

ABSTRACT

Title of Dissertation: REGULATION OF THE
BIOCHEMICAL SYNERGY
BETWEEN MITOCHONDRIAL
OXIDATIVE NETWORKS AND *DE*
NOVO LIPOGENESIS IN THE LIVER

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The liver is a key metabolic organ responsible for whole-body energy metabolism by coordinating the regulation of carbohydrate, lipid, and amino acid metabolism across fed and fasted states. Hepatic mitochondria play a central role in regulating these networks that integrate nutrient utilization, biosynthetic pathways, and energy production. The metabolic flux through these networks dynamically adapts to nutritional and hormonal stimuli, which is essential for supporting the energy needs during animal growth and during the onset of metabolic challenges. Understanding how the flow of nutrients through mitochondrial oxidative networks establishes biochemical signatures that determine the metabolic phenotype of the liver is critical for identifying mechanisms underlying metabolic health during developmental periods and the onset of disease. This dissertation utilizes a combination of *in-vivo* (mice and chickens) and *in-vitro* (isolated mitochondria) systems coupled with stable

isotope-based methodologies and molecular biology-based techniques to study the metabolic regulation of mitochondrial function.

Metabolic dysfunction associated steatotic liver disease (MASLD) is the leading cause of chronic liver disease worldwide and is characterized by excess lipid accumulation in the liver. Dysregulated mitochondrial metabolism, along with high and sustained rates of *de novo* lipogenesis (DNL) are a characteristic feature of people suffering from MASLD. Due to the close biochemical relation of DNL with mitochondria, I examined how a carbohydrate-rich diet that induces DNL can alter mitochondrial metabolism during fatty liver condition. Using murine models, I demonstrate that fructose-induced upregulation of lipogenesis occurs concurrently with upregulation of mitochondrial oxidative pathways. My results suggest that chronic induction of mitochondrial oxidative capacity can contribute to the progressive pathophysiology of MASLD.

The embryonic to post-hatch development in chickens is characterized by a dramatic metabolic transition from lipolytic metabolism in the embryonic stages to lipogenic metabolism after hatch. While the provision of diet is known to induce this metabolic shift, the role of individual macronutrients in initiating this metabolic shift was unclear. My findings revealed that individual dietary macronutrients differentially influenced the initiation of this transition, highlighting nutrient-specific control of hepatic metabolic remodeling during early post-hatch development. Furthermore, hepatic mitochondria coordinate this metabolic switch through dynamic regulation of oxidative pathways along with nutrient transport mechanisms.

In summary, this dissertation highlights the requirement for coordinated nutrient flux through mitochondrial metabolic networks for the establishment of specific metabolic phenotypes in the liver during development and during the onset of metabolic disease. My findings 1) establish a mechanistic link between upregulated lipogenesis and dysregulated mitochondrial oxidative pathways during MASLD and 2) identify the key nutritional regulator responsible for the metabolic switch during the embryonic to post-hatch transition. Overall, this dissertation provides a framework for understanding how the dynamic nature of mitochondrial networks shapes liver function, which in turn could be leveraged to optimize growth and development, and also for developing strategies to manage metabolic defects during MASLD.

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MITOCHONDRIAL OXIDATIVE NETWORKS AND *DE NOVO* LIPOGENESIS
IN THE LIVER

by

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List of Abbreviations

Abbreviation	Full Form
ACP	Acyl Carrier Protein
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AU	Arbitrary Unit
BCA	Bicinchoninic Acid Assay
BDMCS	Tert-Butyldimethylchlorosilane
BHB	Beta Hydroxybutyrate
BSA	Bovine Serum Albumin
COX IV	Cytochrome c Oxidase subunit IV
CPT1A	Carnitine Palmitoyl Transferase 1A
CS	Citrate Synthase
DGAT	Diacylglycerol O-acyltransferase
DNA	Deoxyribonucleic Acid
DNL	<i>De novo</i> Lipogenesis
DOH	Day of Hatch
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol-bis(β -aminoethyl ether)-N,N,N',N'-Tetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ELOVL6	Elongation of Very Long Chain Fatty Acids Protein 6

FADS2	Fatty Acid Desaturase 2
FASN	Fatty Acid Synthase
FCCP	Carbonyl Cyanide-p-Trifluoromethoxyphenylhydrazone
FW	Fructose Water
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GC-MS	Gas Chromatography–Mass Spectrometry
GOT2	Glutamate Oxaloacetate Transaminase 2
H₂O₂	Hydrogen Peroxidase
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HF	High Fat
HF/HFr	High Fat/ High Fructose
HFCS	High Fructose Corn Syrup
HRP	Horse Radish Peroxidase
IDH1	Isocitrate Dehydrogenase 1
IL	Interleukin
LC-MS	Liquid Chromatography–Mass Spectrometry
LCAD	Long-Chain Acyl-CoA Dehydrogenase
LF	Low Fat
MAFL	Metabolic Dysfunction Associated Fatty Liver
MAS-3	Mitochondrial Assay Solution 3
MASH	Metabolic dysfunction-Associated Steatohepatitis
MASLD	Metabolic Dysfunction-Associated Steatotic Liver Disease

MCAD	Medium-Chain Acyl-CoA Dehydrogenase
MDH	Malate Dehydrogenase
ME	Malic Enzyme
MHSE	Mannitol, Sucrose, HEPES, EGTA
MPC	Mitochondrial Pyruvate Carrier
NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Nonalcoholic Fatty Liver Disease
ND1	NADH Dehydrogenase 1
NEFA	Non-Esterified Fatty Acid
NW	Normal Water
OCR	Oxygen Consumption Rate
OGC	Oxoglutarate Carrier
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate-Buffered Saline
PC	Pyruvate Carboxylase
PCK	Phosphoenolpyruvate CarboxyKinase
PDH	Pyruvate Dehydrogenase
PMS	Pyruvate Malate Shuttle
RIPA	RadioImmunoprecipitation Assay buffer
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species

SCD1	Stearoyl-CoA Desaturase-1
SEM	Standard Error of the Mean
SLC	Solute Carrier
SOD1	Superoxide Dismutase 1
TCA	Tricarboxylic Acid
TLR4	Toll-like Receptor 4

Chapter 1

Literature Review

1.1. Introduction

The liver is a key metabolic organ with central roles in orchestrating whole-body energy metabolism [1]. During the postprandial phase, when dietary carbohydrates are abundant, the liver converts excess glucose into glycogen and lipids for storage [2]. In contrast, during fasting, the liver maintains circulating glucose levels by producing and releasing glucose into circulation via gluconeogenesis and glycogenolysis [3]. Additionally, the liver upregulates lipid oxidation during fasting, generating ketone bodies that serve as alternative fuel sources for several extrahepatic tissues [4, 5]. This metabolic transition between fed and fasted states is tightly regulated by systemic hormones, particularly insulin and glucagon [6]. With the onset of metabolic disorders such as obesity, insulin resistance, and fatty liver disease, the biochemical networks essential for maintaining hepatic metabolic plasticity progressively become dysfunctional, which exacerbates disease severity [7].

Hepatic mitochondria play a critical role in supporting central biochemical fluxes that facilitate these metabolic adaptations, regulating tissue homeostasis [8]. Mitochondria are the metabolic hub of the cell as they integrate important biochemical processes, including the tricarboxylic acid (TCA) cycle, fatty acid oxidation (β -oxidation), oxidative phosphorylation, and ATP synthesis. They uniquely integrate and coordinate multiple pathways of carbohydrate, lipid, and amino acid metabolism while simultaneously maintaining a balance of the generation of reducing equivalents through substrate metabolism mediated via several mitochondrial nutrient shuttle systems [9, 10]. The integration of several metabolic pathways, along with nutrient influx and efflux through shuttle systems, determines the flux of metabolites through mitochondrial networks [11-13]. The metabolic flux through these networks dynamically adapts to nutritional and hormonal stimuli, essential for supporting energy needs during animal growth and also during the onset of metabolic challenges [14-17]. **The overarching hypothesis of my research is that the flow of nutrients through mitochondrial pathways and nutrient shuttle systems establishes biochemical signatures that determine the metabolic phenotype of the liver during various physiological states.**

Metabolic dysfunction-associated steatotic liver disease (MASLD; formerly known as non-alcoholic fatty liver disease, NAFLD) [18] is the leading cause of chronic liver disease across the world and is a common comorbidity of obesity and type 2 diabetes [19]. It is characterized by excess hepatic lipid accumulation, exceeding 5-10% of liver weight, in individuals with little to no alcohol consumption. Very often, affected individuals progress from simple steatosis to steatohepatitis, which later progresses to cirrhosis and to ultimate liver failure [20]. Dysregulation in mitochondrial oxidative networks in the liver is a central feature in individuals suffering from MASLD, which includes aberrant nutrient fluxes via oxidative processes, including β -oxidation, tricarboxylic acid cycle, oxidative phosphorylation, ketogenesis, and ATP synthesis [21]. Examining the factors that drive the continuous remodeling of mitochondrial oxidative functions during the progressive severity of MASLD is important in strategizing therapeutic and management strategies against this disease. Furthermore, high and sustained levels of hepatic *de novo* lipogenesis (DNL; new lipid formation) are a hallmark of individuals suffering from MASLD, along with dysfunctional mitochondrial metabolism [22, 23]. The classical hypercaloric Western diets that are closely aligned with the increasing incidence of metabolic disorders usually have high levels of fructose, which is the most common sweetener used in processed foods [24-26]. Fructose is a potent inducer of hepatic lipogenesis and is also a significant contributor to the development of MASLD due to its high lipogenic potential [27, 28]. The first half of my dissertation examines whether higher rates of lipogenesis, through its close biochemical relationships with mitochondria, can contribute to the dysfunctional induction of oxidative networks in murine livers. My findings highlight that fructose administration to mice led to the concurrent upregulation of mitochondrial oxidative networks and lipogenesis in the liver. Sustained induction of both lipogenesis and mitochondrial oxidative functions could accelerate cellular stress and metabolic dysfunction during MASLD [29].

The second half of my dissertation investigates mechanisms regulating macronutrient metabolism and mitochondrial function, utilizing the embryonic-to-post-hatch transition period in a chicken as a metabolic model [30-32]. Chickens undergo a dramatic metabolic transition during the embryonic-to-post-hatch transition period, where they shift from lipid-dependent metabolism in the embryos to carbohydrate-based

metabolism in the hatchling to derive energy. During embryonic development, chicks rely heavily on oxidizing the free fatty acids from the egg yolk to derive energy [33]. Following hatching, chickens shift towards utilizing carbohydrates for energy, and they initiate the process of *de novo* lipogenesis in the liver [34-36]. This metabolic switching during the embryonic to post-hatch stages in chickens involves significant remodeling of metabolic networks governing macronutrient metabolism. The first 3-4 days post-hatch are a critical period with the highest mortality rates in broiler chickens due to the inability to successfully undergo the metabolic transition, resulting in a significant economic burden for the poultry industry [37, 38]. Identifying the metabolic and molecular mediators of the early post-hatch growth and development is the key to improving the viability and health of chickens. This developmental transition provides an ideal platform to study how liver biochemistry adapts to facilitate an efficient metabolic switch, mediated by specific or a group of macronutrients. While it is established that providing diet to the newly hatched chicks is essential for initiating this metabolic shift, the role of specific macronutrients (carbohydrate vs. proteins vs. lipids) towards this metabolic switch remains unclear. My findings show that hepatic mitochondria, through the coordinated flux through their oxidative networks and nutrient shuttle systems, play a central role in facilitating the metabolic transition in the liver of the newly hatched chickens. My results also highlight the differential impact of individual macronutrients on this metabolic shift when administered to neonatal chickens.

1.2. Overall Hypothesis

A coordinated regulation of nutrient flow between mitochondrial metabolic networks and lipid biosynthetic pathways facilitates the development of a lipogenic phenotype in the liver across various physiological stages.

This central hypothesis will be tested using the following two objectives:

Objective 1. To determine whether high rates of hepatic *de novo* lipogenesis (DNL), through its biochemical relationship with mitochondria, contribute to the dysfunctional induction of mitochondrial oxidative networks in the liver during MASLD in mice.

Hypothesis: A higher flux through DNL during MASLD occurs concurrently with the remodeling of the mitochondrial oxidative machinery and thereby contributes to the disease progression.

Objective 2. To utilize the ‘embryonic to post-hatch’ transition model in chickens to examine the contribution of individual dietary macronutrients and the role of mitochondrial nutrient shuttle systems in initiating the metabolic transition in neonatal chickens.

Hypothesis: Individual dietary macronutrients (carbohydrates vs. proteins vs. lipids) differentially influence the initiation of the lipolytic to lipogenic metabolic switch during the early post-hatch transition in chickens, and this transition is mediated by a coordinated nutrient flux through mitochondrial oxidative networks and nutrient shuttle systems across the embryonic to post-hatch period.

To delineate hepatic metabolism, I utilized a combination of stable isotope-based approaches coupled with mass spectrometry (GC-MS) based metabolic flux analysis and targeted metabolomics. Gene and protein expression analyses were conducted to complement and substantiate the metabolic outcomes.

1.3. Metabolic dysfunction-associated fatty liver disease & its risk factors

Metabolic dysfunction-associated steatotic liver disease (MASLD) [18], formerly known as non-alcoholic fatty liver disease (NAFLD), is the most common chronic liver disease worldwide, afflicting more than one-third of the population [39]. The global prevalence of MASLD is estimated at approximately 24%; the highest rates are reported from South America and the Middle East, followed by Asia, the USA, and Europe [40]. It is the fastest-growing cause of hepatocellular carcinoma, and its prevalence is estimated to go up by 56% globally in the next ten years. The epidemiology and demographic characteristics of MASLD vary worldwide, usually parallel to the prevalence of obesity and diabetes, but a substantial proportion of patients are lean too. The deleterious impact of MASLD is multifaceted, often causing end-stage liver disease, cancer, and the need for liver transplantation in some cases [41].

MASLD is a multisystem metabolic disorder involving a complex interaction of hormonal, nutritional, and genetic factors that affects several extrahepatic organs and regulatory pathways [42, 43]. It comprises a continuum of liver conditions varying in the severity of the liver injury and the progression

encompasses the spectrum of simple steatosis, metabolic dysfunction-associated steatohepatitis (MASH) with fibrosis, cirrhosis, and hepatocellular carcinoma [44]. While the metabolic dysfunction-associated fatty liver (MAFL) is characterized by steatosis of the liver with no hepatic injury, metabolic dysfunction-associated steatohepatitis (MASH) is characterized by steatosis, lobular inflammation, and hepatocellular ballooning [45]. MASH is the more aggressive form of MASLD and has an increased risk of developing into progressive fibrosis, which can ultimately cause hepatocellular carcinoma, the major cause of morbidity and mortality induced by liver failure [46]. With the current trend in high-calorie fast foods and the preponderance of a sedentary lifestyle, the prevalence of MASLD is increasing worldwide with each passing year. Due to the complex pathophysiology and substantial heterogeneity of disease phenotype, there is currently no approved drug therapy for MASLD [47]. Healthy food habits and weight reduction remain the crucial preventive measures for MASLD [48].

MASLD is a common co-morbidity of obesity and type-2 diabetes [49]. Obesity can play a significant role both in the development of the initial stage of simple steatosis and in its progression to MASH [50]. Excess circulating free fatty acid availability from accelerated adipose tissue lipolysis and reduced uptake by subcutaneous adipose tissue leads to lipid accumulation in the liver [51]. This leads to the development of simple steatosis and causes lipotoxicity from the accumulation of triglyceride-derived toxic metabolites. Insulin resistance impairs the ability of insulin to suppress adipose tissue lipolysis, increasing the delivery of free fatty acid to the liver, and stimulating lipogenic enzymes to increase *de novo* lipogenesis [52]. These collectively contribute to further lipid accumulation and progressive severity of MASLD.

1.3.1. Dysfunctional hepatic metabolism during MASLD

The liver is a hub of intermediary metabolism, and its metabolic activity is tightly controlled by insulin and other hormones. It is a very plastic organ which efficiently switches metabolic networks from feeding to fasting conditions and ensures that the normal blood sugar level is maintained [2]. In the post-prandial state, signaled by high insulin levels and the presence of dietary carbohydrates, the liver uses glucose as the main metabolic fuel and suppresses its production [6]. Excess glucose enters the blood via

the portal vein, which is rapidly taken up by the liver and sequestered as glycogen and/or converted into fatty acids or amino acids [2]. Fatty acids obtained through diet are esterified in hepatocytes to generate triacylglycerol and are either stored in the liver in lipid droplets or secreted into the circulation. During fasting, the liver takes up and oxidizes the circulating free fatty acids produced by adipose tissue lipolysis to provide energy and produces ketones as a byproduct (ketogenesis). It also breaks down glycogen (glycogenolysis) and amino acids (gluconeogenesis) to produce glucose, which is used by the other tissues [2]. Liver-generated glucose and ketone bodies provide essential metabolic fuels for extrahepatic tissues during starvation and exercise. With the onset of insulin resistance, insulin signaling and hepatic metabolism progressively become dysfunctional, and the metabolic flexibility of the mitochondrial biochemistry becomes blunted. Together with unsuppressed lipolysis, increasing influx of free fatty acids into the liver, in results in increased hepatic triglyceride accumulation [53]. Accumulation of lipids such as triglycerides, free fatty acids, and cholesterol in hepatocytes and serum is a characteristic feature of MASLD [54].

The three major sources of hepatic lipid accumulation are dietary lipids, adipose tissue-derived fatty acids, and *de novo* synthesis of fatty acids from other nutrients, especially carbohydrates (*de novo* lipogenesis). The rate of *de novo* lipogenesis, which otherwise has a low contribution to hepatic lipid accumulation, increases during MASLD [55, 56]. High levels of plasma insulin activate the endoplasmic reticulum membrane-bound transcription factor sterol regulatory element binding protein 1C (SREBP1c), the N-terminus of which translocate to the nucleus and upregulates all genes in the hepatic *de novo* lipogenesis pathway. The hepatic uptake of excess plasma glucose during MASLD promotes the nuclear translocation of carbohydrate response element binding protein (ChREBP), a transcription factor that also upregulates transcription of the majority of fatty acid biosynthetic genes plus pyruvate kinase, which increases the availability of citrate for *de novo* lipogenesis [57].

In the early stages of MASLD, there are multiple hepatic adaptations to regulate fat accumulation caused by the continuous inflow of fatty acids to the liver. Upregulation of mitochondrial fatty acid oxidation, tricarboxylic acid cycle, and oxidative phosphorylation has been reported in patients suffering

from MASLD [58]. This ultimately results in oxidative stress, lipid peroxidation, reactive oxygen species formation, and mitochondrial dysfunction [59]. The innate immune response of MASLD involves resident Kupffer cells, recruited macrophages derived from bone marrow cells, neutrophils, and natural killer T cells. These contribute to the progression of MASH by inducing inflammation and promoting the production of cytokines, chemokines, nitric oxide, and ROS. Also, excessive free fatty acids and cholesterol cause hepatic lipotoxicity and stimulate macrophage activation and the production of proinflammatory cytokines [59]. Together, these factors contribute to adipose tissue inflammation and insulin resistance and the progression of MASLD.

1.3.2. Mechanisms contributing to mitochondrial dysfunction during MASLD

Mitochondria are complex organelles that play a central role in energy metabolism and are a hub for several macronutrient catabolic and anabolic networks to integrate (**Fig. 1.1**) [60]. They play a crucial role in energy production in the form of ATP through the process of oxidative phosphorylation and hold a central role in cellular biogenetics. They also play an essential role in the biosynthesis of macromolecules such as nucleotides, lipids, heme, and iron-sulfur clusters. Beyond metabolism, mitochondria are also involved in regulating signals for the propagation of diverse biological functions, including calcium homeostasis, cellular senescence, inflammation, and age-dependent stem cell activity [61].

The liver plays a predominant role in whole-body energy homeostasis and hence is rich in mitochondria, which are the major actors in the integration of hepatic lipid metabolism [62]. Under physiological conditions, glucose is metabolized into pyruvate through glycolysis and then to acetyl-CoA, which is subsequently oxidized to generate ATP by the TCA cycle and oxidative phosphorylation in the hepatocyte mitochondria. In addition, mitochondria can oxidize the fatty acids and amino acids to produce ATP and ketones or urea for gluconeogenesis during the fasting states. In addition to energy production, mitochondria also provide the carbon intermediates for anabolic reactions, such as lipid biosynthesis. Specifically, mitochondria-derived citrate is converted into acetyl-CoA in the hepatocyte cytosol, where acetyl-CoA is consumed to synthesize fatty acids.

Mitochondrial dysfunction is mainly characterized by excessive ROS production, oxidative stress, and alterations in respiratory chain complexes [63]. These changes are generally due to hepatic lipid accumulation, inflammation, and hepatic cell death [64]. Increased mitochondrial mass and biogenesis in liver tissues have often been seen in patients suffering from MASLD. In simple steatosis, there is an overactive mitochondrial TCA cycle, respiration, β -oxidation, and induction of ketogenesis; a compensatory mechanism to dispose of excess acetyl-CoA units through complete oxidation [65]. However, during progressive and severe liver disease, there is a reduction in the flux through ketogenesis, mitochondrial respiratory chain activity, and ATP synthesis, but an increase in TCA cycle flux (**Fig. 1.1**). This reflects an eventual failure of the compensatory mechanism to optimally adapt to the chronic nutrient overload [66]. To compensate for the massive energy supply from the chronically upregulated TCA cycle, induction of TCA cycle flux could be obligatory to high energy high-energy-demanding processes (e.g., gluconeogenesis, lipogenesis) during MASLD. However, the sustained induction of TCA cycle flux and its uncoupling from mitochondrial respiration and ATP synthesis fuels reactive oxygen species (ROS) production and the development of tissue inflammation. Defects in mitochondrial morphology, electron transport chain, and ATP production have been documented in MASLD along with high levels of ROS and other mediators of the inflammation [67].

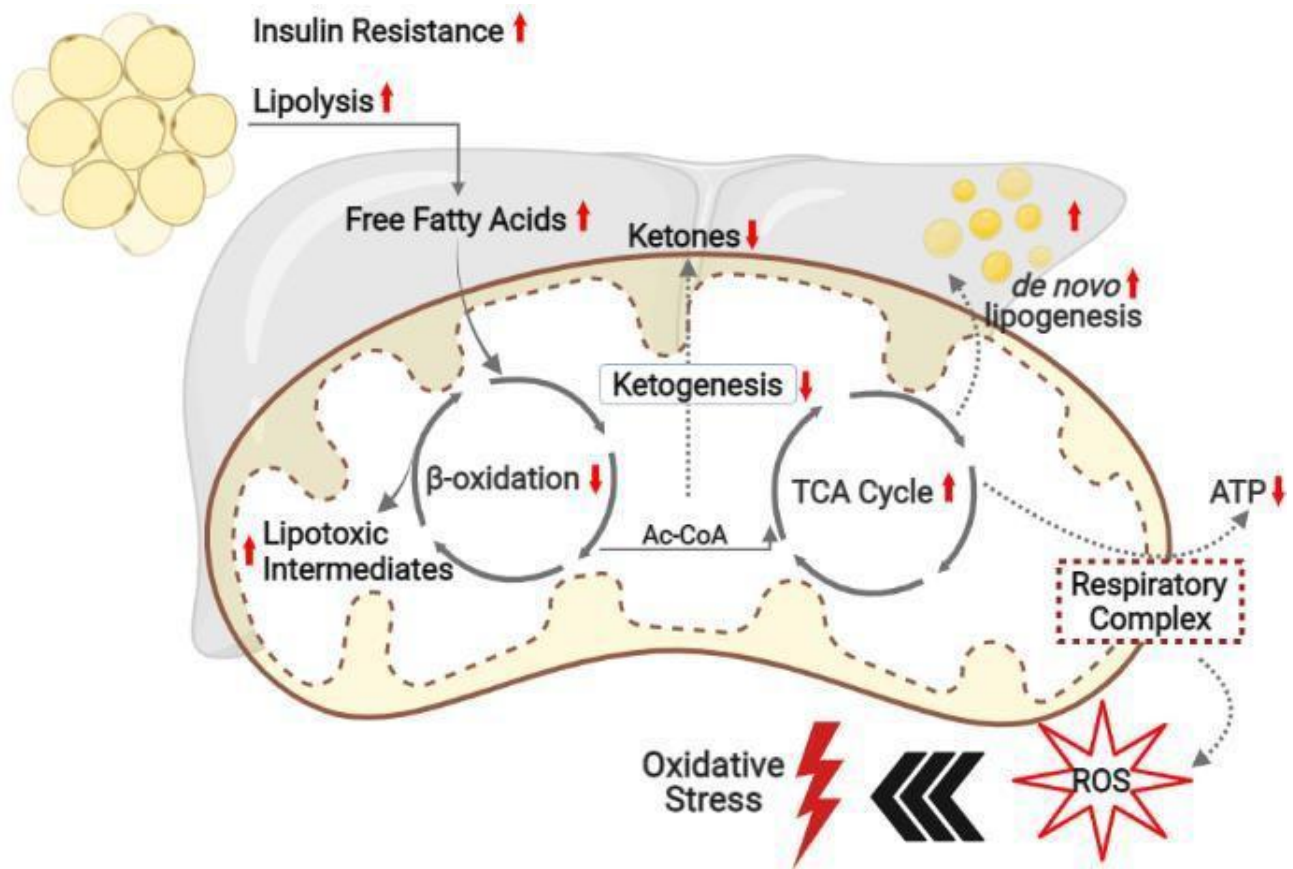


Figure 1.1. Hepatic mitochondrial dysfunction is a characteristic feature of MASLD.

Insulin resistance and increased lipolysis increase the free fatty acid flux into the liver. Due to incomplete β -oxidation, lipotoxic intermediates accumulate in the liver, causing liver injury. The chronically elevated oxidative flux through the TCA cycle results in induction of *de novo* lipogenesis and uncoupled mitochondrial respiration. The increased release of ROS further exacerbates liver injury by inducing oxidative stress. Figure created with BioRender.com.

1.3.3. Interaction between mitochondrial metabolism and oxidative stress during MASLD

Mitochondria are considered a major site of ROS production, and under normal physiologic conditions, cells can efficiently remove ROS through various enzymatic components [68, 69]. During MASLD, the increased mitochondrial fatty acid oxidation and TCA cycle activity persistently supply reducing equivalents to the electron transport chain. This prolonged activity of mitochondrial networks and respiratory complex dysfunction results in the overproduction of superoxide [70]. Uncoupling of the β -oxidation and TCA cycle from the electron transport chain results in inefficient lipid metabolism and ROS overproduction in the liver. The discrepancy between ROS generation and antioxidant defense causes oxidative stress, which leads to DNA and tissue damage. Oxidative stress is an important mediator that triggers low-grade inflammation and accelerates the progression of MASLD to MASH [71]. Oxidative damage to the nuclear DNA impairs mitochondrial function and hinders the transcription of nuclear-encoded mitochondrial genes [71]. It also contributes to the activation of stellate cells and chronic inflammatory response by upregulating proinflammatory cytokines, apoptosis, and the development of hepatic fibrosis. Overall, during MASLD, there is an increased formation of reducing equivalents, which results in an overflow of electrons from the mitochondrial respiratory chain, which induces ROS production [72]. ROS overproduction suppresses the capacity of the antioxidant defense system and causes further oxidative damage [73].

1.3.4. Impact of dietary carbohydrates on hepatic *de novo* lipogenesis and MASLD

Hepatic *de novo* lipogenesis plays an important role in lipid accumulation in the liver and is a significant pathway contributing to the development of MASLD. It is a complex cytosolic polymerization process by which excess acetyl-CoA generated by glycolysis can be converted into fatty acids and triglycerides [74]. Several studies have reported that calorie surplus diets rich in carbohydrates increase the rate of *de novo* lipogenesis in humans [75]. Even in the absence of overfeeding, the rate of *de novo* lipogenesis changes in response to alterations in the macronutrient composition in the diet, specifically increasing in response to an increase in the proportion of carbohydrate in the diet [76]. Additionally, not

only the quantity but also the type of carbohydrate in the diet affects the rate of DNL. While glucose is the main substrate for *de novo* lipogenesis, fructose is highly lipogenic and can bypass the critical rate-limiting steps in glycolysis [55].

Dietary fructose increases levels of enzymes involved in DNL even more strongly than a high-fat diet [27]. Countries that use high fructose corn syrup (HFCS) in their food supply have a 20% higher prevalence of diabetes compared to countries that do not use HFCS, independent of obesity [77]. In research studies, consumption of fructose, but not glucose-sweetened drinks, has been associated with increased visceral adiposity, insulin resistance, and increased hepatic DNL [78]. A potential reason why fructose is more lipogenic is that fructose, like all other carbohydrates, is absorbed from the intestine into the portal vein and delivered directly to the liver [27]. It is only after the first-pass metabolism through the liver that dietary carbohydrates may enter the systemic circulation [27]. Macronutrient and insulin concentration in the portal circulation may be 10 times higher than in the systemic circulation. Thus, the liver is exposed to a much higher carbohydrate load than the rest of the metabolically active tissues. By contrast, long-chain fatty acids are absorbed from the intestine as chylomicron particles, first enter the lymphatic circulation from which they eventually enter systemic circulation via the thoracic duct [79]. Thus, the concentration of dietary fat that the liver is exposed to is the same as that of any other metabolically active tissue. This, taken together with the distribution of lipoprotein lipase in capillaries of muscle and fat tissue, results in chylomicrons being largely metabolized by non-hepatic tissue. Also, carbohydrates are metabolized to the two-carbon intermediate acetyl-CoA and activate the lipogenic transcriptional factors SREBP1c and ChREBP in the liver, stimulating every step of DNL, which converts the acetyl-CoA into triglycerides [74]. By contrast, fatty acids may be directly incorporated into triglycerides through the action of a single enzyme, diacylglycerol acyltransferase (DGAT), thus bypassing most enzymes involved in DNL and failing to activate the same lipogenic transcriptional factors. Taken together, because of these metabolic differences, fructose induces hepatic lipogenesis more powerfully than glucose or fatty acids.

1.3.5. Interactions between mitochondrial biochemistry and lipid biosynthesis

Fatty acid synthesis is an important anabolic process, and it provides a major component for membrane synthesis, energy storage, and possesses a variety of other physiological functions. The fundamental process of fatty acid synthesis is highly conserved amongst the majority of species. Excess acetyl-CoA formed from increased carbohydrate levels in the body initiates lipid formation through lipogenesis [80]. Lipogenesis encompasses both fatty acid synthesis and triglyceride synthesis [81]. The availability of acetyl-CoA is of significance as it initiates lipogenesis.

Lipogenesis begins with acetyl-CoA and advances by the subsequent addition of two carbon atoms from another acetyl-CoA, and this continues until a fatty acid of appropriate length is synthesized [82]. The acetyl-CoA essential for this cytosolic process is produced in the mitochondria and is transferred out to the cytosol via the citrate transporter. Mitochondrial pyruvate gets converted to oxaloacetate and acetyl-CoA; these two combine to form citrate, which travels out from the mitochondria to the cytosol. The key feature of lipogenesis is the sequential extension of an alkyl chain, two carbons at a time, by a series of decarboxylative condensation reactions. Acetyl-CoA is carboxylated to yield malonyl-CoA; the malonyl group is then transferred to an acyl carrier protein to yield malonyl-ACP. Malonyl-ACP is then condensed with acetyl-CoA, reduced, and dehydrated, and reduced once again, yielding an acyl-ACP. The elongation of the chain occurs by condensing another malonyl-ACP with the acyl-ACP and repeating the reaction cycle. NADPH, an essential electron donor, plays an important role in lipogenesis. Fatty acid synthesis utilizes two molecules of NADPH for each molecule of acetate incorporated into long-chain fatty acids. In the liver, the pentose phosphate pathway and malic enzyme furnish the NADPH used for fatty acid synthesis. The activities of the two dehydrogenases of the pentose phosphate pathway and of the malic enzyme correlate positively with the rate of fatty acid synthesis under a wide variety of conditions. Thus, the biosynthesis of lipids requires a coordinated interaction of the mitochondrial and cytosolic nutrients [83].

1.4. Biochemical relationships between mitochondrial nutrient shuttles, anabolic networks, and hepatocellular redox

Mitochondrial nutrient shuttle systems play an essential and indispensable role in maintaining the redox balance between the mitochondrial and cytoplasmic biochemical networks [84]. The compartmentalization of metabolites and enzymes across the mitochondrial membrane and the cytosol is essential for the regulation of energy metabolism in the cell [85]. Mitochondria are involved in the production of some molecules that have a critical role in the conversion of nutrients into usable energy. However, not much is known about how these mitochondrial nutrient shuttle systems are altered during various physiological states, during the period of rapid growth, and the onset of metabolic disorders in animals [86].

1.4.1. The Malate-Aspartate Shuttle (MAS)

Malate-aspartate shuttle plays an important role in moving electrons from the cytosol to the mitochondria through the impermeable mitochondrial membrane [12]. Cytosolic reducing equivalent, NADH, produced by glycolysis, must be transferred to the mitochondrial matrix to enter the electron transport chain and fuel energy production. As the inner mitochondrial membrane is relatively impermeable to NADH molecules, the MAS transports cytosolic reducing equivalents from the cytosol into the mitochondria in the form of malate. Eventually, these electrons enter the electron transport chain for ATP production [84]. MAS compartmentalizes the NAD⁺/NADH balance essential for cellular energy metabolism and for driving NAD⁺ dependent cytosolic reactions. MAS comprises a transport-transamination-redox cycle, which has parts in both cytosolic and mitochondrial domains (**Fig. 1.2**) [11]. It has two antiporters in the inner membrane of the mitochondria, namely the malate- α -ketoglutarate transporter and the glutamate-aspartate transporter. While the former helps to move malate inside the mitochondrial matrix after glycolysis in exchange for α -ketoglutarate, the latter helps aspartate to move out of the mitochondrial matrix in exchange for glutamate. Malate in the mitochondrial matrix is oxidized to oxaloacetate, transferring a pair of electrons to the NADH molecule, which enters the electron transport

chain. The inner mitochondrial membrane being impermeable to oxaloacetate, it is transaminated to aspartate, which then moves out of the mitochondrial matrix. Thus, using the two antiporters, MAS works as a redox shuttle supporting oxidative pathways as well as oxidative phosphorylation.

1.4.2. The Pyruvate-Malate Shuttle (PMS)

There has also been previous evidence that a pyruvate-malate shuttle exists to circumvent the impermeability of the mitochondrial membranes to essential reducing equivalents [87]. While the pyruvate enters the mitochondria using the mitochondrial pyruvate carrier (MPC), the malate molecules move out of the mitochondria to the cytoplasm through the mitochondrial dicarboxylate carrier (**Fig. 1.2**). In this shuttle, oxaloacetate is reduced to malate by mitochondrial malate dehydrogenase, which exits from the mitochondria to the cytosol, where it is decarboxylated to pyruvate in a reaction catalyzed by malic enzyme to produce NADPH [88]. The pyruvate then re-enters the mitochondria to participate in another turn of the shuttle or undergo decarboxylation to acetyl-CoA and oxidation. NADPH produced during the oxidation of malate to pyruvate plays a central role in polymerizing acetyl-CoA molecules during *de novo* lipogenesis [89].

1.4.3. The Citrate Carrier (CiC)

The citrate carrier is a nuclear-encoded protein located in the mitochondrial inner membrane and is a member of the mitochondrial carrier family. CiC plays a pivotal role in intermediary metabolism by connecting carbohydrate catabolism to lipogenesis. It plays a role in removing excess citrate, a TCA cycle intermediate, from the mitochondria to the cytoplasm (**Fig. 1.2**). Cytoplasmic citrate is the prime carbon source for fatty acid, triglyceride, and cholesterol biosynthesis, and it plays a role in regulating glucose metabolism by allosteric inhibitor of phosphofructokinase [90]. Additionally, cytosolic citrate provides, via malic enzyme, NADPH for lipogenesis. The CiC sits at the center of the metabolic landscape of cells, serving a key role in the generation and optimal utilization of resources needed to meet the energetic demands of tissue under physiological conditions [14]. Therefore, understanding the change in activity of CiC during periods of animal development and the onset of metabolic disorders is of key importance.

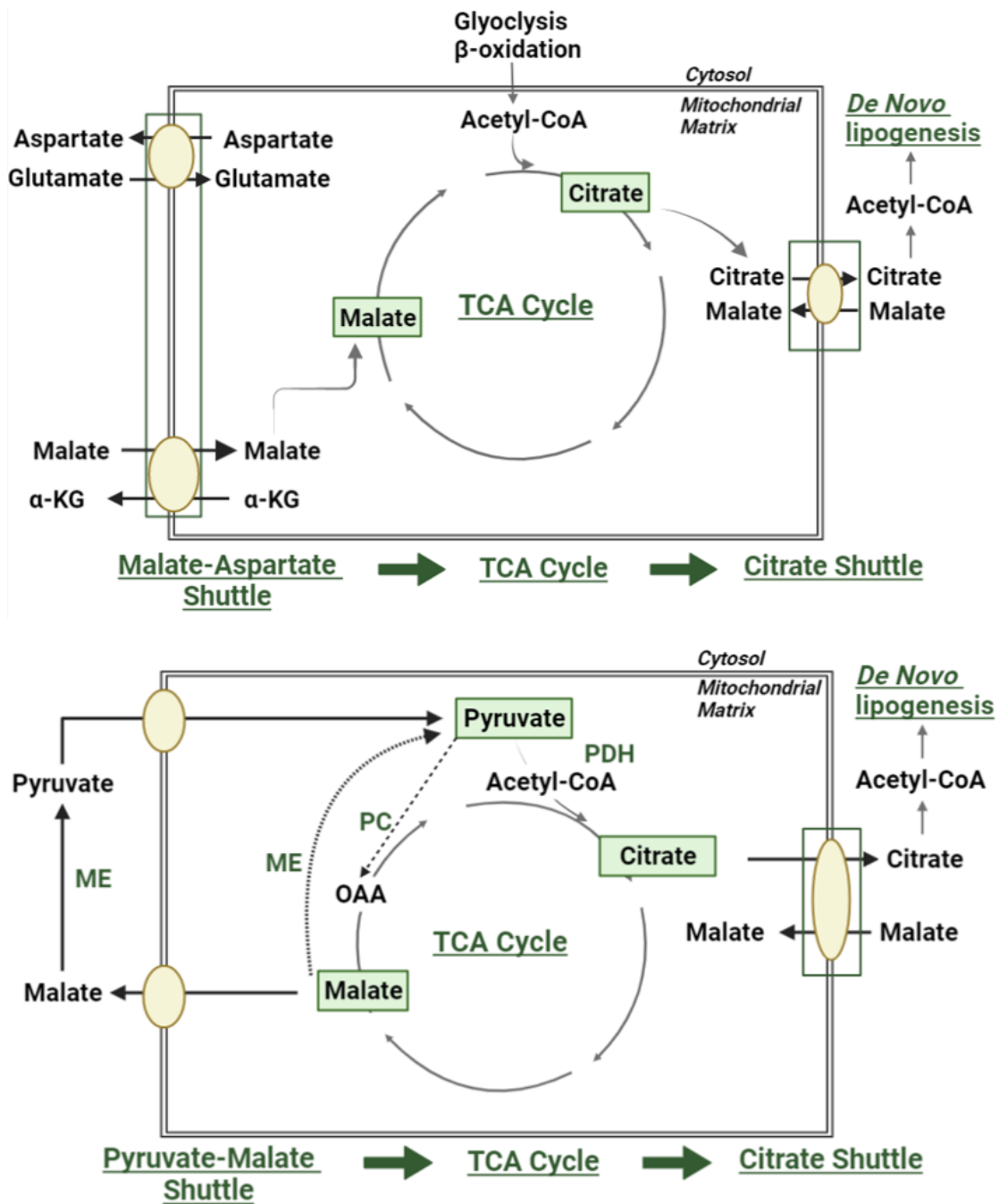


Figure 1.2. The malate-aspartate shuttle, pyruvate-malate, and the citrate carrier play a key role in mediating the biochemical crosstalk between the mitochondria and the cytosol.

ME; Malic Enzyme, PC; Pyruvate carboxylase, PDH; Pyruvate dehydrogenase. Figure created with BioRender.com.

1.4.4. Metabolic interactions between Malate-Aspartate shuttle, Pyruvate-Malate shuttle, and central mitochondrial networks

Active storage of nutrients and efficient disposal of glucose after a meal are characteristics of healthy metabolism. How mitochondria contribute to imbalanced energy homeostasis and metabolic inflexibility is sparsely known. Often, mitochondrial deficiencies limit the capacity of fat oxidation and promote fat storage in tissues [91], but how the mitochondria remodel their nutrient shuttles during such disorders has not been systematically investigated. Nutrients that enter the cytosolic metabolic pathways are catalyzed by different enzymes that use NAD⁺ as a cofactor [92]. These pathways produce NADH as the reducing equivalents, which enter mitochondria for oxidative phosphorylation. As the inner mitochondrial membrane is impermeable to NADH, mitochondrial shuttles help to bring in such molecules inside the mitochondria for ATP production. Mitochondrial shuttles also play a vital role in the flux of the end products of carbohydrate metabolism from the mitochondrial matrix to the cytosol [93]. These products are part of the TCA cycle and also play an important role in lipogenesis.

While the malate-aspartate shuttle plays an important role in fueling the TCA cycle flux, the citrate carrier plays a role in removing excess TCA cycle intermediates from the mitochondria to the cytoplasm. Citrate acts both as a precursor for lipogenesis and an allosteric regulator, binding to several enzymes and modulating their function [94]. There has been previous evidence indicating differential activity of citrate carriers under different hormonal and nutritional conditions. A group of researchers used mitochondrial citrate carrier inhibitors to reverse pathologies related to MASLD [94]. They showed a reduction of pro-inflammatory environment systematically and in the liver, and prevention of fibrosis and hepatocellular carcinoma. Pyruvate-malate shuttle plays an important role in producing NADPH, which is essential for lipogenesis. However, how the coordinated activity of these mitochondrial shuttle systems and the central mitochondrial metabolic pathway changes during *de novo* lipogenesis, which is a major contributor to hepatic lipid accumulation, has not been systematically examined.

1.4.5. Impact of mitochondrial MAS/PMS-TCA-CiC metabolic axes on *de novo* lipogenesis

The oxidative capacity of mitochondria is maintained through coordinated interactions among multiple biochemical networks and nutrient transport systems. While these networks collectively sustain cellular homeostasis, my work highlights the functional interconnectivity between the tricarboxylic acid (TCA) cycle and key mitochondrial shuttle systems, including malate–aspartate shuttle (MAS), pyruvate–malate shuttle (PMS), and citrate shuttle (CiC). These integrated biochemical pathways provide a framework to understand how mitochondrial metabolism is linked to downstream cytosolic processes.

Reducing equivalents generated during glycolysis are transferred into mitochondria via the malate–aspartate shuttle in the form of malate, a key intermediate of the TCA cycle. The pyruvate–malate shuttle facilitates the entry of pyruvate into mitochondria, where it is converted to acetyl-CoA and subsequently fuels TCA cycle activity. In contrast, the citrate shuttle mediates the export of citrate from the mitochondria to the cytosol, where it serves as a primary substrate for *de novo* lipogenesis.

Based on these metabolic relationships, I hypothesized that increased nutrient flux through mitochondrial shuttle systems enhances the delivery of substrates such as malate and pyruvate into mitochondria, thereby stimulating TCA cycle activity and increasing citrate production. Elevated mitochondrial citrate availability would, in turn, promote citrate export into the cytosol, thereby enhancing lipid synthesis via *de novo* lipogenesis. Consistent with this hypothesis, the activity of these interconnected metabolic pathways was upregulated under conditions of increased *de novo* lipogenesis.

These mitochondrial interactions play a critical role in establishing the metabolic phenotype of liver tissue. A key strength of this work lies in the application of stable isotope-based metabolic flux analysis to map these pathways using gas chromatography–mass spectrometry [95-97]. Specifically, enrichment patterns of intermediates derived from ^{13}C -labeled substrates (e.g., [$^{13}\text{C}_3$]pyruvate and [$^{13}\text{C}_4$]malate) were used to quantify the activity of mitochondrial networks. Understanding the interplay between mitochondrial shuttle systems and the TCA cycle in supporting hepatic *de novo* lipogenesis provides important insights for developing therapeutic strategies targeting metabolic dysfunction-

associated steatotic liver disease in humans, as well as identifying metabolic targets to optimize healthy development in neonatal domestic chicken.

1.5. The embryonic to post-hatch transition period in chickens: a metabolic model critical to identify biochemical drivers of lipid metabolism and mitochondrial function

The embryonic to post-hatch transition in chickens is associated with a profound metabolic reprogramming where the animal switches from lipolytic metabolism in the embryonic stage to a carbohydrate-driven lipogenic metabolism in the post-hatch stage [30-32]. During embryonic development, chickens mainly rely on oxidizing yolk lipids as their primary source of energy [33, 36]. These lipids, mostly packaged as lipoproteins, are transported from the yolk sac to embryonic tissues, particularly in the liver, where they are extensively oxidized to meet the energetic demands for growth. Throughout embryogenesis, circulating glucose concentrations are maintained at relatively low but stable levels through the process of gluconeogenesis [98]. As the carbohydrate level of the egg is limited, gluconeogenesis is fueled primarily by glycerol, which is a by-product of lipid metabolism. As the embryonic development progresses, yolk lipid utilization increases substantially, and towards the end of the embryonic phase, around 80% of yolk lipids are absorbed by the embryo [34, 35, 99]. During the final few days of embryonic development, the chickens grow exponentially, and 90% of the energy required for this growth is derived from β -oxidation of these yolk lipids.

Despite the extensive utilization of the yolk lipids during embryogenesis, a residual amount of the yolk lipids remains during hatch, and it serves as a transient energy source for the chickens for the first few days post-hatch [35, 100]. These residual yolk lipids are rapidly used up and typically depleted within the first two days post-hatch. Concurrent with the yolk depletion, hatchlings undergo a metabolic transition following feeding, where they upregulate, the pathways related to carbohydrate metabolism, particularly *de novo* lipogenesis [101]. They are administered with corn-based diets, rich in starch, which then act as the main source of energy for them. This dietary shift drives a coordinated reorganization of

hepatic metabolic pathways, characterized by increased glucose uptake, enhanced glycolytic flux, replenishment of hepatic glycogen stores, and the induction of *de novo* lipogenesis in the liver [30, 31].

At the center of this metabolic transition lies the liver. During embryonic development, the liver acts as the major site of lipid uptake and fatty acid oxidation, supporting the energetic demands of the embryo [33]. After hatching, the liver undergoes reprogramming that favors carbohydrate utilization and lipid synthesis [34]. This transition from lipid oxidation to carbohydrate oxidation, accompanied by active lipogenesis, is stimulated by the administration of diets and is necessary for long-term thriving on a corn-based diet, promoting proper post-hatch growth. This embryonic to post-hatch transition occurs under conditions of high lipid flux in the liver, encompassing both elevated rates of lipid oxidation during embryonic stages and high levels of hepatic lipid accumulation during the post-hatch development [30]. Interestingly, this metabolic transition is very healthy, and it occurs with no apparent symptoms of metabolic dysfunction, hepatocellular stress, or inflammation of the liver. This is in sharp contrast to what happens in humans and mouse models of fatty liver disease, where high rates of lipid oxidation and elevated lipid accumulation in the liver are concurrent with hepatocellular stress, inflammation, and the development of metabolic disorders [102, 103]. Previous research from our lab has demonstrated that the absence of hepatocellular stress and inflammation during this metabolic switch in chicken is associated with optimal coupling between hepatic mitochondrial oxidative networks and lipogenesis, facilitated by a dynamic remodeling of these pathways [30, 31]. This remodeling promotes a coordinated upregulation of β -oxidation, ketogenesis, TCA cycle, along with induction of lipogenic pathways and antioxidant defense mechanisms. These adaptations allow the liver to accommodate the dramatic changes in substrate availability while maintaining cellular homeostasis and fulfilling the metabolic demands.

While some aspects of this transition have been well studied, critical gaps remain in our understanding of the nutritional, metabolic, and molecular factors that mediate this process. Precisely, the role of specific dietary macronutrients and their intracellular metabolic signals that trigger the hepatic reprogramming after hatch has not been systematically investigated. Investigating these mechanisms would help form strategies to improve the early-life health of chickens and improve the productivity of

the poultry industry. Furthermore, this period of metabolic switch provides a valuable opportunity to understand the regulatory factors mediating the induction and downregulation of various facets of lipid metabolism and mitochondrial function.

1.5.1. Composition of an avian egg

The avian egg is a self-contained biological system that is capable of nurturing an embryo from fertilization to hatch and therefore contains all the necessary nutrients required to support growth, differentiation, and energy metabolism [104, 105]. The primary constituent of the egg includes water (74%), proteins (12%), lipids (12%), with a small amount of carbohydrates (1%), minerals, trace elements, vitamins, and antioxidants [105]. While for the most part this composition remains the same, there can be variations in the composition depending on the chicken strain, age, and environmental factors.

A fertile chicken egg has four major structural components, namely the eggshell (9%), the eggshell membrane (<0.5%), egg white (62%), and egg yolk (29%) [106]. While these layers collectively provide physical protection, regulate gas and water exchange, and supply an array of nutrients essential for healthy embryonic development, each component serves a unique purpose in maintaining embryonic viability and supporting the metabolic demands throughout the incubation period.

The eggshell forms the outermost protective barrier of the egg, which is a thin, hard, but brittle covering primarily composed of calcium carbonate. Around 95% of the eggshell consists of inorganic minerals, with calcium carbonate accounting for nearly all of this fraction (98.5%), while the remaining components include organic materials (3.5%) and water (1.5%) [107]. The eggshell primarily serves to provide structural integrity and protect the inner layers of the egg from mechanical impact, microbial contamination, and dehydration [108]. In addition to the mechanical protection, this layer has a porous architecture that allows diffusion of oxygen, carbon dioxide, and water vapor, enabling respiration and maintaining hydration of the developing embryos.

The layer right beneath the eggshell is the eggshell membrane, a thin, fibrous protein network composed primarily of structural proteins (90%), along with minor amounts of lipids (3%) and

carbohydrates (2%) [109]. This layer acts as a structural foundation for the formation and calcification of the shell during egg development and acts as an additional barrier against microbial invasion. It also helps to compartmentalize the egg contents, separating the mineralizing shell from the underlying albumen and preventing exposure of the inner egg components to high calcium concentration.

Below the eggshell membrane is the egg white, or albumin, which makes up the majority of the egg by mass (62%) and accounts for over half of the total protein content of the egg (56%) [110]. The egg white is a translucent, gel-like component composed of water and soluble proteins and serves as a physical barrier against bacterial infiltration and has buffering capacities [111]. It is structurally organized into two phases: an inner thick layer of albumin surrounding the egg yolk and an outer thin layer of albumin surrounding the thick albumen. While the thick albumin is viscous and provides a mechanical cushioning around the egg yolk, the thin albumin has higher water content and provides moisture to the yolks and the developing fertilized embryos.

The innermost layer of the egg is the egg yolk, the central nutrient reserve of the egg, which is enclosed by a transparent proteinaceous membrane called the vitelline membrane. The egg yolk is composed of 50% water, 30% lipids, 16% proteins, 4% carbohydrates, and various micronutrients, including vitamins and essential minerals [112]. Functionally, the egg yolk serves as a principal source of energy and nutrients for the fertilized developing embryo. The egg yolk has two distinct phases: one being a clear yellow aqueous phase, which contains a majority of the yolk's lipids and soluble proteins, and the other being an insoluble phase [113]. The aqueous phase contains 90% of the egg yolk's total lipids, a majority of it being low-density lipids, and approximately 50% of the egg yolk's total proteins. In contrast, the insoluble part contains the remaining 10% lipids, mostly as high-density lipids, and the rest 50% of the egg yolk's total proteins. This structural and biochemical organization of the yolk enables efficient mobilization and delivery of lipids, amino acids, and micronutrients to support the high energetic demands during embryogenesis.

1.5.2. Nutrient metabolism during embryonic development

The embryonic development in chickens is characterized by a progressive shift in energy metabolism that closely follows changes in substrate availability and oxygen supply within the egg [114]. As carbohydrates are present in very limited amounts in the egg (1%), the developing embryo relies predominantly on utilizing the yolk-lipids to derive energy, with fatty acid oxidation accounting for the majority of ATP production during incubation [34, 101]. Studies suggest that around 85-90% of the energy required for growth is derived from the oxidation of yolk lipids, particularly in the latter half of embryogenesis [100]. In general, embryogenesis can be divided into three phases: early embryonic establishment, embryonic development and organogenesis, and the final maturation phase leading to hatching [115]. During the early periods of embryogenesis, oxygen availability within the egg is relatively low due to immature red blood cells and an underdeveloped chorioallantoic membrane [116]. Under these hypoxic conditions, glucose serves as the main source of energy substrate, and glycolysis is the predominant pathway to derive energy during this period [114]. Glucose used during this phase comes from small carbohydrate stores in the egg and limited glycogen stores, promoting slow growth under low oxygen conditions. Pyruvate generated from the glycolysis of glucose is largely reduced to lactate, which accumulates in the embryonic tissues due to restricted oxidative capacity. Later, when the oxygen levels go up, the stored lactate is transported to the liver, where it is recycled to glucose via gluconeogenesis [117].

As the development progresses into the second phase, or the embryonic completion phase, a fully functional chorioallantoic membrane is formed, ensuring adequate oxygen supply for transitioning towards oxidative metabolism for the faster and energy-demanding phase of the embryonic development [114]. Yolk lipid mobilization increases substantially during this period, and fatty acids derived from triglycerides and phospholipids in the yolk sac become the dominant energy source for the developing embryo [100]. These fatty acids are exported to different tissues, especially the liver, where they undergo mitochondrial β -oxidation to produce acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle to

produce energy in terms of ATP. The glycerol released from triglyceride hydrolysis serves as a major substrate for hepatic gluconeogenesis, contributing to maintaining the circulating glucose levels [98]. High rates of lipid oxidation during the mid to late embryonic incubation are accompanied by an increase in ketone production, indicating an increase in both hepatic fatty acid flux and the metabolic demands of the extrahepatic tissues [118]. A systemic reliance on lipid-based metabolism is observed, as highlighted by elevated activities of enzymes involved in fatty acid oxidation and ketogenesis during this stage. During this phase, yolk contents are rapidly mobilized, with the yolk mass declining from 60-65% of egg content around embryonic day 13 to less than 45% by the time of hatch.

In the final few days before hatch, a distinct metabolic shift occurs as the embryo prepares to hatch. An increase in embryonic movements leads to the rupture of the chorioallantoic membrane, which reduces oxygen availability and causes a shift in metabolism [119]. Due to less oxygen availability, the energy demands cannot be fulfilled exclusively by fatty acid oxidation, and this causes an increase in anaerobic glucose catabolism to meet immediate energy demands. Glucose is supplied by hepatic glycogen stores and gluconeogenesis from lactate, amino acids, and glycerol [117]. Despite high fatty acid oxidation capacity, its relative contribution to ATP production declines due to reduced oxygen availability, which emphasizes the metabolic flexibility of the developing embryo. Taken together, these tightly regulated metabolic adaptations allow the chicken embryo to efficiently utilize yolk-derived nutrients across changing environmental and physiological conditions [119]. The ability to dynamically adjust substrate utilization in response to oxygen availability and developmental stage is essential for successful embryogenesis and establishes the metabolic framework that supports the rapid transition to exogenous feeding and carbohydrate-based metabolism following hatch.

1.5.3. Nutrient metabolism in the hatchlings

Although the majority of the yolk lipids are absorbed during the final few days of embryonic development, some amount of the yolk sac remains unabsorbed, and this remaining yolk sac serves as a source of energy for the hatchlings in the first several hours post-hatch, and until feed is available [118].

Hepatic glycogen stores accumulated during late embryogenesis contribute to maintain blood glucose homeostasis during this transitional phase [114].

As the hatchlings are supplemented with external feeds, a marked shift in substrate utilization occurs, characterized by a transition from reliance on yolk-derived lipids to carbohydrate-based metabolism [35]. Commercial starter diets are typically enriched in starch, and dietary carbohydrates become the primary source of metabolizable energy in the hatchlings [118]. This transition in substrate utilization is accompanied by coordinated changes in hepatic metabolic pathways, including increased glucose uptake, enhanced glycolytic flux, and replenishment of glycogen stores [120]. These adaptations work towards facilitating the efficient utilization of dietary carbohydrates and support the high energetic demands associated with rapid post-hatch growth. These changes also happen at the molecular level, where the embryonic to post-hatch transition is associated with dynamic changes in expression and activity of key metabolic regulators in the liver [121]. Transcription factors that promote fatty acid oxidation, for example, peroxisome proliferator-activated receptor alpha (PPAR α), are highly upregulated during embryogenesis but decline in activity post-hatch. Contrastingly, transcriptional regulators that drive carbohydrate utilization and lipogenesis, including carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP-1), are upregulated following exposure to commercial diets in the hatchlings. These transcriptional shifts support the induction of hepatic *de novo* lipogenesis, a hallmark of post-hatch hepatic metabolism in chickens.

The provision of carbohydrate-rich diets post-hatch initiates robust *de novo* lipogenesis in the liver, resulting in increased hepatic lipid accumulation [35]. This lipid accumulation reflects a physiological anabolic response, highlighting a healthy metabolic transition in the hatchlings. It is interesting to note that despite the high rates of lipid oxidation during the embryonic stages and high rates of lipid accumulation during the post-hatch stages, the embryonic to neonatal transition occurs without any apparent symptoms of metabolic dysfunction, cellular stress, or inflammation in the liver [30]. This physiological resilience contrasts sharply with humans or rodents, where high rates of lipid oxidation and hepatic lipid accumulation are accompanied by hepatocellular stress, inflammation, and the development

of metabolic disorders [102, 103]. In chickens, the absence of such pathology during the embryonic to neonatal transition is facilitated by the optimal coupling between mitochondrial oxidative functions and lipogenesis [30]. A dynamic remodeling of the hepatic mitochondrial oxidative functions, including β -oxidation, ketogenesis, and the TCA cycle, along with the pathway of lipogenesis, is observed along with the simultaneous upregulation of antioxidant defense mechanisms during this transition period in chickens.

Early access to feed is known to accelerate the metabolic transition towards carbohydrate utilization and lipogenesis, but the precise nutritional and intracellular signals that initiate this hepatic reprogramming in the hatchlings remain incompletely understood. Specifically, the contributions of specific dietary nutrients and their downstream metabolic intermediates in initiating the mitochondrial and lipogenic remodeling have not been systematically investigated. Addressing these gaps is essential for understanding the mechanisms that enable healthy metabolic adaptation during early life in chickens.

1.5.4. Commercial diets supplemented to the chicken post-hatch

Early post-hatch mortality, during the first 3-4 days after hatch, is a critical challenge for the poultry industry, with losses ranging from approximately 0.5% to 3% [122]. This early period coincides with the metabolic switch during the embryonic to neonatal transition period, during which the hatchlings switch from lipid-based metabolism to carbohydrate-based metabolism. Given that the United States has one of the world's largest poultry production systems, improving the metabolic adaptations during this window has significant implications for chick health, production efficiency, and economic sustainability. Identifying the nutritional and metabolic factors influencing this metabolic transition in chickens is of significant interest for the poultry industry, as it would, in turn, reduce the mortality rates after hatch. Typically, broiler chickens are provided with a pre-starter diet during the first several days post-hatch. These pre-starter diets are formulated to meet the nutritional and physiological requirements of the neonatal chicken, whose gastrointestinal tract and digestive systems are still under development. These diets are highly digestible and have high amounts of readily available nutrients to maximize nutrient absorption and support rapid early growth (**Table 1.1**) [123]. The commercial pre-starter diets generally

provide around 3000-3200kcal/kg of metabolizable energy and are rich in carbohydrates. Around 55-60% of dietary dry matter is composed of dietary carbohydrates, most of which comes from starch-rich grains like corn. These carbohydrates serve as a primary source for the hatchlings, facilitate rapid hepatic glycogen repletion, and rapid growth of chickens during the early post-hatch periods. Dietary protein constitutes approximately 20–25% of feed dry matter and is commonly supplied by soybean meal, providing essential amino acids required for tissue accretion, muscle development, and organ growth. Lipids are present at lower levels in pre-starter diets, around 4–6% of dietary dry matter and are primarily derived from added vegetable oils. These dietary lipids supply essential fatty acids necessary for membrane biogenesis, eicosanoid synthesis, and fat-soluble vitamin absorption, while also contributing to overall dietary energy density. The balance among carbohydrates, proteins, and fats in the commercial diets helps to meet the high energy and anabolic demands of the broiler chickens during the early growth periods while supporting the optimal metabolic adaptations required post-hatch [124].

Despite the widespread use of standardized pre-starter diets for the hatchlings, the specific roles of individual macronutrients in initiating and regulating hepatic metabolic reprogramming during the post-hatch period remain poorly understood. A mechanistic understanding of how early dietary composition influences hepatic metabolism will help the development of optimized feeding strategies that enhance metabolic health, reduce early mortality, and improve long-term productivity in broiler chickens.

Table 1.1. Nutrient composition of commercial broiler starter diets.

Category	Nutrient	Range/Amount	Role in growth
<i>Macronutrients</i>	Carbohydrates (mainly starch)	55-60%	Primary energy for tissue growth
	Proteins (crude proteins)	20-25%	Muscle, organ, and feather development
	Fat (crude fat)	4-6%	Source of concentrated energy, and essential fatty acids
	Metabolizable Energy	3000-3200 kcal/kg	Energy for growth, activity, and metabolism
<i>Essential Amino Acids</i>	Lysine	1.2-1.5%	Key for muscle growth
	Methionine and Cysteine	0.9-1.1%	Feather and tissue development
	Threonine	0.9-1.1%	Supports protein synthesis and gut health
<i>Key Minerals</i>	Calcium (Ca)	0.9-1.0%	Essential for bone development
	Phosphorus (P)	0.45-0.5%	Key for bone formation, and energy metabolism
	Sodium (Na)	0.18-0.22%	Maintains electrolyte balance for metabolism
	Chloride (Cl)	0.15-0.20%	Maintains acid-base balance
<i>Vitamins</i>	Vitamin A	10000-15000 IU/kg	Supports growth, immunity, and tissue repair
	Vitamin D3	3000-4000 IU/kg	Promotes calcium absorption and skeletal growth
	Vitamin E	30-50 IU/kg	Antioxidant, and protects growing tissues
	B-Vitamins	50-60 mg/kg	Helps in energy metabolism and protein utilization

1.6. Metabolic approaches to study mitochondrial oxidative networks and *de novo* lipogenesis in the liver

For investigating the hepatic mitochondrial oxidative functions and *de novo* lipogenesis in the liver, I used an integrated experimental framework combining stable-isotope labelled tracers, gas chromatography-mass spectrometry (GC-MS) based targeted metabolomics, and complementary molecular biology techniques. This multi-level approach helped us to characterize hepatic metabolism across transcriptional, translational, and metabolic levels, providing a comprehensive understanding of pathway activity and regulation.

Metabolic pathways are complex and are regulated by coordinated changes in gene expression, protein abundance and activity, substrate availability, and environmental factors [125]. For that, reliance on a single analytical approach is often insufficient to examine the metabolic states of animals. To address this, I primarily utilized targeted metabolomics approaches based on GC-MS, which allow sensitive and quantitative analysis of metabolites relevant to mitochondrial oxidative metabolism and lipogenesis [125-127]. I also used stable isotope-labeled tracers to assess metabolic flux through specific mitochondrial pathways of interest. Isotope tracing provides insight into pathway activity and substrate fate that cannot be inferred from metabolite pool sizes alone, particularly for highly dynamic processes. To complement the metabolite measurements, expression and abundance of key genes and proteins involved in mitochondrial oxidative networks and lipogenic pathways were quantified using molecular biology techniques. Together, the integration of i) isotope-based metabolic tracing, ii) targeted GC-MS metabolomics, and iii) molecular analyses enabled a mechanistic study of hepatic metabolic remodeling under the physiological conditions examined in this study.

1.7. Use of stable isotopes to study mitochondrial oxidative networks in the liver

Mass spectrometry-based analysis of stable isotope incorporation into metabolic intermediates is a robust approach for analyzing nutrient utilization and metabolic fluxes at the tissue, organ, or whole-animal levels. Non-radioactive stable isotopes like ^{13}C , ^2H , ^{15}N , ^{18}O , and ^{34}S are powerful for biological

applications because they are less naturally occurring, chemically stable, and do not perturb normal cellular functions [125, 128]. When these tracers are incorporated into metabolites, these isotopes help towards the precise tracking of substrate fate through interconnected metabolic pathways.

In gas chromatography-mass spectrometry (GC-MS) based analyses, stable-isotopes introduction creates predictable mass shifts in derivatized metabolites and allows labeled and unlabeled metabolites to be distinguished based on mass differences while retaining identical chemical properties. Isotope-enriched metabolites generate GC-MS spectra similar to those of their naturally abundant counterpart, only differing in isotopic composition. Stable isotope-based approaches provide information about metabolic pathways through i) isotope enrichment of pathway intermediates and ii) quantifying the concentration of the metabolite pool. While metabolite concentration indicates the steady abundance of the metabolite in the tissue/ organ, isotopic enrichment indicates the activity and carbon flow through the pathways, which is very useful for dynamic metabolic processes.

To exemplify, uniformly labeled $^{13}\text{C}_6$ -glucose is used to evaluate glycolytic and TCA cycle activities. As all six carbons of the glucose are uniformly labeled, its conversion through glycolysis yields $^{13}\text{C}_3$ pyruvate, and that helps with the assessment of glucose-derived carbon entry into the downstream oxidative pathways [129-131]. After entering the mitochondria, $^{13}\text{C}_3$ pyruvate provides ^{13}C into the TCA cycle intermediates, and an isotopomeric distribution analysis of the metabolites provides insight into the extent of TCA cycle activity [132]. In addition to enrichment analysis, stable isotope-labeled internal standards are used to quantify the metabolite pool size. By spiking samples with a known amount of uniformly labeled internal standards (eg. $^{13}\text{C}_3$ pyruvate), the ratio of unlabeled to labeled species is used to calculate endogenous metabolite concentrations. Integration of isotopic enrichment data with metabolite abundance measurements helped us to get a more complete interpretation of carbon turnover and metabolic flux through mitochondrial pathways.

1.8. Summary

The liver is a central metabolic organ that plays a vital role in nutrient metabolism, energy production, and coordinating anabolic processes to maintain whole-body energy homeostasis. Hepatic mitochondria, the metabolic hub of the cell, play a critical role in supporting these functions as it orchestrates a variety of biochemical processes, including the TCA cycle, β -oxidation, ketogenesis, and oxidative phosphorylation. Through this dissertation, I explored the biochemical frameworks governing the metabolic coupling of mitochondrial oxidative pathways with the hepatic lipogenic pathway across two animal models, mice and chickens.

Dysregulation of the mitochondrial networks is a characteristic feature of people suffering from metabolic disorders like metabolic dysfunction-associated steatotic liver disease (MASLD). Furthermore, high rates of hepatic *de novo* lipogenesis, particularly induced by dietary fructose, are a hallmark of individuals suffering from MASLD. However, how high rates of *de novo* lipogenesis, through its close biochemical relationship with mitochondria, can contribute to dysfunctional induction of mitochondrial oxidative functions remains poorly understood.

A characteristic feature of the embryonic to post-hatch transition in chickens is the metabolic shift from relying heavily on yolk lipid oxidation in the embryonic stage to relying on carbohydrate metabolism in the post-hatch stage, along with a dramatic increase in *de novo* lipogenesis in the liver. This transition occurs without the onset of hepatocellular stress and inflammation, highlighting an optimal coupling of mitochondrial networks and the lipogenic pathways. While it is known that the provision of diet to the hatchlings immediately after hatch drives this metabolic transition, the role of specific dietary macronutrients in initiating this metabolic transition has not been systematically investigated. How mitochondrial nutrient shuttles in the liver play a role in the development of the lipogenic phenotype in the liver has also not been well examined.

This dissertation investigates two major aspects of hepatic mitochondrial metabolism and lipogenesis: (i) the impact of high and sustained levels of hepatic *de novo* lipogenesis on mitochondrial oxidative network remodeling in mice, and (ii) the differential role of specific dietary macronutrients and

hepatic mitochondrial nutrient shuttle systems in facilitating the lipolytic to lipogenic switch in neonatal chickens. Using a combination of stable isotope-based flux analyses, targeted metabolomics, and gene and protein expression studies, this work delineates how mitochondrial networks coordinate nutrient flux to establish the hepatic metabolic phenotype across distinct physiological and pathological conditions.

Chapter 2

Induction of fructose mediated *de novo* lipogenesis coexists with the upregulation of mitochondrial oxidative functions in mice livers¹

¹ This chapter is based on the following published work:

Bhattacharjee, P., et al. (2025). *Induction of fructose-mediated de novo lipogenesis coexists with the upregulation of mitochondrial oxidative functions in mouse liver. Journal of Nutrition*, 155(6), 1768-1781.

2.1. Abstract

Dysfunctional mitochondrial metabolism and sustained *de novo* lipogenesis (DNL) are characteristics of metabolic dysfunction-associated steatotic liver disease (MASLD), a comorbidity of obesity and type 2 diabetes. Fructose, a common sweetener and a potent inducer of lipogenesis, contributes to the etiology of MASLD. My goal was to determine whether higher rates of DNL, through its biochemical relationships with mitochondria, can contribute to dysfunctional induction of oxidative networks in the liver. Male C57BL/6JN mice were given a low-fat (10% fat kcal, 49.9% corn starch kcal), high-fat (HF; 60% fat kcal), or HF/high-fructose diet (HF/HFr; 25% fat kcal, 34.9% fructose kcal) for 24 weeks. In a follow-up study, mice on normal feed pellets were provided either 30% fructose in drinking water (FW) to induce hepatic DNL or regular water (NW) for 14 days. Hepatic mitochondria and liver tissue were used to determine oxygen consumption, reactive oxygen species (ROS) generation, tricarboxylic acid (TCA) cycle activity, and gene/protein expression profiles. Hepatic steatosis remained similar between HF and HF/HFr fed mice livers. However, lipogenic and lipid oxidation gene expression profiles and the induction of TCA cycle metabolism were all higher ($P \leq 0.05$) in HF/HFr livers. Under fed conditions, the upregulation of DNL in FW livers occurred in concert with higher mitochondrial oxygen consumption (basal; 1.7 ± 0.21 compared with 3.3 ± 0.14 nmoles/min, $P \leq 0.05$), higher ROS (0.87 ± 0.09 compared with 1.25 ± 0.12 μM , $P \leq 0.05$) and higher flux through TCA cycle components ($P \leq 0.05$). Furthermore, TCA cycle activity and lipid oxidation remained higher during fasting in the FW livers ($P \leq 0.05$). My results show that fructose administration to mice led to the concurrent induction of mitochondrial oxidative networks and DNL in the liver. Sustained induction of both DNL and mitochondrial oxidative function could accelerate cellular stress and metabolic dysfunction during MASLD.

2.2. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) [133] is characterized by high rates of lipid accumulation and dysfunctional mitochondrial oxidative function [134-139] and is a highly prevalent comorbidity of obesity [140, 141] and type-2 diabetes mellitus [142-144]. The spectrum of this liver disease ranges from simple steatosis to steatohepatitis, with a subpopulation advancing to cirrhosis and eventual liver failure [145, 146]. Dysregulation in mitochondrial oxidative networks is a hallmark of MASLD [136-138, 147], which includes aberrant nutrient fluxes via oxidative processes, including β -oxidation, tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), ketogenesis, and ATP synthesis [67, 135, 148, 149]. The nutrient flux through these major mitochondrial oxidative networks is linked together through nutrient shuttles, some of which transport cytosolic reducing equivalents into the mitochondria (for example, malate-aspartate shuttle) [12] and others which generate and transfer metabolite precursors from the mitochondria to the cytosol for lipid biosynthesis (for example, citrate/isocitrate carrier) [150]. Understanding the mechanisms responsible for the continuous remodeling of mitochondrial oxidative functions during the progressive severity of MASLD is of major interest in designing therapeutic and management strategies against this disease.

The classical hypercaloric Western diets, which are high in saturated fats and carbohydrates, are closely aligned with the increasing incidence of obesity and MASLD [24, 25]. Most of the Western diets have high levels of fructose, which is also the most common sweetener used in soft drinks and baked goods [26]. Diets rich in carbohydrates, especially fructose, are known to induce *de novo* lipogenesis (DNL) in the liver, which is a central feature of MASLD, along with dysfunctional mitochondrial metabolism [27, 139, 151]. Apart from being lipogenic, increased fructose consumption has also been shown to aggravate cellular inflammation and metabolic dysfunction during MASLD [152-154]. Furthermore, high fructose-induced metabolic dysfunction is also correlated to the degree of hepatic fibrosis during steatohepatitis [155, 156].

Most of the rodent models of the diet-induced MASLD are generated on either high-fat or high-fructose or a combination of high-fat/high-carbohydrate dietary backgrounds [135, 136, 148, 157].

Although all these diet-induced rodent models of MASLD have been shown to result in hyperinsulinemia and hepatic steatosis and varying degrees of insulin resistance, the biochemical networks responsible for lipid accumulation in the liver can greatly vary based on the dietary macronutrient composition. For example, a HF diet rich in saturated fats significantly suppressed DNL from carbohydrates in mice [148, 158-161], a characteristic feature of fatty liver, despite promoting lipid accumulation through esterification and storage of dietary fatty acids in the liver [157, 162, 163]. However, mice consuming high amounts of fructose, either as dietary fructose or in drinking water, or in combination with a HF diet, all had a greater proportion of hepatic lipid accumulation arising via DNL [28, 148, 151, 157]. Furthermore, livers of animal models sustaining high rates of DNL from a high-carbohydrate dietary environment also showed a higher degree of inflammation and hepatocyte ballooning, in comparison to steatosis arising from a HF diet [25, 164-166]. Together, these signify the need to understand whether the higher flux through DNL during MASLD can occur concurrently with the remodeling of the mitochondrial oxidative machinery, thus contributing to the severity of the disease.

2.3. Materials and Methods

2.3.1. Study Design

The animal study protocols were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park. For all the studies, male mice aged 4–6 weeks were procured from Jackson Laboratory and were housed at 20–22°C on a 12-h light/12-h dark cycle with *ad libitum* access to food and water.

For study 1, 8 weeks old C57BL/6NJ mice were randomly assigned for 24 weeks on either a low-fat diet (LF; 10% kcal fat, 20% kcal protein, and 70% kcal carbohydrate; D12450J), a high-fat diet (HF; 60% kcal fat, 20% kcal protein, and 20% kcal carbohydrate; D12492), or a high-fat/high-fructose diet (HF/HFr; 25% kcal fat, 20% kcal protein, and 34.9% kcal fructose; D19013101) (semi purified diets from Research Diets Inc.; **Appendix Table 2.1**). The HF and HF/HFr diets were formulated to test whether the onset of MASLD through a high-fat compared with a HF/HFr macronutrient environment will

differentially modulate hepatic lipid accumulation and mitochondrial oxidative functions [148]. The HF diet, containing 60% fat calories, is one of the most commonly used to generate rodent models of MASLD [135, 148]. The HF/HFr diet was specifically formulated to reflect a Western dietary milieu where both the lipid and carbohydrate (fructose) calories are high [148]. Metabolic profiling was conducted under both fed and overnight fasted (~16 h) conditions. After 24 weeks on the diets, freshly isolated liver mitochondria were used to estimate reactive oxygen species production (ROS), oxygen consumption rate (OCR), and to analyze metabolite concentrations.

For study 2, C57BL/6NJ mice aged around 6 weeks were randomly assigned to either normal feed pellets (Diet # 7022, Inotiv Inc., **Appendix Table 2.2**) with regular drinking water (NW; control diet) or normal feed pellets with 30% (w/v) fructose in drinking water (FW; lipogenic diet). The 30% fructose treatment was selected based on previous rodent studies, which demonstrated that 30% fructose significantly induced hepatic DNL in mice [157]. Fructose is a common sweetener in foods and beverages and is a significant contributor to the development of MASLD, in part due to its high lipogenic potential. Study 2 tested whether a higher rate of DNL occurs concurrently with the induction of mitochondrial oxidative function. All the analyses were conducted under both fed and overnight fasted (~16 h) conditions. At the end of the 14 days of treatments, hepatic mitochondria were isolated to determine ROS production, OCRs, and metabolite levels. All the metabolic profiling was carried out under fed and fasting conditions.

2.3.2. Metabolic profiling of freshly isolated liver mitochondria

Hepatic mitochondria isolation

Following dietary treatments in studies 1 and 2, hepatic mitochondria were isolated for metabolic profiling. Mice were anesthetized under isoflurane, and fresh liver samples were collected and washed in ice-cold 1X-phosphate-buffered saline (PBS) 2 times. Liver tissues were weighed, and hepatic mitochondria were isolated from 600 to 800 mg liver samples. Liver tissue was minced in 3 mL of Mannitol-sucrose-HEPES-EDTA (MSHE) buffer [70 mM sucrose, 210 mM mannitol, 5 mM HEPES (4-

(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA, and 0.5% fatty acid-free bovine serum albumin (BSA); pH 7.2] and homogenized using a Dounce homogenizer with 4 strokes (5–10 turns per stroke). The homogenized tissue was transferred to a 15 mL ice-cold Falcon tube. The Dounce homogenizer was rinsed with 4 mL of MSHE buffer, which was transferred to the 15 mL Falcon tube and centrifuged for 10 minutes at 4°C at 800 g. The supernatant was passed through a double-layered cheesecloth presoaked in MSHE and further centrifuged at 8,000 x g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 3 mL of MSHE buffer and centrifuged for 10 minutes at 4°C at 8,000 g. This process was repeated, and the final mitochondrial pellet was resuspended in 100 µL of BSA-free MSHE buffer. The mitochondrial suspension was diluted (3µL in 57 µL of 1 RIPA (Radio-Immunoprecipitation Assay) buffer) to estimate protein concentration using the Pierce protein assay kit (Thermo Fisher Scientific).

Incubation of freshly isolated mitochondria with stable isotopes

Freshly isolated hepatic mitochondria (500 µg) were incubated in 1 mL MAS (Mitochondrial Assay Solution)-3 buffer (115 mM KCl, 10 mM KH₂PO₄, 2 mM MgCl₂, 3 mM HEPES, 1 mM EGTA, and 0.2% fat-free BSA, 5 mM glutamate; pH 7.2) containing either 1) 1 mM [¹³C₃] pyruvate and 2.5 mM malate, 2) 1 mM pyruvate and 2.5 mM [¹³C₄] malate, or 3) unlabeled 1 mM pyruvate and 2.5 mM malate. Mitochondria were allowed to respire in these incubation buffers for 10 minutes at 37°C with occasional tapping, and the changes in the metabolite levels/tracer enrichments were measured using GC-MS protocols as detailed below. At the end of the incubation period, respiration was stopped by placing samples on ice, followed by centrifugation at 3,000 x g for 5 minutes at 4°C. The supernatant was collected, and the mitochondrial pellet was washed with 500 µL of ice-cold PBS and centrifuged again at 3000 g for 2 minutes at 4°C. The mitochondrial pellet was stored at –80°C for GC-MS-based metabolite analysis.

2.3.3. Analysis of metabolic intermediates by GC-MS

For measuring metabolite concentrations in the mitochondria, liver, and serum samples, the samples were spiked with a known amount of stable isotope-labeled internal standards of the respective metabolites. No internal standards were added to the mitochondrial samples while estimating the enrichment of metabolic intermediates from [$^{13}\text{C}_3$] pyruvate or [$^{13}\text{C}_4$] malate stable isotopes. Blood collected from the posterior vena cava of the anesthetized mice was centrifuged at 2,500 x g for 10 minutes to isolate serum. Serum (25 μL) samples were deproteinized with 750 μL of cold acetonitrile, and the metabolic intermediates were converted to their corresponding oximes with the addition of 20 μL of 2% methoxamine hydrochloride in pyridine, followed by tertbutyldimethylsilyl (TBDMS) derivatization, for GC-MS analysis.

The liver (~15 mg) and the mitochondrial (500 μg) samples were extracted with 750 μL of chloroform: methanol (2:1) and 250 μL of water, shaking for 1 hour. The aqueous phase was dried under nitrogen, and the metabolic intermediates were converted to their corresponding oximes with 20 μL of 2% methoxamine hydrochloride in pyridine (w/v) by microwaving for 90 sec, followed by conversion to their respective TBDMS derivatives by heating at 90°C for 1 hour. The derivatized samples were injected onto a HP-5MS UI column (30 m 0.25 mm 0.25 μm ; Agilent) for metabolite separation, followed by fragmentation under electrical ionization for GC-MS analysis. Fragments of interest were detected using single ion monitoring on a GC-MS (5973N-Mass Selective Detector, 6890-Series GC, Agilent) [167, 168].

2.3.4. ROS production by isolated hepatic mitochondria

Oxidation of Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase (HRP) was used to determine the rate of hydrogen peroxide (H_2O_2) produced by mitochondria. Mitochondria (32 or 64 μg) were incubated with HRP (0.2 U/mL) and Amplex red reagent (100 μM ; 10-acetyl-3,7-dihydroxyphenoxazine) prepared in MAS-3 buffer with 5 mM glutamate and 2.5 mM malate.

Real-time oxidation of Amplex red to the fluorescent resorufin indicated the rate of H₂O₂ produced by the isolated mitochondria. Changes in fluorescence were recorded using the Cytation 5 spectrophotometer (BioTek Instruments, Inc.) with 530 nm excitation and 590 nm emission wavelengths. The rate of H₂O₂ production was calculated by constructing a standard curve by adding known amounts of H₂O₂ to the assay [168].

2.3.5. Oxygen consumption by the isolated hepatic mitochondria

For studies 1 and 2, isolated mitochondria (500 µg) were suspended and allowed to respire in 1 mL of MAS-3 buffer containing 5 mM glutamate, 1 mM pyruvate, and 2.5 mM malate. An oxygraph oxygen electrode (Hansatech Instruments) was utilized to measure basal, ADP-stimulated (with 100 µM ADP), and ADP-depleted oxygen consumption. These mitochondrial respiration rates were normalized for expression of COX IV protein per µg crude hepatic mitochondrial protein pellet [148, 167, 168].

2.3.6. Liver glycogen analysis (study 1)

For estimating liver glycogen content, frozen liver tissues (~20–25 mg) were deproteinized with 8% sulfosalicylic acid, and glycogen was precipitated with 1 mL of ice-cold ethanol (95%). The glycogen pellet was washed 2 times with ice-cold ethanol, and the pellet was air-dried overnight. The glycogen pellet was incubated with amyloglucosidase in acetate buffer at pH 5.0 (250 µL; 1-unit enzyme/mg glycogen) for 1 hour at 55°C. Amyloglucosidase acts on liver glycogen to form glucose molecules. This incubation mixture was lyophilized, and di-O-isopropylidene acetate (a derivative of glucose) was formed and used for GC-MS analysis. The glucose present in the sample was calculated relative to the amount of uniformly labeled [¹³C₆] glucose internal standard [98].

2.3.7. Hepatic gene expression profiles

Approximately 20–25 mg snap-frozen liver samples were aliquoted for mRNA extraction using 500 µL Trizol reagent (Invitrogen) and using the mRNA mini-prep kit (Bio-Rad Laboratories Inc.). 1 µg mRNA was used to make cDNA using the cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using 25 ng of cDNA, 150 nM of forward and reverse primers each, and 5 µL of SYBR

green PCR master mix (Invitrogen) in a 10 μ L reaction. Cyclophilin (Ppib) was used as the housekeeping gene. Samples were analyzed in triplicate on a Bio-Rad CFX Real-Time system C1000 Touch Thermal Cycler (Bio-Rad). The list of primers is provided in **Appendix Table 2.3**.

2.3.8. Western blot analysis

Frozen liver samples (~25 mg) and mitochondrial samples (~250 μ g) were lysed in a 1X RIPA lysis buffer with protease and phosphatase inhibitors. Samples were centrifuged at 16,708 x g for 15 minutes at 4°C, and the supernatant was collected for protein estimation. Protein content was measured using a Pierce BCA (Bicinchoninic acid) protein assay kit (Thermo Fischer Scientific). Proteins were separated using Bolt 8% Bis-tris Plus gels (Invitrogen) and transferred to a polyvinylidene fluoride membrane. The membranes were blocked with 5% milk and probed overnight with the primary antibodies of interest, including OXPHOS, Slc25A11 (Abcam plc), Slc25A1 (Proteintech), and Cox IV, GAPDH, and β -actin (Cell Signaling Technology Inc.). Images were collected using a Gel Doc XR β system (Bio-Rad).

2.3.9. Liver histology (study 1)

Livers (~150 mg) were fixed in 4% paraformaldehyde for 18–24 h, washed thrice with water, and stored in 70% ethanol at 4°C. Masson's Trichrome staining of liver tissue (n=4 per group) was performed by Histoserv, Inc. to visualize lipid droplets.

2.3.10. Biochemical assays

Nonesterified fatty acid (NEFA)-HR2 analytical kit (WAKO diagnostics) was used to determine serum NEFA concentrations. Ultrasensitive mouse insulin ELISA kit (Crystal Chem) was used to determine serum insulin levels. A total of 20 mg of liver tissue was used to extract and analyze liver triglyceride levels using the triglyceride determination kit (Sigma Aldrich) with triolein as the standard. All the assays were conducted according to the kit protocols.

2.3.11. LC-MS/MS analysis of liver acylcarnitine (study 1)

Liver tissues (20–25 mg) were homogenized and transferred to micro centrifuge tubes containing ice-cold acetonitrile, which had a known amount of stable isotope-labeled internal standards of acylcarnitine. Samples were vacuum dried and reconstituted in 50 μ L of 50% acetonitrile, followed by centrifugation at room temperature for 1 minutes at 5000 g. Waters ACQUITY UPLC I-Class with a PFP (pentafluorophenyl) C-18 reversed-phase column was used to separate the analytes in the supernatant. Water and acetonitrile containing 0.1% formic acid were used as mobile phases for gradient elution. Electrospray ionization (ESI) was used to ionize the separated compounds, and these were detected by a Bruker Daltonics quadrupole time-of-flight high-resolution tandem mass spectrometer. The data for acylcarnitine identification and quantification were collected using single-stage MS followed by a series of transitions using multiple reaction monitoring.

2.3.12. Statistical analysis

Data reported are presented as means SEM. Comparisons of the results between groups in studies 1 and 3 were analyzed using 1-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Comparisons in the results from study 2 were analyzed using an unpaired Student's t-test. Means were considered significantly different if $P \leq 0.05$, and trends were considered significant if $P \leq 0.1$. All statistical analyses were conducted, and the graphs were plotted utilizing Prism 7 (Graph Pad Software Inc.).

2.4. Results

2.4.1. *De novo* lipogenesis and lipid oxidation were simultaneously upregulated in the livers of insulin-resistant mice reared on HF/HFr diet

Following 24 weeks of dietary treatments, mice reared on both HF and HF/HFr diets had significantly higher ($P \leq 0.05$) body weight, liver weight, and adipose tissue weight, compared with their LF counterparts (**Table 2.1**). Caloric intake has previously been reported to be similar between the HF and HF/HFr groups, but higher than their LF counterparts [148]. The liver-to-body weight ratio was higher across all 3 groups under “fed” conditions compared with overnight fasting ($P \leq 0.05$; **Fig. 2.1A**), where these ratios remained similar between groups. The lower ($P \leq 0.05$) fed-to-fasted fold change of the plasma nonesterified fatty acids in both HF and HF/HFr mice indicated the blunted induction of adipose tissue lipolysis during fasting (**Fig. 2.1B** and **Table 2.1**). Masson’s Trichrome staining of the liver (**Fig. 2.1C**; scale bar 50 μm) demonstrates the significantly higher accumulation of lipid droplets in both the HF and HF/HFr groups compared with their LF counterparts. The accumulation of lipid droplets and liver triglycerides in the HF and HF/HFr groups, however, remained similar (**Fig. 2.1C–E**), indicating a similar degree of hepatic steatosis during the early stages of diet-induced MASLD in HF and HF/HFr groups. HF dietary conditions resulted in significantly higher plasma insulin levels ($P \leq 0.05$) compared with both LF and HF/HFr groups, both during fed and fasting conditions (**Table 2.1**). Similarly, liver glycogen levels were also elevated in HF mice ($P \leq 0.05$) compared with both LF and HF/HFr groups, during fed and fasting conditions (**Table 2.1**). As expected, *de novo* lipogenic gene expression (*Scd1*, *Acyl*, *Fasn*) under fed conditions was higher in HF/HFr livers compared with HF group ($P \leq 0.07$; **Fig. 2.1F**). Overnight fasting significantly increased the plasma β -hydroxybutyrate levels across all 3 groups ($P \leq 0.05$; **Fig. 2.1G**). However, the fed-to-fasted fold induction of plasma β -hydroxybutyrate concentration was the highest in HF/HFr mice compared with the other 2 groups ($P \leq 0.05$; **Fig. 2.1G**). Fasting levels of hepatic long-chain acylcarnitines (octanoyl-, myristoyl-, palmitoyl-) tended to be the highest in the HF group,

while being lower in the HF/HFr livers (**Fig. 2.1H**). The lower levels of long-chain acyl- carnitines in the HF/HFr livers could be reflective of higher rates of lipid oxidation in the HF/HFr livers, compared with the HF group. Furthermore, the upregulation of genes involved in hepatic lipid oxidation (*Cpt1a*, *Lcad*) and ketogenesis (*Hmgcs2*) with fasting was significantly higher in HF/HFr liver compared with their HF counterparts ($P \leq 0.05$; **Fig. 2.1I**). Taken together, these results suggest that although diet-induced insulin resistance from HF and HF/HFr resulted in a similar degree of hepatic steatosis, it differentially impacted the remodeling of genes involved in DNL and lipid oxidation networks in the liver.

Table 2.1. Characteristics of mice reared on LF, HF, HF/HFr diets for 24 weeks.

	Feeding Status	LF	HF	HF/HFr
Body Weight (g)	Fed	34.8 ± 0.4	51.3 ± 0.8 ^a	39.6 ± 1.1 ^{bc}
	Fasted	31.4 ± 0.8*	44.1 ± 1.8 ^{a*}	38.0 ± 1.7 ^{bc}
Liver Weight (g)	Fed	1.8 ± 0.1	2.9 ± 0.2 ^a	2.0 ± 0.1 ^c
	Fasted	1.2 ± 0.0*	1.8 ± 0.2 ^{a*}	1.6 ± 0.2*
Inguinal Adipose Tissue Weight (g)	Fed	0.8 ± 0.1	1.6 ± 0.1 ^a	1.9 ± 0.2 ^b
	Fasted	1.1 ± 0.2	1.3 ± 0.1*	1.8 ± 0.1 ^b
Liver to body weight ratio (%)	Fed	5.0 ± 0.3	5.7 ± 0.3	5.1 ± 0.2
	Fasted	3.8 ± 0.1*	4.1 ± 0.3*	4.0 ± 0.3*
Adipose to body weight ratio (%)	Fed	2.4 ± 0.4	3.1 ± 0.2	4.8 ± 0.3 ^{bc}
	Fasted	3.3 ± 0.5	3.1 ± 0.2	4.7 ± 0.2 ^{bc}
Blood Glucose (mg/dL)	Fed	188 ± 10	225 ± 22	211 ± 7
	Fasted	143 ± 4*	158 ± 5*	149 ± 4*
Plasma NEFA (mmoles/L)	Fed	0.23 ± 0.02	0.33 ± 0.02 ^a	0.31 ± 0.02 ^b
	Fasted	0.72 ± 0.06*	0.67 ± 0.07*	0.65 ± 0.05*
	Fold Change (AU)	3.06 ± 0.25	2.05 ± 0.20 ^a	2.10 ± 0.15 ^b
Plasma Insulin (ng/ml)	Fed	3.71 ± 0.69	13.44 ± 2.12 ^a	6.41 ± 1.33 ^c
	Fasted	1.30 ± 0.46*	6.65 ± 2.33 ^{a*}	1.55 ± 0.45 ^{c*}
Liver Glycogen (mg/whole liver)	Fed	76 ± 10	127 ± 7 ^a	92 ± 16 ^c
	Fasted	1.6 ± 1*	18.0 ± 4 ^{a*}	5.0 ± 1 ^{bc*}
Liver Triglyceride (mg/g liver)	Fed	19.0 ± 4.2	27.4 ± 1.7	33.0 ± 3.2 ^b
	Fasted	34.8 ± 0.7*	34.6 ± 2.0*	30.0 ± 1.4 ^b

Results ($n = 8-11/\text{group}$) were considered significant at $P \leq 0.05$ following pairwise mean comparisons (one-way ANOVA), which are represented by the following alphabets. ‘a’—LF vs. HF; ‘b’—LF vs. HF/HFr; ‘c’—HF vs. HF/HFr. ‘*’ indicates significance at $P \leq 0.05$ following a t-test between fed and fasted conditions. For all the fed-to-fasted comparisons, the normalization is relative to their respective “fed” LF, “fed” HF or “fed” HF/HFr counterparts.

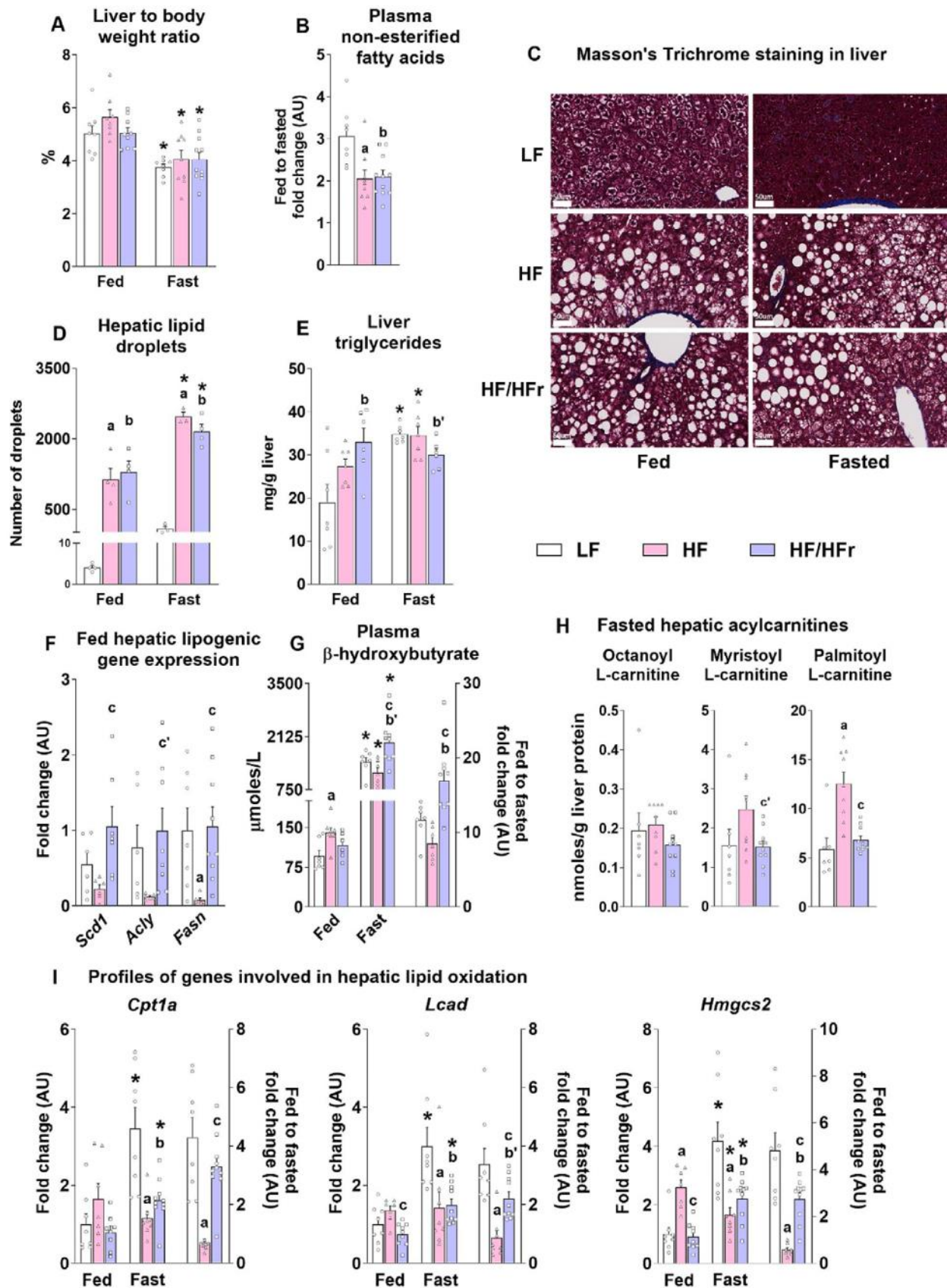
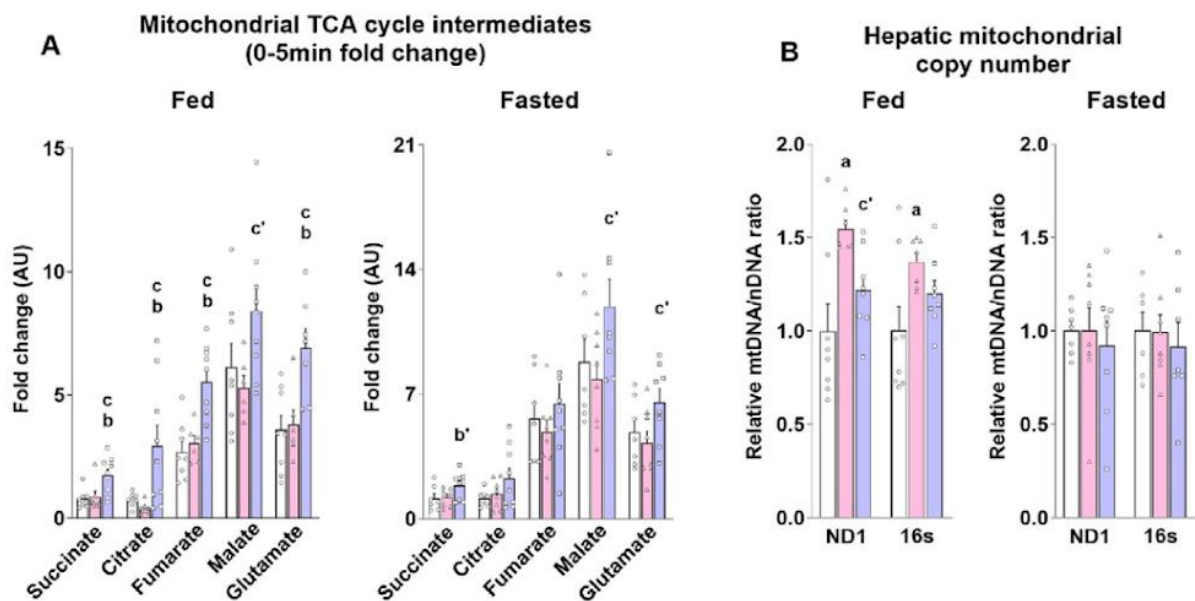


Figure 2.1. *De novo* lipogenesis and lipid oxidation were both higher in high-fat/high-fructose-fed mice.

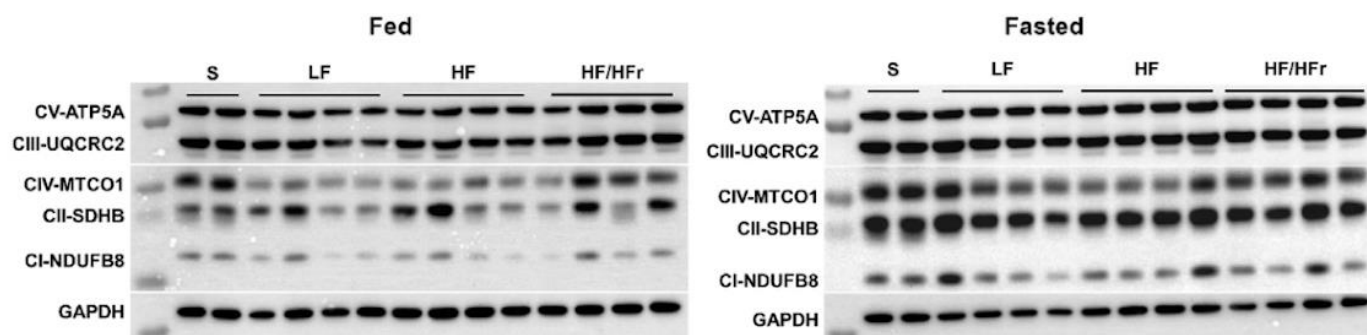
(A) Liver-to-body weight ratio in mice reared on LF, HF, or HF/HFr diets for 24 weeks. (B) Fed-to-fasted fold change in plasma nonesterified fatty acids. (C) Fed and fasted livers stained with Masson's Trichrome stain (scale bar 50 μ m, n=4) and (D) the abundance of lipid droplets in the liver sections (n=4). (E) Liver triglyceride levels after dietary treatments in fed and fasted mice. (F) Fed hepatic lipogenic gene expression (*Scd1*, *Acyl*, *Fasn*) profiles. (G) Circulating levels of β -hydroxybutyrate under fed and fasted conditions. (H) Fasting levels of longer-chain acylcarnitines (octanoyl-, myristoyl-, and palmitoyl-) in the liver. (I) Fed-to-fasted changes in the expression of genes involved in lipid oxidation (*Cpt1a*, *Lcad*, *Hmgcs2*) in the liver. Sample size ranged from 8 to 11/group unless stated otherwise. Following 1-way ANOVA and pairwise mean comparisons, significance at $P \leq 0.05$ are represented by a—LF compared with HF; b—LF compared with HF/HFr; c—HF compared with HF/HFr and significance at $P \leq 0.1$ are represented by a'—LF compared with HF; b'—LF compared with HF/HFr; c'—HF compared with HF/HFr. Furthermore, * indicates significance at $P \leq 0.05$ following an unpaired Student's t-test between fed and fasted groups. ANOVA, analysis of variance; AU, arbitrary units; *Scd1*, stearyl-CoA desaturase; *Acyl*, ATP citrate lyase; *Cpt1a*, carnitine palmitoyl transferase 1; *Lcad*, long-chain acyl dehydrogenase; HF, high-fat; HFr, high-fructose; *Hmgcs2*, 3-hydroxy-3-methylglutaryl-CoA synthase 2; LF, low-fat.

2.4.2. Indices of mitochondrial metabolism were upregulated in the liver from mice reared on HF/HFr diet

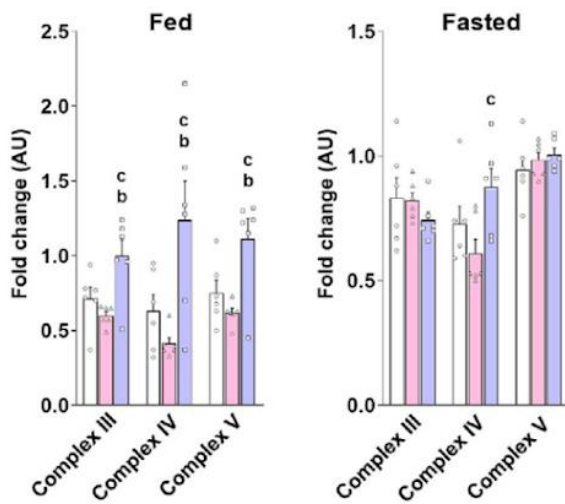
Freshly isolated liver mitochondria from fed and overnight fasted mice were allowed to respire for 5 minutes in a respiration buffer. Under fed conditions, the generation of TCA cycle intermediates during 5 minutes of respiration, which reflects mitochondrial TCA cycle activity, was the highest in the HF/HFr group ($P \leq 0.05$; **Fig. 2.2A**). A similar pattern was observed with the hepatic mitochondria from the HF/HFr fasted mice, even although significant differences were evident only for succinate, malate, and glutamate ($P \leq 0.05$; **Fig. 2.2A**). Under fed conditions, the mitochondrial copy number was higher in the HF livers compared with both LF and HF/HFr livers ($P \leq 0.05$; **Fig. 2.2B**), but the mitochondrial copy number remained similar across the 3 groups after overnight fasting (**Fig. 2.2B**). Expression of proteins involved in OXPHOS was higher (complex III, IV, V; $P \leq 0.05$; **Fig. 2.2C, D**) in the HF/HFr mice livers during fed conditions. After overnight fasting, significant differences only persisted for complex IV protein expression ($P \leq 0.05$; **Fig. 2.2C, D**). Liver mitochondria from the overnight fasted HF/HFr group had a higher rate of ADP-stimulated OCRs ($P \leq 0.05$; **Fig. 2.2E**), whereas these remained similar between groups under fed conditions. Taken together, these results point to an overall induction of mitochondrial metabolism in the liver of the mice reared on HF/HFr diets.



C OXPHOS protein expression in the liver



D OXPHOS protein quantification



E ADP stimulated respiration

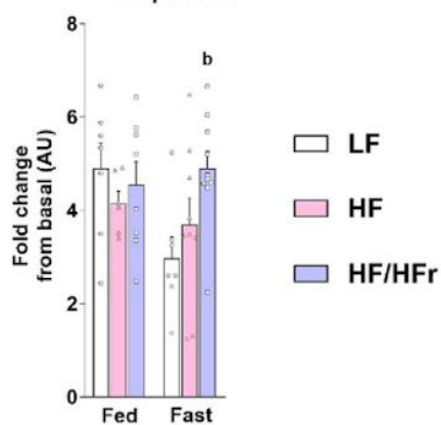


Figure 2.2. Mitochondrial activity and OXPHOS protein profiles in high-fat/high-fructose fed mice livers.

(A) Changes in TCA cycle intermediates (0–5 minutes fold change) in isolated mitochondria from LF, HF, HF/HFr groups under fed and fasted conditions. (B) Mitochondrial copy number, under fed and fasted conditions, was determined from the mtDNA/nDNA ratio by amplification of stable mitochondrial genes, *DN1* and *16S*, and normalized to hexokinase gene expression. (C) Expression profiles of the OXPHOS proteins across the 3 dietary treatments under fed and fasted conditions and (D) their densitometric analysis. (E) Fed-to-fasted changes in ADP-stimulated oxygen consumption by the hepatic mitochondria following the 3 dietary treatments. Results (n = 8–11/group) were considered significant at $P \leq 0.05$ following pairwise mean comparisons (1-way ANOVA), which are represented by the following alphabets a—LF compared with HF; b—LF compared with HF/HFr; c—HF compared with HF/HFr and $P \leq 0.1$ is indicated by a'—LF compared with HF; b'—LF compared with HF/HFr; c'—HF compared with HF/HFr. ANOVA, analysis of variance; AU, arbitrary units; HF, high-fat; HFr, high-fructose; LF, low-fat; ND1, NADH dehydrogenase 1; OXPHOS, oxidative phosphorylation; TCA, tricarboxylic acid.

2.4.3. FW induced hepatic DNL and mitochondrial respiration under fed conditions

To determine whether the changes in mitochondrial metabolism observed in **Fig. 2.2** can be recapitulated following fructose-mediated lipogenic induction, mice were reared on normal feed pellets with either regular drinking water (NW; control) or 30% FW (lipogenic) for a period of 14 days. Mice reared on FW tended to have higher body weight ($P \leq 0.09$) and significantly higher liver ($P = 0.04$) and adipose ($P \leq 0.00$) tissue weights compared with mice on normal drinking water (**Fig. 2.3A**). The mice on fructose drinking water consumed lower calories from normal feed pellets, with a significantly higher proportion of their caloric intake ($P \leq 0.00$) coming from the fructose in their drinking water (**Fig. 2.3B**). The daily total caloric intake was higher in FW compared with NW mice ($P \leq 0.05$; **Fig. 2.3B**). Fed levels of plasma β -hydroxybutyrate were lower in the FW group compared with their NW counterparts ($P \leq 0.05$; **Fig. 2.3C**). Plasma insulin concentration was higher in the FW group compared with NW under fed conditions ($P \leq 0.05$; **Fig. 2.3D**). Hepatic triglycerides were higher in the FW group after 14 days of treatment ($P \leq 0.05$; **Fig. 2.3E**). As expected, 30% FW upregulated hepatic lipogenic gene expression (*Acc*, *Acly*, *Elovl6*, *Fasn*, *Scd1*; $P \leq 0.05$; **Fig. 2.3F**). Interestingly, the basal and ADP-stimulated OCRs by the liver mitochondria were higher in the FW mice (**Fig. 2.3G**). Isolated liver mitochondria from the FW mice also showed higher rates of ROS production (**Fig. 2.3H**). Collectively, these results demonstrate that the lipogenic induction with 30% FW also accompanied an induction in mitochondrial activity.

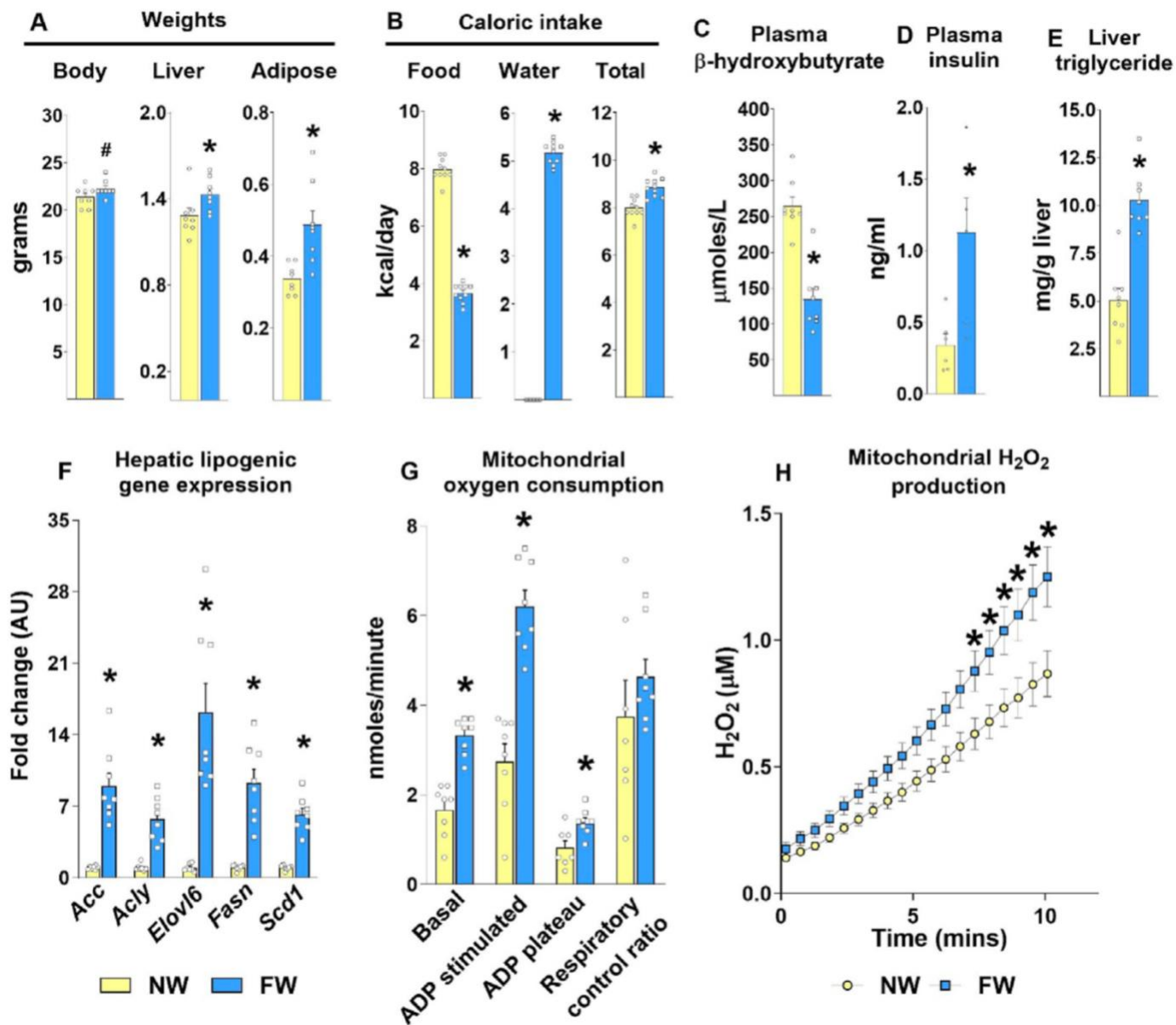


Figure 2.3. Fructose in drinking water induced lipogenesis and mitochondrial respiration in the liver of fed mice.

(A) Body weight, liver weight, and adipose tissue weight after 14 days on normal drinking water (NW) or 30% fructose in drinking water (FW). (B) Daily caloric intake from food and water. (C) Plasma ketone (β -hydroxybutyrate) concentration, (D) plasma insulin, and (E) liver triglyceride in fed mice after 14 days of treatment. (F) Changes in the expression pattern of genes involved in lipogenesis (*Acc*, *Acly*, *Elovl6*, *Fasn*, *Scd1*) in the liver. (G) Indices of hepatic mitochondrial oxygen consumption rate, including basal respiration, ADP-stimulated respiration, plateau after ADP consumption, and respiratory control ratio. (H) Generation of reactive oxygen species by isolated hepatic mitochondria in NW and FW groups. Results (n = 8/group) were considered significant at $P \leq 0.05$ following a t-test between NW and FW groups, which is indicated by “*”. AU, arbitrary units; *Acc*, acetyl-CoA carboxylase; *Acly*, ATP citrate lyase; *Elovl6*; ELOVL fatty acid elongase 6; *Fasn*, fatty acid synthase; *Scd1*, stearyl-CoA desaturase.

2.4.4. FW resulted in the upregulation of multiple components of hepatic TCA cycle metabolism in the fed state

Circulating levels of plasma metabolites (pyruvate, lactate, alanine, succinate, citrate), profiled using GC-MS, were higher in FW mice ($P \leq 0.05$; **Fig. 2.4A**). Metabolite levels in freshly isolated liver mitochondria were measured under both basal conditions (0 min) and after 5 minutes of respiration. Under basal conditions (0 min), mitochondrial metabolites were higher in the FW livers compared with their NW counterparts ($P \leq 0.05$; **Fig. 2.4B**). Furthermore, following 5 minutes of incubation in a respiration buffer, the generation of mitochondrial TCA cycle intermediates, and amino acids (pyruvate, lactate, α -ketoglutarate, alanine, and aspartate) was higher, as evidenced by the higher 0–5 minutes fold change in the FW group ($P \leq 0.05$; **Fig. 2.4B**). Expression patterns of proteins involved in OXPHOS in the fed livers were analyzed and quantified using densitometric analysis. Lipogenic FW livers had higher expression of proteins involved in OXPHOS ($P \leq 0.05$; complexes V and IV) compared with the NW counterpart (**Fig. 2.4C, D**). Additionally, protein and gene expression profiles of several components involved in mitochondrial nutrient transport systems were analyzed. Expression profile of the oxoglutarate carrier (OGC; *Slc25A11*), which plays an important role in the malate-aspartate shuttle, was higher in lipogenic livers ($P \leq 0.05$; **Fig. 2.4C, D**). Furthermore, the gene expression of several components involved in mitochondrial malate-aspartate shuttle (*Slc25A13*, *Mdh1*, *Mdh2*, *Me1*) and pyruvate cycle (*Mpc1*, *Mpc2*, *Pdh*) was also higher in the livers from mice reared on drinking water containing 30% fructose ($P \leq 0.05$; **Fig. 2.4E**). These results indicate the overall induction of mitochondrial TCA cycle metabolism, nutrient transport, and OXPHOS, together with the fructose-driven upregulation of DNL in the liver.

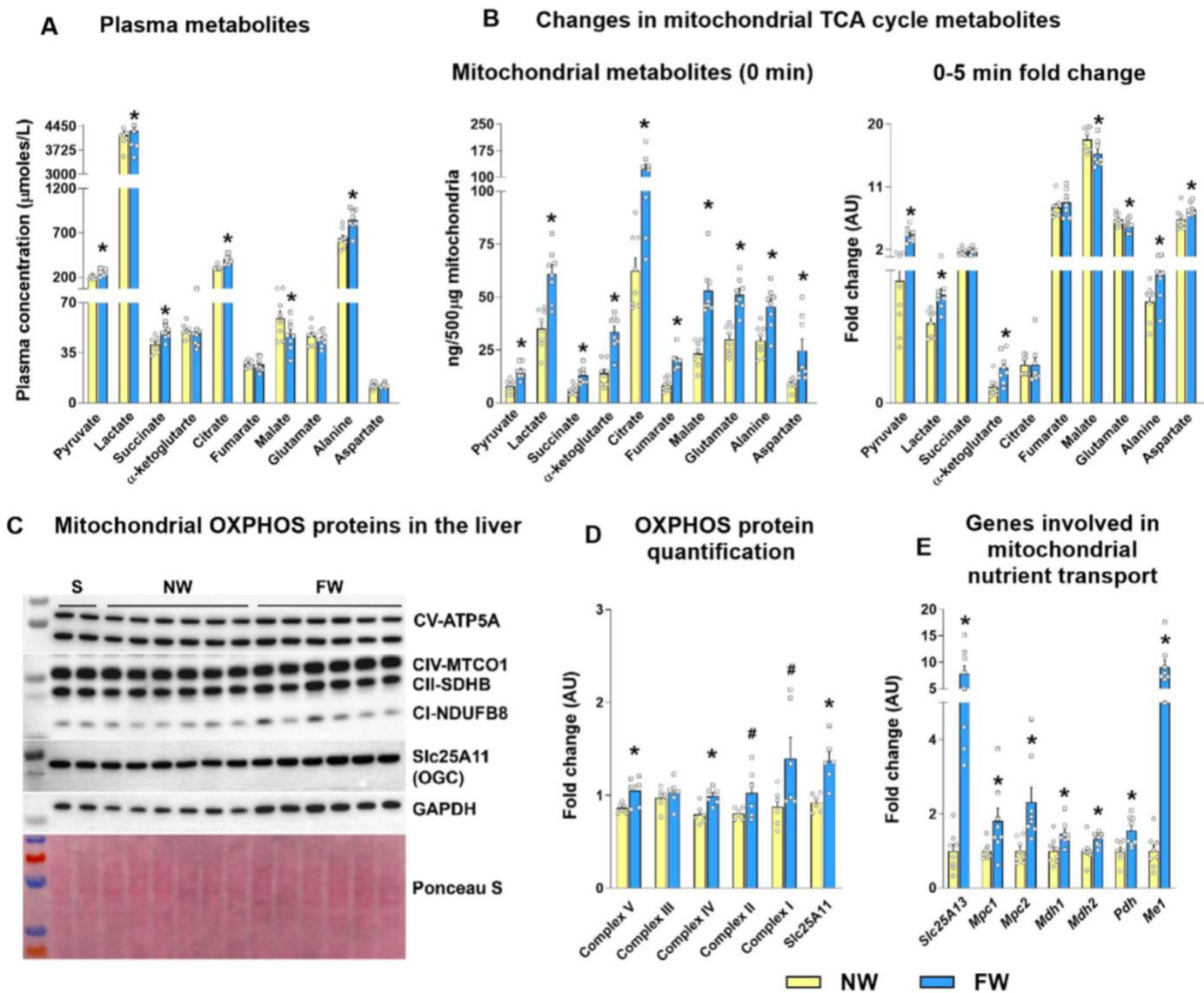


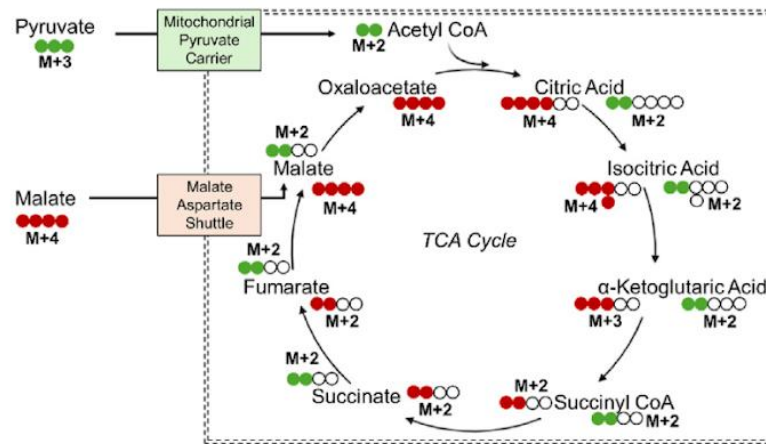
Figure 2.4. Alterations in mitochondrial oxidative networks associated with fructose in drinking water, under fed conditions.

(A) Plasma metabolite levels following fructose (FW) compared with normal drinking water (NW). (B) Metabolite levels in freshly isolated mitochondria under basal conditions (0 min) and after 5 minutes (0–5 minutes fold change) of mitochondrial incubations in a respiration buffer. (C) Expression patterns of hepatic mitochondrial proteins in NW compared with FW groups and (D) the densitometric analysis of the protein bands. (E) Expression profiles of genes involved in mitochondrial nutrient transport in the liver between the NW and FW groups. Results ($n = 8/\text{group}$) were considered significant at $P \leq 0.05$ following a t-test between NW and FW groups, which is indicated by “*”. “#” represents $P \leq 0.1$ following the t-test between NW and FW groups. AU, arbitrary units; *OGC* (*Slc25A11*), 2-oxoglutarate carrier; *Slc25A13*, solute carrier family 25 member 13; *Mpc1*, mitochondrial pyruvate carrier 1; *Mpc2*, mitochondrial pyruvate carrier 2; *Mdh1*, malate dehydrogenase 1; *Mdh2*, malate dehydrogenase 2; *Pdh*, pyruvate dehydrogenase; *Me1*, malic enzyme 1; S, standard (mixture of pooled samples).

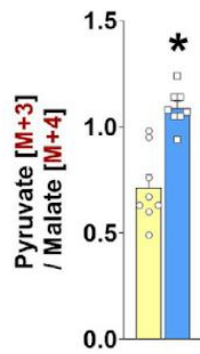
2.4.5. Stable isotope-based metabolic profiling validated the induction of fluxes through the hepatic mitochondrial TCA cycle of mice reared on FW

To determine multiple fluxes through the TCA cycle metabolism, freshly isolated liver mitochondria were allowed to respire in a buffer containing either [$^{13}\text{C}_4$] malate (M+4) or [$^{13}\text{C}_3$] pyruvate (M+3) for a period of 5 min. The incorporation of these stable isotopes into the intermediates of the TCA cycle and the resulting isotopomers (**Fig. 2.5A**) provided us with indices of the activity of multiple biochemical steps in the TCA cycle. As such, incorporation of [M+4] malate into pyruvate [M+3] in the isolated hepatic mitochondria is an indirect index of malic enzyme (ME2) activity and was upregulated in the FW group ($P \leq 0.05$; **Fig. 2.5B**). Furthermore, malate dehydrogenase (MDH2) catalyzes the conversion of malate to oxaloacetate, which is converted to aspartate by glutamic-oxaloacetic transaminase (GOT2). Thus, incorporation of malate [M+4] into aspartate [M+4] reflects MDH2 and GOT2 activities, which were also upregulated in the FW group ($P \leq 0.05$; **Fig. 2.5C**). In the first half of the TCA cycle, citrate gets converted to isocitrate by aconitase and further to α -ketoglutarate by isocitrate dehydrogenase. Higher incorporation of citrate [M+4] into α -ketoglutarate [M+3] is reflective of higher flux through the initial 3 steps of the TCA cycle ($P \leq 0.05$; **Fig. 2.5D**), which was also higher in the liver mitochondria derived from the mice provided with FW. Incorporation of pyruvate [M+3] into citrate [M+2] is an indirect index of pyruvate dehydrogenase activity, which tended to be higher in lipogenic livers from the FW group (**Fig. 2.5E**). Finally, the incorporation of pyruvate [M+3] into oxaloacetate [M+3] is catalyzed by pyruvate carboxylase, which can then be converted to citrate by citrate synthase ($P \leq 0.05$; **Fig. 2.5F**) or to aspartate [M+3] by GOT2 ($P \leq 0.05$; **Fig. 2.5G**). Incorporation of pyruvate [M+3] into citrate [M+3] and aspartate [M+3] can be a crude index of these fluxes and was higher in the fructose-fed mice (**Fig. 2.5F, G**). Taken together, these results provide strong evidence for a general induction of the TCA cycle metabolism in lipogenic livers following FW.

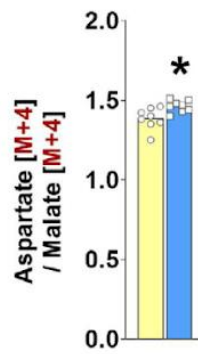
A Stable isotope labeling schemes



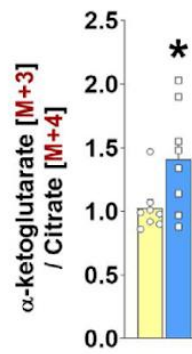
B Malic enzyme activity



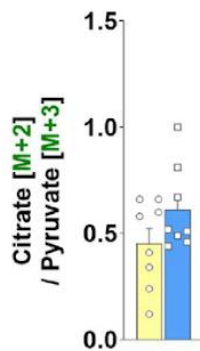
C Malate dehydrogenase activity



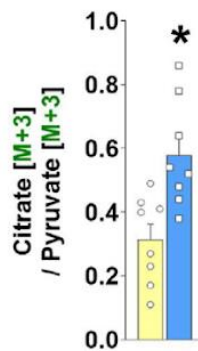
D TCA cycle flux - citrate to α -ketoglutarate



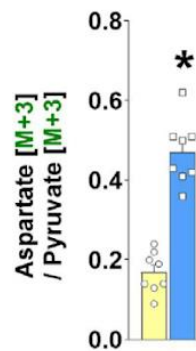
E Pyruvate dehydrogenase activity



F Pyruvate carboxylase activity



G



■ NW ■ FW

Figure 2.5. Stable isotope-based profiling of the mitochondrial TCA cycle function.

(A) Stable isotope labeling scheme illustrating the incorporation of the ^{13}C from [$^{13}\text{C}_4$] malate (M+4) or [$^{13}\text{C}_3$] pyruvate (M+3) into the intermediates of the TCA cycle. (B) Pyruvate [M+3] to malate [M+4] malate ratio in isolated mitochondria as an indirect index of malic enzyme activity. (C) Aspartate [M+4] to malate [M+4] ratio as a reflection of flux through malate dehydrogenase (MDH2) and glutamic-oxaloacetic transaminase (GOT2). (D) α -ketoglutarate [M+3] to citrate [M+4] ratio as an index of flux through the initial 3 steps of the TCA cycle. (E) Incorporation of pyruvate [M+3] into citrate [M+2] as an indirect index of pyruvate dehydrogenase activity. (F) Incorporation of pyruvate [M+3] into aspartate [M+3] as a crude index of flux through pyruvate carboxylase and GOT2. Results ($n = 8/\text{group}$) were considered significant at $P \leq 0.05$ following a t-test between NW and FW groups, which is indicated by “*”. “#” represents $P \leq 0.1$ following the t-test between NW and FW groups. TCA, tricarboxylic acid.

2.4.6. Fasting sustains the induction of lipid oxidation observed during fed conditions in the livers of mice kept on fructose drinking water

We investigated whether overnight fasting would normalize the changes in lipid oxidation and mitochondrial oxidative function observed under fed conditions in the mice reared on 30% FW (lipogenic diet). Body weights under fed conditions remained similar between groups, whereas the FW mice were heavier after an overnight fast ($P \leq 0.05$; **Fig. 2.6A**). A similar trend was observed for the liver weights, with the FW livers heavier after fasting ($P \leq 0.05$; **Fig. 2.6B**). Although the blood glucose levels remained similar between the groups under fed conditions, contrary to expectations, overnight fasting resulted in lower blood glucose levels in the FW mice ($P \leq 0.05$; **Fig. 2.6C**). This trend in blood glucose paralleled the lower endogenous glucose production after fasting in the FW group compared with their NW counterparts ($P \leq 0.07$; **Fig. 2.6D**). Fasting failed to efficiently suppress the circulating plasma branched chain amino acid levels in the FW group ($P \leq 0.05$), highlighting a greater degree of insulin resistance in the FW mice (**Fig. 2.6E**). Expression pattern of genes involved in inflammatory responses, including *TLR4* (toll-like receptor 4) and *IL-1 β* (IL-1 β) and antioxidant enzyme *SOD1* (superoxide dismutase 1) was significantly higher in the FW liver during the feeding-to-fasting transition ($P \leq 0.05$; **Fig. 2.6F**). Overnight fasting normalized lipogenic gene expressions in the liver of FW and NW mice (**Fig. 2.6G**). Despite this, the induction of lipid oxidation was higher in the FW livers during fasting, as evidenced through the higher fold increase in plasma ketones (β -hydroxybutyrate) ($P \leq 0.01$; **Fig. 2.6H**) and higher fasting expression of hepatic *Cpt1a*, *Lcad*, *Ppara*, and *Hmgcs2* ($P \leq 0.05$; **Fig. 2.6I**). Taken together, these results suggest that the FW treatment programmed the lipid oxidation machinery to be chronically active during feeding and fasting.

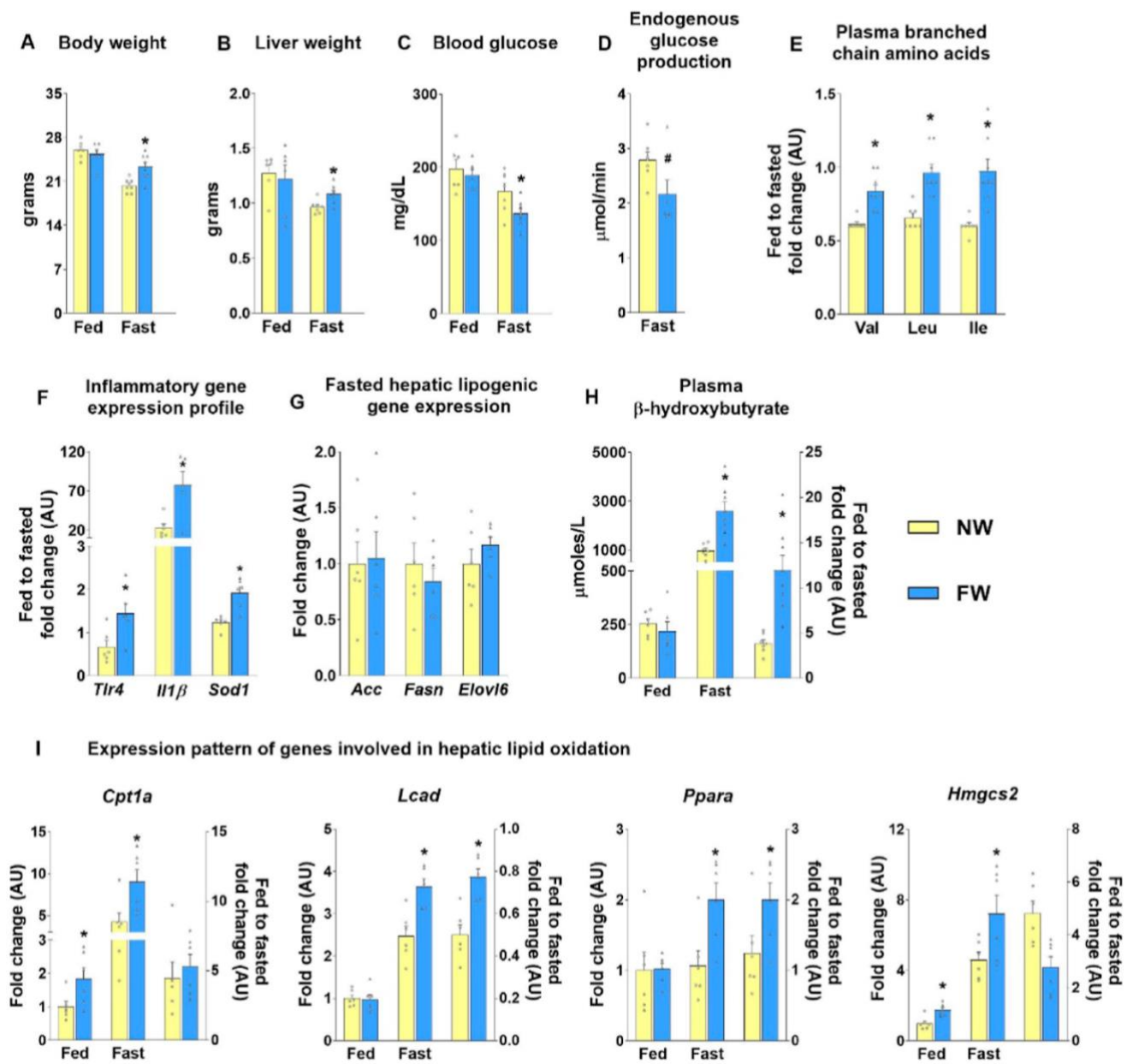


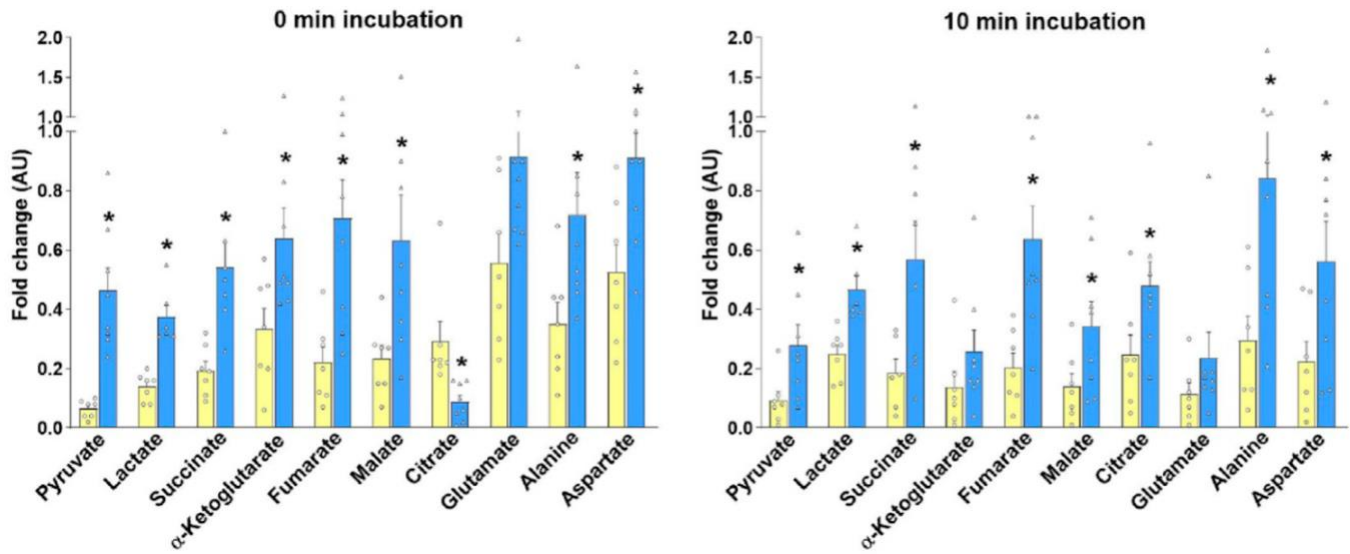
Figure 2.6. Higher induction of lipid oxidation in the livers of mice reared on fructose drinking water is sustained during fasting.

Changes in (A) body weight, (B) liver weight, and (C) blood glucose concentration in NW and FW groups following 14 days of dietary treatments under fed and fasted conditions. (D) Change in endogenous glucose production following overnight fasting. (E) Fed-to-fasted fold change of plasma branched chain amino acid levels (valine, leucine, isoleucine). Expression profile of hepatic genes involved in (F) inflammation (*Tlr4*, *Ilβ*), antioxidant defense (*Sod1*), and (G) lipogenesis (*Acc*, *Fasn*, *Elovl6*). (H) Plasma β-hydroxybutyrate levels under fed and fasted conditions. (I) Expression pattern of genes involved in hepatic lipid oxidation (*Cpt1a*, *Lcad*, *Ppara*) and ketogenesis (*Hmgcs2*) under fed and fasted conditions. Results (n = 5–8/group) were considered significant at $P \leq 0.05$ following a t-test between NW and FW groups, which is indicated by “*”. “#” represents $P \leq 0.1$ following the t-test between NW and FW groups. AU, arbitrary units; Val, valine; Leu, leucine; Ile, isoleucine; *Tlr4*, toll-like receptor 4; *Ilβ*, IL 1β; *Sod1*, superoxide dismutase 1; *Acc*, acetyl-CoA carboxylase; *Elovl6*; ELOVL fatty acid elongase 6; *Fasn*, fatty acid synthase; *Cpt1a*, carnitine palmitoyltransferase 1A; *Lcad*, long-chain acyl-CoA dehydrogenase; *Ppara*, peroxisome proliferator-activated receptor alpha; *Hmgcs2*, 3-hydroxy-3-methylglutaryl-CoA synthase 2.

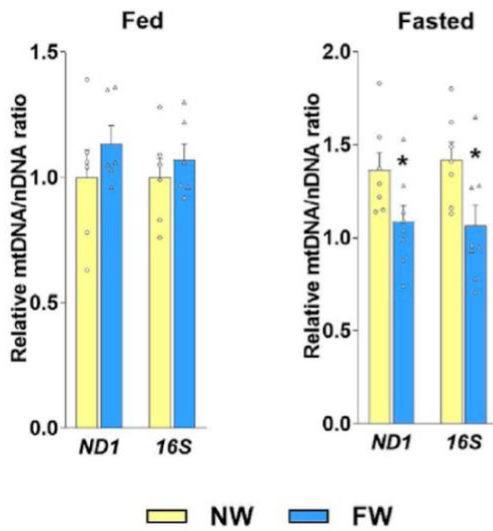
2.4.7. Mitochondrial TCA cycle activity during feeding-to-fasting transition is higher in the liver of mice reared on FW

Fed-to-fasting fold changes in metabolites measured in freshly isolated liver mitochondria (0 minutes of incubation) were higher in the FW group ($P \leq 0.05$; **Fig. 2.7A**). Furthermore, a similar pattern was observed in isolated mitochondria, which were allowed to respire in a buffer for 10 min, with the FW mitochondria generating more TCA cycle metabolites on the feeding-to-fasting transition ($P \leq 0.05$; **Fig. 2.7A**). Mitochondrial copy number in the liver remained similar between the 2 groups under fed conditions, but after an overnight fast, the FW livers had lower mitochondrial copy number ($P \leq 0.05$; **Fig. 2.7B**). These results point to the fact that the mitochondria isolated from the FW livers had higher rates of TCA cycle activity, compared with their NW counterparts. In summary (**Fig. 2.7C**), fructose consumption upregulated DNL under fed conditions, as expected. However, this also accompanied an induction in the overall mitochondrial oxidative machinery, which sustained through the feeding-to-fasting transition. This chronic induction of mitochondrial oxidative capacity could eventually contribute to the pathophysiology of MASLD by producing more ROS and activating canonical inflammatory networks.

A Generation of TCA cycle intermediates by the liver mitochondria (fed to fasted fold change)



B Mitochondrial copy number



C Fructose supplementation chronically upregulates hepatic mitochondrial oxidative function

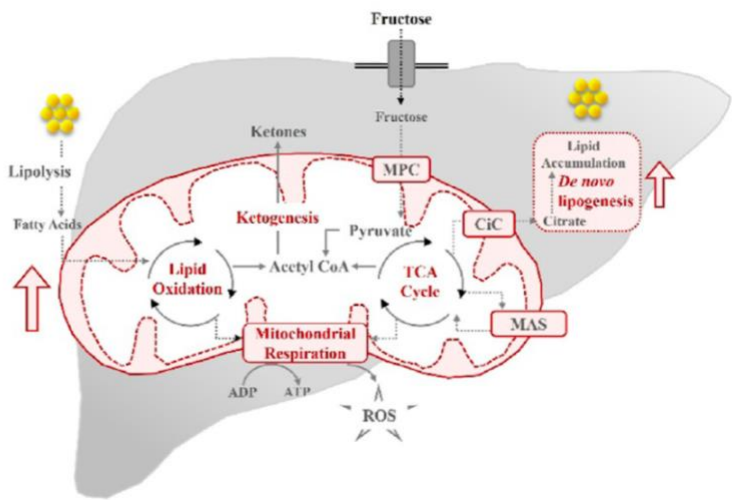


Figure 2.7. Greater induction of mitochondrial TCA cycle metabolism during a feeding-to-fasting transition in mice reared on fructose in drinking water.

(A) Levels of metabolites in freshly isolated mitochondria from the NW and FW livers after 0 and 10 minutes of respiration, expressed as fed-to-fasted fold change. (B) Mitochondrial copy number under fed and fasted states, determined from the mtDNA/nDNA ratio by amplification of stable mitochondrial genes, *ND1* and *16S*, and normalized to hexokinase gene expression. (C) Schematic representation of changes in mitochondrial function in the liver after fructose consumption. Results (n = 8/group) were considered significant at $P \leq 0.05$ following a t-test between NW and FW groups, which is indicated by “*”. AU, arbitrary units; *ND1*, NADH dehydrogenase 1; *16S*, 16S ribosomal RNA; TCA, tricarboxylic acid.

2.5. Discussion

Under insulin sensitivity, the biochemistry of the liver is highly plastic and responds to insulin and glucagon levels to efficiently switch from “fed” glucose utilization/storage and DNL to “fasting” gluconeogenesis and lipid oxidation. With the onset of insulin resistance, these biochemical networks gradually lose their insulin and glucagon responsiveness, leading to the accumulation of triglycerides and their lipotoxic intermediates, dysfunctional lipid oxidation, and hepatocellular stress, and inflammation [137, 147]. MASLD encompasses this consortium of events associated with chronic insulin resistance, with mitochondrial dysfunction and hepatic steatosis as its central features [137, 139]. We and others have shown that aspects of mitochondrial oxidative metabolism, especially TCA cycle activity and ketogenesis, are upregulated during early and milder stages of insulin resistance and MASLD [134, 135, 147], presumably to support the chronic and dysfunctional induction of gluconeogenesis and lipid accumulation. Hepatic steatosis during MASLD can occur from either dietary lipid overload or higher hepatic uptake of circulating free fatty acids derived from adipose tissue lipolysis or higher rates of DNL from carbohydrates [169]. Among these, the contribution of hepatic DNL to total lipid accumulation is low under insulin sensitivity (5%–10%) but is significantly upregulated (>20%) in people suffering from MASLD [139, 169] and in rodent models of MASLD reared on Western diets with greater proportions of carbohydrates [148]. In contrast, administration of diets rich in mostly saturated and monounsaturated fatty acids (commonly referred to as HF diets) to rodents significantly lowers the rates of hepatic DNL, while inducing hepatic steatosis and MASLD symptoms [158-161]. Thus, it remains unclear whether the etiology of MASLD is different when the major sources of hepatic lipid accumulation arise from dietary or adipose tissue-derived free fatty acids compared with carbohydrate-mediated DNL.

I investigated whether a macronutrient milieu that upregulates hepatic DNL can concomitantly impact the remodeling of hepatic mitochondrial oxidative function, compared with a high-fat-rich macronutrient environment. Diet-induced obesity, insulin resistance, and MASLD in rodent models are usually attained through the provision of either excess calories from lipids (45%– 60%) or excess calories from carbohydrates (for example, fructose) or excess calories from a combination of lipids and

carbohydrates. The latter 2 macronutrient regimes include features of the Western diets [170-172], known to initiate insulin resistance and MASLD in humans. To determine the impact of varying macronutrient compositions on liver metabolism, I customized the rodent diets to contain either 25% calories from lipids plus 35% calories from fructose (HF/HFr diet) or 60% of calories from lipids (HF diet). The provision of both the HF/HFr and HF diets for 24 week resulted in similar degrees of hepatic steatosis, with no major differences in lipid droplet accumulation. However, and not surprisingly, indices of DNL were higher in the HF/HFr livers [148] due to their higher fructose content and contributed significantly to lipid accumulation. In the HF-fed mice livers, DNL remained significantly lower compared with their LF controls and their HF/HFr counterparts. Taken together with the results that hepatic steatosis remained similar between the HF and HF/HFr groups, we concluded that the esterification and re-esterification of dietary and/or adipose tissue-derived fatty acids were the dominant source of hepatic lipids in the HF livers. More interestingly, although lipid accumulation in the liver remained similar between the HF/HFr and HF mice, multiple aspects of mitochondrial oxidative function were induced in the HF/HFr livers during the feeding-to-fasting transition. This included a greater induction of hepatic genes involved in lipid oxidation during fasting and a higher fed-to-fasted induction of ketogenesis in mice reared on HF/HFr diet. Furthermore, freshly isolated mitochondria from fed and fasted HF/HFr livers generated more TCA cycle intermediates on 5 minutes of incubation in a respiration buffer, compared with their HF counterparts. Oxygen consumption (ADP-stimulated) by the isolated mitochondria and the abundance of proteins involved in OXPHOS were also higher in the HF/HFr livers. Taken together, these results suggest that the induction of hepatic DNL during the initial stages of MASLD is accompanied by higher activity of mitochondrial oxidative networks in the liver.

Hepatic mitochondria play a pivotal role in central metabolism, orchestrating the catabolic processes of β -oxidation, TCA cycle metabolism, and ketogenesis with anabolic processes of gluconeogenesis and lipogenesis. As such, the prevailing thought is that, during early stages of MASLD, and especially diet- induced induction of insulin resistance, mitochondria remodel their overall activity to be higher, possibly as a compensatory adaptation to the caloric burden from either lipids or carbohydrates

[136, 137, 147]. In line with this thought, several studies have demonstrated higher TCA cycle activity, or higher rates of fatty acid oxidation and ketogenesis in mouse models and human subjects with MASLD [134, 135, 147]. At the same time, it's also been demonstrated that components of mitochondrial oxidative machinery can gradually become blunted to the nutritional overload with progressive severity of MASLD, which could be manifested as impairments or decreased activity of those metabolic networks [67, 137, 138, 149]. It has been rather difficult to predict the exact nature and timeline of these mitochondrial adaptations, considering 1) the variety of dietary regimens utilized to induce MASLD in animal models, 2) the genetic and environmental conditions involved, and 3) the species-specific differences in lipid oxidation compared with synthesis in the liver of humans compared with other animal models of MASLD. Considering the complex nature of MASLD and the major role of macronutrient compositions in remodeling mitochondrial biochemistry, I further determined whether exclusive induction of DNL in the liver resulted in a parallel upregulation of mitochondrial oxidative function. Furthermore, given the potent *de novo* lipogenic capacity of fructose and its significance as a major contributor to the prevalence of MASLD, I determined the changes in hepatic mitochondrial metabolism that occurred together with fructose-induced DNL. Mice reared on drinking water supplemented with 30% fructose (w/v) for 14 days had higher body and liver weights, along with higher liver triglyceride and plasma insulin levels compared with their regular water drinking counterparts. As expected, *de novo* lipogenic gene expression was significantly higher in the fructose-drinking mice livers. More interestingly, multiple aspects of the hepatic mitochondrial biochemistry were remodeled to be higher in the mice reared on FW. The induction of mitochondrial activity with fructose was clear from the higher rates of oxygen consumption, higher generation of ROS by the isolated mitochondria, and higher generation of TCA cycle intermediates by the isolated mitochondria. We substantiated the induction of mitochondrial activity utilizing stable isotope tracers [$^{13}\text{C}_4$] malate and [$^{13}\text{C}_3$] pyruvate. Specific incorporation of ^{13}C from malate and pyruvate into TCA cycle intermediates was able to provide us with indirect, but reliable indices of the activities of various enzymatic steps involved in mitochondrial biochemistry. As elaborated in **Figure 2.5**, flux through several key steps of the mitochondrial TCA cycle

metabolism was upregulated in the liver mitochondria from mice reared on FW. These included higher flux through the initial steps of the TCA cycle (pyruvate to citrate to α -ketoglutarate via pyruvate carboxylase, citrate synthase, and isocitrate dehydrogenase), higher activities of pyruvate dehydrogenase, malic enzyme, and malate dehydrogenase in fructose-fed livers. Together, these results provide strong evidence for the concurrent induction of both DNL index and mitochondrial TCA cycle function after fructose feeding.

The overactive components of mitochondrial biochemistry in my studies included overlapping facets of mitochondrial nutrient transport (for example, malate-aspartate, pyruvate-malate, and citrate shuttles). This was highlighted by the expression levels of several genes involved in mitochondrial malate-aspartate shuttle (*Slc25A13*, *Mdh1*, *Mdh2*, *Me1*) and pyruvate cycle (*Mpc1*, *Mpc2*, *Pdh*), which were upregulated in the livers of mice reared on FW. Indeed, mitochondrial oxidative capacity is maintained through the synergy between biochemical networks involved in nutrient transport, substrate oxidation, OXPHOS, and ATP synthesis. This includes the interconnectivity of the TCA cycle with the malate-aspartate, the pyruvate-malate, and the citrate shuttle systems encompassing 1) the transfer of cytosolic NADH into the mitochondria as malate during high rates of carbohydrate oxidation [12], 2) malate also serving as an intermediate of the TCA cycle, inducing its activity and resulting in generation of mitochondrial citrate [173], and 3) the citrate shuttle transporting the mitochondrial citrate to the cytoplasm, providing carbon skeletons for acetyl-CoA for DNL [174]. Thus, it is plausible that the coordinated induction of several of these components of mitochondrial biochemistry could be responsible for the abnormal induction of DNL, which warrants further investigation.

Although the concurrent induction of lipogenic genes and mitochondrial TCA cycle metabolism was clear under “fed” conditions, I further determined whether the residual impacts of fructose feeding on mitochondrial oxidative function persisted during fasting. Fasting is known to downregulate hepatic DNL in response to lower levels of insulin, while upregulating lipid oxidation. Surprisingly, fasting blood glucose levels and endogenous glucose production were lower in mice reared on FW compared with the NW counterparts. Although the mechanism behind this is not clear, a possible explanation for this

observation could be the rapid conversion of fructose to fructose-1-phosphate, depletion of phosphate stores, and a consequent drop in liver ATP stores and glycogen depletion, leading to reduced blood glucose levels [153, 175, 176]. Furthermore, fructose 1-phosphate can stimulate glucokinase indirectly, stimulate glucose uptake and glycogen storage, and contribute to the lower hepatic glucose production [177, 178]. Despite the lower fasting plasma glucose levels, mice reared on FW induced fasting plasma ketone production and lipid oxidation gene expression, significantly higher than their normal drinking water counterparts. Furthermore, fed-to-fasted fold changes in TCA cycle intermediates were higher in liver mitochondria from mice reared on fructose, both under basal conditions (0 minutes of incubation) and after 10 minutes of incubation in a respiration buffer. These changes were independent of mitochondrial copy number in the liver of these mice. Overall, these results suggest that the exposure to fructose also resulted in the remodeling of mitochondrial oxidative machinery to be chronically active during fed and fasted states.

In summary, we demonstrate that fructose-mediated induction of hepatic lipogenesis coexists with higher activities of several mitochondrial oxidative networks, including ketogenesis, TCA cycle metabolism, and lipid oxidation. A limitation of my studies is that the lipogenic indices I have utilized are indirect measures, even though we have previously determined rates of lipogenesis in mice reared on HFr diets using ^2H -nuclear magnetic resonance spectroscopy [148]. Furthermore, with the provision of fructose in the diet (HF/HFr) and in the drinking water (FW), the caloric intake by those mice was higher, compared with their respective control counterparts, which could account for the metabolic changes in the liver. The duration of fructose exposure in my studies was not long or severe enough to clearly establish the signatures of hepatic inflammation and metabolic dysfunction typical to MASLD. However, we conclude that sustained activity of both mitochondrial oxidative networks and DNL during the initial stages of MASLD etiology could accelerate hepatocellular damage and metabolic dysfunction.

Chapter 3

Macronutrient-specific initiation of metabolic switch in newly hatched chickens

3.1. Abstract

The embryonic to post-hatch transition in chickens is characterized by a dynamic metabolic shift from reliance on yolk lipid oxidation during embryogenesis to rapid activation of hepatic *de novo* lipogenesis after hatch. Although this switch is broadly attributed to the provision of the starter diet immediately after hatching, the specific contribution of individual dietary macronutrients to initiating this metabolic switch remains unclear. I hypothesized that dietary carbohydrates alone are sufficient to initiate this metabolic shift in post-hatch chickens. Newly hatched Ross 708 broiler chickens (n=8/group) were sacrificed on the day of hatch (DOH), without feeding, or were given starter diet *ad libitum* (SD), or fasted for 48 hours (Fast). Further, groups of newly hatched chickens were orally gavaged (3 times/day for 2 days post-hatch) with isocaloric amounts of semi-purified diets, with either complete with all three macronutrients provided in the form of dextrin, casein hydrolysate, and corn oil (Com), or 85% of the calories derived from dextrin (Dex), casein (Cas), or corn-oil (Oil). In a follow-up study, diets were refined and reformulated into solid feeds to avoid gavage, with 100% of the calories derived from specific macronutrients, and were administered *ad libitum* for 48-hrs. Plasma and tissue metabolites were quantified using gas chromatography-mass spectrometry, and hepatic gene expression was assessed by qPCR analysis. Plasma β -hydroxybutyrate concentrations were elevated in Oil, Cas, Fast, and DOH groups but was significantly reduced in Dex, Com, and SD treatments ($P \leq 0.05$), indicating robust suppression of lipid oxidation. Conversely, plasma pyruvate, lactate, and alanine concentrations were increased in Dex, Com, and SD groups ($P \leq 0.05$), indicating enhancement of carbohydrate utilization. Similar metabolic patterns were observed in liver and muscle tissues. Dextrin-enriched diets upregulated hepatic lipogenic gene expression (e.g., *SCD1*, *ELOVL6*; $P \leq 0.05$) and downregulated lipid oxidation gene expression (e.g., *CPT1A*, *PPAR α* ; $P \leq 0.05$). Taken together, these findings demonstrate that dietary carbohydrate in the form of dextrin alone is sufficient to initiate the post-hatch metabolic transition in broiler chicks. These results could have implications for optimizing early nutritional strategies to support neonatal growth and development and reduce post-hatch morbidity and mortality rates.

3.2. Introduction

Chickens undergo a dramatic metabolic transition from lipolytic to lipogenic metabolism during the embryonic to post-hatch developmental period [30, 32, 33, 36, 101]. During embryogenesis, the developing embryo relies heavily on utilizing yolk lipids via β -oxidation as its primary energy source [33]. A clear and rapid metabolic switch is observed at hatching, as the neonatal chicken transitions to utilizing carbohydrates from the starter diets to derive energy and upregulates lipogenic pathways [33-35]. This shift from fatty acid oxidation during embryonic stages to carbohydrate utilization and lipid synthesis is accompanied by extensive remodeling of the biochemical pathways governing macronutrient metabolism, particularly in the liver [30, 36, 179-181]. A failure to successfully undergo this metabolic transition results in higher morbidity and mortality during the first week post-hatch. Few effective strategies exist to improve the metabolic transition during this critical developmental period, despite the reported mortality rates reaching approximately 3% in this window and the associated economic losses representing a significant burden to the poultry industry [37, 38].

At the center of this metabolic transition lies the liver [30-33, 99]. The liver represents a rapidly adapting and highly plastic metabolic environment during this transition, facilitating the shift from fatty acid oxidation in the embryonic stage to new lipid synthesis in the hatchlings. Interestingly, despite the high rates of lipid oxidation during embryogenesis and the elevated lipid accumulation in the post-hatch livers, this transition takes place without any symptoms of insulin resistance, metabolic dysfunction, cellular stress, or inflammation [30-32]. This is in contrast to the condition of fatty liver in rodents or humans, where elevated lipid oxidation and hepatic lipid accumulation are accompanied by insulin resistance, hepatocellular stress, and inflammation [67, 134, 135]. Hepatic mitochondria play a central role in the efficient orchestration of the integration of macronutrient metabolism in the liver. Previous research from our lab has highlighted that optimal coupling between mitochondrial oxidative networks and lipogenic pathways, along with their dynamic remodeling during this transition, is essential for a successful initiation of the metabolic switch in the liver [30, 31].

While it is established that the provision of feed immediately after hatch initiates the metabolic transition in the hatchlings, the role of individual dietary macronutrients (carbohydrates, proteins, and lipids) in driving this metabolic shift in the liver remains poorly understood. Given that the liver is the primary organ governing the metabolic switch [30-32], and that early life mortality causes significant economic losses, identifying the metabolic and molecular drivers of early hatchling development is essential for designing strategies to improve poultry health. In this study, we aimed to identify which dietary macronutrient, when administered as the sole macronutrient immediately after hatch, can initiate the metabolic switch and establish the lipogenic phenotype in post-hatch livers. We hypothesized that carbohydrates, proteins, and lipids would elicit distinct biochemical adaptations in the liver and differentially impact the initiation of an efficient metabolic switch. Furthermore, we hypothesized that coordinated flux through hepatic mitochondrial oxidative networks and nutrient shuttles, to optimally upregulate TCA cycle activity and mitochondrial nutrient transport systems, is essential for establishing the lipogenic phenotype in the liver. My results highlight that carbohydrates alone are sufficient to successfully initiate the metabolic transition in hatchlings when administered in the first 48 hours post-hatch. Additionally, distinct alterations in hepatic mitochondrial oxidative functions and nutrient shuttle systems accompanied the induction of hepatic *de novo* lipogenesis.

3.3. Materials and Methods

3.3.1. Study Design

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland, College Park. Fertilized eggs (64 ± 0.5 g) were obtained from Perdue Farms Inc. (Salisbury, MD) from a Ross 708 broiler breeder flock (26-28 weeks of age) and incubated at 37°C and 60% relative humidity. At hatch (day 21), chicks were transferred to floor pens maintained at 37°C and either provided a starter diet (Diet S-G 5065, LabDiet Co., MO; **Table 3.1**) *ad libitum* or assigned to experimental dietary treatments.

For the **Study 1**, on the day of hatch, chicks were randomly assigned to dietary treatments for 48 hours (**Table 3.2**). Chickens were orally gavaged three times daily with isocaloric semi-purified liquid diets consisting of (i). balanced macronutrients (dextrin, casein hydrolysate, and corn oil; **SP-Complete**), (ii). 85% of calories from dextrin (**SP-Dextrin**), (iii). 85% of calories from casein hydrolysate (**SP-Casein**), and (iii). 85% of calories from corn oil (**SP-Oil**). Additional groups included chickens provided (iv). *ad libitum* starter diet (**Starter Diet**) or (v). fasted for 48 hours (**Fasted**). Further, a separate group was sampled on the (vi). **day of hatch** (DOH) to establish baseline metabolic measurements. However, chicks assigned to the SP-Casein group exhibited poor viability and high mortality rates due to an unknown reaction from casein gavage and were excluded from subsequent analyses.

Subsequent **Study 2** was designed and conducted to eliminate potential confounding effects of gavage. For this, diets were formulated into baked cookies and crushed into pellets similar to the size and texture of the starter diet (**Table 3.3**). Diets were optimized such that ~100% of caloric content in experimental groups was derived from a single macronutrient. Due to technical limitations in making an oil-dominant baked cookie, the SP-Oil diet was administered via oral gavage. A corresponding control gavage group received all components except oil (**Control Gavage**). Further, a separate group received a cellulose enriched diet (**SP-Cellulose**) that has minimal metabolizable energy but served as a feeding control to assess metabolic changes associated with the initiation of the feeding behaviour independent of the caloric intake. Chicks were assigned for 48 hours post-hatch to one of the following treatments: SP-Complete, SP-Dextrin, SP-Casein, Starter Diet, Fasted, SP-Cellulose, SP-Oil (gavaged), or Control Gavage.

In a separate **Study 3**, we examined the specific contribution of hepatic mitochondria to the metabolic transition from lipid oxidation toward lipogenesis and carbohydrate utilization in post-hatch chickens. For this, on the day of hatch, neonatal chickens were either reared on a regular starter diet *ad libitum* (**Table 3.1**) or fasted for 48 hours. On post-hatch day 2, liver tissue was collected for the isolation of intact mitochondria. Freshly isolated mitochondria were used to assess the generation of reactive

oxygen species (ROS), oxygen consumption rates (OCR), and the abundance of TCA cycle intermediates.

For all the studies, neonatal chickens were euthanized by decapitation following isoflurane anesthesia. Plasma, liver, and muscle tissues were collected and stored at minus 80°C for subsequent biochemical and molecular analyses. Body weights were recorded throughout each study. Detailed methodologies for individual assays are described in the sections below.

Table 3.1. Composition of the starter diet for the chickens (Diet S-G 5065, LabDiet Co.)

Component	Composition (as-fed basis)	Caloric Contribution (% of total energy)
Carbohydrates	NFE: 54.8%	
	Starch: 34.2%	63.47%
	Sucrose: 1.58%	
Protein	Crude Protein: ≥ 21.0%	
	(analyzed: 22.1%)	25.62%
	Crude Fat: ≥ 3.0%	
Fat	Ether Extract: 4.2%	
	Acid Hydrolysis Fat: 6.6%	10.91%

Table 3.2. Ingredients for the custom isocaloric gavage diets for the chickens.

	SP-Complete	SP-Dextrin	SP-Casein	SP-Oil
Ingredients	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
Casein	3.45	1.40	11.90	1.05
Dextrin	8.97	11.90	1.40	1.05
Corn Oil	0.55	0.31	0.31	5.29
Cellulose	0.21	0.21	0.21	0.21
Vitamin Mix	0.14	0.14	0.14	0.14
Mineral Mix	0.14	0.14	0.14	0.14
Total Kcal	56	56	56	56
Final Volume (ml)	22	22	22	22
Kcal/gavage	2.33	2.33	2.33	2.33
Kcal/day	7	7	7	7

Note: In SP-Dextrin, SP-Casein, and SP-Oil diets, 85% of the total calories were derived from dextrin, casein, and oil, respectively.

Table 3.3. Ingredients for the custom-baked and liquid gavage diets for the chickens.

	SP- Complete	SP- Dextrin	SP- Casein	Cellulose Control	SP-Oil (gavage)	Control Gavage
Ingredients	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>	<i>grams</i>
Casein	12.5	-	47.5	-	-	-
Dextrin	32.5	47.5	-	-	-	-
Corn Oil	2.5	-	-	-	47.5	-
Cellulose	0.75	0.75	0.75	0.75	0.75	0.75
Starter Diet	2.5	2.5	2.5	2.5	2.5	2.5

3.3.2. Metabolic profiling of freshly isolated live hepatic mitochondria

Hepatic mitochondrial isolation

Following dietary treatments in study 3, live mitochondria were isolated from the liver for metabolic profiling [29]. Chickens were euthanized by decapitation and fresh liver samples were collected and washed in ice-cold 1X Phosphate Buffered Saline (PBS) 2 times. Whole liver tissue weight was measured, and mitochondria were isolated from ~800mg of the liver tissue. Mannitol-sucrose-HEPES-EDTA (MSHE) buffer [70 mM sucrose, 210 mM mannitol, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 1 mM EGTA, and 0.5% fatty acid-free bovine serum albumin (BSA); pH 7.2] was used for isolating live mitochondria. Liver tissues were minced in 3ml of MSHE buffer, homogenized using a Dounce homogenizer with 4 strokes (5-10 turns per stroke), and transferred into a 15ml ice-cold Falcon tube. The Dounce homogenizer was rinsed with 4ml of the MSHE buffer, which was transferred into the same Falcon tube and centrifuged at 4°C at 800 x g for 10 minutes. A double layered cheesecloth presoaked in MSHE buffer was used to strain the supernatant and further centrifuge at 4°C at 8,000 x g 10 minutes. The pellet was resuspended in 3ml of MSHE buffer and centrifuged at 4°C at 8,000 x g 10 minutes. This process was repeated twice, and the final mitochondrial pellet was resuspended in 100µl of BSA-free MSHE buffer. The mitochondrial stock was diluted (3µl in 57 µl of 1X Radio-Immunoprecipitation Assay (RIPA) buffer) to measure protein concentration using the Pierce protein assay kit (Thermo Fisher Scientific) for further downstream analyses.

ROS generated by the isolated hepatic mitochondria

The rate of hydrogen peroxide (H_2O_2) produced by mitochondria was determined by the oxidation of Amplex red (10-acetyl-3,7-dihydroxyphenoxazine) in the presence of horseradish peroxidase (HRP). Mitochondria (32 µg and 64 µg) were incubated with HRP (0.2 U/mL) and Amplex red reagent (100 µM) prepared in MAS-3 buffer with 5mM glutamate and 2.5mM malate. The oxidation of Amplex Red to fluorescent resorufin in real-time indicated the rate of H_2O_2 produced by the isolated mitochondria and the

changes in fluorescence were recorded using the Cytation 5 spectrophotometer (BioTek Instruments, Inc.) with 530 nm excitation and 590nm emission wavelengths. A standard curve with known amounts of H₂O₂ was constructed to calculate the rate of H₂O₂ production [29].

Oxygen consumption by the isolated hepatic mitochondria

Oxygraph oxygen electrode (Hansatech Instruments, Norfolk, England) was utilized to measure oxygen consumption by isolated mitochondria (250 µg) suspended in 1 ml of MAS-3 buffer containing 5mM glutamate and 2.5mM malate. Basal, ADP stimulated (150µM), ADP depleted, and FCCP (5µM) added respiration rates were determined. The mitochondrial respiration rates were normalized for expression of COX IV protein per µg crude hepatic mitochondrial protein [29, 148, 168].

Incubation of live mitochondria with stable isotopes

Freshly isolated live hepatic mitochondria (500 µg) were incubated in 1 mL MAS (Mitochondrial Assay Solution)-3 buffer (115mM KCl, 10 mM KH₂PO₄, 2 mM MgCl₂, 3 mM HEPES, 1 mM EGTA, and 0.2% fat-free BSA, 5 mM glutamate; pH 7.2) containing (i). 1 mM [¹³C₃] pyruvate and 2.5 mM malate, (ii). 1mM pyruvate and 2.5 mM [¹³C₄] malate, or (iii). unlabeled 1 mM pyruvate and 2.5 mM malate. Mitochondria were allowed to respire in these incubation buffers for 5 minutes at 37°C with occasional tapping, and the changes in the metabolite levels/tracer enrichments were measured using GC-MS protocols as detailed. At the end of the incubation period, mitochondrial respiration was stopped by placing samples on ice, followed by centrifugation at 3,000 x g for 5 minutes at 4°C. The supernatant was collected, and the mitochondrial pellet was washed with 500 µL of ice-cold PBS and centrifuged again at 3,000 x g for 2 minutes at 4°C. 100 µL of ice-cold methanol was added to the mitochondrial pellets and were stored at -80°C for GC-MS-based metabolite analysis [29].

3.3.3. Analysis of metabolic intermediates by GC-MS

A known amount of stable isotope-labeled internal standards were added to the mitochondrial, liver, muscle and serum samples for measuring the concentrations of the respective metabolites. Internal

standards were not added to the mitochondrial samples where the enrichment of the metabolic intermediates from [$^{13}\text{C}_3$] pyruvate or [$^{13}\text{C}_4$] malate stable isotopes were estimated.

Blood collected from the chickens were centrifuged at 2,500 x g for 10 minutes to isolate serum. Serum samples (25 μL) were spiked with the cocktail of internal standards and then were deproteinized with 750 μL of cold acetonitrile (-20°C). The samples were dried under nitrogen gas, the metabolic intermediates were converted to their corresponding oximes with the addition of 20 μL of 2% methoxamine hydrochloride in pyridine, followed by TBDMS (Tert-butyldimethylsilyl) derivatization. The samples were further heated at 90°C for one hour, cooled to room temperature, and loaded onto a GC-MS machine

Aliquots of ground liver and muscle tissue (~15 mg), and mitochondrial (500 μg) samples were spiked with an internal standard cocktail, followed by the addition of hot 70% ethanol. Samples were bead-milled for 120 seconds, incubated on a heating block at 90°C for 10 minutes, bead-milled for an additional 60 seconds, cooled on ice for 10 minutes, bead-milled once more for 60 seconds, and then centrifuged at 13,500 rpm for 10 minutes at 4°C. The supernatant was extracted, dried under nitrogen gas, and mixed with 30 μL of 2% methoxamine HCl in pyridine. 40 μL of 1:1 mixture of MTBSTFA with 1% t-BDMCS and pyridine was added to each sample and vortexed. Following one hour of heating at 90°C, metabolites were separated within an HP-5MS UI column, fragmented by electron ionization, and detected by single ion monitoring using GC-MS (5973N-Mass Selective Detector, 6890-Series GC, Agilent, CA) [29, 168].

3.3.4. Glycogen content in the liver (Study 2)

Liver samples (~25mg) were deproteinized with 8% sulfosalicylic acid, and glycogen was precipitated in 1ml ice-cold ethanol (95%). The glycogen pellet was twice washed with 500 μL of ice-cold ethanol (95%), and the pellet was air-dried overnight. Amyloglucosidase in acetate buffer (250 μL ; pH 5.0; 1-unit enzyme/mg glycogen) was added to the glycogen pellet and the reaction mixture was incubated for 1 hour at 55°C. Amyloglucosidase breaks up glycogen moiety to form several glucose molecules, which is

then further derivatized. The incubation mixture was lyophilized, converted into di-O-isopropylidene acetate (a derivative of glucose) and analyzed via GC-MS. The glucose present in the sample was calculated relative to the amount of labeled [$^{13}\text{C}_6$, C-d $_7$] glucose internal standard [29].

3.3.5. Gene expression profiles in the liver

Liver samples (~25mg) were aliquoted for mRNA extraction using 500 μL Trizol reagent (Invitrogen) and using the mRNA mini-prep kit (Bio-Rad Laboratories Inc.). 1 μg mRNA was used to make cDNA using the cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using 25 ng of cDNA, 150 nM of forward and reverse primers each, and 5 μL of SYBR green PCR master mix (Invitrogen) in a 10 μL reaction. B-tubulin was used as the housekeeping gene. Samples were analyzed in triplicate on a Bio-Rad CFX Real-Time system C1000 Touch Thermal Cycler (Bio-Rad). The list of primers is provided in **Appendix Table 3.1**.

3.3.6. Statistical analysis

Data reported are presented as means $\pm$ SEM. Comparisons of the results between groups in studies 1 and 2 were analyzed using 1-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Comparisons of the results from study 3 were analyzed using an unpaired Student's t-test. Means were considered significantly different if $P \leq 0.05$. All statistical analyses were conducted, and the graphs were plotted utilizing Prism 10.4 (Graph Pad Software Inc.).

3.4. Results

3.4.1. Effects of oral gavage of macronutrients on body and tissue weights in neonatal chickens

In study 1, dietary treatments (as listed in **Table 3.2**) were administered by oral gavage to the chickens during the first 48 hours post-hatch. Chickens administered SP-Casein diet showed poor viability with high mortality rates following gavage and were excluded from further analysis. On post-hatch day 2, chickens reared on a starter diet (SD) exhibited significantly higher body weights compared to all other dietary treatment groups ($P < 0.00$; **Fig. 3.1A**). Body weights of chickens in SP-Complete (Com), SP-Dextrin (Dex), SP-Oil (Oil), and day of hatch (DOH) groups were similar but lower compared to SD group, whereas fasted (Fast) chickens had the lowest body weights. A similar trend was observed for liver weights. The SD liver weighed significantly higher compared to all other dietary treatments ($P < 0.00$; **Fig. 3.1B**). Liver weights in Com, Com, Dex, Oil, and Fast were similar, while DOH livers were the smallest. Heart weights followed a similar pattern (**Fig. 3.1C**). These findings indicate that administration of diets via oral gavage impacted the body and the tissue weights of the chickens.

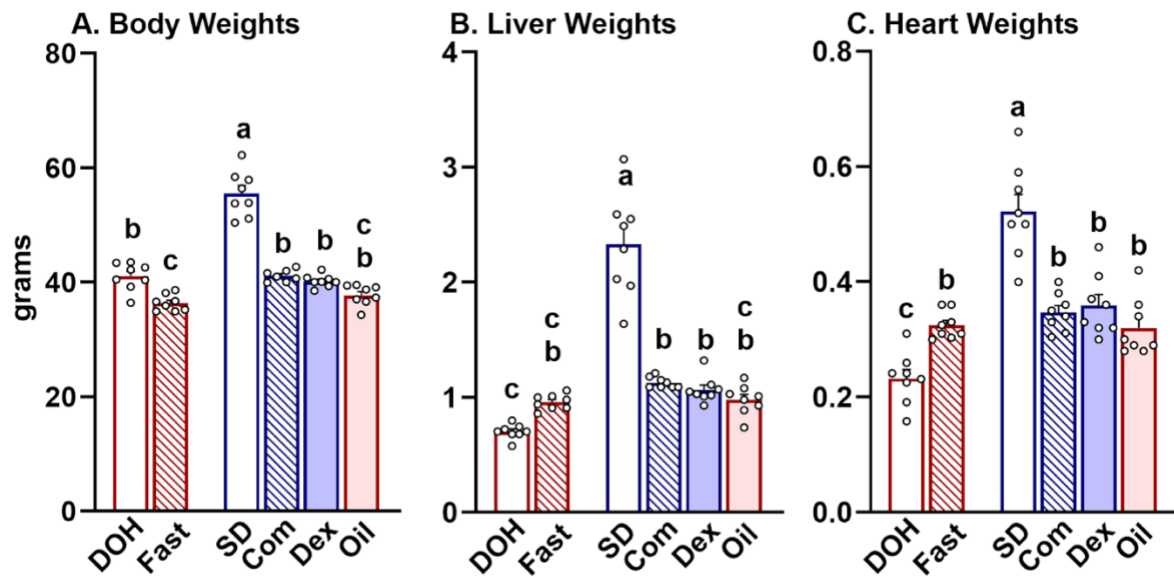


Figure 3.1. Effect of dietary treatments by oral gavage on tissue weights in neonatal chickens.

Changes in (A) body, (B) liver, and (C) heart weights in the neonatal chickens following 48 hours of post-hatch dietary interventions. Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

3.4.2. Higher circulating levels of plasma by-products of carbohydrate metabolism and lower ketone levels in SD, Com, and Dex chickens following oral gavage

Plasma metabolites were measured to establish substrate utilization across dietary treatments (Table 3.4; Study 1). During carbohydrate oxidation, glucose undergoes glycolysis to form pyruvate. Pyruvate can either enter mitochondria via the mitochondrial pyruvate carrier to enter the tricarboxylic acid (TCA) cycle or be converted in the cytosol to lactate by lactate dehydrogenase or to alanine via alanine transaminase. A schematic representation of these metabolic interconversions is shown in Fig. 3.2A. Plasma concentrations of pyruvate, lactate, and alanine (by-products of carbohydrate oxidation) were significantly higher in the SD, Com, and Dex groups compared to the Oil, Fast, and DOH groups ($P \leq 0.05$; Fig. 3.2B), indicating enhanced carbohydrate oxidation in the former groups. In contrast, plasma β -hydroxybutyrate (by-product of lipid oxidation) concentrations were significantly lower in the SD, Com, and Dex groups relative to the Oil, Fast, and DOH groups ($P \leq 0.01$; Fig. 3.2C), indicating reduced lipid oxidation. Correlation analysis revealed a significant negative association between plasma β -hydroxybutyrate and the abundance of pyruvate, lactate, and alanine ($P \leq 0.01$; Fig. 3.2D), further supporting an inverse relationship between carbohydrate oxidation and lipid oxidation. The correlation analysis indicates that chickens in the Dex group could effectively transition toward carbohydrate utilization, similar to the SD and Com groups. However, chickens in the Oil group exhibited a metabolic profile similar to Fast and DOH groups and were unable to undergo the embryonic to post-hatch metabolic transition successfully.

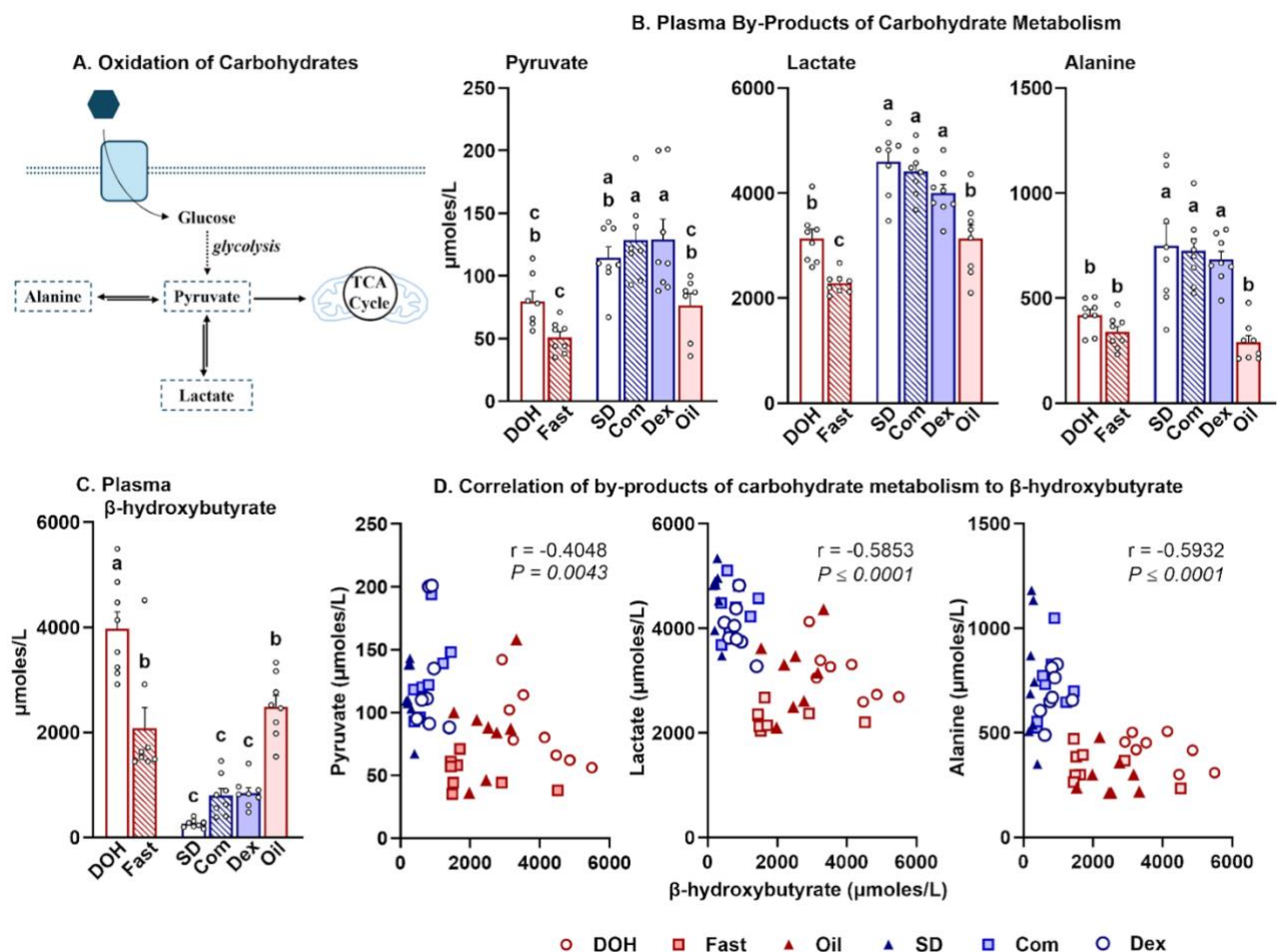


Figure 3.2. Enhanced carbohydrate oxidation and reduced ketone production in SD, Com, and Dex gavaged groups.

(A) Schematic depiction of the by-products formed during carbohydrate oxidation. Plasma levels of the (B) by-products of carbohydrate oxidation (pyruvate, lactate, and alanine) and (C) β -hydroxybutyrate across the dietary treatments. (D) Correlation between plasma β -hydroxybutyrate and by-products of carbohydrate metabolism. Data are represented as mean $\pm$ SEM ($n = 7-9$ /group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

Table 3.4. Abundance of plasma metabolites (organic acids and amino acids) in chickens administered dietary treatments by oral gavage.

A. TCA Cycle Intermediates						
Plasma Concentration (μmoles/L ± SEM)						
	DOH	Fast	SD	Com	Dex	Oil
Pyruvate	87 ± 10 ^{cb}	51 ± 4 ^c	115 ± 9 ^{ab}	129 ± 11 ^a	129 ± 16 ^a	87 ± 13 ^{cb}
Lactate	3142 ±	2279 ±	4600 ±	4408 ±	3998 ±	3138 ±
	177 ^b	70 ^c	213 ^a	159 ^a	163 ^a	254 ^b
Succinate	27 ± 2 ^a	29 ± 5 ^a	41 ± 14 ^a	34 ± 2 ^a	37 ± 7 ^a	28 ± 4 ^a
Fumarate	9 ± 1 ^a	12 ± 5 ^a	17 ± 5 ^a	11 ± 1 ^a	12 ± 2 ^a	8 ± 1 ^a
α-ketoglutarate	47 ± 4 ^c	67 ± 4 ^{cb}	83 ± 7 ^{ab}	108 ± 9 ^a	90 ± 7 ^{ab}	72 ± 7 ^{cb}

B. Amino Acids

Plasma Concentration (μmoles/L ± SEM)						
	DOH	Fast	SD	Com	Dex	Oil
Alanine	419 ± 28 ^b	338 ± 28 ^b	750 ± 105 ^a	725 ± 59 ^a	683 ± 40 ^a	289 ± 33 ^b
Glycine	497 ± 24 ^{ab}	583 ± 41 ^a	410 ± 33 ^b	514 ± 27 ^{ab}	454 ± 21 ^c	449 ± 19 ^b
Valine	597 ± 20 ^a	670 ± 41 ^a	417 ± 43 ^{bc}	339 ± 20 ^{bc}	319 ± 20 ^c	442 ± 16 ^b
Leucine	238 ± 6 ^b	386 ± 17 ^a	403 ± 41 ^a	181 ± 18 ^b	184 ± 15 ^b	249 ± 11 ^b
Isoleucine	361 ± 8 ^b	467 ± 26 ^a	318 ± 34 ^b	192 ± 19 ^c	178 ± 14 ^c	296 ± 17 ^b
Proline	463 ± 29 ^a	356 ± 26 ^{ab}	444 ± 69 ^a	282 ± 30 ^b	256 ± 18 ^b	253 ± 13 ^b
Methionine	109 ± 5 ^a	111 ± 9 ^a	71 ± 6 ^b	85 ± 4 ^{ab}	92 ± 7 ^{ab}	89 ± 5 ^{ab}
Serine	744 ± 52 ^a	486 ± 32 ^b	448 ± 44 ^b	762 ± 44 ^a	870 ± 52 ^a	412 ± 33 ^b
Threonine	762 ± 67 ^{bc}	1341 ± 200 ^a	535 ± 81 ^c	1025 ± 107 ^{ab}	557 ± 33 ^c	799 ± 92 ^{bc}
Phenylalanine	133 ± 3 ^b	129 ± 5 ^b	424 ± 52 ^a	132 ± 9 ^b	145 ± 8 ^b	112 ± 6 ^b

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

3.4.3. Hepatic lipogenic gene expression induction following oral gavage of Dextrin was similar to the observed induction in the SD and Com groups

During embryonic development, the chicken embryo relies heavily on lipid oxidation to derive energy. Following hatch, there is a shift in metabolism, and the post-hatch chickens transition towards *de novo* lipogenesis. To assess this metabolic shift, the relative mRNA expression pattern of genes involved in hepatic *de novo* lipogenesis was measured (**Fig. 3.3A**; Study 1). Expression of *ELOVL6* was significantly higher in SD, Com, and Dex livers compared to DOH, Fas, and Oil groups ($P \leq 0.03$). Similarly, *SCD1* expression was significantly higher in the SD liver compared to the other dietary treatments ($P < 0.00$), with Com and Dex groups exhibiting higher *SCD1* expression compared to DOH, Fas, and Oil livers. The relative mRNA expression pattern of genes involved in hepatic lipid oxidation was also measured (**Fig. 3.3B**). Both *LCAD* and *PPAR α* showed a similar expression pattern (**Fig. 3.3B**). Fasted livers had higher expression of *LCAD* and *PPAR α* ($P < 0.09$), while the remaining dietary treatments showed a similar expression pattern. Collectively, these data established that with 48 hours of Dex gavage, chicken livers could efficiently downregulate the lipid oxidation genes and upregulate the expression of lipogenic genes in the liver, similar to what was observed in SD and Com-treated livers.

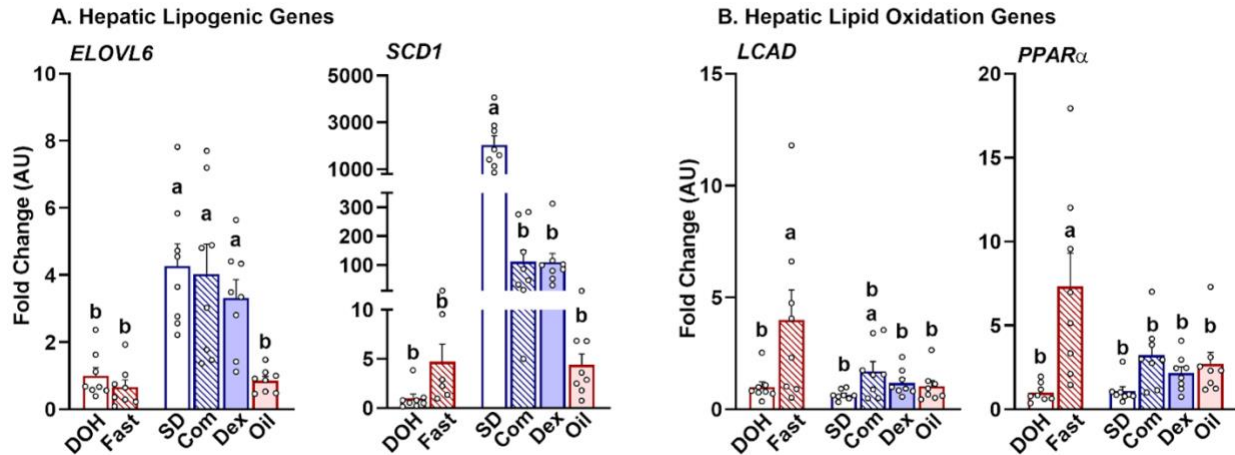


Figure 3.3. Hepatic expression of genes regulating lipid metabolism following dietary macronutrient administration by oral gavage.

(A) Relative mRNA expression of genes involved in lipogenic pathways in the liver. (B) Relative mRNA expression of genes involved in lipid oxidation pathways in the liver. Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. *ELOVL6*, Elongation of Very Long Chain Fatty Acids Protein 6; *SCD1*, Stearoyl-CoA desaturase-1; *LCAD*, Long-Chain Acyl-CoA Dehydrogenase; *PPARα*, Peroxisome Proliferator-Activated Receptor Alpha, DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil

3.4.4. Dextrin treatment by oral gavage efficiently promoted the metabolic shift towards carbohydrate metabolism in the liver

To determine if hepatic metabolite profiles paralleled the changes in the gene expression patterns observed in **Figure 3.3**, liver concentrations of pyruvate, lactate, and alanine, which reflect the end products of carbohydrate metabolism (**Table 3.5**; Study 1). Pyruvate, lactate, and alanine levels tended to be higher in SD, Com, and Dex livers compared to DOH, Fast, and Oil groups (**Fig. 3.4A**). In contrast, hepatic β -hydroxybutyrate levels were significantly lower in SD, Com, and Dex livers compared to DOH, Fast, and Oil groups ($P < 0.01$; **Fig. 3.4B**). Correlation analysis suggested a significant negative association between hepatic β -hydroxybutyrate levels vs. pyruvate, lactate, and alanine ($P \leq 0.03$; **Fig. 3.4C**), supporting an inverse relationship between carbohydrate metabolism and lipid oxidation in the liver. Dextrin administered chickens showed correlation patterns similar to SD and Com groups, whereas Oil administered chickens showed patterns similar to DOH and Fast groups. Taken together, these findings suggest that dextrin administration facilitates the transition from lipid oxidation to carbohydrate utilization in post-hatch livers, whereas oil administration maintains a metabolic state that is similar to the pre-switch conditions.

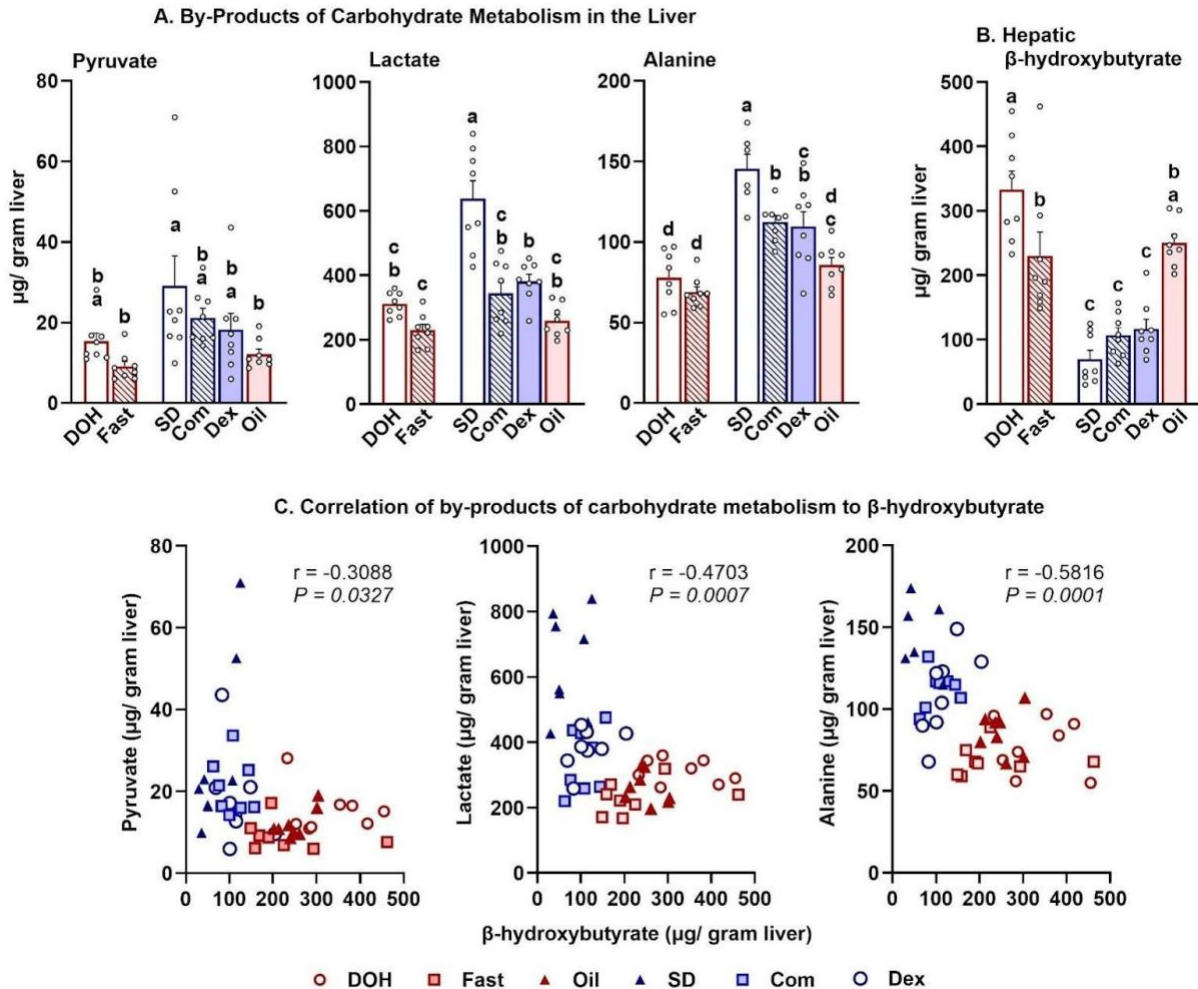


Figure 3.4. Hepatic metabolite profiles following oral gavage of dietary macronutrients.

(A) Hepatic abundance of pyruvate, lactate, and alanine. (B) Level of β -hydroxybutyrate in the livers. (C) Correlation between by-products of carbohydrate metabolism and β -hydroxybutyrate across the dietary treatments. Data are represented as mean $\pm$ SEM ($n = 7-9/\text{group}$) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

Table 3.5. Abundance of liver metabolites (organic acids and amino acids) in chickens administered dietary treatments by oral gavage.

A. TCA Cycle Intermediates						
Hepatic Concentration (µg/ gram liver ± SEM)						
	DOH	Fast	SD	Com	Dex	Oil
Pyruvate	15 ± 2 ^{ab}	9 ± 1 ^b	29 ± 7 ^a	21 ± 2 ^{ab}	18 ± 4 ^{ab}	12 ± 1 ^b
Lactate	312 ± 13 ^{bc}	230 ± 18 ^c	638 ± 56 ^a	344 ± 35 ^{bc}	382 ± 22 ^b	260 ± 18 ^{bc}
Succinate	98 ± 9 ^c	91 ± 12 ^c	231 ± 30 ^a	143 ± 7 ^b	150 ± 8 ^{ab}	112 ± 10 ^{bc}
Fumarate	139 ± 4 ^{cd}	112 ± 10 ^d	280 ± 43 ^a	207 ± 10 ^{ab}	176 ± 12 ^{bc}	182 ± 19 ^{ac}
α-keto glutarate	36 ± 5 ^a	38 ± 8 ^a	39 ± 10 ^a	52 ± 6 ^a	30 ± 5 ^a	38 ± 3 ^a
Malate	227 ± 26 ^{cd}	150 ± 14 ^d	435 ± 52 ^a	281 ± 22 ^{bc}	309 ± 17 ^{ab}	204 ± 13 ^{cd}
Citrate	15 ± 2 ^{ab}	7 ± 1 ^b	19 ± 2 ^a	15 ± 1 ^{ab}	18 ± 4 ^a	11 ± 2 ^{ab}

B. Amino Acids

Hepatic Concentration ($\mu\text{g/ gram liver} \pm \text{SEM}$)						
	DOH	Fast	SD	Com	Dex	Oil
Alanine	$78 \pm 6^{\text{d}}$	$69 \pm 3^{\text{d}}$	$191 \pm 33^{\text{a}}$	$112 \pm 4^{\text{b}}$	$110 \pm 9^{\text{bc}}$	$86 \pm 4^{\text{cd}}$
Glycine	$87 \pm 5^{\text{b}}$	$94 \pm 3^{\text{b}}$	$137 \pm 17^{\text{a}}$	$102 \pm 6^{\text{b}}$	$90 \pm 6^{\text{b}}$	$105 \pm 4^{\text{ab}}$
Valine	$82 \pm 4^{\text{a}}$	$87 \pm 5^{\text{a}}$	$65 \pm 9^{\text{c}}$	$62 \pm 3^{\text{bc}}$	$65 \pm 3^{\text{bc}}$	$76 \pm 3^{\text{ab}}$
Leucine	$71 \pm 7^{\text{a}}$	$84 \pm 4^{\text{a}}$	$92 \pm 12^{\text{a}}$	$78 \pm 5^{\text{a}}$	$86 \pm 5^{\text{a}}$	$87 \pm 6^{\text{a}}$
Isoleucine	$62 \pm 3^{\text{ab}}$	$72 \pm 3^{\text{a}}$	$59 \pm 8^{\text{bd}}$	$48 \pm 2^{\text{d}}$	$50 \pm 2^{\text{cd}}$	$61 \pm 3^{\text{bc}}$
Proline	$61 \pm 4^{\text{b}}$	$59 \pm 4^{\text{b}}$	$102 \pm 14^{\text{a}}$	$52 \pm 7^{\text{bc}}$	$37 \pm 2^{\text{c}}$	$67 \pm 8^{\text{b}}$
Methionine	$18 \pm 2^{\text{b}}$	$17 \pm 1^{\text{b}}$	$17 \pm 2^{\text{b}}$	$22 \pm 2^{\text{ab}}$	$27 \pm 2^{\text{a}}$	$22 \pm 2^{\text{ab}}$
Serine	$167 \pm 12^{\text{b}}$	$73 \pm 11^{\text{c}}$	$291 \pm 30^{\text{a}}$	$293 \pm 43^{\text{a}}$	$259 \pm 14^{\text{a}}$	$118 \pm 12^{\text{bc}}$
Threonine	$135 \pm 6^{\text{ab}}$	$210 \pm 39^{\text{a}}$	$150 \pm 18^{\text{ab}}$	$214 \pm 31^{\text{a}}$	$114 \pm 4^{\text{b}}$	$166 \pm 15^{\text{ab}}$
Phenyl alanine	$36 \pm 3^{\text{cd}}$	$35 \pm 2^{\text{d}}$	$83 \pm 13^{\text{a}}$	$47 \pm 2^{\text{bc}}$	$49 \pm 2^{\text{b}}$	$43 \pm 3^{\text{bd}}$
Aspartate	$282 \pm 32^{\text{bc}}$	$224 \pm 13^{\text{c}}$	$330 \pm 36^{\text{bc}}$	$501 \pm 56^{\text{a}}$	$369 \pm 28^{\text{ab}}$	$315 \pm 16^{\text{bc}}$
Glutamate	$518 \pm 26^{\text{b}}$	$549 \pm 38^{\text{ab}}$	$644 \pm 57^{\text{ab}}$	$767 \pm 100^{\text{a}}$	$624 \pm 41^{\text{ab}}$	$535 \pm 22^{\text{b}}$
Tyrosine	$68 \pm 5^{\text{a}}$	$54 \pm 3^{\text{a}}$	$69 \pm 11^{\text{a}}$	$59 \pm 3^{\text{a}}$	$63 \pm 4^{\text{a}}$	$60 \pm 4^{\text{a}}$

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

3.4.5. Dextrin administration by oral gavage induces metabolic reprogramming in skeletal muscle

In order to determine if the metabolic shifts observed in the liver were mirrored in the muscle tissues, muscle concentrations of metabolites associated with carbohydrate and lipid oxidation were measured (**Table 3.6**). Muscle pyruvate level was significantly higher in dextrin-administered chickens compared to all other dietary treatments ($P < 0.01$; **Fig. 3.5A**). This was in contrast to muscle lactate abundance, where dextrin-administered chickens had significantly lower muscle lactate levels compared to SD and Com groups ($P < 0.03$; **Fig. 3.5A**) and were comparable to Oil and DOH groups. While muscle alanine concentration was highest in the SD group, both Com and Dex groups had significantly higher alanine levels compared to Oil, fasted, and DOH groups ($P < 0.00$; **Fig. 3.5A**). Muscle β -hydroxybutyrate levels were lower in SD, Com, and Dex groups but were significantly higher in Oil, Fast, and DOH chickens ($P < 0.00$; **Fig. 3.5B**). Correlation analyses revealed a trend towards a negative association between β -hydroxybutyrate and pyruvate. Further, lactate and alanine showed a significant negative correlation with β -hydroxybutyrate ($P < 0.00$; **Fig. 3.5C**). The correlation plots suggested that dextrin treatments could initiate a metabolic transition in the muscle tissue, unlike the oil treatment. Collectively, these findings suggested that administration of the dextrin-enriched diet for 48hours to the hatchlings promoted metabolic reprogramming in the skeletal muscle, similar to the hepatic transition from lipid oxidation towards carbohydrate utilization as observed in the earlier figures.

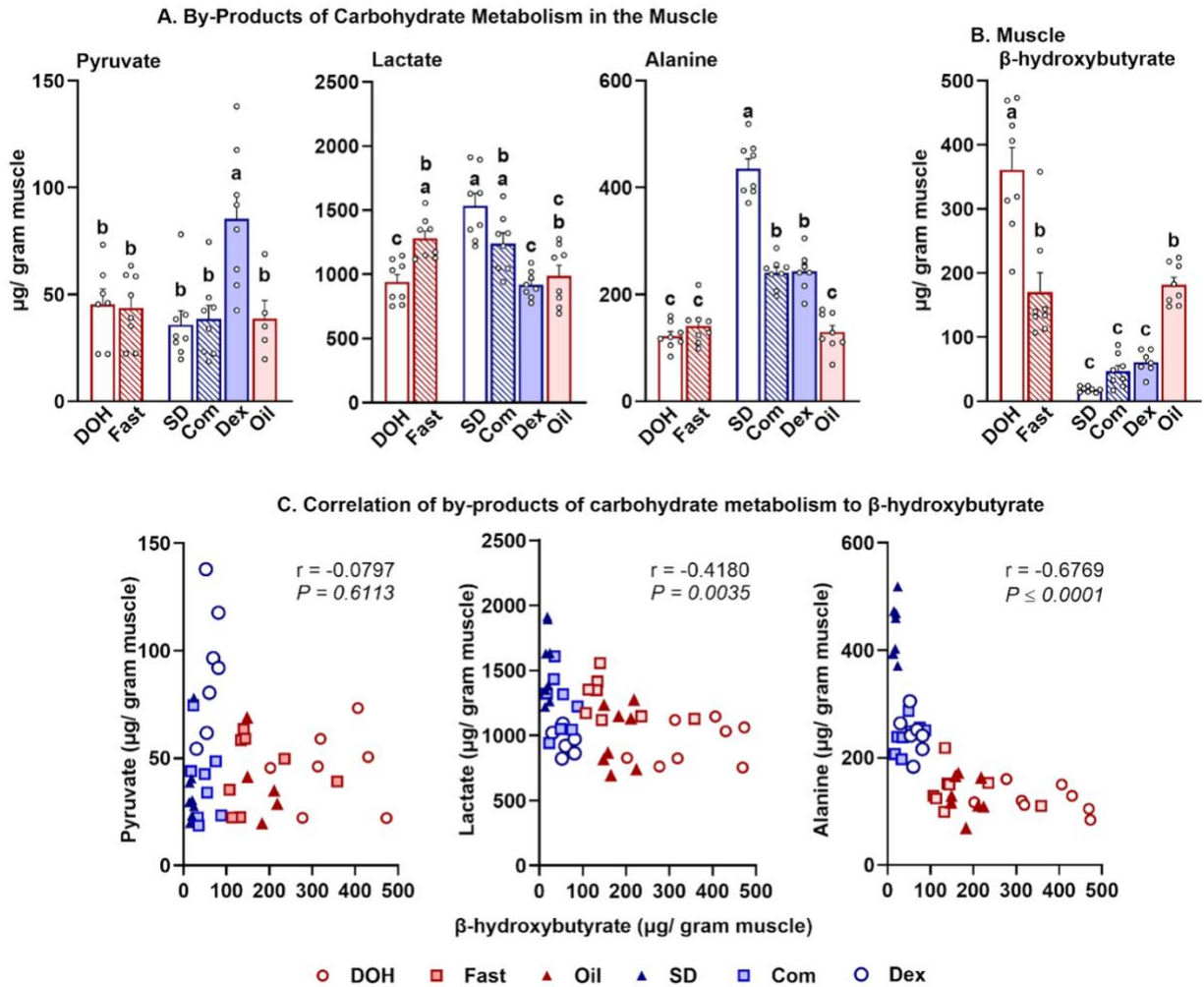


Figure 3.5. Muscle metabolite profiles following oral gavage of dietary macronutrients.

(A) Muscle abundance of pyruvate, lactate, and alanine. (B) Level of β -hydroxybutyrate in the muscle.

(C) Correlation between by-products of carbohydrate metabolism and β -hydroxybutyrate across the dietary treatments. Data are represented as mean $\pm$ SEM ($n = 7-9/\text{group}$) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

Table 3.6. Abundance of muscle metabolites (organic acids and amino acids) in chickens administered dietary treatments by oral gavage.

A. TCA Cycle Intermediates						
Muscle Concentration ($\mu\text{g}/\text{gram muscle} \pm \text{SEM}$)						
	DOH	Fast	SD	Com	Dex	Oil
Pyruvate	56 ± 12^b	44 ± 6^b	36 ± 6^b	38 ± 6^b	85 ± 11^a	39 ± 9^b
Lactate	$941 \pm$	$1280 \pm$	$1536 \pm$	1242 ± 79^{ab}	$919 \pm$	$990 \pm$
	58^c	57^{ab}	96^a		37^c	82^{bc}
Succinate	32 ± 3^a	23 ± 1^b	30 ± 1^{ab}	27 ± 6^{ab}	27 ± 3^{ab}	26 ± 2^{ab}
Fumarate	37 ± 3^a	40 ± 4^a	35 ± 2^a	38 ± 3^a	43 ± 4^a	40 ± 5^a
α-keto glutarate	68 ± 12^a	28 ± 3^b	35 ± 5^{ab}	24 ± 3^b	58 ± 5^{ab}	54 ± 13^{ab}
Malate	55 ± 2^c	79 ± 3^a	75 ± 3^a	70 ± 4^{ab}	56 ± 4^{bc}	64 ± 3^{ac}
Citrate	52 ± 6^a	29 ± 3^b	31 ± 1^b	25 ± 3^b	39 ± 4^{ab}	40 ± 4^{ab}

B. Amino Acids

Muscle Concentration ($\mu\text{g/gram muscle} \pm \text{SEM}$)						
	DOH	Fast	SD	Com	Dex	Oil
Alanine	122 ± 8^c	142 ± 13^c	435 ± 18^a	241 ± 10^b	225 ± 22^b	130 ± 12^c
Glycine	169 ± 17^a	138 ± 11^a	178 ± 22^a	142 ± 6^a	158 ± 24^a	115 ± 6^a
Valine	64 ± 3^b	79 ± 6^a	44 ± 2^c	37 ± 2^c	38 ± 3^c	50 ± 3^{bc}
Leucine	29 ± 1^b	55 ± 4^a	49 ± 2^a	26 ± 1^b	28 ± 2^b	35 ± 2^b
Isoleucine	43 ± 2^b	62 ± 5^a	32 ± 2^{bd}	24 ± 1^d	26 ± 2^{cd}	38 ± 3^{bc}
Proline	81 ± 6^b	54 ± 5^{bc}	228 ± 16^a	78 ± 23^{bc}	52 ± 6^{bc}	48 ± 6^c
Methionine	13 ± 1^b	22 ± 2^a	14 ± 1^b	17 ± 2^b	17 ± 1^{ab}	17 ± 1^{ab}
Serine	166 ± 13^b	122 ± 12^b	249 ± 18^a	304 ± 39^a	303 ± 15^a	159 ± 21^b
Threonine	116 ± 9^b	186 ± 20^a	151 ± 12^{ab}	183 ± 18^a	107 ± 8^b	128 ± 13^{ab}
Phenyl alanine	21 ± 1^b	27 ± 2^b	72 ± 6^a	23 ± 1^b	26 ± 2^b	21 ± 1^b
Aspartate	89 ± 9^b	143 ± 10^{ab}	121 ± 8^{ab}	111 ± 14^{ab}	112 ± 11^{ab}	150 ± 20^a
Glutamate	423 ± 33^b	332 ± 21^b	540 ± 19^a	345 ± 25^b	317 ± 28^b	369 ± 29^b
Tyrosine	28 ± 1^{bc}	26 ± 2^{bc}	50 ± 4^a	20 ± 1^{bc}	29 ± 3^b	18 ± 1^c

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. DOH, day of hatch; Fast, fasted; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Oil, SP-Oil.

3.4.6. *Ad libitum* feeding of dextrin pellets replicated the suppression of circulating plasma ketone profiles as observed in the oral gavage experiments

To minimize potential confounding effects associated with repeated oral gavage of macronutrients, in study 2, the experimental diets were prepared by baking them into cookies, crumbling them into pellets, and feeding them *ad libitum*. Because of the impracticality of preparing pellets, the oil-enriched diet was still administered via oral gavage, accompanied by a control gavage (CG) group to account for gavage-related changes. After 48 hours of dietary treatments, caloric intake was comparable in SD, Com, and Dex groups but lower in the Casein (Cas) group (**Fig. 3.6A**). Oral gavage administration of the Oil diet ensured consistent caloric delivery in that group, whereas Fast, Cel, and CG groups had negligible caloric intake, nearing zero, as expected for control treatments. SD group weighed the highest ($P \leq 0.00$), followed by Com, Dex, and Cas groups ($P \leq 0.04$), whereas Oil, Fast, Cellulose (Cel), and Control Gavage (CG) had similar and lower body weights (**Fig. 3.6B**). Liver weights were significantly higher in the SD and Dex groups compared to the other dietary treatments ($P \leq 0.00$, **Fig. 3.6C**). Heart weight tended to be higher in the SD group but was comparable across the other treatments (**Fig. 3.6D**).

Plasma levels of ketone bodies, β -hydroxybutyrate, and acetoacetate were measured. The interconversion of β -hydroxybutyrate to acetoacetate, catalyzed by β -hydroxybutyrate dehydrogenase (**Fig. 3.6E**), has a critical role in energy production, metabolic signaling, and maintaining redox balance. While β -hydroxybutyrate is the primary ketone body produced and exported by the liver, it must be converted into acetoacetate in the peripheral tissues for fueling the TCA cycle for ATP production. Plasma β -hydroxybutyrate levels were significantly lower in the SD, Com, and Dex groups compared to the other dietary treatments ($P \leq 0.00$; **Fig. 3.6F**). Plasma acetoacetate levels were measured and were normalized to plasma norleucine levels. Although SD, Com, and Dex tended to have lower plasma acetoacetate levels, Dex had the lowest level overall, whereas Cas and Oil had significantly higher acetoacetate compared to Dex treatment ($P \leq 0.03$; **Fig. 3.6F**). Plasma β -hydroxybutyrate to acetoacetate ratio, indicative of hepatic mitochondrial redox state, is typically high under high NADH environment in

the hepatic mitochondria. This ratio tended to be lower in the SD, Com, and Dex groups compared to the other dietary treatments. Collectively, these findings indicate that dextrin administration reduces circulating ketone levels and induces a metabolic profile consistent with decreased utilization of lipids.

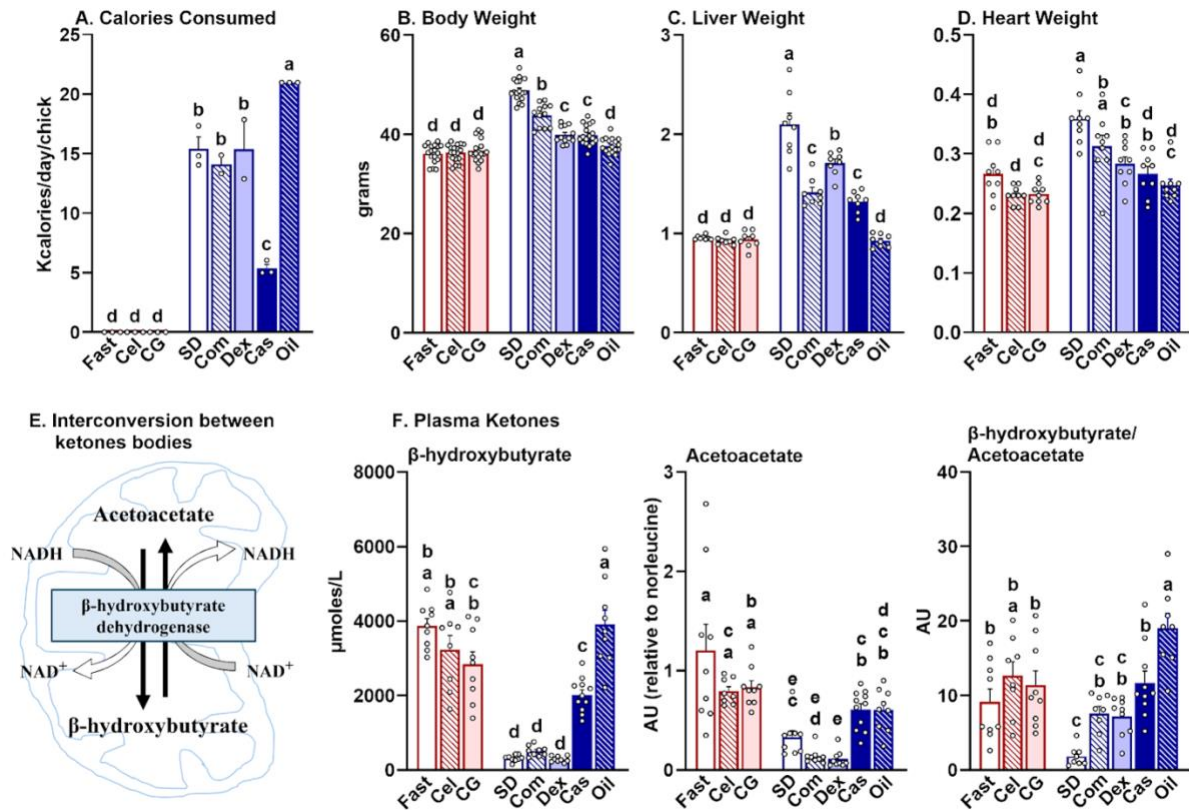


Figure 3.6. Changes in body and organ weights and circulating ketone profiles following dietary treatments under *ad libitum* feeding.

(A) Calories consumed in 48 hours. (B) Body weights, (C) liver weights, and (D) heart weights across the dietary groups. (E) Schematic illustration of the interconversion between β -hydroxybutyrate and acetoacetate. (F) Circulating levels of plasma ketones β -hydroxybutyrate and acetoacetate, and their ratios. Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil; AU, Arbitrary Units.

3.4.7. Circulating levels of markers of carbohydrate metabolism were elevated in chickens reared on dextrin-enriched diets *ad libitum*

Plasma metabolite concentrations were quantified across all dietary treatments (**Table 3.7**; Study 2). Plasma pyruvate level was highest in the chickens reared with dextrin-enriched diets (**Fig. 3.7A**). Similarly, plasma lactate levels were significantly elevated in the Dex group compared to the other dietary treatments ($P \leq 0.00$; **Fig. 3.7A**). Plasma alanine levels were significantly higher in SD and Com compared to the other dietary treatments ($P \leq 0.00$; **Fig. 3.7A**). While correlation analysis between β -hydroxybutyrate and the products of carbohydrate metabolism (pyruvate, lactate, and alanine) showed a significant negative association ($P \leq 0.00$; **Fig. 3.7B**), the distribution pattern of Dex group indicated a successful transition from lipid oxidation to carbohydrate utilization upon dextrin treatment. This transition was similar to what was observed in SD and Com groups. In contrast, chickens administered casein and oil-enriched diets exhibited metabolic profiles that indicated inefficient metabolic transition in these groups.

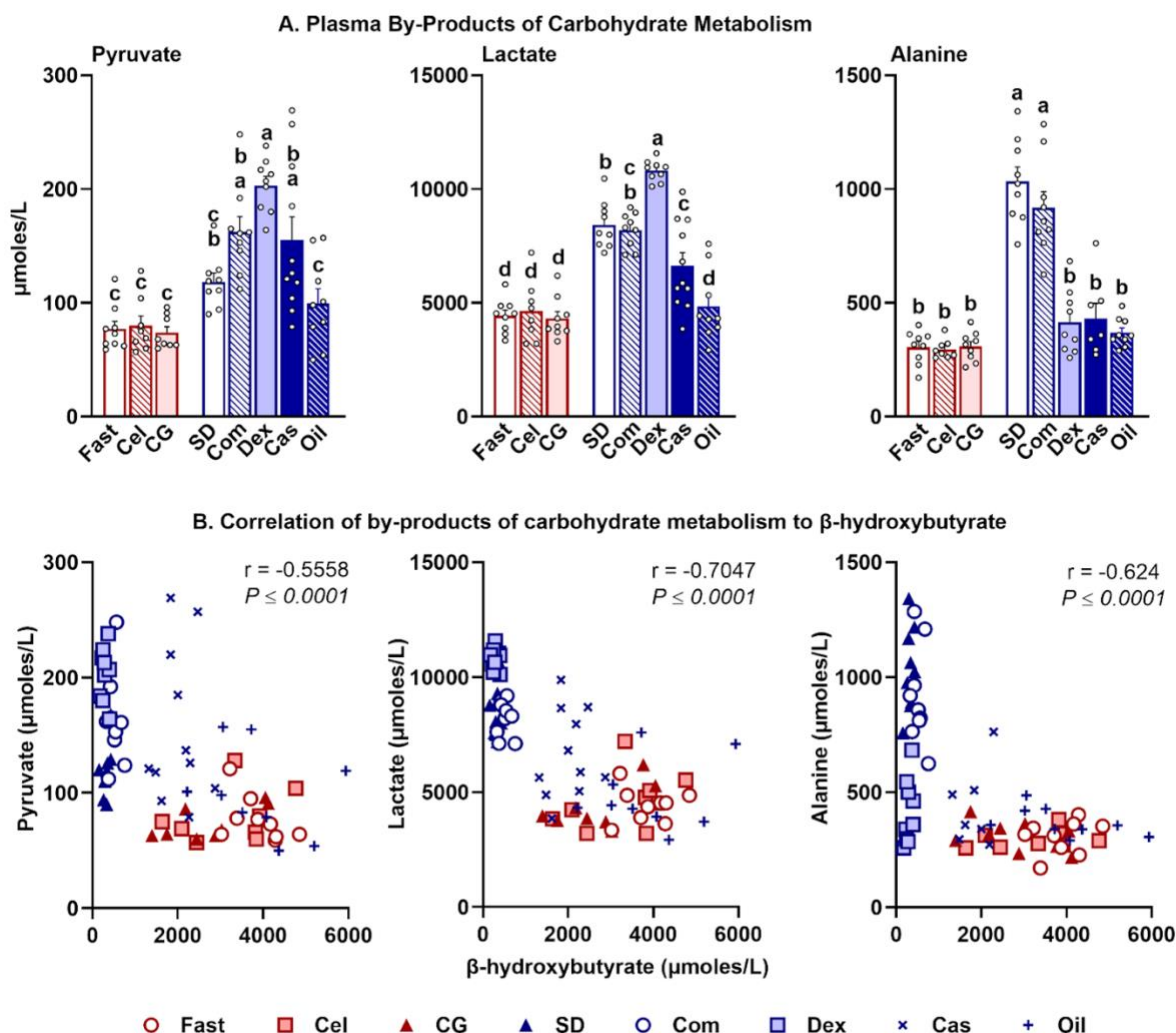


Figure 3.7. Plasma levels of by-products of carbohydrate metabolism in chickens reared on dietary macronutrients *ad libitum*.

(A) Plasma concentrations of pyruvate, lactate, and alanine. (B) Correlation between plasma β -hydroxybutyrate and pyruvate, lactate, and alanine. Data are represented as mean $\pm$ SEM ($n = 7-9$ /group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

Table 3.7. Abundance of plasma metabolites (organic acids and amino acids) in chickens reared on dietary macronutrients *ad libitum*.

A. TCA Cycle Intermediates								
Plasma Concentration (μmoles/L ± SEM)								
	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Pyruvate	77 ± 6 ^c	80 ± 8 ^c	82 ± 9 ^c	119 ± 7 ^{cb}	163 ± 13 ^{ba}	203 ± 7 ^a	152 ± 22 ^{ba}	100 ± 13 ^c
Lactate	4425 ± 247 ^d	4630 ± 472 ^d	4315 ± 299 ^d	8430 ± 349 ^b	8204 ± 256 ^{cb}	10804 ± 156 ^a	6468 ± 652 ^c	4844 ± 519 ^d
Succinate	12 ± 1 ^b	11 ± 1 ^b	16 ± 3 ^b	19 ± 2 ^b	20 ± 3 ^b	17 ± 1 ^b	29 ± 5 ^b	15 ± 1 ^b
Fumarate	5 ± 0.4 ^c	7 ± 1.2 ^{cb}	6 ± 0.9 ^c	9 ± 0.8 ^{ab}	7 ± 0.6 ^{cb}	7 ± 0.6 ^{cb}	10 ± 1.6 ^a	6 ± 0.3 ^{bc}
α-keto glutarate	50 ± 3 ^a	61 ± 6 ^a	55 ± 4 ^a	70 ± 5 ^a	75 ± 3 ^a	71 ± 6 ^a	104 ± 18 ^a	63 ± 4 ^a
Malate	17 ± 0.9 ^d	20 ± 2.3 ^{dc}	17 ± 1 ^d	37 ± 2.4 ^a	31 ± 2.2 ^{ba}	28 ± 1.4 ^{cba}	31 ± 4.0 ^{ba}	25 ± 1.7 ^{db}
Citrate	236 ± 16 ^{cb}	238 ± 21 ^{cb}	196 ± 22 ^c	429 ± 23 ^a	314 ± 20 ^b	426 ± 19 ^a	214 ± 17 ^c	322 ± 25 ^b

B. Amino Acids

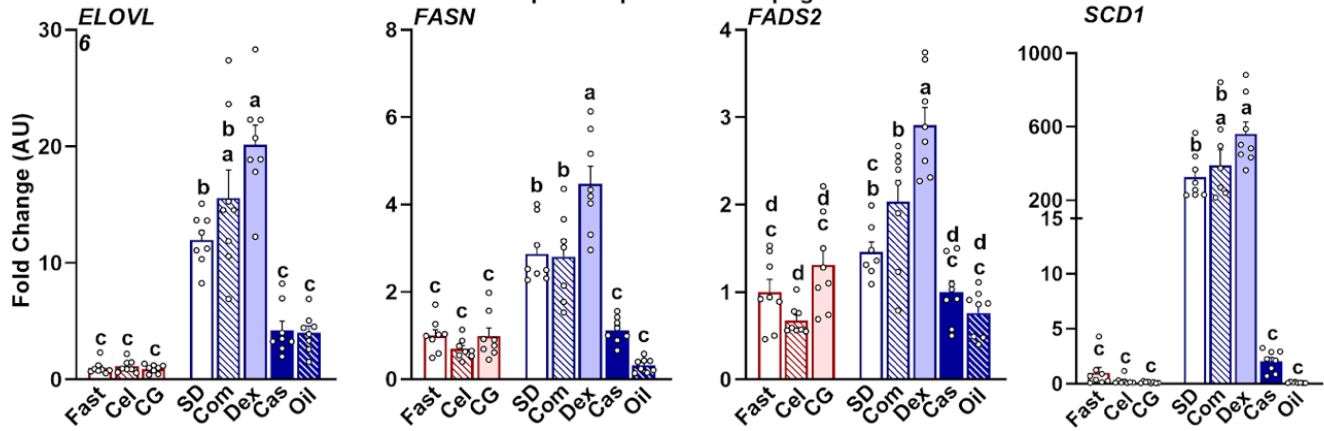
Plasma Concentration (μmoles/L ± SEM)								
	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Alanine	305 ± 24 ^b	296 ± 14 ^b	309 ± 21 ^b	1035 ± 61 ^a	917 ± 70 ^a	414 ± 47 ^b	432 ± 65 ^b	369 ± 21 ^b
Glycine	533 ± 22 ^{cb}	532 ± 28 ^{ca}	485 ± 33 ^{cb}	723 ± 32 ^a	723 ± 75 ^a	248 ± 27 ^d	663 ± 59 ^{ba}	435 ± 23 ^c
Valine	582 ± 30 ^b	516 ± 27 ^{cb}	550 ± 30 ^b	482 ± 30 ^{cb}	645 ± 88 ^b	229 ± 13 ^c	1363 ± 160 ^a	464 ± 23 ^{cb}
Leucine	329 ± 16 ^{cb}	300 ± 13 ^c	302 ± 18 ^c	432 ± 26 ^b	348 ± 32 ^{cb}	159 ± 7 ^d	651 ± 76 ^a	244 ± 12 ^{dc}
Isoleucine	398 ± 20 ^b	361 ± 28 ^b	355 ± 20 ^b	332 ± 26 ^b	313 ± 26 ^b	149 ± 4 ^c	669 ± 68 ^a	287 ± 24 ^b
Proline	290 ± 19 ^b	305 ± 6 ^b	283 ± 15 ^b	771 ± 45 ^a	727 ± 74 ^a	119 ± 12 ^b	1024 ± 136 ^a	238 ± 14 ^b
Methionine	87 ± 5 ^{cb}	111 ± 4 ^{ba}	91 ± 5 ^{cb}	97 ± 6 ^{cb}	107 ± 10 ^{ba}	53 ± 4 ^d	127 ± 11 ^a	74 ± 4 ^{dc}
Serine	443 ± 43 ^{cb}	506 ± 26 ^{cb}	461 ± 57 ^{cb}	663 ± 30 ^b	1077 ± 118 ^a	321 ± 28 ^c	421 ± 33 ^{cb}	599 ± 33 ^{cb}
Threonine	1085 ± 100 ^b	1066 ± 115 ^b	946 ± 76 ^b	1078 ± 99 ^b	2087 ± 205 ^a	212 ± 36 ^c	1359 ± 140 ^b	901 ± 106 ^b
Phenyl alanine	126 ± 6 ^{dc}	112 ± 3 ^d	124 ± 9 ^{dc}	311 ± 25 ^a	182 ± 10 ^b	145 ± 5 ^{db}	172 ± 9 ^{cb}	112 ± 6 ^d
Aspartate	96 ± 17 ^a	84 ± 16 ^a	85 ± 17 ^a	52 ± 4 ^a	61 ± 11 ^a	38 ± 5 ^a	81 ± 23 ^a	94 ± 13 ^a
Glutamate	104 ± 9 ^{db}	93 ± 7 ^{dc}	98 ± 6 ^{db}	155 ± 8 ^a	165 ± 25 ^{ba}	74 ± 5 ^d	129 ± 21 ^{cba}	96 ± 5 ^{dc}
Tyrosine	67 ± 4 ^d	79 ± 3 ^{dc}	79 ± 8 ^{dc}	155 ± 8 ^a	105 ± 9 ^{cb}	136 ± 13 ^{ba}	51 ± 4 ^d	76 ± 7 ^{dc}

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

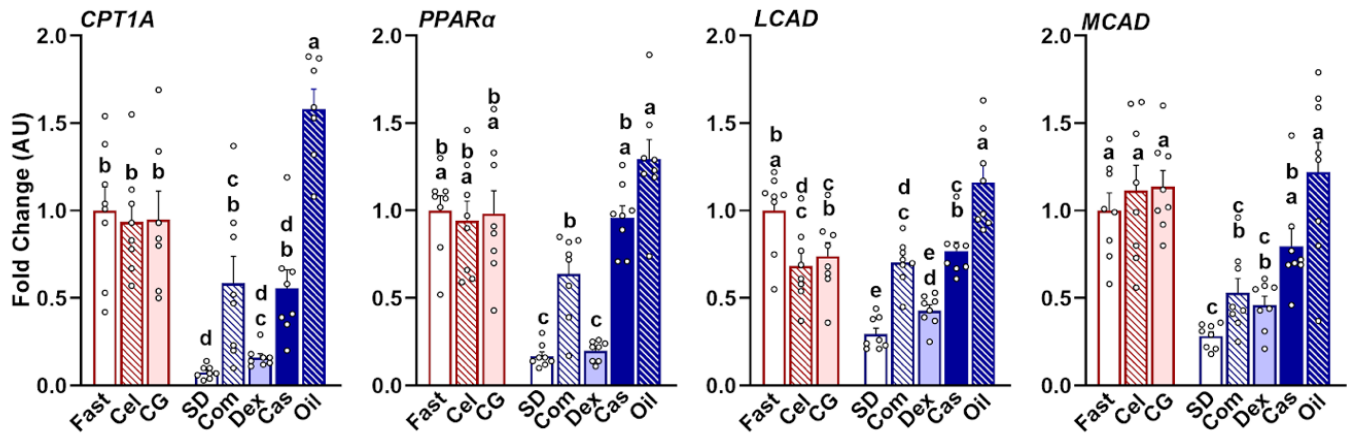
3.4.8. *Ad libitum* feeding of dextrin-enriched diet upregulated hepatic *de novo* lipogenic genes while suppressing lipid oxidation pathways

The expression profiles of genes involved in hepatic *de novo* lipogenesis were examined (Study 2). Hepatic ELOVL6 expression was significantly elevated in SD, Com, and Dex groups ($P < 0.00$; **Fig. 3.8A**) compared to the remaining dietary treatments, with the highest expression observed in the Dex livers. Similarly, FASN expression was highest in dextrin-treated livers ($P < 0.00$; **Fig. 3.8A**), followed by SD and Com groups, both of which were significantly higher than the other dietary treatments ($P < 0.00$). This expression pattern was consistently observed for *FADS2* and *SCD1*, with Dex exhibiting the greatest hepatic abundance, followed by SD and Com groups (**Fig. 3.8A**). Expression of genes associated with hepatic fatty acid oxidation was measured. *CPT1A* expression was highest in Oil-treated livers, followed by Fast, Cel, CG, Com, and Cas groups, whereas SD and Dex livers exhibited the lowest expression levels (**Fig. 3.8B**). A similar trend was observed for *PPAR α* , *LCAD*, and *MCAD*, with these lipid oxidation-associated genes significantly downregulated in SD and Dex groups relative to the other dietary treatments (**Fig. 3.8B**). Correlation analysis between *PPAR α* and lipogenic genes (*ELOVL6*, *FASN*, *FADS2*, and *SCD1*) revealed a significant negative association ($P < 0.00$; **Fig. 3.8C**), highlight the inverse regulation between hepatic lipogenesis and lipid oxidation pathways. These plots suggested that dextrin administration to the post-hatch chickens for 48hours, induced the expression of the lipogenic genes in the livers while concomitantly suppressing lipid oxidation gene expression. Collectively, these data highlight that while Dex livers could successfully transition from lipolytic phenotype to lipogenic phenotype, Cas and Oil treated livers were not able to make this transition.

A. Hepatic Expression of Lipogenic Genes



B. Hepatic Expression of Lipid Oxidation Genes



C. Correlation of lipogenic genes with lipid oxidation gene

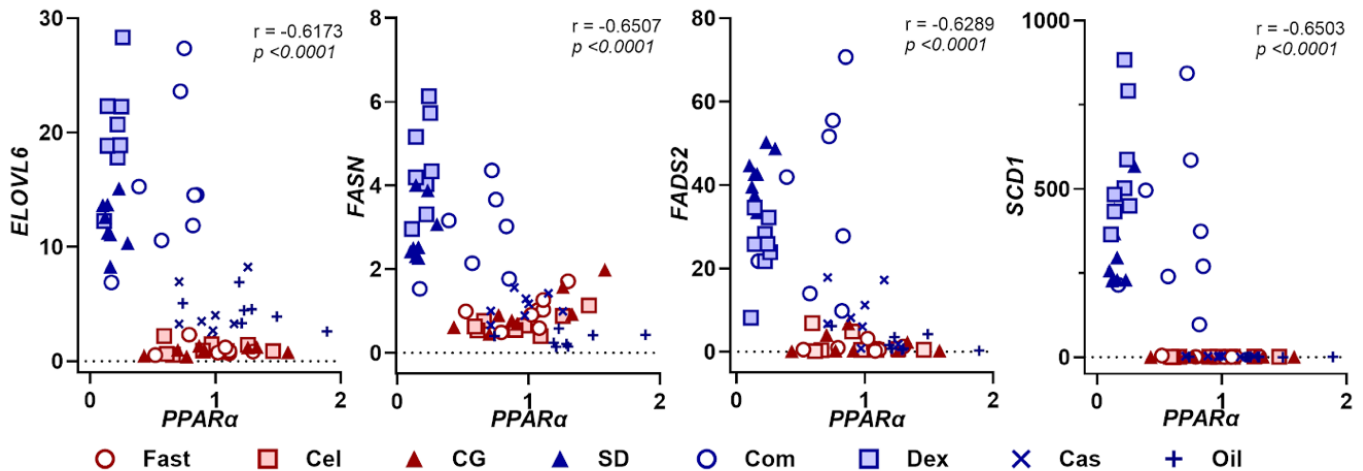


Figure 3.8. Alterations in hepatic lipogenic and lipolytic gene expression in chickens fed dextrin-enriched diet *ad libitum*.

(A) Relative mRNA expression of genes involved in lipogenic pathways, including *ELOVL6*, *FASN*, *FADS2*, and *SCD1*. (B) Relative mRNA expression of genes involved in hepatic lipid oxidation pathways, including *CPT1A*, *PPAR α* , *LCAD*, and *MCAD*. (C) Correlation between lipogenic genes and lipid oxidation genes in the liver across the dietary treatments. Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. *ELOVL6*, Elongation of Very Long Chain Fatty Acids Protein 6; *FASN*, Fatty Acid Synthase; *FADS2*, Fatty Acid Desaturase 2; *SCD1*, Stearoyl-CoA desaturase-1; *CPT1A*, Carnitine Palmitoyltransferase 1A; *LCAD*, Long-Chain Acyl-CoA Dehydrogenase; *Ppara*, Peroxisome Proliferator-Activated Receptor Alpha, *MCAD*, Medium-Chain Acyl-CoA Dehydrogenase; Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

3.4.9. Increased hepatic carbohydrate utilization and storage in chickens fed dextrin-enriched diets *ad libitum*.

Hepatic metabolite concentrations were quantified across dietary treatments (**Table 3.8**; Study 2). Liver glycogen content, the primary storage form of glucose, was significantly elevated in the chickens reared on the dextrin-enriched diet ($P < 0.00$; **Fig. 3.9A**) compared to other dietary treatments. This was followed by SD and Com livers ($P < 0.00$), whereas Cas, Oil, CG, Cel, and Fast livers exhibited similar and lower hepatic glycogen content. Hepatic pyruvate concentrations were significantly higher in the Dex, SD, and Com-treated groups compared to their counterparts ($P < 0.02$; **Fig. 3.9B**). Lactate levels in the liver were significantly elevated in the SD and Dex groups ($P < 0.03$; **Fig. 3.9B**), followed by Com, with lower levels observed in the other dietary treatments. Hepatic alanine concentrations did not differ significantly among the dietary treatments, but the lowest levels were observed in the Fast group (**Fig. 3.9B**). Hepatic β -hydroxybutyrate analysis showed significantly lower levels in SD, Com, and Dex-treated livers compared to the other dietary groups ($P \leq 0.004$; **Fig. 3.9C**). Correlation analysis revealed a significant negative association between hepatic β -hydroxybutyrate and pyruvate and lactate ($P < 0.0001$; **Fig. 3.9D**), whereas this relationship was less evident with alanine. Clustering patterns in the correlation plots showed Dex, SD, and Com livers grouping together, characterized by higher carbohydrate utilization and lower β -hydroxybutyrate levels. In contrast, Cas- and Oil-treated livers clustered with Fast, Cel, and CG groups, indicating sustained lipid oxidation and comparatively lower carbohydrate utilization. Taken together, these data indicate that dextrin administration for 48 hours promotes hepatic carbohydrate utilization and glycogen storage while suppressing lipid oxidation in the post-hatch chickens.

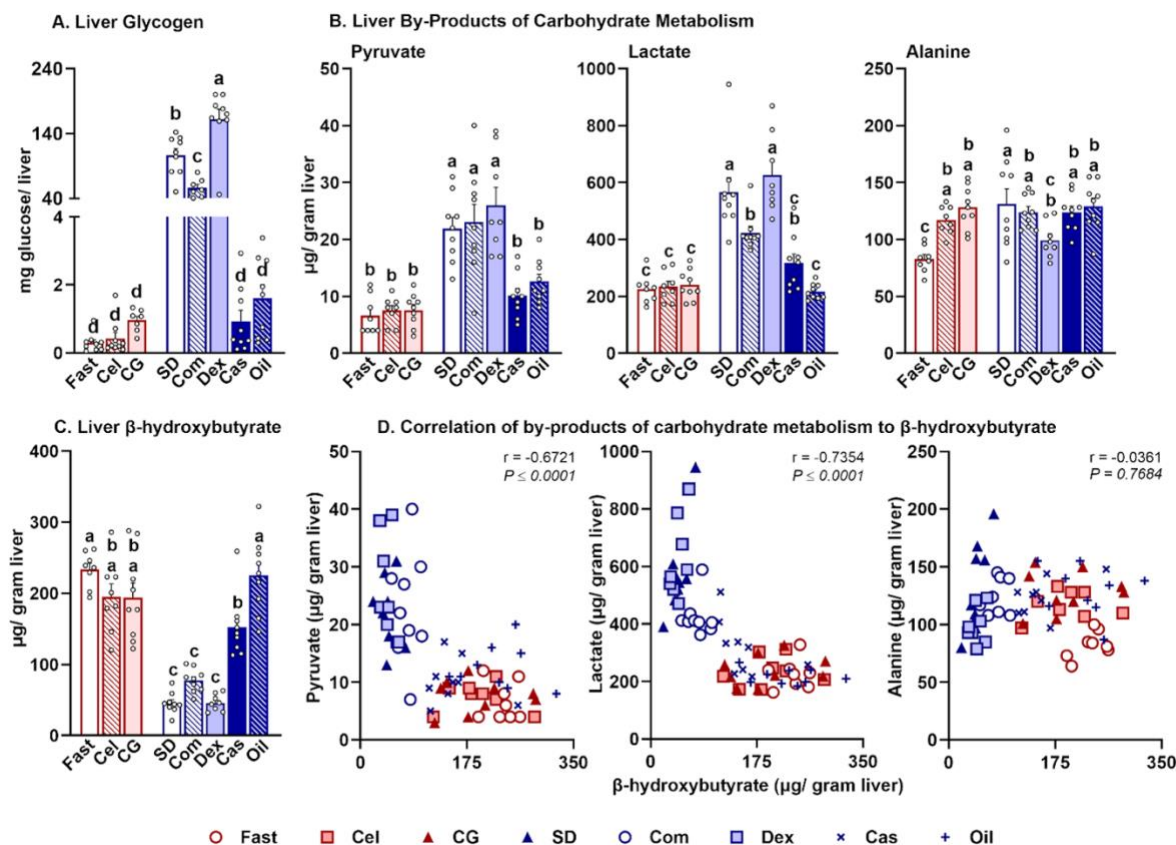


Figure 3.9. Hepatic metabolite levels following *ad libitum* dietary treatments.

(A) Hepatic glycogen content across dietary treatments. (B) Hepatic concentrations of pyruvate, lactate, and alanine. (C) Liver β -hydroxybutyrate levels across the dietary treatments. (D) Correlation analysis between hepatic β -hydroxybutyrate and products of carbohydrate metabolism. Data are represented as mean $\pm$ SEM ($n = 7-9$ /group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

Table 3.8. Abundance of liver metabolites (organic acids and amino acids) in chickens reared on dietary treatments *ad libitum*.

A. TCA Cycle Intermediates								
Hepatic Concentration (µg/ gram liver ± SEM)								
	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Pyruvate	7 ± 1 ^b	7 ± 1 ^b	8 ± 1 ^b	22 ± 2 ^a	23 ± 3 ^a	26 ± 3 ^a	10 ± 1 ^b	13 ± 1 ^b
Lactate	225 ±	234 ±	240 ±	566 ±	424 ±	627 ±	317 ±	217 ±
	18 ^c	19 ^c	20 ^c	52 ^a	25 ^b	49 ^a	23 ^{cb}	10 ^c
Succinate			112 ±	140 ±	106 ±	103 ±	123 ±	113 ±
	90 ± 6 ^c	88 ± 6 ^c	7 ^{ca}	10 ^a	8 ^{cb}	5 ^{cb}	5 ^{ba}	7 ^{ca}
Fumarate				141 ±			112 ±	100 ±
	85 ± 7 ^{db}	70 ± 5 ^{dc}	74 ± 4 ^{dc}	13 ^a	69 ± 3 ^{dc}	59 ± 7 ^d	10 ^{ba}	8 ^{bc}
α-keto		29 ±						
glutarate	29 ± 5 ^{ba}	2.3 ^{ba}	32 ± 4 ^{ba}	34 ± 4 ^{ba}	41 ± 5 ^a	20 ± 2 ^b	36 ± 3 ^{ba}	34 ± 4 ^{ba}
Malate	151 ±	115 ±		298 ±		120 ±	198 ±	162 ±
	9 ^{cb}	10 ^c	133 ± 8 ^c	26 ^a	126 ± 6 ^c	13 ^c	10 ^b	15 ^{cb}
Citrate				11 ±	9 ±			
	4 ± 0.2 ^c	4 ± 0.3 ^c	4 ± 0.2 ^c	0.9 ^a	0.8 ^{ba}	8 ± 0.8 ^b	4 ± 0.3 ^c	5 ± 0.3 ^c

B. Amino Acids

Hepatic Concentration (µg/ gram liver ± SEM)								
	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Alanine	83 ± 4 ^c	117 ± 4 ^{ba}	128 ± 6 ^{ba}	131 ± 14 ^a	124 ± 5 ^{ba}	99 ± 5 ^{cb}	124 ± 6 ^{ba}	129 ± 8 ^{ba}
Glycine	108 ± 5 ^b	135 ± 6 ^b	135 ± 5 ^b	127 ± 6 ^b	137 ± 5 ^b	67 ± 2 ^c	203 ± 16 ^a	125 ± 5 ^b
Valine	82 ± 3 ^{cb}	97 ± 3 ^{cb}	103 ± 5 ^b	56 ± 3 ^{ed}	77 ± 2 ^{dc}	47 ± 1 ^e	170 ± 10 ^a	97 ± 4 ^{cb}
Leucine	97 ± 5 ^c	130 ± 5 ^b	135 ± 6 ^b	82 ± 7 ^c	97 ± 4 ^c	75 ± 2 ^c	174 ± 10 ^a	138 ± 7 ^b
Isoleucine	65 ± 3 ^{cb}	78 ± 3 ^b	79 ± 4 ^b	46 ± 3 ^{ed}	55 ± 2 ^{dc}	38 ± 1 ^e	117 ± 7 ^a	76 ± 3 ^b
Proline	66 ± 5 ^{dc}	76 ± 3 ^{cb}	81 ± 4 ^{cb}	114 ± 6 ^b	116 ± 10 ^b	29 ± 1 ^d	188 ± 24 ^a	64 ± 4 ^{dc}
Methionine	22 ± 2 ^c	35 ± 2 ^{ba}	38 ± 2 ^a	19 ± 1 ^c	22 ± 2 ^c	24 ± 1 ^{cb}	34 ± 3 ^{ba}	43 ± 3 ^a
Serine	82 ± 6 ^c	134 ± 15 ^{cb}	150 ± 18 ^{ca}	210 ± 18 ^{ba}	212 ± 30 ^{ba}	230 ± 20 ^a	166 ± 19 ^{ba}	167 ± 19 ^{ba}
Threonine	180 ± 12 ^{cb}	217 ± 24 ^{ba}	204 ± 17 ^b	170 ± 21 ^{cb}	322 ± 33 ^a	74 ± 5 ^c	188 ± 19 ^{ba}	160 ± 11 ^{cb}
Phenyl alanine	61 ± 5 ^a	76 ± 5 ^a	83 ± 4 ^a	71 ± 7 ^a	62 ± 4 ^a	80 ± 11 ^a	67 ± 4 ^a	115 ± 30 ^a
Aspartate	218 ± 20 ^a	224 ± 10 ^a	248 ± 8 ^a	242 ± 22 ^a	280 ± 19 ^a	90 ± 7 ^b	267 ± 8 ^a	276 ± 13 ^a
Glutamate	613 ± 63 ^a	534 ± 37 ^a	492 ± 59 ^a	655 ± 39 ^a	645 ± 29 ^a	251 ± 17 ^b	612 ± 34 ^a	552 ± 82 ^a

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

3.4.10. Skeletal muscle metabolite profiles reflect reduced ketone abundance in dextrin-fed chickens under *ad libitum* feeding

Muscle metabolite concentrations were quantified across dietary treatments (**Table 3.9**; Study 2). Pyruvate and lactate levels did not differ significantly in the muscle across the dietary treatments (**Fig. 3.10A**). However, alanine concentration in the muscle was significantly higher in the SD and Dex-treated chickens ($P \leq 0.008$; **Fig. 3.10A**), followed by the Com group, whereas Cas, Oil, Fast, Cel, and CG groups had lower and similar alanine levels. Muscle β -hydroxybutyrate levels were significantly lower in SD, Com, and Dex groups ($P < 0.001$; **Fig. 3.10B**), while higher levels were observed in the remaining dietary treatments. Correlation analysis between muscle β -hydroxybutyrate and pyruvate or lactate did not show any clear association (**Fig. 3.10C**). In contrast, β -hydroxybutyrate showed a significant negative correlation with alanine ($P \leq 0.0001$, **Fig. 3.10C**). SD, Com, and Dex groups clustered with higher alanine and lower β -hydroxybutyrate levels, whereas Cas and Oil groups displayed an opposing pattern, similar to that observed in Fast, Cel, and CG groups. These findings suggest that dextrin and starter diet treatments promote a metabolic transition in skeletal muscles, highlighted by reduced ketone abundance and elevated alanine levels, indicating a decreased reliance on lipid oxidation for deriving energy.

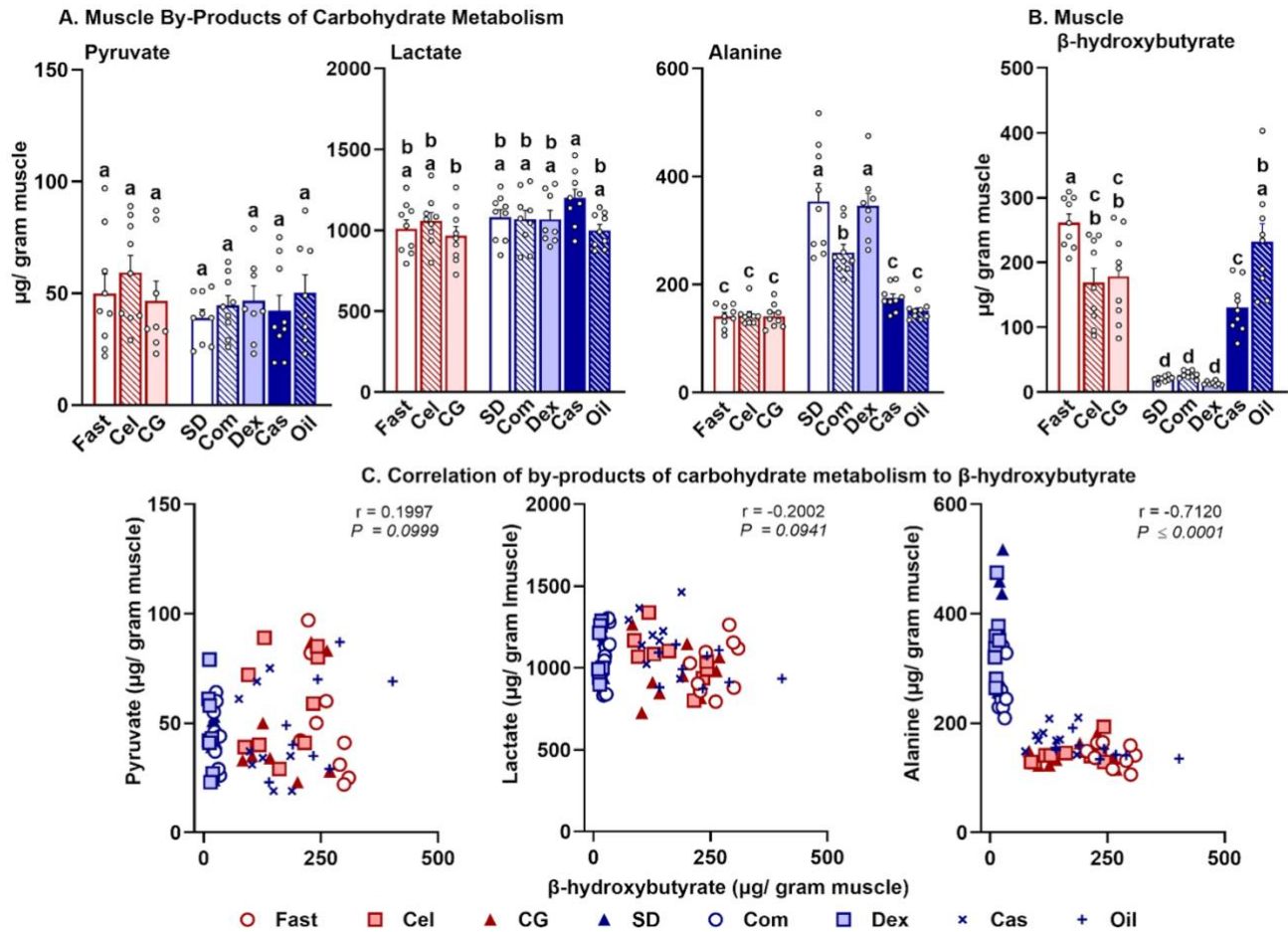


Figure 3.10. Skeletal muscle metabolite levels following *ad libitum* dietary treatments.

(A) Abundance of pyruvate, lactate, and alanine in the muscles. (B) Muscle β-hydroxybutyrate levels across dietary treatments. (C) Correlation between muscle β-hydroxybutyrate and products of carbohydrate metabolism. Data are represented as mean ± SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

Table 3.9. Abundance of muscle metabolites (organic acids and amino acids) in chickens reared on dietary treatments *ad libitum*.

A. TCA Cycle Intermediates								
Muscle Concentration ($\mu\text{g/gram muscle} \pm \text{SEM}$)								
	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Pyruvate	50 $\pm$ 8 ^a	59 $\pm$ 7 ^a	47 $\pm$ 9 ^a	39 $\pm$ 4 ^a	44 $\pm$ 4 ^a	47 $\pm$ 6 ^a	42 $\pm$ 7 ^a	50 $\pm$ 8 ^a
Lactate	1011 $\pm$	1058 $\pm$	968 $\pm$	1080 $\pm$	1068 $\pm$	1067 $\pm$	1200 $\pm$	1001 $\pm$
	53 ^{ba}	50 ^{ba}	56 ^b	47 ^{ba}	56 ^{ba}	56 ^{ba}	54 ^a	35 ^{ba}
Succinate	35 $\pm$ 1 ^{ab}	25 $\pm$ 3 ^b	27 $\pm$ 1 ^b	40 $\pm$ 3 ^a	31 $\pm$ 2 ^{ab}	28 $\pm$ 2 ^{ab}	31 $\pm$ 4 ^{ab}	31 $\pm$ 1 ^{ab}
Fumarate	40 $\pm$ 3 ^{ab}	42 $\pm$ 2 ^a	39 $\pm$ 1 ^{ab}	34 $\pm$ 1 ^{ab}	36 $\pm$ 1 ^{ab}	29 $\pm$ 2 ^b	41 $\pm$ 3 ^a	40 $\pm$ 1 ^{ab}
α-keto glutarate	33 $\pm$ 5 ^{ab}	39 $\pm$ 6 ^a	34 $\pm$ 5 ^{ab}	29 $\pm$ 4 ^{ab}	24 $\pm$ 3 ^{ab}	26 $\pm$ 4 ^{ab}	17 $\pm$ 3 ^b	38 $\pm$ 5 ^{ab}
Malate	77 $\pm$ 4 ^{ab}	74 $\pm$ 3 ^{ab}	79 $\pm$ 4 ^{ab}	70 $\pm$ 3 ^b	67 $\pm$ 4 ^{cb}	55 $\pm$ 3 ^c	87 $\pm$ 2 ^a	75 $\pm$ 1 ^{ab}
Citrate	24 $\pm$ 2 ^{ab}	22 $\pm$ 3 ^{ac}	24 $\pm$ 2 ^{ab}	31 $\pm$ 2 ^a	21 $\pm$ 3 ^{ac}	15 $\pm$ 2 ^{bc}	13 $\pm$ 1 ^c	25 $\pm$ 2 ^{ab}

B. Amino Acids

Muscle Concentration ($\mu\text{g/ gram muscle} \pm \text{SEM}$)

	Fast	Cel	CG	SD	Com	Dex	Cas	Oil
Alanine	141 $\pm$ 7 ^c	143 $\pm$ 6 ^c	141 $\pm$ 7 ^c	354 $\pm$ 33 ^a	259 $\pm$ 15 ^b	346 $\pm$ 22 ^a	175 $\pm$ 7 ^c	151 $\pm$ 5 ^c
Glycine	124 $\pm$ 5 ^{cb}	129 $\pm$ 7 ^{cb}	116 $\pm$ 5 ^c	150 $\pm$ 9 ^{ac}	175 $\pm$ 13 ^a	163 $\pm$ 14 ^{ab}	173 $\pm$ 9 ^a	112 $\pm$ 5 ^c
Valine	69 $\pm$ 3 ^b	59 $\pm$ 3 ^{bc}	63 $\pm$ 3 ^b	42 $\pm$ 2 ^{cd}	57 $\pm$ 3 ^{cb}	35 $\pm$ 1 ^d	158 $\pm$ 9 ^a	52 $\pm$ 2 ^{bd}
Leucine	46 $\pm$ 1 ^b	41 $\pm$ 2 ^{bc}	42 $\pm$ 2 ^{bc}	43 $\pm$ 3 ^{bc}	39 $\pm$ 2 ^{bc}	30 $\pm$ 1 ^c	103 $\pm$ 7 ^a	34 $\pm$ 1 ^{bc}
Isoleucine	55 $\pm$ 3 ^b	47 $\pm$ 3 ^{cb}	46 $\pm$ 2 ^{cb}	28 $\pm$ 2 ^{de}	34 $\pm$ 2 ^{ce}	23 $\pm$ 1 ^e	98 $\pm$ 6 ^a	38 $\pm$ 2 ^{cd}
Proline	44 $\pm$ 2 ^b	48 $\pm$ 3 ^b	45 $\pm$ 2 ^b	192 $\pm$ 21 ^a	151 $\pm$ 15 ^a	89 $\pm$ 6 ^b	197 $\pm$ 15 ^a	41 $\pm$ 2 ^b
Methionine	17 $\pm$ 1 ^{cb}	20 $\pm$ 1 ^{cb}	17 $\pm$ 1 ^{cb}	14 $\pm$ 1 ^c	17 $\pm$ 1 ^{cb}	22 $\pm$ 2 ^{ab}	29 $\pm$ 2 ^a	16 $\pm$ 1 ^{bc}
Serine	108 $\pm$ 7 ^c	147 $\pm$ 13 ^{cb}	111 $\pm$ 9 ^c	220 $\pm$ 19 ^b	348 $\pm$ 27 ^a	323 $\pm$ 33 ^a	119 $\pm$ 5 ^c	181 $\pm$ 11 ^{bc}
Threonine	161 $\pm$ 12 ^{bd}	158 $\pm$ 11 ^{bd}	145 $\pm$ 10 ^{bd}	203 $\pm$ 16 ^{bc}	362 $\pm$ 27 ^a	100 $\pm$ 8 ^d	214 $\pm$ 25 ^b	132 $\pm$ 11 ^{cd}
Phenyl alanine	28 $\pm$ 2 ^{bc}	26 $\pm$ 2 ^{bc}	27 $\pm$ 2 ^{bc}	54 $\pm$ 4 ^a	37 $\pm$ 1 ^b	35 $\pm$ 3 ^{bc}	36 $\pm$ 1 ^b	25 $\pm$ 2 ^c
Aspartate	130 $\pm$ 8 ^{ac}	128 $\pm$ 15 ^{ac}	140 $\pm$ 11 ^{ab}	107 $\pm$ 8 ^{bc}	92 $\pm$ 5 ^{cd}	56 $\pm$ 5 ^d	158 $\pm$ 11 ^a	110 $\pm$ 6 ^{bc}
Glutamate	432 $\pm$ 23 ^{cb}	467 $\pm$ 37 ^{ab}	447 $\pm$ 19 ^{ac}	557 $\pm$ 23 ^a	382 $\pm$ 35 ^{bc}	336 $\pm$ 36 ^c	354 $\pm$ 23 ^{bc}	462 $\pm$ 29 ^{ab}
Tyrosine	20 $\pm$ 0.9 ^c	22 $\pm$ 0.4 ^{cb}	22 $\pm$ 1.9 ^{cb}	30 $\pm$ 1.6 ^b	23 $\pm$ 2.5 ^{cb}	45 $\pm$ 5.9 ^a	13 $\pm$ 1.2 ^c	20 $\pm$ 1.5 ^{bc}

Data are represented as mean $\pm$ SEM (n= 7-9/group) and statistical significance was determined by one-way ANOVA followed by pairwise mean comparisons, with significance set at $p \leq 0.05$. Different alphabetical letters indicate significant differences among dietary treatments. Fast, fasted; Cel, SP-Cellulose; CG, Control Gavage; SD, starter diet; Com, SP-Complete; Dex, SP-Dextrin; Cas, SP-Casein; Oil, SP-Oil.

3.4.11. Distinct plasma and hepatic metabolic profiles in fed and fasted post-hatch chickens

In Study 3, to further investigate the role of hepatic mitochondria in mediating the lipolytic to lipogenic metabolic shift observed in hatchlings, chickens were either fasted or fed starter diets for the first 48 hours post-hatch. Plasma and hepatic metabolic profiles were subsequently analyzed. As expected, plasma β -hydroxybutyrate (BHB) concentrations were significantly elevated in the fasted group compared to its fed counterpart ($P < 0.00$; **Fig. 3.11A**). Most circulating amino acids, including alanine, glycine, leucine, proline, serine, phenylalanine, glutamate, and tyrosine, were higher in the fed conditions ($P < 0.00$). In contrast, valine, isoleucine, and aspartate were significantly lower in the fed group ($P < 0.05$; **Fig. 3.11B**). Branched-chain keto acids, products of branched-chain amino acid catabolism, were also significantly higher in the fasted condition compared to their fed counterparts ($P < 0.05$; **Fig. 3.11C**). Plasma organic acids, including pyruvate, lactate, succinate, fumarate, α -ketoglutarate, malate, and citrate, were higher in the fed condition ($P < 0.01$; **Fig. 3.11D**). Liver metabolite analysis revealed a similar pattern. Liver β -hydroxybutyrate (BHB) level was significantly higher in the fasted group compared to its fed counterpart ($P < 0.00$; **Fig. 3.11E**). Hepatic amino acids, including alanine, glycine, proline, and serine, were significantly higher in the fed conditions ($P < 0.03$; **Fig. 3.11F**), but branched-chain amino acids, valine, leucine, and isoleucine, were significantly lower in the fed conditions ($P < 0.03$). Further, hepatic organic acids, including pyruvate, lactate, succinate, fumarate, malate, and citrate, were significantly higher under fed conditions ($P < 0.00$; **Fig. 3.11G**).

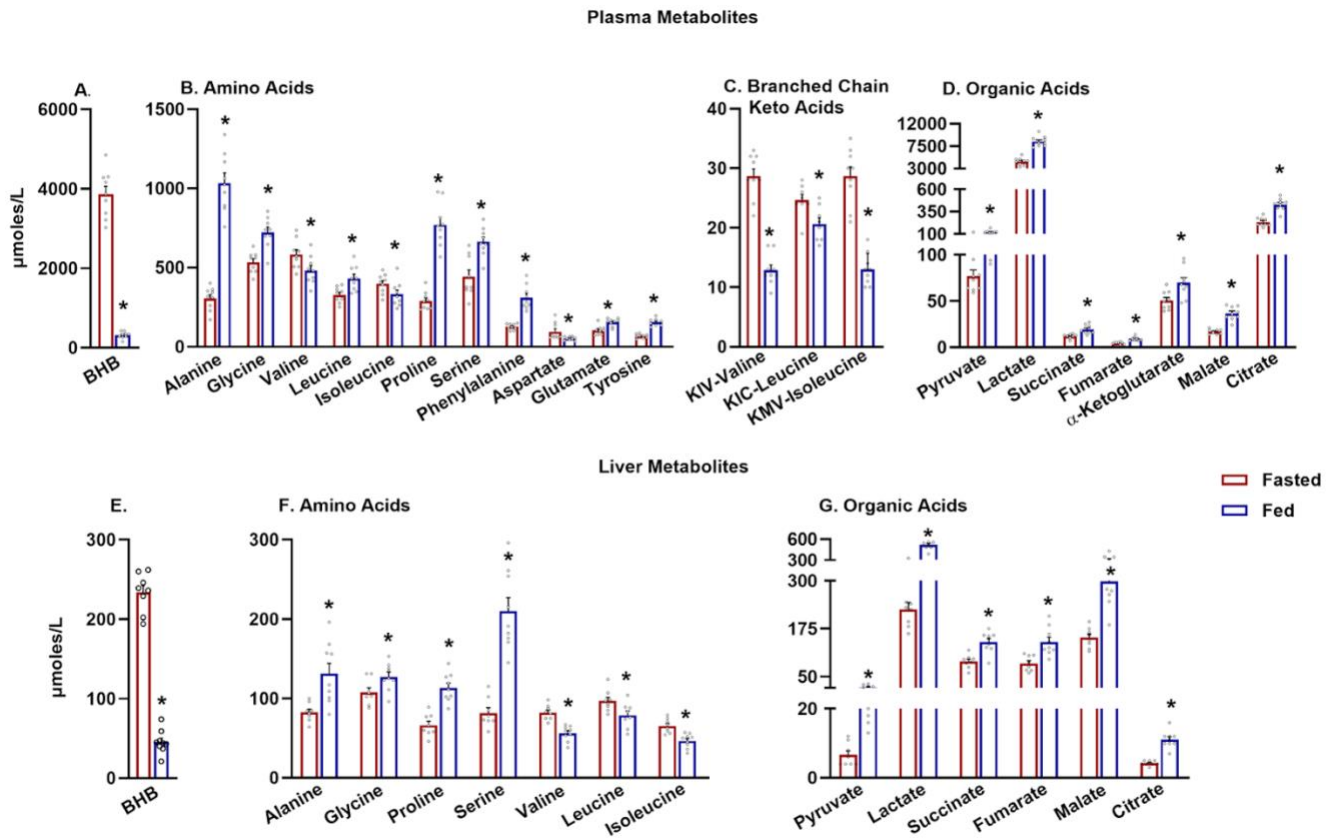


Figure 3.11. Differences in plasma and liver metabolic profiles with feeding and fasting conditions.

Plasma (A) β -hydroxybutyrate (BHB), (B) amino acids, (C) branched-chain keto acids, and (D) organic acid concentrations. Hepatic (E) β -hydroxybutyrate (BHB), (F) amino acids, and (G) organic acid levels.

Data are represented as mean $\pm$ SEM (n= 7-9/group). Statistical significance was determined using an unpaired Student's t-test, with significance set at $P < 0.05$. Asterisks (*) indicate significant differences between fed and fasted groups.

3.4.12. Enhanced mitochondrial TCA cycle activity in hepatic mitochondria of fasted chickens

Isolated hepatic mitochondria were used to estimate basal (0 minutes) metabolite levels and the concentration after 5 minutes of respiration. Under basal conditions, abundance of TCA cycle intermediates, including pyruvate, succinate, α -ketoglutarate, aspartate, citrate, fumarate, malate, and glutamate, was significantly higher in the hepatic mitochondria isolated from the fed group ($P < 0.04$; **Fig. 3.12A**). In contrast, the abundance of other amino acids, including glycine, valine, leucine, isoleucine, proline, methionine, serine, threonine, and phenylalanine, is significantly lower in the fed mitochondria ($P < 0.05$; **Fig. 3.12B**). After 5 minutes of respiration, TCA cycle intermediates, including pyruvate, succinate, aspartate, fumarate, and malate (**Fig. 3.12C**), were lower in the fed group, whereas α -ketoglutarate, citrate, and glutamate remained significantly higher in the fed group ($P < 0.03$; **Fig. 3.12C**). Amino acid abundance in fed mitochondria remained lower relative to fasted controls ($P < 0.05$; **Fig. 3.12D**). Interestingly, fold-change analysis (0 to 5 minutes) revealed a higher abundance of TCA cycle intermediates, including pyruvate, succinate, α -ketoglutarate, aspartate, fumarate, and malate in the fasted chickens ($P < 0.03$; **Fig. 3.12E**), indicating a greater induction of the abundance of the TCA cycle metabolites in the fasted state after 5 minutes of respiration. In contrast, citrate exhibited a significantly greater fold increase in the fed group ($P < 0.02$; **Fig. 3.12E**), indicating that the fed mitochondria are primed to use the mitochondrial citrate to form the building block for fatty acid production via *de novo* lipogenesis in the cytosol. Fold changes of the other amino acids did not differ significantly except for alanine, glycine, and phenylalanine levels (**Fig. 3.12F**). Taken together, these data indicate that mitochondria isolated from fasted livers are primed to induce the TCA cycle activity, which resulted in higher fold change in metabolite levels after 5 minutes of respiration.

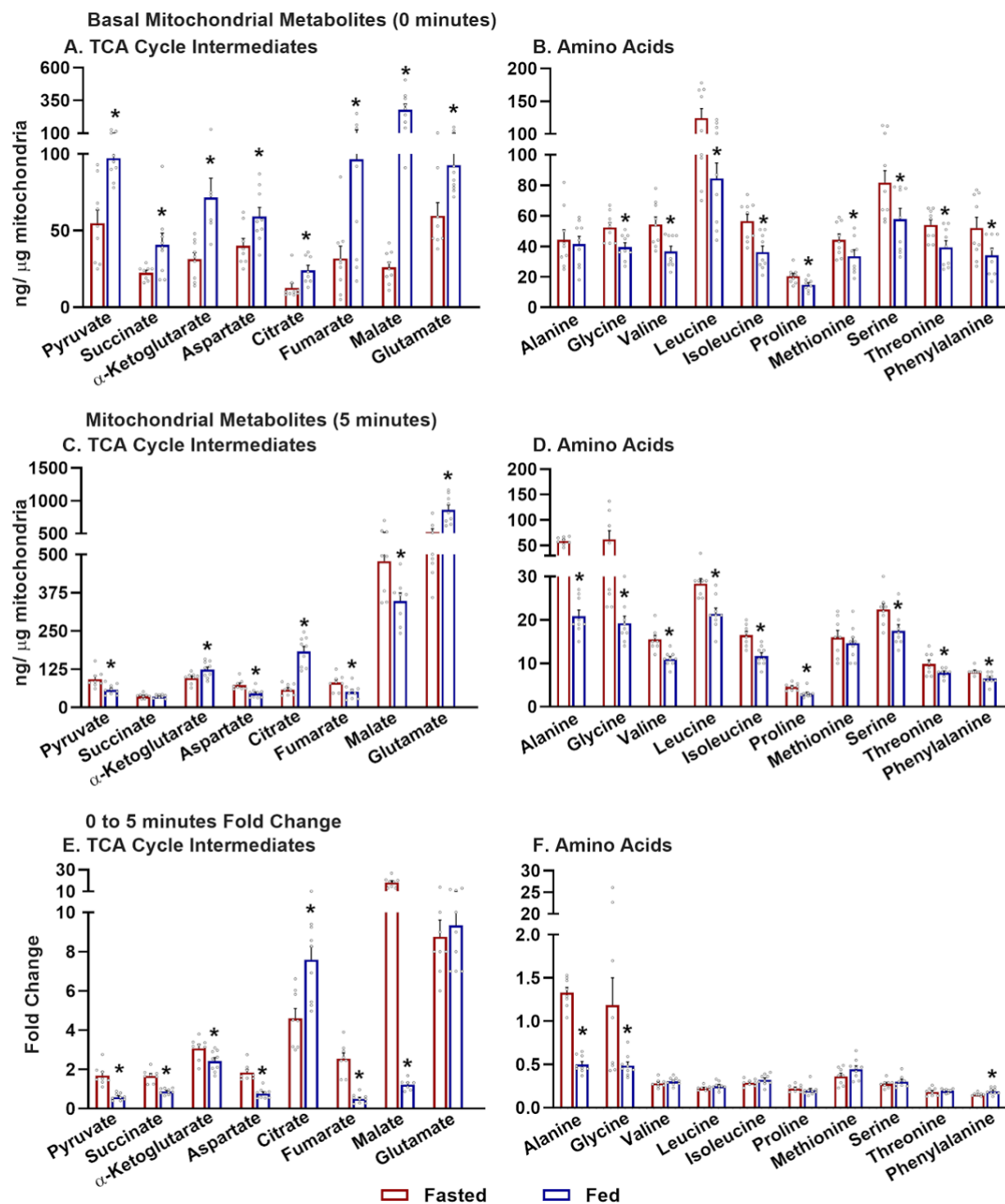


Figure 3.12. Hepatic mitochondrial metabolite levels under fed and fasted conditions.

Abundance of (A) TCA cycle intermediates and (B) other amino acids under basal conditions (0 minutes) in isolated hepatic mitochondria. Abundance of (C) TCA cycle intermediates and (D) other amino acids in isolated mitochondria following 5 minutes of respiration. Fold change (0 to 5 minutes) in mitochondrial (E) TCA cycle intermediates and (F) other amino acids. Data are represented as mean $\pm$ SEM (n= 7-9/group). Statistical significance was determined using an unpaired Student's t-test, with significance set at $P < 0.05$. Asterisks (*) indicate significant differences between fed and fasted groups.

3.4.13. Distinct alterations in hepatic mitochondrial oxidative functions between fed and fasted conditions

Reactive oxygen species (ROS) production was measured using isolated hepatic mitochondria. Mitochondria from chickens reared on the starter diet exhibited significantly higher ROS production compared to those from the fasted group (**Fig. 3.13A**). Maximal oxygen consumption following FCCP stimulation was also significantly elevated in mitochondria isolated from the fed group ($P = 0.03$; **Fig. 3.13B**) compared to their fasted counterparts. To understand the molecular basis of these changes, the expression pattern of several genes involved with mitochondrial metabolism and nutrient shuttle systems was measured (**Fig. 3.13C**). Expression of genes involved in mitochondrial substrate transport, MPC2 and SLC25A13, was significantly downregulated under fed conditions ($P < 0.04$). Expression of ME1, responsible for the conversion of malate to pyruvate in the pyruvate-malate shuttle, was highly upregulated in the fed group ($P < 0.00$). Expression of PDH, a critical link between glycolysis and the TCA cycle, was also highly upregulated in the fed group ($P=0.008$). A similar pattern was observed for CS, which catalyzes the rate-limiting step of the TCA cycle ($P < 0.00$). In contrast, IDH1 and PCK2, encoding enzymes involved in the TCA cycle, were downregulated under fed conditions ($P=0.02$). Collectively, these findings indicate the presence of distinct alterations of hepatic mitochondria activity between fed and fasted conditions. This alteration is accompanied by coordinated changes in the expression pattern of genes encoding TCA cycle enzymes and nutrient shuttle components.

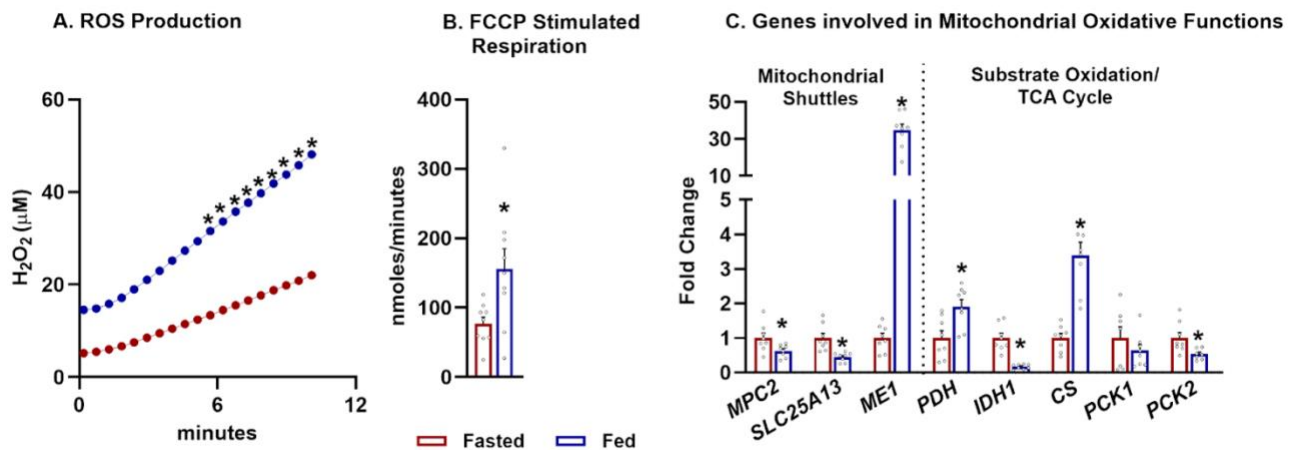


Figure 3.13. Alterations in hepatic mitochondrial activity and nutrient shuttle gene expression under fed and fasted conditions.

(A) Reactive oxygen species (ROS) production by isolated hepatic mitochondria. (B) Oxygen consumption by isolated hepatic mitochondria. (C) Relative mRNA expression of genes involved in mitochondrial oxidative functions under fed and fasted conditions. Data are represented as mean $\pm$ SEM (n= 7-9/group). Statistical significance was determined using an unpaired Student's t-test, with significance set at $P < 0.05$. Asterisks (*) indicate significant differences between fed and fasted groups. MPC2, mitochondrial pyruvate carrier 2; SLC25A13, solute carrier family 25 member 13; ME1, Malic Enzyme 1; PDH, pyruvate dehydrogenase; IDH1, isocitrate dehydrogenase 1; CS, citrate synthase; PCK1, Phosphoenolpyruvate Carboxykinase 1, PCK2; Phosphoenolpyruvate Carboxykinase 2.

3.5. Discussion

The embryonic to post-hatch development in chickens is characterized by a dramatic metabolic transition, characterized by a shift from lipolytic metabolism during embryonic stages to a lipogenic phenotype and carbohydrate utilization in the post-hatch stages [30, 32, 101]. During embryogenesis, the developing chick relies almost exclusively on oxidizing yolk lipids as the primary source of energy [33, 36]. Around 80% of the yolk lipids are absorbed during the final few days of egg incubation, and nearly 90% of the total energy produced is derived from β -oxidation of the yolk lipids during the last week to support rapid embryonic development [100]. During this period, due to the absence of dietary carbohydrates, glycerol formed as a by-product of lipid oxidation is used to fuel gluconeogenesis to sustain circulating glucose levels in the embryo [98]. A residual portion of the yolk is retained at hatch, which acts as a transient nutrient reserve but is rapidly depleted within the first 3 days post-hatch [33-35]. Embryonic metabolism is largely sustained by lipid metabolism to derive energy, with glycerol generated from lipolysis contributing to gluconeogenesis and maintenance of the basal blood glucose level [98]. Following hatching, hatchlings are administered a corn-based exogenous diet, and they rapidly transition towards carbohydrate utilization for deriving energy. This sudden abundance of carbohydrates post-hatch also provides substrates for new lipid synthesis. The neonatal liver undergoes profound biochemical reprogramming wherein lipid oxidation is suppressed, glycolytic flux is upregulated, and *de novo* lipogenesis is robustly induced [30, 31]. Proper initiation of this metabolic switch is essential for the growth of the hatchlings, as delayed feeding for 48 hours suppresses lipogenic gene expression and compromises long-term performance [182]. This underscores the importance of an efficient transition from yolk lipid metabolism to post-hatch lipogenesis and highlights it as a critical determinant of early-life survival and productivity.

The liver serves as a central organ orchestrating this transition, wherein the embryonic liver is responsible for extensive lipid oxidation, and the neonatal liver serves as the site of lipid synthesis, accumulation, and export [99]. Importantly, the rate of hepatic lipid accumulation in neonatal chickens

occurs at around twice the threshold associated with fatty liver conditions in rodents and humans, but without the signs of hepatocellular stress and inflammation [102, 103]. This contrast in physiological tolerance suggests the presence of adaptive regulatory mechanisms that are unique to neonatal chicken livers. Previous research from our lab suggested the dynamic and synergistic remodeling of mitochondrial oxidative networks that enabled the metabolic switching during the embryonic to neonatal transition [30, 31]. In the embryonic liver, acetyl-CoA derived from fatty acid β -oxidation is efficiently channeled toward ketogenesis, preventing lipotoxic accumulation. In the post-hatch liver, the same mitochondrial networks are reconfigured such that acetyl-CoA generated from carbohydrate oxidation is simultaneously directed toward the TCA cycle and lipogenic pathways [30]. This coordinated shift in mitochondrial substrate handling distinguishes the physiological hepatic lipid accumulation in neonatal chickens from the pathological steatosis seen in metabolic disease models.

Although the provision of feed after hatch is known to initiate the metabolic switch, the differential contribution of dietary macronutrients in triggering this metabolic switch remains unclear. Through my study, I systematically examined whether dietary carbohydrates, proteins, or lipids independently drove the metabolic transition and led to the biochemical adaptations required to establish the lipogenic phenotypes in post-hatch livers. I also hypothesized that a coordinated flux through mitochondrial oxidative pathways and nutrient shuttle systems underlies the successful metabolic reprogramming, and that dietary carbohydrates would be both necessary and sufficient to initiate this transition. To address this research gap, two complementary feeding studies were designed in which newly hatched broiler chicks received diets formulated to deliver the majority (85%; by gavage, **Table 3.2**) or the entirety (100%; *ad libitum* feeding, **Table 3.3**) of the caloric content from a single macronutrient, carbohydrates (dextrin), proteins (casein), or fats (corn oil) for the first 48 hours post-hatch. In the initial gavage study, casein-administered chickens did not thrive well, and regurgitated jelly-like, foul-smelling liquid, likely due to microbial overgrowth or fermentation in the crop due to the high protein load. This group was excluded from the downstream analysis in the first study. Additionally,

gavage-related handling stress introduced confounding variability in body and tissue weights across groups (**Fig. 3.1**), which was later addressed in the follow-up experiment, through the provision of custom diets *ad libitum*. Despite these limitations, the metabolic and transcriptomic profiles generated from the dietary treatments in both studies provided clear evidence of macronutrient-specific effects, prominently mediated by dextrin, on the initiation of the metabolic switch.

These findings demonstrate that dietary carbohydrates, dextrin, alone are sufficient and necessary to initiate multiple aspects of the metabolic transition in post-hatch chickens. Administration of dextrin during the first 48 hours post-hatch elevated circulating levels of end products of carbohydrate oxidation, namely pyruvate, alanine, and lactate, and simultaneously suppressed ketone body abundance, indicating reduced lipid oxidation but enhanced carbohydrate metabolism (**Fig. 3.2, 6-7**). This metabolic signature was similar to those observed in chickens administered a starter diet and a complete diet, both of which had a balanced mix of all three macronutrients. On the contrary, casein and oil-enriched diets failed to induce similar metabolic signatures, and instead they resembled metabolic profiles of fasted and day of hatch chickens. This metabolic pattern was consistent in the plasma profiles and both liver (**Fig. 3.4, 9**) and muscle tissues (**Fig. 3.5, 10**).

Consistent with the tissue metabolite data, dextrin feeding upregulated hepatic lipogenic networks as indicated by increased expression pattern of genes involved in *de novo* lipogenesis (ELOVL6 and SCD1) and lowered the expression of genes involved in lipid oxidation (LCAD and PPAR α) (**Fig. 3.3,8**). Hepatic glycogen abundance, indicative of enhanced carbohydrate oxidation and utilization, was also higher in dextrin-treated livers (**Fig. 3.9**). Importantly, β -hydroxybutyrate to acetoacetate ratios remained higher in the casein and oil-administered chickens (**Fig. 3.6**), suggesting the presence of a fasting-like mitochondrial redox environment in these groups, which favors lipid oxidation over carbohydrate utilization. These results together indicate that dietary carbohydrates are the primary driver of the lipolytic to lipogenic transition in neonatal chickens.

Hepatocellular mitochondrial metabolism plays a central role in this transition by mediating the flux of nutrients from the cytoplasmic oxidation of carbohydrates into the mitochondria for the synthesis and export of carbon units necessary for lipid synthesis. I examined the remodeling of hepatic mitochondrial networks under fed and fasted conditions to determine whether a coordinated induction of mitochondrial oxidative machinery is a necessary factor for the induction of lipogenesis after feeding. My results show that feeding induced a shift towards anabolic metabolism as evidenced by increased circulating and hepatic amino acids and organic acids, but a suppression of plasma ketones (**Fig. 3.11**). Investigation of freshly isolated hepatic mitochondrial metabolism indicated that fasted mitochondria exhibited a greater fold increase in several TCA cycle intermediates following 5 minutes of respiration, indicating that fasted mitochondria were primed to activate TCA cycle machinery under this condition (**Fig. 3.12**). Contrastingly, fed mitochondria had higher levels of citrate abundance following respiration, highlighting the anabolic priming to support cytosolic lipid synthesis in this group. Functional analyses demonstrated differential alterations in mitochondrial activity between fed and fasted nutritional states. Mitochondria from fed chicks exhibited higher reactive oxygen species production and elevated maximal oxygen consumption, accompanied by coordinated transcriptional remodeling of nutrient shuttle and oxidative pathways. Upregulation of ME1, PDH, and CS, alongside downregulation of mitochondrial substrate transporters (MPC2 and SLC25A13), indicates remodeling of mitochondrial networks. These findings suggest that feeding induces remodeling of mitochondrial function, aligning mitochondrial metabolism with anabolic lipid synthesis.

In summary, my study demonstrates that dietary carbohydrates, administered as dextrin, alone can initiate the metabolic switch required for post-hatch adaptation in chickens. Carbohydrate availability drives coordinated systemic, hepatic, and muscle metabolic remodeling and promotes lipogenic gene expression while suppressing lipolytic genes. As early post-hatch mortality represents a major economic burden to the poultry industry, my findings suggest that administration of carbohydrate-enriched pre-starter diets during the first 48 hours post-hatch is an important nutritional stimuli/ strategy to improve

metabolic adaptation and early growth performance of the hatchlings. Furthermore, understanding that remodeling of mitochondrial oxidative networks is an integral component of this transition and will further help us to target specific nutritional interventions to optimize neonatal metabolic health in broiler production.

Overall Summary and Future Directions

4.1. Overall Summary

The liver functions as a central metabolic organ responsible for coordinating whole-body energy metabolism through the tightly regulated integration of carbohydrates, lipids, and amino acid metabolism across nutritional states of the animal [1, 2, 183]. Hepatic metabolism is characterized by its metabolic plasticity, the ability to dynamically drift between catabolic and anabolic processes depending on the hormonal and nutritional cues [184-186]. Hepatic mitochondria play a central role in this metabolic adaptability by integrating oxidative metabolism, biosynthetic pathways, and cellular energy production [62, 187]. Despite the extensive knowledge about how individual metabolic pathways operate, how coordinated nutrient flux through mitochondrial networks establishes distinct metabolic phenotypes across physiological and pathological conditions remains poorly understood.

The overarching hypothesis of this dissertation was that coordinated nutrient flux through mitochondrial oxidative networks and nutrient transport systems is required to establish biochemical signatures that upregulate the process of lipogenesis in the liver across diverse physiological states [29]. To test my hypothesis, two distinct biological models were utilized: i) diet-induced metabolic dysfunction associated steatotic liver disease (MASLD) in mice and ii) the embryonic to post-hatch metabolic transition in developing chickens. Collectively, these two models allowed us to investigate how mitochondrial oxidative machinery in the liver interacts and changes cytoplasmic processes like carbohydrate oxidation and lipid synthesis during disease progression and during rapid phases of growth and development.

The first half of the dissertation examined whether elevated rates of hepatic *de novo* lipogenesis (DNL), induced by fructose consumption, contribute to the remodeling of mitochondrial oxidative networks during MASLD. Using diet induced mouse models of MASLD and also fructose in drinking water challenges, this work demonstrated that fructose-induced *de novo* lipogenesis occurred concurrently with higher mitochondrial oxidative activities during fatty liver conditions in mice [29].

Despite similar degrees of hepatic steatosis between high-fat and high-fat/high-fructose diets, dietary fructose resulted in i) increased expression of lipogenic and lipolytic genes, ii) elevated

mitochondrial oxygen consumption, iii) greater flux through the TCA cycle, iv) increased ketone production during fasting, and v) higher mitochondrial reactive oxygen species production [29]. Further, stable isotope-based analyses revealed increased flux through mitochondrial networks and nutrient shuttle systems, including malate-aspartate shuttle, pyruvate-malate shuttle, and citrate-isocitrate shuttle. These findings demonstrate that elevated lipogenesis is tightly coupled with increased mitochondrial oxidative capacity, indicating that overactive mitochondria are an early adaptive response to the nutritional load during fatty liver condition. Collectively, the data support that mitochondrial oxidative networks are initially upregulated to accommodate excess nutrients in the liver, potentially leading to chronic oxidative stress and metabolic disarray. Sustained activation of lipogenesis along with mitochondrial oxidative pathways can lead to hepatocellular stress and exacerbate the pathophysiology during MASLD progression.

The second half of the dissertation examined the embryonic to post-hatch transition in chickens as a physiological model to study rapid metabolic reprogramming [30, 32, 33]. Unlike fatty liver conditions in rodents and humans with MASLD, embryonic and neonatal chickens undergoing rapid hepatic lipid oxidation and lipid synthesis do not develop typical symptoms of inflammation and metabolic dysfunction [102, 103]. This highlights the significance and relevance of the embryonic to post-hatch period in the chicken as a model to investigate mechanisms favoring efficient metabolic adaptations.

The major macronutrient stimuli responsible for the efficient shift from embryonic lipid metabolism to post-hatch carbohydrate metabolism was investigated. My work demonstrated that dietary carbohydrates alone can initiate the metabolic switch from embryonic lipid oxidation to post-hatch lipogenesis. Administration of dextrin in the first 48 hours post-hatch i) robustly suppressed ketogenesis and hepatic lipid oxidation, ii) increased circulating and tissue metabolites associated with carbohydrate utilization, iii) upregulated hepatic lipogenic gene expression, and iv) increased glycogen accumulation in the liver. Contrastingly, protein and lipid administration failed to initiate the lipogenic pathways and maintained fasting-like metabolic conditions. Furthermore, mitochondrial analyses suggested that feeding induced anabolic processes characterized by increased mitochondrial citrate production, altered oxygen

consumption, elevated reactive oxygen species generation, and transcriptional remodeling of mitochondrial oxidative pathways. These findings establish dietary carbohydrate availability as the primary nutritional signal driving early post-hatch metabolic adaptation and demonstrate that mitochondrial metabolic networks actively coordinate this transition by redirecting substrate flux toward biosynthetic pathways (e.g., lipid synthesis).

In summary, this dissertation demonstrates that mitochondrial oxidative networks have a predominant role in determining the hepatic metabolic phenotype. A coordinated nutrient flux through mitochondrial pathways determines the lipogenic or lipolytic state of the liver across both disease and developmental contexts. Hepatic mitochondrial remodeling events underlie both physiological and pathological lipid accumulation; in neonatal chickens, coordinated mitochondrial adaptation supports efficient metabolic switching without cellular stress, but in MASLD, chronic nutrient stress leads to sustained mitochondrial activation, leading to disease progression. Together, these findings highlight mitochondrial metabolic coordination as a central determinant of hepatic metabolic health.

4.2. Future Directions

The findings from the dissertation open several avenues for further investigation with an aim to advance mechanistic understanding of hepatic mitochondrial metabolism, its relationship with several other cytoplasmic networks, and the overall cellular redox, and translate these insights into therapeutic and nutritional strategies to optimize growth and development or to manage metabolic diseases.

Based on the findings from my studies on mice with MASLD, it is clear that the coordinated flux through mitochondrial oxidative networks and nutrient shuttle systems plays a central part in reprogramming the liver during the progression of insulin resistance, lipid accumulation, and metabolic dysfunction. However, the causal relationship and the precise interactions between the cytoplasmic and mitochondrial networks contributing to these remodeling remains to be systematically investigated. Genetic manipulation or pharmacological inhibition of mitochondrial nutrient transport systems, including malate-aspartate shuttle, pyruvate-malate shuttle, and citrate-isocitrate shuttle, would help determine whether disrupting the nutrient flux through these networks would affect TCA cycle

metabolism and hepatic lipogenesis. Here, identifying the directionality of flux through these networks is critical to determine whether upregulation or downregulation of any one or more of these networks has any benefits on overall liver metabolic function. These future studies will further help us to strategize and develop specific therapeutic targets for or against one or more of these pathways to reduce unwanted lipid accumulation in the liver during insulin resistance and MASLD.

The results from the post-hatch chicken studies identified carbohydrates as the major initiator of the metabolic switch in neonatal chicks. The practical application of these results for the poultry industry is towards using this information to improve embryonic to post-hatch transition and in turn, reduce early-stage morbidity and mortality rates. In the future, we can evaluate how variation in carbohydrate composition influences the efficiency of this metabolic switch, with insights into specific sources and types of carbohydrates and their impact on mitochondrial networks. Evaluating the impact of the carbohydrate source, their digestibility, and the duration of feeding immediately post-hatch will help to optimize mitochondrial adaptations in the liver and improve early growth performance and survivability. Furthermore, the mechanistic interactions between the cytoplasmic and mitochondrial networks as described in the previous paragraph still need to be investigated in the embryonic to post-hatch period in the chicken. To better understand the macronutrient-specific effects on mitochondrial function and lipid accumulation, future studies need to be conducted using microencapsulated diets to minimize palatability and intake differences and to reduce variability associated with animal handling and feed preference between treatments. This would allow precise control of nutrient uptake while maintaining equivalent caloric consumption and palatability across dietary treatment groups, enabling clearer interpretation of the contribution of individual macronutrients towards mitochondrial remodeling and lipid synthesis.

Overall, this dissertation establishes coordinated mitochondrial nutrient flux as a fundamental regulator of hepatic metabolic activity across early stages of growth and development and during the onset of metabolic disease. By linking lipogenesis, mitochondrial oxidative function, and nutrient availability within a unified framework, this work advances our understanding of how metabolic networks adapt and remodel, or fail to adapt, during changing physiological demands or during insulin resistance

and metabolic dysfunction. Continued investigation of mitochondrial mechanisms regulating various aspects of cellular redox and function holds significant promise for improving strategies to prevent metabolic disease and to optimize developmental metabolic health.

Appendices

Appendix Table 2.1. Composition of the rodent diets from Research Diets Inc.

	Low Fat (LF)		High Fat (HF)		High Fat/ High Fructose (HF/HFr)	
	g%	kcal%	g%	kcal%	g%	kcal%
Protein	19	20	26	20	21	20
Carbohydrate	67	70	26	20	57	55
Fat	4	10	35	60	12	25
Total		100		100		100
kcal/g	3.8		5.2		4.2	
Ingredient	g	kcal	g	kcal	g	kcal
Casein	200	800	200	800	200	800
L-Cystine	3	12	3	12	3	12
Corn Starch	506	2025	0	0	0	0
Maltodextrin 10	125	500	125	500	125	500
Sucrose	68.8	275	68.8	275	68.8	275
Fructose	0	0	0	0	353.8	1415
Cellulose, BW200	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225
Lard	20	180	245	2205	87.7	789
Mineral Mix, S10026	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0
Potassium Citrate, 1 H ₂ O	16.5	0	16.5	0	16.5	0
Vitamin Mix, V10001	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0
Total	1055	4057	773.8	4057	970.3	4057

Appendix Table 2.2. Composition of the NIH-07 mice/rodent diets (7022) from Inotiv Inc.

Normal Chow

Macronutrients		
Crude Protein	%	22.5
Fat (ether extract)	%	5.2
Carbohydrate (available)	%	42.7
Crude Fiber	%	3.7
Neutral Detergent Fiber	%	11.7
Ash	%	6.7
Energy Density	kcal/g (kJ/g)	3.1 (13.0)
Calories from Protein	%	29
Calories from Fat	%	15
Calories from Carbohydrate	%	56

Appendix Table 2.3. Primer sequences used for qPCR analysis in mice tissues (C57BL/6NJ)

<i>Acc1</i>	Forward: 5'-GGACAGACTGATCGCAGAGAAAG-3' Reverse: 5'-TGGAGAGCCCCACACACA-3'
<i>Acly</i>	Forward: 5'-TGGATGCCACAGCTGACTAC-3' Reverse: 5'-GGTTCAGCAAGGTCAGCTTC-3'
<i>Cd36</i>	Forward: 5'-TGGTCAAGCCAGCTAGAAA-3' Reverse: 5'-CCCAGTCTCATTTAGCCAC-3'
<i>Cpt1a</i>	Forward: 5'-CAAAGATCAATCGGACCCTAGAC-3' Reverse: 5'-CGCCACTCACGATGTTCTTC-3'
<i>Elovl6</i>	Forward: 5'-TGCCATGTTTCATCACCTTGT-3' Reverse: 5'-TGCTGCATCCAGTTGAAGAC-3'
<i>Fasn</i>	Forward: 5'-GCTGCGGAAACTTCAGGAAAT-3' Reverse: 5'-AGAGACGTGTCACTCCTGGACTT-3'
<i>Lcad</i>	Forward: 5'-TCAATGGAAGCAAGGTGTTCA-3' Reverse: 5'-GCCACGACGATCACGAGAT-3'
<i>Mcad</i>	Forward: 5'-GATGCATCACCTCGTGTAAC-3' Reverse: 5'-AAGCCCTTTTCCCCTGAAG-3'
<i>Pgc1a</i>	Forward: 5'-AGACAAATGTGCTTCCAAAAAGAA-3' Reverse: 5'-GAAGAGATAAAGTTGTTGGTTTGGC-3'
<i>Ppara</i>	Forward: 5'-ACAAGGCCTCAGGGTACCA-3' Reverse: 5'-GCCGAAAGAAGCCCTTACAG-3'
<i>Hmgcs2</i>	Forward: 5'-GACTTCCTGTCATCCAGC-3' Reverse: 5'-GGTGTAGGTTTCTTCCAGC-3'
<i>Ppib</i>	Forward: 5'-GGAGATGGCACAGGAGGAA-3'

	Reverse: 5'-GCCCCGTAGTGCTTCAGCTT-3'
<i>Slc25A13</i>	Forward: 5'-GGTGCAATCCAACCAGAGAG-3' Reverse: 5'-TGGGCCTCGGCCAAGTTGA-3'
<i>Slc25A11</i>	Forward: 5'-CCCTCACCAGCATCCTGAA-3' Reverse: 5'-GCGCAGTAGACCAGCTGACA-3'
<i>Mpc1</i>	Forward: 5'-TTTCGCCCTCTGTTGCTATT-3' Reverse: 5'-TCGTAGTTGATAAGTCGTCCTCC-3'
<i>Mpc2</i>	Forward: 5'-CTGGGCTCCAATAATGAAATG-3' Reverse: 5'-AAATAAACCCCTGTAGCCATCAAC-3'
<i>Slc25A1</i>	Forward: 5'-CCCCATGGAGACCATCAAG-3' Reverse: 5'-CCTGGTACGTCCCCCTTCAG-3'
<i>Got1</i>	Forward: 5'-CCGGTTCTGGTCTTTAAGCTC-3' Reverse: 5'-CAGGTACTCGTGGTTGAGG-3'
<i>Mdh1</i>	Forward: 5'-AGATTGCCTTCAAAGACCTGG-3' Reverse: 5'-GCAGTTCGTATTGGCTGGGT-3'
<i>Mdh2</i>	Forward: 5'-TTGGGCAACCCCTTTCCTC-3' Reverse: 5'-GCCTTTCACATTTGCTCTGGTC-3'
<i>Pdh</i>	Forward: 5'-CCATGAGAAGAGCCCAGAAGA-3' Reverse: 5'-GAACTTTGACCAGCGTGTCTACAA-3'
<i>Me1</i>	Forward: 5'-AGTATCCATGACAAAGGGCACAT-3' Reverse: 5'-GCCAGTTTACCCACAGGGG-3'
<i>CS</i>	Forward: 5'-AGCTGTAGCTCTCTCCCTTC-3' Reverse: 5'-TGCTTCAGTCCCGGTCATCCTA-3'
<i>Idh1</i>	Forward: 5'-ATGCAAGGAGATGAAATGACACG-3'

	Reverse: 5'-GCATCACGATTCTCTATGCCTAA-3'
<i>Scd1</i>	Forward: 5'-GCGATACACTCTGGTGCTCA-3' Reverse: 5'-CCCAGGGAAACCAGGATATT-3'
<i>ND1</i>	Forward: 5'-CTAGCAGAAACAAACCGGGC-3' Reverse: 5'-CCGGCTGCGTATTCTACGTT-3'
<i>16S</i>	Forward: 5'-CCGCAAGGGAAAGATGAAAGAC-3' Reverse: 5'-TCGTTTGGTTTCGGGGTTTC-3'
<i>H2K</i>	Forward: 5'-GCCAGCCTCTCCTGATTTTAGTGT-3' Reverse: 5'-GGGAACACAAAAGACCTCTTCTGG-3'

Appendix Table 3.1. Primer sequences used for qPCR analysis in chicken tissues (Ross 708)

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>ELOVL6</i>	CCG GGC CAA TGA ACA TGT	TCA TGC TCG TTG AAC TGC TTC T
<i>SCD1</i>	CAA TGC CAC CTG GCT AGT GA	CGG CCG ATT GCC AAA C
<i>LCAD</i>	GGG AGA GCA CCA GGA AGT TCT	GCC ATC CTT CTC CCA TTC TG
<i>PPARα</i>	ATA ACA GAC ACC CTT TCA CCA GC	TGT TTT CTG CTC CAC TGG GT
<i>FASN</i>	GGC TAC ACA CTA GTT GGC ACT	CAC TGT GTT CCC ATG CCT GA
<i>FADS2</i>	TCT TAA TGG GGA GGG AAC AGG T	ACC AGC TTC TTG CAT TTT CAC A
<i>CPT1A</i>	TGC TCA CTA CCG AGA CAT GG	GGT ACA TGA CCG GAC GGT TT
<i>MCAD</i>	CAC GCA GTA GGC ACA CAT GAT	CGG CAG CAG CGC AAA T
<i>MPC2</i>	GCA GTG CGA CCG AGC GGT AC	GGG TGG TTG TAG AAG GGC CGG
<i>SLC25A13</i>	ACT TAG CGC GTG TCT GTA GA	ATT CCA AAC ACC AGC AAC CC
<i>ME1</i>	GCC AGC ATT ACG GTT TAG CAT	CCA TAA CAG CCA AGG TCT CCA
<i>PDH</i>	GCC ATA AAG CCT ACA GAC	CGA CAA TAC CAT TAC CAC C
<i>IDH1</i>	CTC TGT TGC ACA AGG TTA TGG C	AAT GGG GTT CGT GGA GGT TT
<i>CS</i>	AGG GAT TTC ATC TGG AAC ACA CT	CAC CGT GTA GTA CTT CAT CTC CCT
<i>PCK1</i>	GCA GGG GTT ATG ATG AGA AGT	ACG GAT CAC AGT TTT GAA GAC
<i>PCK2</i>	CCT TCG CCA TGA GCC CCT TTT TC	CAG CTC CGC CAT GAC ATC CCT

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