Mechanisms Of Vitamin A Delivery To Mammary Milk And Offspring In Mice

by

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Abstract

Background: Vitamin A status is an important health determinant. Deficiency of this micronutrient is a serious global health issue in the developing nations as the leading cause of preventable blindness in children and is a significant contributor to long-term morbidity and mortality. Nutritional demands for vitamin A are highest during pregnancy and lactation. This period is important for establishing offspring's vitamin A reserves critical for long-term health.

Aim: Our overall objective was to better understand the mechanisms underlying the delivery of vitamin A to maternal milk and offspring in mice. This included an analysis of: 1) maternal vitamin A homeostasis during lactation, with a focus on the mammary gland, and 2) genetic, and 3) dietary intervention studies designed to dissect the contribution of maternal vitamin A stores and dietary vitamin A intake in the establishment of vitamin A reserves in offspring.

Methods: All studies were conducted in mice. In Chapter 3, the mammary gland and other tissues were collected from virgin, lactating and involuting mice, followed by an assessment of gene expression (qPCR) and tissue vitamin A concentrations (HPLC). In Chapter 4, wild-type pregnant mice were split into two groups, one receiving a diet with 25 IU vitamin A/g, and one receiving a vitamin A deficient diet (0 IU vitamin A/g), during pregnancy and lactation. Tissues were collected from offspring at (postnatal day [P]1), during lactation (P7), at weaning (P21), two weeks post-weaning (P35), P70 and P105 with vitamin A concentrations measured by HPLC. In Chapter 5, tissues were collected from the offspring of wild-type and *Lrat*$^{-/-}$ female mice at P1, P7 and P21 vitamin A concentrations were measured (HPLC).

Results: In Chapter 3 we observed a shift in the expression pattern of the vitamin A metabolic pathway in the mammary gland of lactating mice. Most prominently, there was a significant

increase in the expression of the retinyl ester-synthesizing enzyme *Lrat* during lactation, which returned to baseline following involution. In Chapter 4, our results show a direct relationship between maternal vitamin A intake and offspring liver retinol and retinyl ester reserves. Offspring from dams consuming a vitamin A deficient diet had consistently lower hepatic vitamin A levels than control at P7, P21, and P35, which returned to control group concentrations at P70 (adulthood). In Chapter 5, we observed no significant differences in the hepatic vitamin A status of heterozygous offspring born to wild-type and $Lrat^{-/-}$ female, despite $Lrat^{-/-}$ female lacking hepatic vitamin A stores.

Conclusion: We have characterized pronounced changes in the vitamin A metabolic pathway in the mammary gland of lactating mice, consistent with the role of LRAT as the primary enzyme responsible for retinyl ester incorporation into milk. Our study further supports the hypothesis that maternal dietary vitamin A intake is critical in establishing adequate vitamin A reserves in offspring and suggests that maternal hepatic stores cannot compensate for a lack of maternal dietary vitamin A. Moreover, vitamin A sufficient pups accumulate hepatic vitamin A stores with time, which is hindered in pups with dams on a vitamin A deficient diet and persists until early adulthood despite the introduction of adequate dietary intake after weaning.

Preface

This thesis is an original work by Jenna Richardson. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Experimental Manipulation of Vitamin A Homeostasis”, No. 2966. Some of the data collected in chapters 3 and 4 was conducted previously by Annette Sanchez-Enkerlin at the University of Alberta. General technical support and data collection for this thesis were assisted by Sam Kinney. No part of this thesis has been previously published.

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List of Abbreviations

Adequate Intake (AI)

American Institute of Nutrition (AIN)

Aldehyde dehydrogenase (RALDH)

Beta-carotene (BC)

β-carotene-15,15′-monooxygenase (BCMO1)

Casein gamma (CSNG)

Cellular retinol binding protein (CRBP)

Cellular retinoic acid binding protein (CRABP)

Chylomicron (CM)

Cluster of differentiation 36 or fatty acid translocase (CD36)

Cytochrome P450 family 26 subfamily A member 1 (Cyp26a1)

Cytochrome P450 family 26 subfamily B member 1 (Cyp26b1)

Dehydrogenase/reductase 3 (Dhrs3)

Developmental Origins of Health and Disease (DOHaD)

Diglyceride acyltransferase (DGAT)

Dietary Reference Intakes (DRIs)

Enzyme-linked immunosorbent assay (ELISA)

Estimated Average Requirement (EAR)

Hepatic stellate cell (HSC)

High Efficiency Particulate Air (HEPA)

High Performance Liquid Chromatography (HPLC)

Lactalbumin alpha (LALBA)

Lecithin retinol acyl transferase (LRAT)

Lipoprotein lipase (LPL)

Low-density lipoprotein (LDL)

LDL-related protein (LRP)

Long-chain fatty acid (LCFA)

Phosphate-buffered saline (PBS)

Postnatal day (P)

Quantitative Polymerase Chain Reaction (qPCR)

Recommended Dietary Allowances (RDAs)

Retinal (RAL)

Retinaldehyde dehydrogenase (Raldh)

Retinoid X receptor (RXR)

Retinoic acid (RA)

Retinoic acid receptor (RAR)

Retinoic acid response element (RARE)

Retinol (ROL)

Retinol activity equivalent (RAE)

Retinol binding protein 4 (RBP4)

Retinol binding protein receptor 2 (RBPR2)

Retinol dehydrogenase (RDH)

Retinyl ester hydrolase (REH)

Retinyl esters (RE)

Revolutions per minute (RPM)

Signaling receptor and transporter of retinol (STRA6)

Tolerable Upper Intake Level (UL)

Transthyretin (TTR)

Visceral white adipose tissue (VWAT)

World Health Organization (WHO)

Whey acidic protein (WAP)

CHAPTER 1 - General

Introduction

1.1 Vitamin A

Vitamin A is an essential liposoluble micronutrient required for many physiological processes, including embryonic development and various postnatal physiological events (1–3). Commonly defined as all-trans-retinol, vitamin A belongs to a family of molecules called retinoids, which are individually named according to their terminal functional group. This includes retinol and its various metabolites, as well as its storage form; retinyl ester and its active forms; retinaldehyde and retinoic acid (Figure 1.1). Retinol and retinyl esters are highly abundant, making up the largest quantity of retinoids in the body, whereas retinoic acid is tightly regulated in concentrations 100 to 1,000 times lower (2,4–6). This is largely due to its role as an intracellular messenger, which elicits the transcriptional regulation of over 500 genes (1,2,7). Humans and other mammals are unable to produce vitamin A de novo (2,4,8,9), therefore dietary intake of vitamin A is required, which is taken up by intestinal enterocytes to be stored and metabolized by various tissues, such as the liver, visceral white adipose tissue, lung, and mammary gland (2,5,10). Due to the extensive influence of vitamin A on various physiological processes, it is important to recognize that both minimal and excessive reserves of vitamin A can cause adverse health outcomes, and therefore concentrations of this micronutrient must be optimally regulated.

1.1.1 Dietary Sources of Vitamin A

Dietary vitamin A is available in foods in two prominent forms: either as preformed retinoids or as provitamin A carotenoids, both of which can be hydrolyzed endogenously to form retinol following absorption (2,9,11,12). Preformed retinoids are comprised of long-chain fatty acid retinyl esters, commonly found in animal products (largely retinyl palmitate) such as, organ meats (particularly liver), eggs, dairy products, and fatty fish.

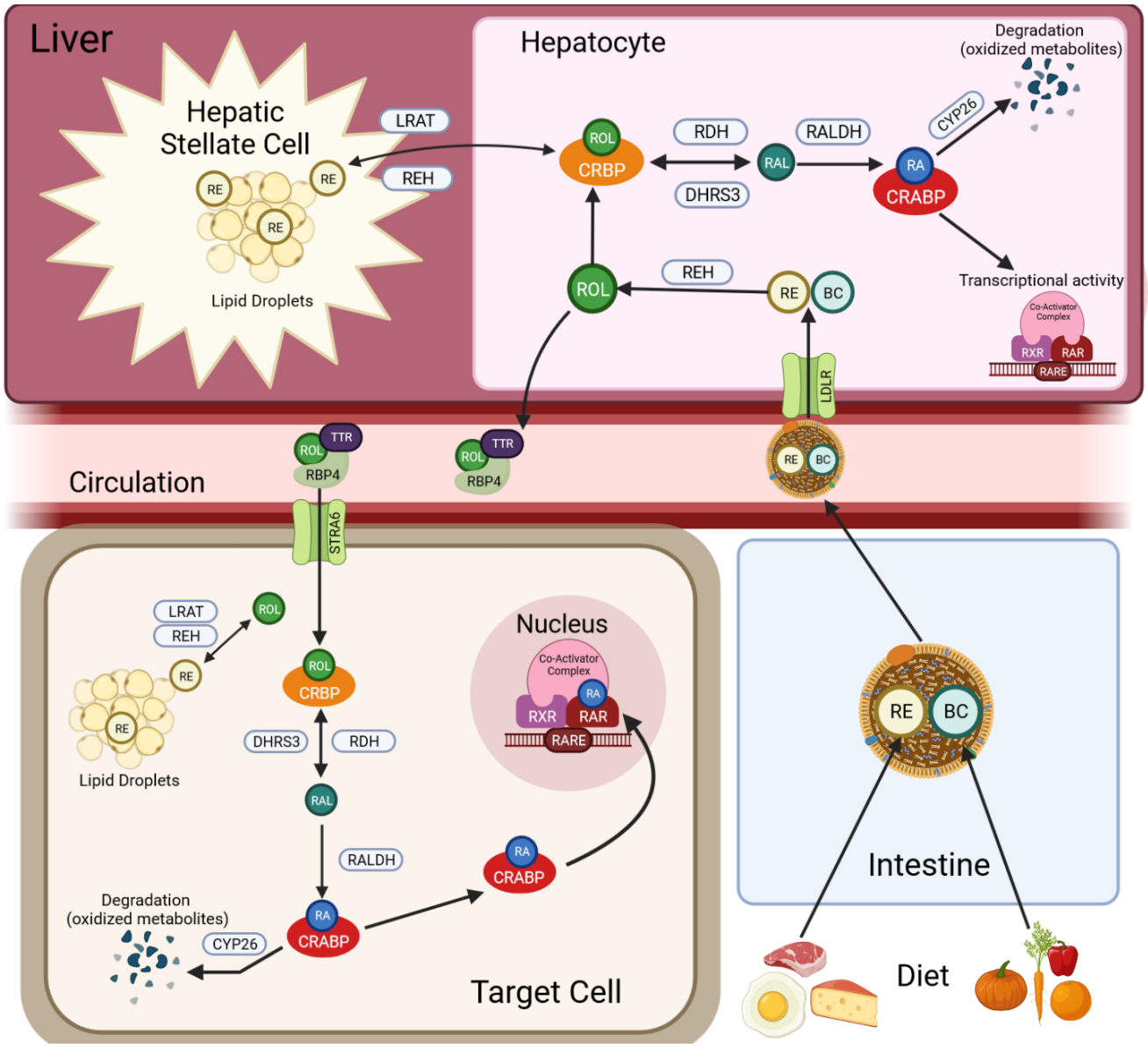


Figure 1.1 Schematic representation of vitamin A storage, mobilization, and metabolism. After ingestion from the diet, carotenes (BC; from plant products) and retinyl esters (RE; from animal products) are packed into chylomicrons (CM) for transport into the lymph and blood. Once chylomicron RE and BC are internalized by the hepatocytes and hydrolyzed to form retinol (ROL), they can 1) be stored in hepatic stellate cells (HSC) as RE in lipid droplets, 2) undergo metabolism into retinal (RAL) and subsequently retinoic acid (RA) for transcriptional activity or degradation, or 3) become re-mobilized in the circulation bound to the carrier protein retinol binding protein 4 (RBP4) and transthyretin (TTR). This circulating ROL may then be utilized by a variety of target tissues, including the mammary gland. See main text for details regarding the mechanisms involved in each pathway. Created with BioRender.com.

Provitamin A carotenoids are predominantly found in dark leafy greens and colorful fruits and vegetables (specifically those with a yellow to orange hue) such as spinach, carrots, broccoli, sweet potato, mangos, apricots, pumpkin, etc. (2,11,13,14). It is important to note that not all carotenoids exhibit properties conducive to vitamin A activity. In fact, over 750 carotenoids have been identified in nature, yet only 10% are estimated to be metabolically cleaved into retinol (9,11,15,16). Of these, β-carotene is the most abundant.

At physiological concentrations, efficiency of vitamin A absorption in a healthy individual spans 70 to 90% in those with substantial fat consumption (11,17). Although both sources of vitamin A are essential to the global population, bioavailability of vitamin A is higher in preformed retinoids, which have a conversion factor of 1 μg retinyl ester:1 μg of all-*trans*-retinol (11,16,18,19). Despite the ability for β-carotene to be cleaved into two molecules of vitamin A, it is rather estimated to have a conversion factor of 6 μg β-carotene to 1 μg of all-*trans*-retinol due to its reduced efficiency of absorption. However, recent findings emphasize this to be a gross underestimate for mixed diets of provitamin A carotenoids, suggesting an approximate 21:1 ratio (12:1 for fruits and 26:1 for vegetables), which is more reflective of other considerations that may reduce conversion efficiency (18).

Economic, geographical and cultural influences all serve as determinants for a population's primary dietary source of vitamin A (9,20–22). In westernized countries, where ingestion of fortified and animal-based products is high, preformed retinoids make up 75% of consumed vitamin A. In developing countries, 70–90% of vitamin A is utilized by provitamin A carotenoids, thereby increasing the risk of vitamin A deficiency because a higher quantity of provitamin A carotenoids is required to meet daily vitamin A requirements. Other factors that influence vitamin A bioavailability include the prevalence of infections and parasites, intestinal

or liver disease, iron and zinc status, dietary fat and protein consumption, and alcohol intake (9,16,22,23).

1.1.2 Dietary Vitamin A Requirements

As a liposoluble micronutrient, vitamin A homeostasis is strictly maintained to support numerous physiological functions, hence too much or too little dietary vitamin A intake can be detrimental to the development and maintenance of many physiological processes in the body. Dietary Reference Intakes (DRIs) were therefore established by the Food and Nutrition Board (National Academies of Sciences) to provide a set of reference recommendations for vitamin A intakes of healthy individuals of a diverse range of ages and sexes (16). Retinol activity equivalent (RAE) is currently the preferred unit to express vitamin A requirements because it accounts for the specific bioactivities of both retinyl esters and provitamin A carotenoids (9,16). As such, 1 μg of RAE = 1 μg of all-*trans*-retinol and is equivalent to 3.33 IU vitamin A. On average, the Recommended Dietary Allowances (RDAs) of preformed vitamin A for males and females are 900 and 700 μg RAE/day, respectively, with a Tolerable Upper Intake Level (UL) of 3,000 μg RAE/day (9,16,24). During pregnancy and lactation, the nutritional demand of mothers increases, requiring 750 μg of RAE/day and 1300 μg of RAE/day, respectively. In children, vitamin A requirements vary according to age and sex, ranging from 300–600 μg RAE/day (Table 1.1). Current vitamin A intake guidelines in infants are based on the assumption that they are exclusively breastfed by a well-nourished and healthy mother (24,25). As such, it is estimated that 400 μg RAE /day for infants 0-6 months and 500 μg RAE /day for infants 7-12 months is adequate.

AGE	Estimated Average Requirement (EAR)[1]	Recommended Dietary Allowance (RDA)[2]	Tolerable Upper Intake Level (UL)[3]
Infants			
0-6 months	NA	400 RDA	600 UL
7-12 months	NA	500 RDA	600 UL
Children			
1-3 years	210 EAR,	300 RDA	600 UL
4-8 years	275 EAR	400 RDA	900 UL
Males			
9-13 years	445 EAR	600 RDA	1700 UL
14-18 years	630 EAR	900 RDA	2800 UL
19-30 years	625 EAR	900 RDA	3000 UL
31-50 years	625 EAR	900 RDA	3000 UL
51-70 years	625 EAR	900 RDA	3000 UL
>70 years	625 EAR	900 RDA	3000 UL
Females			
9-13 years	420 EAR	600 RDA	1700 UL
14-18 years	458 EAR	700 RDA	2800 UL
19-30 years	500 EAR	700 RDA	3000 UL
31-50 years	500 EAR	700 RDA	3000 UL
51-70 years	500 EAR	700 RDA	3000 UL

>70 years	500 EAR	700 RDA	3000 UL
Pregnancy			
<18 years	530 EAR	750 RDA	2800 UL
19-30 years	550 EAR	770 RDA	3000 UL
31-50 years	550 EAR	770 RDA	3000 UL
Lactation			
<18 years	885 EAR	1200 RDA	2800 UL
19-30 years	900 EAR	1300 RDA	3000 UL
31-50 years	900 EAR	1300 RDA	3000 UL

[1]Estimated Average Requirement (EAR): mean daily vitamin A intake estimated to meet the requirements of 50% of healthy individuals (used largely to assess groups of people)
[2]Recommended Dietary Allowance (RDA): mean daily vitamin A intake sufficient to meet the requirements of 97%–98% of all healthy individuals.
[3]Tolerable Upper Intake Level (UL): maximum daily intake of vitamin A unlikely to cause adverse health effects.

Table 1.1 A summary of the Health Canada Dietary Reference Intakes (2010). This is adapted from the Canada Health Dietary Reference Intakes article (24) available from https://www.canada.ca/en/health-canada/services/food-nutrition/healthy-eating/dietary-reference-intakes/tables/reference-values-vitamins-dietary-reference-intakes-tables-2005.html.

This is based on evidence suggesting that infants ingest a mean of 0.78 L breast milk containing 485 μg/L of retinol per day and 0.60 L of breast milk containing 291 μg/L of retinol in addition to 244 μg of retinol from physical food sources per day, respectively. Although these recommended dietary allowances are intended to fulfill the vitamin A requirements of virtually all infants, in populations where dietary vitamin A absorption is impaired (ex. a high prevalence of infections) they may not suffice. There are no well-defined vitamin A intake recommendations for provitamin A carotenoids for any age or population group (9,24,26).

1.1.3 Mammary Milk Composition

During the first six months of life, mammary milk from breast feeding is exclusively recognized as the optimal nutritional source for infants and is continually encouraged for upwards of one to two years of age as the most ideal feeding practice (27–30). This is because human milk is highly specified to infant nutritive, immunological, and developmental requirements for promoting proper health and overall survival. As such, mammary milk has a unique and complex composition which is not homogeneous between individuals, populations of mothers, the stage of lactation, and time of the day (27). Mammary milk is composed of a diversity of macronutrients, micronutrients, growth factors, oligosaccharides, immunoglobulins, lysozymes, cytokines, hormones, etc., which originate from maternal dietary intake, stores, and lactocyte nutrient production. Oligosaccharides (primarily lactose), proteins and fats make up the primary macronutrient component of mature human mammary milk, with a mean composition of 66 to 78 g/L lactose, 32 to 36 g/L protein, and 32 to 36 g/L fat, respectively (31–33). Unlike human studies, there is very limited published research regarding mouse milk composition because of its ability to vary considerably between strains and challenging analysis due to its limited sample

volume (34,35). It is, however, understood that mice have a much higher fat concentration in mammary milk, which increases throughout lactation (in addition to lactose content), while protein levels remain largely unchanged (34,36,37).

In humans, high concentrations of retinoids are prevalent in breast milk to aid in the development of offspring vitamin A reserves, which vary according to the milk fat content, dietary source of vitamin A (retinyl esters versus provitamin A carotenoids) and vitamin A status of the mother (11,25). Almost exclusively, retinyl palmitate and retinyl stearate (long-chain fatty acid retinyl esters) make up the vitamin A proportion present in the lipid fraction of breast milk, contributing to over 60-80% of the retinoid content (9,11,38). The exact lactational proportions of vitamin A originating from dietary chylomicrons versus maternal hepatic stores (retinol-RBP4) has been highly contested, although it is accepted that both pathways are important in establishing vitamin A content in mammary milk. Additionally, depending on the stage of lactation, retinoid content (and overall milk composition) will differ considerably to accommodate offspring requirements (25).

Vitamin A concentration is highest in the colostrum, which is the initial nutritional source for newborns (first stage of lactation) produced for the first 4 ± 6 days postpartum (9,11,27). Despite its lower quantity and reduced fat and lactose content, colostrum is rich in various immunological and developmental factors, and contains enhanced concentrations of proteins, vitamin A, B12, and K (9,27). In healthy, nourished mothers, human colostrum contains 1510 μg vitamin A/L (25,39), which quickly declines and plateaus to less than half its original amount after one month. As such, the colostrum allows newborns to rapidly develop vitamin A reserves after limited gestational transplacental transfer (9). Therefore, in populations where discarding colostrum is commonplace (usually due to its thick yellow appearance), incidence of childhood

vitamin A deficiency may be elevated. Transitional milk is secreted during the second stage of lactation, at 7 ± 21 days postpartum (25,34). During this period, breast milk production significantly elevates and undergoes extensive alterations in composition, as it transitions from the colostrum into mature milk (11,27). On average, human transitional milk contains 880 μg vitamin A/L, which is elevated in comparison to mature milk but less than the colostrum (11,25,39). By one month postpartum, milk concentrations plateau at 750 μg vitamin A/L and remain constant for the remainder of the lactational period. This third and final stage of lactation is defined as mature milk, which begins at approximately 15 days after birth and reaches full maturity by 4 weeks (25,27).

In pregnant and breastfeeding mothers, dietary intake plays an important role in meeting the nutritional demands of the infant and has been shown to have a strong correlation to the vitamin A content secreted in breast milk (9). It has been reported that malnourished mothers and dams have altered compositions of protein, fat, water, and nutrient composition which may hinder perinatal growth (40–43). As such, vitamin A in mature milk is known to reduce to less than half its quantity in un-supplemented mothers from low vitamin A environments (largely developing countries) compared to those with adequate vitamin A intake (25,40). This is emphasized by the accepted default value of 300 μg vitamin A /L of in the milk from inadequately nourished mothers without intervention efforts, compared to 700-750 μg vitamin A /L of from properly nourished mothers (25,34,44). Furthermore, vitamin A concentration in milk is positively correlated with the fat content of the milk. Therefore, vitamin A varies over the course of the day (greatest in the mid-morning when fat content in milk is high), and in each individual feeding period, where it appears greatest towards the end of the feed and least at the beginning of a feed when distribution of fat in the milk is highest and lowest, respectively (23,34). Due to this

positive relationship between dietary vitamin A, fat intake, and breast milk vitamin A concentration, it is highly advised that breastfeeding mothers ensure their diet includes adequate proportions of both fat content and vitamin A, for optimal vitamin A secretion to offspring.

1.1.4 Vitamin A Storage, Mobilization and Metabolism

Upon ingestion of a vitamin A rich meal, dietary preformed retinoids (largely retinyl esters) are taken up by the lumen of the small intestine and hydrolyzed at the intestinal brush boarder by retinyl ester hydrolase to yield free retinol (4,6,9). Once in its free retinol state, vitamin A can enter the enterocytes by utilizing both passive diffusion and carrier mediated processes. In contrast, provitamin A dietary carotenoids directly cross into the epithelial cells by utilizing membrane bound transporters, then are absorbed in their original form or oxidized into retinal by utilizing the enzyme β-carotene-15,15′-monooxygenase (BCMO1) (4,9,45). With the assistance of CRBP2 (cellular retinol binding protein 2; an intracellular carrier protein), retinal is reduced into retinol by retinal reductase activity. This retinol, along with the preformed free retinol, are re-esterified by lecithin retinol acyl transferase (LRAT) with long chain fatty acids for incorporation into chylomicrons as retinyl ester (4,6,9,46). Retinyl ester containing chylomicrons are then secreted into the lymphatic system and taken into the general circulation where they undergo remodeling into chylomicron remnants via hydrolysis by lipoprotein lipase (LPL) (2,47). These remnants are taken up by various tissues, with the most prominent being the liver (6). This process of endocytosis is facilitated by the low-density lipoprotein (LDL) receptor, or via the LDL-related protein (LRP) (2,13). Once the vitamin A is in liver hepatocytes, dietary retinyl ester is hydrolyzed and bound to carrier protein CRBPI (cellular retinol binding protein 1) as retinol, where it is either 1) transported to the hepatic stellate cells (HSC) for vitamin A

storage as retinyl ester, 2) bound to retinol binding protein 4 (RBP4) and released back into the circulation for transfer to extrahepatic tissues or 3) catabolized into retinoic acid for transcriptional regulation (Figure 1.1) (2,13,48,49).

1) **Hepatic Stellate Cell Vitamin A Storage**: It is widely accepted that the liver is the predominant storage site of vitamin A, with approximately 70 to 85% of the body's retinoids found in this organ (2,4). More specifically, these retinoids are stored in HSC lipid droplets as retinyl ester. The primary retinyl ester found in HSCs is retinyl palmitate, with fractions of retinyl linoleate, retinyl oleate, and retinyl stearate. Although it is known that LRAT enzymatic activity is required for the esterification of retinol into retinyl ester within the HSCs, the molecular processes of transfer for retinol from the hepatocytes into the hepatic stellate cell lipid droplets is undetermined and poorly characterized. It is only certain that retinyl ester must be hydrolyzed into retinol in the hepatocytes prior to its transportation into the HSC (50).

2) **Vitamin A Mobilization by RBP4:** When retinoids are required in the circulation, or for extrahepatic tissues, HSC retinyl ester reserves are utilized to produce free retinol via retinyl ester hydrolase (REH) (2,4,9). This free retinol is then mobilized from the liver as the complex all-*trans*-retinol–RBP4 (holo-RBP4) and secreted into the circulation for utilization by peripheral tissues. RBP4 is a plasma transport protein predominantly synthesized at the endoplasmic reticulum within the hepatocytes of the liver. Moreover, to prevent glomerular filtration of retinol by the kidneys, the retinol-RBP4 complex binds to the protein transthyretin (TTR) within the hepatocyte (51).

3) **Retinoic Acid Metabolism and Gene Transcription:** At extrahepatic cells, cell surface receptor, STRA6 (signaling receptor and transporter of retinol) interacts with RBP4 to facilitate both the cellular uptake and efflux of retinol (52,53). Intracellular retinol is then oxidized through the retinoid metabolic pathway, where it is converted into its active products: retinaldehyde and retinoic acid. The oxidation of retinol into retinaldehyde (retinal) is a reversible reaction requiring the enzymatic activity of the retinol dehydrogenase (RDH) family and aid of cellular binding protein CRBP (1,9,54). Retinoic acid is formed when retinal is further oxidized irreversibly by retinaldehyde dehydrogenase (RALDH) and subsequently bound to cellular retinoic acid binding protein (CRABP) for enhanced mobilization to its target receptors. Retinoic acid elicits its transcriptional properties through the nuclear receptors; retinoic acid receptor (RAR) and retinoid X receptor (RXR). When retinoic acid binds to RAR, these receptors heterodimerize and bind to the retinoic acid response element (RARE), which is found at the promoter region of genes of interest. This allows retinoic acid to transcriptionally modulate gene expression of over 532 genes; 27 of which are identified to be directly controlled through the classical retinoic acid pathway and the remaining for intermediary gene expression for an indirect target (1). Alternatively, if retinoic acid is not required for transcriptional activity, it is further oxidized via isoforms of CYP26 mono-oxygenase, into polar metabolites for excretion (55,56).

1.2 Vitamin A Deficiency

According to the World Health Organization (WHO), vitamin A deficiency is one of the most common nutritional disorders worldwide and is regarded as a public health issue in 50% of

countries – particularly the developing world (20). The examination of circulating concentrations of plasma retinol is the primary means for measuring vitamin A deficiency in individuals, however, this methodology is limited because retinol concentrations are tightly regulated to a maintain a constant homeostatic balance in the plasma (3,20,57). As a result, its sensitivity is restricted to only severely deficient amounts of vitamin A and thus is not a reliable indicator of individual vitamin A status (3,11). Nonetheless, measuring circulating levels of vitamin A is valuable for estimating the incidence of vitamin A deficiency in larger populations, where a cut-off of < 0.7 μmol/L is indicative of vitamin A deficiency and concentrations < 0.35 μmol/L are suggestive of severe vitamin A deficiency (57–59). Because vitamin A deficiency is uniquely associated with a large spectrum of ocular disorders known as xerophthalmia, vision diseases/impairments serve as an important clinical indicator of minimal vitamin A content in individuals (20). Consuming a poor vitamin A diet is the main cause of vitamin A deficiency, which can be exacerbated by poor absorption due to parasites and infection (ex. diarrhea and measles), intestinal or liver diseases, status of iron and zinc, amount of dietary fat, alcohol intake, and food processing (20,60–63). Societal factors such as economic constraints, sociocultural limitations, and geographical features also contribute to the source and quantity of vitamin A received in the diet, naturally predisposing developing nations to an increased risk of vitamin A deficiency (3,64).

The influence of vitamin A in regulating various physiological systems has been extensively examined, shedding light on the importance of this micronutrient. Vitamin A status is an important health determinant as vitamin A deficiency is associated with a higher incidence of morbidity and mortality due to its adverse effects on vital physiological processes throughout all stages of life (3,14,65,66). As such, vitamin A deficiency induces degeneration of the eye

through the keratinization of a conjunctival and corneal structures, which ranges from night blindness and Bitot's spots, to corneal xerosis, ulceration, and necrosis – resulting in blindness (64,67). Vitamin A is also critical for the maintenance, growth, and differentiation of epithelial cells in mucosal tissues. Therefore, in the gastrointestinal tract, keratinization and dysregulation of mucosal membranes is prominent and brush border enzyme activity is hindered in vitamin A deficiency (63,66,68–70). This damage to protective mucosal epithelium subsequently exacerbates inflammation and increases the precipitation of infectious diseases which may result in reduced intake, absorption, and metabolism of vitamin A and other essential nutrients (namely iron – resulting in anemia) (3,66,71). Likewise, vitamin A deficiency diminishes the immune system by affecting macrophage, neutrophil, and natural killer cell function and significantly impairs the adaptive immune response by compromising Th2 cell and B-cell antibody-mediated responses, while supporting inflammatory T cell reactions (66,72). Furthermore, vitamin A deficiency disrupts respiratory function by inducing histopathological changes in the pulmonary epithelial lining of the lung, prompting severe tissue dysregulation, and increasing risk of death from infections (63,66). Taken together, these and additional clinical manifestations of vitamin A deficiency underlie the consequences of insufficient dietary vitamin A intake and its significance as a serious threat to global health.

1.2.1 Perinatal and Childhood Vitamin A Deficiency

Vitamin A deficiency is largely prevalent during stages of life where nutritional demands are higher, such as pregnancy, lactation, and early childhood development (3,9,65). Despite this, pregnant and lactating women exhibit the highest risk of vitamin A deficiency, resulting in around one-third of preschool aged infants < 5 years considered sub-clinically vitamin A

deficient and is attributed to 2% of all deaths at this age (73,74). The World Health Organization (WHO) emphasizes the global impact of childhood vitamin A deficiency by estimating 45 and 122 countries experience moderate and severe public health outcomes from vitamin A deficiency, respectively (22). Current data suggests this childhood prevalence of vitamin A deficiency persists at high rates in developing countries, namely in sub-Saharan Africa (48%) and South Asia (44%), while it has moderately improved in East Asian populations (3,20,22,74–77). Moreover, it is consistently observed that 10-20% of pregnant woman exhibit vitamin A deficiency within these settings (3,76,78). Elevated incidence of vitamin A deficiency among infants, children, and pregnant women exacerbates morbidity and mortality rates, especially in countries that are commonly impacted by infectious diseases like measles, diarrhea, and malaria (22,79,80). In 2013, vitamin A deficiency was credited to over 11,200 deaths from measles and 94,000 deaths from diarrhoea, accounting for 1.7% of deaths in children 6-59 months old (81). Although prominent in developing nations, vitamin A deficiency is not exclusive to these populations and has been shown to have a sub-clinical effect in developed countries, including those in North America. According to the most recent National Health and Nutrition Examination Survey (NHANES) performed in the United States, 31% of American pregnant women do not meet their estimated average requirement of vitamin A. Despite 78% utilizing supplements, 18% remain below the estimated average requirement, which is exacerbated in those with poor dairy ingestion (82). Within Canada, perinatal vitamin A deficiency disproportionately affects indigenous and minority communities (83). It has been observed that the average vitamin A intake and plasma concentration of Indigenous (First Nations and Inuit) mothers is considerably lower compared to non-Indigenous mothers, with a significantly elevated risk of vitamin A deficiency without supplementation (83). Subsequently, infants born

from Indigenous mothers had a plasma retinol concentration substantially reduced in comparison to infants from Caucasian mothers, and overall increased risk of sub-clinical vitamin A deficiency.

During the nine months of gestation, mothers tend to the nutritional needs of the fetus through transplacental transfer, which is tightly regulated to enable proper embryonic development (9,27,84). However, in cases of maternal malnutrition, fetal growth and placental development is compromised, resulting in reduced maternal-fetal exchange of vitamin A (40,85). Breastfeeding mothers also have a greater susceptibility for vitamin A deficiency due to their higher nutritional demand as the neonate utilizes their vitamin A reserves through the mammary milk to establish their own vitamin A stores (9). In the event of milk retinol concentrations ≤ 1.0 μmol vitamin A/L or ≤ 300 μg vitamin A/L resulting from poor maternal vitamin A status, infants are expected to become sub clinically vitamin A deficient within the first 6 months of life and have the potential to fall under the critical requirement of 0.018 μmol vitamin A/g for basic physiological needs (11,86–88). This is further exacerbated by sub-optimal breastfeeding practices and colostrum disposal, which deprives infants of their first rich source of vitamin A (9,27).

Vitamin A exerts an important role during embryonic development and postnatal physiological events including cell differentiation and proliferation, lung function and maturation of alveoli, establishment of immune function, ocular function, reproduction, and maintenance of epithelial development, integrity and growth (63,66,89–91). Vitamin A's criticality in these functions is heightened by the fact that deficiency during embryogenesis and the first few months of life may not be compensated in the postnatal period and is associated with preterm birth (third trimester), organ malformations, fetal resorption and reduced neonatal reserves (84,92). During

this period vitamin A is critical for neural differentiation during somitogenesis, molecular patterning and spinogenesis of the prefrontal and motor cortex, and proper cardiovascular, renal, facial and diaphragmatic development (3,90,93–95). It is still uncertain if the impact of vitamin A deficiency during gestation is attributed to dysregulation of placental function (with subsequent effects on the fetus), or through direct effects on the fetus itself (84). Postpartum, vitamin A deficiency is known to be the leading cause of preventable blindness in children worldwide (xerophthalmia), which is a significant contributor to long term morbidity and mortality in preschool aged children (73,96). Given the necessity of vitamin A for the development of all cell types, vitamin A deficiency is also known to exert negative effects on health lasting later in life by limiting the secretion of nocturnal growth hormone – therefore stunting growth in individuals (97) and has a significant influence in delaying puberty and dysregulating both male and female reproductive functions (98–100).

1.3 Supplementation Programs and Interventions for Vitamin A Deficiency

Numerous approaches have been implemented to limit and treat global vitamin A deficiency, including periodic supplementation with high-dose capsules, dietary diversification to increase vitamin A rich food intake, and the incorporation of micronutrient powders and fortifying staple foods with vitamin A during processing (14). In populations where vitamin A deficiency is common, UNICEF estimates that these intervention programs save over 350,000 children's lives annually (67). Consequently, high-dose supplementation programs and fortification of staple food products are the most pervasive interventions and are the predominant strategies recommended by the WHO to improve vitamin A status in the global population (101–104).

Several human and animal studies have aimed to elucidate the impact of high-dose supplementation programs in enhancing vitamin A reserves in mothers and babies at risk of vitamin A deficiency. These programs are primarily implemented ≥ 8-12 weeks postpartum due to the risk of teratogenicity (by exceeding 10,000 IU of vitamin A) during embryogenesis (105). In animal studies, lactational vitamin A supplementation programs have indicated that while single high dose administration of vitamin A in dams within the first month postpartum is readily acquired in pups, it is not effectively sustained in their peripheral tissues (106,107). Likewise, high dose vitamin A supplementation trials in lactating human mothers from Bangladesh (108–110), Indonesia (110), and India (111) indicate improved breast milk vitamin A status and a transient enhancement of vitamin A status of both mothers and breast-fed infants.

Aside from postnatal maternal high-dose supplementation programs, direct supplementation of children 6–59 months, every 6 months with high-dose vitamin A capsules, is implemented through national campaigns in more than 80 countries in response to childhood vitamin A deficiency mortalities (25,73,77,112). Despite both human maternal and childhood trials offering a protective dose against vitamin A deficiency, it is uncertain if this intervention alone will be successful in meeting chronic vitamin A requirements, due to its temporary effect on serum retinol concentrations (77). Complementary frequent food-based supplementation approaches (ex. vitamin A biofortification initiatives) as well as enhanced dietary diversity (rich in preformed vitamin A) and promotion of nutritional education may therefore be essential to sustain adequate vitamin A status for neonatal development (14,73,113).

Biofortification is an effective and low-cost process which improves the nutritional value of centrally processed foods and crops (via plant breeding or recombinant DNA technology) to mass deliver low quantities of vitamin A to broad populations (102,113,114). Prioritized staple

foods for fortification by national nutritional programs include: milk and milk products, vegetable oil and hydrogenated oil products (Morocco and Philippines), maize flour (Venezuela, South Africa), refined sugars (Zambia and several Central American countries), and wheat flour, cereal flours and rice (Philippines, North America) (14,102,113,113,115). In populations where biofortified foods are poorly accepted, modifying traditional dishes to include more provitamin A sources is a promoted approach (102). Moreover, multivitamin trials by Sânzio Gurgel *et al.* (116) and the implementation of the Healthy Foods North Inuit and Inuvialuit community-based intervention program by Pakseresht *et al.* (117), indicate the potential success of multivitamin supplements and culturally appropriate educational awareness programs, respectively, in reducing the risk of vitamin A deficiency in infants.

Investigations into the means of supplementation have alluded to which mode of transfer is most beneficial to offspring vitamin A status and retinol kinetics (25,107). This involves direct dosage to newborn humans versus indirect dosage of enhanced dietary vitamin A intake by mothers shortly after delivery. While both are observed to improve the vitamin A status of offspring, indirect maternal supplementation during lactation depicts a gradual and sustained effect, contrary to the dramatic yet transient influence of direct pup supplementation. As a result, interventions aimed at improving maternal vitamin A status during lactation and promoting optimal and exclusive breastfeeding practices may be the most ideal means of improving the vitamin A status of offspring, (10,25,107,113). However, in cases where offspring suffer severe vitamin A deficiency and/or breastfeeding practices are partial or inadequate, combining direct high dose supplementation and food fortification methods may be necessary to extend to those not sufficiently covered by breastfeeding alone and should be further explored. As such, future chronic low dose versus transient high-dose studies are required to better grasp the degree which

vitamin A dosing successfully elevates liver status above deficient levels to act as a buffer for vitamin A depletion (59).

1.4 Establishment of Vitamin A reserves in Offspring

There are two ways in which vitamin A reserves are established in newborns; by transplacental transfer and through breast milk secretion (40,65,118,119). During the gestational period the nutritional demands of the fetus are met by maternal retinol transfer through the transplacental route (9,27,120). It has been observed that this transport process is tightly regulated and largely prioritized during the late stages of pregnancy (2). Despite this, the development of neonatal vitamin A reserves through transplacental transfer are quite limited, amassing only 0–0.34 μmol/g in the liver of typical birth weight babies, which can be easily depleted in the postnatal period (27,30,61,121). Therefore, during the lactational period, early initiation of breastfeeding is crucial for the establishment of infant vitamin A stores, which are reliant on maternal vitamin A intake stored in the mammary breast milk (11,25). Infants exclusively breastfed for the first 6 months of life by mothers with optimal vitamin A intake are known to accumulate around 310 μmol of hepatic vitamin A, which is 90-fold higher than that of the transplacental pathway (18,25,122,123). As a result, analysis of breast milk vitamin A is commonly used as a metric to determine the extent of vitamin A deficiency within a given population of lactating women and their infants (11,76).

In addition to the two ways in which vitamin A reserves are established, there are two potential sources of vitamin A transfer to offspring. This includes existing maternal stores and maternal dietary intake. Roughly 70-85% of maternal stores of vitamin A appear in the liver as retinyl esters in hepatic stellate cells (124,125). As mentioned, when circulatory or peripheral

tissue retinoid levels are low, vitamin A is mobilized from hepatic stores into the circulation as retinol bound to the carrier protein; RBP4 and TTR (2,120,126). According to Goodman and colleagues (127), holo-RBP4 is the dominant source of retinoid present in the fasting circulation and is regarded as a crucial source of fetal vitamin A during transplacental transfer. Because maternal RBP4 cannot cross the fetal-maternal interface, retinol is thought to diffuse through the yolk sac and placenta, where it is taken up by zygotic RBP4 (128). During lactation, vitamin A delivery to the mammary gland by RBP4 has been regarded as relatively constant, given maternal vitamin A status is adequate – however its exact quantitative contribution remains unknown (119). Maternal dietary intake has been observed to alter milk vitamin A composition more dramatically, despite plasma retinol concentrations remaining unchanged (118,129). Unlike maternal stores, postprandial dietary retinoids are packaged into chylomicrons (within the intestinal enterocytes) as retinyl esters and pro-retinoid carotenoids, then circulated directly to the lactating mammary gland for utilization in the milk during breastfeeding (2,10,119,125) (Figure 1.2). Ross *et al.* (119) suggests this dietary uptake response to be relatively immediate on milk vitamin A content, which increases linearly in accordance with the quantity of chylomicron vitamin A.

1.5 Overview of Mammary Gland Differentiation Across Life Stages

Due to the importance of the mammary milk as a vessel of vitamin A transfer to offspring during lactation, it is important to have an understanding of the morphogenesis and differentiation of the mammary gland within its different stages to gain insight into the mechanisms involved in this process. The mammary gland is a complex organ comprised of a tubulo-alveolar gland enclosed

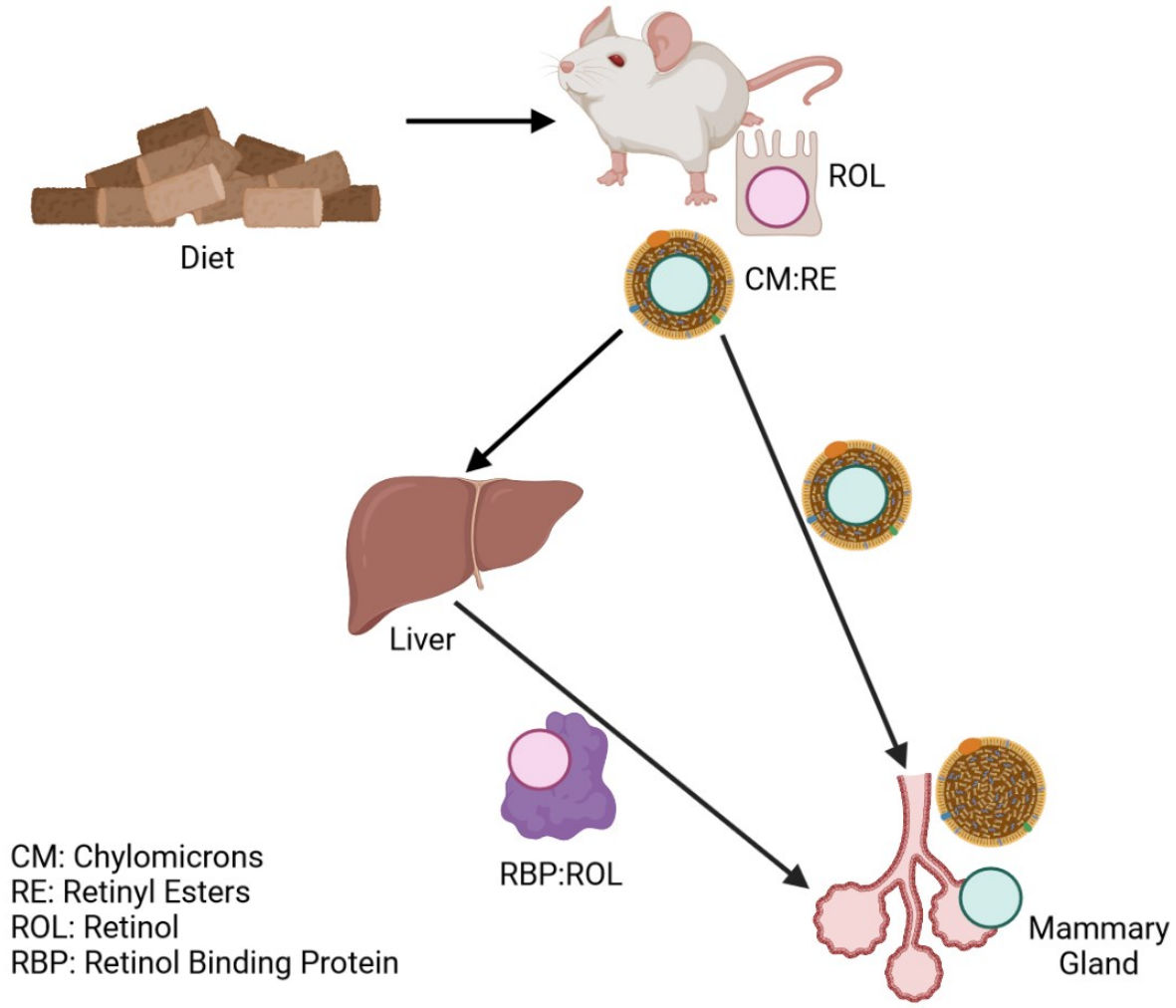


Figure 1.2 Vitamin A mode of transfer from the diet to the hepatocytes and lactating mammary gland. After ingestion from the diet, retinyl esters (RE) are converted to retinol (ROL) inside of the intestine or at the intestinal epithelial cell 'brush border' layer. These retinol molecules are then re-esterified and packed into chylomicrons (CM) for transport into the circulation. Once CM:RE is internalized by the hepatocytes and hydrolyzed, retinol can bind to a carrier protein called retinol binding protein (RBP). RBP:ROL may then be released back into the circulation for transport to a variety of target tissues, including the mammary gland. During lactation, retinyl esters packed into chylomicrons from the intestine can also directly travel through the circulation to target the mammary gland for vitamin A uptake. Created with BioRender.com.

by irregular connective tissue (9). This organ undergoes profound structural and functional differentiation during the physiological stages of gestation, lactation, and involution which are regulated by hormonal activity (primarily estrogen, progesterone, and prolactin) and other environmental factors. As such, significant proliferation, branching morphogenesis, differentiation, regression, and apoptosis of subpopulations of mammary cells has been extensively examined in both humans and mice models throughout these stages (130–134). Development of mammary tissue during embryogenesis is comparable across mammalian species (135). In its initial stages (embryonic day 10–11 in mice), the mammary gland is an epithelial bud attempting to penetrate its surrounding mesenchyme (135,136). As embryonic development progresses, this bud sprouts into the underlying mammary fat pad precursor tissue and branches-out until the end of gestation, where it becomes an established rudimentary ductal structure. This process is not observed in male embryos, as production of androgens by the testes initiates the mild necrosis of the mammary epithelium.

After birth, the mammary gland in female newborns comprises epithelial cords and the stroma as well as a rudimentary ductal tree that will remain quiescent until puberty (9,135–137). At puberty, sex hormones (estrogen and prolactin) and growth hormone, are released to initiate branching morphogenesis. This process involves the invasion of the mammary fat pad by terminal end buds, which undergo ductal elongation and branching to form secondary and tertiary ducts. Furthermore, with each menstrual cycle, the mammary gland proliferates and regresses to develop alveolar buds. Therefore, in the virgin, post-pubertal female, the mammary gland encompasses a fully developed bilayered ductal tree structure with epithelial derived alveoli bordered by myoepithelial contractile cells and a dense stromal layer of adipocytes (9,136).

During gestation and lactation, alveolar epithelium in the mammary gland proliferates and differentiates, taking on a secretory and milk producing lobuloalveolar structure containing lipid droplets. These lipid droplets serve as a crucial storage site for esterified vitamin A to be delivered to offspring during nursing (9,135,136). The differentiation of these alveolar buds to become capable of milk secretory activity occurs because of progesterone, estrogen and prolactin secretion during pregnancy, and results in the progressive disappearance of adipose tissue to make room for the proliferating epithelial alveoli cells (135,136,138). After parturition, the simultaneous elevation of prolactin and glucocorticoids and reduction in progesterone trigger the alveoli to begin copious milk secretion and establish lactation. When milk is required for nursing, a suckling stimulus by the newborn triggers oxytocin release and subsequent contraction of myoepithelial basal cells to eject milk out of the alveoli and through the ducts toward the nipple (9,136).

Following the weaning of offspring, a process known as involution occurs due to reduced stimulation of secretory epithelial cells for milk removal by the newborn. Involution restores the mammary gland to its virgin-like state and involves the regression and apoptosis of epithelial structures and re-differentiation of the mammary tissue from a lobuloalveolar to largely adipocytic structure (130,134,136,139). This period is coordinated by programmed cell death and remodeling of the extracellular matrix and mammary tissue. Akin to the prepregnant state, the mammary gland reverts to encompass a high percentage of unilocular adipocytes, in place of epithelial structures.

1.5.1 Vitamin A Incorporation into Mammary Milk

During lactation, widespread metabolic changes occur to ensure adequate vitamin A transfer to the mammary gland during milk production to meet offspring demands (9,140). This includes enhancing vitamin A availability by increasing maternal food intake, as well as a redistribution of these retinoids directly to the mammary tissue for milk secretion. Sources of vitamin A in mammary milk consist of stored retinoids in the liver mobilized as serum retinol-RBP4 and direct delivery of esterified dietary retinyl ester (preformed retinoids) and/or carotenoids (provitamin A) packed in postprandial chylomicrons (2,9,125). Although it has been established that dietary chylomicron vitamin A absorption occurs in a similar fashion to other dietary lipids (2), much remains to be understood regarding how retinoids are transported to, taken up by, and enzymatically incorporated into mammary milk during lactation. Knowledge of the esterification of retinol by lecithin retinol acyltransferase (LRAT) is well documented in the liver and most peripheral tissues (6,46,141,142), yet its role in the mammary gland has been disputed in prior literature, and has been proposed that an acyl-CoA dependent enzyme known as ARAT primarily facilitates this activity (119,143). Using an *Lrat*$^{-/-}$ mouse model, O'Byrne *et al.* (125) have since identified LRAT as the most critical enzyme responsible for the majority of retinyl ester synthesis in mammary milk droplets, offering novel insights concerning vitamin A incorporation into milk during lactation.

Investigations with retinol-binding protein null mice (*Rbp*$^{-/-}$) have affirmed the importance of the postprandial pathway in retinoid delivery for milk formation, suggesting a LPL-dependent pathway may have evolved to compensate retinoid uptake into mammary tissue to allow the maintenance of milk retinoid concentrations akin to wild-type mice (119,125). LPL is an enzyme known for facilitating the hydrolysis of chylomicrons and their remnants for uptake of fatty acids and fat-soluble nutrients from the postprandial circulation to the liver and

peripheral tissues (2,47,119,125). Several lines of evidence by Jensen *et al.* (144) and (145) using fluorescence immunohistochemistry suggest that in the lactating mammary gland, interstitial cells located in the mammary adipocytes adjacent to mammary alveoli serve as the primary production source of LPL. It has been hypothesized that during the lipolysis of chylomicron triglycerides, a membrane continuum between the chylomicrons and the underlying mammary parenchymal cells forms to facilitate the uptake of vitamin A into the lactating tissue, which is then transported to the milk via transcytosis (144,146–148). Jensen *et al.* (145) also notes that in mice 48 hours after parturition, LPL expression at mammary tissue increases tenfold, which could be attributed to the requirement for rapid and fat vitamin A uptake into milk for delivery to offspring (119,145,146). This elevation in mammary LPL expression during lactation is supported by a simultaneous depression of LPL activity in peripheral adipose tissue, allowing for the redirection of vitamin A incorporation into milk lipid droplets (144,145,147,149). Continued analysis is required to further understand the influence of the retinoid delivery pathway to vitamin A incorporation in mammary milk.

1.5.2 Retinoid Signaling Pathway Influences on Mammary Gland Morphogenesis

Although vitamin A is a critical micronutrient stored in the mammary milk for nursing, the retinoid signaling pathway has been shown to extensively influence the proper morphogenesis and maintenance of the mammary gland at virgin, lactating, and involuted states. This occurs largely through the regulation of retinoic acid concentrations that are critical for mammary tissue maintenance and regulates significant morphological alterations via epithelial cell proliferation, differentiation, and apoptosis (131). As such, precise signaling of retinoic acid and associated

retinoid genes (RALDH2 and RARβ) are involved in the initial development of the mammary epithelium during embryonic development (150). At birth, activation of RARγ may be responsible for regulating mammary development during adolescence and puberty by having a major effect on neonatal ductal tree expansion and branching morphogenesis, which is inhibited in the absence of these receptors (132,133,151). Likewise, literature has alluded to a universal expression of RARγ in adult mammary epithelial cell nuclei. Several mediators within the retinoic acid signaling pathway: carrier proteins CRABP2 and CRBP1, and various isoforms (α, β, and γ) of nuclear receptors RAR and RXR, are activated during lumen formation and mammary gland differentiation during pregnancy and lactation (largely RARβ and RARγ) and matrix remodeling at involution (predominantly RARα) (133,134,152). Transgenic mouse models which have tried to emulate vitamin A deficiency by terminating retinoic acid or specific mediators of the retinoic acid signaling pathway have exhibited reduced lactational efficiency in dams resulting from excessive branching morphogenesis and hyperplasia of the mammary gland via the upregulation of *metalloprotease 3* (*MMP-3*) and *cyclin D1* expression (132,133,153). However, some studies have observed that this may be restored upon supplementation of retinoic acid, affirming the criticality of vitamin A for mammary gland development. During involution, retinoids are critical for tissue remodeling via apoptosis of mammary secretory cells, extracellular matrix degradation, and adipogenesis. This is thought to occur via significant increases in CRABP2 and CRBP1 protein levels modulating increased retinoic acid signaling, which binds RARα/p300 to the *metalloprotease 9* (*MMP9)* gene promoter to regulate proteinase activity (9,132,153).

Retinoids can also exert chemopreventive and chemotherapeutic activity by inhibiting cell cycle G1/S progression or arresting the S-phase in breast cancer cell line production (132),

whereas loss of retinoic acid biosynthesis and subsequent signaling has been associated to breast cancer development (154,155). Specifically, studies using *Crbp1*$^{-/-}$ mice have exhibited dysregulated epithelial proliferation and depleted endogenous mammary retinoid acid, which have contributed to excess collagen secretion and hypercellularity, consistent with mammary models of oncogenesis (131,134,150,151,156). Due to its anti-proliferative properties, *Crbp1* expression is observed to be epigenetically silenced in around 25% of human breast cancers (131,134), resulting in abnormal fibrotic tissue which increases breast density and contributes to a dysfunctional microenvironment. This suggests that restoring *Crbp1* expression and endogenous retinoic acid may be of interest as a therapeutic strategy for tumorigenesis (156).

Activity of retinaldehyde dehydrogenase 1 (RALDH1; a retinoid enzyme important for oxidating aldehydes into retinoic acid) shows potential as a functional marker to identify and isolate cancer stem and progenitor cells from multiple tissues including the mammary gland (157). This is because excessive RALDH1 expression has been found to correlate with early metastasis and the tumorigenic cell portion in breast carcinomas (153,158,159). This is thought to occur via RALDH1 mediation of NOTCH1 signaling in the primary differentiation of stem cells. High RALDH1 activity has also been associated with decreased survival in multiple myeloma and acute myeloid leukemia stem cell populations (160,161) and thus could provide a predictive marker for clinically aggressive breast cancer behavior.

Collectively, these insights examining the influence of the retinoid signaling pathway on mammary gland homeostasis, point to the dual role of retinoids in the mammary gland as both a critical nutrient in mammary milk composition and its requirement for proper morphogenesis, regression, and maintenance of mammary structures.

1.6 Animal Models in the Study of Lactation

In biomedical research, mice are a model species for experimental examination because of their physiological, anatomical, and genetic resemblance to humans (162,163). They also carry the advantage of their ease of maintenance, small size, shorter lifespan, and ample genomic resources available. Regardless of their limited lifespan compared to humans, mice have been shown to express various organ and systemic physiological processes, disease pathogenesis patterns, and molecular mechanisms of ageing analogous to humans (163,164). Gestational and lactational periods are no exception, where rodent models are commonly used to assess the effects of a diverse nature of maternal physiological conditions and related pathologies (34,163). Although mice only contain one canal per teat in the mammary gland compared to the extensive number of canals in the human mammary gland, it has been noted that gross mammary anatomy is not a highly critical factor in lactational experiments (34). Because some aspects of the mouse mammary gland closely resemble the physiological function and lactogenesis process in humans, it is considered a suitable model of these processes. This is demonstrated in the conservation of the prolactin and oxytocin hormonal pathways involved in epithelial cell differentiation into lobuloalveolar structures and myoepithelial cell contraction for milk injection, respectively.

The maximum life span between mice (4 years) and humans (120 years) differs immensely (162,163). Therefore, having an established age comparison with regards to both the lifespan and individual life stages across both species is required to interpret mice as an experimental model for human development (Figure 1.3). It has been noted that one human year is considered equivalent to 9 mice days over the span of a lifetime, however, developmental stages of mice are not uniform compared to humans, and therefore each life stage needs to be examined separately (34,162,163):

Weaning: Mice in controlled animal rearing environments are typically weaned at postnatal day 21-28. This is considered to correlate to the weaning age for humans at postnatal day 180 or 6 months.

Puberty: Mice reach sexual maturity around age 35-42 days which is equivalent to 11.5-12.5 years in humans. This is characterized by a maturation of the hypothalamo–pituitary–gonadal axis and marked by balanopreputial separation in males and vaginal cornification and/or onset of the oestrous cycle in females (165).

Adulthood: This has been experimentally identified as after postnatal day 70 to 6 months in mice. This is because sexual maturity rapidly continues until this age and is considered similar to the period of growth plate closure which is used to identify adulthood in humans. Therefore, the human age equivalent is 20-30 years.

Middle-Aged Adult: In mice this is characterized by senescent changes in some biological processes and involves greater collagen cross linking and accumulation of activated T cells. Mice in this age group will be at least 10 months old with an upper limit of 14-15 months old. Reproductive functions cease at 15-16 months. This stage is equivalent to 38 to 47 years in humans, where adults begin to express similar biomarkers of aging and most women begin to undergo the process of menopause [traditionally occurring between 45 and 55 years (166)].

1.7 Overall Hypothesis and Aims

Although key processes involved in retinoid storage, mobilization, and delivery have been examined, much remains to be understood regarding the mechanistic relationship amongst dietary vitamin A intake and maternal milk vitamin A status.

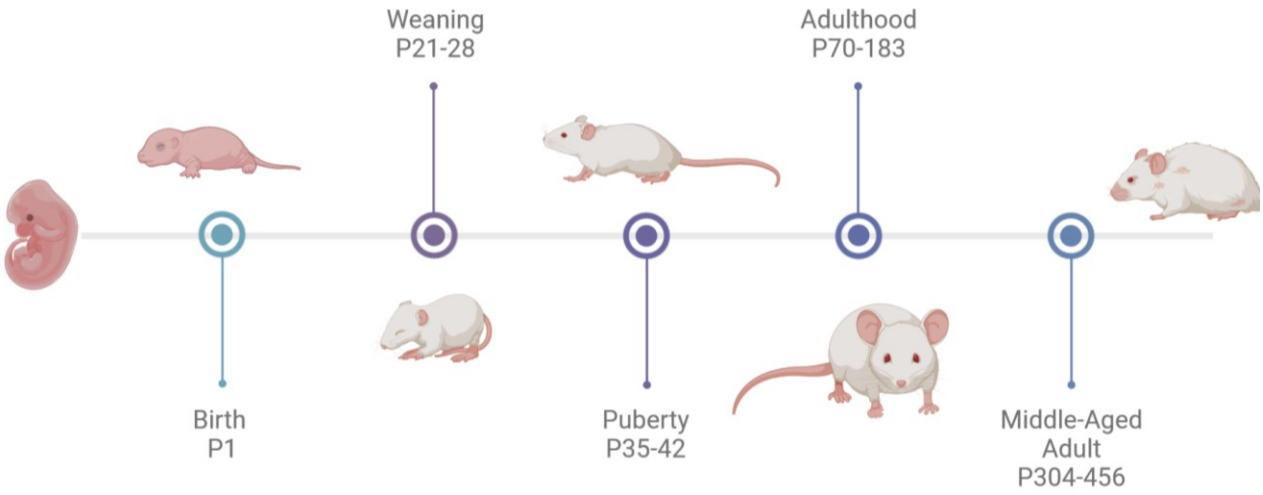


Figure 1.3 Timeline of the mouse lifespan. This is depicted in human days, beginning with birth [postnatal day (P)1] to middle-aged adult mice (P304 to 456). Figure and caption adapted from (34,162,163). Created with BioRender.com.

While knowledge of chylomicron vitamin A (dietary vitamin A) and retinol-RBP4 (maternal hepatic stores of vitamin A) are largely accepted as important physiologic carriers of vitamin A to maternal milk, their quantitative contributions have been highly disputed and have not been studied extensively in relation to offspring vitamin A status. Given the importance of establishing sufficient vitamin A reserves early in life, our overall objective is to gain insight into the mechanisms underlying the delivery of vitamin A to maternal milk and offspring in mice (Figure 1.4). This includes an analysis of:

1) Maternal vitamin A homeostasis during lactation by assessing changes in retinoid metabolizing genes (with an emphasis on mammary tissue) between virgin, lactating and involuted stages.

2) The role of maternal dietary intake (postprandial chylomicron retinoids) during gestation and lactation in establishing offspring vitamin A reserves. This is accomplished with a dietary intervention model using varied concentrations of vitamin A provided in the diet.

3) The influence of maternal hepatic reserves (mobilization of retinol-RBP4) to offspring vitamin A status during gestation and lactation. This is accomplished using a genetic intervention model (via transgenic *Lrat* $^{-/-}$ mice) to prevent maternal hepatic storage of vitamin A.

This research will assess the overall hypothesis that maternal dietary vitamin A intake is the primary determinant of vitamin A reserves in offspring during gestation and lactation, independent of maternal vitamin A reserves.

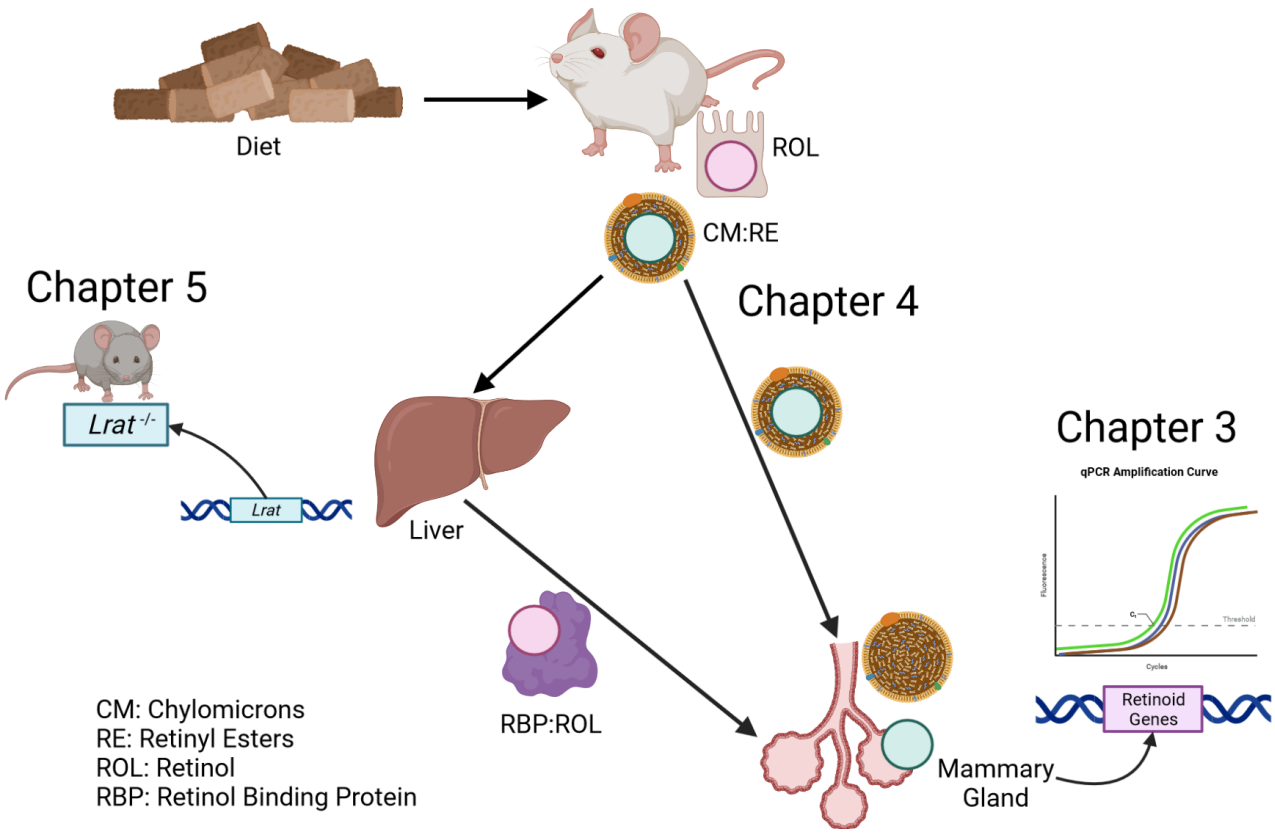


Figure 1.4 Summary schematic displaying the respective aims of our research program. Created with BioRender.com

CHAPTER 2 - General Methods

The following chapter discusses general methodology common to the research described in Chapters 3, 4 and 5. Procedural details specific to the research discussed within these chapters is located in their respective specific methods sections.

2.1 Animal Model and Tissue Collection

Research protocols were approved by the University of Alberta Animal Research Ethics Committee (AUP 00002966) and conducted in accordance with the Canadian Council on Animal Care. All mice were housed in the University of Alberta's conventional animal facility with a 12-h light–dark cycle and temperature controlled environmental conditions (21 ± 2 °C and 40-70% humidity). Animals were housed using standard caging with environmental enrichment (hiding structures, bedding, and nesting material) in individually HEPA ventilated cages. Studies in Chapters 3, and 4 were conducted using BALB/c mice purchased from Jackson Labs (Bar Harbor, ME, USA). Experiments in Chapter 5 were conducted using *Lrat*$^{-/-}$ and wild-type mice on a C57BL/6 background produced as described in (167) and were generously donated from William Blaner (Columbia University, NY, USA). The assignment of pregnant dams and offspring into their respective experimental groups was done randomly.

In all studies, body weight and other physical parameters of pups and dams were recorded prior to tissue collection. Liver tissue was collected from pups on postnatal day (P)1 following decapitation. Pups and dams had tissues collected on P7 through P105 following anesthetization via an isoflurane chamber (5% Isoflurane delivered in 1L/M of air) and death by cervical dislocation. Liver and stomach were collected from P7 pups, where the stomach 'milk curd' served as a proxy for mammary milk measurements (168). Blood was collected by cardiac puncture in P21 through P105 pups and dams prior to cervical dislocation, and plasma was

acquired via separation by centrifugation at max speed for 10 minutes (Sorvall Legend Micro 21R centrifuge; Thermo Fisher Scientific, Waltham, Massachusetts, USA). In dams, thoracic mammary glands, visceral white adipose tissue, liver, and plasma were collected for analysis on P21. All tissues collected were weighed, flash frozen in liquid nitrogen, and stored at -80°C until use.

2.2 Experimental Diets

General dietary composition and vitamin A content provided to mice varied according to the study conducted. In Chapter 3 all mice were housed in the conventional animal facility which provided a standard mouse chow diet (5L0D PicoLab® Laboratory Rodent Diet; LabDiet®), containing 18 IU vitamin A/g for the duration of the study. In Chapter 4, all dams received a purified AIN-93G diet (F3156 Rodent Diet; Bio-Serv, Flemington, New Jersey, USA) throughout gestation and lactation containing custom amounts of vitamin A (25 IU vitamin A/g or 0 IU vitamin A/g) and an otherwise standard vitamin and mineral mix, and macronutrient composition. Following weaning, all offspring were provided the standard conventional chow diet containing 18 IU vitamin A/g until tissue collection. In Chapter 5, dams were housed in the barrier animal facility and received breeder chow (5058 PicoLab® Mouse Diet 20; LabDiet®) containing 15 IU vitamin A/g throughout gestation and until weaning of the offspring. Despite variations in vitamin A content, all diets utilized a copious amount of vitamin A (apart from the 0 IU diet) in the form of retinyl palmitate, which are expected to establish adequate vitamin A stores in healthy mice under normal conditions. Given that The American Institute of Nutrition (AIN) has emphasized that an intake of 4 IU vitamin A/g is necessary to provide a marginal vitamin A diet for mice (169,170), the difference between the amounts of vitamin A provided in 15 IU, 18 IU and 25 IU copious diets are not expected to significantly influence our findings. For

a complete analysis of diet composition please refer to the manufacturer. All diets were irradiated rather than autoclaved. Table 2.1 details the dietary macronutrient composition and vitamin A content of these respective diets.

2.3 Vitamin A Quantification by High Performance Liquid Chromatography

HPLC was used to quantify vitamin A derivatives (retinol and various molecular species of retinyl ester) from tissue samples and plasma using standard methods (168). Approximately 0.090-0.120 g of each tissue sample was thawed, weighed, and homogenized for 30 seconds (Speed 20; Dremel ® 8220 Homogenizer, Mt. Prospect, Illinois, USA) in a glass test tube (12 x 75 mm) containing 1 ml phosphate-buffered saline (PBS). For liver samples ≥ P21, 500 µL of the homogenate was extracted into a 12 x 100 mm test tube containing 200 µL retinyl acetate (internal standard; Sigma-Aldrich, St Louis, MO, USA) and 300 µL ethanol, then briefly vortexed and placed on ice. For mammary, visceral white adipose and ≤ P7 liver samples, 900 µL of homogenate was extracted into 100 µL retinyl acetate and 800 µL ethanol. This homogenization step was not required for plasma samples.

Due to the light-sensitivity of retinoids, the remaining protocol was performed in a dark room with yellow light bulbs to avoid sample degradation. Here, 3 mL of hexane (Fisher Chemical, Hampton, New Hampshire, USA) was added to each sample and vortexed for 30 seconds, followed by centrifugation (Sorvall ST8 Centrifuge; Thermo Fisher Scientific) for 10 minutes at 10,000 RPM (max speed). This allowed for the separation of the organic (top) and aqueous (bottom) phases for extraction. Using a glass Pasteur pipette, the upper phase of each sample (containing the extracted retinoids) was transferred into a new glass test tube containing 500µL

	AIN-93G Vitamin A Deficient Diet (Chapter 4)	AIN-93G Vitamin A Copious Diet (Chapter 4)	Standard Mouse Chow (Chapter 3 and 4)	Breeder Chow (Chapter 5)
Fat	19.3%	19.3%	13.6%	21.6%
Protein	17.1%	17.1%	28.9%	23.2%
Carbohydrate	63.4%	63.4%	57.5%	55.2%
Vitamin A	0 IU/g	25 IU/g	18 IU/g	15 IU/g

Table 2.1 Macronutrient composition and vitamin A content in each diet. This table depicts the percentage of total calories obtained from macronutrients in each respective diet, as specified in the manufacture's product sheet. Vitamin A content is shown as the number of international units (IU) per gram of purified diet.

of PBS, vortexed for 30 seconds and centrifuged for another 10 minutes at 10,000 RPM. The remaining supernatant was then extracted into an empty test tube, followed by evaporation under a gentle stream of nitrogen. Once dry, the sample was resuspended in 40 µL of HPLC mobile phase (70% acetonitrile /15% methanol /15% methylene chloride; Fisher Scientific), vortexed and transferred into amber HPLC vials.

Analysis of vitamin A content involved a 20 µL injection of each sample through a Zorbax Eclipse Plus C18 separating column (4.6 × 250 mm, 5 µm particle size; Agilent, Santa Clara, California, USA) for 40 min using the Agilent 1200 HPLC machine. Pump flow rate was set at 1.8 mL/min at 27℃. The retention time of each vitamin A derivative was analyzed at an absorbance of 325 nm using a diode array detector (Agilent). Relative quantities of retinol and retinyl ester were determined by integrating the area under the curve of chromatogram peaks using ChemStation software (Agilent) and adjusted to the recovery of the internal standard (Figure 2.1).

2.4 Gene Expression Analysis

Liver and mammary tissues were assessed by qPCR analysis to examine the expression of genes associated with the retinoid metabolic pathway. In mammary tissue, genes associated with milk protein production and triglyceride and/or fatty acid metabolism were additionally examined.

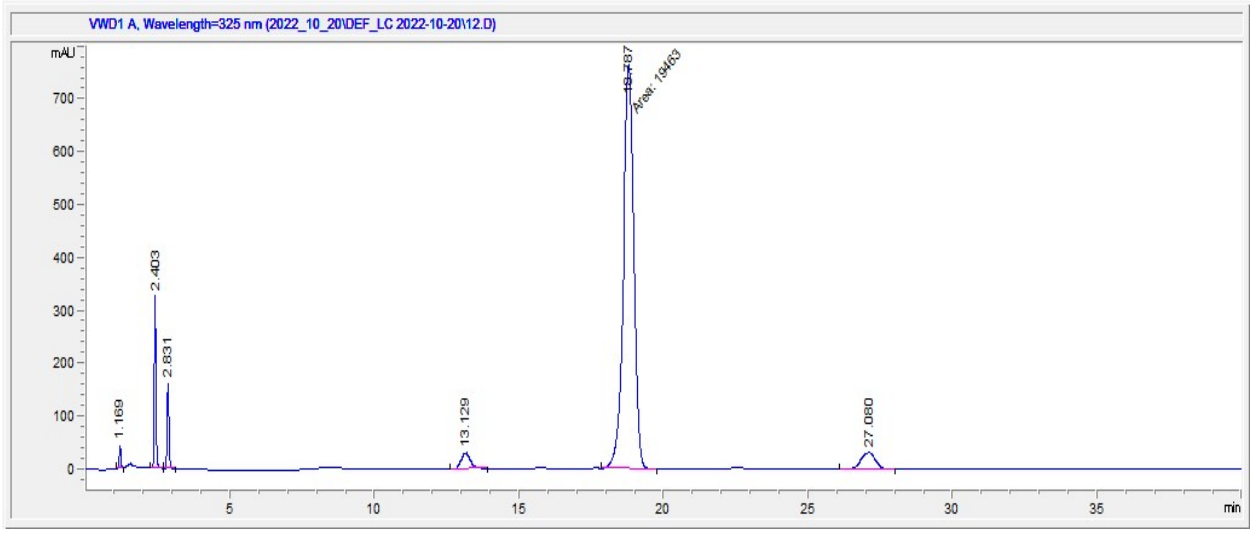


Figure 2.1 Representative HPLC chromatogram of mouse liver retinol and retinyl esters. Peak UV absorbance is depicted on the y axis expressed as absorbance units (mAU) with a value of 325 nm (the peak absorbance for vitamin A). The x axis indicates the retention time in minutes. The following peaks were used for the analysis of retinoid content in liver tissue: ~2.4 minutes, retinol; ~2.8 minutes, retinyl acetate (internal standard), ~13.1 minutes, retinyl linoleate, ~18.8 minutes, retinyl oleate/palmitate, and 27.1 minutes, retinyl stearate.

2.4.1 Tissue RNA Extraction

RNA was extracted from homogenized tissue samples using TRIzol (Invitrogen, Burlington, Ontario, Canada) and the Qiagen RNAeasy Plus mini kit (Hilden, Germany) according to the manufacturers protocol. In brief, 100 mg of each tissue sample was homogenized in 1 ml TRIzol reagent and put on ice. Homogenates were incubated at room temperature for 5 minutes, after which, 1 mL was added to 0.2 ml of chloroform in a 1.5 mL tube. Tubes were capped tightly and shaken vigorously for 15 seconds. Following a 2-3 min incubation at room temperature, samples were centrifuged (Sorvall Legend Micro 21R centrifuge; Thermo Fisher Scientific) at 2-8°C for 10 minutes at max speed to separate into a lower red, phenol-chloroform phase and a colorless upper aqueous phase. Approximately 450 µL of the upper aqueous phase (the portion containing extracted nucleic acids) was transferred into a gDNA eliminator column and spun for 30 sec at max speed. After discarding the inner column, an equal volume of 70% ethanol was added to the column flow through and mixed thoroughly by pipetting. Next, 700 µL of each sample were transferred into RNeasy mini spin columns (Qiagen), then repeatedly spun down and washed with buffers to remove salt and other contaminants. Finally, tissue RNA samples were eluted into a 1.5 ml collecting tube with 30-50 µL of RNase-free water and centrifuged to extract RNA. Quantification of the concentration of 2 µL extracted RNA and assessment of its quality were performed by the NanoDrop1000 spectrophotometer (Thermo Fisher Scientific).

2.4.2 cDNA Synthesis

Using the high-capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Waltham, Massachusetts, USA) 2 µg of purified RNA was reverse transcribed into cDNA according to the manufacturer's protocol. In 200 µL PCR tubes, 10 µL of RNA reaction mixture (consisting of

H_2O and a total of 2 µg RNA) and 10 µL Master Mix (2 µL 10X RT Buffer, 0.8 µL 25X dNTPs, 2 µL 10X random primers, 1 µL reverse transcriptase and 4.2 µL RNase free water; Roche Diagnostics, Basel, Switzerland) were combined for each sample, then run in the ProFlex PCR System (Thermo Fisher Scientific). Samples were run for 10 minutes at 25℃, then incubated for 120 minutes at 37℃, followed by denaturation for 5 minutes at 85℃. cDNA was then diluted by a factor of 1:40 with nuclease-free water and stored in three aliquots at -80°C until use.

2.4.3 Quantitative PCR Analysis

Quantitative real-time PCR analysis was performed to assess mRNA expression levels of genes of interest using the Applied Biosystems Quantstudio 3 thermocycler (Thermo Fisher Scientific), with data collected via Quantstudio software (Thermo Fisher Scientific). Each reaction contained 5 µL of experimental cDNA and 15 µL of reaction mixture made up of 10 µL SYBR Green qPCR Master Mix (Roche Diagnostics), 3 µL RNase free water, and 1µL forward and reverse primers for the target gene. Duplicates of each sample were loaded onto a plate, including a negative control blank (containing only master mix and RNase free water). All reactions were performed in duplicates. qPCR cycling conditions involved two phases, with the first requiring a cycle of 2 minutes at 50°C followed by 10 minutes at 95 °C and the second requiring 45 cycles of denaturing (10 seconds at 95°C) followed by annealing/extension (10 seconds at 60°C). This second phase was concluded by a final extension step (12 seconds at 72°C). Gene expression was quantified (after performing a melt curve ramp) by the cycle threshold (CT) recorded for each sample and normalized to *18s* and *CycloA* as reference genes for liver and mammary tissues, respectively. All primer sets used are listed in Table 2.1.

Genes	Primer sequence	Amplicon size
18s	Forward: CCATCCAATCGGTAGTAG CG Reverse: GTAACCCGTTGAACCCCA TT	150
Cyclo A	Forward: ATTTCCGACTGTGGACAGCTCTA Reverse: GAGCAGAGATTACAGGACATTGCGA	126
Wap	Forward: CCATGTGCTGTCCCGGT Reverse: ATCATTTGGAGTAGATTCCAAGGGC	100
CsnG	Forward: GCCAGAAGAAGAGAGAGTGGGATA Reverse: TGCAGTTAATACGGCTCCACAGTC	100
Lalba	Forward: GCAAGGTGTCCCATGCCATTA Reverse: TTGTGTGTCGTAGCCACTGGTAT	101
Cd36	Forward: GCTGTGTTTGGAGGCATTCT Reverse: CCTTGATTTTGCTGCTGTTC	196
Lpl	Forward: ATCAACTGGATGGAGGAGGAGTTT Reverse: TTGGTCAGACTTCCTGCTACGC	104
Dgat1	Forward: GAGTCTATCACTCCAGTGGG Reverse: GGCGGCACCACAGGTTGACA	100
Lrat	Forward: CAGGCATCGAAGAGATGACTCCG Reverse: GCTGCTGGTAACTAAATCCTGGTCC	84
Crbp1	Forward: CTTACTGTCCCTACTGTGTGTCAAGCACTA Reverse: CCTGAGATGAACCTCCTGAGATGGTTTA	73
Crbp3	Forward: CCCGCTTGAGGCAACTACT Reverse: GTTTCTCATACAGGCTGTGTGACAT	119
Stra6	Forward: GTTCAGGTCTGGCAGAAAGC Reverse: CAGGAATCCAAGACCCAGAA	102
Rbpr2	Forward: ATCGTGCCTCTGGACTTCG Reverse: GCCCACTGAGGAACCGT	131
Rbp4	Forward: AGACACGGAGGCTGGTGA Reverse: TTTCTGGAGGGCCTGCTTTG	103
Dhrs3	Forward: CCGCCTGATGTGCATCTACTATTT Reverse: TGCTGTGTAACCAGTTTGCACGA	106
Rdh1	Forward: AACCCACACACTCCTGCTT Reverse: AGCAGAGGATGGCCTTGTT	113
Raldh1	Forward: TGGGAATACCGTGGTTGTCAAGC Reverse: TTGGCCCATAACCAGGGACAAT	125
Raldh3	Forward: ACCACATTCAATAACGGGCACG Reverse: CATAAATGAAAGCCCAGACGGGAT	149
Cyp26a1	Forward: GGCACTGTGATTGGCAGCTTCTAA Reverse: TGCAGGGAGATTGTCCACAGGGTA	73
Cyp26b1	Forward: GCAGTATATGCTTATGACATCTGAATC Reverse: CCTGACCACTCACCAACAAA	77
Rara	Forward: CACGCCTGAGCAAGACACAATGA Reverse: GAAGGCAAAGACCAAGTCGGTGA	106
Rarb	Forward: GGGCATGTCCAAAGAGTCTGTTAG Reverse: CTAGCTCCGCTGTCATCTCATAG	101

Crabp1	Forward: AAGGCGTTGGGTGTGAACGC Reverse: TGCGTCCGTCCACTGTCTCCT	181
Crabp2	Forward: CTTGCTGCCACTATGCCTAA Reverse: TGTTTGATCTCGACTGCTGG	209

Table 2.2 List of gene and primer sequences used in qPCR analysis. This was performed in of liver and mammary tissue in Chapters 3 and 4.

2.5 Statistical Analysis

Graphical illustrations and statistical analysis were performed using GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA). All values depict the means ± standard deviations (SD). Statistical analysis of data varied according to the type of data being evaluated. For experimental data with two groups, results were compared using an unpaired Student's *t*-test. When comparing experimental data with three groups, statistical analysis was generated with an ordinary one-way ANOVA and Tukey's multiple comparisons test. Experimental data with four groups was compared using a two-way ANOVA followed by a Tukey's multiple comparisons post-test. In all statistical analysis, statistical significance was denoted as $p < 0.05$.

CHAPTER 3 – Markers of Vitamin A Metabolism at Different Stages of Mammary Gland Development

3.1 Introduction

The mammary gland is a complex organ which undergoes profound structural and metabolic changes during the physiological stages of gestation, lactation, and involution. During lactation, the mammary tissue is comprised of a tubulo-alveolar gland enclosed by irregular connective tissue and myoepithelial contractile cells (9). The formation of this structure is critical for establishing adequate vitamin A status in newborns, as it allows for milk production, which retinoids are distributed into for offspring uptake during breastfeeding (9,140). Although there is some knowledge surrounding the molecular mechanisms involved in vitamin A incorporation into milk and the influence of retinoid gene expression in mammary tissue morphology, its focus is narrow and primarily applied to models of cancer biology (131–133,153). With this in mind, we developed a research program aimed at providing a comprehensive analysis of maternal vitamin A homeostasis and retinoid metabolizing gene expression at virgin, lactating and involuted stages, with a focus on the mammary gland.

The purpose of this study was to examine if alterations in mammary morphology during lactation influences changes in gene expression relating to vitamin A retinyl ester production for inclusion into mammary milk droplets, and if this is supported by alterations in hepatic vitamin A homeostasis. As a result, the whole mammary gland was assessed to examine the expression of genes of interest pertaining to mammary milk proteins, triglyceride and/or fatty acid metabolism, and retinoid pathway genes across virgin, lactation, and involution life stages. Given that the requirements for vitamin A increase during lactation (24), we anticipate a shift in the expression pattern of the vitamin A metabolic pathway in support of retinyl ester synthesis and uptake into milk for secretion, instead of vitamin A metabolism into retinoic acid in the mammary gland of lactating mice.

3.2 Experimental Design

With regards to our research program, we conducted our experiment using adult female Balb/c mice under standard conditions as described in Chapter 2. All mice were provided a vitamin A sufficient chow diet of 18 IU vitamin A/g diet throughout their respective lifespans. Liver, mammary, and visceral white adipose tissue samples were collected from virgin, lactating (postnatal day [P]7), and involuting (P42) dams (Figure 3.1). Analysis of dam plasma and circulating RBP4 (by enzyme linked immunosorbent assay) were additionally conducted. Dams in our lactation and involution groups were bred with Balb/c male counterparts, while virgin mice did not undergo mating. Moreover, virgin female tissue collection was age matched to both lactation and involution dam groups to eliminate potential discrepancies in data due to age. This involved collecting half the mice at P7 and the other half at three weeks post weaning (P42). Assessing dams in these three groupings allowed us to examine retinoid homeostasis and gene expression differences across all primary mammary tissue developmental stages, which was confirmed by histological analysis of mammary tissue. Quantitative amounts and molecular species of vitamin A were assessed by high performance liquid chromatography analysis (HPLC) and gene expression analysis was examined by quantitative polymerase chain reaction (qPCR). In addition, physical characteristics of all dams (maternal age, litter size, body weight, and liver weight) were evaluated on the respective day their tissues were collected to assess for discrepancies between study groups.

3.3 Methods

A description of the animal husbandry and tissue collection process, vitamin A quantification by high performance liquid chromatography, gene expression analysis and statistical analyses are

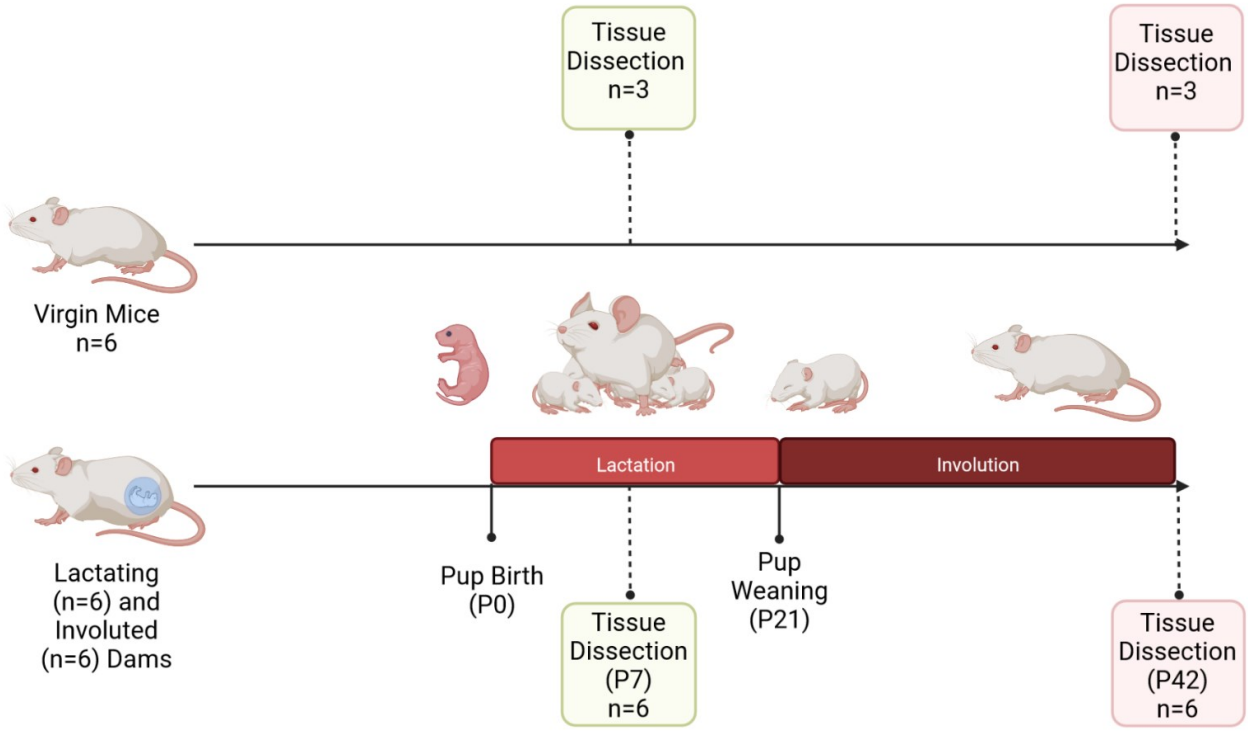


Figure 3.1 Summary figure displaying the experimental design for the role of vitamin A metabolism during stages of mammary gland development. Tissue samples were collected from virgin (split between P7 and P42), lactating (P7), and involuting (P42) dams. Vitamin A content was examined using HPLC analysis of various dam tissues, complemented by hepatic and mammary qPCR analysis of retinoid gene expression. Created with BioRender.com.

provided as general methods in Chapter 2. A comprehensive list of genes and primers sequences used in mammary and liver qPCR analysis are shown in Table 2.1.

3.3.1 RPB4 Quantification by Enzyme-Linked Immunosorbent Assay

Concentrations of dam RBP4 were measured in duplicate plasma samples (with a dilution factor of 1:10,000) according to the manufacturer's protocol using the Dual Mouse/Rat RBP4 ELISA kit from AdipoGen (Seoul, South Korea) and BioTek EPOCH2 microplate reader (Agilent, Santa Clara, California, USA).

3.3.2 Histological Analysis of Mammary Tissue

Histology sections were prepared by the Faculty of Medicine and Dentistry Histocore using standard methods as depicted in Figure 3.2. After fixation with formalin, mammary tissues were dehydrated and embedded in paraffin, then cut into 5 μm sections, which were stained with hematoxylin and eosin. Slides were digitally imaged using a B-292 Optika Microscope (Ponteranica, BG, Italy) and Optika Vision Lite version 2.13 software, with a scale bar of 25 μm. Modifications to image formatting and contrast were performed using Adobe Photoshop (version 23.0.0; Mountain View, California, USA).

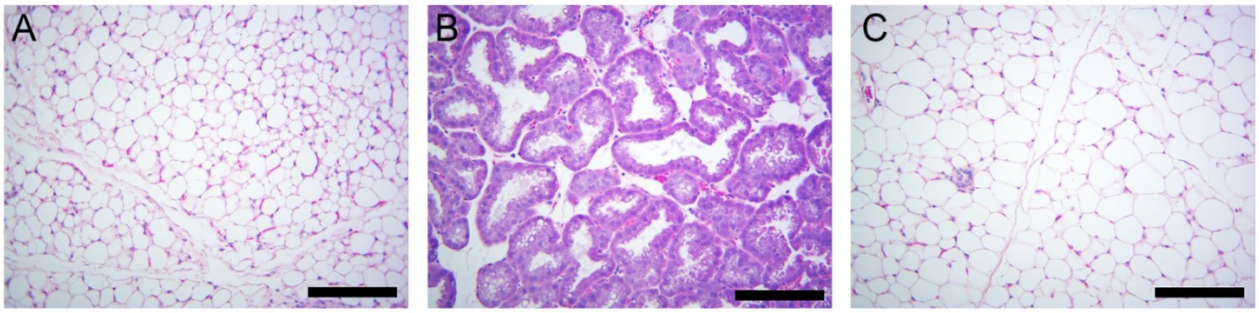


Figure 3.2 Histological examples of mouse mammary tissue morphology. Representative histological images of (A) virgin, (B) lactating and (C) involuted mammary tissue. Scale bar = 25 μm.

3.4 Results

3.4.1 Physical Characteristics of Virgin, Lactating and Involuted Dams

Physical parameters of dams were measured during lactation (P7, n=15) and involution (P42, n=15) time points, with the virgin mice assessed across both ages (Figure 3.3, Table 3.1). This was done to ensure no significant discrepancies were present between dams which may affect result outcomes. As expected, maternal age (days) displayed a significant ($p < 0.01$) variation between lactation and involution groups (113.40 ± 21.10 days, and 139.00 ± 12.46 days, respectively), while the virgin group remained non-significant when compared to both, due to the split of mice collections across both dissection time points (127.00 ± 19.04 days) (Figure 3.3 B). Litter size did not significantly differ between lactating and involuted groups of dams (7.16 ± 1.17 pups, and 7.00 ± 1.83 pups, respectively) ensuring the number of pups in each litter did not impact our findings (Figure 3.3 A). Assessment of maternal body weight (Figure 3.3 C) showed that lactating (28.37 ± 1.33 g) and involuted (26.73 ± 1.68 g) dams were significantly heavier than virgin mice (22.74 ± 2.76 g) but did not differ between each other ($p < 0.0001$). Hepatic enlargement during pregnancy and increased accumulation of adipose deposits may have attributed to this. Maternal liver weights at involution and lactation were also significantly larger ($p < 0.0001$) than virgin mice (1.07 ± 0.19 g), however, livers from involuted dams (1.49 ± 0.19 g) were significantly smaller than that of lactating dams (1.97 ± 0.16 g), as shown in Figure 3.3 D. This remarkable growth of the liver is driven by hepatocyte proliferation and size increase, which is characterized as an adaptation to the demands of pregnancy (171). Comparison of the ratio of maternal liver weight to body weight parallels this data by displaying significant discrepancies ($p < 0.0001$) between the virgin (0.047 ± 0.0049), lactating (0.069 ± 0.0043) and involuted (0.056 ± 0.0059) groups of mice (Figure 3.3 E).

	Virgin	Lactation	Involution	p-value
Number of Females (n)	10	10	10	-
Litter Size (n)	NA	7.16 ± 1.17	7.00 ± 1.83	n.s.
Maternal Age (days)	127.00 ± 19.04 ^{ab}	113.40 ± 21.10 ^{a}	139.00 ± 12.46 ^{b}	$p < 0.01$
Maternal Body Weight (g)	22.74 ± 2.76 ^{a}	28.37 ± 1.33 ^{b}	26.73 ± 1.68 ^{b}	$p < 0.0001$
Maternal Liver Weight (g)	1.07 ± 0.19 ^{a}	1.97 ± 0.16 ^{b}	1.49 ± 0.19 ^{c}	$p < 0.0001$
Maternal Liver Weight : Body Weight Ratio	0.047 ± 0.0049 ^{a}	0.069 ± 0.0043 ^{b}	0.056 ± 0.0059 ^{c}	$p < 0.0001$

Table 3.1 Maternal physical characteristics of virgin, lactating and involuted mice. All data is analyzed by one-way ANOVA, with the exception of litter size. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

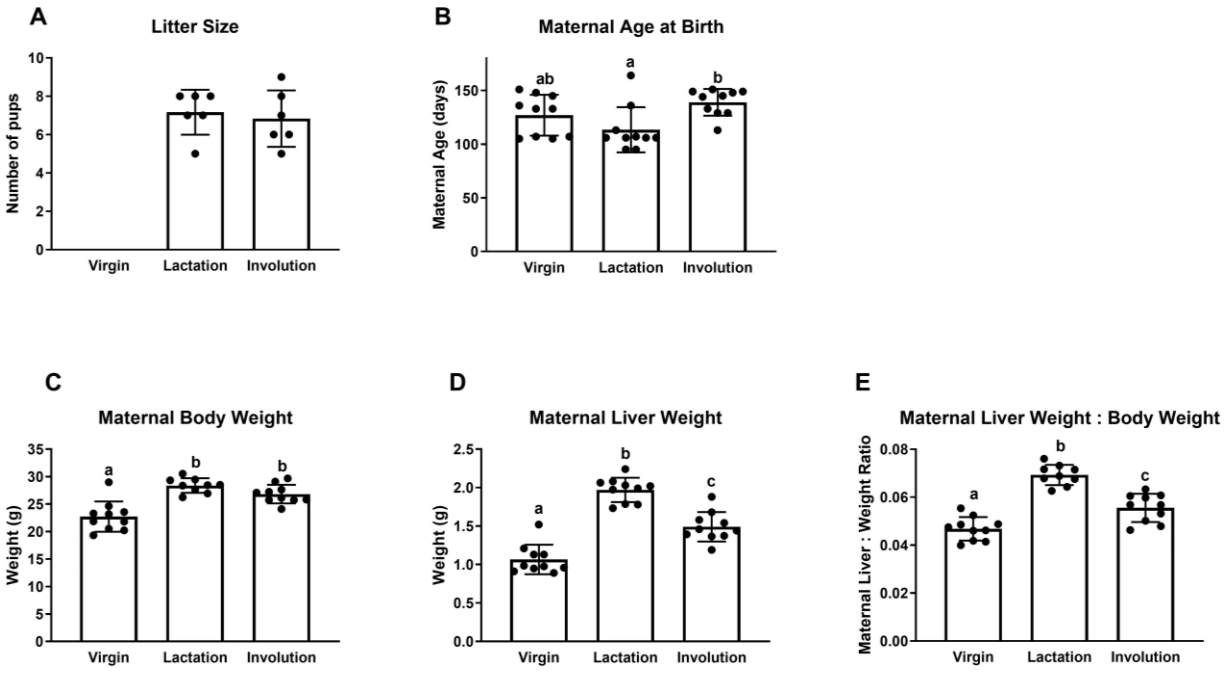


Figure 3.3 Maternal physical characteristics of virgin, lactating and involuted mice. (A) Litter size at parturition, (B) maternal age at the birth of the offspring, (C) maternal body weight, (D) maternal liver weight, (E) maternal liver to body weight ratio. All data is analyzed by one-way ANOVA, except for litter size. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

3.4.2 Retinol and Retinyl Ester Content in Dams at Virgin, Lactating, and Involuted Stages

To elucidate the impact of lactation on maternal vitamin A reserves and subsequent maternal vitamin A homeostasis, mean retinol and retinyl ester content of dams was examined by HPLC analysis in mammary tissue, visceral white adipose tissue (VWAT), liver tissue, and plasma extracted at virgin, lactating and involuted stages (Figure 3.4). Analysis of mammary retinol content showed an ~81% reduction ($p < 0.0001$) from virgin (1.39 ± 0.36 nmol ROL/g) to lactating (0.26 ± 0.10 nmol ROL/g) mice, which recovered to virgin levels once mice reached involution (1.69 ± 0.31 nmol ROL/g; Figure 3.4 G). This was paralleled by retinyl ester content (Figure 3.4 H), which dropped ~69% ($p < 0.0005$) in lactating mice compared to their virgin counterparts (1.78 ± 0.13 nmol RE/g and 5.73 ± 0.52 nmol RE/g, respectively), then returned to baseline upon involution (5.13 ± 1.37 nmol RE/g). It can therefore be assumed that these findings reflect the loss of maternal vitamin A stores to mammary milk which are utilized by the offspring during the lactational period.

During the HPLC quantification of vitamin A content in VWAT tissue (Figure 3.4 E), mean concentrations of retinol did not significantly vary amongst virgin, lactating and involuted mice (1.86 ± 0.53 nmol ROL/g, 2.33 ± 0.86 nmol ROL/g, and 1.92 ± 0.79 nmol ROL/g, respectively). Despite this, VWAT retinyl ester concentrations were higher in ($p < 0.05$) lactating (17.95 ± 16.87 nmol RE/g) than involuted (5.63 ± 3.05 nmol RE/g) dams, but did not differ in virgin (8.26 ± 6.30 nmol RE/g) dams to both lactating and involuted groups (Figure 3.4 F). This data emphasizes an opposite trend to mammary tissue, whereby retinyl ester increases in VWAT during lactation, which declines just below virgin levels at involution.

Hepatic HPLC analysis revealed a significant reduction ($p < 0.0001$) in retinol concentrations in lactating mice compared to virgin but not involuted mice, with 13.54 ± 5.90

nmol ROL/g versus 28.26 ± 5.78 nmol ROL/g, and 10.63 ± 3.69 nmol ROL/g, respectively (Figure 3.4 C). Mean liver retinyl ester concentrations in virgin dams (1,721.62 ± 670.46 nmol RE/g) however, were significantly smaller (~29%, $p < 0.05$) than involuted (2,430.15 ± 422.96 nmol RE/g) but not lactating (1,731.76 ± 195.39 nmol RE/g) dams as shown in Figure 3.4 D. This suggests that hepatic vitamin A retinyl ester reserves was largely unaffected by the change to a lactating state and therefore may not significantly contribute to establishing mammary milk vitamin A composition during this process. Finally, evaluation of mean plasma retinol concentrations, as shown in Figure 3.4 A, depicted no statistically significant differences across all stages in dams (0.68 ± 0.18 µmol ROL/L, 0.64 ± 0.17 µmol ROL/L, and 0.81 ± 0.26 µmol ROL/L, respectively). This affirms that retinol status is tightly maintained in the circulation, which is indicative of maternal retinyl ester reserves not being depleted.

3.4.3 Mammary Gland Retinoid Metabolizing Genes Expression at Virgin, Lactating, and Involuted Stages

Whole mammary gland homogenates were used to examine the expression of genes pertaining to milk proteins, triglyceride and/or fatty acid metabolism, and the retinoid pathway in virgin, lactation, and involution mice life stages. Genes of interest were analyzed to determine if changes in mammary morphology influenced changes in retinoid gene expression, particularly those pertaining to the retinoic metabolic pathway. Prominent milk proteins: whey acidic protein (*Wap*), casein gamma (*Csng*) and lactalbumin alpha (*Lalba*), were first assessed as a control to confirm the differentiation of the mammary gland to a glandular ductal structure producing milk droplets (from the prior adipocytic structure) (Figure 3.5). As anticipated, all genes increased significantly ($p < 0.001$, $p < 0.0001$, $p < 0.0001$, respectively) in expression during lactation,

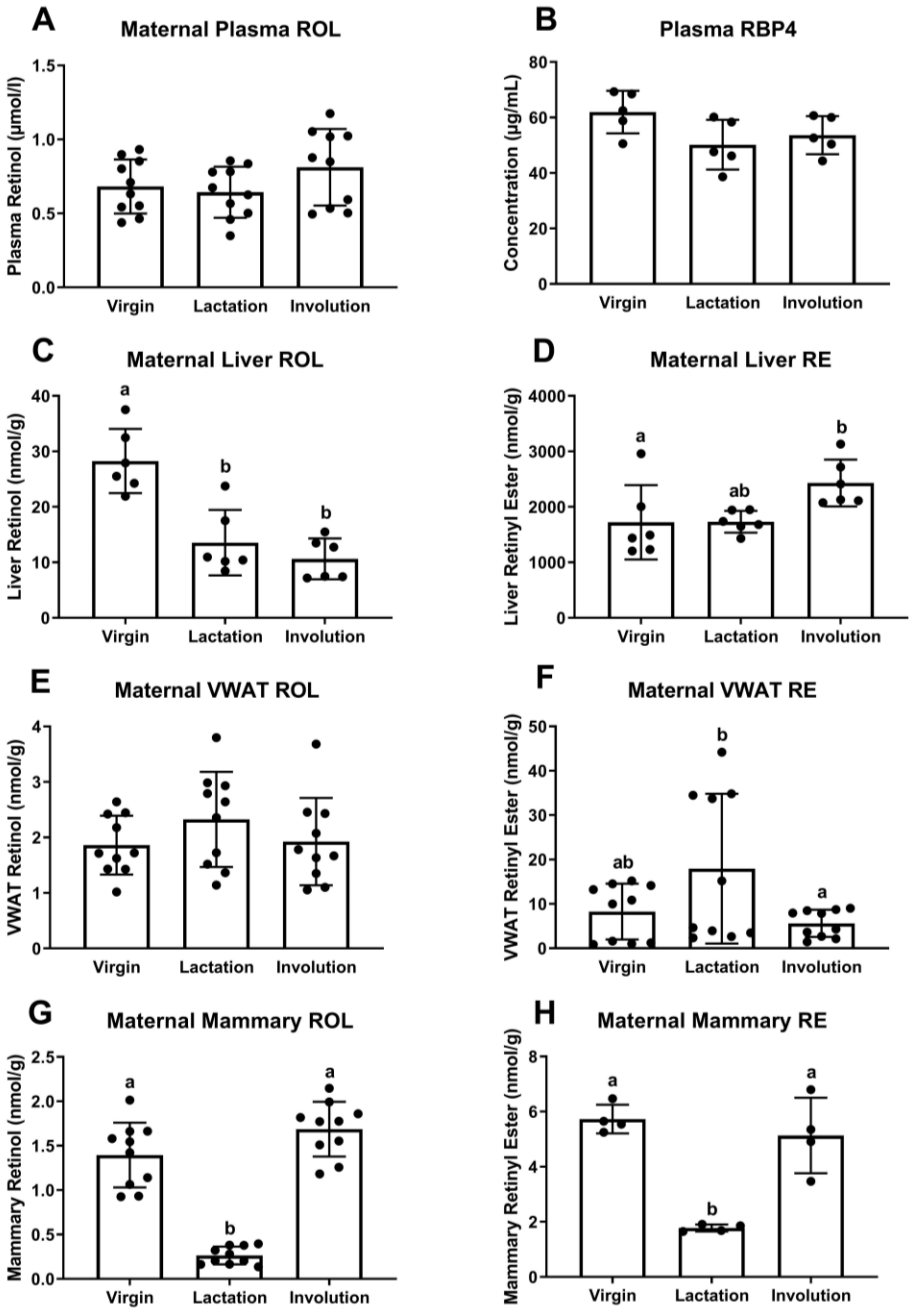


Figure 3.4 HPLC quantification analysis of retinol and retinyl ester concentrations in tissues of virgin, lactating and involuted mice. (A) Maternal plasma retinol, (B) RBP4 plasma ELISA content, (C) retinol content in the dam liver, (D) retinyl ester in the dam liver, (E) retinol content in the dam visceral white adipose tissue, (F) retinyl ester content in the dam visceral white adipose tissue, (G) retinol content in the dam mammary tissue, and (H) retinyl ester content in the dam mammary tissue. All data is analyzed by one-way ANOVA. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

then reduced to around virgin levels upon involution, therefore affirming these morphological changes in the mammary tissue. *Wap* expressed a fold change of 1.00 ± 0.80 to 13,388.03 ± 8,509.48 to 119.37 ± 99.61, respectively (Figure 3.5 A), while *Csng* expressed a fold change of 1.00 ± 0.93 to 2,654.08 ± 794.36 to 18.34 ± 15.10, respectively (Figure 3.5 B). Similarly, a fold change of 1.00 ± 1.38 to 1,555.72 ± 564.64 to 21.45 ± 10.29, respectively, was expressed by *Lalba* (Figure 3.5 C). This examination of mammary morphology was supplemented by a histological analysis of mammary tissue at virgin, lactating and involuted stages (Figure 3.2). These findings affirmed the differentiation of the mammary tissue from a largely adipocytic structure in virgin and involuted states, into a functional glandular structure containing milk lipid droplets at lactation.

Genes associated with triglyceride and/or fatty acid metabolism were next analyzed to assess changes in the translocation, degradation, and synthesis of chylomicron triglycerides at the mammary gland. This involved the evaluation of cluster of differentiation 36 or fatty acid translocase (*Cd36*), lipoprotein lipase (*Lpl*), and diglyceride acyltransferase (*Dgat1*). Expression of *Cd36* showed no significant difference from virgin to lactating to involuted dams: 1.00 ± 0.52 to 0.92 ± 0.57 to 1.57 ± 0.66, respectively (Figure 3.6 A). Similarly, *Lpl*, an enzyme involved in chylomicron metabolism and distribution of triglycerides into mammary milk, was not statistically different across virgin (1.0 ± 0.60), lactating (1.40 ± 1.30), and involuted dams (Figure 3.6 B). Furthermore, *Dgat1*, which catalyzes the production of triacylglycerol and in certain circumstances may catalyze the production of retinyl ester (4), also displayed non-significant gene expressions across virgin, lactation, and involution mammary stages (1.00 ± 0.490, 0.45 ± 0.0.11, 1.31 ± 0.75, respectively) as shown in Figure 3.6 C.

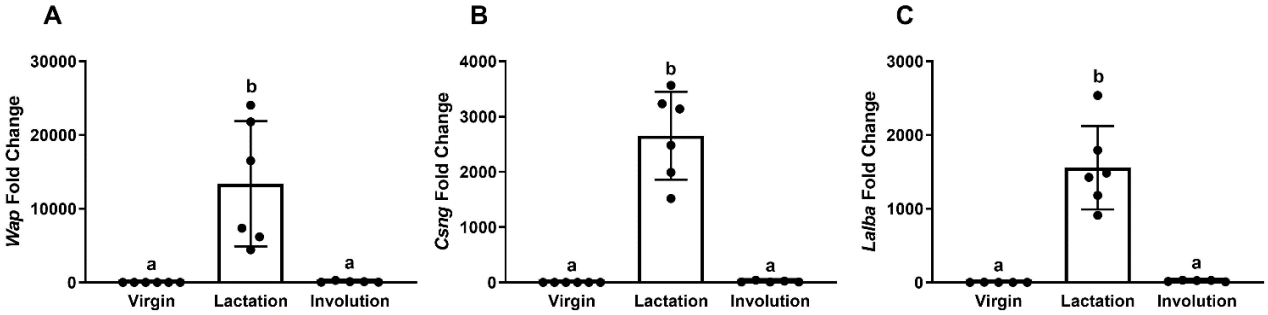


Figure 3.5 qPCR analysis of mammary milk protein (control group) expression during virgin, lactation (P7) and involution (P42) time points. All genes are normalized to their respective virgin state and depict a fold-change relative to the reference gene *CycloA*. (A) Whey adipose protein fold change, (B) casein gamma fold change, and (C) lactalbumin alpha fold change. All data is analyzed by one-way ANOVA. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

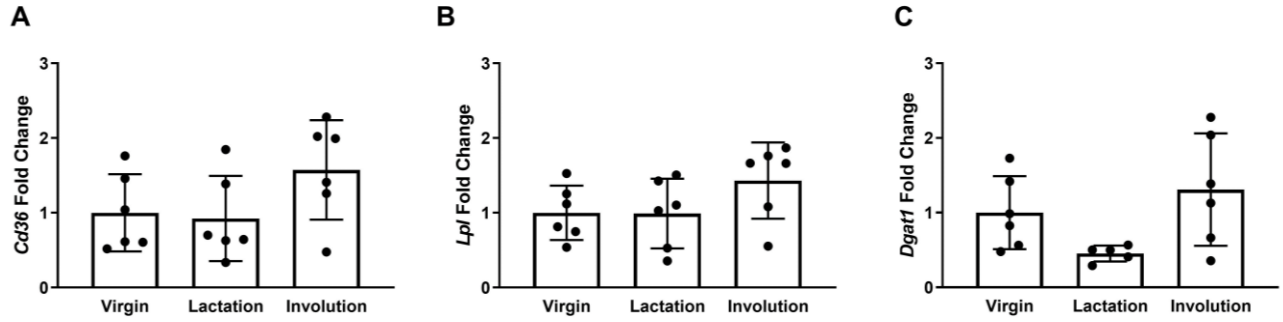


Figure 3.6 qPCR analysis of mammary expression of genes associated with triglyceride and/or fatty acid metabolism during virgin, lactation (P7) and involution (P42) time points. All genes are normalized to their respective virgin state and depict a fold-change relative to the reference gene *CycloA*. (A) Cluster of differentiation 36 or fatty acid translocase, (B) lipoprotein lipase and (C**)** diglyceride acyltransferase-1 fold change. All data is analyzed by one-way ANOVA. $p < 0.05$ depicts statistical significance between virgin, lactation, and involution results.

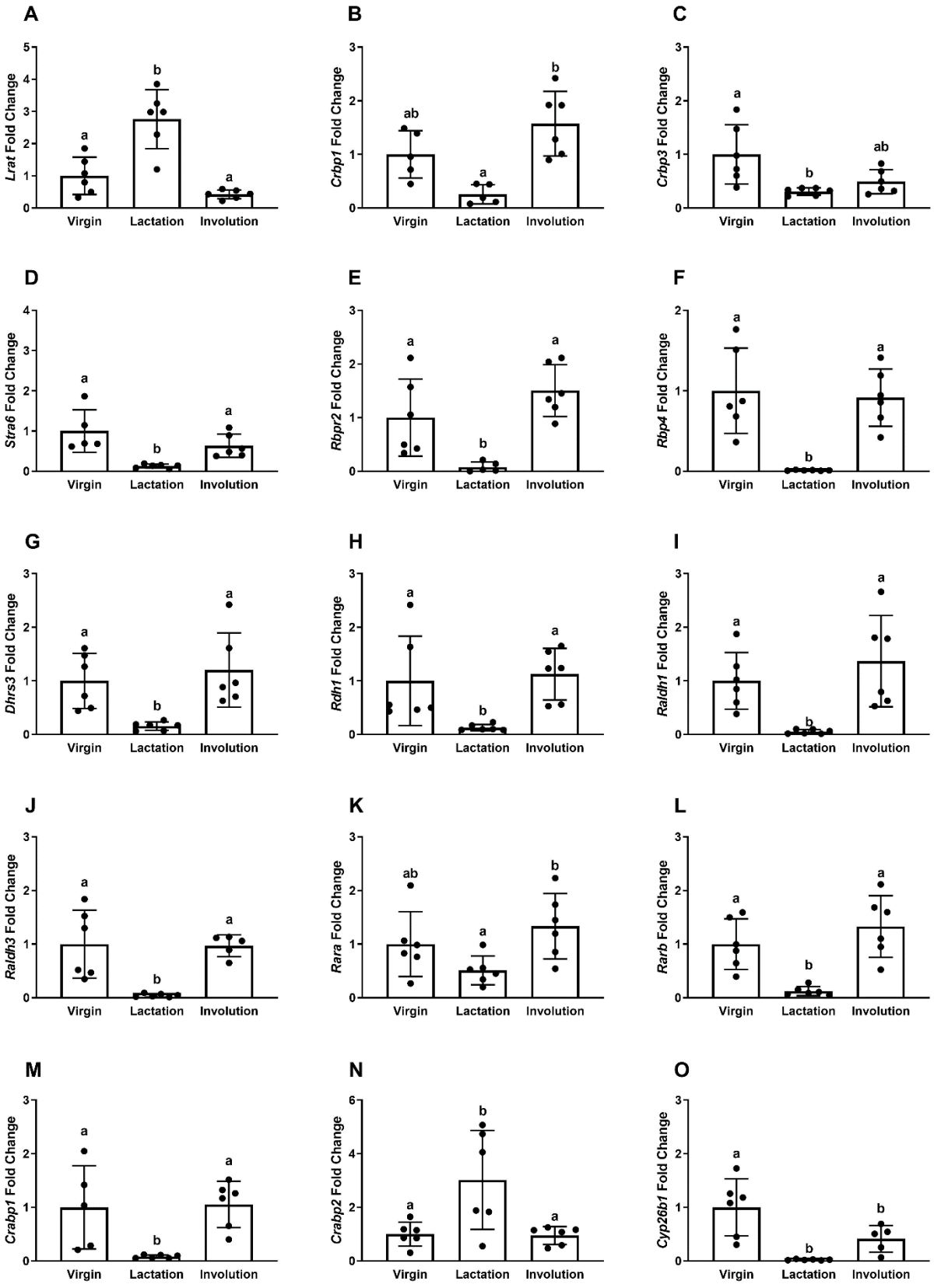
Retinoid related gene expression was next evaluated to assess alterations in the expression of genes directly associated with the retinoid metabolic pathway, including those involved in retinoid transport and uptake as well as mediators of retinoid metabolism to retinoic acid. The following genes were examined and compared by one-way ANOVA and are referred to in Figure 1.1: cellular retinol binding protein 1 (*Crbp1*), retinol binding protein receptor 2 (*Rbpr2*), signaling receptor and transporter of retinol 6 (*Stra6*), lecithin retinol acyltransferase (*Lrat*), retinol binding protein 4 (*Rbp4*), cellular retinol binding protein 3 (*Crbp3*), dehydrogenase/reductase 3 (*Dhrs3*), retinol dehydrogenase 1 (*Rdh1*), retinaldehyde dehydrogenase 1 (*Raldh1*), retinaldehyde dehydrogenase 3 (*Raldh3*), cytochrome P450 family 26 subfamily B member 1 (*Cyp26b1*), retinoic acid receptor alpha (*Rara*), retinoic acid receptor beta (*Rarb*), cellular retinoic acid-binding protein 1 (*Crabp1*), and cellular retinoic acid-binding protein 2 (*Crabp2*).

As shown in Figure 3.7, expression of *Crbp1*, which encodes an intracellular retinoid transport protein, displayed a reduction ($p < 0.001$) in lactating (0.26 ± 0.18) mammary tissue compared to virgin (1.00 ± 0.44) and involuted (1.57 ± 0.60) mice, but was only statistically significant between lactation and involution groups (Figure 3.7 B). Likewise, expression of genes associated with retinol transport in the circulation and cellular uptake: *Rbpr2*, *Stra6*, and *Rbp4* were shown to decline significantly in expression ($p < 0.005$, $p < 0.005$ and $p < 0.0005$, respectively) from virgin to lactating dams, which increased to around virgin levels upon involution. *Rbpr2* expressed a fold change of 1.00 ± 0.72 to 0.079 ± 0.092 to 1.51 ± 0.48, respectively (Figure 3.7 E), while *Stra6* expressed a fold change of 1.00 ± 0.53 to 0.13 ± 0.050 to 0.633 ± 0.289, respectively (Figure 3.7 D). Similarly, a fold change of 1.00 ± 0.53 to 0.011 ± 0.0047 to 0.92 ± 0.36, respectively was expressed by *Rbp4* (Figure 3.7 F). Figure 3.7 C also

depicts a statistically significant reduction of *Crbp3* expression, from 1.00 ± 0.55 in virgin mice to 0.31 ± 0.069 in lactating mice, with $p < 0.01$. Expression in involuted mice (0.49 ± 0.23) increased slightly, however, remained non-significant to both virgin and lactating groups.

We additionally measured values of genes encoding *Lrat,* a gene responsible for esterifying retinol into retinyl ester for incorporation into mammary milk droplets. Of note, we observed a significant increase in *Lrat* expression during lactation, which returned to baseline following involution with values of 1.00 ± 0.58 to 2.76 ± 0.92 to 0.43 ± 0.14, respectively ($p < 0.0001$, Figure 3.7 A). This suggests that morphological changes in mammary tissue which support the development of ductal lipid droplets during lactation may correlate with increased retinyl ester reserve formation in the mammary milk. Upon further examination of the expression of mediators of retinoid metabolism, *Rdh1* and *Dhrs3* were assessed to observe any changes in the oxidation of retinol to retinaldehyde, and reduction of retinaldehyde back into retinol, respectively (172). Following the change from virgin to lactating mammary structure, dams had significantly reduced expressions of both *Dhrs3* (1.00 ± 0.51 to 0.16 ± 0.078, respectively) and *Rdh1* (1.00 ± 0.83 to 0.13 ± 0.059, respectively), with both exhibiting a significant increase in expression upon involution (1.20 ± 0.69 and 1.13 ± 0.48, $p < 0.01$ and $p < 0.001$, respectively; Figure 3.7 G and H). *Raldh1* and *Raldh3* displayed a similar pattern of mammary expression across virgin, lactating and involution stages (Figure 3.7 I and J). *Raldh1* and *Raldh3* are known to encode isoenzymes of retinaldehyde dehydrogenase, which irreversibly oxidize retinal into retinoic acid (173). When we examined these genes across virgin (1.00 ± 0.53 and 1.00 ± 0.63, respectively), lactating (0.052 ± 0.041 and 0.043 ± 0.032, respectively) and involuted (1.37 ± 0.85 and 0.97 ± 0.20, respectively) stages in mice, we observed a significant decline in mammary expression during lactation which returned to levels comparable to the virgin state upon

involution. These values were statistically significant with $p < 0.005$ and $p < 0.005$ respectively. Additionally, when we examined mammary *Cyp26b1* expression, we observed a significant reduction ($p < 0.001$) from virgin to lactating dams, however, this expression did not significantly change in involuted dams. This was depicted by a fold change from virgin to lactation to involution of 1.00 ± 0.53 to 0.022 ± 0.011 to 0.41 ± 0.25, respectively (Figure 3.7 O). *Cyp26b1* encodes a hydroxylase enzyme which catabolizes retinoic acid into polar metabolites for disposal (174) and is known as a highly sensitive marker of retinoic acid regulation. Therefore, these results propose that during the lactational period, mammary tissue may undergo a decline in retinoic acid biosynthesis and subsequent transcriptional activity. *Rara* and *Rarb*, genes responsible for isoforms of the nucleic retinoic acid receptor, also express sensitivity to retinoic acid levels (175) and affirm this finding in lactating mammary gland. As such, both genes were observed to decline in expression from virgin to lactating dams, with a significant increase during involution ($p < 0.05$ and $p < 0.001$, respectively). *Rara* expressed a fold change of 1.00 ± 0.60 to 0.51 ± 0.27 to 1.33 ± 0.61, respectively (Figure 3.7 K), while *Rarb* expressed a fold change of 1.00 ± 0.47 to 0.12 ± 0.085 to 1.33 ± 0.58, respectively (Figure 3.7 L). Finally, we examined *Crabp1* and *Crabp2*; retinoic acid binding protein isoforms involved in the intracellular transport of retinoic acid to cytochrome P450 family enzymes for catabolism or retinoic acid receptors in the nucleus to elicit transcriptional activity (176,177). Interestingly, dams displayed significant variations in the expression of both genes ($p < 0.05$, $p < 0.01$, respectively). *Crabp1* presented a significant reduction in expression during lactation with a fold change of 1.00 ± 0.78 to $0.082 \pm 0.0.030$ to 1.05 ± 0.43, respectively (Figure 3.7 M), while *Crabp2* depicted a significant rise in gene expression in lactating dams with a fold change of 1.00 ± 0.45 to 3.02 ± 1.85 to 0.95 ± 0.33, respectively (Figure 3.7 N).


A
Lrat Fold Change
a
b
a
Virgin
Lactation
Involution
B
Crbp1 Fold Change
ab
a
b
C
Crbp3 Fold Change
a
b
ab
D
Stra6 Fold Change
a
b
a
E
Rbpr2 Fold Change
a
b
a
F
Rbp4 Fold Change
a
b
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G
Dhrs3 Fold Change
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Rara Fold Change
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Rarb Fold Change
a
b
a
M
Crabp1 Fold Change
a
b
a
N
Crabp2 Fold Change
a
b
a
O
Cyp26b1 Fold Change
a
b
b

Figure 3.7 qPCR analysis of mammary retinoid pathway protein expression during virgin, lactation (P7) and involution (P42) time points. All genes are normalized to their respective virgin state and depict a fold-change relative to the reference gene *CycloA*. (A) Lecithin retinol acyltransferase, (B) cellular retinoid binding protein 1, (C) cellular retinol binding protein 3, (D) signaling receptor and transporter of retinol 6, (E) retinol binding protein receptor 2, (F) retinol binding protein 4, (G) dehydrogenase/reductase 3, (H) retinol dehydrogenase 1, (I) retinaldehyde dehydrogenase 1, (J) retinaldehyde dehydrogenase 3, (K) retinoic acid receptor alpha, (L) retinoic acid receptor beta, (M) cellular retinoic acid-binding protein 1, (N) cellular retinoic acid-binding protein 2, (O) cytochrome P450 family 26 subfamily B member 1. All data is analyzed by one-way ANOVA. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

3.4.4 Expression of Retinoid Metabolizing Genes in the Dam Liver at Virgin, Lactating, and Involuted Stages

Hepatic gene expression of key mediators of retinoid metabolism were analyzed to evaluate the influence of lactation on the formation and mobilization of hepatic vitamin A stores for delivery to mammary milk. Expression of hepatic lecithin retinol acyltransferase (*Lrat*), cellular retinoid binding protein 1 (*Crbp1*), retinol binding protein 4 (*Rbp4)*, retinoic acid receptor beta (*Rarb*), cytochrome P450 family 26 subfamily A member 1 (*Cyp26a1*) and cytochrome P450 family 26 subfamily B member 1 (*Cyp26b1*) were examined (Figure 3.8). Unlike mammary tissue, the relative expression of *Lrat* across virgin (1.00 ± 0.32), lactation (1.47 ± 0.80), and involution (1.32 ± 0.79) time points depicted no significant variation, which suggests that esterification of all-trans-retinol into retinyl ester stores remained unchanged in the dam liver (Figure 3.8 A). *Crbp1* rather showed a significantly higher ($p < 0.01$) expression during lactation (2.20 ± 0.91) compared to virgin and involuted dams which were expressed by a fold change of 1.0 ± 0.51 and 0.900 ± 0.65, respectively (Figure 3.8 B). Since *Crbp1* encodes an important binding protein responsible for intracellular retinoid chaperoning (178), this proposes an increase in cytosolic transport of retinol during this stage. *Rarb*, a nucleic retinoic acid receptor, also significantly increased ($p < 0.05$) in expression during lactation (1.45 ± 0.32) compared to virgin mice (1.00 ± 0.41), yet was non-significant compared to mice at involution (1.02 ± 0.40) (Figure 3.8 D). As shown in Figure 3.8 E and F, relative expression of *Cyp26a1* and *Cyp26b1* in the liver of virgin, lactating and involuted dams were not significantly altered. *Cyp26a1* expressed a fold change of 1.00 ± 0.34 to 1.50 ± 0.38 to 1.54 ± 0.79, respectively, while *Cyp26b1* expressed a fold change of 1.00 ± 0.33 to 0.92 ± 0.59 to 0.44 ± 0.51, respectively. Likewise, expression of *Rbp4* was unaltered across virgin (1.00 ± 0.39), lactating (0.95 ± 0.30) and involuted dams (1.38 ± 0.41), indicative of unaltered production of *Rbp4* in the liver and subsequent transport of retinol in the

circulation (4) (Figure 3.8 C). This was further confirmed by ELISA RBP4 analysis of plasma, which revealed no significant alterations in circulating RBP4 (Figure 3.4 B).

3.5 Discussion

Although the current literature has alluded to the significant role of vitamin A as both a regulator of mammary tissue morphology and a critical nutrient stored in the mammary milk, a comprehensive understanding of maternal retinoid homeostasis and gene expression across virgin, lactation, and involution life stages has not been well characterized. According to our findings, during lactation the expression pattern of the vitamin A metabolic pathway at the mammary gland undergoes a shift in favor of retinyl ester synthesis for incorporation into milk, rather than vitamin A oxidation into active metabolites (Figure 3.9). This allows high concentrations of retinoids that are prevalent in breast milk (almost exclusively as retinyl esters) to be made available to offspring, helping to establish their vitamin A reserves during lactation (9,11,38). Despite this insight, many breast cancer models also emphasize the regulatory role of retinoid pathway in promoting proper maintenance and morphogenesis of mammary structures. As a result, we aimed to better understand how alterations in mammary morphology influence retinoid pathway signaling, by comprehensively assessing maternal vitamin A homeostasis and retinoid gene expression at virgin, lactating and involuted stages.

As mentioned, the mammary gland undergoes profound structural and functional differentiation during the physiological stages of gestation, lactation, and involution.

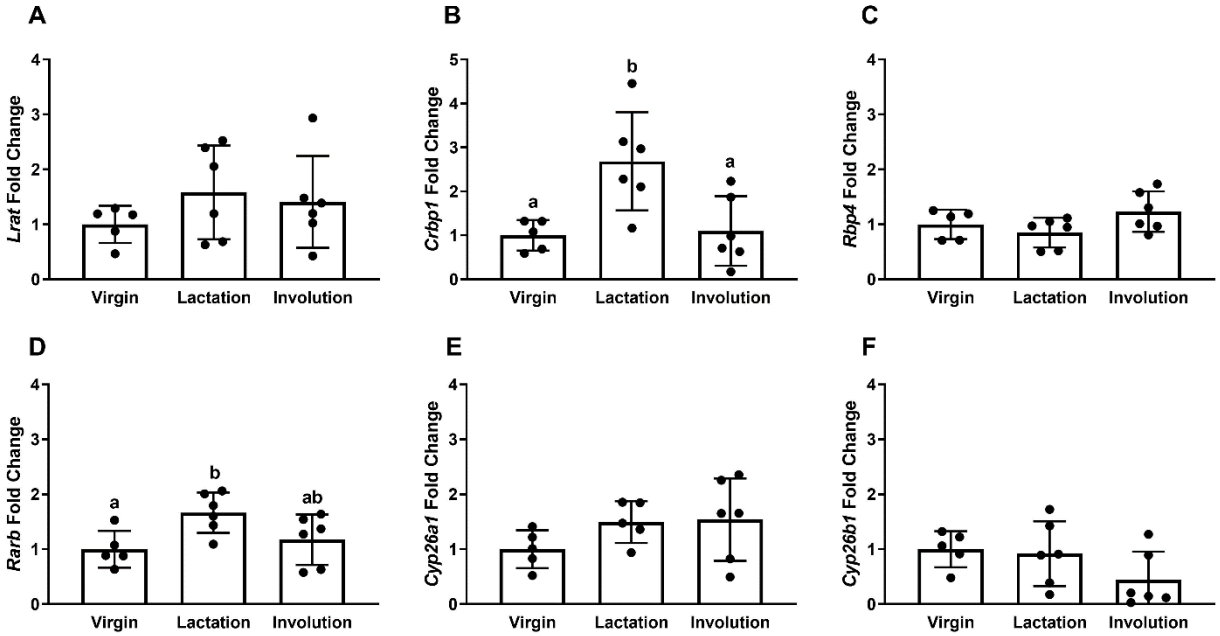

Figure 3.8 qPCR analysis of retinoid pathway protein expression in the liver during virgin, lactation (P7) and involution (P42) time points. All genes are normalized to their respective virgin state and depict a fold-change relative to the reference gene *18s*. (A) Lecithin retinol acyltransferase, (B) cellular retinoid binding protein 1, (C) retinol binding protein 4, (D) retinoic acid receptor beta, (E) cytochrome P450 family 26 subfamily A member 1, (F) cytochrome P450 family 26 subfamily B member 1. All data is analyzed by one-way ANOVA. Groups that do not share a common letter are statistically significant from each other, which is denoted by $p < 0.05$.

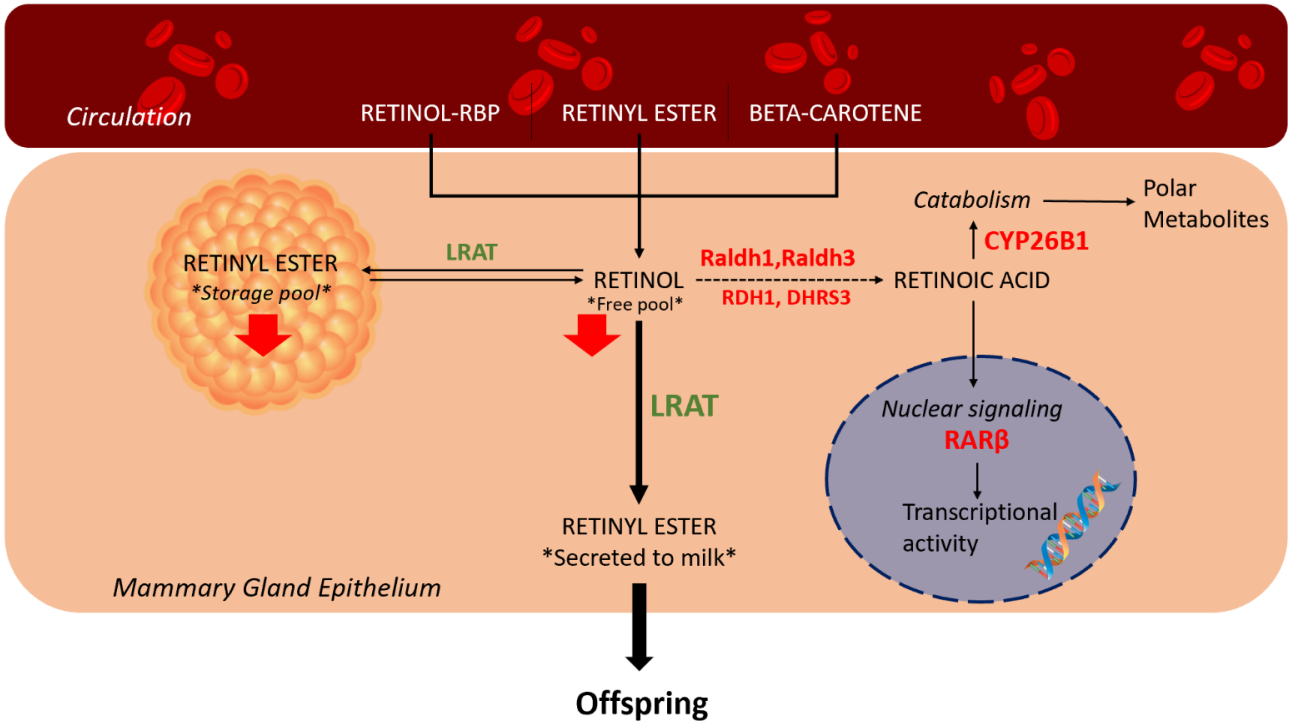

Figure 3.9 Summary figure displaying main conclusions drawn from Chapter 3. Vitamin A mammary homeostasis and retinoid gene expression of enzymes involved in vitamin A esterification and metabolism during lactation were examined. Green and red lettering denote an increase and decrease in gene expression, respectively. Created with BioRender.com.

Throughout gestation and lactation, alveolar epithelium in the mammary gland proliferates and differentiates, allowing it to take on a secretory and milk producing lobuloalveolar structure containing lipid droplets which serve as storage sites for esterified vitamin A (9,135,136). This is important because the accumulation of vitamin A in these lipid droplets during milk production ensures adequate vitamin A transfer to meet offspring demands (9,140). Therefore, prior to assessing retinoid homeostasis and gene expression, we needed to first ensure proper epithelial differentiation in the mammary tissue in our study model. Depicted in the work of Robinson (179) *et al.*, examining the expression of milk proteins is an accurate means of assessing for this. Prominent milk proteins *Wap*, *Csng* and *Lalba* were therefore assessed as controls to confirm these morphological changes. As expected, we observed a dramatic increase in all three genes during the lactational period, which returned to virgin levels upon involution, suggestive of the mammary tissue losing its adipocytic structure for a glandular ductal structure producing milk droplets. These results were additionally confirmed by histological analysis of virgin, lactating and involuting mammary tissue, which depicted identical morphological changes with defined glandular epithelial structures observed during lactation in place of the dense cluster of unilocular adipocytes observed during virgin and involution states (Figure 3.2). Analysis of physical characteristics also showed that lactating dams had significantly heavier body weight and maternal liver weights in comparison to virgin mice, which can also be indicative of our mice achieving pregnant and lactating states. This is because hepatic enlargement driven by hepatocyte proliferation and increased accumulation of adipocyte deposits are known adaptations in mice to the demands of pregnancy and therefore could contribute to this increase in weight (171).

After affirming the appropriate differentiation of our mouse model across virgin, lactating, and involuted states, we aimed to examine if these variations in mammary morphology favor the conversion of retinol directly into retinoic acid for transcriptional activity versus retinyl ester incorporation into the mammary milk. Whole mammary gland was assessed to investigate the expression of genes pertaining to retinoid related lipids and retinoid pathway genes. As mentioned previously, LRAT is accepted as the primary enzyme responsible for esterifying retinol into retinyl ester for storage in the mammary milk droplets during lactation (125). As a result, we were keen to explore the prevalence of this gene as an indicator for milk vitamin A retinyl ester accumulation. Interestingly, we observed a significant elevation in the expression of *Lrat* during lactation, which returned to baseline following involution (Figure 3.7 A). This suggests that the differentiation of the mammary gland during lactation, which supports breastmilk production through the development of ductal lipid droplets, may act parallel to increased vitamin A esterification, to meet the retinoid demands of the offspring. As such, we are one of the first research programs to directly examine increases in *Lrat* expression in lactating mice compared to virgin and involuted mice. This finding is consistent with the work of O'Byrne *et al.* (125), who note a significant increase in the expression of genes in the mammary epithelium associated with retinoid uptake and esterification during the lactational period, with an emphasis on LRAT catalytic activity being critical for the majority of this storage activity with *Lrat* knockout mouse models. Retinoid related lipid genes; *Cd36*, *Lpl*, and *Dgat1*, were analyzed to assess changes in the translocation, hydrolysis, and synthesis of triglycerides at the mammary gland. The mammary gland stores significant quantities of triglycerides which are synthesized through the enzymatic actions of DGAT1, and serve as substrates for milk production (180,181). Moreover, $Dgat1^{-/-}$ dams have exhibited a failure to lactate (182) as well

as display impaired epithelial proliferation and alveolar development (181). This emphasizes the requirement of DGAT1 not only in milk production, but proper functional differentiation of the mammary gland. Similarly, CD36 and LPL are required for long-chain fatty acid (LCFA) incorporation into milk (148,183,184), however, LPL also acts to hydrolyze vitamin A containing chylomicrons from the postprandial circulation to allow retinoid uptake into mammary milk (2,47,119,125). Contrary to what we anticipated, all three genes displayed non-significant changes in expression between virgin, lactating and involuted dams, suggesting that retinoid and fatty acid delivery to mammary milk reserves is maintained across these three physiological states. This was an unexpected finding, as we predicted an upregulation in the expression of these genes during lactation to support milk lipid composition as shown in lactating murine mammary models by Han *et al*. (185) who depicted significant increases in mRNA expression of these genes. We were particularly surprised by the maintained expression of LPL, as it is well-established that this gene increases throughout the entire period of lactation [as much as ten-fold (145)], while being simultaneously depressed in adipose tissue to allow for the redirection of vitamin A incorporation into milk fat (144,145,147,149). Therefore, we attribute a limitation of our study being that we assessed whole mammary tissue instead of exclusively assessing epithelial cells with an organoid model and could have benefited from assessing protein expression in addition to the mRNA level of genes of interest, which may have provided us greater insight, which we plan to examine in future studies.

Aside from the mammary gland's capacity to esterify and store retinoids, vitamin A can also be utilized by the retinoid metabolic pathway as a precursor for the synthesis of its active metabolite retinoic acid. Through the action of its nuclear receptors, retinoic acid elicits transcriptional activity important for physiological homeostasis, including processes specific to

mammary gland maintenance and differentiation (133,151,154,175). Therefore, we were curious to know if this pathway is prioritized during the lactational period, given its investigation in various breast cancer models. Retinoid gene expression was subsequently evaluated by qPCR analysis to assess alterations in the expression of genes directly associated with the retinoid metabolic pathway, including those involved in retinoid transport, uptake, and mediators of retinoid metabolism into retinoic acid. First, we assessed the expression of *Crbp1* and *Crbp3*, cellular retinoid transport proteins responsible for efficiently channeling retinol to enzymes required for its oxidation into retinoic acid or esterification into retinyl ester (48,178,186). Work by Piantedosi *et al.* (186) and Pierzchalski *et al.* (131), have alluded to CRBP3 protein levels being significantly increased in mammary tissue during lactation for retinoid incorporation into milk and the requirement for CRBP1 for the maintenance of mammary morphological structures to prevent the incidence of breast oncogenesis. Therefore, we were surprised that the expression of *Crbp3* was significantly reduced during lactation in mammary tissue, given that *Crbp3* knockout have mice previously been shown to have diminished amounts of milk retinyl esters (186). Additional studies have alluded to increased CRABP2 and CRBP1 protein levels during involution to modulate increased retinoic acid signaling for tissue remodeling via apoptosis (9,132,153). This may explain the elevated expression of *Crbp1* we observed in involuted mammary tissue in comparison to lactating mammary tissue, although this is uncertain without further analysis dissecting the expression of *Crbp1*. Furthermore, this unexpected expression of *Crbp3* may have simply resulted from the mentioned limitation of our model using whole mammary tissues and allocated time points in which we examined these tissues. Expression of genes associated with retinol transport in the circulation and cellular uptake: *Rbpr2*, *Stra6*, and *Rbp4* were also shown to decline significantly in expression from virgin to lactating dams, which

increased to around virgin levels upon involution. At extrahepatic cells, cell surface receptor STRA6 interacts with RBP4 to facilitate both the cellular uptake and efflux of retinol (52,53). Since it has been well established that vitamin A uptake at the mammary tissue increases significantly during lactation, we were again unsure if this may result from the limitation of our model or could be an indicator of the prioritization of the chylomicron lipoprotein lipase pathway in utilizing direct dietary vitamin A uptake over circulating retinol-RBP4 (which these genes are heavily involved with) (126). With regards to the direct mediation of the retinoid metabolic pathway, we examined the expression of *Dhrs3, Rdh1, Raldh1,* and *Raldh3* to observe any changes in the oxidation of retinol to retinoic acid. Interestingly, expression of all four genes were shown to significantly reduce in lactating dams compared to their virgin and involuted counterparts. Given that *Rdh1, Raldh1,* and *Raldh3* are required for the oxidation of retinol into retinaldehyde, and irreversible oxidation of retinaldehyde into retinoic acid, respectively (1,54,172), we conclude that the metabolism of intracellular retinol into retinoic acid is significantly hindered during the lactational period, potentially in support of vitamin A esterification and utilization in milk, as alluded to in prior results. Additionally, *Dhrs3* is required for the reduction of retinaldehyde back into retinol and is a negative regulator which acts to prevent the over-production of retinoic acid. Given we see a reduction in this gene's expression, we assume that retinoic acid isn't being heavily produced to require an increase in the regulatory activity of DHRS3. Upon examining *Cyp26b1,* a gene responsible for the catabolism of retinoic acid into polar metabolites for excretion (55,56,174), we observed an analogous reduction in expression from virgin to lactating dams, consistent with a decline in retinoic acid biosynthesis. Similar to DHRS3, this gene is a highly sensitive marker of retinoic acid regulation to prevent excessive amounts of retinoic acid production. Because we observe a

reduction in its expression during lactation, we can assume retinoic acid production during lactation is minimal. This is reinforced by the examined decline in nucleic retinoic acid receptors (*Rara* and *Rarb*) and retinoic acid binding protein *Crabp1* gene expression, indicative of reduced intracellular transport of retinoic acid to its target nuclear receptors (178) and loss of subsequent transcriptional activity. Although not well understood why these genes are expressed minimally during lactation, data from Zaragoza *et al.* (134) proposes that increased retinoic acid levels are likely responsible for tissue remodeling and the initiation of involution via RARα elevating metalloproteinase activity. Combined with the findings of other authors, we may speculate that retinoic acid activity is more heavily involved in the transition of the virgin mammary tissue into lactating tissue during late pregnancy (a time point we did not assess) and subsequent regression and milk inhibition during involution (133,134,152). While cancer studies have referred to the requirement of this pathway for the maintenance of lactating tissue, this may appear limited in comparison to gene expression involved in the vitamin A esterification and incorporation into milk during this time. As a result, these findings broadly suggest that during the lactational period, mammary tissue undergoes a decline in retinoic acid biosynthesis and subsequent transcriptional activity in favor of vitamin A esterification and utilization in the mammary milk produced for offspring uptake.

Hepatic gene expression of key mediators of retinoid metabolism were additionally analyzed to evaluate influences of the lactational period on the formation of hepatic stores or utilization of vitamin A for retinoic acid synthesis. Unlike in the mammary tissue, *Lrat* expression remained constant regardless of the mammary morphological state, which suggests that the storage of vitamin A through the esterification of retinol into retinyl ester remained unaltered in the liver. This observation paired with the non-significant change in hepatic retinyl

ester content, affirms that during lactation there is a switch to prioritize the mammary gland as the prominent site of vitamin A uptake and storage, instead of the liver. With regards to the retinoid metabolic pathway, *Crbp1* and *Rarb* expression increased upon lactation, indicative of an increase in cytosolic transport of retinol and nucleic retinoic acid receptor binding in the liver during lactation. Despite this insight, relative expressions of Cyp26a1 and Cyp26b1 compared across virgin, lactating and involuted dams were not significantly altered, indicating that while certain members of the retinoid pathway may be elevated, it is unlikely that retinoic acid synthesis significantly increased at the liver during lactation. This is because we would otherwise expect a substantial up-regulation of the CYP26 enzymes, which are highly sensitive to retinoic acid levels and are known to elicit a negative autoregulatory feedback that catabolizes retinoic acid into polar metabolites to regulate its cellular levels (55,56). Therefore, we conclude that the change in mammary morphology across virgin, lactating, and involution states did not significantly influence alterations in hepatic vitamin A storage and retinoic acid synthesis. Finally, evaluation of mean plasma retinol concentrations and hepatic *Rbp4* expression were unaltered across virgin, lactating and involuted dams. This proposes there to be unaltered production of *Rbp4* in the liver and subsequent maintained transport of retinol into and within the circulation, indicative of maternal retinyl ester reserves not being substantially utilized for mammary milk vitamin A. This is because if the liver was significantly delivering retinol to the mammary tissue for incorporation into milk, *Rbp4* expression would rather be upregulated. ELISA RBP4 analysis of plasma further confirm this observation by depicting no significant alterations in circulating RBP4 proteins.

Apart from qPCR analysis, dam mammary retinol and retinyl ester content were examined by HPLC to elucidate the impact of virgin, lactating and involuted stages on maternal

vitamin A reserves and subsequent maternal vitamin A homeostasis. Consistent with the increased recommended dietary allowance of vitamin A in pregnant and lactating women [requiring 750 μg of RAE/day and 1300 μg of RAE/day, respectively (9,16,24)] to supply adequate offspring vitamin A reserves, we predicted retinoid content in mammary tissue to be significantly impacted, particularly with a reduction in the lactating dams. As anticipated, mammary retinol and retinyl ester content declined ~81% and ~69%, respectively in lactating mice, which recovered to virgin levels upon involution. We assume these findings reflect the loss of maternal mammary vitamin A stores to meet the nutritional requirements of the pups, through the milk secreted during feeding. Interestingly, HPLC analysis of retinoid content in the visceral white adipose tissue, plasma, and the liver remained unaltered from virgin to lactating mice. If dams were reliant on using these body stores for milk retinyl ester synthesis, we would expect liver and VWAT vitamin A retinyl esters to be significantly decreased. Therefore, the maintenance in vitamin A status from non-mammary tissues, taken together with our mammary HPLC results, affirms that the development of offspring vitamin A reserves during lactation is primarily attributed to the accumulation of vitamin A in the milk via local vitamin A stores in the mammary gland, rather than the contribution of whole-body vitamin A reserves, which do not seem to significantly contribute to this process. This is a novel finding which has not been reported in prior literature. Our mammary and hepatic qPCR results mentioned above parallel this thinking, which reveal that the lactating mammary gland prioritizes vitamin A uptake and esterification via increased LRAT activity for secretion in milk, while *Lrat* and *Rbp4* expression are unchanged in the liver, suggesting these retinoid reserves are not being utilized to deliver significant concentrations of retinol to the mammary milk.

Taken together, we conclude that alterations in mammary gland morphology during lactation shifts retinoid metabolism in this tissue to prioritize retinoid incorporation into the milk during lactation for offspring uptake, above its role as a transcriptional regulator through the oxidation of retinol via the retinoid signalling pathway (Figure 3.9).

CHAPTER 4 - Role of Maternal Dietary Vitamin A Intake in Establishing Offspring Vitamin A Reserves

4.1 Introduction

Vitamin A is a critical micronutrient throughout gestation and lactation, particularly in the neonate for the establishment of foundational vitamin A reserves and development of various physiological processes. Despite its importance, vitamin A deficiency is known to be highly prevalent during stages of life where nutritional demands are higher, due to inadequate dietary intake and poor maternal vitamin A status (3,9,65). As previously discussed in Chapter 1, there are currently two basic mechanisms of vitamin A distribution to the placenta and mammary gland during gestation and lactation, respectively. This involves the transfer of retinoids from maternal hepatic stores or dietary chylomicrons (Figure 1.2). When postprandial dietary retinoids are packaged into chylomicrons as retinyl esters, instead of going to the liver to be taken up for storage and recirculated as retinol-RBP4, they are directly transported to the lactating mammary gland for utilization in the milk (2,10,119,125). While both have been established as important pathways contributing to offspring vitamin A reserves, the relationship between mammary milk vitamin A reserves and dietary vitamin A obtained from chylomicrons has not been extensively examined.

As a result, this chapter aims to elucidate the importance of maternal dietary vitamin A intake with the focus of clarifying its contribution as a determinant of a vitamin A reserves in offspring. This was accomplished by manipulating the dietary vitamin A intake of dams during gestation and lactation and assessing offspring hepatic reserves from birth until adulthood. Since a truly vitamin A deficient model is difficult to establish in mice [involving the eradication of vitamin A in the diet from birth or over multiple generations (84,187,192,193)], we were able to ensure that maternal hepatic stores were present if dams were raised on a normal chow diet, prior to manipulating vitamin A concentrations in their diet during gestation and lactation. Utilizing a

model where existing maternal hepatic reserves are normal, therefore allowed us to assess the contribution of diet to maternal and offspring retinol and retinyl ester reserves. Should maternal dietary intake of vitamin A be the primary determinant of offspring vitamin A reserves, we would anticipate that pups from mothers on the vitamin A deficient diet to have significantly lower hepatic retinol and retinyl ester stores. Therefore, we hypothesize that maternal mice with normal vitamin A reserves, but inadequate vitamin A intake will generate offspring with poor vitamin A reserves. This is a novel perspective and suggests that regardless of maternal vitamin A stores, adequate vitamin A intake during pregnancy and breastfeeding is essential.

4.2 Experimental Design

In our study design, Balb/c dams were separated into two dietary groups and mated with male counterparts, with one group receiving a control purified diet (25 IU vitamin A/g diet) and the other receiving a deficient diet (0 IU vitamin A/g diet), throughout gestation and lactation. Offspring tissues were collected at birth (postnatal day [P]1), during lactation (P7), at weaning (P21), two weeks post-weaning (P35), P70 and P105 (Figure 4.1). Pups remained on their respective dam's diet until weaning (P21), at which point all pups were provided with a sufficient chow diet with 18 IU vitamin A until their respective tissue collections. Using this model allowed us to ensure the maintenance of normal maternal hepatic stores, while manipulating dietary vitamin A as our experimental group to directly assess the role of maternal dietary intake on the development of offspring reserves through transplacental transfer during gestation and mammary milk during lactation. Evaluating pups after weaning also allowed us to observe if any alterations in pup vitamin A status resulting from maternal dietary intake were significant enough to persist into adolescence and later adulthood, despite mice ingesting a

vitamin A sufficient diet. Quantitative amounts and molecular species of vitamin A were assessed by HPLC analysis and gene expression analysis was examined by qPCR as described in Chapter 2. Liver tissues were assessed in all offspring, while lung, visceral white adipose tissue and plasma were additionally examined in P21, P35, P70 and P105 mice. Dam liver and plasma were collected at P21. Physical characteristics of pups and dams were evaluated during tissue collection to investigate for indications of vitamin A deficiency as well as differences in body and liver weight between mice of the same age in different study groups.

4.3 Specific Methods

A description of the animal husbandry and tissue collection process, vitamin A quantification by high performance liquid chromatography, gene expression analysis and statistical analyses are provided as general methods in Chapter 2. A comprehensive list of genes and primers sequences used in liver qPCR analysis are shown in Table 2.1.

4.3.1 Animal Study Diets

This study was conducted using two groups of pregnant Balb/c mice, with differing dietary vitamin A intake. Specialized research diets containing custom amounts (25 IU and 0 IU) of retinyl palmitate (vitamin A) in purified pellets were utilized and acquired from Bio-Serv (Flemington, New Jersey, USA). Apart from varying quantities of vitamin A, the pellets contained identical AIN-93G vitamin and mineral mix with a standard macronutrient composition. During gestation, dams were placed on either a vitamin A control (25 IU vitamin A/g diet) or vitamin A deficient (0 IU vitamin A/g diet) purified diet.

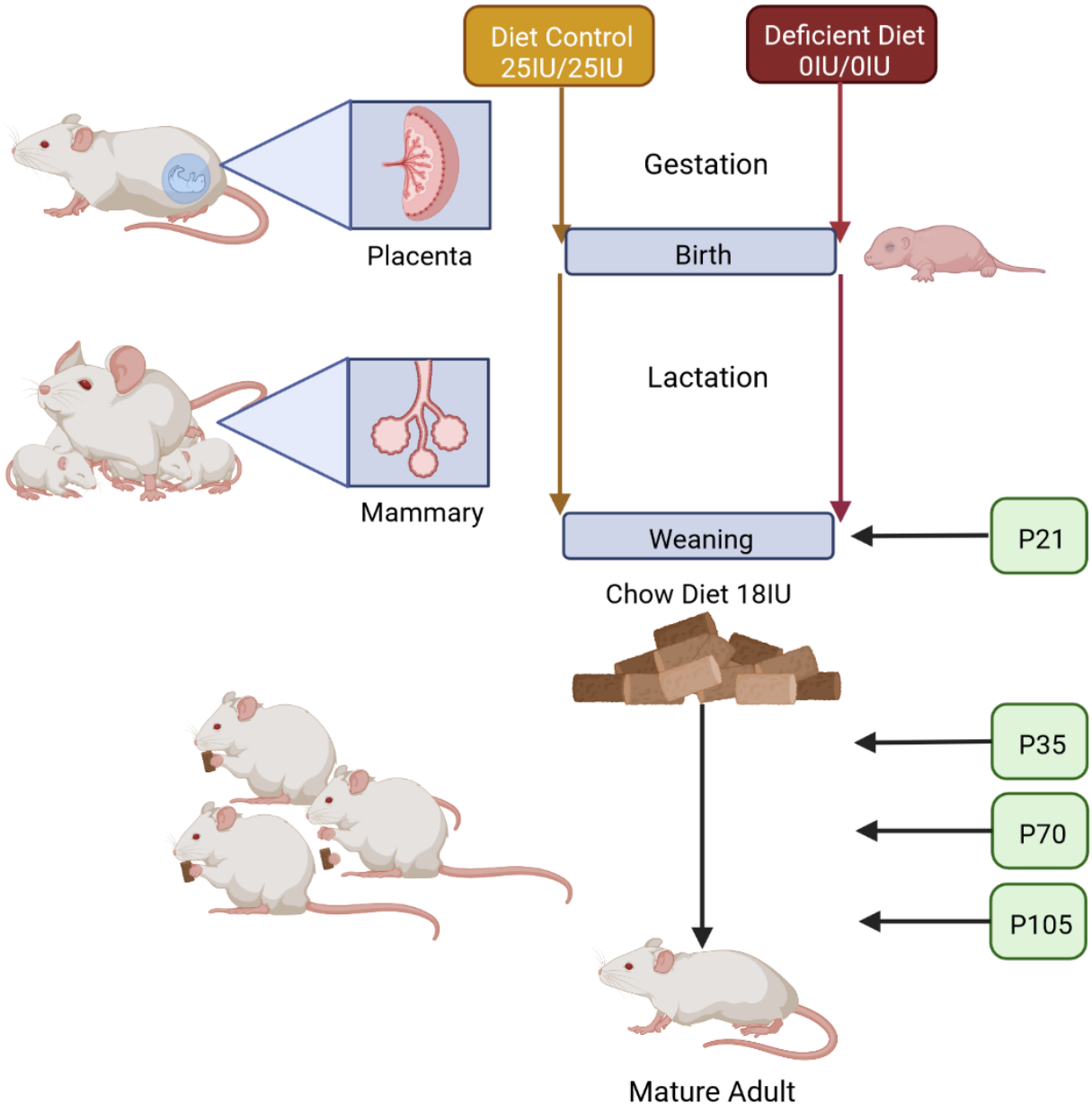

Figure 4.1 Summary figure displaying the experimental design for the role of maternal dietary intake vitamin A stores in establishing offspring vitamin A reserves. Dams were split into two diet group manipulations, with one receiving 25 IU vitamin A and 0 IU vitamin A, throughout gestation and lactation. Hepatic vitamin A content from neonates was examined at P1 (birth), P7 (lactation), P21 (weaning), P35 (two weeks post-weaning), P70 and P105 by HPLC analysis. Created with BioRender.com.

Following parturition, dams were kept on their respective diets (control; 25 IU or experimental; 0 IU) until postnatal day 21, when standard chow containing 18 IU vitamin A/g was introduced. Therefore, IU is representative of vitamin A content consumed during gestation and lactation time points. Table 4.2 details the dietary macronutrient composition and vitamin A content of these respective diets.

4.4 Results

4.4.1 Impact of a Vitamin A Deficient Diet on Maternal Physical Characteristics

Despite manipulations in dietary vitamin A, neither maternal body weight nor maternal liver weight differed significantly. Control group dams placed on the 25 IU diet measured a mean weight of 25.85 ± 2.80 g. This data was consistent with that collected from our experimental group where vitamin A was removed from the diet during both lactation and gestation (0 IU; 25.36 ± 1.47 g) (Figure 4.2 C). Likewise, mean liver weight values did not display a statistically significant change, as dams on the control diet (1.30 ± 0.22 g) displayed comparable liver weight values to those on the deficient diet (1.39 ± 0.17 g; Figure 4.2 D). Comparison of the ratio of maternal liver weight to body weight supports this data by indicating no significant differences between the mean values of dams in the 25 IU control group (0.053 ± 0.012) and 0 IU (0.055 ± 0.0023) experimental group (Figure 4.2 E). Maternal age (days) at the litter's birth was also investigated for any variations which may impact findings. As shown in Figure 4.2 B, there was no significant difference in number of days between control (25 IU) and (0 IU) experimental groups (137.00 ± 25.57 days and 136.00 ± 24.04 days, respectively).

	Vitamin A Deficient Diet	Vitamin A Copious Diet
Fat	19.3%	19.3%
Protein	17.1%	17.1%
Carbohydrate	63.4%	63.4%
Vitamin A	0 IU/g	25 IU/g

Table 4.1 Macronutrient composition and vitamin A content of AIN-93G purified diets. This table depicts the percentage of total calories obtained from macronutrients in each AIN-93G purified diet. Vitamin A content is shown as the number of international units (IU) per gram of purified diet.

Similarly, there was no significant difference in litter size between the mean number of pups in the control (5.78 ± 2.33 pups) and deficient (7.45 ± 1.86 pups) groups (Figure 4.2 A). No obvious differences in appearance suggestive of vitamin A deficiency or pathology (eg. based on activity level, fur quality, moistness of epithelial tissue, growth and size, etc.) were apparent in the dams. Furthermore, dams on a vitamin A deficient diet did not indicate any physical differences in comparison to dams on the control diet.

4.4.2 Effect of a Vitamin A Deficient Diet on Offspring Physical Characteristics

Pup physical characteristics were also assessed to see if manipulations of vitamin A concentrations in the diet would elicit variations in development (Figure 4.3). When examining pup body weight at P1 (Figure 4.3 A), no significant discrepancies were observed between the control and experimental groups (25 IU; 1.40 ± 0.13 g and 0 IU; 1.40 ± 0.23 g, respectively). Similarly, pup body weight values during P7 did not display a statistically significant change. Rather, pups on the control diet (5.83 ± 0.23 g) showed comparable values to the 0 IU (5.48 ± 1.1 g) experimental group (Figure 4.3 B). P21 pups also had similar mean body weight values across control (12.34 ± 1.52 g) and 0 IU (12.00 ± 0.92 g) experimental groups, which were not statistically significant (Figure 4.3 C). Post-weaning, after which pups had been introduced to a 18 IU chow diet, body weight did not significantly differ in P35, P70 and P105 pups born to dams on a 25 IU versus 0 IU diet (Figure 4.3 D, E, F). As such, it was observed that control group pups weighed 17.83 ± 1.42 g, 20.65 ± 1.29 g, and 24.93 ± 2.04 g and experimental group pups weighed 18.36 ± 1.93 g, 21.53 ± 2.44 g, and 24.55 ± 2.98 g, at P35, P70 and P105, respectively. Liver weight was also examined in pups from P7 to P105 in Figure 4.4.

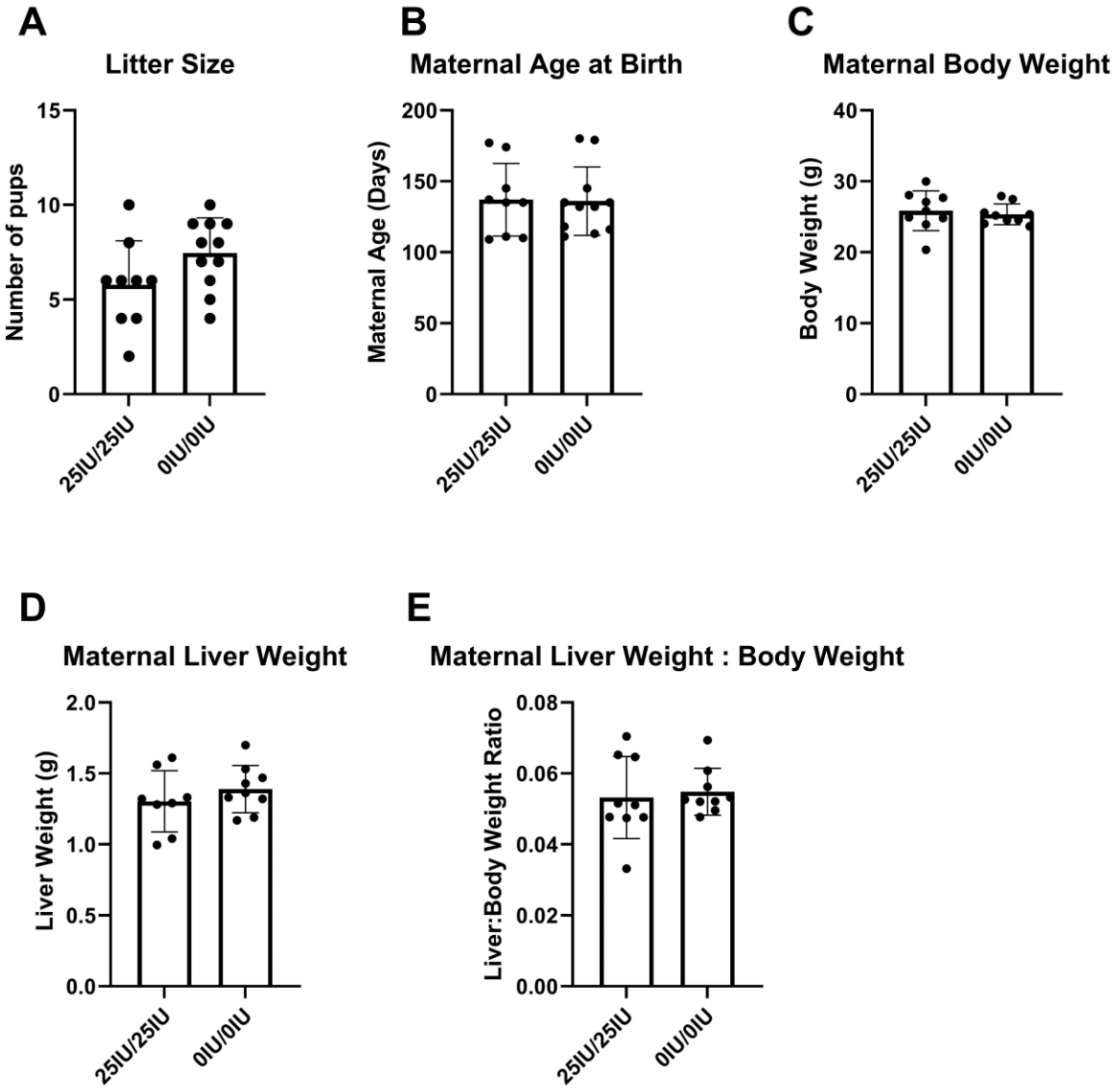

Figure 4.2 Maternal physical characteristics of 25 IU control versus 0 IU mice. (A) Litter size at parturition, (B) maternal age at the birth of the offspring, (C) maternal body weight, (D) maternal liver weight, (E) maternal liver to body weight ratio. All characteristics C - E were assessed upon weaning at postnatal day 21. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

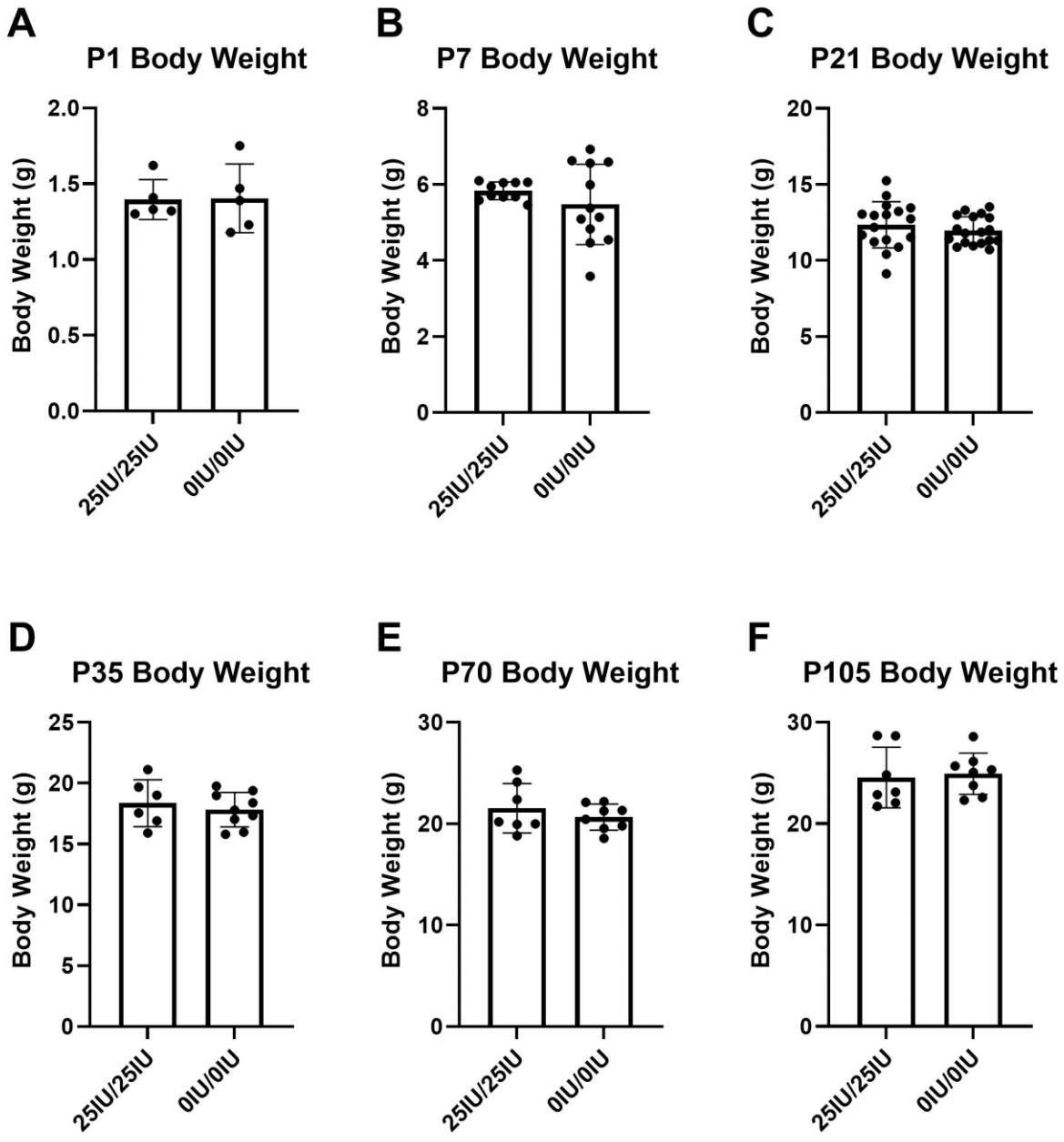

Figure 4.3 Pup body weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup body weight at birth (P1), (B) pup body weight at lactation (P7), (C) pup body weight at weaning (P21), (D) pup body weight at two weeks post-weaning (P35), (E) pup body weight at P70, (F) pup body weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

Liver weight in pups at P7 were not significant between control and 0 IU experimental groups (0.224 ± 0.018 g and 0.217 ± 0.040 g, respectively; Figure 4.4 A). Likewise, pups at P21 had no significant differences in liver weight upon comparison between these diet groups (0.61 ± 0.10 g and 0.57 ± 0.082 g, respectively; Figure 4.4 B). Moreover, liver weight was maintained in P35, P70 and P105 pups regardless of being born to a dam on a 25 IU (0.89 ± 0.14 g, 1.04 ± 0.20 g, and 1.19 ± 0.21 g, respectively) versus a 0 IU diet (0.93 ± 0.13 g, 1.02 ± 0.12 g, and1.31 ± 0.23 g, respectively) as depicted in Figure 4.4 C, D and E. Assessment of the pup liver weight to body weight ratio in Figure 4.5 shows that all mice did not significantly vary between the control group (25 IU) with 0.039 ± 0.0036, 0.049 ± 0.0059, 0.052 ± 0.0042, 0.049 ± 0.0043, and 0.052 ± 0.0054 and the experimental group (0 IU) with 0.040 ± 0.0029g, 0.048 ± 0.0041, 0.048 ± 0.0031, 0.047 ± 0.0048 and 0.048 ± 0.0037 for pups at P7, P21, P35, P70 and P105, respectively. As such, no obvious physical manifestations of vitamin A deficiency were apparent in mice from mothers consuming a vitamin A deficient diet (eg. based on activity level, fur quality, moistness of epithelial tissue, growth, and size, etc.). Lung and VWAT weight also depicted no significant differences amongst pups as shown in Supplementary Figures 4.1 and 4.2. Furthermore, there was no major effect of sex on the physical characteristics and tissue vitamin A content data presented for both male and female offspring of all ages and dietary subgroups (Supplementary Figures 4.3, 4.4, 4.5, 4.6, and 4.7).

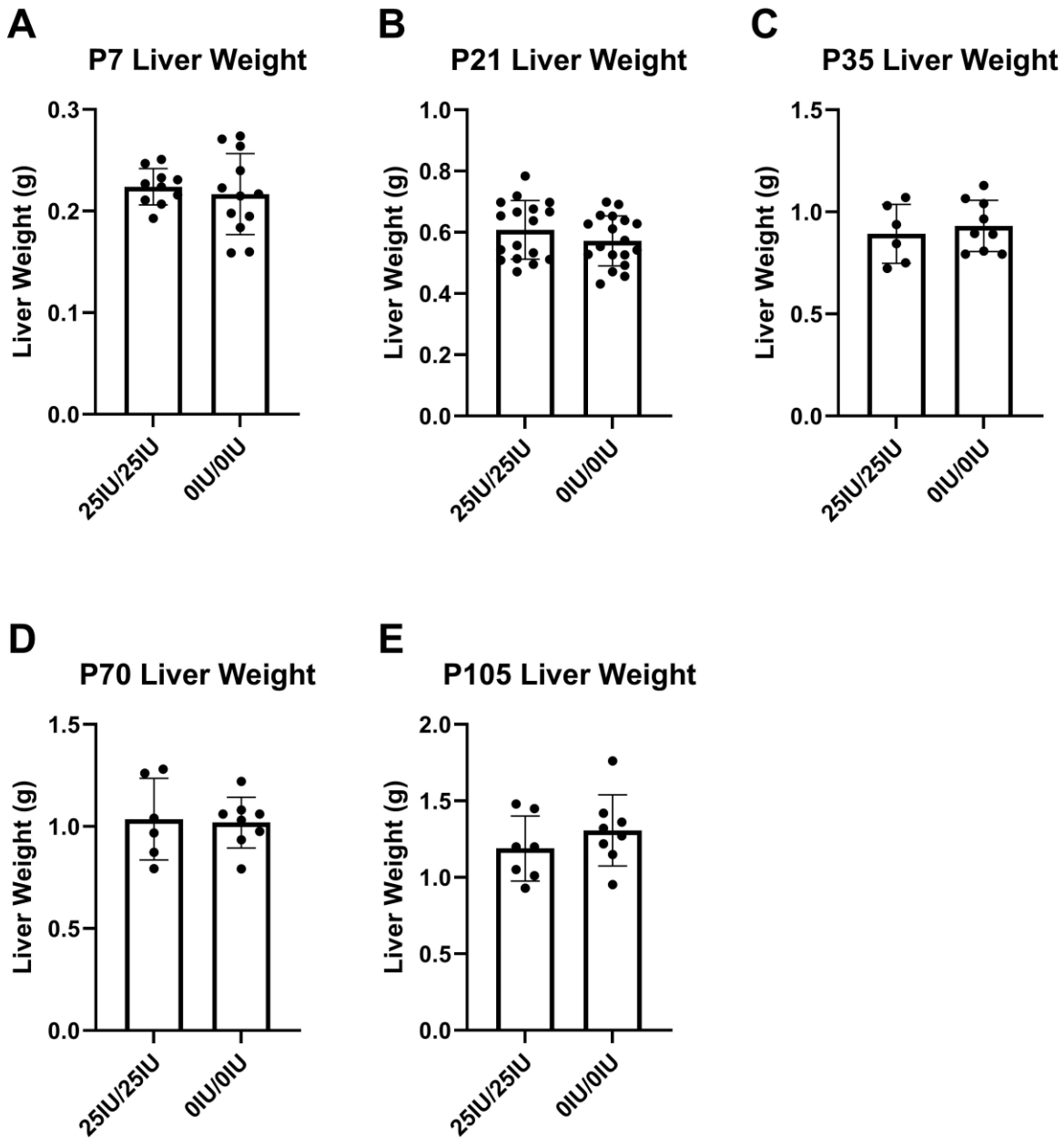

Figure 4.4 Pup liver weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup liver weight at birth (P1), (B) pup liver weight at lactation (P7), (C) pup liver weight at weaning (P21), (D) pup liver weight at two weeks post-weaning (P35), (E) pup liver weight at P70, (F) pup liver weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

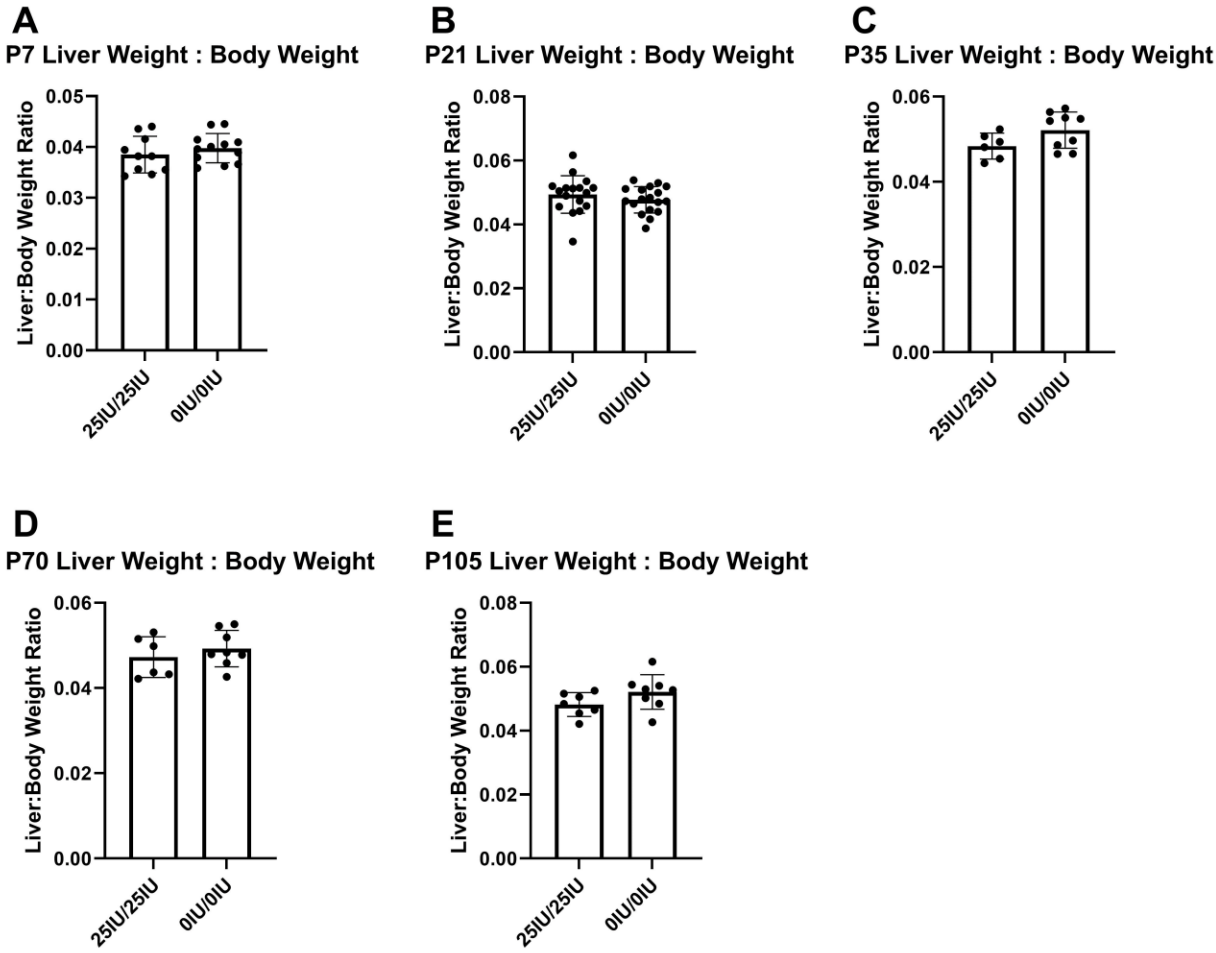

Figure 4.5 Pup liver weight : body weight ratio analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup liver weight : body weight ratio weight at birth (P1), (B) pup liver weight : body weight ratio weight at lactation (P7), (C) pup liver weight : body weight ratio weight at weaning (P21), (D) pup liver weight : body weight ratio weight at two weeks post-weaning (P35), (E) pup liver weight : body weight ratio weight at P70, (F) pup liver weight : body weight ratio weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

4.4.3 Dam Retinol and Retinyl Ester Content with Manipulations of Dietary Vitamin A

HPLC quantification of maternal liver and plasma retinol and retinyl ester concentrations were performed at P21 to elucidate alterations in retinoid status between dams consuming a 25 IU (control) versus 0 IU (deficient) vitamin A diet throughout gestation and lactation (Figure 4.6). Dams consuming a vitamin A deficient diet had a ~81% decrease in hepatic retinol content and ~49% reduction in hepatic retinyl ester reserves, respectively, compared with those consuming the control diet. This was depicted by mother's consuming the 0 IU diet having 9.40 ± 3.29 nmol ROL/g, compared to mother's consuming the 25 IU with 49.22 ± 24.33 nmol ROL/g ($p < 0.005$; Figure 4.6 A). Similarly, hepatic retinyl ester concentrations of dams consuming the deficient diet (749.94 ± 329.41 nmol RE/g) were significantly reduced ($p < 0.01$) in comparison to dams consuming the control diet (1458.83 ± 335.88 nmol RE/g; Figure 4.6 B). Taken together, we assume this to reflect the loss of retinoid reserves in the maternal liver used to maintain circulating levels of vitamin A due to the absence of vitamin A in the diet. This assumption was confirmed through analysis of mean plasma retinol concentrations (Figure 4.6 C), which depicted no significant alterations between dams consuming a 25 IU versus 0 IU vitamin A diet (0.91 ± 0.19 µmol ROL/L and 0.95 ± 0.20 µmol ROL/L, respectively). Therefore, despite the presence or lack of dietary vitamin A intake in dams throughout gestation and lactation, plasma retinol concentrations remain tightly regulated at their normal concentrations in the circulation, which is suggestive of the absence of vitamin A deficiency.

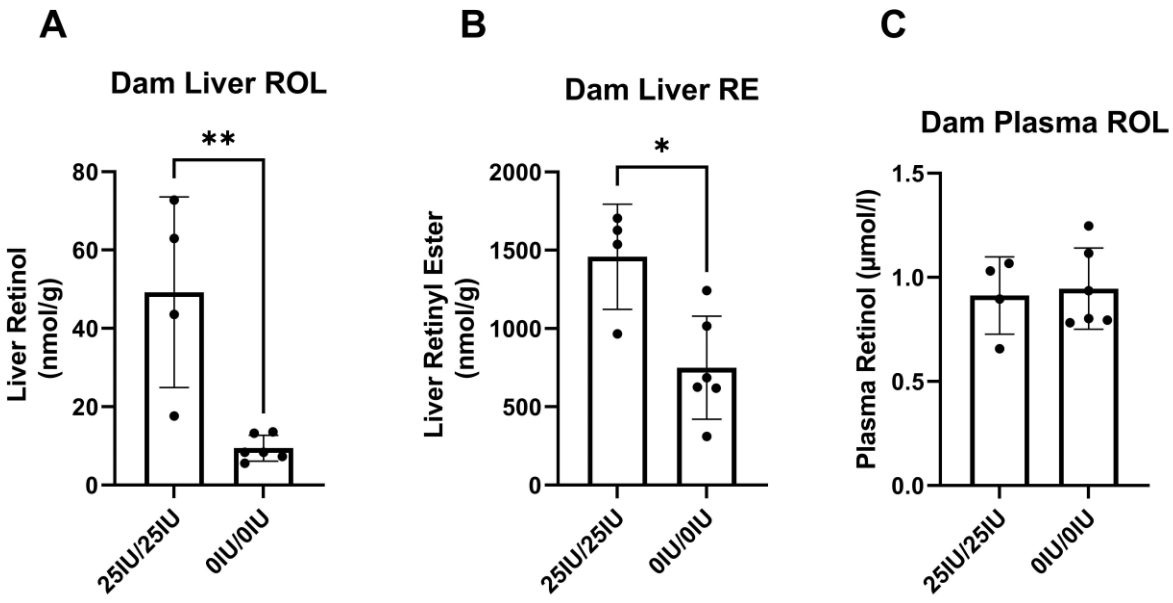

Figure 4.6 HPLC quantification analysis of retinol and retinyl ester concentrations (nmol/g) in dam plasma and liver. Retinoid content was assessed in dams consuming a control (25 IU/25 IU, sufficient) versus deficient (0 IU/0 IU) dietary vitamin A. (A) Retinol content in the dam liver (nmol ROL/g liver), (B) retinyl ester in the dam liver (nmol RE/g liver), (C) retinol content in dam plasma (nmol ROL/g). Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

4.4.4 Comparison of Liver Retinoid Content in Offspring from Dams with Manipulated Amounts of Dietary Vitamin A

Following the analysis of maternal vitamin A status, we examined mean retinol and retinyl ester content in the liver of pups by HPLC analysis. This was conducted in pups born to dams on either a control (25 IU) or deficient (0 IU) vitamin A diet, with tissues examined at P1 (birth), P7 (lactation), P21 (weaning), P35 (two weeks post-weaning), P70 and P105 (Figure 4.7).

P1 evaluation of mean liver retinol and retinyl ester levels, depict that offspring vitamin A status developed normally through transplacental transfer of vitamin A during gestation (Figure 4.7 A and B). As such, we observed no statistically significant differences in pups from dams consuming the 25 IU control (1.44 ± 0.355 nmol ROL/g and 12.1 ± 5.54 nmol RE/g, respectively) versus the 0 IU (1.16 ± 0.255 nmol ROL/g and 13.45 ± 4.90 nmol RE/g, respectively) diet. Livers from P7 pups were next assessed to determine the influence of vitamin A transfer from the dam to offspring hepatic reserves through the mammary milk during lactation. These mice displayed a statistically significant ($p < 0.005$) difference between retinol 25 IU (1.78 ± 0.469 nmol ROL/g) and 0 IU (0.87 ± 0.226 nmol ROL/g) groups, depicting a 51% decline in pups whose mother were given a vitamin A deficient diet (Figure 4.7 C). Interestingly, in Figure 4.7 D, mean retinyl ester concentrations in the P7 liver showed no significant alterations, regardless if pups were born to dams on the control (25.56 ± 7.76 nmol RE/g) or deficient (18.27 ± 10.47 nmol RE/g) diets. This indicates that the dietary influence of the mother was not quite significant to alter the establishment of offspring retinyl ester reserves, but enough to have a significant impact on hepatic reservoirs of retinol. However, at P21 both mean retinol and retinyl ester groups displayed significant results ($p < 0.01$ and $p < 0.0001$, respectively). Retinol concentrations were reduced 60% from 5.67 ± 3.94 nmol ROL/g in 25 IU pups, to 2.25 ±

1.23 nmol ROL/g in 0 IU pups (Figure 4.7 E), and mean retinyl ester concentrations declined 88% from 46.99 ± 13.13 nmol RE/g in 25 IU pups, to 5.46 ± 2.77 nmol RE/g in 0 IU pups (Figure 4.7 F). This suggests that dietary manipulations of dam vitamin A intake until weaning significantly alter pup vitamin A content in the liver. Assessment of mean retinol and retinyl ester levels in P35 mice livers shown in Figure 4.8 A and B respectively, emphasize a sustained effect of insufficient maternal vitamin A intake on offspring vitamin A reserves, despite pups being weaned onto a vitamin A sufficient chow diet for two weeks. Interestingly, this data continued to depict a statistically significant reduction in both retinol and retinyl ester reserves in pups from dams on a vitamin A deficient diet ($p < 0.05$ and $p < 0.001$ respectively), which declined 23% and 41%, respectively. As such, retinol concentrations dropped from 28.44 ± 5.823 nmol ROL/g in 25 IU group mice, to 21.80 ± 4.37 nmol ROL/g in 0 IU group mice. Similarly, retinyl ester values in 25 IU and 0 IU groups shifted from 271.41 ± 66.82 nmol RE/g to 159.67 ± 23.40 nmol RE/g, respectively. A t P70 (around early adulthood) we observed this gap in vitamin A reserves of pups who had a mother with a vitamin A deficient diet to catch-up to their counterparts, as depicted in the nonsignificant variation in retinol and retinyl ester content in pups from the 25 IU (30.23 ± 8.70 nmol ROL/g and 558.64 ± 138.78 nmol RE/g, respectively) and 0 IU (25.93 ± 3.11 nmol ROL/g and 447.92 ± 110.94 nmol RE/g, respectively) groups (Figure 4.8 C and D). This is consistent with the vitamin A status of P105 offspring, which were observed to have no statistically significant differences in retinyl ester concentrations in pups from dams consuming the 25 IU control (935.69 ± 310.52 nmol RE/g) versus the 0 IU deficient (762.20 ± 205.34 nmol RE/g) diet.

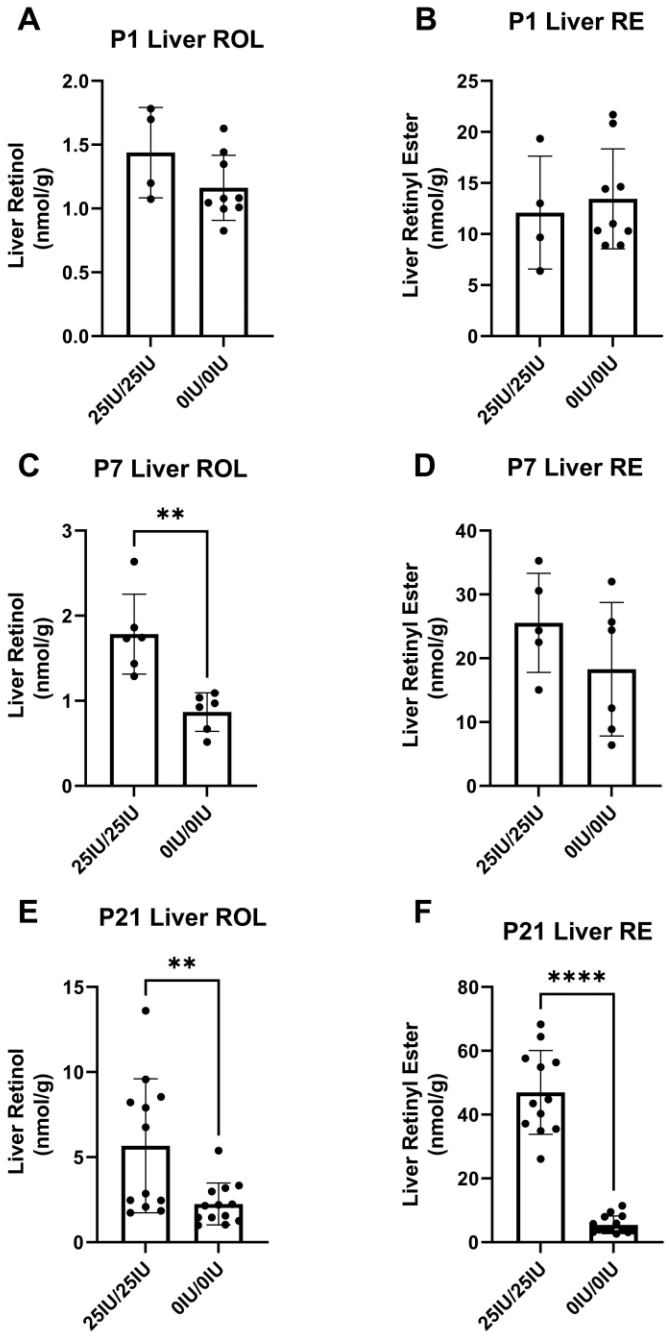

Figure 4.7 HPLC quantification analysis of retinol and retinyl ester concentrations in P1 – P21 offspring livers from 25 IU/25 IU control and 0 IU/0 IU deficient maternal dietary vitamin A intake. This occurred during P1 (birth), P7 (lactation) and P21 (weaning). (A) Retinol content in the P1 pup liver, (B) Retinyl ester in the P1 pup liver, (C) Retinol content in the P7 pup liver, (D) Retinyl ester in the P7 pup liver, (E) Retinol content in the P21 pup liver, (F) Retinyl ester in the P21 pup liver. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

Despite this, evaluation of hepatic retinol content was shown to be significantly reduced ($p < 0.005$) in pups from the 0 IU (32.98 ± 7.39 nmol ROL/g) group in comparison to pups from the 25 IU group (46.61 ± 6.19 nmol ROL/g; Figure 4.8 E and F). Sex of the offspring did not have a significant influence on the data presented for dietary subgroups (Supplementary Figure 4.8).

4.4.5 Analysis of Vitamin A Concentrations in the Extrahepatic Tissues of Offspring

In addition to analyzing hepatic retinol and retinyl ester concentrations, visceral white adipose tissue (VWAT), lung, and plasma were assessed in offspring to determine if manipulations in maternal vitamin A dietary intake would influence extrahepatic vitamin A reserves in pups, and if these tissues are affected in a similar fashion to the liver. This was observed in P21, P35, P70, and P105 pups, whereby assessment of vitamin A reserves was determined strictly from retinoid transfer in the mammary milk during lactation, and the subsequent vitamin A sufficient chow diet pups were placed on after weaning. If these tissues are affected in a similar regard to the liver, we would expect a significant reduction in both vitamin A retinol and retinyl ester reserves until two weeks post weaning (P35), with a return to control group concentrations of vitamin A into adulthood (after P70). Interestingly, VWAT - which is the second-largest storage site of vitamin A (188), depicted no substantial changes in vitamin A status regardless of the dam diet (with the exception of P35; Figure 4.9). As such, VWAT from P21 pups displayed non-significant retinol values between those with a 0 IU fed dam (0.33 ± 0.044 nmol ROL/g) versus a 25 IU fed dam (0.48 ± 0.25 nmol ROL/g), as well as an absence of retinyl ester accumulation which was not significant enough to be determined by HPLC analysis in both groups (Figure 4.9 A).

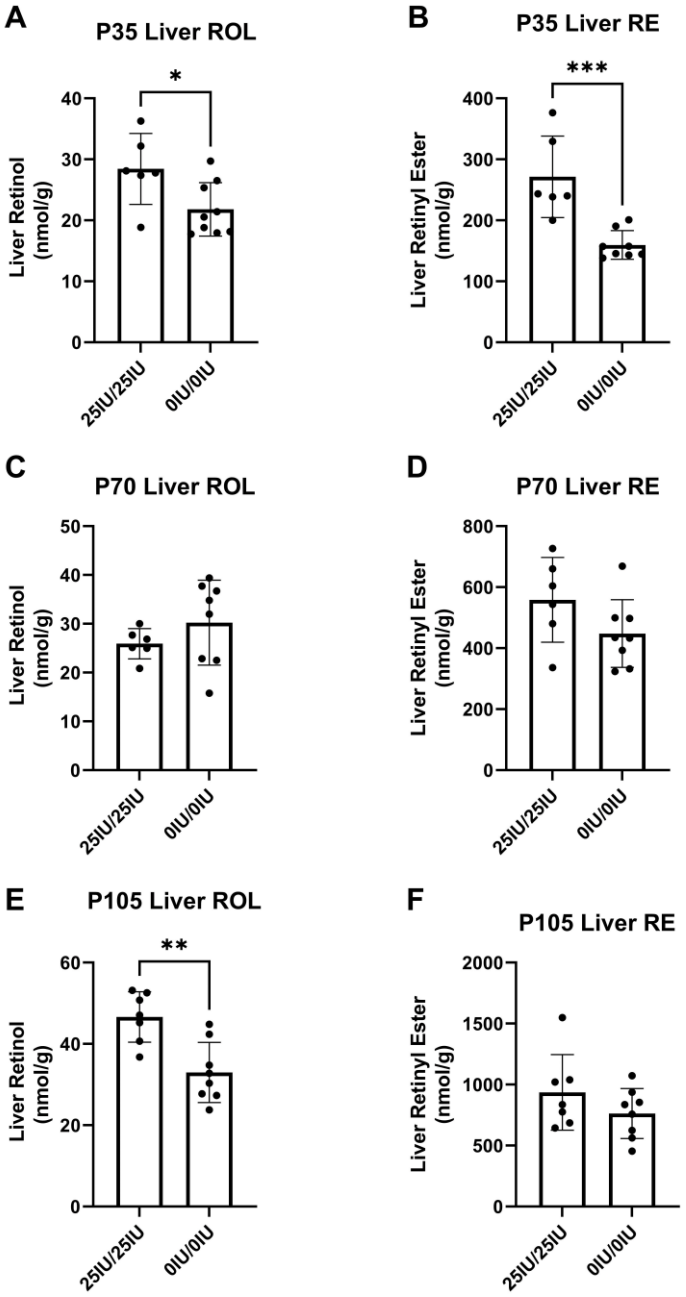

Figure 4.8 HPLC quantification analysis of retinol and retinyl ester concentrations in P35 – P105 offspring livers from 25 IU/25 IU control and 0 IU/0 IU deficient maternal dietary vitamin A intake. This occurred during P35 (two weeks post weaning), P70 and P105. (A) Retinol content in the P35 pup liver, (B) Retinyl ester in the P35 pup liver, (C) Retinol content in the P70 pup liver, (D) Retinyl ester in the P70 pup liver, (E) Retinol content in the P105 pup liver, (F) Retinyl ester in the P105 pup liver. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

However, in Figure 4.9 B and C, mean retinol and retinyl ester concentrations in the P35 offspring were statistically significant ($p < 0.05$, $p < 0.005$, respectively) with a ~31% decline in retinol and ~70% decline in retinyl ester, respectively, in pups born to a mother on a 0 IU diet (1.54 ± 0.40 nmol ROL/g and 2.25 ± 0.62 nmol RE/g, respectively), compared to those from a mother on a 25 IU diet (2.24 ± 0.63 nmol ROL/g and 6.70 ± 3.55 nmol RE/g, respectively).Akin to the P21 offspring, P70 and P105 pups depicted no significant difference in VWAT retinol and retinyl ester concentrations, regardless of the dietary intake of mother. This is represented in Figures 4.9 D, E, F and G which shows P70 pups having retinol and retinyl ester concentrations of 2.19 ± 0.58 nmol ROL/g and 3.36 ± 1.23 nmol RE/g, respectively, in the 25 IU diet group and 2.03 ± 0.24 nmol ROL/g and 3.68 ± 1.43 nmol RE/g, respectively, in the 0 IU diet group, while P105 pups had 2.64 ± 0.84 nmol ROL/g and 4.42 ± 0.89 nmol RE/g, respectively, in the 25 IU diet group and 2.12 ± 0.72 nmol ROL/g and 3.82 ± 1.86 nmol RE/g, respectively, in the 0 IU diet group.

Offspring lung tissue was next assessed for HPLC concentrations of retinol and retinyl ester (Figure 4.10). This tissue was examined because proper development and maintenance of the lung is highly reliant upon vitamin A status, and therefore gives us an indication of how maternal dietary intake affects extrahepatic organs influenced by this micronutrient. Similar to VWAT analysis, the majority of pups depicted nonsignificant changes in the retinol and retinyl ester content, apart from P21. At P21, both retinol and retinyl ester content, reduced significantly by ~57% ($p < 0.0001$) and ~69% ($p < 0.0001$), respectively, in offspring born to 0 IU fed mothers compared to their control counterparts (1.42 ± 0.28 nmol ROL/g to 3.32 ± 0.39 nmol ROL/g, and 9.32 ± 1.71 nmol RE/g, to 30.50 ± 10.2 nmol RE/g, respectively; Figure 4.10 A and B). Meanwhile, at P35, assessment of lung retinol and retinyl ester levels in offspring from control

versus vitamin A deficient fed mothers (Figure 4.10 C and D) depicted non-significant results for both retinol concentrations (13.49 ± 2.54 nmol ROL/g and 11.38 ± 2.35 nmol ROL/g, respectively), and retinyl ester content (118.82 ± 28.27 nmol RE/g and 123.45 ± 27.14 nmol RE/g, respectively). P70 and P105 evaluation of mean lung retinol and retinyl ester levels shown in Figure 4.10 E, F, G and H, respectively, also depict non-significant differences in offspring retinol concentrations regardless of the dietary intake of the mother. Retinol content in pups from 0 IU dams had 22.89 ± 4.93 nmol ROL/g and 16.59 ± 3.11 nmol ROL/g, respectively, while pups from 25 IU dams had 19.07 ± 3.53 nmol ROL/g and 19.49 ± 3.14 nmol ROL/g, respectively. Retinyl ester content in pups from 0 IU dams had 22.89 ± 58.67 nmol RE/g and 237.59 ± 89.47 nmol RE/g, respectively, while pups from 25 IU dams had 213.23 ± 72.20 nmol RE/g and 270.65 ± 92.80 nmol RE/g, respectively. This indicates that despite the important role for retinoid activity at the lung, offspring reserves in this tissue are not significantly impacted from the loss of vitamin A intake by dams during gestation and lactation.

Mean plasma retinol concentrations across P21, P35, P70 and P105 pups followed similar trends to the lung, whereby only P21 retinol content significantly varied ($p < 0.05$) due to maternal dietary vitamin A manipulations (25 IU; 1.47 ± 0.35 μmol ROL/L and 0 IU; 1.15 ± 0.32 μmol ROL/L, respectively). Plasma retinol was maintained in P35, P70 and P105 pups regardless of being born to a dam on a 25 IU (1.00 ± 0.19 μmol ROL/L, 0.93 ± 0.20 μmol ROL/L, and 0.92 ± 0.17 μmol ROL/L, respectively) versus 0 IU diet (0.93 ± 0.14 μmol ROL/L, 0.87 ± 0.20 μmol ROL/L, and 0.92 ± 0.25 μmol ROL/L, respectively) as depicted in Figure 4.11, suggestive of regulated circulating levels of vitamin A remaining maintained into adulthood in

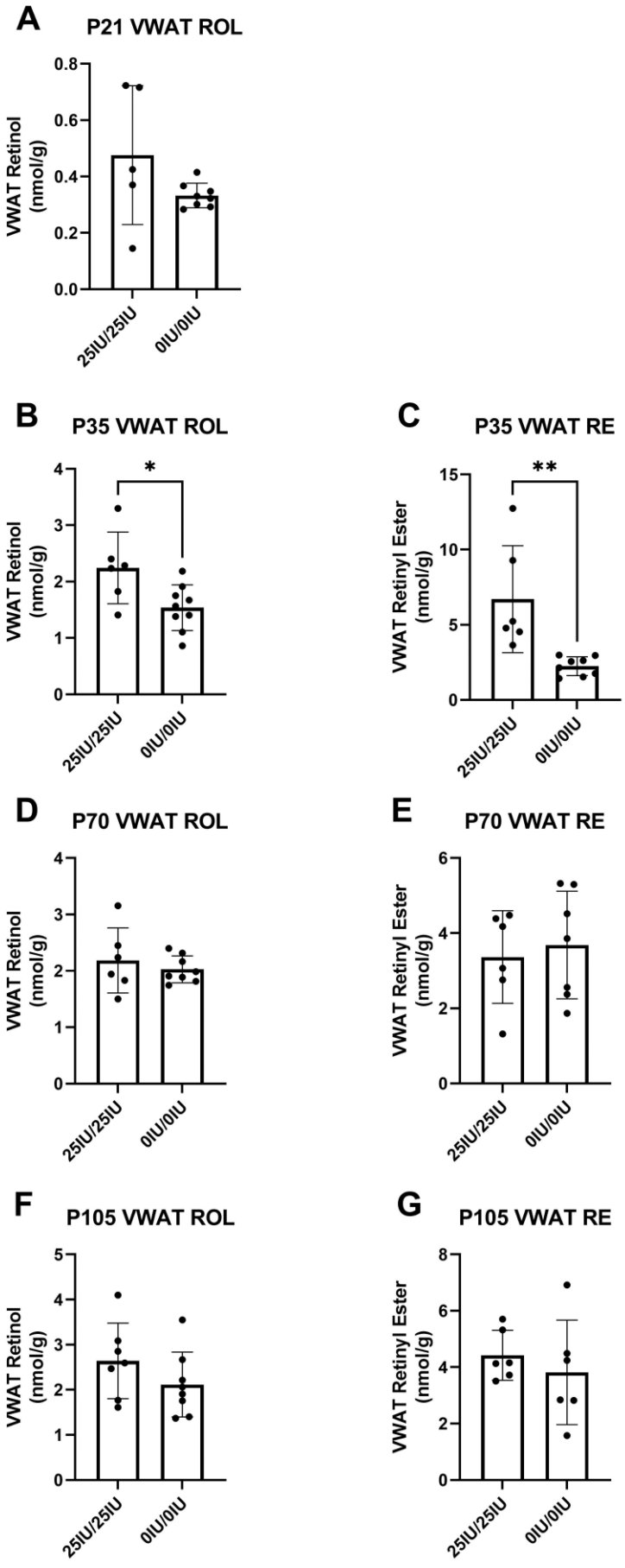
A
P21 VWAT ROL
VWAT Retinol (nmol/g)
0.8
0.6
0.4
0.2
0.0
25IU/25IU
0IU/0IU
B
P35 VWAT ROL
*
VWAT Retinol (nmol/g)
4
3
2
1
0
25IU/25IU
0IU/0IU
C
P35 VWAT RE
**
VWAT Retinyl Ester (nmol/g)
15
10
5
0
25IU/25IU
0IU/0IU
D
P70 VWAT ROL
VWAT Retinol (nmol/g)
4
3
2
1
0
25IU/25IU
0IU/0IU
E
P70 VWAT RE
VWAT Retinyl Ester (nmol/g)
6
4
2
0
25IU/25IU
0IU/0IU
F
P105 VWAT ROL
VWAT Retinol (nmol/g)
5
4
3
2
1
0
25IU/25IU
0IU/0IU
G
P105 VWAT RE
VWAT Retinyl Ester (nmol/g)
8
6
4
2
0
25IU/25IU
0IU/0IU

Figure 4.9 HPLC quantification analysis of retinol and retinyl ester concentrations in offspring visceral white adipose tissue (VWAT) from 25 IU/25 IU control and 0 IU/0 IU deficient maternal dietary vitamin A intake. This occurred during P21, P35, P70 and P105 ages in pups. (A) Retinol content in P21 pup VWAT, (B) Retinol content in P35 pup VWAT, (C) Retinyl ester in P35 pup VWAT, (D) Retinol content in P70 pup VWAT, (E) Retinyl ester in P70 pup VWAT, (F) Retinol content in P105 pup VWAT, (G) Retinyl ester in P105 pup VWAT. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

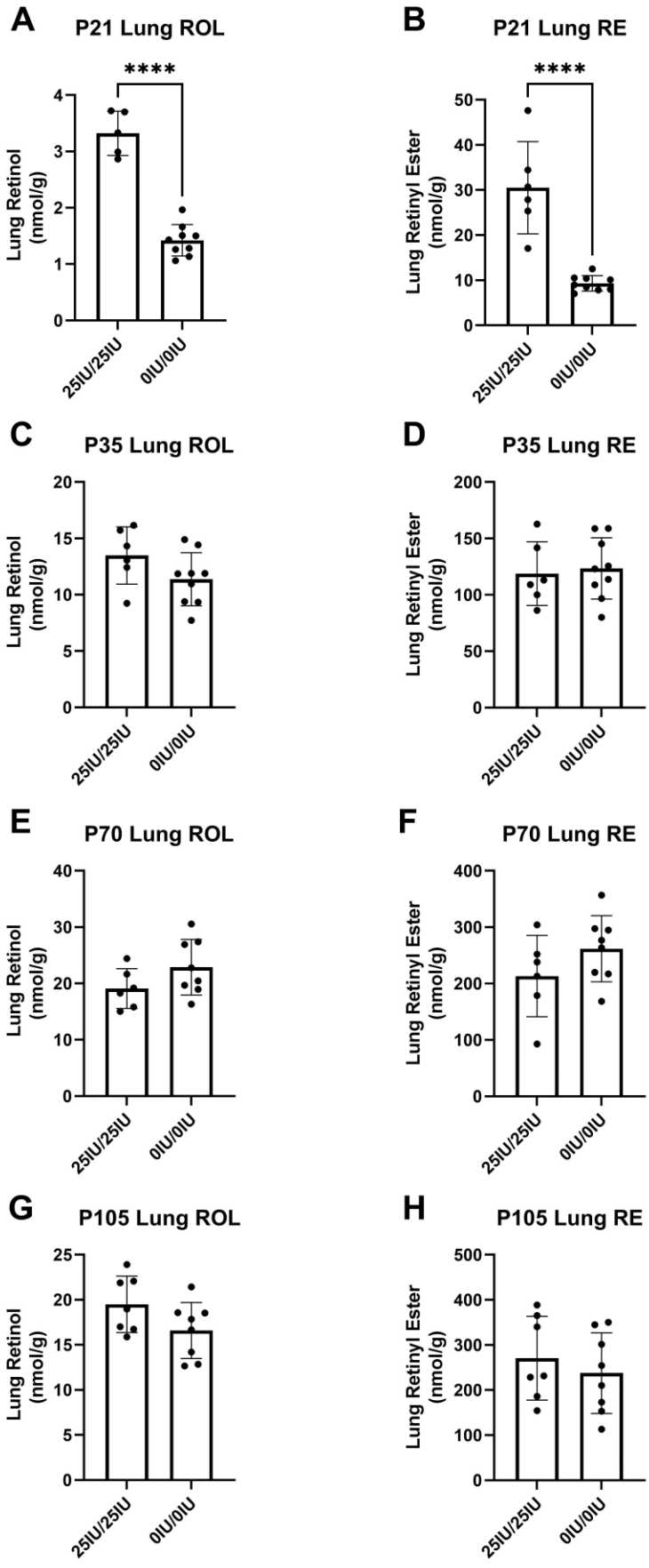
A
P21 Lung ROL
****
Lung Retinol (nmol/g)
0
1
2
3
4
25IU/25IU
0IU/0IU
B
P21 Lung RE
****
Lung Retinyl Ester (nmol/g)
0
10
20
30
40
50
25IU/25IU
0IU/0IU
C
P35 Lung ROL
Lung Retinol (nmol/g)
0
5
10
15
20
25IU/25IU
0IU/0IU
D
P35 Lung RE
Lung Retinyl Ester (nmol/g)
0
50
100
150
200
25IU/25IU
0IU/0IU
E
P70 Lung ROL
Lung Retinol (nmol/g)
0
10
20
30
40
25IU/25IU
0IU/0IU
F
P70 Lung RE
Lung Retinyl Ester (nmol/g)
0
100
200
300
400
25IU/25IU
0IU/0IU
G
P105 Lung ROL
Lung Retinol (nmol/g)
0
5
10
15
20
25
25IU/25IU
0IU/0IU
H
P105 Lung RE
Lung Retinyl Ester (nmol/g)
0
100
200
300
400
500
25IU/25IU
0IU/0IU

Figure 4.10 HPLC quantification analysis of retinol and retinyl ester concentrations in offspring lung from 25 IU/25 IU control and 0 IU/0 IU deficient maternal dietary vitamin A intake. This occurred during P21, P35, P70 and P105 pup ages. (A) Retinol content in the P21 pup lung, (B) Retinyl ester in the P21 pup lung, (C) Retinol content in the P35 pup lung, (D) Retinyl ester in the P35 pup lung, (E) Retinol content in the P70 pup lung, (F) Retinyl ester in the P70 pup lung, (G) Retinol content in the P105 pup lung, (H) Retinyl ester in the P105 pup lung. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

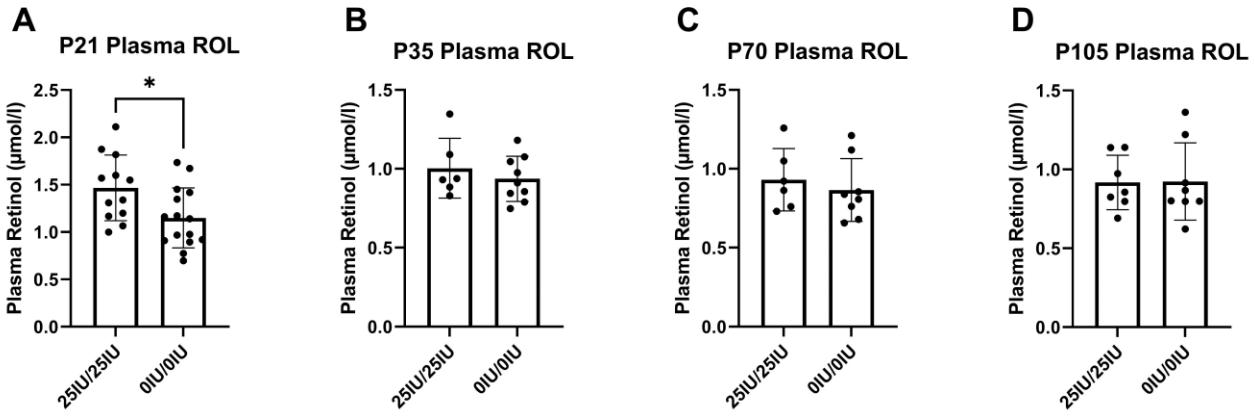

Figure 4.11 HPLC quantification analysis of pup plasma retinol concentrations born from from dams on 25 IU/25 IU control and 0 IU/0 IU deficient dietary vitamin A intake. Retinol content in pup plasma, was assessed in pups during (A) P21, (B) P35, (C) P70 and (D) P105 time points. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

the presence of a vitamin A adequate chow diet. Furthermore, sex of the offspring had no major effect on the data presented for plasma retinol, and VWAT and lung vitamin A content (Supplementary Figures 4.9, 4.10, and 4.11).

Finally, P7 stomach retinol and retinyl ester content was assessed as a proxy for mammary milk measurements (168) to gain insight into the retinoid composition of the mammary milk (Figure 4.12). Due to analytical problems, we could not directly measure stomach vitamin A content in our samples. However, unpublished data previously collected from the Clugston lab comparing mice consuming a vitamin a deficient diet (0 IU) vs chow diet (18 IU) shows similar trends depicted by P21-P35 and dam hepatic HPLC analysis described above. As such, retinyl ester content significantly declined by ~89% ($p < 0.0001$) in offspring born to 0 IU fed mothers compared to their control counterparts (2.17 ± 1.92 nmol RE/g to 19.30 ± 5.89 nmol RE/g, respectively; Figure 4.12 B). Interestingly, stomach retinol concentrations in P7 pups depicted nonsignificant findings, with 5.88 ± 2.39 nmol ROL/g, to 4.89 ± 1.31 nmol ROL/g, in 0 IU and 18 IU fed dams, respectively (Figure 4.12 A). Despite this, it is worth noting that in control mice, retinyl esters makes up 80% of content, so this data is meaningful regardless if retinol is unchanged.

4.4.6 Impact of Vitamin A Intake on Retinoid Gene Expression in the Pup Liver

Gene expression of several key mediators of retinoid storage and metabolism were assessed in the liver of P21 offspring to examine the influence of maternal dietary vitamin A intake during the lactational period on the expression of retinoid pathway genes in newly weaned pups. Expression of hepatic lecithin retinol acyltransferase (*Lrat*), retinoic acid receptor beta (*Rarβ*),

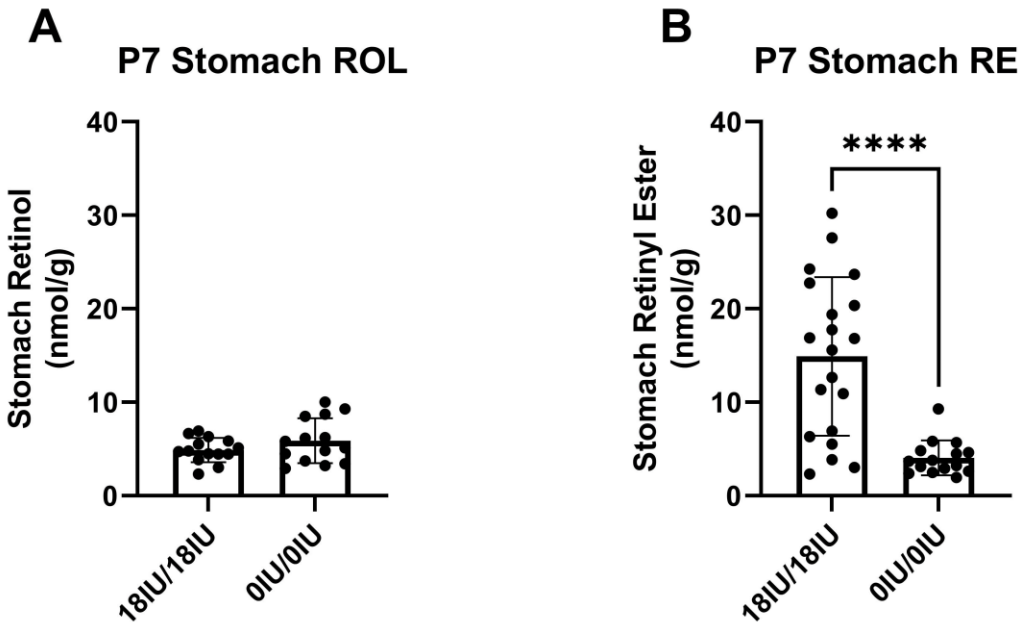

Figure 4.12 HPLC quantification analysis of P7 stomach retinol and retinyl ester concentrations born from dams on 18 IU/18 IU control and 0 IU/0 IU deficient dietary vitamin A intake. (A) Retinol content in the pup stomach, (B) Retinyl ester in pup stomach. Statistical significance, $p < 0.05$ between 18 IU/18 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

cytochrome P450 family 26 subfamily A member 1 (*Cyp26a1*) and cytochrome P450 family 26 subfamily B member 1 (*Cyp26b1*) were all examined against the reference gene *18s* (Figure 4.13). As mentioned in Chapter 3, LRAT is involved in the esterification of retinol into retinyl ester and is critical for the storage of vitamin A in the hepatic stellate cells of the liver. Interestingly, when we assessed the expression of *Lrat* in offspring born to mothers on a 0 IU vitamin A diet (0.60 ± 0.077), it had significantly ($p < 0.01$) declined compared to offspring born to mothers on a 25 IU vitamin A diet (1.00 ± 0.37) (Figure 4.13 A). *Rarb*, a nucleic retinoic acid receptor involved in the transcriptional activity of retinoic acid (175,189), also significantly reduced ($p < 0.05$) in expression in pups with dams on a 0 IU vitamin A diet compared to a control 25 IU vitamin A diet, depicted as a fold change of 0.73 ± 0.11 and 1.00 ± 0.29, respectively (Figure 4.13 B). Furthermore, both *Cyp26a1* and *Cyp26b1* expression was examined in offspring from 0 IU and 25 IU groups, showing a significant decline ($p < 0.01$, $p < 0.05$, respectively) in the expression of mice with a mother on a vitamin A deficient diet (Figure 4.13 C and D). *Cyp26a1* expressed a fold change of 0.069 ± 0.37 to 1.00 ± 1.00, respectively, while *Cyp26b1* expressed a fold change of 0.41 ± 0.23 to 1.00 ± 0.60, respectively. Given that both genes encode important enzymes responsible for the catabolism of retinoic acid into its active metabolites, this is indicative of reduced retinoic acid synthesis and its subsequent excretion from the liver.

4.5 Discussion

Although it is well recognized that maternal hepatic stores and dietary maternal intake contribute to the development of offspring vitamin A reserves, a controversial question remains regarding the extent to which vitamin A from each pathway is acquired by the placenta and mammary

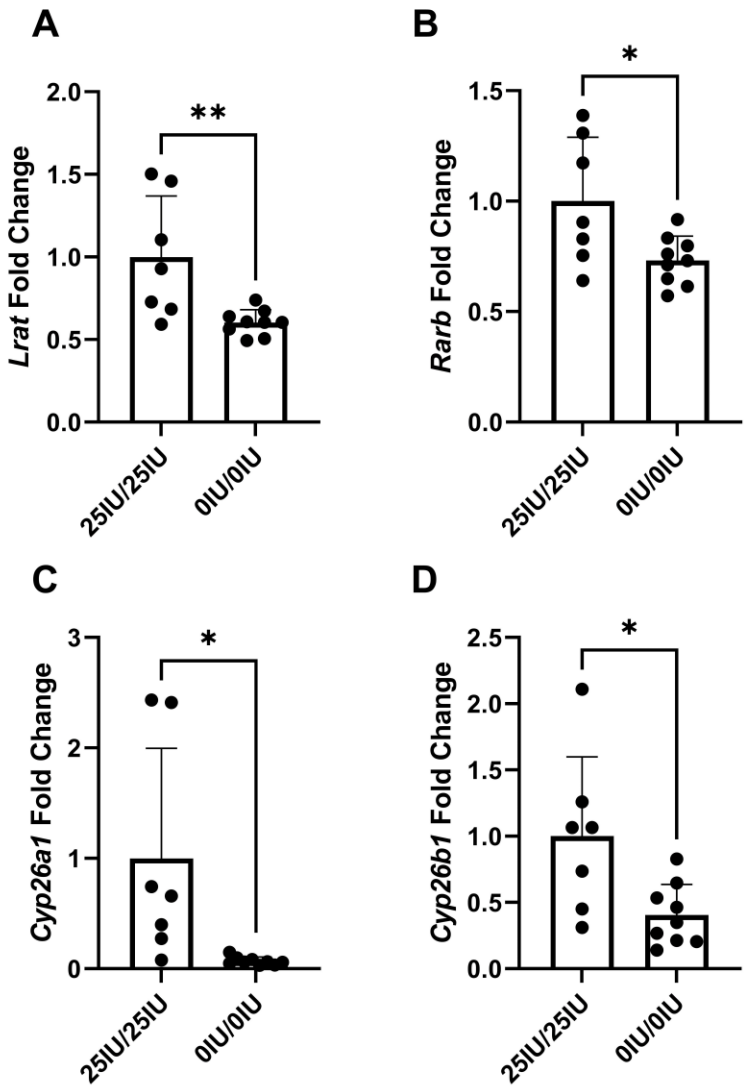

Figure 4.13 qPCR analysis of retinoid pathway protein expression in the P21 liver born from dams on 25 IU/25 IU control and 0 IU/0 IU deficient dietary vitamin A intake. All genes depict a fold-change relative to the reference gene *18s*. (A) Lecithin retinol acyltransferase, (B) retinoic acid receptor beta, (E) cytochrome P450 family 26 subfamily A member 1, (F) cytochrome P450 family 26 subfamily B member 1. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

gland during gestation and lactation, respectively, to meet perinatal retinoid demands. This is because information regarding the direct postprandial chylomicron uptake of vitamin A into the mammary milk has not been recognized in the literature as extensively as alternative pathways, and therefore its quantitative input is unknown (2). Additionally, the consequences of vitamin A deficient intake during the perinatal period on maternal vitamin A metabolism and its extension to offspring retinoid status have yet to be addressed.

According to our results, we reason that chylomicron dietary vitamin A intake is essential to establishing offspring vitamin A reserves, and that maternal stores are incapable of compensating for loss of vitamin A in the maternal diet. Thus far, several authors have confirmed that increasing dietary vitamin A intake or utilizing vitamin A supplementation increases the proportion of vitamin A distributed into the milk postpartum, which they speculate occurs through the contribution of chylomicron retinyl esters in postprandial conditions (10,118,147,190). This is supported by compartmental analysis studies which attribute the rapid lipolysis of chylomicrons at mammary tissue with the immediate postprandial accumulation of vitamin A in breast milk (119). As a result, we emphasize the importance of this direct dietary retinoid pathway as a highly efficient and effective mode of vitamin A transfer to the mammary milk, and confirm that it is a significant contributor to offspring retinoid reserves.

As previously discussed, upon ingesting a vitamin A rich meal, retinoids are esterified and incorporated into chylomicrons at the intestine for secretion into the general circulation (4,6,9,46). Apart from being utilized at the liver for hepatic stores, these circulating chylomicron remnants can directly be transported to the mammary tissue where they are taken up into the milk after undergoing hydrolysis by an enzyme known as lipoprotein lipase (LPL) (2,47). It is this direct postprandial LPL-mediated pathway which we aimed to examine in this study, to

determine the influence of maternal diet as a determinant in establishing offspring vitamin A reserves. This required us to first ensure our maternal model only reflected experimental changes in diet, while maternal hepatic stores of vitamin A were maintained as a control. We did this by utilizing two groups of dams, one on a vitamin A deficient diet with 0 IU vitamin A and the other on a control diet of 25 IU vitamin A [which is considered to reflect optimal vitamin A intake in humans (191,192)] throughout gestation and lactation. Because dams were raised on a vitamin A sufficient chow diet prior to breeding, we were able to ensure that their livers had accumulated adequate vitamin A reserves for gestational and lactational periods. Likewise, it has been established that developing a truly vitamin A deficient model in mice can only occur after they are put on a diet absent of vitamin A at birth and for extended durations (or sometimes over multiple generations) (84,187,192,193). Therefore, because our dams were put on a deficient diet only for the duration of pregnancy and lactation, we can justify that their hepatic retinyl ester reserves are likely not depleted by this point in time.

Assessment of maternal HPLC analysis also reflects this conservation of maternal hepatic retinyl ester reserves by depicting the maintenance of circulating plasma retinol concentrations in both groups of dams regardless of the diet they consumed. This suggests that maternal hepatic reserves largely remain intact, as we would only expect to see a significant reduction in the plasma retinol of 0 IU fed dams (indicative of vitamin A deficiency) if maternal reserves were completely diminished (3,20,57). Likewise, we saw no significant difference in physical characteristics between dams consuming a 25 IU versus 0 IU vitamin A diet, such as litter size, bodyweight, liver weight, and body to liver weight ratio, as well as no qualitative differences in appearance suggestive of pathology or vitamin A deficiency. Therefore, we can confirm that maternal stores were maintained, consequently affirming our model to assess the contribution of

diet on maternal and offspring retinol and retinyl ester reserves. Upon assessment of hepatic vitamin A content by HPLC analysis, we revealed a ~81% decrease in retinol and ~49% reduction in retinyl ester in dams consuming a vitamin A deficient (0 IU) diet compared to their counterparts on a control diet (25 IU). Taken together, we assume this to reflect the reduction of retinoid reserves in the maternal liver used to maintain circulating concentrations of vitamin A (due to the absence of dietary vitamin A intake), but not the depletion of these stores as circulating levels of retinol were maintained.

In addition to hepatic vitamin A, we assessed P7 pup stomach which is a known proxy for mammary milk measurements of vitamin A during lactation (168). Consistent with our liver HPLC findings, we observed a significant reduction in retinyl ester content in the milk from 0 IU versus 18 IU pup stomachs. As such, the mammary milk from dams consuming a deficient vitamin A diet had an 89% decline in retinyl ester, compared to dams with an optimal vitamin A diet. It is important to note that in such animals, milk retinoid content is solely derived from maternal hepatic stores because postprandial chylomicron vitamin A would be absent with the loss of vitamin A in the diet (129). Therefore, we observe that inhibiting dietary vitamin A intake can significantly impact the retinoid composition in mammary milk, regardless of the presence of maternal hepatic stores. This is consistent with the work of Green *et al.* (147), who utilized continuous infusion of varying amounts of labelled vitamin A to determine postprandial chylomicron contribution to milk vitamin A during lactation. As a result, they observed that increases in dietary vitamin A intake led to reciprocal increases in milk vitamin A content, and therefore speculate that a large proportion of this incoming dietary chylomicron vitamin A is directed to the mammary tissue to be utilized for secretion in the mammary milk during lactation. This is supported by Ross *et al*. (146), who used compartmental analysis to show that active

lipolysis of chylomicrons containing [3H] vitamin A at the mammary tissue was responsible for the substantial and rapid (2–3 min) vitamin A uptake and accumulation in breast milk postprandially, which was shown to increase linearly in accordance with the quantity of chylomicron vitamin A injected. As mentioned, Jensen *et al.* (145), notes that 48 hours after parturition, LPL expression at mammary tissue in mice increased tenfold (and depressed in peripheral adipose tissue), which is likely attributed to this requirement for rapid vitamin A uptake into milk lipid droplets for delivery to offspring (119,145,146). This knowledge paired with our findings support the notion that maternal dietary intake via chylomicron lipolysis is favored in establishing milk vitamin A reserves more significantly than maternal hepatic retinyl ester stores.

Following the analysis of maternal vitamin A status, we integrated pup retinol and retinyl ester HPLC data from tissues examined at P1 (birth), P7 (lactation), P21 (weaning), P35 (two weeks post-weaning), P70 and P105 pup. This was most importantly examined in liver tissues, which are known to be indicative of mean vitamin A levels in mice (119). The establishment of these reserves was analyzed in offspring born to dams fed either a control (25 IU) or deficient (0 IU) vitamin A diet throughout gestation and lactation, to determine the prominence of maternal dietary intake in establishing adequate offspring hepatic retinyl ester reserves. Should maternal dietary intake of vitamin A in fact be the primary determinant of vitamin A reserves in offspring, we anticipated that pups from mothers on the 0 IU diet to have significantly lower hepatic retinol and retinyl ester stores.

Interestingly, upon evaluating P1 hepatic retinol and retinyl ester reserves, we observed no significant discrepancies in offspring born to dams consuming the 25 IU vitamin A versus the 0 IU vitamin A diet. This is an important finding as offspring vitamin A status at P1 (birth) is

strictly indicative of transplacental transfer of vitamin A from the dam to pup during gestation. Vitamin A from maternal hepatic reserves mobilized as retinol-RBP4 has been well regarded as the primary source of fetal vitamin A during transplacental transfer (127). This is congruent with our results, which agree that maternal stores are essential during gestation by depicting pups from dams on a vitamin A deficient diet still having liver stores comparable to the control group. Additionally, our results emphasize limited transplacental transfer of vitamin A to offspring during gestation, likely prioritized to meet physiological demands but not yet required for the accumulation of retinyl ester stores. This is because our findings depicted only a marginal presence of liver retinyl ester reserves in both dietary groups of pups. Current literature similarly indicates transplacental transfer of vitamin A to be a highly regulated process and thus limited in its transmission from mother to fetus (40,65,119,194,195). As such, experiments in mammalian newborns have alluded to the requirement for an external vitamin A supply for the developing neonate, having examined low hepatic vitamin A content in the fetal liver regardless of maternal dietary intake during gestation (40,118,194) Conclusions from Pasatiempo and Ross (40) have displayed this, where regardless of the dam's total retinol liver reserves, newborn pups averaged only 63 nmol/g vitamin A during gestation. Likewise, experiments by Dann (194) using rats and rabbits fed varying amounts of carotene, determined that the amount of vitamin A in offspring liver at birth is not reliant on the amount of vitamin A ingested by the mother during pregnancy. Therefore, current thinking proposes colostrum and milk are critical channels of transmission for vitamin A and thus may compensate for the reduced vitamin A status at birth prior to weaning (10,65,118,119,194). Taken together, these findings suggest that while the fetus acquires reduced but adequate vitamin A reserves during gestation, they are highly reliant on breastmilk vitamin A

levels postnatally to build protective stores, which emphasizes the dependence on the dam's dietary vitamin A intake during lactation.

Livers from P7 pups were next assessed to determine the influence of vitamin A transfer during the lactational period, whereby offspring vitamin A reserves are established through mammary milk secretion from dams during nursing. Unlike the P1 mice, pups at P7 displayed a ~51% reduction in liver retinol content in those with mothers on a 0 IU dietary intake of vitamin A, compared to control fed dams. Contrastingly, mean retinyl ester concentrations were not significantly impacted, regardless of if pups had dams on the 25 IU versus 0 IU diet. This indicates that at this point in lactation, dietary influences on mammary milk vitamin A composition were not quite significant enough to alter the establishment of offspring retinyl ester reserves, but enough to have a significant impact on hepatic reservoirs of retinol. Given that both liver retinol and retinyl ester HPLC quantification trend downwards in offspring from mothers on a vitamin A deficient diet, we assume that this reduction in hepatic retinol is being utilized to maintain circulating levels of vitamin A, while hepatic retinyl esters are starting to be re-mobilized to support free retinol pools, but not enough at this time to appear significant. Complementary to these results, Akohoue *et al*. (10) investigated the effect of maternal dietary vitamin A intake on milk vitamin A concentrations and subsequent pup vitamin A status during pregnancy and lactation. Congruent with our findings, they observed that rats fed a vitamin A supplemented diet during lactation had significantly higher retinol concentrations in dam milk (1.5–3 times) at day 7 of lactation and pup liver (1.1–6.7 times) at lactation day 13, compared to mothers consuming a vitamin A deficient diet. Similarly, this observation is supported by Gardner and Ross (195) who used dams on either low marginal, high marginal or sufficient vitamin A diets and measured the total retinol concentrations in the stomachs (milk curd) of their

P9 offspring. Their results emphasized a significant decline in retinol content among pup stomachs from the low marginal (16% reduction) and high marginal (35% reduction) groups as a percentage of the sufficient group. This can be extrapolated to what we would expect to see in the hepatic retinol status of offspring, which combined with our findings suggests that a maternal vitamin A deficient or low marginal diet during lactation compromises the quantity of retinol content accumulated in dam milk, thus limiting the ability for their offspring to establish adequate stores. It is important to note that unlike our findings, these studies only assessed stomach and hepatic retinol content, without an evaluation of hepatic retinyl ester reserves. This is important because retinyl ester is indicative of the true storage form of vitamin A and therefore gives us a better understanding of vitamin A reserves, while retinol fluctuates more readily according to circulating vitamin A concentrations. As a result, utilizing a paired analysis with both forms of retinoid gives us a more comprehensive and accurate understanding of hepatic vitamin A content and subsequently whole-body vitamin A status.

When examining P21 pups, we observed that maternal mice with inadequate vitamin A intake during gestation and lactation, generated offspring with limited hepatic retinol and retinyl ester reserves. These significant findings were depicted with retinol concentrations being reduced over ~60% in 0 IU group pups, while mean retinyl ester concentrations declined ~88%. This suggests that dietary manipulations of dam vitamin A intake until weaning allowed for substantial discrepancies in mammary milk content, which significantly reduced pup vitamin A retinyl ester reserves in the liver that were likely re-mobilized to maintain circulating vitamin A levels within the normal range. This is consistent with experimental rodent studies previously performed by Davila and colleagues (118), who examined dams on high vitamin A content or a low vitamin A content diets during gestation until day 14 of lactation (at which point pups and

dams were collected). As a result, these authors showed that the quantity of retinol stored in the neonatal liver was markedly increased during nursing in pups from supplemented mothers and is thus speculated to be influenced by the enrichment of retinyl ester in chylomicrons in the maternal diet during lactation. Collectively with our results, it is affirmed that a reduction in vitamin A stores in offspring from dams on a vitamin A deficient diet persists during weaning and continues to emphasize the prominence of proper maternal dietary intake as a primary source of vitamin A reserves. This emphasis on the presence of sustained dietary vitamin A intake by the mother during lactation, in contrast to reliance on maternal stores, is consistent with our hypothesis, especially given maternal stores inadequately compensate in its absence.

Since an examination of the effect of maternal dietary intake during lactation has not been examined in literature in postweaning pups, we wanted to understand if this outcome was consistent in P35 mice. Interestingly, our findings continued to depict a statistically significant reduction in both mean retinol and retinyl ester levels between pups born to control (25 IU) versus experimental (0 IU) group mothers at two weeks post-weaning (P35). As such, retinol concentrations declined ~23% in pups from 0 IU fed mothers, while retinyl ester values declined ~41% from 25 IU to 0 IU groups. This indicates that even after pups had been weaned onto a vitamin A sufficient chow diet for two weeks, mice from dams with no dietary vitamin A intake during lactation were unable to replenish their hepatic stores in a timely fashion. Given that P35 is considered around puberty in mice (34,162,163), this emphasizes that mothers with deficient diets during gestation and lactation may give rise to a sustained inability to accumulate normal vitamin A reserves in offspring, whereby they have a prolonged risk of developing vitamin A deficiency until pubertal years.

Given these findings, we extended our investigation into early- and mid-adulthood in P70 and P105 mice, respectively, to further understand if pups would continue to be hindered in accumulating hepatic reserves of vitamin A after puberty, or if this resolves around adulthood. Interestingly, upon quantification of P70 vitamin A content by HPLC analysis, we observed this gap in hepatic vitamin A reserves of pups who had a mother with a vitamin A deficient diet to largely catch-up to the vitamin A stores present in the liver of their counterparts. This was depicted by no significant variations in retinol and retinyl ester content in pups from the 25 IU and 0 IU groups. Similarly, this is consistent with the vitamin A status of P105 offspring, which were observed to have no statistically significant differences in retinyl ester concentrations in pups from dams consuming a 25 IU versus 0 IU diet. It is therefore understood that upon adulthood, the influence of maternal dietary intake during gestation and lactation no longer significantly affects pup hepatic reserve accumulation, which is now mediated exclusively by vitamin A content in the chow diet. Furthermore, interpreting our findings drawn from hepatic retinol and retinyl ester content in pups from both diet groups as a cumulative comparison over time (Figure 4.14), allowed us to observe a progressive linear increase in vitamin A content as the 25 IU offspring aged, which was not consistent in the 0 IU group pups. This suggests that pups with sufficient maternal dietary intake during gestation and lactation accumulate hepatic vitamin A stores with time, which is delayed by pups with vitamin A deficient fed dams.

Due to the influence of maternal vitamin A intake on offspring vitamin A reserves, we aimed to examine physical characteristics across both diet groups in all ages of pups to determine the presence of any significant alterations between both groups of mice, particularly those indicative of vitamin A deficiency. Like dams, we examined body weight, liver weight, and calculated liver to body weight ratio. In all experiments no significant differences were shown

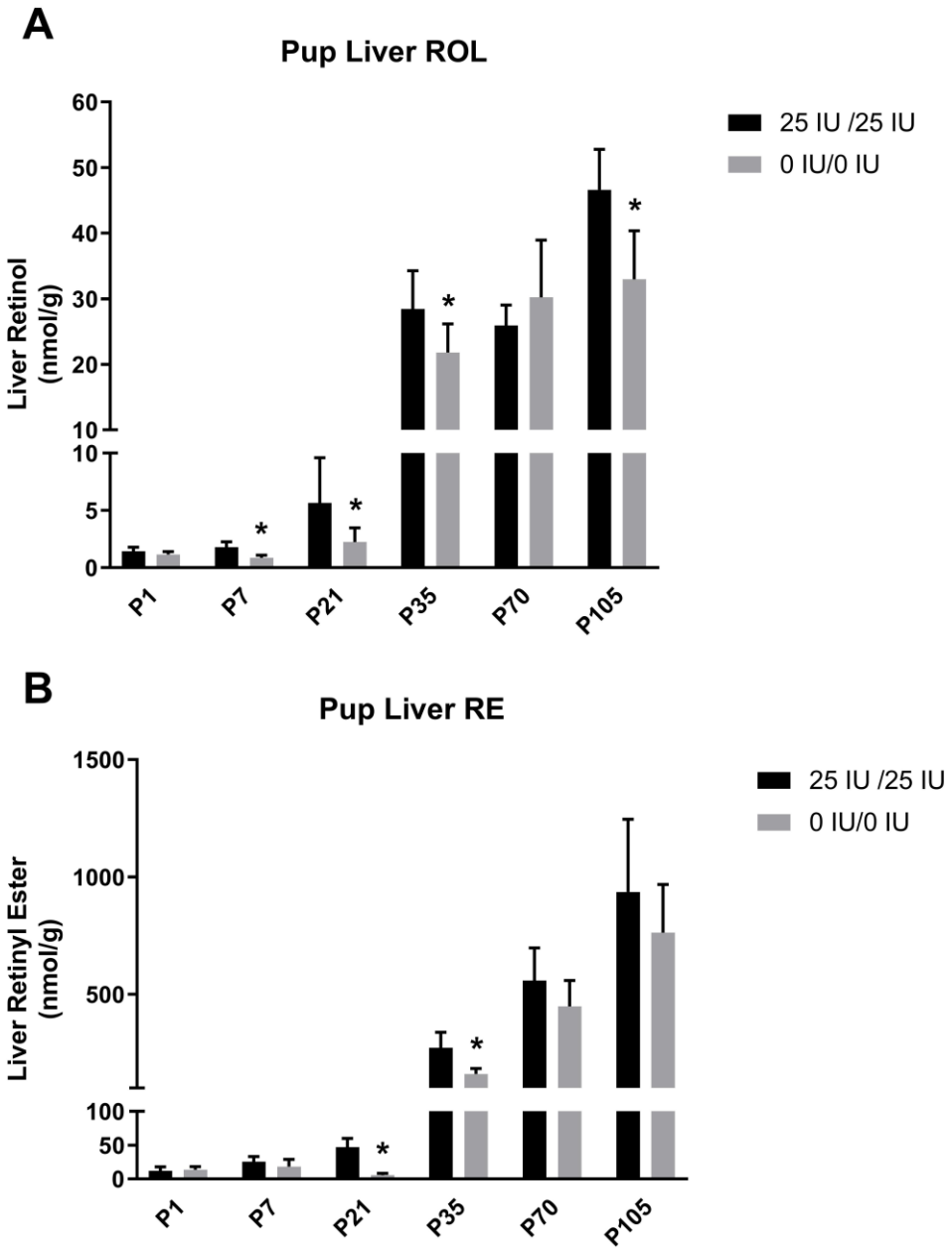

Figure 4.14 Cumulative comparisons of hepatic HPLC quantification analysis of retinol and retinyl ester concentrations. This was observed at P1 (birth), P7 (lactation), P21 (weaning) and P35 (two weeks post-weaning), P70 and P105, across control (25 IU/25 IU) and experimental (0 IU/0 IU; deficient) dietary groups. (A) Hepatic retinol content comparison in offspring, (B) Hepatic retinyl ester content comparison in offspring. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.

between 25 IU versus 0 IU diet groups, emphasizing that the amount of maternal dietary vitamin A intake and subsequent chow intake did not impede these physical parameters. We additionally assessed lung and VWAT weights at P21, P35, P70 and P105, which consistently displayed no significant changes. Moreover, no manifestations of pathology or vitamin A deficiency (eg. based on activity level, fur quality, moistness of epithelial tissue, growth and size, etc.) were physically apparent in all groups of pups.

Absence of vitamin A deficiency was further confirmed by a HPLC analysis of plasma retinol concentrations in P21, P35, P70 and P105 pups, which displayed no significant discrepancies, regardless if pups were born to dams receiving a 25 IU versus 0 IU diet during gestation and lactation. In addition to liver HPLC analysis, we conducted an examination of retinol and retinyl ester content in P21, P35, P70 and P105 lung and VWAT. As we anticipated, vitamin A stores predominantly remained unchanged in these tissues regardless of the dam's dietary vitamin A intake (with the exception of P21 lung and P35 VWAT). This emphasizes the role of the liver in being the principal storage site of vitamin A in the body - given it was the tissue most severely affected by changes in milk vitamin A status, and subsequently provides the best indication of whole-body vitamin A status.

In addition to HPLC analysis, hepatic gene expression of key mediators of the retinoid pathway were examined in P21 pups to observe if deficient maternal dietary intake of vitamin A during lactation alters the formation of hepatic retinyl ester stores or the oxidation of retinol for retinoic acid synthesis. This involved the assessment of lecithin retinol acyltransferase (*Lrat*), retinoic acid receptor *β* (*Rarb)*, cytochrome P450 family 26 subfamily A member 1 (*Cyp26a1*) and cytochrome P450 family 26 subfamily B member 1 (*Cyp26b1*) gene expression. As predicted, *Lrat* expression significantly declined in pups who had a mother with the vitamin A

deficient diet during gestation and lactation. Given we know that LRAT is required for the esterification of retinol into retinyl ester, this finding aligns with our hepatic HPLC analysis depicting a reduction in P21 liver retinyl ester reserves. Therefore, we assume that because these pups are likely receiving less dietary vitamin A in the mammary milk compared to control group pups, vitamin A utilization at the liver for establishing retinyl ester reserves is reduced and consequently *Lrat* expression declines because it is not required as extensively. Likewise, retinoid metabolic pathway gene expression by *Rarb* and *Cyp26a1* and *Cyp26b1* significantly declined in pups from mothers on the 0 IU diet compared to those on the 25 IU diet. As established in our Chapter 3 discussion, these genes are highly sensitive to retinoic acid levels, with the Cyp26 enzymes being known to elicit a negative autoregulatory feedback to regulate cellular levels of retinoic acid via catabolizing it into polar metabolites (56,174). As a result, we speculate that the significant decline in retinoid content in the liver of P21 pups with 0 IU fed dams is responsible for the reduced expression of these genes. This is because less hepatic vitamin A is present and therefore retinoic acid production is reduced and not as readily available to bind to nuclear receptors to elicit transcriptional activity or be oxidized for excretion, respectively. Ross *et al.* (142), affirms this thinking by observing a strong response of *Lrat* and *Cyp26* mRNA expression in the liver to differences in vitamin A nutrition, whereby vitamin A deficient < vitamin A marginal < vitamin A adequate < vitamin A supplemented diets. Furthermore, they suggest that regulation of *Lrat* and *Cyp26* expression serves as a response mechanism to maintain retinoid homeostasis and avoid retinoid excess by modifying vitamin A metabolism accordingly to what is received in the diet. Recent work by Borel *et al.* (196), conversely emphasizes that dams consuming a vitamin A deficient diet during the perinatal period may cause offspring to increase their metabolism of vitamin A in favor of survival in an

environment with insufficient vitamin A. As such, these authors note that this prenatal programming of vitamin A metabolism involves altered retinoid gene expression to support higher hepatic accumulation of vitamin A, which directly contrasts our observations. Upon further examination of this study, we question the validity of these statements, given the authors depict *Lrat* expression to show no significant difference regardless of the diet parents were on, as well as some of the genes examined in the liver are not prominently known to be expressed in this location for vitamin A metabolism eg. *Rbp2* (197). Moreover, their analysis of hepatic vitamin A content showed no true significant effect of the dam diet, rather they make assumptions claiming it is systematically higher in the vitamin A deficient fed dam group. Furthermore, the substantially lower quantities of hepatic retinol and retinyl ester content measured in this data are questionable, as they should be much higher given the adult age of the offspring.

Altogether, our data affirms our hypothesis that maternal mice with normal vitamin A reserves, but inadequate dietary vitamin A intake will generate offspring with limited vitamin A reserves. This study emphasizes that maintaining adequate maternal dietary intake of vitamin A from postprandial chylomicrons is critical during lactation as it significantly influences the amount of vitamin A received by the offspring through mammary milk and their subsequent liver reserves. As such, we assume this dramatic change in offspring hepatic reserves to not be significantly influenced by maternal hepatic stores, which may be insufficiently utilized given they are unable to compensate for a loss of dietary vitamin A intake. This is supported with vitamin A sufficient pups accumulating hepatic vitamin A stores with time, which is hindered by pups (until early adulthood) with dams on a vitamin A deficient diet during lactation. Furthermore, this study emphasizes that dietary intake during lactation is essential for

establishing offspring vitamin A status and therefore serves as a primary determinant for vitamin A reserves in offspring.

CHAPTER 5 – Role of Maternal Hepatic Vitamin A Stores in Establishing Offspring Vitamin A Reserves

5.1 Introduction

As discussed in Chapter 4, following gestation, newborns have minimal reserves of retinoid and are highly dependent on maternal milk vitamin A for proper growth and development (25,125). Vitamin A from maternal hepatic reserves is a primary source of mammary milk vitamin A and is regarded as a crucial source of fetal vitamin A during transplacental transfer (2,46,127). In the fasting circulation or when peripheral tissue retinoid levels are low, this source of vitamin A is mobilized from the liver as retinol bound to retinol-binding protein 4 (RBP4) and taken into target cells via the RBP receptor, STRA6 (52,53,120,198). During lactation, mothers have a higher nutritional demand to help establish foundational reserves of vitamin A in their offspring through their mammary milk. While retinol-RBP4 may be a central component to this process, its contribution is unknown and highly contested in current literature. Indeed, it is generally assumed that this source of vitamin A is the sole mechanism responsible for establishing offspring reserves, with an estimate that ~40–90% of milk retinoid from lactating rats comes from holo-RBP, independent of dietary retinoid from chylomicrons (2,147). With this in mind, we developed a research program aimed at assessing the main determinant of vitamin A reserves in offspring by genetically dissecting the influence of maternal hepatic stores using *Lrat*$^{-/-}$ dams.

Since the majority of vitamin A is esterified and stored in the liver through lecithin retinol acyltransferase (LRAT) activity (46,167), utilizing *Lrat*$^{-/-}$ dams ensured no maternal hepatic retinyl ester stores would be present, allowing us to examine diet as the exclusive contributor to offspring reserves. As such, we examined if maternal mice with depleted hepatic retinyl ester reserves and sufficient dietary vitamin A intake would generate heterozygous offspring with normal vitamin A reserves. If maternal hepatic vitamin A stores are the primary contributing source to vitamin A reserves in offspring, we would expect a significant reduction in the vitamin

A status of offspring from *Lrat*$^{-/-}$ dams, despite them having adequate vitamin A intake. However, we hypothesize that maternal mice with depleted vitamin A reserves and adequate vitamin A intake will generate offspring with normal vitamin A reserves.

5.2 Experimental Design

To address this research question, we developed an experimental design using *Lrat*$^{-/-}$ and wild-type mice on a C57BL/6 background produced as described in (167) which were generously donated from William Blaner (Columbia University, NY, USA). Wild-type dams were bred with *Lrat*$^{-/-}$ males, while *Lrat*$^{-/-}$ dams were bred with wild-type males to ensure all offspring were generated with the same genotype. Both groups of mice were placed on an adequate vitamin A (15 IU vitamin A/g diet) chow diet throughout gestational and lactational periods, until weaning at postnatal day 21. Since diet remained as a control in this experiment, we were able to solely assess the influence of maternal hepatic stores on the development of offspring reserves. Hepatic tissues were collected from offspring at birth (postnatal day [P]1), during lactation (P7), and weaning (P21), therefore pup reserves were contingent on maternal transplacental transfer and through mammary milk only (Figure 5.1). Dam liver and plasma were collected at P21. Quantitative amounts and molecular species of vitamin A were assessed by high performance liquid chromatography analysis as described in Chapter 2. Supplemental to this data, physical characteristics (body weight, liver weight, etc.) of pups and dams were evaluated on the respective day their tissues were collected to assess for indications of vitamin A deficiency and discrepancies between study groups.

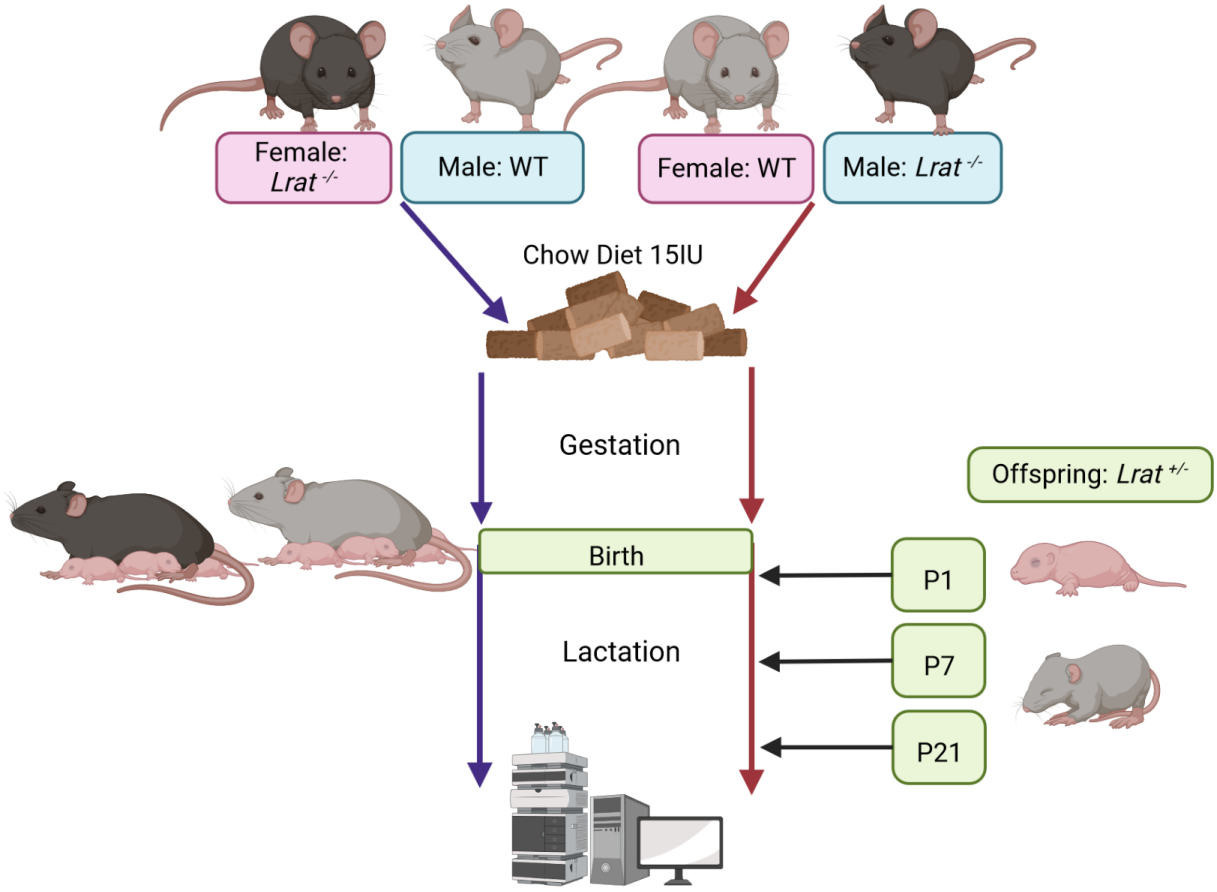

Figure 5.1 Summary figure displaying the experimental design for the role of maternal hepatic vitamin A stores in establishing offspring vitamin A reserves. Loss of maternal hepatic stores was evaluated by comparing *Lrat* $^{-/-}$ dams mated with wild-type (WT; *Lrat* $^{+/+}$) males and *Lrat* $^{-/-}$ dams mated with WT (*Lrat* $^{+/+}$) males, respectively, fed a regular chow diet throughout gestation and lactation. Hepatic vitamin A content from heterozygous neonates was examined after parturition at P1 (birth), P7 (lactation), and P21 (weaning) by HPLC analysis. Created with BioRender.com.

5.3 Methods

A description of the animal husbandry and tissue collection process, vitamin A quantification by high performance liquid chromatography (HPLC), and statistical analyses are provided as general methods in Chapter 2.

5.3.1 Genotyping for Lecithin Retinol Acyltransferase

The *Lrat* genotype was determined in offspring using tail clip DNA and PCR analysis.

5.3.1.1 DNA Extraction

The protocol for DNA extraction was performed as described in (199). In short, DNA was extracted using 75 µL of lysis buffer and run twice (first with a large piece of tail, then repeated with a piece cut to 0.2 cm) in the ProFlex PCR System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for a one hour run at 95℃. Afterwards, samples were cooled to 4°C and 75 µL of neutralization buffer was added.

5.3.1.2 PCR Genotyping

Genotyping for *Lrat* wild-type and knockout alleles in transgenic mice was determined by PCR analysis. In separate tubes, 2 µL of DNA extract and 18 µL of reaction mixture (10 µL SYBR, 6.5 µL H_2O, 0.5 µL of primers 1, 2 and 3 respectively; Table 5.1), were combined. Amplification from primers 1 and 3 produced a 300 bp band, corresponding to the wild-type *Lrat* allele, and amplification from primers 2 and 3 produced a 370 bp band, corresponding to the *Lrat* null allele. All samples and positive and negative controls were run in a ProFlex PCR System

Primer	Primer Sequence
1 Wild-type allele (LRATWT1)	Reverse: 5'-AAGTGCTGGGCATGGTGACTTGTG-3'
2 Knockout allele (NEO1)	Reverse: 5′- TGCGAGGCCAGAGGCCACTTGTGTAGC-3′
3 (LRAT1S)	Forward: 5'-TCCAGTTCCAGACTCTTTCCACCCAC-3'

Table 5.1 List of alleles and primer sequences used in *Lrat* PCR genotyping. Primer 3 is a common forward primer for both primers 1 and 2. Amplification from primers 1 and 3 produced a 300 bp band, corresponding to the wild-type *Lrat* allele, while amplification from primers 2 and 3 produced a 370 bp band, corresponding to the *Lrat* null allele.

(Thermo Fisher Scientific). PCR reaction conditions for *Lrat* genotyping occurred over 3 stages. Stage 1 had a 3-minute cycle at 94℃, followed by stage 2 which required 35 cycles of 30 seconds at 94℃ proceeded by 30 seconds at 61℃ and 1 minute at 72℃. Stage 3 concluded the PCR reaction with a cycle at 72℃ for 10 minutes.

5.3.1.3 Agarose Gel Electrophoresis

A 1.2% gel (1.2 g agarose / 100 ml TAE / 5 µL Red Safe nucleic acid staining solution) was set for 15 minutes and wells were loaded, starting with a 100 bp DNA Ladder (Invitrogen, Thermo Fisher Scientific), followed by 18 µL of each PCR product sample and positive and negative controls. Gels were run in a horizontal electrophoresis system (Owl EasyCast B2; Thermo Fisher Scientific) at 150V for 50 minutes, then bands were analyzed on the ChemiDoc Touch Imaging System (BioRad, Hercules, California, USA) to assess for the presence or absence of *Lrat.* Mice with a single band at 300 bp were considered wild-type for *Lrat*, mice with a single band at 370 bp were considered knockout for *Lrat*, and mice with two bands, one at 300 bp and at 370 bp, were denoted as heterozygous for *Lrat* (Figure 5.2).

5.4 Results

5.4.1 Maternal Physical Characteristics of Lrat-/- Versus Wild-Type mice

Physical characteristics of dam tissues were analyzed at postnatal day 21 (weaning) to assess for any significant differences between wild-type and $Lrat^{-/-}$ dams which may affect result outcomes (Figure 5.3).

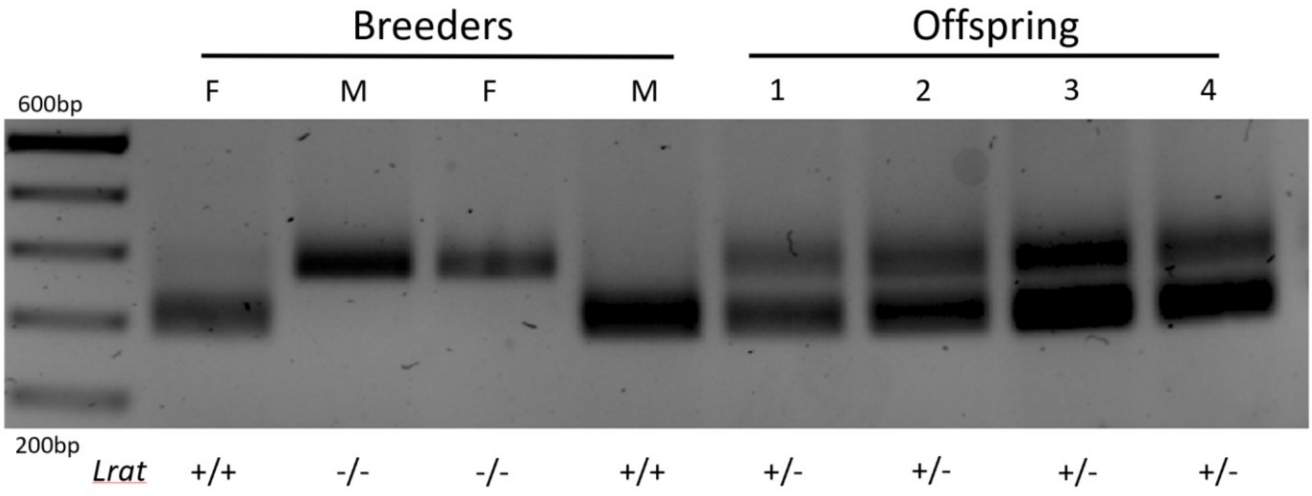

Figure 5.2 Representative genotyping of *Lrat* transgenic mice. Mutant allele ($Lrat^{-/-}$) breeders display one band at 370 bp. Wild-type allele ($Lrat^{+/+}$) breeders display one band at 300 bp. Heterozygosity of *Lrat* expression ($Lrat^{+/-}$) in offspring born from a mutant breeder mated with a wild-type breeder is denoted by the presence of two bands at 370 bp and 300 bp.

Regardless of the presence of *Lrat*, both wild-type and *Lrat*$^{-/-}$ dam groups displayed no significant changes in maternal mean liver weight nor maternal visceral white adipose tissue weight (1.52 ± 0.68 g and 1.82 ± 0.30 g Liver, and 0.46 ± 0.25 g and 0.88 ± 0.35 g VWAT, respectively; Figure 5.3 D and F). Maternal body weight, however, was shown to be significantly increased ($p < 0.01$) in *Lrat*$^{-/-}$ dams (33.32 ± 2.78 g) compared to wild-type dams (27.23 ± 2.74 g) (Figure 5.3 C). This was visually evident with *Lrat*$^{-/-}$ mice appearing considerably larger than their wild-type counterparts. Mean mammary gland weight values were not statistically significant in *Lrat*$^{-/-}$ dams (0.37 ± 0.20 g) versus wild-type dams (0.26 ± 0.14 g), as depicted in Figure 5.3 G. Comparison of the ratio of maternal liver weight to body weight suggests that the difference in dam body weight correlates to the difference in liver weight, therefore retinoid content in HPLC data should be unaffected by the difference in dam size. This is depicted by the non-significant difference in ratio values of the *Lrat*$^{-/-}$ dams (0.055 ± 0.0075) and wild-type (0.055 ± 0.0043) control dams (Figure 5.3 E). Litter size and maternal age (days) at the litter's birth were also examined for deviations resulting from the loss of *Lrat* expression. As shown in Figures 5.3 A and B, these parameters remained constant between wild-type control and *Lrat*$^{-/-}$ groups, where we observed each littering 8.25 ± 2.22 pups to 8.00 ± 1.87 pups, respectively, at ages 87.25 ± 31.94 days to 119.50 ± 38.56 days respectively. No obvious differences in appearance suggestive of vitamin A deficiency (eg. based on activity level, fur quality, moistness of epithelial tissue, growth and size, etc.) were apparent in either group of dams.

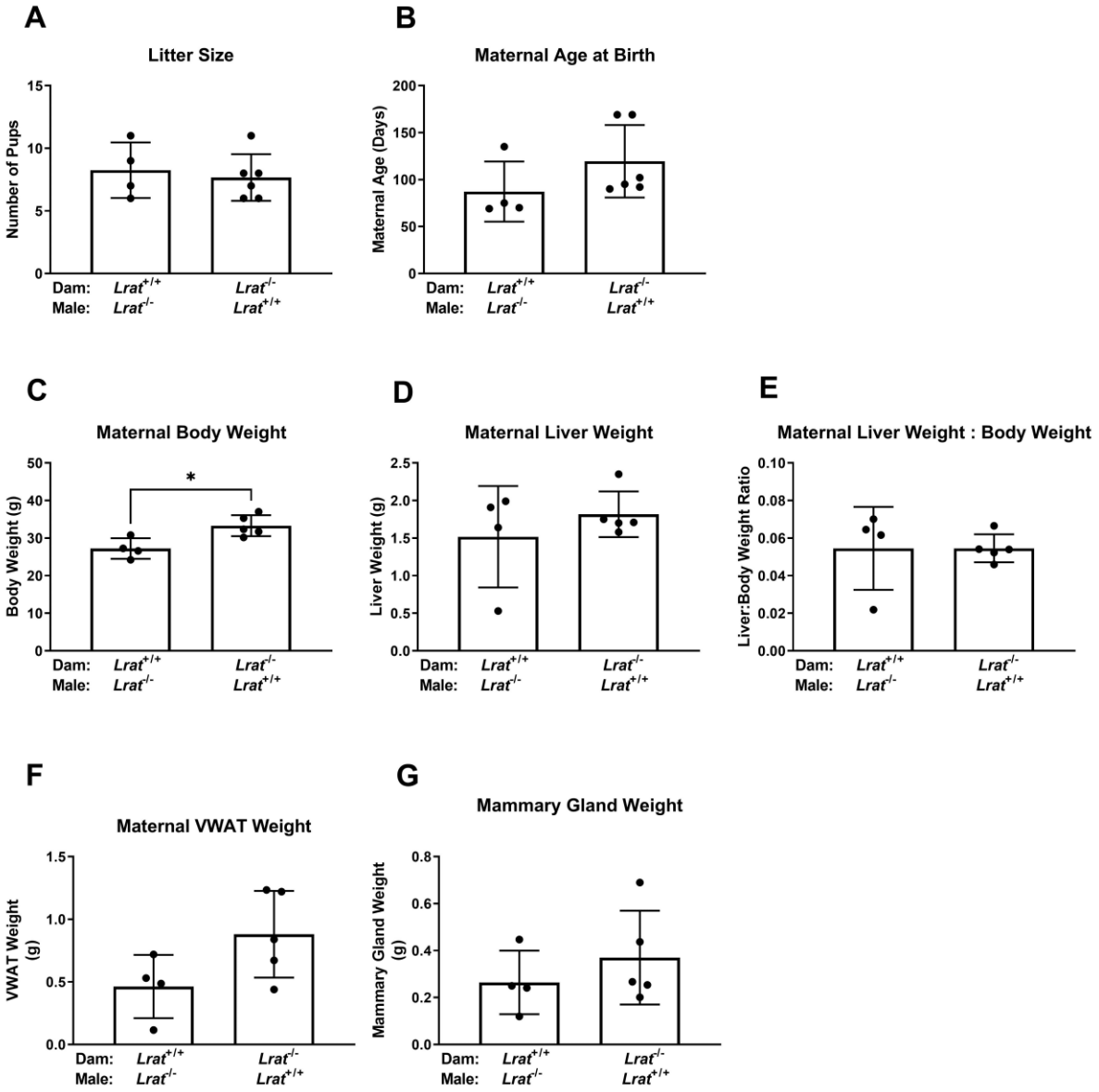

Figure 5.3 Maternal physical characteristics of *Lrat*$^{-/-}$ versus wild-type mice. (A) Litter size at parturition, (B) maternal age at the birth of the offspring, (C) maternal body weight, (D) maternal liver weight, (E) maternal liver to body weight ratio, (F) maternal visceral white adipose weight, (G) maternal mammary gland weight. All characteristics C - G were assessed upon weaning at postnatal day 21. Statistical significance, $p < 0.05$ between *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ groups is denoted by * and was determined using an unpaired Student's T-test.

5.4.2 Physical Characteristics of Offspring from Lrat-/- Versus Wild-Type Dams

In addition to *Lrat*$^{-/-}$ and wild-type dams, physical characteristics of pups were assessed to understand if the presence or loss of *Lrat* in the mother would have a significant influence on pup physical development (Figures 5.4, 5.5 and 5.6). At postnatal day 1 (birth), it was determined that pups with a *Lrat*$^{-/-}$ mother had a significantly greater ($p < 0.005$) mean body weight (1.70 ± 0.16 g) compared to pups with a wild-type mother (1.45 ± 0.089 g; Figure 5.4 A). Mean body weight values of P7 offspring (Figure 5.4 B) also displayed a statistically significant ($p < 0.05$) increase in those with a *Lrat*$^{-/-}$ dam (5.46 ± 1.44 g) in comparison a wild-type dam (4.52 ± 0.67 g). At P21, however, this difference in pup size was not present (Figure 5.4 C), with mean body weight values showing 14.35 ± 1.58 g and 13.19 ± 1.34 g for offspring with a *Lrat*$^{-/-}$ versus wild-type dam, respectively. Mean liver weight was also examined in pups across all three time points (P1, P7 and P21) in Figure 5.5 A, B and C, respectively. These findings indicated no significant differences in liver weight between *Lrat*$^{-/-}$ and wild-type mothered offspring at all pup ages; P1 (0.071 ± 0.016 g and 0.057 ± 0.0092 g, respectively), P7 (0.17 ± 0.059 g and 0.15 ± 0.034 g, respectively), and P21 (0.69 ± 0.075 g and 0.61 ± 0.11 g, respectively). Assessment of the liver weight to body weight ratio of pups in Figure 5.6 A, B and C parallel this finding, indicating pups from wild-type dams (0.040 ± 0.040 g, 0.033 ± 0.0041 g, 0.046 ± 0.0016 g) verses Lrat$^{-/-}$ dams (0.042 ± 0.0083 g, 0.031 ± 0.0038 g, 0.048 ± 0.0016 g) had no significant variations at P1, P7 and P21, respectively. Physical indications of vitamin A deficiency (eg. based on activity level, fur quality, moistness of epithelial tissue, growth and size, etc.) were not apparent in any pups.

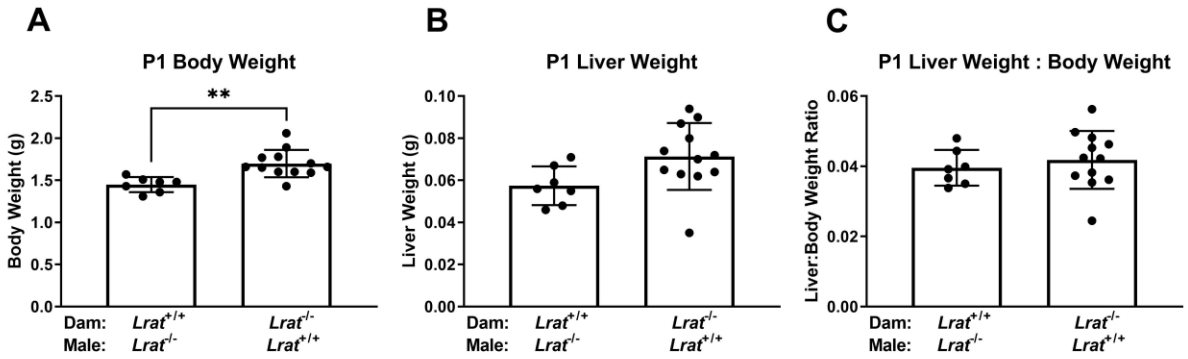

Figure 5.4 P1 pup physical characteristics from *Lrat*$^{-/-}$ versus wild-type dams. (A) Body weight at birth, (B) liver weight at birth, (C) liver to body weight ratio at birth. Statistical significance, $p < 0.05$ between pups from *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ dams is denoted by * and was determined using an unpaired Student's T-test.

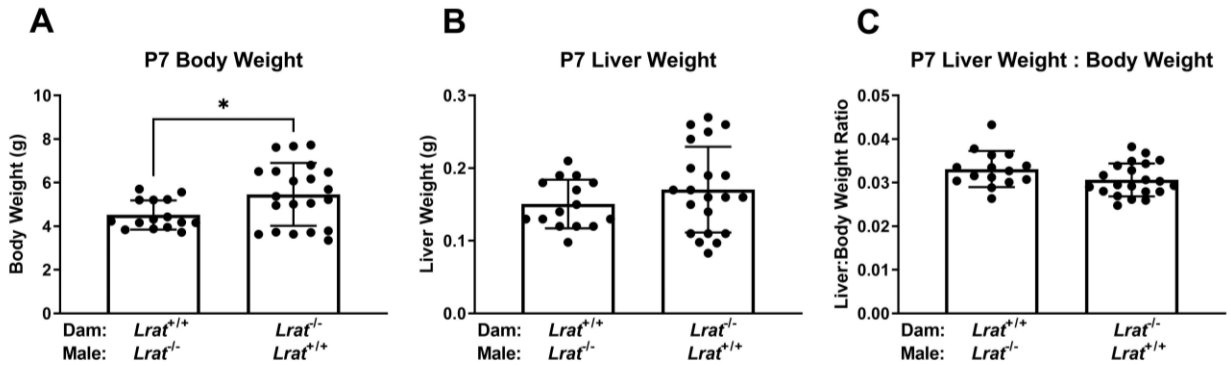

Figure 5.5 P7 pup physical characteristics from *Lrat*$^{-/-}$ versus wild-type dams. (A) Body weight during lactation, (B) liver weight during lactation, (C) liver to body weight ratio during lactation. Statistical significance, $p < 0.05$ between pups from *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ dams is denoted by * and was determined using an unpaired Student's T-test.

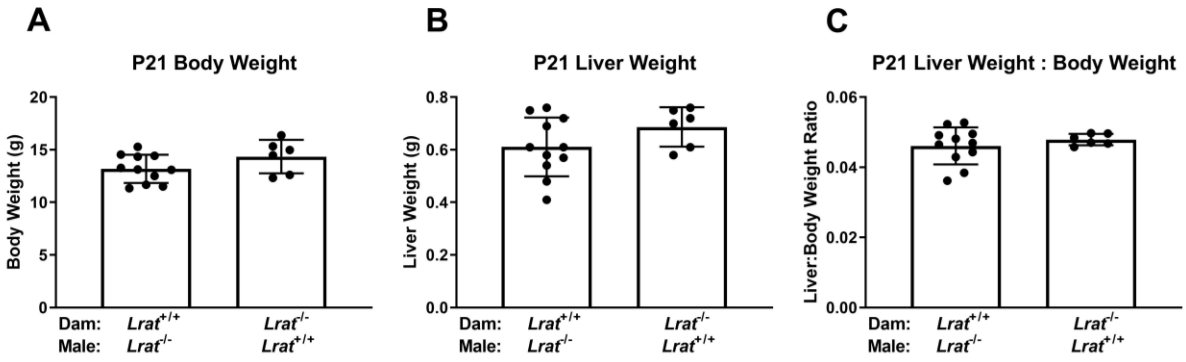

Figure 5.6 P21 pup physical characteristics from *Lrat*$^{-/-}$ versus wild-type dams. (A) Body weight at weaning, (B) liver weight at weaning, (C) liver to body weight ratio at weaning. Statistical significance, $p < 0.05$ between pups from *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ dams is denoted by * and was determined using an unpaired Student's T-test.

5.4.3 Retinol and Retinyl Ester Content in Dams with Genetic Manipulations of Lrat

Maternal vitamin A status was confirmed by HPLC quantification of wild-type and *Lrat*$^{-/-}$ dam retinol and retinyl ester concentrations assessed at postnatal day 21 (Figure 5.7). As predicted, loss of *Lrat* expression contributed to a ~92% reduction in hepatic retinol, and complete loss of hepatic retinyl ester reserves. Liver HPLC analysis revealed a significant decrease ($p < 0.0001$) in retinol concentrations in *Lrat*$^{-/-}$ mice compared to wild-type mice with 1.26 ± 0.052 nmol ROL/g versus 16.07 ± 3.56 nmol ROL/g, respectively (Figure 5.7 B). Evaluation of mean liver retinyl ester concentrations in *Lrat*$^{-/-}$ dams (0.00 ± 0.00 nmol RE/g) were significantly reduced ($p < 0.0001$) in comparison to wild-type dams (283.87 ± 69.86 nmol RE/g) as shown in Figure 5.7 C, emphasizing the complete depletion of maternal hepatic retinyl ester reserves due to the removal of *Lrat* expression. Mean plasma retinol concentrations were additionally examined (Figure 5.7 A) to determine if *Lrat*$^{-/-}$ dams were unable to maintain regulated concentrations of vitamin A in the circulation, indicative of vitamin A deficiency. Interestingly, plasma retinol remained unaltered, depicting no significant changes between wild-type and *Lrat*$^{-/-}$ dams (1.08 ± 0.14 µmol ROL/L and 1.24 ± 0.20 µmol ROL/L, respectively).

5.4.4 HPLC Comparison of Retinol and Retinyl Ester Content in Offspring

Mean liver retinol and retinyl ester concentrations were determined by HPLC analysis of heterozygous offspring from a wild-type male bred with a *Lrat*$^{-/-}$ female, or a wild-type female bred with a *Lrat*$^{-/-}$ male (Figure 5.8). To determine if maternal hepatic retinyl ester stores are the primary contributing source to vitamin A reserves in offspring, we would expect a significant reduction in the vitamin A reserves of offspring from *Lrat*$^{-/-}$ dams, despite them having adequate

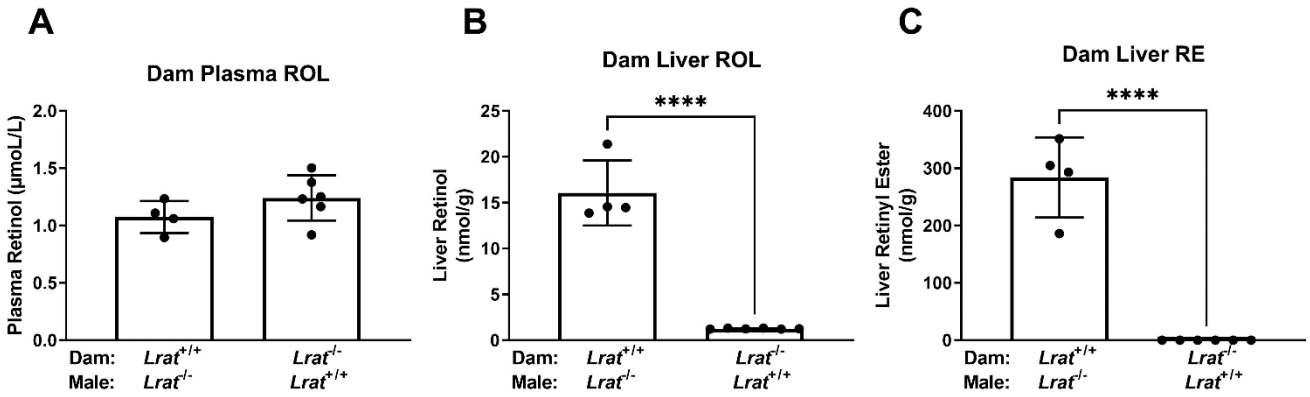

Figure 5.7 HPLC quantification analysis of retinol and retinyl ester concentrations (nmol/g) in dam plasma and liver, with (*Lrat*$^{+/+}$) and without (*Lrat*$^{-/-}$) *Lrat* expression. (A) Retinol content in dam plasma, (B) retinol content in the dam liver, and (C) retinyl ester in the dam liver. Statistical significance, $p < 0.05$ between *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ groups is denoted by * and was determined using an unpaired Student's T-test.

vitamin A intake. Interestingly, no significant reductions in the hepatic vitamin A status of heterozygous offspring born to *Lrat*$^{-/-}$ females versus wild-type females was observed. Livers from P1 pups displayed non-significant retinol values between those with a wild-type (3.71 ± 1.08 nmol ROL/g) versus a *Lrat*$^{-/-}$ (2.80 ± 1.02 nmol ROL/g) dam (Figure 5.8 A). However, in Figure 5.8 B, mean retinyl ester concentrations in the P1 liver were statistically significant ($p < 0.05$) with a ~17% increase in pups with an *Lrat*$^{-/-}$ dam (31.2 ± 8.97 mol RE/g), compared to those with a wild-type dam (25.9 ± 11.9 nmol RE/g). P7 evaluation of mean liver retinol and retinyl ester levels shown in Figure 5.8 C and D respectively, also depicted no significant differences in offspring retinol concentrations regardless of the *Lrat* status of the mother. Pups from wild-type dams had 1.62 ± 0.28 nmol ROL/g, while pups from *Lrat*$^{-/-}$ dams had 3.38 ± 2.87 nmol ROL/g (Figure 5.8 C). Retinyl ester content rose significantly ($p < 0.05$) by ~47% in offspring from *Lrat*$^{-/-}$ mothers compared to their wild-type counterparts (39.19 ± 16.16 nmol RE/g, to 24.89 ± 10.64 nmol RE/g, respectively; Figure 5.8 D). Assessment of mean liver retinol and retinyl ester levels in wild-type versus *Lrat*$^{-/-}$ mothered P21 pups (Figure 5.8 E and F respectively) depicted similar non-significant changes in retinol concentrations (8.43 ± 2.97 nmol ROL/g and 7.57 ± 2.00 nmol ROL/g, respectively), yet ~45% greater hepatic retinyl ester content (51.53 ± 7.70 nmol RE/g and 93.59 ± 20.01 nmol RE/g; $p < 0.001$, respectively) indicative of the offspring from the dam *Lrat*$^{-/-}$ group developing greater hepatic retinyl ester reserves despite the loss of vitamin A transfer from maternal hepatic stores. Finally, mean plasma retinol concentrations between P21 pups from wild-type (0.72 ± 0.055 µmol ROL/L) versus *Lrat*$^{-/-}$ dams (1.03 ± 0.18 µmol ROL/L) increased, suggesting circulating levels of vitamin A were maintained (Figure 5.9).

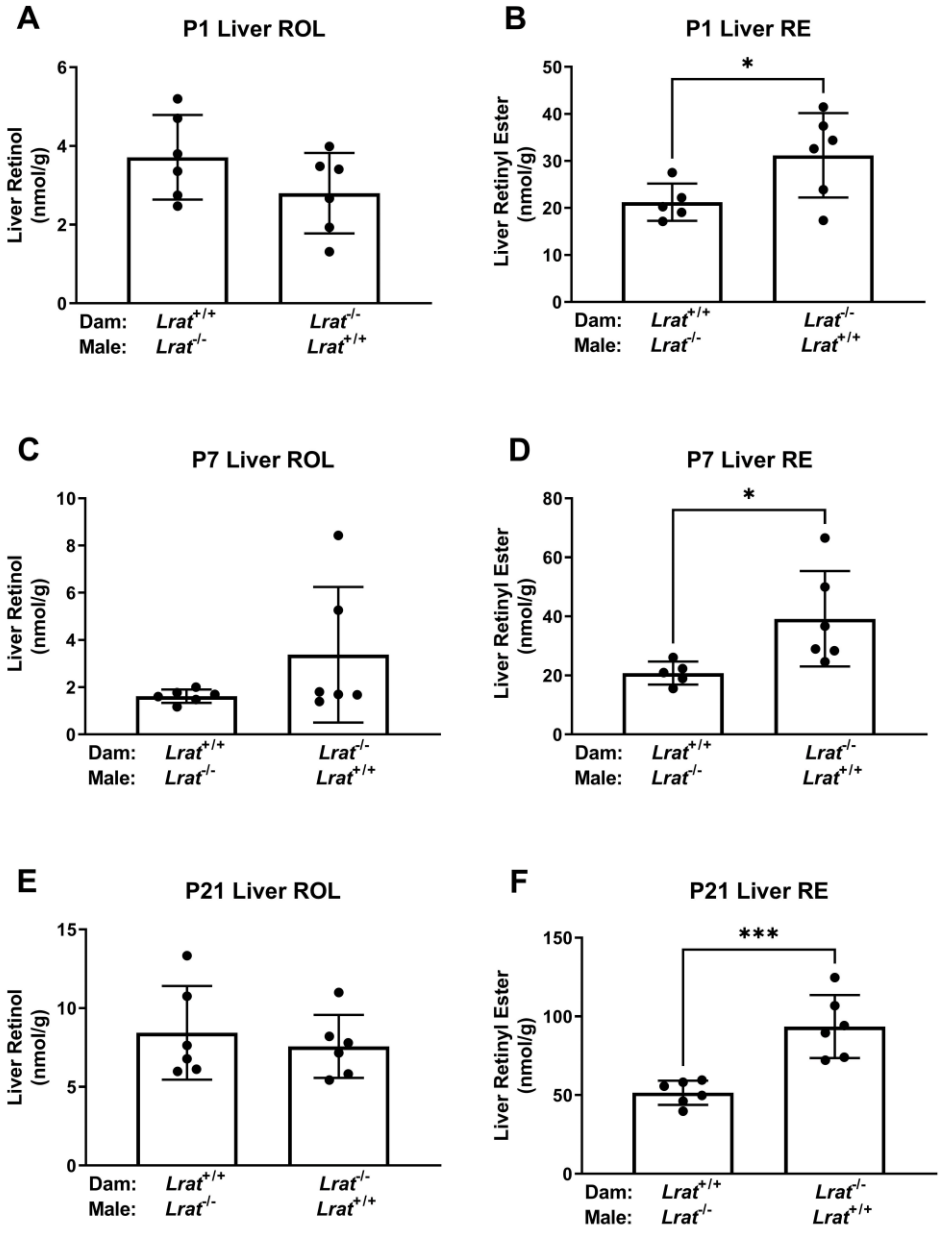

Figure 5.8 HPLC quantification analysis of retinol and retinyl ester concentrations in pup plasma and liver from *Lrat*$^{+/+}$ dams mated with *Lrat*$^{-/-}$ males and *Lrat*$^{-/-}$ dams mated with *Lrat*$^{+/+}$ males, respectively. This occurred during P1 (birth), P7 (lactation), and P21 (weaning). (A) Retinol content in the P1 pup liver, (B) retinyl ester content in the P1 pup liver, (C) retinol content in the P7 pup liver, (D) retinyl ester content in the P7 pup liver, (E) retinol content in the P21 pup liver, (F) retinyl ester in the P21 pup liver. Statistical significance, $p < 0.05$ between *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ groups is denoted by * and was determined using an unpaired Student's T-test.

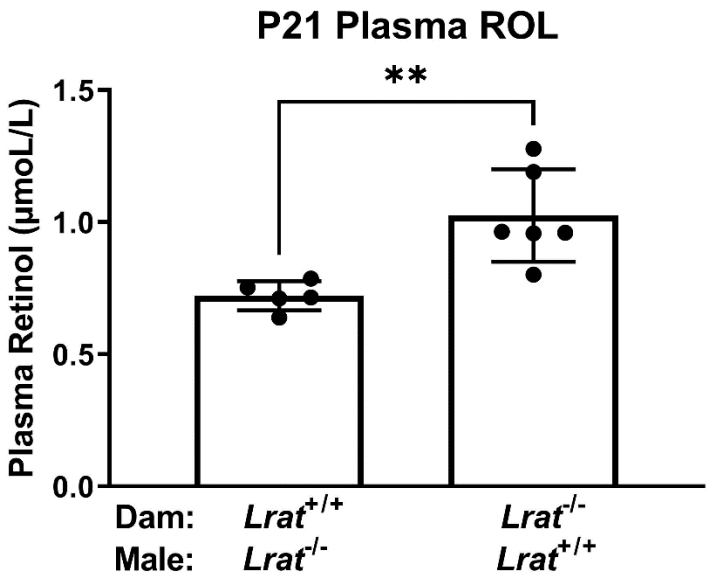

Figure 5.9 HPLC quantification analysis of P21 pup plasma retinol concentrations. Retinol content in the P21 pup plasma, was assessed in pups from *Lrat*$^{+/+}$ dams mated with *Lrat*$^{-/-}$ males and *Lrat*$^{-/-}$ dams mated with *Lrat*$^{+/+}$ males, respectively. Statistical significance, $p < 0.05$ between *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ groups is denoted by * and was determined using an unpaired Student's T-test.

5.5 Discussion

Despite progress in understanding the molecular mechanisms of retinoid incorporation into mammary milk, it is highly controversial whether circulating retinol-RBP4 or postprandial chylomicron retinyl ester is the primary determinant of vitamin A reserves in offspring. Based on our findings, we speculate maternal hepatic reserves to be less involved than previously thought, contrary to the work of Green *et al.* (147) and by Vahlquist and Nilsson (201) who estimate ~40–90% of milk retinoid is derived from this pathway. While we agree that retinol-RBP4 is a key component in this process which allows consistent transfer of vitamin A to the mammary tissue, we question the emphasis on its role as a sole contributor to mammary milk vitamin A status and subsequent offspring vitamin A reserves, independent of dietary postprandial vitamin A.

As previously mentioned, the liver HSCs are the predominant storage site of vitamin A (2,4) which is mobilized as retinol-RBP4 for utilization at mammary tissue (2,4,9). With this knowledge, we aimed to genetically dissect the importance of maternal liver vitamin A stores towards mammary milk vitamin A status in lactating dams by generating an *Lrat* knockout mouse model. In the liver, hepatic stellate cells express high levels of *Lrat,* which catalyzes the trans-esterification of retinol [transferring an acyl group from the *sn*-1 position of phosphatidylcholine to retinol (202)] into its storage form retinyl ester (167,203,204). Therefore, in using a $Lrat^{-/-}$ model, we aimed to inhibit the ability for dams to establish hepatic retinyl ester reserves of vitamin A by removing this esterification process.

HPLC analysis was used to determine the hepatic retinol and retinyl ester concentrations of wild-type and $Lrat^{-/-}$ dams at postnatal day 21. As predicted, loss of *Lrat* expression contributed to a ~92% reduction in hepatic retinol, and complete loss of hepatic retinyl ester reserves

compared to wild-type dams. This is consistent with studies conducted by Batten *et al.* (167), who observed a 10,000-fold reduction of hepatic retinyl ester reserves in *Lrat*$^{-/-}$ mice, unlike in their wild-type counterparts. Moreover, additional findings from *Lrat* knockout experiments assessing the effect of excess dietary vitamin A intake, have demonstrated that livers from *Lrat*$^{-/-}$ mice do not contain or only show trace levels of retinyl ester (205). Taken together, these findings emphasize the success of our model in completely depleting maternal hepatic retinyl ester reserves, allowing us to solely dissect the role of retinol-RBP4 vitamin A incorporation into the mammary milk. This observed eradication of maternal hepatic reserves of vitamin A also affirms that there is no alternative enzymatic pathway compensating for the loss of LRAT activity in the liver. This is important because both *Lrat* and diacylglycerol acyltransferase (*Dgat1*) are known to be expressed in liver, however, consistent with our data, *Dgat1* does not appear to contribute to the synthesis of hepatic retinyl ester reserves (206,207). Therefore, we conclude that LRAT accounts for most if not all enzymatic retinyl ester formation in the liver. Mean plasma retinol concentrations were additionally examined to verify if *Lrat*$^{-/-}$ dams could maintain tightly circulating levels of vitamin A protective against vitamin A deficiency. Interestingly, plasma retinol remained unaffected between wild-type and *Lrat*$^{-/-}$ dams. Given the absence of maternal hepatic vitamin A reserves, we predict that the postprandial dietary vitamin A pathway is compensating as the primary source of vitamin A in this circumstance. It is also possible that the free retinol pools in the maternal liver are contributing to the preservation of circulating retinol levels in the plasma, given they were revealed to significantly decline in *Lrat*$^{-/-}$ mice. Finally, comparison of physical characteristics displayed no significant discrepancies between wild-type and *Lrat*$^{-/-}$ dams (with the exception of body weight) and no clinical indications of vitamin A deficiency. This is consistent with several *Lrat* knockout models which

affirm no apparent phenotypic differences or histological signs of liver fibrosis in these mice, despite the absence of liver retinyl ester stores (46,167), and alludes to chylomicron retinoids accounting for the viability of the mice.

To determine the impact of maternal hepatic vitamin A stores on the vitamin A status of offspring, mean liver retinol and retinyl ester content was assessed in heterozygous offspring from a wild-type male bred with a *Lrat*$^{-/-}$ female, or a wild-type female bred with a *Lrat*$^{-/-}$ male. It is important to note that only one allele of *Lrat* is required for the full function of this enzyme in offspring, therefore all pups had the same enzymatic capacity to establish hepatic reserves of vitamin A. In line with current thinking, if maternal hepatic stores of retinyl ester are the primary contributing source to vitamin A reserves in offspring, a significant decline in pup vitamin A reserves from *Lrat*$^{-/-}$ dams would be prevalent, despite consuming adequate vitamin A in the diet. However, as we anticipated the hepatic vitamin A status of heterozygous offspring born to *Lrat*$^{-/-}$ females compared to wild-type females displayed no such reduction in vitamin A (Figure 5.10).

In fact, livers taken from pups at birth (P1) depicted non-significant discrepancies in retinol values yet a ~17% increase in retinyl ester content in those with an *Lrat*$^{-/-}$ versus wild-type dam. As mentioned, assessing pup tissues at birth is important because it solely emphasizes the transplacental transfer of vitamin A content to the fetus during gestation. Current literature highlights this period to involve limited transfer of vitamin A, primarily to prioritize the physiological demands for adequate fetal development, rather than establishing vitamin A reserves in the liver (27,30,61,121). As such, maternal retinol-RBP4 has been regarded as a crucial source of fetal vitamin A during transplacental transfer (2,46,127). Given we see no significant reduction in retinyl ester reserves in pups from *Lrat*$^{-/-}$ dams versus wild-type dams, it is assumed that the maintenance of these reserves is compensated by chylomicron dietary

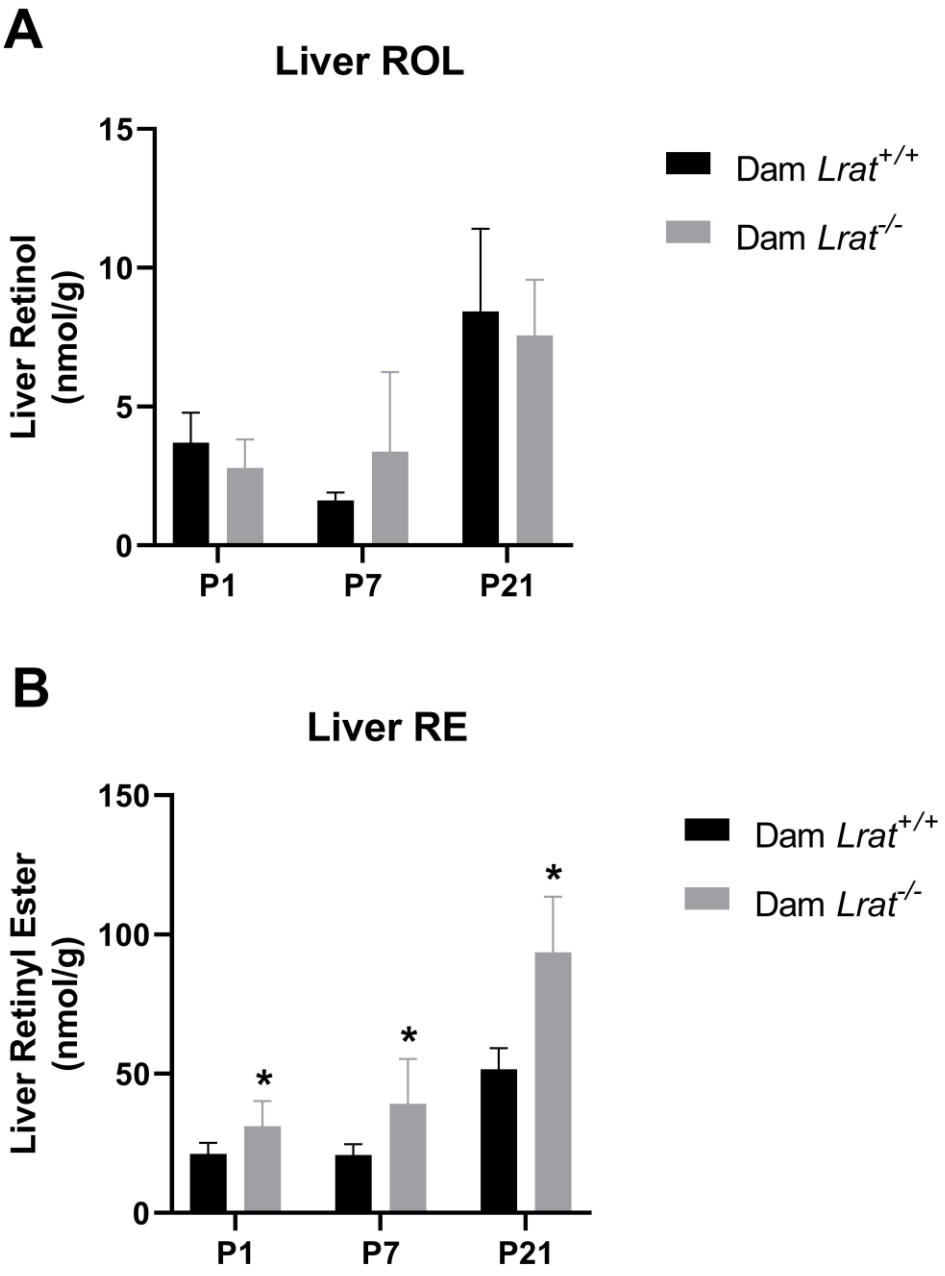

Figure 5.10 Cumulative comparisons of HPLC quantification analysis of retinol and retinyl ester concentrations at P1, P7, and P21. This is examined between pups from *Lrat*$^{+/+}$ dams mated with *Lrat*$^{-/-}$ males and *Lrat*$^{-/-}$ dams mated with *Lrat*$^{+/+}$ males, respectively**.** (A) Hepatic retinol content comparison in the pup, (B) hepatic retinyl ester content comparison in the pup. Statistical significance, $p < 0.05$ between *Lrat*$^{+/+}$ and *Lrat*$^{-/-}$ groups is denoted by * and was determined using an unpaired Student's T-test.

vitamin A, which Lesley *et al.* (208) notes can be taken up directly by the placenta to affect fetal development. This thinking can be extended to explain why there is no observed change in hepatic retinol content, as well as the viability of the offspring with no apparent pathology, suggesting the fetus is adequately supported by dietary vitamin A to deter manifestations of vitamin A deficiency.

Assessment of mean liver retinol and retinyl ester levels in wild-type versus *Lrat*$^{-/-}$ mothered P7 and P21 pups depict comparable trends with non-significant changes in retinol concentrations, yet ~47% and ~45% greater hepatic retinyl ester content, respectively, despite the loss of vitamin A transfer from maternal hepatic retinyl ester stores. Assessment of pup hepatic tissues at these time points is relevant for understanding vitamin A transfer to the mammary milk during the lactational period, until the point of weaning. This assumes pup hepatic reserves were established purely from mammary milk vitamin A content, independent of direct dietary vitamin A intake from chow pellets. Similar to the P1 offspring, it is predicted that chylomicron vitamin A is compensating for the absence of maternal hepatic retinyl ester reserves, allowing for adequate vitamin A accumulation in the milk and subsequent uptake in the offspring during nursing. This assumption is supported by our findings which exhibit no significant reductions in retinol and retinyl ester content in pups from *Lrat*$^{-/-}$ mothers, indicative of an alternative pathway meeting their vitamin A requirements. HPLC analysis of P21 plasma retinol further emphasizes this conclusion in depicting maintained circulating levels of vitamin A between pups from wild-type versus *Lrat*$^{-/-}$ dams. Moreover, physical characteristics showed no significant changes other than body weight in P1 and P7 pups, which we speculate may have resulted from Lrat$^{-/-}$ dams having a significantly larger body size and weight than their wild-type counterparts, subsequently allowing them to birth larger offspring (200). Taken together with these physical

observations of the pups, we can affirm both P7 and P21 pups retained sufficient vitamin A status preventative against complications associated with vitamin A deficiency.

Although various experiments using an *Lrat* knockout model have been implemented, none have been used to examine the role of maternal hepatic stores in establishing vitamin A status in milk and subsequent vitamin A reserve accumulation in offspring. As a result, this research program is the first of its kind in utilizing a genetic mouse model to assess the primary determinant of vitamin A reserves in offspring. Nonetheless, analysis of breastmilk vitamin A has been used to determine the extent of vitamin A deficiency within a given population of lactating women and their infants (76,125). Findings by O'Byrne *et al.* (125) affirm our logic, which highlights that milk retinyl ester content in *Lrat*$^{-/-}$ mice depict no significant difference in total retinoid concentration compared to wild-type milk, which can be extrapolated to what we would expect to find in offspring vitamin A reserves. Similarly, findings from *Rbp4* $^{-/-}$ dam studies observe that dams maintain milk retinoid concentrations similar to those in matched wild-type mice and are viable aside from visual defects (125,209,210). In agreement with our predictions, these authors speculate this maintenance of milk vitamin A status to result from a greater postprandial delivery of vitamin A, which is mediated by heightened lipoprotein lipase enzymatic activity at the mammary tissue.

While our findings reflect our expected hypothesis, we were surprised to observe offspring from *Lrat*$^{-/-}$ dams having significantly higher retinyl ester stores (rather than equal retinyl ester quantities) than their counterparts from wild-type dams. Because our *Lrat*$^{-/-}$ model involves a whole-body removal of *Lrat* activity, we assume this to impact the elevation in retinyl ester status in these pups. This is because *Lrat* is broadly expressed in various tissues aside from the liver, notably at the intestine and mammary gland. In the intestine, LRAT is involved in the

esterification and packaging of retinyl ester into nascent chylomicrons (46,211). Given that the intestine possesses DGAT1 enzymatic activity, which can catalyze retinyl ester formation in the absence of LRAT (46,125,212), this process is known to be suboptimal because ~ 60% of the retinoid absorbed in chylomicrons is present as free retinol, compared to less than 10% found in wild-type mice. As a result, we speculate that *Lrat*$^{-/-}$ dams package higher amounts of free retinol into chylomicrons from the diet, which may be more efficiently streamlined for uptake into the milk droplets once at the mammary tissue. At the mammary tissue, LRAT also acts to catalyze the formation of retinyl esters that are incorporated into milk. When this enzymatic activity is abolished, DGAT1 similarly acts in place of LRAT, resulting in a significant reduction in milk retinyl ester concentrations and favors an increase in free retinol content (46,46,125,207,213). Normally, the dominant species of retinyl ester in the mammary milk of wild-type mice is retinyl palmitate followed by retinyl stearate (each accounting for ~40% of the total retinyl esters). However, data from O'Byrne *et al.* (125) suggests *Lrat*$^{-/-}$ mice display altered retinoid composition favoring esters of medium-chain acyl groups with shorter HPLC retention times. Therefore, we propose that the higher presence of free retinol in the *Lrat* knockout dams contributes to higher retinoid reserves in pups via increased efficiency of transfer and subsequent absorption across the placenta in P1 mice and into the mammary milk for uptake during lactation in P7 and P21 pups. Unfortunately, a limitation of our study is that we did not assess P7 stomach (which is a proxy for mammary milk measurements) as well as dam mammary tissue HPLC. This data may have provided us better insight regarding our assumptions for the enriched vitamin A content in pups from *Lrat*$^{-/-}$ dams, as well as confirm the prominent species of retinoid composed in the mammary milk. Given the importance of this knowledge, we plan to include the assessment of these tissues in a following study.

In conclusion, our data affirms our hypothesis that maternal mice with depleted vitamin A reserves and adequate vitamin A intake will generate offspring with normal vitamin A reserves. This emphasizes that delivery of retinoid via the postprandial pathway can compensate in the absence of maternal hepatic retinyl ester stores to ensure optimal incorporation of vitamin A into milk for use by the newborn, and subsequently establishes its criticality as a primary determinant of offspring vitamin A reserves.

CHAPTER 6 - Conclusions and Future Directions

6.1 Introduction

Vitamin A status is an important health determinant, as deficiency of this micronutrient is a significant contributor to long-term morbidity and mortality. This is especially prevalent during pregnancy, lactation and childhood, where nutritional demands for vitamin A are highest (3,9,65). Despite this, significant gaps in understanding remain regarding the mechanistic relationships among dietary vitamin A intake and milk vitamin A status. Particularly, the contribution of maternal vitamin A stores compared to direct dietary chylomicron intake in the development of offspring vitamin A reserves is still uncertain. Therefore, this thesis was developed to gain understanding into the primary determinant of vitamin A reserves in offspring, with an emphasis on the gestational and lactational period. As such, we implemented three research studies aimed at elucidating the mechanisms underlying delivery of vitamin A to maternal milk and offspring in mice, as well as an understanding of vitamin A metabolic modifications during the perinatal period in support of establishing sufficient vitamin A reserves early in life. In our first research study (Chapter 3) we examined maternal mammary vitamin A homeostasis by evaluating changes in retinoid metabolizing gene expression in virgin, lactating and involuted mice. For our second research study (Chapter 4) we explored the influence of maternal chylomicron vitamin A dietary intake in establishing offspring vitamin A reserves. This was achieved with a dietary intervention model using varied concentrations of vitamin A provided to dams during gestation and lactation. Finally, in our third research study (Chapter 5) we examined the role of maternal hepatic reserves in establishing offspring vitamin A status throughout gestation and lactation. This was accomplished using a genetic intervention model of transgenic *Lrat* $^{-/-}$ dams lacking hepatic vitamin A reserves. Taken together, these studies assess our overall hypothesis that maternal dietary vitamin A intake is the primary determinant of

vitamin A reserves in offspring and highlights the requirement for further analysis of the molecular mechanisms involved in establishing offspring vitamin A reserves.

6.2 Summary Discussion

Understanding the underlying mechanisms of vitamin A delivery to maternal milk and offspring are crucial for ensuring the development and maintenance of postnatal vitamin A reserves long-term, and the subsequent physiological well-being of the offspring. We have shown that changes in mammary morphology during lactation influence gene expression pertaining to the retinoid metabolic pathway, in support of vitamin A utilization by mammary milk (Chapter 3). As such, reductions in the expression of genes heavily involved in the metabolism of vitamin A into retinoic acid during lactation emphasizes a decline in the rate of retinoic acid biosynthesis and subsequent transcriptional activity. Increased *Lrat* expression suggests retinol esterification into retinyl esters is rather favored in mammary milk droplets, proposing that morphological changes in mammary tissue during lactation align with increased vitamin A incorporation into the mammary milk. Assessment of vitamin A metabolism and distribution during lactation underlines a demand for mammary retinol and retinyl ester as a local supply of vitamin A to be readily taken up by offspring during nursing. Maternal plasma retinol and hepatic retinyl ester content during the lactational period, further indicate that mammary reserves are more dramatically impacted by maternal dietary vitamin A intake than by existing maternal hepatic vitamin A stores, given the insignificant change in retinoid homeostasis and gene expression in these tissues. Genetic dissection using $Lrat^{-/-}$ dams to assess the primary determinant of vitamin A reserves in offspring (Chapter 5), depict related findings. Maternal mice with absent vitamin A hepatic retinyl ester reserves and sufficient dietary vitamin A intake generated heterozygous

offspring with normal vitamin A reserves, at birth, lactation, and weaning time points. Therefore, the maintenance of these hepatic vitamin A reserves is compensated by chylomicron dietary vitamin A from the postprandial pathway which acts in the absence of maternal hepatic retinyl ester stores to ensure optimal incorporation of vitamin A into mammary milk. This reinforces that appropriate maternal dietary intake of vitamin A is crucial in establishing adequate vitamin A reserves in offspring during suckling, due to its compensation in the absence of maternal hepatic stores. Our dietary intervention study (Chapter 4) supports this idea by showing that pups with a mother on a vitamin A deficient diet accumulate significantly less hepatic vitamin A reserves. This indicates that maternal hepatic stores alone cannot compensate and therefore may be insufficiently utilized in this circumstance. Since we examined the influence of maternal liver stores in Chapter 5, we are able to distinguish that the decrease in hepatic vitamin A in pups is predominantly due to the vitamin A deficient intake of the dams and not the vitamin A deficient diet causing dams to have insufficient stores to upregulate vitamin A for their pups. Consistent with our examination of hepatic retinoid homeostasis and gene expression presented in Chapter 3, we may assume that under normal conditions maternal hepatic vitamin A stores provide a steady but limited release of vitamin A to the mammary milk in response to lactation, which is more dramatically influenced by postprandial chylomicron retinyl ester uptake directly at the mammary gland. This would also explain the reduced but not deficient vitamin A status in the pups from mothers on a vitamin A deficient diet in Chapter 4. Moreover, this study affirms the limitation of transplacental transfer of vitamin A to fetal liver stores, and suggests that the neonate primarily relies on the lactational period via maternal milk to develop reserves adequate for sustaining growth and development. As such, we affirm that during gestation the primary determinant of offspring reserves is retinol-RBP4 mobilized from maternal hepatic stores, which

shifts to maternal dietary retinyl esters during lactation. Furthermore, comparison of liver retinol and retinyl ester content over time suggests vitamin A sufficient pups accumulate hepatic vitamin A stores with time, which is hindered by pups with dams on a vitamin A deficient diet. Together, our studies are significant because they can help to generate new knowledge of vitamin A transfer from mother to pup, and gain insight into mechanisms underlying the delivery of vitamin A to maternal milk. With complementary analysis, this data has the potential to be translated into improved approaches to mitigate the effects of childhood vitamin A deficiency.

6.3 Limitations and Future Directions

Throughout our investigation of the primary determinant of vitamin A reserves in offspring, we recognized multiple scientific limitations and future directions of analysis pertinent to each of our three experimental studies. As previously mentioned in Chapter 3, a current limitation of our model is that we examined the whole mammary gland rather than mammary epithelial cells exclusively, when analyzing markers of vitamin A metabolism in differing stages of mammary gland development. During lactation, the mammary epithelium proliferates and branches out into a ductal alveolar system capable of mammary milk synthesis and secretion (9,136). Given that this differentiation of mammary epithelium is critical during lactation to support milk production, it is our future focus to examine retinoid gene expression in this cell type. Three-dimensional mammary organoid cultures have been recognized as a valuable model for studying mammary epithelial cell development (214,215) and branching morphogenesis (216–218), with recent developments capable of exhibiting physiological processes involved in lactation and involution (219). Through the generous donation of organoid cultures by Han Li and colleagues (Institute Pasteur, Paris, France), our preliminary experiments using mammary epithelium

organoid cDNA have shown it to be a promising model for analyzing retinoid related genes during lactation, which will be examined in future studies. Additionally, we recognize this study could have benefited from assessing protein expression in addition to the mRNA level of genes of interest. Complementary analysis of both molecular populations could provide us with a more comprehensive understanding regarding vitamin A metabolism during lactation and is planned to be completed at a future time.

A recognized limitation in Chapter 4 is that we use a copious amount (25 IU/g diet) of vitamin A in the diet as a control, which is representative of individuals consuming an optimal amount of vitamin A in their diet. While this is an good model to gain a foundational understanding of how maternal dietary vitamin A intake affects offspring vitamin A reserves, it may not be clinically characteristic of what is observed in current day pregnant and lactating mothers. Although vitamin A deficiency is highly prevalent in developing nations, it is not exclusive to these populations and could have a sub-clinical effect in developed nations (82). For example, recent analyses from NHANES suggests that a large proportion of pregnant and lactating women in the USA do not meet the estimated average requirement of vitamin A. This suggests that they ingest marginal amounts of vitamin A in their diet. Therefore, we have extended this research to examine mouse dams consuming a marginal (4 IU/g diet) vitamin A diet throughout gestation and lactation and assessed offspring tissues at identical time points discussed in Chapter 4. Although 4 IU is considered marginal in pregnant mice, due to the elevated demand for vitamin A during lactation, it is likely inadequate to meet the recommended daily allowance for this group (191,192). Therefore, we aim to collectively examine 25 IU, 4 IU, and 0 IU dietary vitamin A intake to gain a comprehensive look at how maternal dietary vitamin A impacts offspring vitamin A status. Preliminary data has shown this model to follow similar

trends with our Chapter 4 data, emphasizing the importance of adequate maternal dietary vitamin A intake during gestation and lactation.

Finally, a key limitation of our study in Chapter 5 is that we did not do an assessment of P7 stomach and dam the mammary tissue with HPLC analysis. As alluded to prior, assessment of P7 stomach milk curd is considered a proxy for mammary milk intake (168). This data, paired with results from dam mammary retinol and retinyl ester content, will provide us with insight regarding mammary milk vitamin A composition, by confirming the prominent species of retinoid in the mammary milk and quantify the amount of vitamin A directly incorporated into milk droplets. Consequently, this will allow us to define why vitamin A content in pups from *Lrat*$^{-/-}$ dams is enriched compared to their counterparts from wild-type dams. Given the importance of this knowledge in understanding the influence of maternal hepatic stores during lactation on offspring vitamin A reserves, assessment of these tissues in a related study is necessary.

6.4 Clinical Relevance

Vitamin A intake is critical for establishing hepatic retinoid reserves and involved in mediating various physiological processes required for normal growth and development during the perinatal period. As mentioned, deficiency of this micronutrient is highly prevalent during stages of life where nutritional demands are higher, therefore pregnant, and lactating mothers, and young children are at a greater risk (3,9,65). In addition to being prevalent in developing countries, insufficient vitamin A intake and subsequent sub-clinical vitamin A deficiency is known to impact mothers in developed nations. According to the most recent National Health and Nutrition Examination Survey, 31% of American pregnant women do not meet the estimated

average requirement of vitamin A and 18% do not supplement for this inadequate intake (220). This is reflected in breast milk vitamin A content, which can negatively impact nursing children by increasing their risk of vitamin A deficiency and subsequently raise the prevalence and severity of infectious disease morbidity and mortality, visual impairments, immune dysregulation, amongst many other physiological pathologies (3,14,63,65,66,91). Our findings are clinically relevant, as they emphasize sufficient maternal dietary vitamin A intake during lactation to be the primary determinant for offspring hepatic vitamin A stores, protective against vitamin A deficiency. Moreover, we highlight that maternal vitamin A consumption during the perinatal period has persisting effects on the development of foundational reserves in offspring until early adulthood, which are not sufficiently compensated by maternal hepatic stores or with the introduction of direct sufficient dietary intake upon weaning.

The concept of Developmental Origins of Health and Disease (DOHaD) hypothesizes that exposure to environmental insults during critical stages of growth and development can impose significant consequences on the long-term health outcomes of an individual (221). Indeed, DOHaD implications can be drawn from this research program, suggesting that impaired maternal vitamin A intake during lactation is directly correlated to mammary milk vitamin A secretion to offspring. Subsequently, this could contribute to a delay in vitamin A reserve accumulation persisting after puberty, therefore increasing the risk of vitamin A deficiency and associated complications during adolescence, including the dysregulation of various physiological processes which may impact these individuals later in life. Of interest to us, vitamin A signaling is highly involved in mammary tissue production, differentiation, maintenance, and incidence of breast cancer. Given we observe this risk of vitamin A deficiency to persist throughout puberty [a period where vitamin A is involved in mammary morphogenesis

(9,135–137)], we hope this research may inspire further investigations underling the effect of maternal dietary intake on offspring mammary fibrosis and the risk of reduced lactational efficiency later in life.

Despite the significant public health threat vitamin A deficiency poses, progress has been variable since 2000, which is suspected to be offset by the recent COVID-19 pandemic (77). Therefore, we recognize multisectoral actions may be required to attenuate this issue. As such, our findings support undertaking additional research to examine breast milk as a screening tool for insight into offspring vitamin A uptake and to identify mothers in need of additional dietary interventions (76). Additionally, since social inequalities are often present in these mothers, increasing access to nutritional counseling services and promotion of optimal breastfeeding practices and policies are highly effective approaches for improving vitamin A status in infants (76,77). Tariku *et al*. (22), notes that maternal literacy is strongly correlated with positive nutritional outcomes in children, yet only half are raised with optimal breastfeeding. Moreover, Ross and Harvey (25), stresses that both replacing colostrum with prelacteal feeds and introducing non-exclusive breastfeeding after one week postpartum can be detrimental to infants because of its inferior nutrient composition compared to breastmilk (222) and capacity to increase exposure to allergens and pathogens. Taken together with our research outcomes, encouraging the promotion of early initiated and exclusive breast-feeding practices in mothers for at least six months postpartum (and supporting policies which allow them to do so) may be vital strategies in alleviating vitamin A deficiency in young children (25). As evidenced by our Chapter 4 findings, the success of these strategies is also reliant on proper maternal dietary vitamin A intake and therefore should be paired with dietary supplementation initiatives. Given we emphasize meeting dietary requirements specifically during lactation to the establish

foundational vitamin A reserves in offspring (regardless of the child's diet after weaning), we support continued utilization of high-dose maternal supplementation in the immediate postpartum period (25,113,223), dietary diversification initiatives for improved dietary quality (102), and biofortification of staple foods where supplementation or food variety are limited (14,102,113,115). Furthermore, continued data regarding vitamin A status is required for countries to make informed decisions that accurately target populations affected by vitamin A deficiency (73) so that supplementation programs can be appropriately utilized and not prematurely removed in areas where vitamin A deficiency poses a significant public health risk.

6.5 Overall Conclusion

In conclusion, this thesis offers significant insight into the mechanisms underlying the delivery of vitamin A to maternal milk and offspring in mice. As such, it successfully supports our novel hypothesis that maternal dietary vitamin A intake is the primary determinant of vitamin A reserves in offspring. Moreover, this research affirms the criticality of the mammary gland and lactational period in establishing foundational vitamin A reserves early in life required for sustaining neonate growth and development. Furthermore, this work is significant because it can generate new knowledge of vitamin A transfer between mother and pup, and with complementary analysis, has the potential to be translated into improved approaches for mitigating the prevalence of vitamin A deficiency in children.

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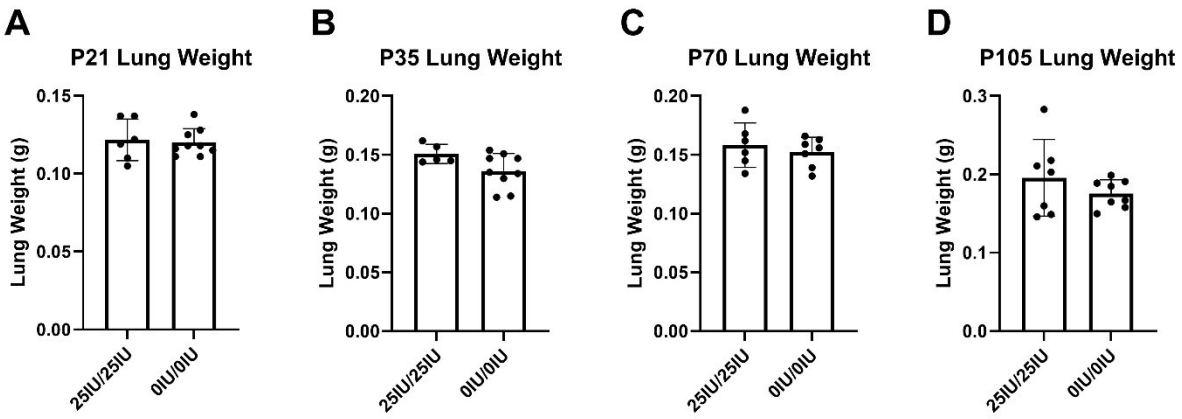
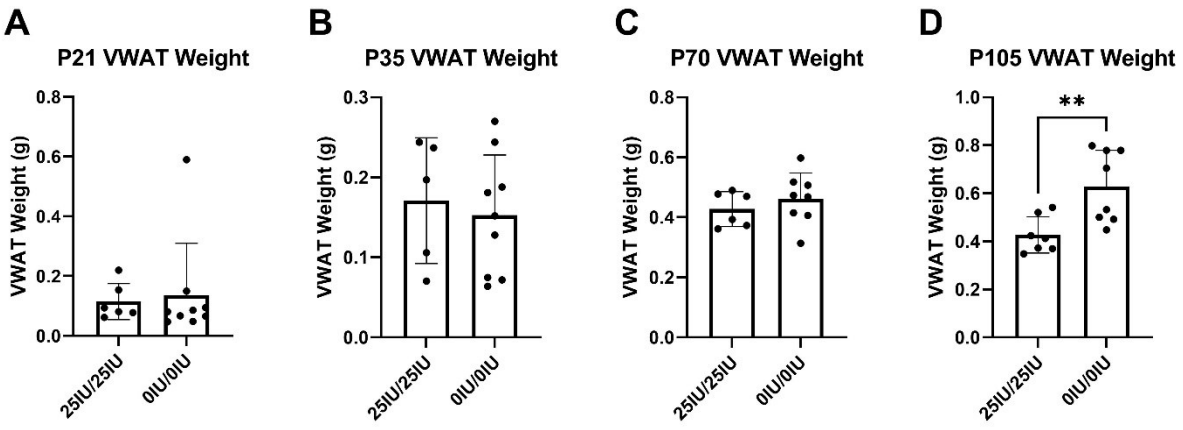
Appendix

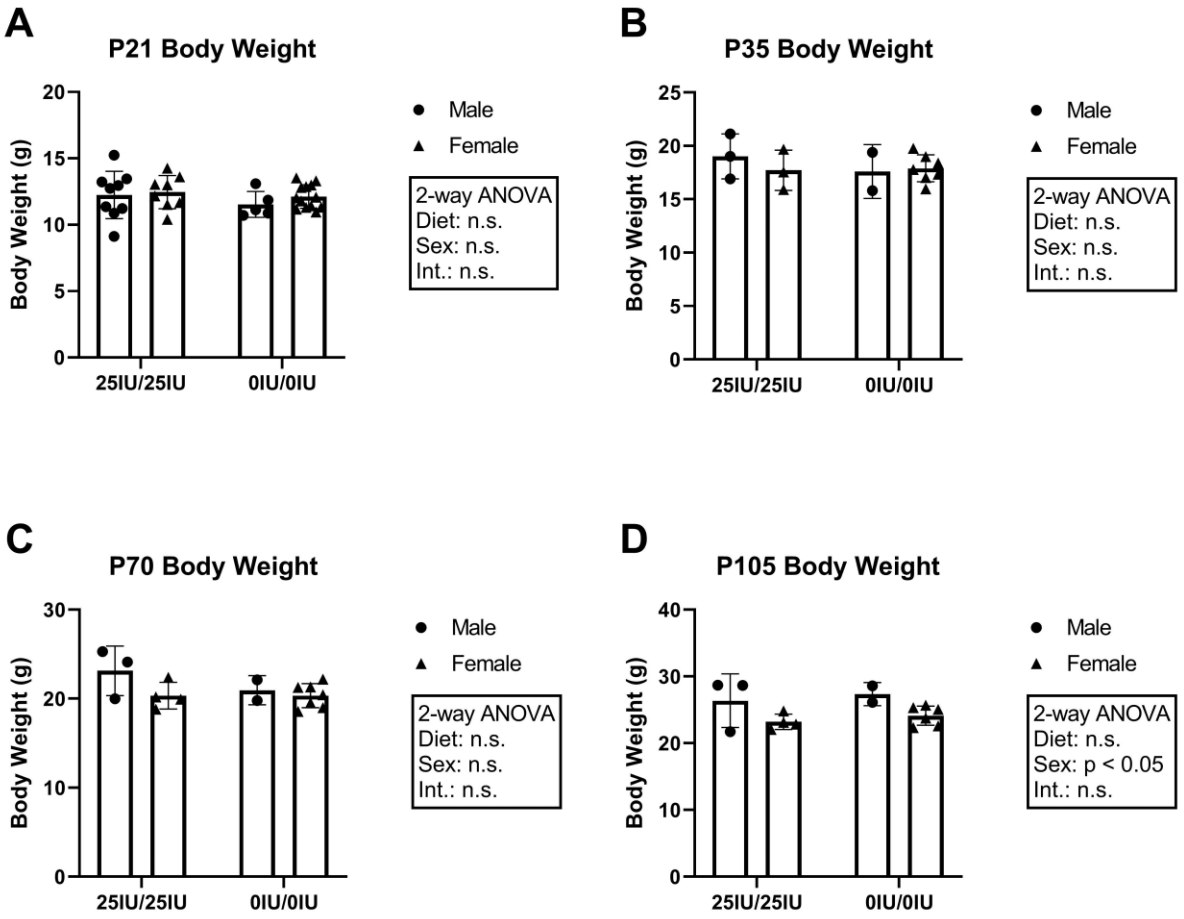
Chapter 4 Supplementary Figures



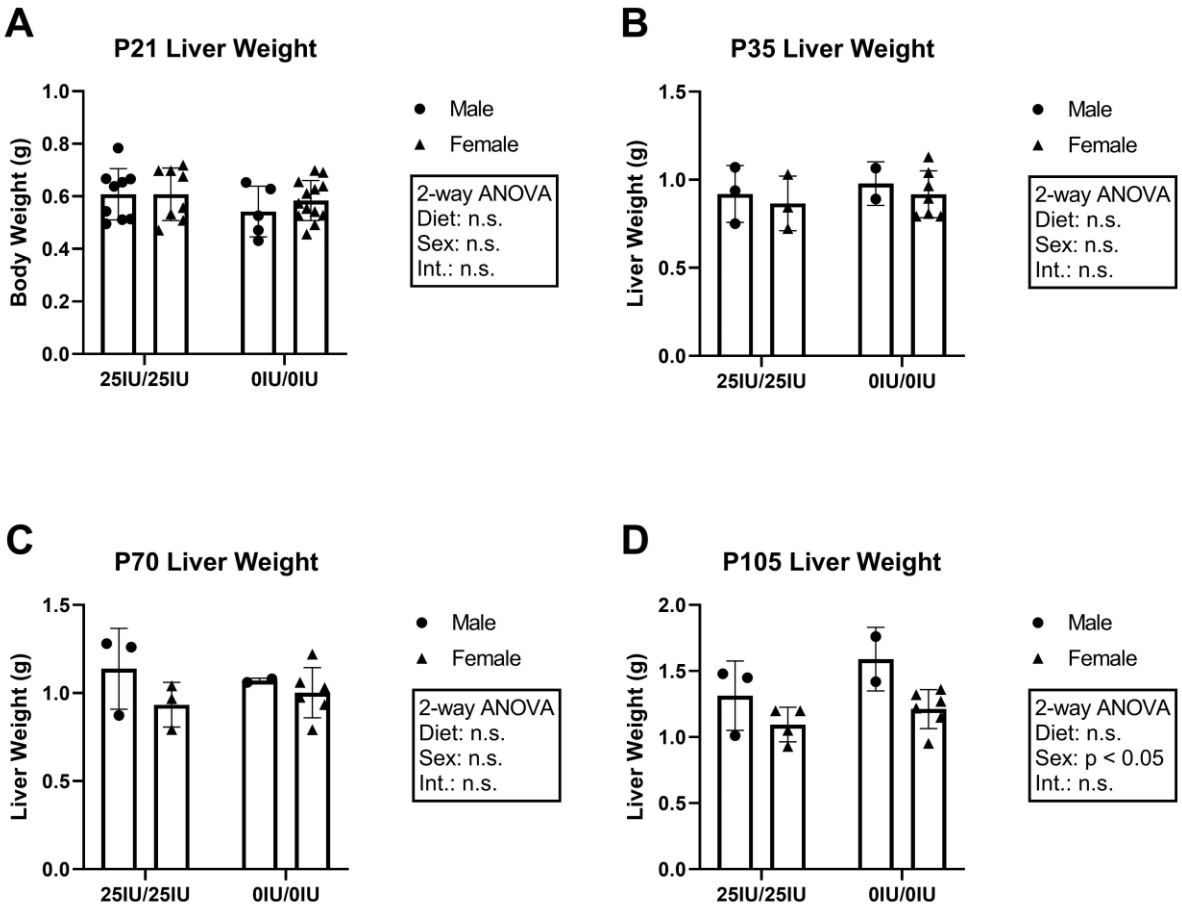
Supplementary Figure 4.1 Pup lung weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup lung weight at weaning (P21), (B) pup lung weight at two weeks post-weaning (P35), (C) pup lung weight at P70, (D) pup lung weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.



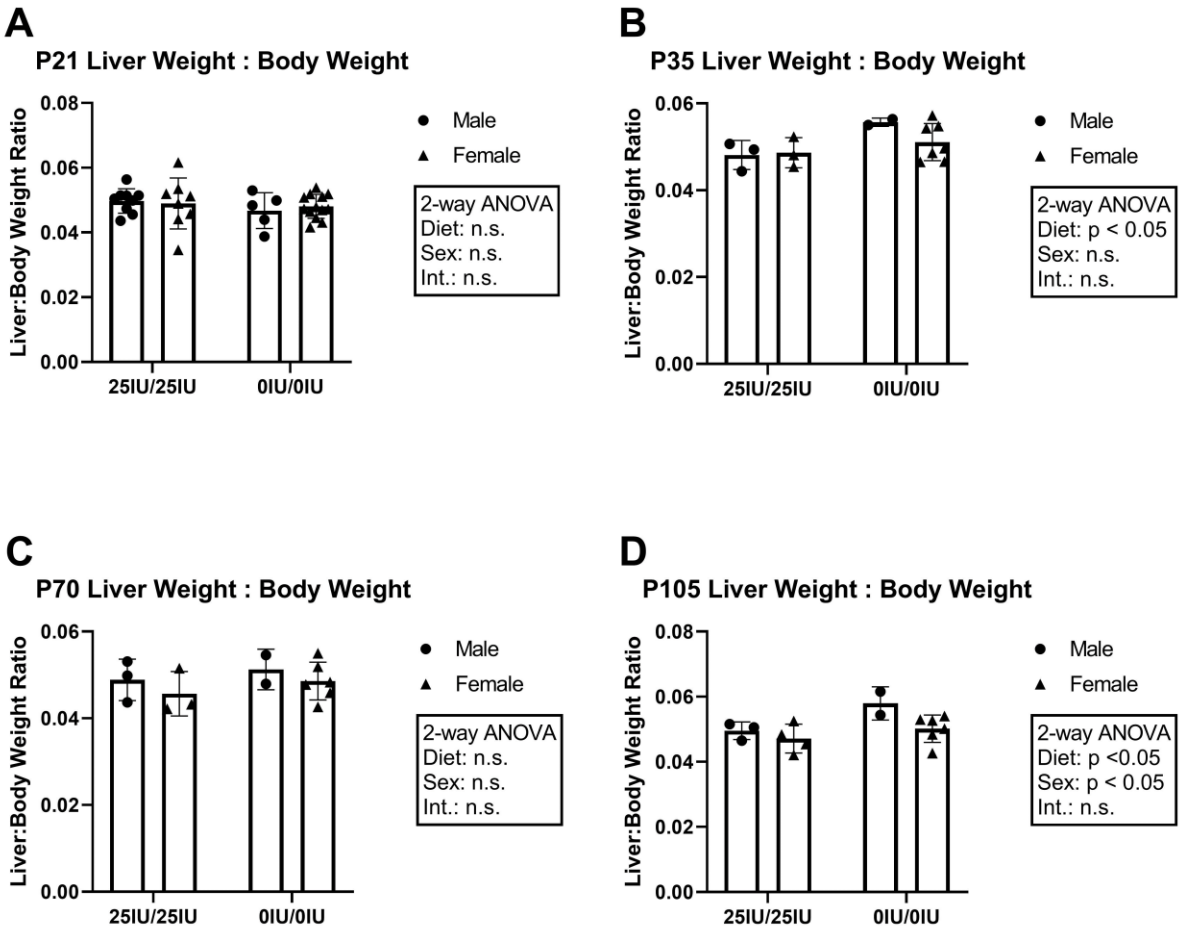
Supplementary Figure 4.2 Pup visceral white adipose tissue weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup visceral white adipose tissue weight at weaning (P21), (B) pup visceral white adipose tissue weight at two weeks post-weaning (P35), (C) pup visceral white adipose tissue weight at P70, (D) pup visceral white adipose tissue weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using an unpaired Student's T-test.



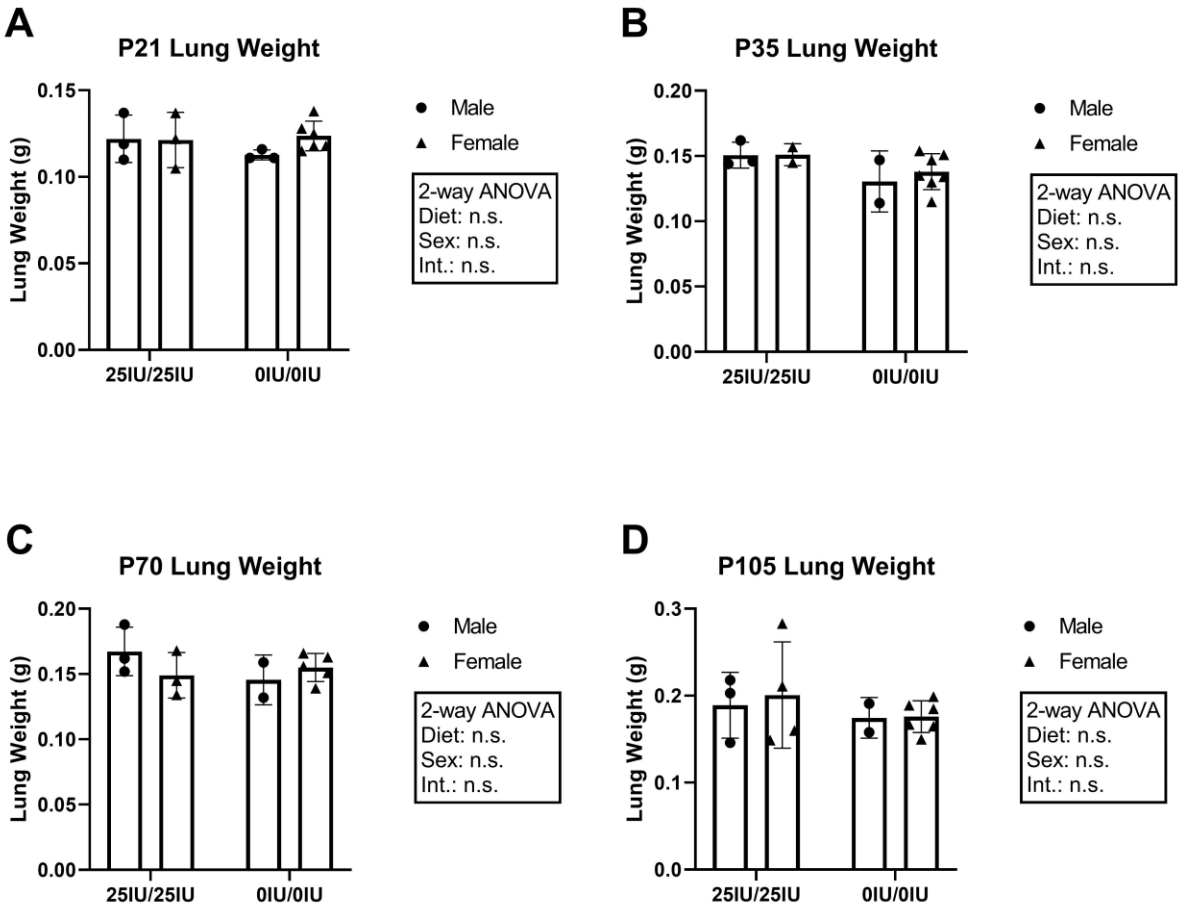
Supplementary Figure 4.3 Influence of sex on pup body weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup body weight at weaning (P21), (B) pup body weight at two weeks post-weaning (P35), (C) pup body weight at P70, (D) pup body weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA



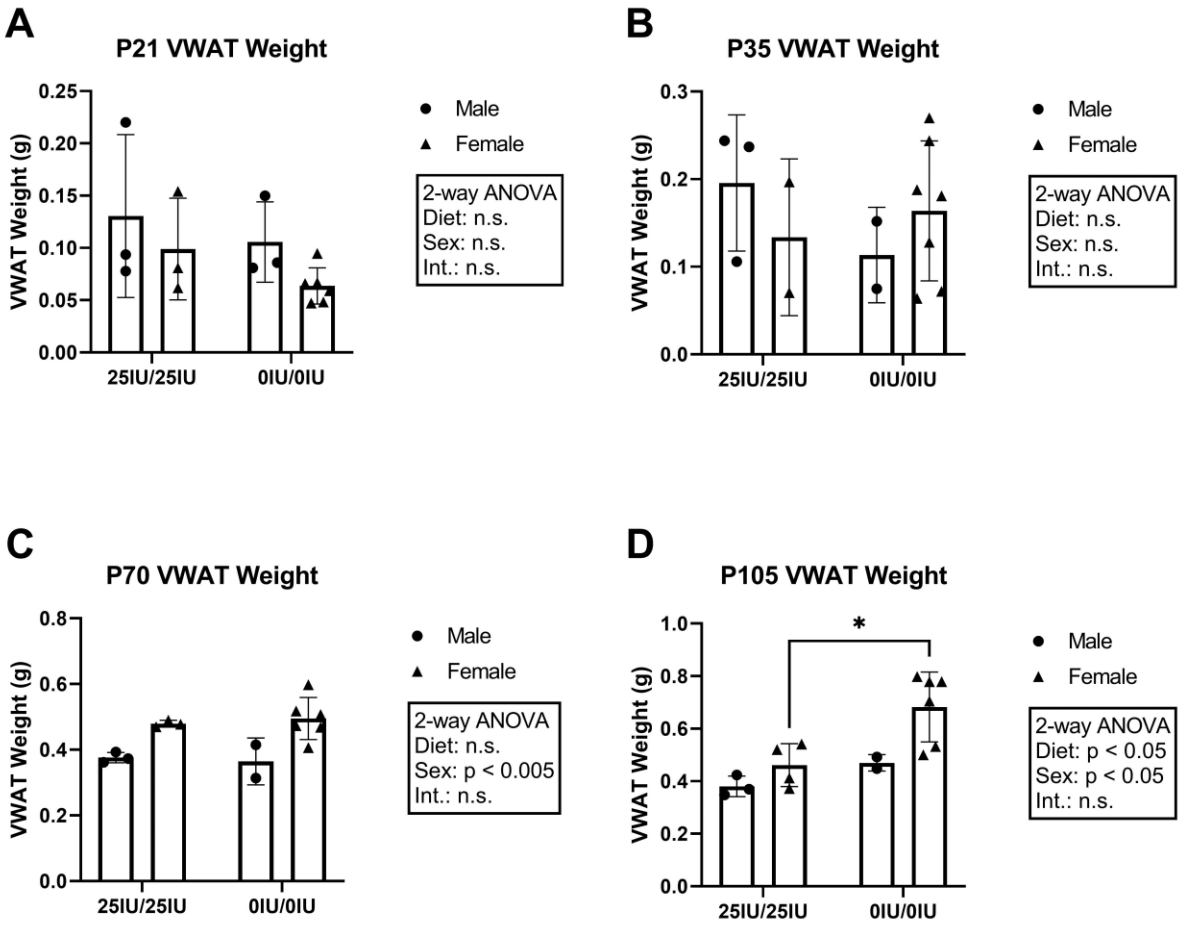
Supplementary Figure 4.4 Influence of sex on pup liver weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup liver weight at weaning (P21), (B) pup liver weight at two weeks post-weaning (P35), (C) pup liver weight at P70, (D) pup liver weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.



Supplementary Figure 4.5 Influence of sex on pup liver weight : body weight ratio from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup liver weight : body weight at weaning (P21), (B) pup liver weight : body weight at two weeks post-weaning (P35), (C) pup liver weight : body weight at P70, (D) pup liver weight : body weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.



Supplementary Figure 4.6 Influence of sex on pup lung weight analysis from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup lung weight at weaning (P21), (B) pup lung weight at two weeks post-weaning (P35), (C) pup lung weight at P70, (D) pup lung weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.



Supplementary Figure 4.7 Influence of sex on pup visceral white adipose tissue weight from dams fed a 25 IU control versus 0 IU deficient diet. (A) Pup visceral white adipose tissue weight at weaning (P21), (B) pup visceral white adipose tissue weight at two weeks post-weaning (P35), (C) pup visceral white adipose tissue weight at P70, (D) pup visceral white adipose tissue weight at P105. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.

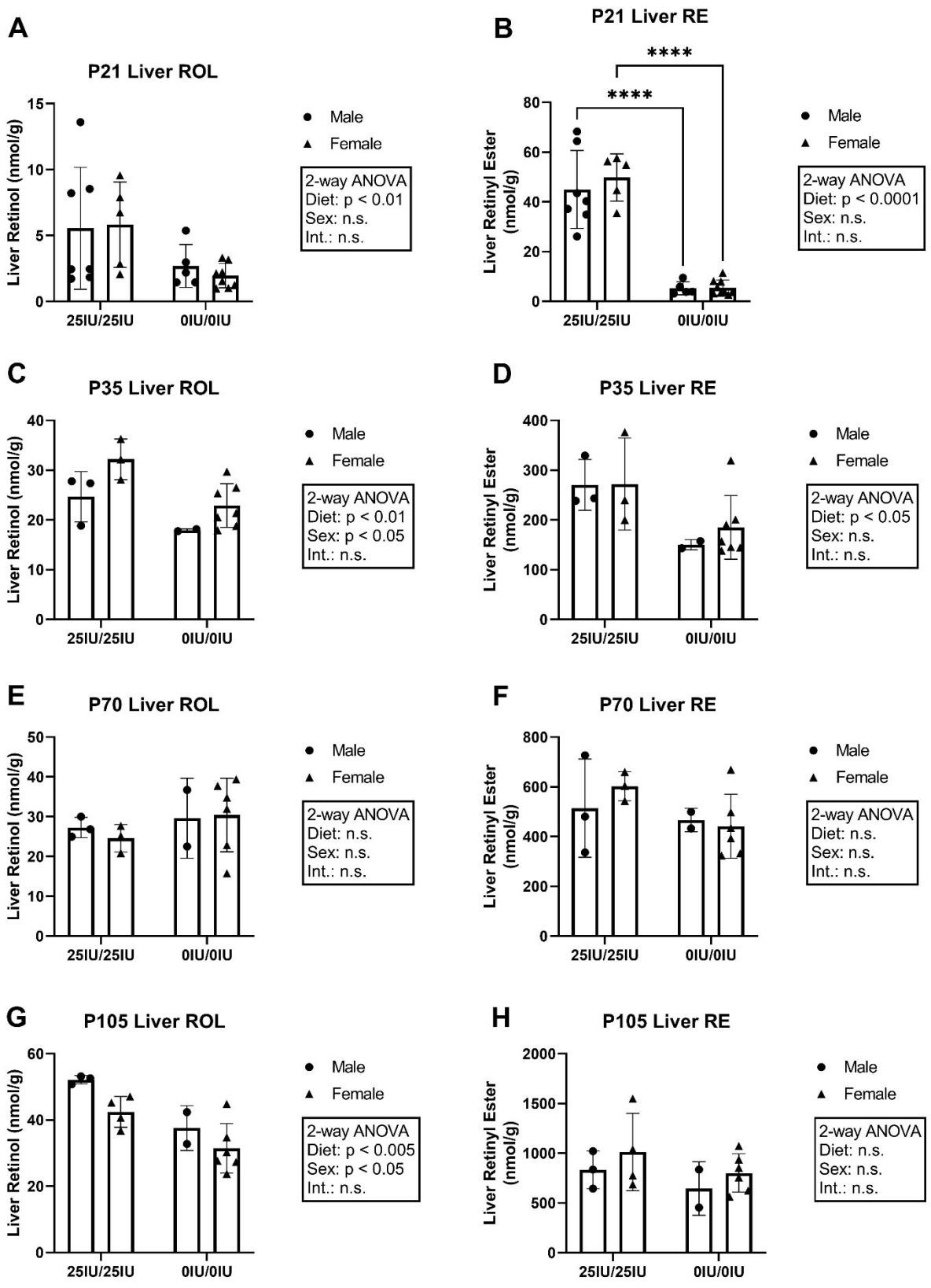

A
P21 Liver ROL
Liver Retinol (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.01
Sex: n.s.
Int.: n.s.
B
P21 Liver RE
****
****
Liver Retinyl Ester (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.0001
Sex: n.s.
Int.: n.s.
C
P35 Liver ROL
Liver Retinol (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.01
Sex: p < 0.05
Int.: n.s.
D
P35 Liver RE
Liver Retinyl Ester (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.05
Sex: n.s.
Int.: n.s.
E
P70 Liver ROL
Liver Retinol (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
F
P70 Liver RE
Liver Retinyl Ester (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
G
P105 Liver ROL
Liver Retinol (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.005
Sex: p < 0.05
Int.: n.s.
H
P105 Liver RE
Liver Retinyl Ester (nmol/g)
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.

Figure 4.8 Influence of sex on pup HPLC quantification analysis of retinol and retinyl ester concentrations in offspring livers from 25 IU/25 IU control and 0 IU/0 IU deficient maternal dietary vitamin A intake. This was examined during P21, P35, P70 and P105 ages in pups. (A) Retinol content in P21 pup liver, (B) Retinol content in P35 pup liver, (C) Retinyl ester in P35 pup liver, (D) Retinol content in P70 pup liver, (E) Retinyl ester in P70 pup liver, (F) Retinol content in P105 pup liver, (G) Retinyl ester in P105 pup liver. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.

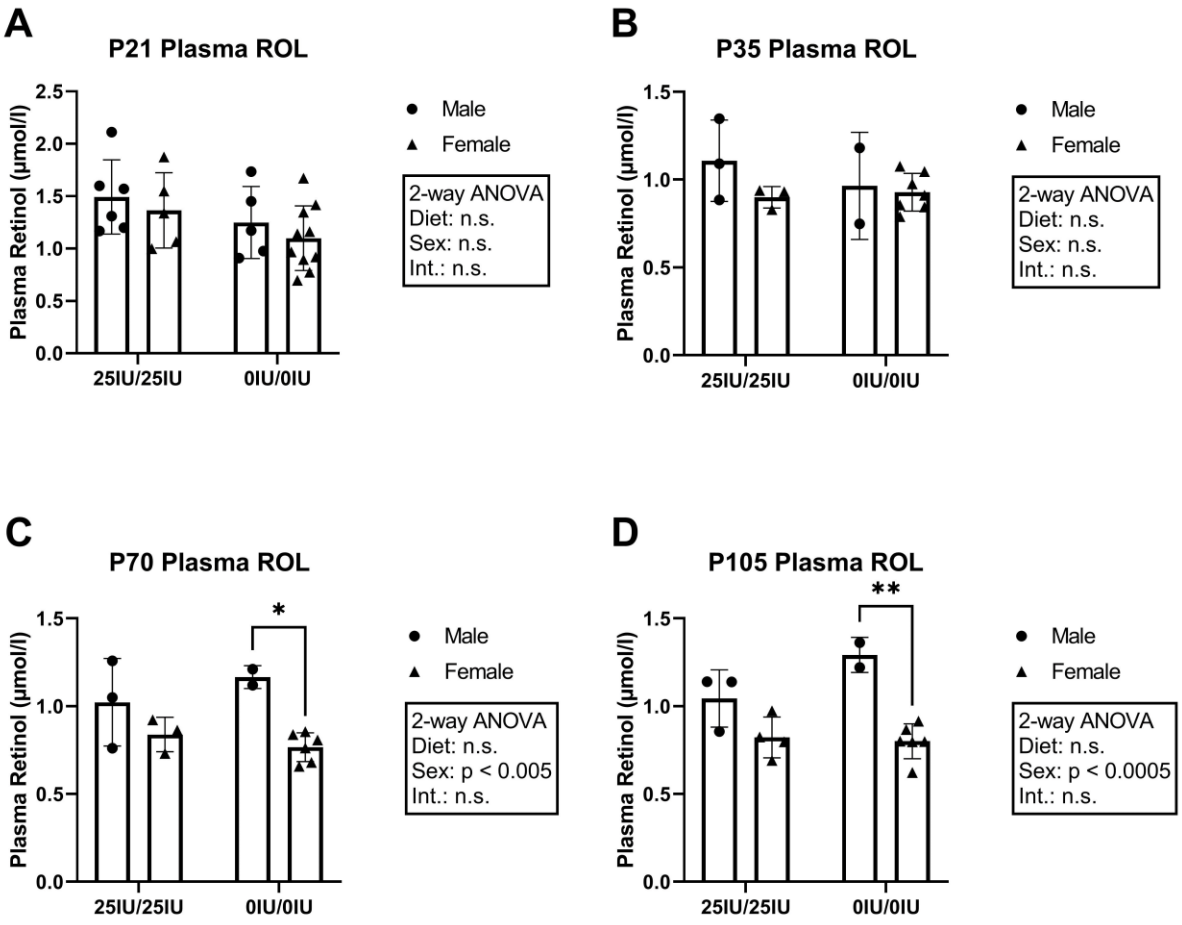


Figure 4.9 Influence of sex on pup HPLC quantification of plasma retinol concentrations born from dams on 25 IU/25 IU control and 0 IU/0 IU deficient dietary vitamin A intake. Retinol content in pup plasma, was assessed in pups during (A) P21, (B) P35, (C) P70 and (D) P105 time points. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.

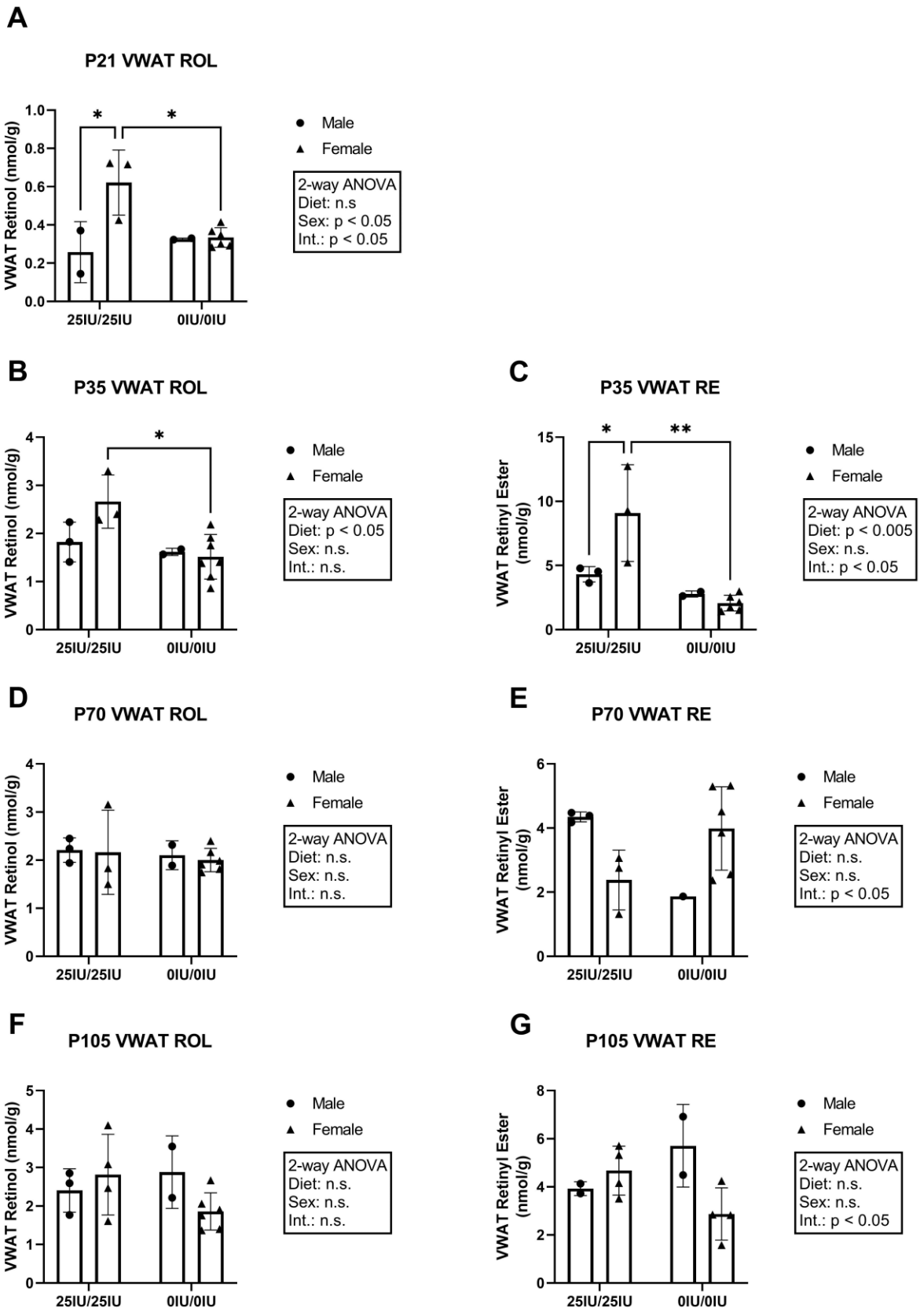

A
P21 VWAT ROL
VWAT Retinol (nmol/g)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s
Sex: p < 0.05
Int.: p < 0.05
B
P35 VWAT ROL
VWAT Retinol (nmol/g)
4
3
2
1
0
*
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.05
Sex: n.s.
Int.: n.s.
C
P35 VWAT RE
VWAT Retinyl Ester (nmol/g)
15
10
5
0
*
**
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.005
Sex: n.s.
Int.: p < 0.05
D
P70 VWAT ROL
VWAT Retinol (nmol/g)
4
3
2
1
0
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
E
P70 VWAT RE
VWAT Retinyl Ester (nmol/g)
6
4
2
0
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: p < 0.05
F
P105 VWAT ROL
VWAT Retinol (nmol/g)
5
4
3
2
1
0
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
G
P105 VWAT RE
VWAT Retinyl Ester (nmol/g)
8
6
4
2
0
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: p < 0.05

Figure 4.10 Influence of sex on pup HPLC quantification of VWAT retinol and retinyl ester concentrations born from dams on 25 IU/25 IU control and 0 IU/0 IU deficient dietary vitamin A intake. This was examined during P21, P35, P70 and P105 ages in pups. (A) Retinol content in P21 pup VWAT, (B) Retinol content in P35 pup VWAT, (C) Retinyl ester in P35 pup VWAT, (D) Retinol content in P70 pup VWAT, (E) Retinyl ester in P70 pup VWAT, (F) Retinol content in P105 pup VWAT, (G) Retinyl ester in P105 pup VWAT. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.

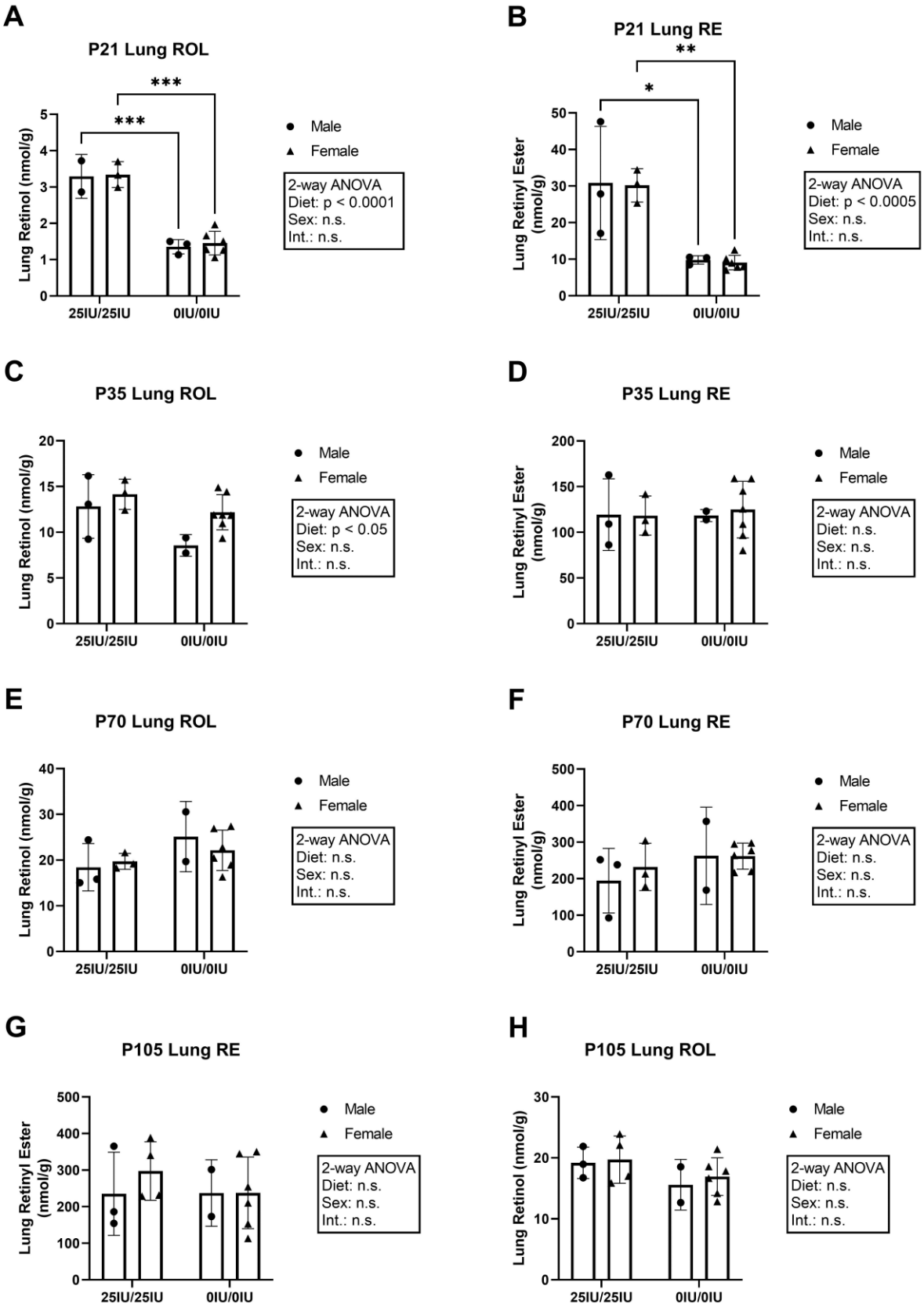

A
P21 Lung ROL
***
***
Lung Retinol (nmol/g)
0
1
2
3
4
5
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.0001
Sex: n.s.
Int.: n.s.
B
P21 Lung RE
**
*
Lung Retinyl Ester (nmol/g)
0
10
20
30
40
50
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.0005
Sex: n.s.
Int.: n.s.
C
P35 Lung ROL
Lung Retinol (nmol/g)
0
5
10
15
20
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: p < 0.05
Sex: n.s.
Int.: n.s.
D
P35 Lung RE
Lung Retinyl Ester (nmol/g)
0
50
100
150
200
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
E
P70 Lung ROL
Lung Retinol (nmol/g)
0
10
20
30
40
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
F
P70 Lung RE
Lung Retinyl Ester (nmol/g)
0
100
200
300
400
500
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
G
P105 Lung RE
Lung Retinyl Ester (nmol/g)
0
100
200
300
400
500
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.
H
P105 Lung ROL
Lung Retinol (nmol/g)
0
10
20
30
25IU/25IU
0IU/0IU
Male
Female
2-way ANOVA
Diet: n.s.
Sex: n.s.
Int.: n.s.

Figure 4.11 Influence of sex on pup HPLC quantification of lung retinol and retinyl ester concentrations born from dams on 25 IU/25 IU control and 0 IU/0 IU deficient dietary vitamin A intake. This was examined during P21, P35, P70 and P105 ages in pups. (A) Retinol content in P21 pup lung, (B) Retinol content in P35 pup lung, (C) Retinyl ester in P35 pup lung, (D) Retinol content in P70 pup lung, (E) Retinyl ester in P70 pup lung, (F) Retinol content in P105 pup lung, (G) Retinyl ester in P105 pup lung. Statistical significance, $p < 0.05$ between 25 IU/25 IU and 0 IU/0 IU groups is denoted by * and was determined using a two-way ANOVA test.