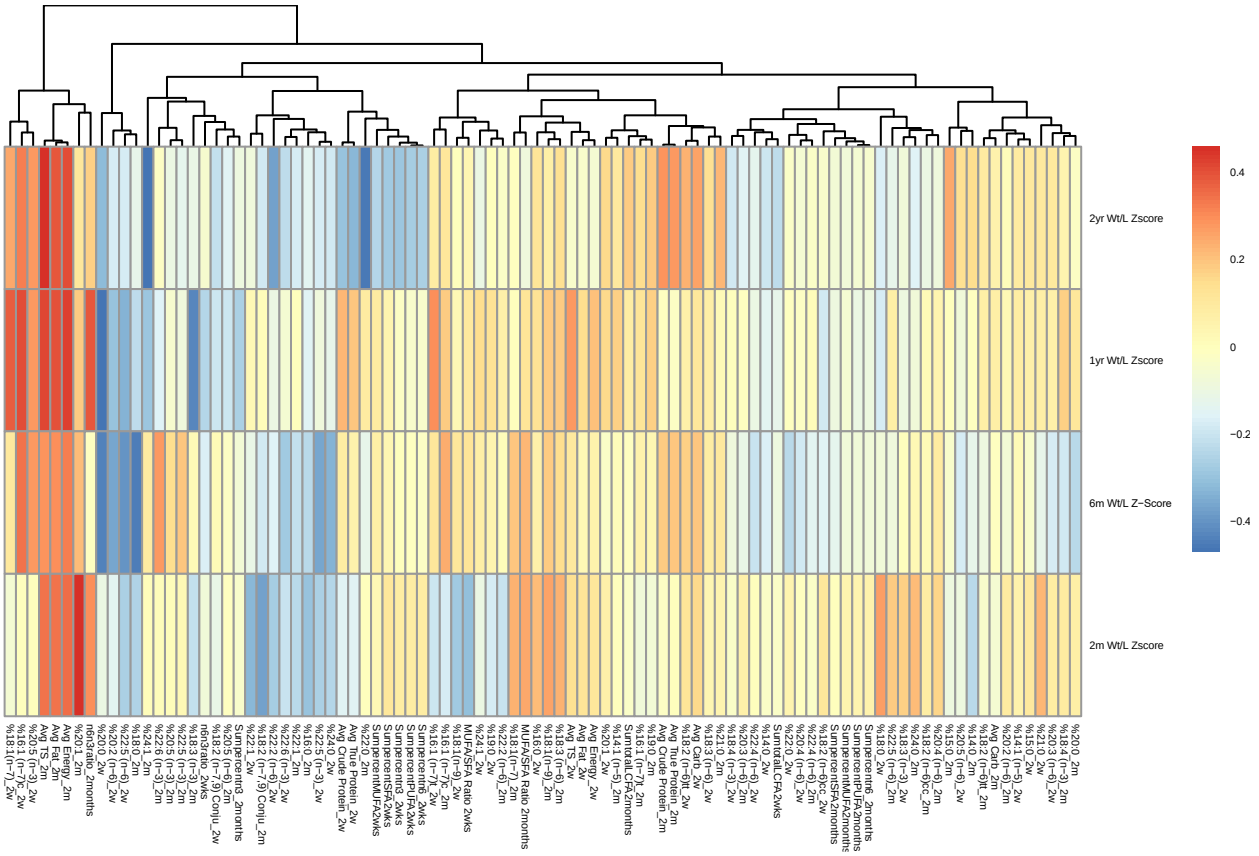


Figure 23: Heatmap showing correlations between milk components at 2 weeks (2w) and 2 months (2m) and weight-for-length z-scores (displayed as Wt/L Zscore) for exclusively breastfed infants clustered by similarity of associations.

Each row represents a time-period when weight-for-length z scores were measured. Positive correlations are represented in red, and negative correlations are represented in blue with the color intensity representing the strength of the correlation.

4.4.6 Various Trends for the Association between Fatty Acid Percentages at 2 Weeks and 2 Months and Weight-for-Length z-Scores

After determining the significant associations between milk fatty acids and infant adiposity, we wanted to better examine the association trends between fatty acids at 2 weeks and 2 months by fatty acid categories and weight-for-length z-scores among all infants. The figures below

(Figures 25-28) highlight the association trends for each fatty acid group and growth, with significant associations noted with an asterisk.

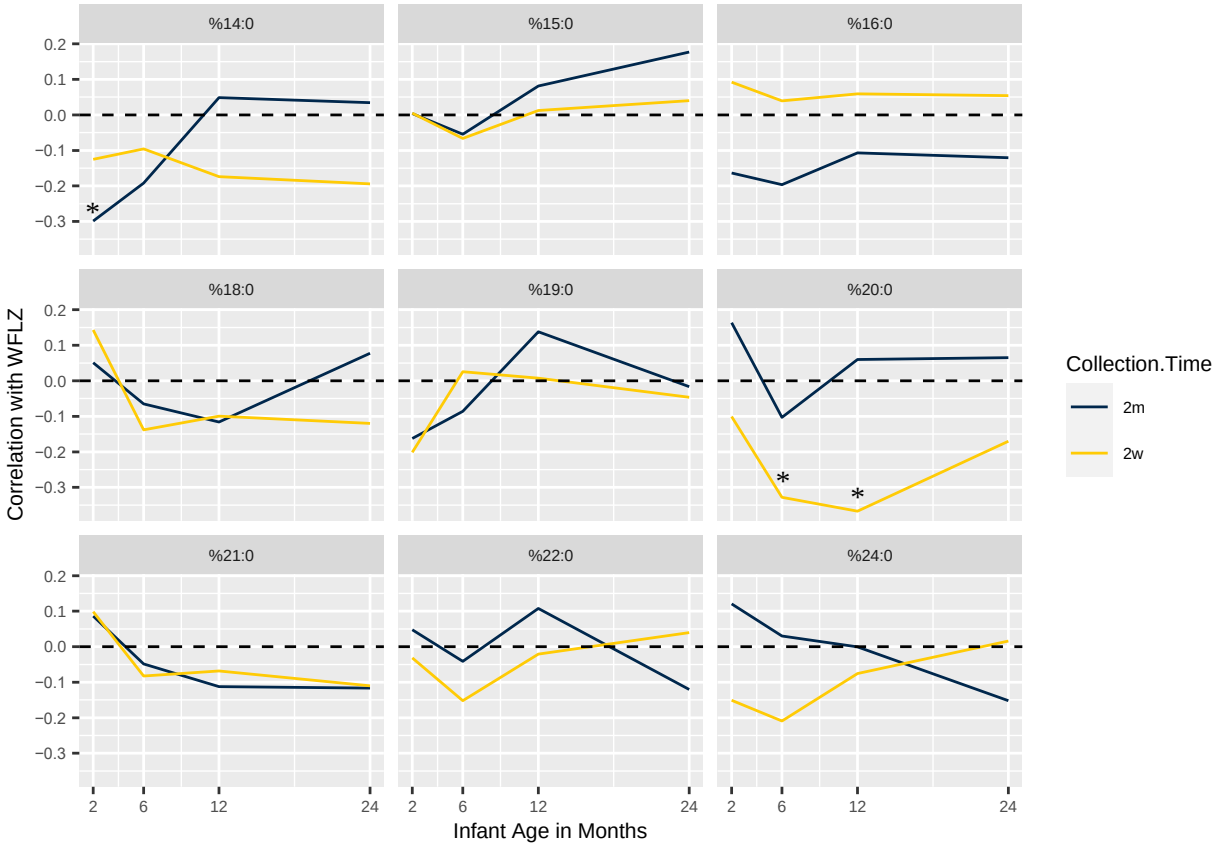


Figure 24: Summary of saturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for saturated fatty acids only using Pearson correlation.

Significant associations from Pearson correlations (nominal $p<0.05$) are highlighted with an asterisk.

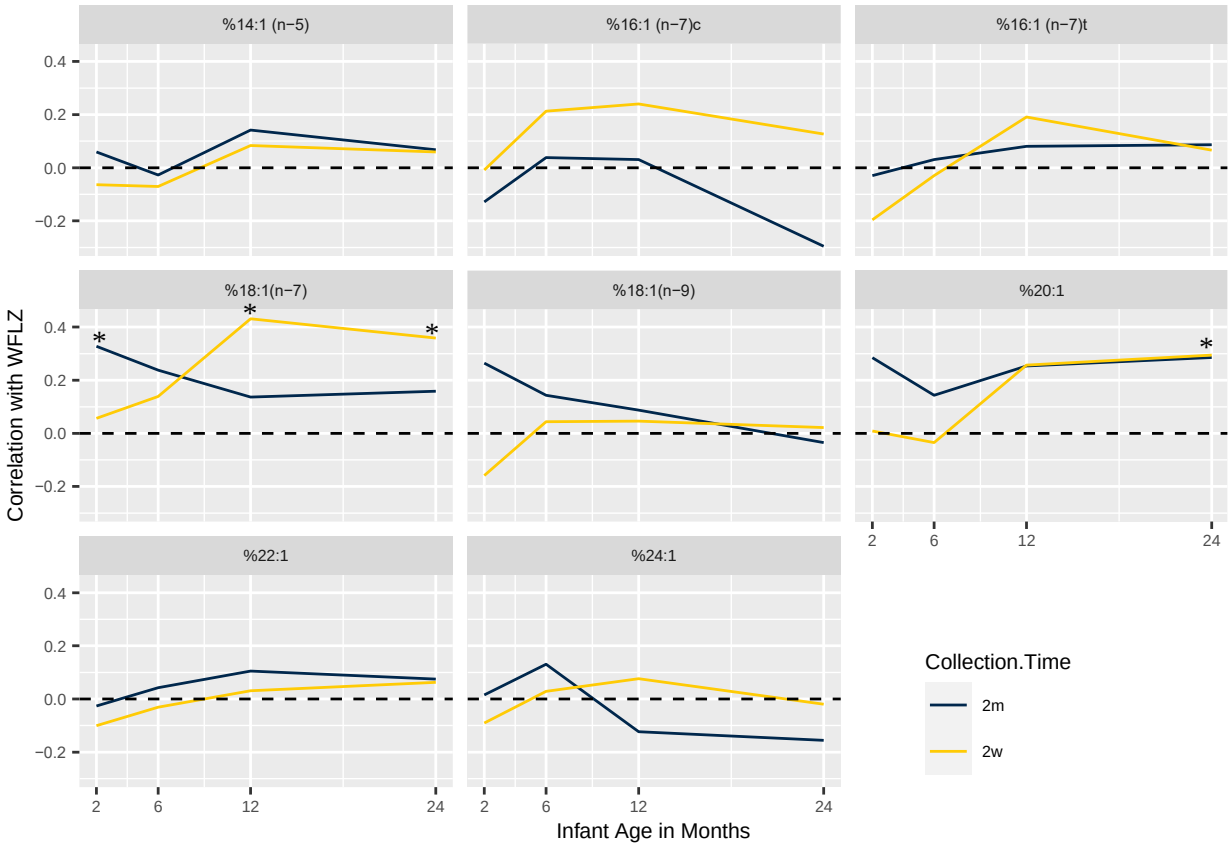


Figure 25: Summary of monounsaturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for monounsaturated fatty acids only.

Significant associations from Pearson correlations (nominal $p<0.05$) are highlighted with an asterisk.

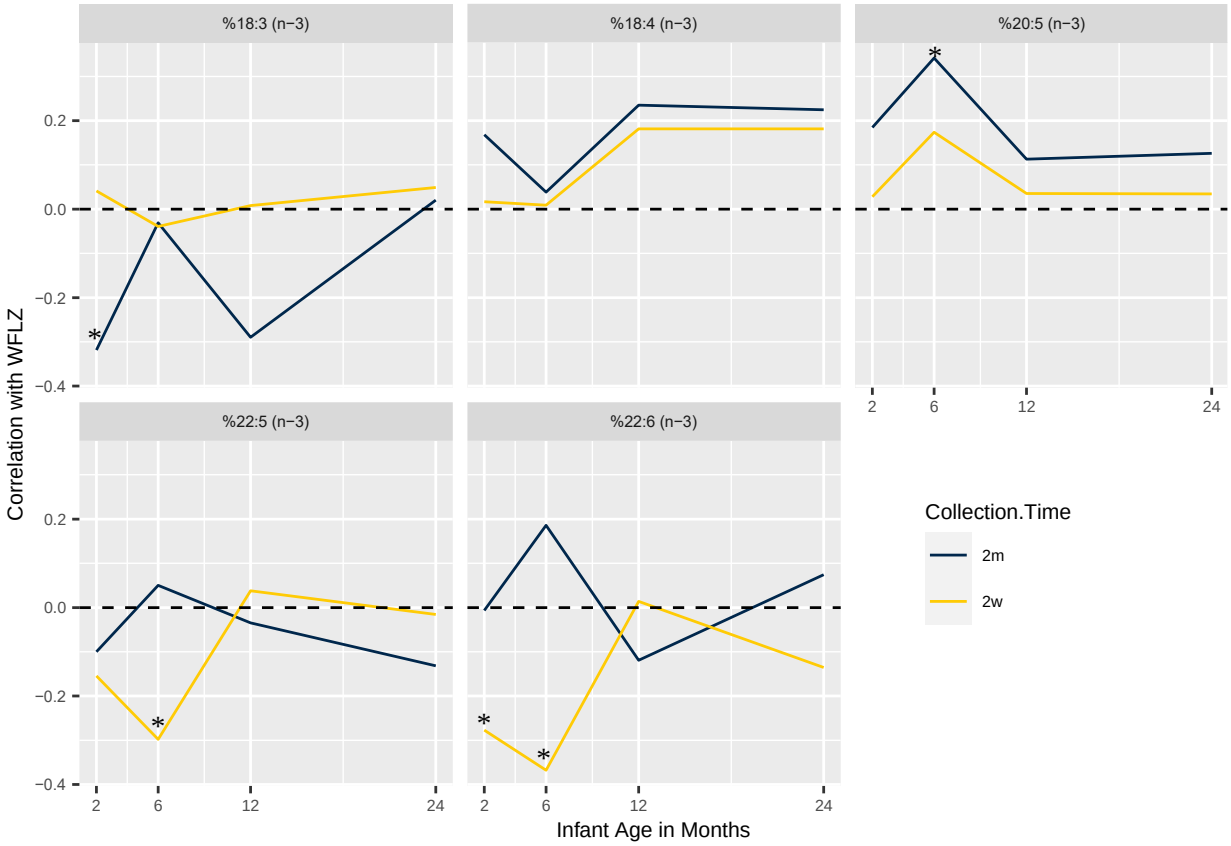


Figure 26: Summary of n-3 polyunsaturated fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for omega-3 polyunsaturated fatty acids only.

Significant associations from Pearson correlations (nominal $p<0.05$) are highlighted with an asterisk.

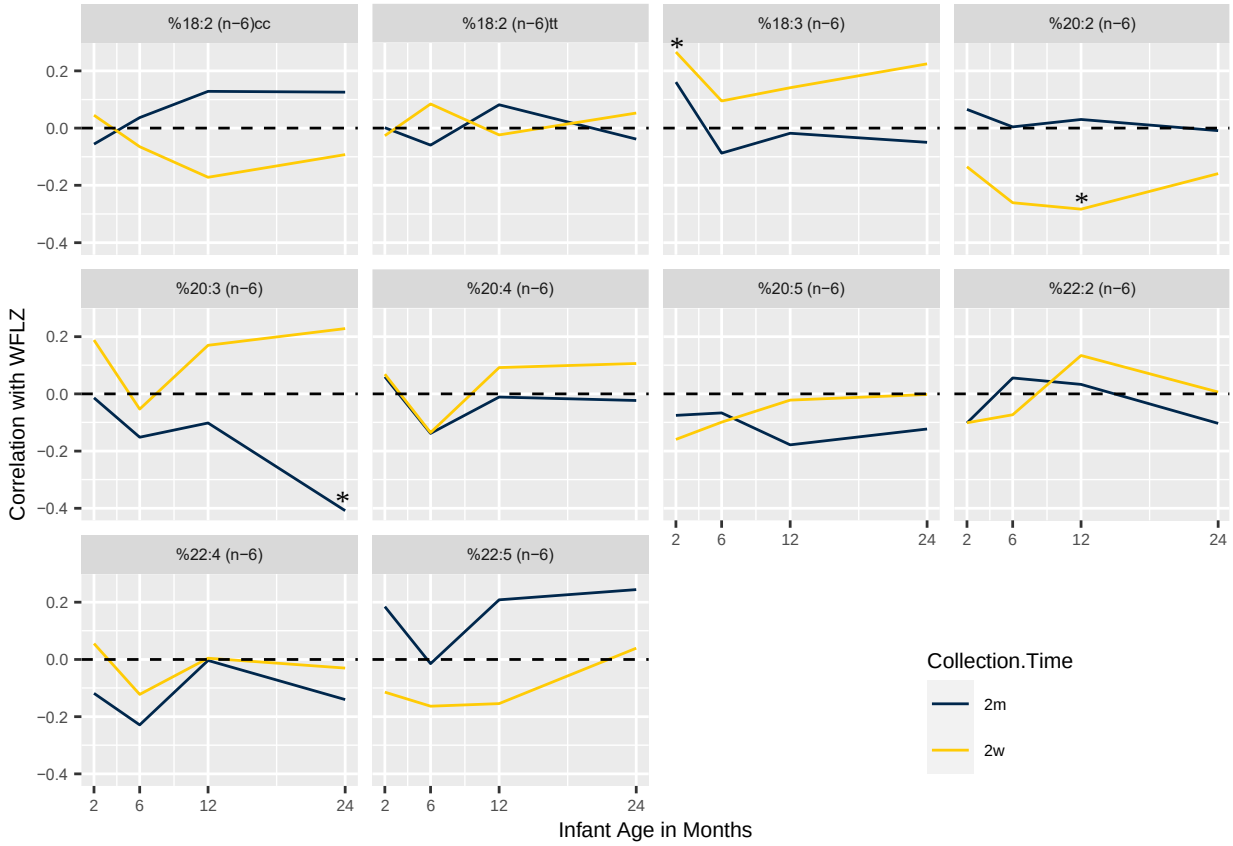


Figure 27: Summary of n-6 fatty acid associations with weight-for-length z-scores by collection period (2 weeks; 2w and 2 months; 2m) and infant age in months for omega-6 polyunsaturated fatty acids only.

Significant associations from Pearson correlations (nominal $p<0.05$) are highlighted with an asterisk.

4.5 Discussion

This study aimed to evaluate the nominal associations between milk fatty acid components at 2 weeks and 2 months and anthropologic markers of infant adiposity. Consistent with the dynamic nature of breastmilk and the changes in fatty acid composition (Khor et al., 2021), we found that several fatty acids decreased in human milk from 2 weeks to 2 months. Additionally, multiple long chain fatty acid percentages were significantly associated with infant weight-for-length z-score at multiple measurement periods in infancy. Interestingly, 18:1 (n-7) (vaccenic acid) had

multiple significant positive nominal associations. Omega-3 fatty acids 22:6 (n-3) (docosahexaenoic acid), 18:3 (n-3) (alpha-linolenic acid), and 22:5 (n-3) (docosapentaenoic acid) showed significant negative correlations with weight-for-length z-scores with the exception of 20:5 (n-3) (eicosapentaenoic acid) which showed a positive correlation. Omega-6 fatty acids showed conflicting results with 20:3 (n-6) (dihomo-gamma-linolenic acid) and 20:2 (n-6) (eicosadienoic acid) being negatively associated and 18:3 (n-6) (gamma-linolenic acid) being positively associated with infant adiposity. However, when evaluating a subsample of exclusively breastfed infants at 2 weeks and 2 months, we found additional stronger associations. The monounsaturated fatty acid 18:1 (n-7) (vaccenic acid) now had a single significant association that was positive with weight-for-length z score. The omega-3 fatty acids 22:5 (n-3) (docosapentaenoic acid) and 18:3 (n-3) (alpha-linolenic acid) showed a stronger negative association with infant adiposity. Omega-6 fatty acids showed a stronger negative nominal association for 20:2 (n-6) (eicosadienoic acid), and new negative nominal associations that were significant for 22:5 (n-6) (osbond acid) and 22:2 (n-6) (docosadienoic acid). Additionally, average milk energy (kcal/100mL), fat (g/100mL), and omega6:omega3 ratio showed significant positive nominal associations with infant adiposity among exclusively breastfed infants. Given that our data showed some new or more pronounced associations between milk components and infant weight-for-length z-scores, it is crucial that future work accounts for infant feeding method to better assess the underlying mechanisms at play and determine if a dose-responsive relationship exists.

Contrary to previous work showing positive associations between alpha-linolenic and docosahexaenoic acids supplementation throughout pregnancy and lactation and infant adiposity

(Helland et al., 2008; Much et al., 2013), we showed a negative nominal association with weight-for-length z-scores, which is consistent with the work of Pedersen et al. (Pedersen et al., 2012). However, it is worth noting that the supplementation during the pregnancy window could account for these differences. Our data also shows a positive nominal association between infant adiposity and eicosapentaenoic acid which is similar to previously reported data (Much et al., 2013). Omega-6 to omega-3 ratio was positively associated with infant adiposity only in exclusively breastfed infants in our study, and this matches previous reports showing a higher ratio corresponding to higher infant fat mass and weight-for-length z-score (Panagos et al., 2016; Rudolph et al., 2017). In transgenic mouse studies where milk was enriched with alpha-linolenic acid and eicosapentaenoic acid, offspring consuming enriched milk weighed less on postnatal day 18 than counterparts consuming control milk (Bongiovanni, Depeters, & Van Eenennaam, 2007). In lamb, a higher milk vaccenic acid content correlated with increased offspring weight during suckling (Ptáček et al., 2019). This is consistent with our findings that vaccenic acid was positively associated with infant adiposity. Furthermore, considering the trends in the nominal association between fatty acids and weight-for-length z-scores at 2, 6, 12, and 24 months, it is worthy to note that the directionality of the correlations vary by time and milk sampling period. Hence, it is important to evaluate the association between milk fatty acid components and infant growth markers at each unique timepoint.

With this exploratory analysis, this study highlights the possible role of milk fatty acids and components in infant adiposity programming. There are several strengths to our approach including a holistic examination of milk components and infant weight-for-length z-score, a detailed examination of milk components and infant adiposity among exclusively breastfed

infants, having multiple infant measurements throughout two years of age, and using consistent methods for synchronized milk collection and analysis. Some limitations to our study include a small sample size, lack of measurements of short and medium chain fatty acids, and lack of diversity in participant demographic characteristics. In addition, a lack of information regarding maternal diet throughout lactation is a limitation since maternal diet can affect milk composition. We also did not have availability of detailed infant diet aside from milk, and this can also affect infant growth and adiposity. The milk samples collected for this study were consistently collected in the morning, and milk fat content has been found to be higher during the day and to increase within feed leading to higher fat content in hindmilk compared to foremilk (Demmelmair & Koletzko, 2018). While we feel this collection strategy is a strength, single daily samples may not be reflective of the overall milk fat composition throughout the day. Future work including a larger sample size and full lipidomic analyses of all fatty acids along with longer childhood follow-up is warranted to better understand the role of milk components in infant adiposity and long-term obesity risk.

In summary, our analysis showed several significant nominal associations between fatty acid levels and infant adiposity. As 8% of infants under two years of age exceed the 95th percentile for WFL z-scores, it is important to assess the role of fatty acids in human milk and infant adiposity. Our work highlights the importance of testing milk fatty acid compositional differences at multiple time points throughout gestation and infant adiposity with time. Future work can include a cohort of exclusively breastfed infants and a higher sample size to determine the role of milk fatty acids on infant development.

Supplementary Table 1: Nominal and FDR-adjusted associations between milk components at 2 weeks and 2 months and weight-for-length z-scores (Wt/L Zscore) at 2, 6, 12, and 24 months of age.

Outcome	Milk Component at 2 weeks (2w) or 2 months (2m)	p-value	Adjusted p-value
1yr Wt/L Zscore	%14:0_2m	0.7597624793998080	0.9991241851760790
1yr Wt/L Zscore	%14:0_2w	0.20932894840493100	0.9991241851760790
1yr Wt/L Zscore	%14:1 (n-5)_2m	0.37030123748682000	0.9991241851760790
1yr Wt/L Zscore	%14:1 (n-5)_2w	0.5471069935923770	0.9991241851760790
1yr Wt/L Zscore	%15:0_2m	0.6095573780794740	0.9991241851760790
1yr Wt/L Zscore	%15:0_2w	0.9295844929981760	0.9991241851760790
1yr Wt/L Zscore	%16:0_2m	0.49915103352985500	0.9991241851760790
1yr Wt/L Zscore	%16:0_2w	0.6708665185929610	0.9991241851760790
1yr Wt/L Zscore	%16:1 (n-7)c_2m	0.8453485649176460	0.9991241851760790
1yr Wt/L Zscore	%16:1 (n-7)c_2w	0.08055084353963070	0.9991241851760790
1yr Wt/L Zscore	%16:1 (n-7)t_2m	0.6106332547980700	0.9991241851760790
1yr Wt/L Zscore	%16:1 (n-7)t_2w	0.1667221508991930	0.9991241851760790
1yr Wt/L Zscore	%18:0_2m	0.46431316078946000	0.9991241851760790
1yr Wt/L Zscore	%18:0_2w	0.47394059918351100	0.9991241851760790
1yr Wt/L Zscore	%18:1(n-7)_2m	0.3882332044552150	0.9991241851760790
1yr Wt/L Zscore	%18:1(n-7)_2w	0.0011326295942391100	0.5323359092923830
1yr Wt/L Zscore	%18:1(n-9)_2m	0.5825773923761200	0.9991241851760790
1yr Wt/L Zscore	%18:1(n-9)_2w	0.7397263172003170	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-6)cc_2m	0.41754322745567900	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-6)cc_2w	0.2148643920540160	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-6)tt_2m	0.6060075842372890	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-6)tt_2w	0.8672651440230320	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-7,9) Conju_2m	0.7954256498466630	0.9991241851760790
1yr Wt/L Zscore	%18:2 (n-7,9) Conju_2w	0.14276579717008200	0.9991241851760790
1yr Wt/L Zscore	%18:3 (n-3)_2m	0.0630868123022844	0.9991241851760790
1yr Wt/L Zscore	%18:3 (n-3)_2w	0.9538874892000650	0.9991241851760790
1yr Wt/L Zscore	%18:3 (n-6)_2m	0.9091550625543030	0.9991241851760790
1yr Wt/L Zscore	%18:3 (n-6)_2w	0.3076047022758760	0.9991241851760790
1yr Wt/L Zscore	%18:4 (n-3)_2m	0.13445796886972500	0.9991241851760790
1yr Wt/L Zscore	%18:4 (n-3)_2w	0.1890955511501930	0.9991241851760790
1yr Wt/L Zscore	%19:0_2m	0.38277966379287000	0.9991241851760790
1yr Wt/L Zscore	%19:0_2w	0.9592195963889170	0.9991241851760790
1yr Wt/L Zscore	%20:0_2m	0.7069039054718710	0.9991241851760790
1yr Wt/L Zscore	%20:0_2w	0.0062974248275242700	0.6516459674236960
1yr Wt/L Zscore	%20:1_2m	0.10511888019631600	0.9991241851760790

1yr Wt/L Zscore	%20:1_2w	0.061129491279634600	0.9991241851760790
1yr Wt/L Zscore	%20:2 (n-6)_2m	0.8465212563204250	0.9991241851760790
1yr Wt/L Zscore	%20:2 (n-6)_2w	0.037849501421150200	0.9632706658262890
1yr Wt/L Zscore	%20:3 (n-6)_2m	0.5209156802499580	0.9991241851760790
1yr Wt/L Zscore	%20:3 (n-6)_2w	0.22091848722779200	0.9991241851760790
1yr Wt/L Zscore	%20:4 (n-6)_2m	0.9423389371594710	0.9991241851760790
1yr Wt/L Zscore	%20:4 (n-6)_2w	0.5093268353068330	0.9991241851760790
1yr Wt/L Zscore	%20:5 (n-3)_2m	0.4763117546896460	0.9991241851760790
1yr Wt/L Zscore	%20:5 (n-3)_2w	0.8003615855693780	0.9991241851760790
1yr Wt/L Zscore	%20:5 (n-6)_2m	0.2588411709861400	0.9991241851760790
1yr Wt/L Zscore	%20:5 (n-6)_2w	0.8751330274920490	0.9991241851760790
1yr Wt/L Zscore	%21:0_2m	0.4781769853283800	0.9991241851760790
1yr Wt/L Zscore	%21:0_2w	0.6221963317411680	0.9991241851760790
1yr Wt/L Zscore	%22:0_2m	0.49667587094628500	0.9991241851760790
1yr Wt/L Zscore	%22:0_2w	0.8822781975525460	0.9991241851760790
1yr Wt/L Zscore	%22:1_2m	0.508980048150751	0.9991241851760790
1yr Wt/L Zscore	%22:1_2w	0.8249432518767130	0.9991241851760790
1yr Wt/L Zscore	%22:2 (n-6)_2m	0.8382490850525410	0.9991241851760790
1yr Wt/L Zscore	%22:2 (n-6)_2w	0.3352124367587950	0.9991241851760790
1yr Wt/L Zscore	%22:4 (n-6)_2m	0.9829322659980600	0.9991241851760790
1yr Wt/L Zscore	%22:4 (n-6)_2w	0.9746836289024330	0.9991241851760790
1yr Wt/L Zscore	%22:5 (n-3)_2m	0.8267405479158240	0.9991241851760790
1yr Wt/L Zscore	%22:5 (n-3)_2w	0.7851357359004790	0.9991241851760790
1yr Wt/L Zscore	%22:5 (n-6)_2m	0.18518525201596000	0.9991241851760790
1yr Wt/L Zscore	%22:5 (n-6)_2w	0.26404717303208800	0.9991241851760790
1yr Wt/L Zscore	%22:6 (n-3)_2m	0.4524364923216800	0.9991241851760790
1yr Wt/L Zscore	%22:6 (n-3)_2w	0.9216825074261750	0.9991241851760790
1yr Wt/L Zscore	%24:0_2m	0.9983743833366350	0.9997817365020370
1yr Wt/L Zscore	%24:0_2w	0.5856170700649440	0.9991241851760790
1yr Wt/L Zscore	%24:1_2m	0.43587604459132800	0.9991241851760790
1yr Wt/L Zscore	%24:1_2w	0.5812784543664370	0.9991241851760790
1yr Wt/L Zscore	Avg Carb_2m	0.7672148139817210	0.9991241851760790
1yr Wt/L Zscore	Avg Carb_2w	0.2585604406119370	0.9991241851760790
1yr Wt/L Zscore	Avg Crude Protein_2m	0.6075388890368530	0.9991241851760790
1yr Wt/L Zscore	Avg Crude Protein_2w	0.9063981823944530	0.9991241851760790
1yr Wt/L Zscore	Avg Energy_2m	0.06381595288104620	0.9991241851760790
1yr Wt/L Zscore	Avg Energy_2w	0.39049654131479200	0.9991241851760790
1yr Wt/L Zscore	Avg Fat_2m	0.0733977547590168	0.9991241851760790
1yr Wt/L Zscore	Avg Fat_2w	0.5560455988024210	0.9991241851760790
1yr Wt/L Zscore	Avg True Protein_2m	0.6228875495222320	0.9991241851760790
1yr Wt/L Zscore	Avg True Protein_2w	0.9366083365008160	0.9991241851760790
1yr Wt/L Zscore	Avg TS_2m	0.05169529253625310	0.9991241851760790
1yr Wt/L Zscore	Avg TS_2w	0.2951547329427290	0.9991241851760790

1yr Wt/L Zscore	MUFA/SFA Ratio 2months	0.580220171998592	0.9991241851760790
1yr Wt/L Zscore	MUFA/SFA Ratio 2wks	0.3204514938654710	0.9991241851760790
1yr Wt/L Zscore	n6n3ratio_2months	0.06261493244923800	0.9991241851760790
1yr Wt/L Zscore	n6n3ratio_2wks	0.028830289088146200	0.9361832953619540
1yr Wt/L Zscore	SumpercentMUFA2months	0.9654754153990330	0.9991241851760790
1yr Wt/L Zscore	SumpercentMUFA2wks	0.4359176228878550	0.9991241851760790
1yr Wt/L Zscore	Sumpercentn3_2months	0.4052709859234920	0.9991241851760790
1yr Wt/L Zscore	Sumpercentn3_2wks	0.5646581399377520	0.9991241851760790
1yr Wt/L Zscore	Sumpercentn6_2months	0.9729166897220740	0.9991241851760790
1yr Wt/L Zscore	Sumpercentn6_2wks	0.9477861832306960	0.9991241851760790
1yr Wt/L Zscore	SumpercentPUFA2months	0.9285342087765330	0.9991241851760790
1yr Wt/L Zscore	SumpercentPUFA2wks	0.9269334597518300	0.9991241851760790
1yr Wt/L Zscore	SumpercentSFA2months	0.8693138372671880	0.9991241851760790
1yr Wt/L Zscore	SumpercentSFA2wks	0.6583396257697120	0.9991241851760790
1yr Wt/L Zscore	SumtotalLCFA2months	0.7848009324778730	0.9991241851760790
1yr Wt/L Zscore	SumtotalLCFA2wks	0.1745942525808120	0.9991241851760790
2m Wt/L Zscore	%14:0_2m	0.04130363482675290	0.9632706658262890
2m Wt/L Zscore	%14:0_2w	0.34440273857710000	0.9991241851760790
2m Wt/L Zscore	%14:1 (n-5)_2m	0.6897015328866690	0.9991241851760790
2m Wt/L Zscore	%14:1 (n-5)_2w	0.6327583786135890	0.9991241851760790
2m Wt/L Zscore	%15:0_2m	0.9796881500646740	0.9991241851760790
2m Wt/L Zscore	%15:0_2w	0.9710850638291040	0.9991241851760790
2m Wt/L Zscore	%16:0_2m	0.2712029030621440	0.9991241851760790
2m Wt/L Zscore	%16:0_2w	0.487738828939675	0.9991241851760790
2m Wt/L Zscore	%16:1 (n-7)c_2m	0.3876085672914490	0.9991241851760790
2m Wt/L Zscore	%16:1 (n-7)c_2w	0.9478030360166450	0.9991241851760790
2m Wt/L Zscore	%16:1 (n-7)t_2m	0.8440015916614630	0.9991241851760790
2m Wt/L Zscore	%16:1 (n-7)t_2w	0.13738266709220400	0.9991241851760790
2m Wt/L Zscore	%18:0_2m	0.7329020659848030	0.9991241851760790
2m Wt/L Zscore	%18:0_2w	0.2802354439356210	0.9991241851760790
2m Wt/L Zscore	%18:1(n-7)_2m	0.02440707572860730	0.9361832953619540
2m Wt/L Zscore	%18:1(n-7)_2w	0.6709026481818620	0.9991241851760790
2m Wt/L Zscore	%18:1(n-9)_2m	0.07240119003418180	0.9991241851760790
2m Wt/L Zscore	%18:1(n-9)_2w	0.22906276208148300	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-6)cc_2m	0.7109276814915750	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-6)cc_2w	0.7338419988039840	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-6)tt_2m	0.9922597446468500	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-6)tt_2w	0.8425390222275900	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-7,9) Conju_2m	0.2592359290386380	0.9991241851760790
2m Wt/L Zscore	%18:2 (n-7,9) Conju_2w	0.8152127018217790	0.9991241851760790
2m Wt/L Zscore	%18:3 (n-3)_2m	0.02909671737024780	0.9361832953619540
2m Wt/L Zscore	%18:3 (n-3)_2w	0.757791328634051	0.9991241851760790
2m Wt/L Zscore	%18:3 (n-6)_2m	0.2798673142881740	0.9991241851760790

2m Wt/L Zscore	%18:3 (n-6)_2w	0.04150261911272840	0.9632706658262890
2m Wt/L Zscore	%18:4 (n-3)_2m	0.2588023863458710	0.9991241851760790
2m Wt/L Zscore	%18:4 (n-3)_2w	0.9004243634879060	0.9991241851760790
2m Wt/L Zscore	%19:0_2m	0.27597655842688200	0.9991241851760790
2m Wt/L Zscore	%19:0_2w	0.12623700575496700	0.9991241851760790
2m Wt/L Zscore	%20:0_2m	0.27223904697052900	0.9991241851760790
2m Wt/L Zscore	%20:0_2w	0.44764789870443000	0.9991241851760790
2m Wt/L Zscore	%20:1_2m	0.052692098311579100	0.9991241851760790
2m Wt/L Zscore	%20:1_2w	0.9471906891995830	0.9991241851760790
2m Wt/L Zscore	%20:2 (n-6)_2m	0.6605129110128090	0.9991241851760790
2m Wt/L Zscore	%20:2 (n-6)_2w	0.30685472395165900	0.9991241851760790
2m Wt/L Zscore	%20:3 (n-6)_2m	0.9252848478220790	0.9991241851760790
2m Wt/L Zscore	%20:3 (n-6)_2w	0.15475058057640700	0.9991241851760790
2m Wt/L Zscore	%20:4 (n-6)_2m	0.6900103356389280	0.9991241851760790
2m Wt/L Zscore	%20:4 (n-6)_2w	0.6072564580555020	0.9991241851760790
2m Wt/L Zscore	%20:5 (n-3)_2m	0.2147084810626100	0.9991241851760790
2m Wt/L Zscore	%20:5 (n-3)_2w	0.8321781872413330	0.9991241851760790
2m Wt/L Zscore	%20:5 (n-6)_2m	0.6149871857624480	0.9991241851760790
2m Wt/L Zscore	%20:5 (n-6)_2w	0.227629072021193	0.9991241851760790
2m Wt/L Zscore	%21:0_2m	0.5649808699102430	0.9991241851760790
2m Wt/L Zscore	%21:0_2w	0.45720485266054000	0.9991241851760790
2m Wt/L Zscore	%22:0_2m	0.750212532628097	0.9991241851760790
2m Wt/L Zscore	%22:0_2w	0.8110021228641380	0.9991241851760790
2m Wt/L Zscore	%22:1_2m	0.8606129793008940	0.9991241851760790
2m Wt/L Zscore	%22:1_2w	0.45063491728613400	0.9991241851760790
2m Wt/L Zscore	%22:2 (n-6)_2m	0.4935877569990790	0.9991241851760790
2m Wt/L Zscore	%22:2 (n-6)_2w	0.4446065482256400	0.9991241851760790
2m Wt/L Zscore	%22:4 (n-6)_2m	0.42629332906805800	0.9991241851760790
2m Wt/L Zscore	%22:4 (n-6)_2w	0.6776051483171160	0.9991241851760790
2m Wt/L Zscore	%22:5 (n-3)_2m	0.5038839865906420	0.9991241851760790
2m Wt/L Zscore	%22:5 (n-3)_2w	0.243366859539374	0.9991241851760790
2m Wt/L Zscore	%22:5 (n-6)_2m	0.2147926923892430	0.9991241851760790
2m Wt/L Zscore	%22:5 (n-6)_2w	0.3871523919241480	0.9991241851760790
2m Wt/L Zscore	%22:6 (n-3)_2m	0.9663287046910860	0.9991241851760790
2m Wt/L Zscore	%22:6 (n-3)_2w	0.033636565294687100	0.9361832953619540
2m Wt/L Zscore	%24:0_2m	0.4197475115347990	0.9991241851760790
2m Wt/L Zscore	%24:0_2w	0.2542511341538430	0.9991241851760790
2m Wt/L Zscore	%24:1_2m	0.9184448263355990	0.9991241851760790
2m Wt/L Zscore	%24:1_2w	0.49427867924704400	0.9991241851760790
2m Wt/L Zscore	Avg Carb_2m	0.7261923534526870	0.9991241851760790
2m Wt/L Zscore	Avg Carb_2w	0.3589155546226140	0.9991241851760790
2m Wt/L Zscore	Avg Crude Protein_2m	0.564871555687132	0.9991241851760790
2m Wt/L Zscore	Avg Crude Protein_2w	0.486464754963537	0.9991241851760790

2m Wt/L Zscore	Avg Energy_2m	0.4222249363785690	0.9991241851760790
2m Wt/L Zscore	Avg Energy_2w	0.8766571041483400	0.9991241851760790
2m Wt/L Zscore	Avg Fat_2m	0.40141383531690500	0.9991241851760790
2m Wt/L Zscore	Avg Fat_2w	0.8836467948779410	0.9991241851760790
2m Wt/L Zscore	Avg True Protein_2m	0.5912214182023250	0.9991241851760790
2m Wt/L Zscore	Avg True Protein_2w	0.4715131671228400	0.9991241851760790
2m Wt/L Zscore	Avg TS_2m	0.4415928246570070	0.9991241851760790
2m Wt/L Zscore	Avg TS_2w	0.6604628162096580	0.9991241851760790
2m Wt/L Zscore	MUFA/SFA Ratio 2months	0.16608066695669000	0.9991241851760790
2m Wt/L Zscore	MUFA/SFA Ratio 2wks	0.28094071572392000	0.9991241851760790
2m Wt/L Zscore	n6n3ratio_2months	0.08681707901942240	0.9991241851760790
2m Wt/L Zscore	n6n3ratio_2wks	0.9773581963497400	0.9991241851760790
2m Wt/L Zscore	SumpercentMUFA2months	0.7976341960330220	0.9991241851760790
2m Wt/L Zscore	SumpercentMUFA2wks	0.6973489579798640	0.9991241851760790
2m Wt/L Zscore	Sumpercentn3_2months	0.5421445787185390	0.9991241851760790
2m Wt/L Zscore	Sumpercentn3_2wks	0.7235267703833760	0.9991241851760790
2m Wt/L Zscore	Sumpercentn6_2months	0.9698139277195410	0.9991241851760790
2m Wt/L Zscore	Sumpercentn6_2wks	0.4974784430008450	0.9991241851760790
2m Wt/L Zscore	SumpercentPUFA2months	0.9293258272838040	0.9991241851760790
2m Wt/L Zscore	SumpercentPUFA2wks	0.5137439623755190	0.9991241851760790
2m Wt/L Zscore	SumpercentSFA2months	0.962249650561342	0.9991241851760790
2m Wt/L Zscore	SumpercentSFA2wks	0.5046786302740790	0.9991241851760790
2m Wt/L Zscore	SumtotalLCFA2months	0.30423292762266200	0.9991241851760790
2m Wt/L Zscore	SumtotalLCFA2wks	0.04747017165575730	0.9991241851760790
2yr Wt/L Zscore	%14:0_2m	0.8475643222280140	0.9991241851760790
2yr Wt/L Zscore	%14:0_2w	0.1807451821268200	0.9991241851760790
2yr Wt/L Zscore	%14:1 (n-5)_2m	0.7036856450578950	0.9991241851760790
2yr Wt/L Zscore	%14:1 (n-5)_2w	0.6870075877058070	0.9991241851760790
2yr Wt/L Zscore	%15:0_2m	0.31663831405641800	0.9991241851760790
2yr Wt/L Zscore	%15:0_2w	0.7860054075652300	0.9991241851760790
2yr Wt/L Zscore	%16:0_2m	0.4963517191714710	0.9991241851760790
2yr Wt/L Zscore	%16:0_2w	0.7107632488625040	0.9991241851760790
2yr Wt/L Zscore	%16:1 (n-7)c_2m	0.08956027965415710	0.9991241851760790
2yr Wt/L Zscore	%16:1 (n-7)c_2w	0.3845446456190790	0.9991241851760790
2yr Wt/L Zscore	%16:1 (n-7)t_2m	0.6260093721422840	0.9991241851760790
2yr Wt/L Zscore	%16:1 (n-7)t_2w	0.6504252658509920	0.9991241851760790
2yr Wt/L Zscore	%18:0_2m	0.6624722198598240	0.9991241851760790
2yr Wt/L Zscore	%18:0_2w	0.41245985795334500	0.9991241851760790
2yr Wt/L Zscore	%18:1(n-7)_2m	0.3701451832053560	0.9991241851760790
2yr Wt/L Zscore	%18:1(n-7)_2w	0.011328609938650800	0.8191456417178260
2yr Wt/L Zscore	%18:1(n-9)_2m	0.8435308151263020	0.9991241851760790
2yr Wt/L Zscore	%18:1(n-9)_2w	0.883348584553725	0.9991241851760790
2yr Wt/L Zscore	%18:2 (n-6)cc_2m	0.4778601542034480	0.9991241851760790

2yr Wt/L Zscore	%18:2 (n-6)cc_2w	0.5279594758126720	0.9991241851760790
2yr Wt/L Zscore	%18:2 (n-6)tt_2m	0.8296467285589690	0.9991241851760790
2yr Wt/L Zscore	%18:2 (n-6)tt_2w	0.7163058163476100	0.9991241851760790
2yr Wt/L Zscore	%18:2 (n-7,9) Conju_2m	0.5854073232532650	0.9991241851760790
2yr Wt/L Zscore	%18:2 (n-7,9) Conju_2w	0.26509807824893400	0.9991241851760790
2yr Wt/L Zscore	%18:3 (n-3)_2m	0.909687088551092	0.9991241851760790
2yr Wt/L Zscore	%18:3 (n-3)_2w	0.7381121279559540	0.9991241851760790
2yr Wt/L Zscore	%18:3 (n-6)_2m	0.7795917152985640	0.9991241851760790
2yr Wt/L Zscore	%18:3 (n-6)_2w	0.12079737440715500	0.9991241851760790
2yr Wt/L Zscore	%18:4 (n-3)_2m	0.20181297752811600	0.9991241851760790
2yr Wt/L Zscore	%18:4 (n-3)_2w	0.21174642267127100	0.9991241851760790
2yr Wt/L Zscore	%19:0_2m	0.9254919781358960	0.9991241851760790
2yr Wt/L Zscore	%19:0_2w	0.7529258248827840	0.9991241851760790
2yr Wt/L Zscore	%20:0_2m	0.7151446071299840	0.9991241851760790
2yr Wt/L Zscore	%20:0_2w	0.24159886508946700	0.9991241851760790
2yr Wt/L Zscore	%20:1_2m	0.1017111053992950	0.9991241851760790
2yr Wt/L Zscore	%20:1_2w	0.03980665652293140	0.9632706658262890
2yr Wt/L Zscore	%20:2 (n-6)_2m	0.9621107019161830	0.9991241851760790
2yr Wt/L Zscore	%20:2 (n-6)_2w	0.2760118104706680	0.9991241851760790
2yr Wt/L Zscore	%20:3 (n-6)_2m	0.01640413669433420	0.9361832953619540
2yr Wt/L Zscore	%20:3 (n-6)_2w	0.11488124589922600	0.9991241851760790
2yr Wt/L Zscore	%20:4 (n-6)_2m	0.8955558866158790	0.9991241851760790
2yr Wt/L Zscore	%20:4 (n-6)_2w	0.4705652126978520	0.9991241851760790
2yr Wt/L Zscore	%20:5 (n-3)_2m	0.47781018837498200	0.9991241851760790
2yr Wt/L Zscore	%20:5 (n-3)_2w	0.8150445830205840	0.9991241851760790
2yr Wt/L Zscore	%20:5 (n-6)_2m	0.48760483741922100	0.9991241851760790
2yr Wt/L Zscore	%20:5 (n-6)_2w	0.9865686101189660	0.9991241851760790
2yr Wt/L Zscore	%21:0_2m	0.5118482542124690	0.9991241851760790
2yr Wt/L Zscore	%21:0_2w	0.4517813831364560	0.9991241851760790
2yr Wt/L Zscore	%22:0_2m	0.4976442961427480	0.9991241851760790
2yr Wt/L Zscore	%22:0_2w	0.7872429391575580	0.9991241851760790
2yr Wt/L Zscore	%22:1_2m	0.673878250232445	0.9991241851760790
2yr Wt/L Zscore	%22:1_2w	0.6684262544401090	0.9991241851760790
2yr Wt/L Zscore	%22:2 (n-6)_2m	0.5608144317904260	0.9991241851760790
2yr Wt/L Zscore	%22:2 (n-6)_2w	0.9669663336406200	0.9991241851760790
2yr Wt/L Zscore	%22:4 (n-6)_2m	0.4271386562377870	0.9991241851760790
2yr Wt/L Zscore	%22:4 (n-6)_2w	0.8375850990220120	0.9991241851760790
2yr Wt/L Zscore	%22:5 (n-3)_2m	0.45713326673350800	0.9991241851760790
2yr Wt/L Zscore	%22:5 (n-3)_2w	0.914110186324255	0.9991241851760790
2yr Wt/L Zscore	%22:5 (n-6)_2m	0.16426101890407100	0.9991241851760790
2yr Wt/L Zscore	%22:5 (n-6)_2w	0.7876545594782190	0.9991241851760790
2yr Wt/L Zscore	%22:6 (n-3)_2m	0.676700083933182	0.9991241851760790
2yr Wt/L Zscore	%22:6 (n-3)_2w	0.3529675593618120	0.9991241851760790

2yr Wt/L Zscore	%24:0_2m	0.39054641884009100	0.9991241851760790
2yr Wt/L Zscore	%24:0_2w	0.913548030558897	0.9991241851760790
2yr Wt/L Zscore	%24:1_2m	0.378641678071709	0.9991241851760790
2yr Wt/L Zscore	%24:1_2w	0.8948024674400050	0.9991241851760790
2yr Wt/L Zscore	Avg Carb_2m	0.24948606027186000	0.9991241851760790
2yr Wt/L Zscore	Avg Carb_2w	0.3383034618640920	0.9991241851760790
2yr Wt/L Zscore	Avg Crude Protein_2m	0.1502801938343300	0.9991241851760790
2yr Wt/L Zscore	Avg Crude Protein_2w	0.21715431673938100	0.9991241851760790
2yr Wt/L Zscore	Avg Energy_2m	0.2821262668585690	0.9991241851760790
2yr Wt/L Zscore	Avg Energy_2w	0.7546667595395540	0.9991241851760790
2yr Wt/L Zscore	Avg Fat_2m	0.2720441628828550	0.9991241851760790
2yr Wt/L Zscore	Avg Fat_2w	0.6283387974172900	0.9991241851760790
2yr Wt/L Zscore	Avg True Protein_2m	0.1329617267891530	0.9991241851760790
2yr Wt/L Zscore	Avg True Protein_2w	0.21447258143736900	0.9991241851760790
2yr Wt/L Zscore	Avg TS_2m	0.20915023393490500	0.9991241851760790
2yr Wt/L Zscore	Avg TS_2w	0.8811006817652800	0.9991241851760790
2yr Wt/L Zscore	MUFA/SFA Ratio 2months	0.8493534177454880	0.9991241851760790
2yr Wt/L Zscore	MUFA/SFA Ratio 2wks	0.5310477715763790	0.9991241851760790
2yr Wt/L Zscore	n6n3ratio_2months	0.7847435496758710	0.9991241851760790
2yr Wt/L Zscore	n6n3ratio_2wks	0.4281328987906430	0.9991241851760790
2yr Wt/L Zscore	SumpercentMUFA2months	0.8281198546329160	0.9991241851760790
2yr Wt/L Zscore	SumpercentMUFA2wks	0.8755369957825340	0.9991241851760790
2yr Wt/L Zscore	Sumpercentn3_2months	0.6970683215950960	0.9991241851760790
2yr Wt/L Zscore	Sumpercentn3_2wks	0.9422323069348030	0.9991241851760790
2yr Wt/L Zscore	Sumpercentn6_2months	0.6616417267281420	0.9991241851760790
2yr Wt/L Zscore	Sumpercentn6_2wks	0.8948992746194880	0.9991241851760790
2yr Wt/L Zscore	SumpercentPUFA2months	0.6682731694329560	0.9991241851760790
2yr Wt/L Zscore	SumpercentPUFA2wks	0.8928542331603510	0.9991241851760790
2yr Wt/L Zscore	SumpercentSFA2months	0.818481344898907	0.9991241851760790
2yr Wt/L Zscore	SumpercentSFA2wks	0.8530696020932000	0.9991241851760790
2yr Wt/L Zscore	SumtotalLCFA2months	0.7197936239474990	0.9991241851760790
2yr Wt/L Zscore	SumtotalLCFA2wks	0.0381539394965537	0.9632706658262890
6m Wt/L Z-Score	%14:0_2m	0.22381176395193000	0.9991241851760790
6m Wt/L Z-Score	%14:0_2w	0.495736814723609	0.9991241851760790
6m Wt/L Z-Score	%14:1 (n-5)_2m	0.8638504663896710	0.9991241851760790
6m Wt/L Z-Score	%14:1 (n-5)_2w	0.6150229122683180	0.9991241851760790
6m Wt/L Z-Score	%15:0_2m	0.7311767961299950	0.9991241851760790
6m Wt/L Z-Score	%15:0_2w	0.6372306614532010	0.9991241851760790
6m Wt/L Z-Score	%16:0_2m	0.21264015411701500	0.9991241851760790
6m Wt/L Z-Score	%16:0_2w	0.7792203175661350	0.9991241851760790
6m Wt/L Z-Score	%16:1 (n-7)c_2m	0.8104595357534	0.9991241851760790
6m Wt/L Z-Score	%16:1 (n-7)c_2w	0.12589766855589900	0.9991241851760790
6m Wt/L Z-Score	%16:1 (n-7)t_2m	0.846090026947684	0.9991241851760790

6m Wt/L Z-Score	%16:1 (n-7)t_2w	0.8329400395281500	0.9991241851760790
6m Wt/L Z-Score	%18:0_2m	0.682407258859798	0.9991241851760790
6m Wt/L Z-Score	%18:0_2w	0.3252266353534320	0.9991241851760790
6m Wt/L Z-Score	%18:1(n-7)_2m	0.12958274553438500	0.9991241851760790
6m Wt/L Z-Score	%18:1(n-7)_2w	0.32048963718119900	0.9991241851760790
6m Wt/L Z-Score	%18:1(n-9)_2m	0.36562352739001000	0.9991241851760790
6m Wt/L Z-Score	%18:1(n-9)_2w	0.756430706028755	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-6)cc_2m	0.8154787764529740	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-6)cc_2w	0.6427818720203290	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-6)tt_2m	0.7094467388918370	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-6)tt_2w	0.5480426583392670	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-7,9) Conju_2m	0.9506258809147110	0.9991241851760790
6m Wt/L Z-Score	%18:2 (n-7,9) Conju_2w	0.76008680933419000	0.9991241851760790
6m Wt/L Z-Score	%18:3 (n-3)_2m	0.846335932876754	0.9991241851760790
6m Wt/L Z-Score	%18:3 (n-3)_2w	0.7808971337015430	0.9991241851760790
6m Wt/L Z-Score	%18:3 (n-6)_2m	0.5832804733833750	0.9991241851760790
6m Wt/L Z-Score	%18:3 (n-6)_2w	0.4988490917002690	0.9991241851760790
6m Wt/L Z-Score	%18:4 (n-3)_2m	0.8085794869696320	0.9991241851760790
6m Wt/L Z-Score	%18:4 (n-3)_2w	0.9478637352547490	0.9991241851760790
6m Wt/L Z-Score	%19:0_2m	0.5885050027478560	0.9991241851760790
6m Wt/L Z-Score	%19:0_2w	0.856719568649386	0.9991241851760790
6m Wt/L Z-Score	%20:0_2m	0.5160059493160570	0.9991241851760790
6m Wt/L Z-Score	%20:0_2w	0.016521890487459600	0.9361832953619540
6m Wt/L Z-Score	%20:1_2m	0.3641412395895760	0.9991241851760790
6m Wt/L Z-Score	%20:1_2w	0.8031999368702940	0.9991241851760790
6m Wt/L Z-Score	%20:2 (n-6)_2m	0.9768366701697980	0.9991241851760790
6m Wt/L Z-Score	%20:2 (n-6)_2w	0.05940736242571740	0.9991241851760790
6m Wt/L Z-Score	%20:3 (n-6)_2m	0.3377328579015460	0.9991241851760790
6m Wt/L Z-Score	%20:3 (n-6)_2w	0.7023266147563910	0.9991241851760790
6m Wt/L Z-Score	%20:4 (n-6)_2m	0.3831393664666780	0.9991241851760790
6m Wt/L Z-Score	%20:4 (n-6)_2w	0.33242407847259700	0.9991241851760790
6m Wt/L Z-Score	%20:5 (n-3)_2m	0.02683992030703080	0.9361832953619540
6m Wt/L Z-Score	%20:5 (n-3)_2w	0.21356902036645600	0.9991241851760790
6m Wt/L Z-Score	%20:5 (n-6)_2m	0.6721180590626340	0.9991241851760790
6m Wt/L Z-Score	%20:5 (n-6)_2w	0.4797355456713230	0.9991241851760790
6m Wt/L Z-Score	%21:0_2m	0.7605396582768410	0.9991241851760790
6m Wt/L Z-Score	%21:0_2w	0.5572342987496670	0.9991241851760790
6m Wt/L Z-Score	%22:0_2m	0.794923735127391	0.9991241851760790
6m Wt/L Z-Score	%22:0_2w	0.27613077531104500	0.9991241851760790
6m Wt/L Z-Score	%22:1_2m	0.7890023575031550	0.9991241851760790
6m Wt/L Z-Score	%22:1_2w	0.8263057702664950	0.9991241851760790
6m Wt/L Z-Score	%22:2 (n-6)_2m	0.7268067190678240	0.9991241851760790
6m Wt/L Z-Score	%22:2 (n-6)_2w	0.6024506739879600	0.9991241851760790

6m Wt/L Z-Score	%22:4 (n-6)_2m	0.14444611660669400	0.9991241851760790
6m Wt/L Z-Score	%22:4 (n-6)_2w	0.382241030460269	0.9991241851760790
6m Wt/L Z-Score	%22:5 (n-3)_2m	0.7524547837106910	0.9991241851760790
6m Wt/L Z-Score	%22:5 (n-3)_2w	0.03030914156538110	0.9361832953619540
6m Wt/L Z-Score	%22:5 (n-6)_2m	0.9243767541121840	0.9991241851760790
6m Wt/L Z-Score	%22:5 (n-6)_2w	0.24169597334374300	0.9991241851760790
6m Wt/L Z-Score	%22:6 (n-3)_2m	0.23864107707247300	0.9991241851760790
6m Wt/L Z-Score	%22:6 (n-3)_2w	0.006737628983309170	0.6516459674236960
6m Wt/L Z-Score	%24:0_2m	0.8508820031418230	0.9991241851760790
6m Wt/L Z-Score	%24:0_2w	0.1326343791386880	0.9991241851760790
6m Wt/L Z-Score	%24:1_2m	0.4083362264594780	0.9991241851760790
6m Wt/L Z-Score	%24:1_2w	0.8380894034727880	0.9991241851760790
6m Wt/L Z-Score	Avg Carb_2m	0.8885264680777450	0.9991241851760790
6m Wt/L Z-Score	Avg Carb_2w	0.164192802488265	0.9991241851760790
6m Wt/L Z-Score	Avg Crude Protein_2m	0.5136456988288820	0.9991241851760790
6m Wt/L Z-Score	Avg Crude Protein_2w	0.7184861459266150	0.9991241851760790
6m Wt/L Z-Score	Avg Energy_2m	0.2045028325818500	0.9991241851760790
6m Wt/L Z-Score	Avg Energy_2w	0.7895833125406820	0.9991241851760790
6m Wt/L Z-Score	Avg Fat_2m	0.2698357073401710	0.9991241851760790
6m Wt/L Z-Score	Avg Fat_2w	0.6334895752228950	0.9991241851760790
6m Wt/L Z-Score	Avg True Protein_2m	0.5063506938360950	0.9991241851760790
6m Wt/L Z-Score	Avg True Protein_2w	0.8449710325179840	0.9991241851760790
6m Wt/L Z-Score	Avg TS_2m	0.20026345798739100	0.9991241851760790
6m Wt/L Z-Score	Avg TS_2w	0.9541209499542360	0.9991241851760790
6m Wt/L Z-Score	MUFA/SFA Ratio 2months	0.3097568590927030	0.9991241851760790
6m Wt/L Z-Score	MUFA/SFA Ratio 2wks	0.6429185040249970	0.9991241851760790
6m Wt/L Z-Score	n6n3ratio_2months	0.8148762563023440	0.9991241851760790
6m Wt/L Z-Score	n6n3ratio_2wks	0.64098756267793	0.9991241851760790
6m Wt/L Z-Score	SumpercentMUFA2months	0.5297516725128150	0.9991241851760790
6m Wt/L Z-Score	SumpercentMUFA2wks	0.8142242395295230	0.9991241851760790
6m Wt/L Z-Score	Sumpercentn3_2months	0.5606734230259130	0.9991241851760790
6m Wt/L Z-Score	Sumpercentn3_2wks	0.7917440343122080	0.9991241851760790
6m Wt/L Z-Score	Sumpercentn6_2months	0.48150073129446	0.9991241851760790
6m Wt/L Z-Score	Sumpercentn6_2wks	0.9386345171972910	0.9991241851760790
6m Wt/L Z-Score	SumpercentPUFA2months	0.4844109177212070	0.9991241851760790
6m Wt/L Z-Score	SumpercentPUFA2wks	0.9321255274104660	0.9991241851760790
6m Wt/L Z-Score	SumpercentSFA2months	0.37488300185336100	0.9991241851760790
6m Wt/L Z-Score	SumpercentSFA2wks	0.9023196044930730	0.9991241851760790
6m Wt/L Z-Score	SumtotalLCFA2months	0.6863738985868490	0.9991241851760790
6m Wt/L Z-Score	SumtotalLCFA2wks	0.10168279442692500	0.9991241851760790

Chapter 5 : Conclusion

Our findings provide invaluable insight into the effects of early life exposures on offspring health in mouse and human data across the early developmental windows of pregnancy and lactation. This chapter will provide a summary of the key findings, public health implications, and future work on areas that need further examining to provide a holistic understanding of the issues presented in this work and to improve the health of infants.

Results from Chapter 2 provided invaluable and novel insights on how maternal dexamethasone exposure affects offspring health *in utero* through looking at placental and fetal development, and postnatally. It is well established that the use of glucocorticoids in high-risk pregnancies is the ideal intervention to promote fetal lung maturation, prevent respiratory distress syndrome, and increase infant survival probability. However, it is also important to assess the adverse effects of these interventions on the short and long term to determine if dosage or timing of the treatment need to be altered to optimize infant health and reduce risk of morbidity and to identify infants most at risk of metabolic disease. In Chapter 2, the novel use of a placenta-specific glucocorticoid receptor knockout provides further knowledge on the sex-specific role of placental glucocorticoid receptor in mediating fetal survival. The results also shed light on how maternal glucocorticoids may affect offspring health through emerging mechanisms not directly involving the placental glucocorticoid receptor. Future work on the effects of maternal glucocorticoid exposure on offspring health should take into account offspring sex, as we noted

sex-specific differences that are not fully elucidated in the literature. Additionally, future work can examine the mechanisms by which glucocorticoids affect placental development, gene expression, and physiology in a sex-specific manner. This work is an important step forward to further our current knowledge on the impacts of antenatal glucocorticoid exposure.

From a public health perspective, the use of maternal glucocorticoids is common for various health conditions. However, elevated maternal glucocorticoids can also occur from stressful life events. Women who are subjected to institutional racism are at risk of delivering preterm infants and have a higher risk of infant mortality (Bower, Geller, Perrin, & Alhusen, 2018; Braveman et al., 2021). This disproportionate impact of stress warrants further examination to determine the best methods of intervention to promote infant health. It is also crucial to note that social determinants of health play a major role in predicting maternal and infant health, and hence, a holistic approach to maternal health should be thoroughly considered in clinical care.

As maternal obesity rates are rising, Chapter 3 explored the role of excess anabolic nutrient sensing in the mammary gland on lactation outcomes. The lactation window is a crucial window of development where offspring are heavily relying on maternal breast milk as a main source of nutrition. Results from Chapter 3 provided a novel approach to understanding the role of mammary gland lipids in altering the milk composition during lactation without the confounding variables associated with an underlying obese phenotype. This work showed that excess nutritional sensing in the mammary gland increased milk fat composition. However, the milk fat showed a healthier composition with lower saturated fatty acids and higher monounsaturated and omega-3 fatty acids. This increase in milk fat caused the offspring to be heavier during lactation.

Further analysis of the mammary gland histology showed that the excess nutrient sensing caused the mammary glands to be smaller but increased the size of the adipocytes within these mammary glands. This could contribute to the increased availability of fat stores surrounding the mammary gland that can contribute to the fat content in the expressed milk. Gene expression analysis showed no significant difference in lipogenic gene expression but an increased expression of downstream targets of PPARγ. However, it is important to note that the excess nutrient sensing was only in the adipocytes and not in the mamamry gland, making our goal to determine underlying mechanisms less unique to changes within the mammary gland epithelial cells and more of a reflection of the holistic changes within the mamamry gland and including adjacent adipocytes. Future work can aim to elucidate the mechanism by which excess nutrient sensing within the mamamry gland epithelial cell compratment may alter milk composition and offspring outcomes. Additionally, lactational programming can be assessed via a longitudinal study that considers offspring health throughout adulthood to determine the role of the lactational window in disease susceptibility and future health.

From a public health perspective, in women who have obesity, it is demonstrated that the breast milk consists of higher fat content (Leghi et al., 2020) which can affect infant health during lactation. Breastfeeding remains the gold standard for infant nutrition, but it is important to examine the role of maternal obesity, health, and diet on infant health. Additionally, almost 20% of infants receive infant formula within the first 2 days of life, and 46.9% of infants are exclusively breastfed within the first 3 months of life (“Breastfeeding Report Card | Breastfeeding | CDC,” n.d.). It is thus important to incorporate our findings into formula milk engineering to ensure optimal infant growth and availability of essential fatty acids that promote

a healthy start of life. In connection to Chapter 2, it is also estimated that infants born to mothers with elevated glucocorticoid levels due to racism and who are born preterm are less likely to breastfeed for the recommended time period (Hackman, Alligood-Percoco, Martin, Zhu, & Kjerulff, 2016; Johnson, Menke, Handelzalts, Green, & Muzik, 2021). Hence, understanding the role of milk components on infant health and growth is crucial in determining optimal early life nutritional composition.

As understanding the role of milk composition is crucial to ensure optimal early nutrition and a healthy lactational window, Chapter 4 aimed to examine the role of specific long-chain fatty acids in infant adiposity through two years of age. Results from Chapter 4 advance our knowledge in the field of lactation by exploring how certain fatty acids can alter infant adiposity. These results highlighted a positive correlation between levels of vaccenic acid in human milk and infant adiposity. However, to our knowledge, there are no studies that examine vaccenic acid and its role in infant fat deposition. Additionally, our results show a negative association between milk levels of docosahexaenoic acid and infant adiposity but a positive association between eicosapentaenoic acid and infant adiposity. In this Chapter, we further assess the associations between fatty acids and infant weight considering infant feeding methods and looking at exclusively breastfed infants. As the rates of formula feeding are increasing, it is crucial to delineate the role of breastmilk fatty acids and supplementary nutrition in infant health. Several associations showed a stronger association when accounting for exclusively breastfed infants, highlighting the confounding role of supplementary nutrition in determining infant health outcomes and possibly suggesting a dose dependent effect. However, our work does not consider infant dietary habits throughout the first two years of life or maternal dietary habits throughout

lactation, making a clear link between the maternal-milk-infant triad less likely to achieve an understanding of the underlying mechanisms. Additionally, recent work has highlighted differences in milk composition and volume based on infant sex. Future work can further determine if the associations between milk components and infant growth vary by the sex of the infant.

From a public health perspective, it is crucial to understand the growth trajectories of infants as they can be predictive of adult health. These findings provide crucial data to ensure formula milk components are matched with infant needs and ensure optimal and gradual infant growth. In Chapter 3, we similarly show that docosahexaenoic acid levels were higher in milk of dams that had excess nutrient sensing and had heavier offspring. As obesity rates are rising, it is also important to determine compositional differences in human milk by maternal health status to further understand the associations between milk components and infant health programming.

Taken together, the data presented in this dissertation demonstrate the important role of early life exposures and nutrition on offspring health. These findings include the role of excess glucocorticoids in offspring *in utero* resorptions, postnatal lethality, and postnatal low birth, the role of excess nutrient sensing in altering milk composition and increasing offspring weight, and the role of specific milk fatty acids in infant adiposity throughout the first two years of age. These findings are an important milestone to advance our understanding of the gestational and lactational windows of development and their role in shaping the health of the offspring. The work provided here also highlights the need for continued research in these areas and

breastfeeding policies to guide future nutritional and therapeutic guidelines and maternal recommendations regarding breastfeeding benefits to ensure optimal offspring health.

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