Impact of maternal high-fat consumption on offspring exercise performance, skeletal muscle energy metabolism, and obesity susceptibility

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„Lehrjahre sind keine Herrenjahre.“

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ABBREVIATIONS

AMPK	AMP-activated protein kinase
Atf3	Activating transcription factor 3
Atf4	Activating transcription factor 4
Atf6	Activating transcription factor 6
ATP	Adenosine triphosphate
Atp5b	Mitochondrial ATP synthase, beta subunit
Bcl6	B-cell lymphoma 6
CD36	Cluster of differentiation 36
Chop	DNA damage-inducible transcription 3
Cpt1b	Carnitine palmitoyltransferase 1b
Ctf1	Cardiotrophin 1
Cytc	Cytochrome c
DEPC	Diethyl pyrocarbonate
Dyrk2	Dual specificity tyrosine phosphorylation-regulated kinase 2
ER	Endoplasmic reticulum
Esrrα	Estrogen-related receptor α
Fatp3	Fatty acid transport protein 3
Fatp4	Fatty acid transport protein 4
Flt1	Vascular endothelial growth factor receptor 1
Fndc5	Fibronectin type III domain containing 5
Gadd34	Growth arrest and DNA damage-inducible protein 34
Glut1	Glucose transporter 1
Glut4	Glucose transporter 4
HFD	High-fat diet
HIF1α	Hypoxia-inducible factor 1α
Il15	Interleukin 15
IL6	Interleukin 6
IRE1α	Inositol-requiring enzyme 1α
LDH	Lactate dehydrogenase
LFD	Low-fat diet
MAPK	Mitogen-activated protein kinase
Mct1	Monocarboxylate transporter 1

Mct4	Monocarboxylate transporter 4
MHC	Myosin heavy chain
Ncoa1	Nuclear receptor coactivator 1
Ndufa5	NADH: Ubiquinone oxidoreductase subunit A5
NMR	Nuclear magnetic resonance spectroscopy
Nr4a1	Nuclear receptor subfamily 4 group A member 1
Ostn	Osteocrin
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PGC1α	Peroxisome proliferating receptor ɣ coactivator 1α
Popdc3	Popeye domain containing 3
Rpl13a	60S ribosomal protein L13a
RW	Running wheel
SERCA	Sarcoplasmic/endoplasmic reticulum calcium ATPase
Sik1	Salt-inducible kinase 1
Tfam	Mitochondrial transcription factor A
TG	Triglyceride
Tnfrsf1a	TNF receptor superfamily member 1a
UPR	Unfolded protein response
VEGFß	Vascular endothelial growth factor ß
wk	Week

ABSTRACT

Background: Obesity is thought to be the consequence of an unhealthy nutrition and a lack of physical activity. Although the resulting metabolic alterations such as impaired glucose homeostasis and insulin sensitivity can usually be improved by physical activity, some obese patients fail to enhance skeletal muscle metabolic health with exercise training. Since this might be largely heritable, maternal nutrition during pregnancy and lactation is hypothesized to impair offspring skeletal muscle physiology.

Objectives: This PhD thesis aims to investigate the consequences of maternal high-fat diet (mHFD) consumption on offspring skeletal muscle physiology and exercise performance. We could show that maternal high-fat diet during gestation and lactation decreases the offspring's training efficiency and endurance performance by influencing the epigenetic profile of their skeletal muscle and altering the adaptation to an acute exercise bout, which in long-term, increases offspring obesity susceptibility.

Experimental setup: To investigate this issue in detail, we conducted several studies with a similar maternal feeding regime. Dams (C57BL/6J) were either fed a low-fat diet (LFD; 10 energy% from fat) or high-fat diet (HFD; 40 energy% from fat) during pregnancy and lactation. After weaning, male offspring of both maternal groups were switched to a LFD, on which they remained until sacrifice in week 6, 15 or 25. In one study, LFD feeding was followed by HFD provision from week 15 until week 25 to elucidate the effects on offspring obesity susceptibility. In week 7, all mice were randomly allocated to a sedentary group (without running wheel) or an exercised group (with running wheel for voluntary exercise training). Additionally, treadmill endurance tests were conducted to investigate training performance and efficiency. In order to uncover regulatory mechanisms, each study was combined with a specific analytical setup, such as whole genome microarray analysis, gene and protein expression analysis, DNA methylation analyses, and enzyme activity assays.

Results: mHFD offspring displayed a reduced training efficiency and endurance capacity. This was not due to an altered skeletal muscle phenotype with changes in fiber size, number, and type. DNA methylation measurements in 6 week old offspring showed a hypomethylation of the Nr4a1 gene in mHFD offspring leading to an increased gene expression. Since Nr4a1 plays an important role in the regulation

of skeletal muscle energy metabolism and early exercise adaptation, this could affect offspring training efficiency and exercise performance in later life.

Investigation of the acute response to exercise showed that mHFD offspring displayed a reduced gene expression of vascularization markers (Hif1a, Vegfb, etc) pointing towards a reduced angiogenesis which could possibly contribute to their reduced endurance capacity. Furthermore, an impaired glucose utilization of skeletal muscle during the acute exercise bout by an impaired skeletal muscle glucose handling was evidenced by higher blood glucose levels, lower GLUT4 translocation and diminished Lactate dehydrogenase activity in mHFD offspring immediately after the endurance test. These points towards a disturbed use of glucose as a substrate during endurance exercise. Prolonged HFD feeding during adulthood increases offspring fat mass gain in mHFD offspring compared to offspring from low-fat fed mothers and also reduces their insulin sensitivity pointing towards a higher obesity and diabetes susceptibility despite exercise training. Consequently, mHFD reduces offspring responsiveness to the beneficial effects of voluntary exercise training.

Conclusion: The results of this PhD thesis demonstrate that mHFD consumption impairs the offspring's training efficiency and endurance capacity, and reduced the beneficial effects of exercise on the development of diet-induced obesity and insulin resistance in the offspring.

This might be due to changes in skeletal muscle epigenetic profile and/or an impaired skeletal muscle angiogenesis and glucose utilization during an acute exercise bout, which could contribute to a disturbed adaptive response to exercise training.

ZUSAMMENFASSUNG

Hintergrund: Übergewicht ist die Folge einer ungesunden Ernährung und einem Mangel an körperlicher Aktivität. Obwohl die daraus resultierenden metabolischen Veränderungen wie die beeinträchtigte Glukose-Homöostase und die Insulinsensitivität in der Regel durch körperliche Aktivität verbessert werden können, sind einige adipöse Patienten nicht in der Lage ihren Skelettmuskel-Metabolismus durch regelmäßiges Training zu verbessern. Da dies weitgehend vererbbar sein könnte, wird vermutet, dass die maternale Ernährung während der Gestation und Laktation einen beeinträchtigten Energiestoffwechsel des Skelettmuskels der Nachkommen begünstigt.

Ziel: Ziel dieser Dissertation war es, den Einfluss der maternalen Hochfett-Diät (mHFD) auf den Skelettmuskel des Nachkommens zu untersuchen. Wir konnten zeigen, dass eine mHFD während der Gestation und Laktation die Trainingseffizienz und die Ausdauerleistung der Nachkommen verringert, verursacht durch die Veränderung des epigenetischen Profils des Skelettmuskels der Nachkommen und der verminderten Anpassung an eine akute Trainingsleistung, was langfristig die Anfälligkeit für die Entwicklung einer Adipositas im Nachkommen erhöht.

Experimentelles Setup: Um dieses Thema ausführlich zu erforschen, hatten wir ein komplexes Studiendesign. Allen vier Studien ging dasselbe maternale Fütterungsregime voraus. Weibliche C57BL/6J Mäuse wurden entweder mit einer Niedrigfett-Diät (NFD; 10 Energie% aus Fett) oder Hochfett-Diät (HFD; 40 Energie% aus Fett) während der Gestation und Laktation gefüttert. Nach Absatz wurden die männlichen Nachkommen beider Gruppen auf eine LFD umgestellt, auf der sie bis zum Ende der jeweiligen Studie in der Woche 6, 15 oder 25 blieben. In einer Studie folgte auf die LFD-Fütterung eine HFD-Versorgung von Woche 15 bis Woche 25, um den Einfluss der maternalen Diät auf die Entwicklung einer Adipositas der Nachkommen aufzuklären. In der 7. Woche wurden alle Mäuse zufällig einer sesshaften Gruppe (ohne Laufrad) oder einer Trainingsgruppe (mit Laufrad für freiwilliges Trainingstraining) zugewiesen. Darüber hinaus wurden Laufband-Ausdauertests durchgeführt, um die Trainingsleistung und -effizienz zu untersuchen. Um die regulatorischen Mechanismen aufzudecken, wurde jede Studie mit einem spezifischen analytischen Aufbau kombiniert, wie z. B. einer

Mikroarray-Analyse, Gen- und Protein-Expressionsanalysen, DNA-Methylierungsanalysen und Enzymaktivitäts-Assays.

Ergebnisse: mHFD Nachkommen zeigten eine reduzierte Trainingseffizienz und Ausdauerkapazität. Dies ist nicht auf einen veränderten Skelettmuskel-Phänotyp mit Veränderungen der Muskelfasergröße, der Anzahl und der Muskelfasertypen zurückzuführen. DNA-Methylierungsmessungen bei 6 Wochen alten Nachkommen zeigten eine Hypomethylierung des Nr4a1-Gens in mHFD-Nachkommen, was wiederum in einer erhöhten Genexpression resultierte. Da Nr4a1 eine wichtige Rolle bei der Regulierung des Skelettmuskel-Energiestoffwechsels und der frühen Trainingsanpassung spielt, könnte dies die Trainingseffizienz und -leistung der Nachkommen im späteren Leben beeinflussen. Bei der Untersuchung der Reaktion auf eine akute Ausdauerleistung zeigten die mHFD-Nachkommen eine reduzierte Genexpression von Vaskularisierungsmarkern (Hif1a, Vegfb usw.), die auf eine reduzierte Angiogenese hindeuteten, welche eine Ursache für ihre verminderte Ausdauerkapazität darstellen könnte. Darüber hinaus wurde eine beeinträchtigte Glucoseverwertung des Skelettmuskels während des akuten Ausdauertrainings durch eine beeinträchtigte Glucose-Nutzung des Skelettmuskels mit erhöhten Blutzuckerwerten, einer verminderten GLUT4-Translokation und Laktatdehydrogenase-Aktivität in mHFD-Nachkommen unmittelbar nach dem Ausdauertest gezeigt. Dies weist auf eine gestörte Verwertung der Glucose als Substrat während des Ausdauertrainings hin. Länger andauernde HFD-Fütterung während des Erwachsenenalters erhöht die Fettmasse in mHFD-Nachkommen im Vergleich zu mLFD-Nachkommen und verringert zudem ihre Insulinsensitivität, was Hinweise auf eine erhöhte Adipositas- und Diabetes-Anfälligkeit gibt. Folglich führt die mHFD zu einer verminderten Anpassung der Nachkommen an die positiven Effekte des freiwilligen Laufrad-Trainings.

Schlussfolgerung: Die Ergebnisse dieser Dissertation zeigen, dass der mHFD-Konsum die Trainingseffizienz und Ausdauerkapazität der Nachkommen beeinträchtigt, was die Adipositas- und Diabetes-Anfälligkeit im Erwachsenenalter erhöhen kann.

Dies könnte auf Veränderungen im epigenetischen Profil des Skelettmuskels und/oder Beeinträchtigungen der Angiogenese und Glukoseverwertung im Skelettmuskel während eines akuten Ausdauertrainings zurückzuführen sein, was zu einer gestörten Anpassung der Nachkommen an das Training beitragen könnte.

1 INTRODUCTION

1.1 Prevalence of obesity and the metabolic syndrome

Since 1980, the prevalence of obesity has more than doubled worldwide, due to an increasing sedentary lifestyle. Unhealthy nutrition rich in energy-dense food with a high-fat content and decreased physical activity are the main reasons for the development of overweight and obesity as well as related chronic diseases [1].

The metabolic syndrome is characterized as a cluster of different risk factors such as dyslipidemia, hypertension, central obesity, cardiovascular disease, and insulin resistance [1, 2]. It is diagnosed by the occurrence of three of these components [3].

Overweight and obesity are characterized by abnormal or excessive fat accumulation, which mainly occurs in industrialized countries, but also increased in developing countries in the last years. It is the consequence of a lack of physical activity, food processing, and education [1]. In 2013 the results of the German health interview and examination survey for adults (DEGS1) were published, which analyzed the prevalence of overweight and obesity in Germany from 2008-2011 based on the body mass index (BMI) [4]. The BMI for adults is defined as body weight [kg] divided by the square of body height [m^2]. A BMI equal or greater than 25 kg/m^2 was used as cut-off value for overweight, while obesity was classified as a BMI of 30 kg/m^2 or higher [1]. Mensink et al. demonstrated for German adults (18-79 years) that the total prevalence of overweight for women is 53.0 % and for men 67.1 %, while 23.9 % of women and 23.3 % of men were obese. Especially in young people, an increase in obesity prevalence was recorded. Thus, women in reproductive age showed an obesity prevalence of 9.6 % (18-29 years) and 17.9 % (30-39 years) [4].

For school-aged children, the prevalence of overweight and obesity doubled/tripled from 1970 until the end of 1990 in Spain, UK, Germany, and the USA. Even in countries with economic growth like Brazil, Chile or Mexico, the prevalence of childhood obesity reached comparable levels to industrialized countries [5]. In 2014, 41 million children worldwide under the age of 5 years were overweight or obese according to the WHO. Childhood obesity is associated with increased future health risks like obesity in adulthood, breathing difficulties, early markers of cardiovascular disease, hypertension, and insulin resistance. Especially children in developing countries are more prone to inadequate prenatal and infant nutrition.

They are exposed to foods rich in sugar, fat, and salt, but poor in nutrients. This energy dense nutrition, in combination with a lack of physical activity, caused the increase of childhood obesity also in low- and middle- income countries [1].
For this reason, prevention is nowadays becoming more and more important, in addition to interventions such as weight reduction. Therefore, research is focusing on the impact of maternal overweight and obesity during pregnancy on offspring/childhood obesity [6].

1.2 Impact of maternal diet on offspring energy metabolism

Since it is known that the individual phenotype is influenced by the perinatal and early postnatal environment, research focuses on the nutrition during early life [7]. The "developmental origins of health and disease hypothesis" by Barker claims that the conditions during a critical window in pregnancy can cause permanent changes to the offspring´s metabolism [8-10]. The most popular example is the Dutch famine of 1944-1945. Epidemiological studies showed that the exposure to the famine during late gestation resulted in offspring with a low birth weight and a small head circumference [11]. In adulthood, these people developed an impaired glucose tolerance and were more prone to type 2 diabetes [12, 13]. The exposure to the famine during early pregnancy caused an atherogenic lipid profile in the offspring and thus, an increased risk of obesity and metabolic diseases (a threefold increase of cardiovascular disease) in adult life [14-16], while caloric restriction during mid-gestation had no impact on birth weight and disease predisposition [17]. Furthermore, maternal obesogenic diet results in the development of low-grade inflammation, associated with an increased release of cytokines, which is proposed to modulate placental growth and function and thereby influence fetal growth [18]. Consequently, maternal nutrition during pregnancy had an important impact on the metabolic future of the offspring that programs the offspring for obesity and the metabolic syndrome in adult life [7, 11, 19].
An association between maternal BMI and gestational diabetes with offspring obesity was reported in many epidemiological studies and was thought to be due to the transmission of genes that program the offspring for overweight and obesity [20-22]. However, this transmission is prevented by maternal weight loss after bariatric surgery, which reduced the risk of offspring metabolic disorders [23] and suggests,

that the maternal environment and not only the genes contribute to offspring obesity [19]. Especially the early life environment plays a decisive role in the development of non-communicable diseases such as coronary heart disease or type 2 diabetes. This is suggested to be influenced by an altered epigenetic regulation of genes. Thus, also epigenetic changes play a role in the development of the metabolic syndrome. Such epigenetic DNA alterations include non-coding RNAs, histone modifications, and DNA methylations. Highly methylated DNA within the promoter region usually results in transcriptional silencing, which is due to the prevention of transcription factor binding to the DNA. Hence, genes important for the maintenance of energy homeostasis are not functional anymore, which contributes to the development of the metabolic syndrome [24].

To investigate the detrimental effects of maternal overnutrition on offspring development, many animal studies were performed. In non-human primates, increased adiposity and hepatic lipotoxicity were shown in offspring of chronically high-fat diet fed dams [25]. Also, Pruis et al. could demonstrate that male mice offspring exposed to prenatal and post-weaning Western-style diet developed a hepatomegaly with an increased accumulation of hepatic triglycerides and cholesterol [26]. In studies with rats, it was shown that a high-fat diet during pregnancy causes offspring hypertension, adiposity and reduced insulin sensitivity [27, 28]. Furthermore, Bayol and colleagues showed that also the skeletal muscle is affected by maternal overnutrition. Hence, they reported a reduced muscle mass and strength for offspring of “cafeteria diet” fed dams [29, 30]. It was also shown that muscle mitochondrial catalytic activity was reduced in offspring of high-fat diet fed dams [31, 32]. Furthermore, it is known that maternal obesity during pregnancy induces insulin resistance in liver and muscle, cardiovascular disease, leptin resistance in the brain, and accelerated pancreatic ß-cell maturation and impaired glucose sensing [11, 18] (Figure 1).

Thus, in this context, it appears to be difficult to distinguish whether the maternal diet per se or the resulting metabolic parameters of the mother are decisive for the phenotypic changes in the offspring.

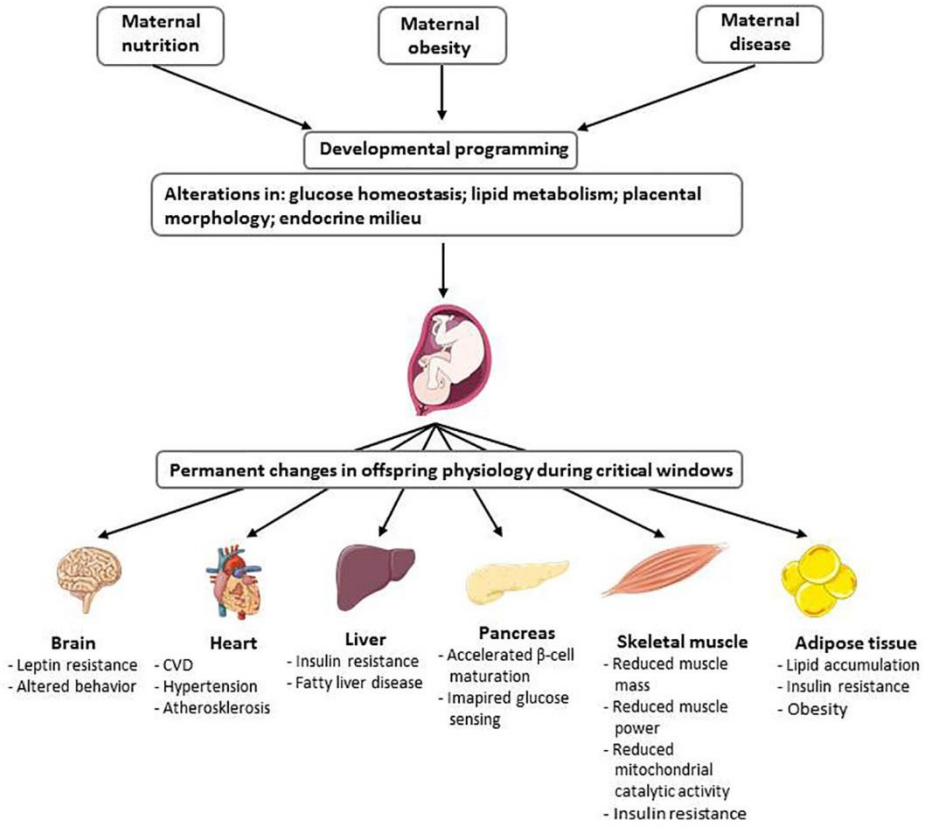

Figure 1: Perinatal impact on the programming of the offspring´s phenotype.
Offspring´s exposure to various risk factors during critical developmental period results in adaptations that cause the offspring's metabolic disorders in adulthood. CVD, Cardiovascular disease (adapted from [11, 19]). This figure was created using Servier Medical Art (http://www.servier.com).

1.3 Impact of physical activity on skeletal muscle energy metabolism

A sedentary lifestyle is known to contribute to the development of the metabolic syndrome. Hallal and co-workers published that about four out of five adolescents and one-third of the adults worldwide did not reach the recommended levels of daily physical activity [33]. Physical activity, such as cycling, endurance and resistance training or walking, has significant beneficial health effects. On one hand, it reduces the risk of hypertension, coronary heart disease, diabetes, and stroke. On the other hand, exercise improves the muscular and coronary fitness as well as bone function and is important for energy balance and weight control [34].

Many studies have shown that the physical activity status of an individual is negatively correlated with the mortality caused by atherosclerotic disease. In the National Health and Nutrition Examination Survey (NHANES I), it was found that

in diabetic patients, physical inactivity is associated with higher rates of coronary-related death [35].
Both the acute and long-term adaptations to physical activity have positive effects on glucose uptake and distribution. Thus, impaired adaptation to physical activity stimulates the development of the metabolic syndrome [36].

1.3.1 Skeletal muscle physiology – molecular background

The skeletal muscle belongs to the striated muscle and consists essentially of the proteins actin and myosin. The cross-striation is required for the contractility of skeletal muscle. The skeletal muscle contractile function is modulated by the Ca^{2+} flux. The interaction between myosin and actin and thereby the rates of muscle relaxation and force generation are controlled by Ca^{2+} release into the myocytes and the subsequent binding with troponin [37-39]. For muscle force generation the key element in calcium handling is the ryanodine receptor, while for relaxation the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) plays an important role. During the excitation-contraction process, the ryanodine receptor interacts with the dihydropyridine receptor in response to an increasing intracellular calcium concentration and acts as the sarcoplasmic reticulum calcium release channel [40, 41]. In response to the increasing calcium concentration in the cytoplasm, Ca^{2+} binds to the C subunit of the troponin-tropomyosin complex, which results in a change in conformation and hence, the binding of myosin to actin. Energy (adenosine triphosphate, ATP) is used for the interaction between myosin and actin leading to the contraction [38, 39]. Thus, ATP supply is a pivotal constraint for skeletal muscle function. ATP supply, produced by mitochondria, and calcium-circulation play an important role for skeletal muscle performance.
Muscle force, contraction speed, oxidative/glycolytic capacity and endurance are characteristics attributed to skeletal muscle fibers. Since skeletal muscles differ in their phenotype which is related to the existence of different fiber types characterized by different myosin heavy chain (MHC) ATPases with different pH optima. Muscle fibers are classified as MHCI, IIa, and IIx and for rodents also IIb, based on histochemical staining. Type I muscle fibers show a high amount of the oxygen storage protein myoglobin, which is responsible for the red staining of these muscle fibers. Furthermore, they are characterized as oxidative, slow-twitch muscle fibers with a slow shortening velocity and a high number of mitochondria. They show

a predominant aerobic metabolism with free fatty acids as an energy substrate. Since the isoforms of SERCA are differentially expressed in the different fiber types, Type I muscle fibers predominantly express SERCA2a, while Type II fibers express SERCA1a. Type II muscle fibers are characterized as glycolytic, fast-twitch muscle fibers with fewer mitochondria and less myoglobin. They display a pale staining and mainly use glucose as an energy substrate which is predominantly metabolized by anaerobic glycolysis [42]. Hence, sprinters have more fast-twitch than slow-twitch muscle fibers, while endurance athletes display more slow-twitch muscle fibers. By influencing the fiber type conversion endurance exercise can result in pronounced changes in the metabolic properties of skeletal muscle [43].

1.3.2 Skeletal muscle energy metabolism

Skeletal muscle plays an essential role in the context of physical activity. Since it is the main site for glucose and fatty acid utilization, it is responsible for up to 30 % of the resting energy expenditure and up to 90 % of the energy expenditure in response to physical activity [44, 45]. Thus, disorders in the regulation of skeletal muscle glucose and lipid metabolism are known to be involved in the development of type 2 diabetes and obesity [46, 47]. Skeletal muscle of healthy individuals rapidly switches between carbohydrate and lipid fuels, while this metabolic flexibility is impaired in patients suffering from obesity or type 2 diabetes [46, 48]. They display increased intramuscular lipid contents and a high glycolytic activity, while the activity of oxidative enzymes is reduced [49].

The energy source used during extensive contractions is ATP. Since the intramuscular ATP store is small it is depleted within the first two seconds of skeletal muscle contraction. Thus, ATP needs to be constantly replenished by other metabolic pathways, which are divided into aerobic and anaerobic pathways [50]. The aerobic pathway predominates during prolonged endurance exercise in which the muscle glycogen is metabolized aerobically by oxidative phosphorylation for ATP production [51, 52]. Depending on the training status and exercise intensity, also skeletal muscle lipid metabolism is increased during physical activity. Free fatty acids derived from muscle triglyceride stores function as a substrate [50]. Additionally, amino acids derived from skeletal muscle protein degradation could be used for aerobic exercise, but account only for a small amount of the generated energy [53].

In the case of high-intensity exercise or at the end of exhaustive endurance exercise oxygen supply to the skeletal muscle can not be increased adequately. Hence, anaerobic pathways have to be used to generate ATP, which is fewer than the ATP amount produced by the aerobic pathway [54]. During the anaerobic metabolism, ATP can be produced by the hydrolysis of creatine phosphate. In rest creatine phosphate results from the transfer of a phosphate residue from ATP to creatine, catalyzed by mitochondrial creatine kinase [55]. During physical activity, ATP is used. Thus, cytosolic creatine kinase catalyzes the transfer of the phosphate residue from creatine phosphate to ADP, which results in ATP and can be used for contraction for approximately ten seconds [53, 56].

During exercise, the main energy substrate for ATP production is muscle glycogen which is converted into lactate due to anaerobic glycolysis. The glycogen breakdown is regulated via the enzyme glycogen phosphorylase that releases glucose which enters the glycolysis and is converted into pyruvate. Pyruvate and $NADH+H^+$ are converted by lactate dehydrogenase (LDH) reaction into lactate and NAD^+. This reaction is important for ATP production [57]. Lactate reaches the liver via the blood stream and is used as a substrate for hepatic gluconeogenesis to resynthesize glucose. Hepatic glucose then can be used as extracellular glucose source during extensive exercise. This mechanism is called Cori-cycle [56, 58]

Hence, also liver metabolism is influenced during physical activity by providing glucose as an energy substrate for the skeletal muscle. Physical activity induces hepatic glycogenolysis and gluconeogenesis to maintain glucose homeostasis. During endurance exercise, hepatic gluconeogenesis is the main glucose-generating pathway for extrahepatic tissues to maintain glucose homeostasis and blood glucose concentrations. The regulation of hepatic gluconeogenesis is mainly controlled on two levels, the increased provision of gluconeogenic substrates and the activity of gluconeogenic enzymes, respectively [59].

1.3.3 Skeletal muscle fatigue and adaptation

The lack of glucose utilization is one explanation for muscle fatigue. This is defined as the activity-induced decline in physical performance due to an accumulation of lactate and a reduced ATP supply in skeletal muscle cells. One distinguishes between central and peripheral fatigue [53]. Central fatigue occurs during the interplay between the central nervous system and skeletal muscle and is of great

importance during low-intensity activities [60, 61], while peripheral fatigue dominates during activities of high intensities and relates to factors within the muscle. If the contractile function of skeletal muscle is impaired during exercise, peripheral fatigue occurs [62, 63].

Regular physical activity and exercise training improve the contractile performance by inducing adaptation processes in skeletal muscle cells [53]. Adaptations in skeletal muscle improve fatigue resistance, substrate delivery (lipid mobilization) and mitochondrial respiration [53, 64]. Regarding the latter, mitochondrial encoded proteins form functional complexes to maintain mitochondrial respiration and ATP production, and the import of metabolites and proteins. Hence, mitochondrial biogenesis is important for the oxidative capacity of skeletal muscle and therefore whole body fitness and metabolic health [65].

Furthermore, oxygen delivery to mitochondria is improved by physical activity stimulating the capillary formation and angiogenesis [66, 67]. The major angiogenic factor is the vascular endothelial growth factor (VEGF), which is known to be induced by exercise in response to low oxygen levels (hypoxia) [68].

Different stimuli, such as hypoxia, ROS or increased calcium ion concentrations, improve muscular oxidative phenotype by the induction of specific signaling factors like hypoxia-inducible factor 1α (HIF1α), AMP-activated protein kinase (AMPK) or mitogen-activated protein kinase (MAPK) (Figure 2). These induce the peroxisome proliferating receptor γ coactivator 1α (PGC1α) that is essential for the establishment of an oxidative phenotype characterized by an enhanced insulin-stimulated glucose uptake in response to endurance exercise [53].

Besides an improved angiogenesis and mitochondrial biogenesis, physical activity also results in an induction of the unfolded protein response (UPR) in skeletal muscle to maintain endoplasmic reticulum (ER) homeostasis [69]. Amongst other things the ER is responsible for the folding of newly synthesized proteins by molecular chaperones. The occurrence of ER stress is characterized by the appearance of misfolded/unfolded proteins. To counteract this, the UPR is induced to increase the expression of chaperones [70]. Since ER stress also occurs during physical activity, activation of the UPR is essential in the adaptation to exercise [71]. This can be induced by three main signal cascades downstream: Protein kinase RNA-like endoplasmic reticulum kinase (PERK), Inositol-requiring enzyme 1α (IRE1α) and Activating transcription factor 6 (ATF6) [69].

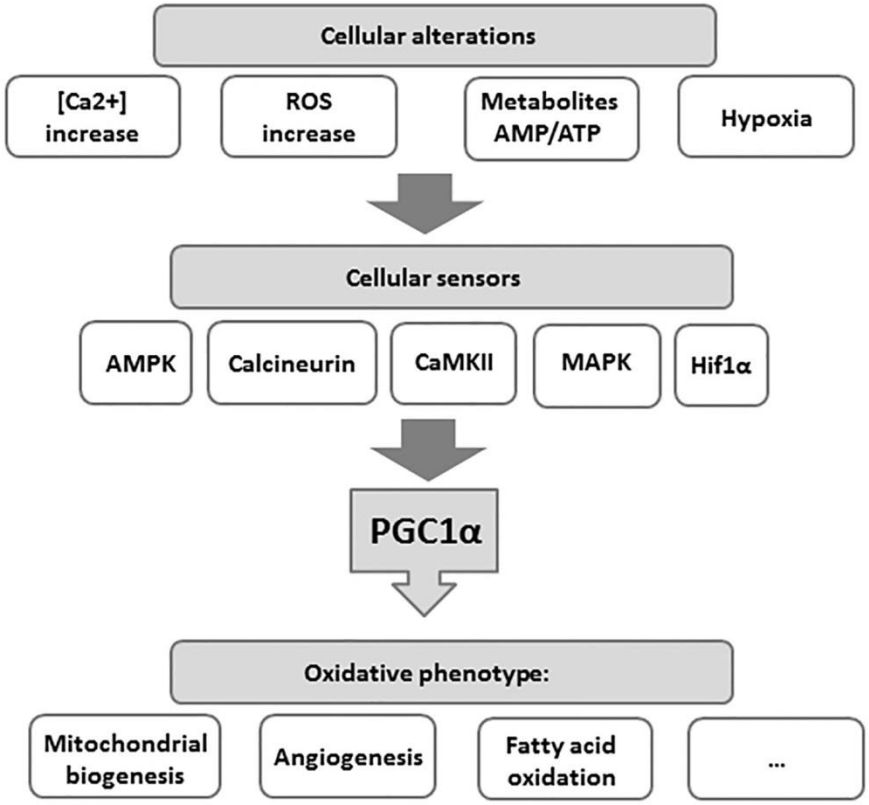

Figure 2: Adaptive response to endurance exercise. The role of signaling factors in improving the muscular oxidative phenotype, due to the induction of PGC1α.
ROS, reactive oxygen species; AMP, adenosine monophosphate; ATP, adenosine triphosphate; AMPK, AMP-activated protein kinase; CaMKII, calcium calmodulin kinase II; MAPK, mitogen-activated protein kinase; HIF1α, hypoxia inducible factor 1α (adapted from [53]).

1.4 Impact of maternal nutrition on offspring exercise performance

Since maternal obesity affects skeletal muscle function by reducing muscle mass, power, and mitochondrial activity [29, 31], skeletal muscle plays a major role in energy expenditure during physical activity [44] and thereby may have consequences for offspring exercise performance.

Rajia and co-workers investigated the connection between maternal obesity, offspring post-weaning exercise training and offspring high-fat intervention in female Sprague-Dawley rats. They hypothesized that offspring voluntary running wheel training improves the negative effects of maternal obesity, such as offspring obesity, insulin resistance, and impaired lipid metabolism. They showed that offspring of maternal obese dams displayed the highest values of hepatic triglycerides, as well as the highest body weight and fat mass. Furthermore, they

found out that voluntary training reversed the metabolic effects caused by maternal obesity in rats fed a control diet, while it did not fully attenuate insulin resistance, adiposity and glucose intolerance in high-fat diet fed offspring. Thus, they conclude, that not only running wheel intervention improved the negative effects of maternal obesity, also post-weaning dietary intervention had an impact on offspring metabolic health [72].

In a study with C3H mice, the impact of maternal high-fat diet feeding during pregnancy and lactation on offspring exercise performance was investigated. Walter and colleagues demonstrated that offspring of high-fat diet fed dams displayed a reduced endurance capacity in response to voluntary running wheel training. Furthermore, skeletal muscle gene expression of cluster of differentiation 36 (CD36), fatty acid synthase and glucose transporter 1 (Glut1) was impaired in mHFD offspring. Hence, they concluded that a maternal high-fat diet during pregnancy and lactation impairs offspring training efficiency and exercise capacity due to an impaired skeletal muscle energy supply [73].

More recently, Bucci and colleagues investigated whether the detrimental effects of maternal obesity during pregnancy can be reversed by exercise training in late adult life. Therefore, a subgroup of the Helsinki Birth Cohort Study (HBSC) was studied. Frail elderly women underwent resistance training sessions three times a week for four months. The groups were matched for BMI and normoglycaemic, but women whose mothers had been overweight/obese displayed insulin resistance and impaired muscle glucose uptake compared to women born to mothers of normal weight. Elderly women, born to overweight or obese mothers, improved their glucose uptake and insulin sensitivity after four months of resistance training, while exercise had no impact on women born to normal weight mothers. Additionally, women born to obese mothers reduced their intracellular lipid content in skeletal muscle with resistance exercise. Thus, the authors suggested that women born to obese mothers might have a more rapid response to exercise training [74].

These studies indicate that maternal overnutrition during pregnancy affects offspring exercise response and the preposition for metabolic diseases in later life. However, it still needs to be elucidated which mechanisms contribute to this effect and whether regular physical exercise can reverse the negative metabolic effects.

1.5 Objectives

The prevalence of childhood overweight and obesity has increased in the last years [1]. Recent studies indicate that parental nutrition and lifestyle play a major role in the development of offspring metabolic disorders like childhood obesity and the metabolic syndrome later in life [75, 76]. It is well established that physical activity can prevent the development of the metabolic syndrome, although some individuals (15 – 20 %) fail to improve their metabolic health by performing physical exercise. In genetic studies, it was demonstrated that the exercise response is largely heritable, while DNA methylations are also linked to the exercise response of skeletal muscle. This indicates that besides genetic regulations of skeletal muscle metabolism also epigenetic modifications contribute to the exercise training response [77].

Therefore, this PhD thesis aimed to investigate the impact of maternal overnutrition during pregnancy and lactation on offspring skeletal muscle energy metabolism and exercise performance using a mouse model (Figure 3).

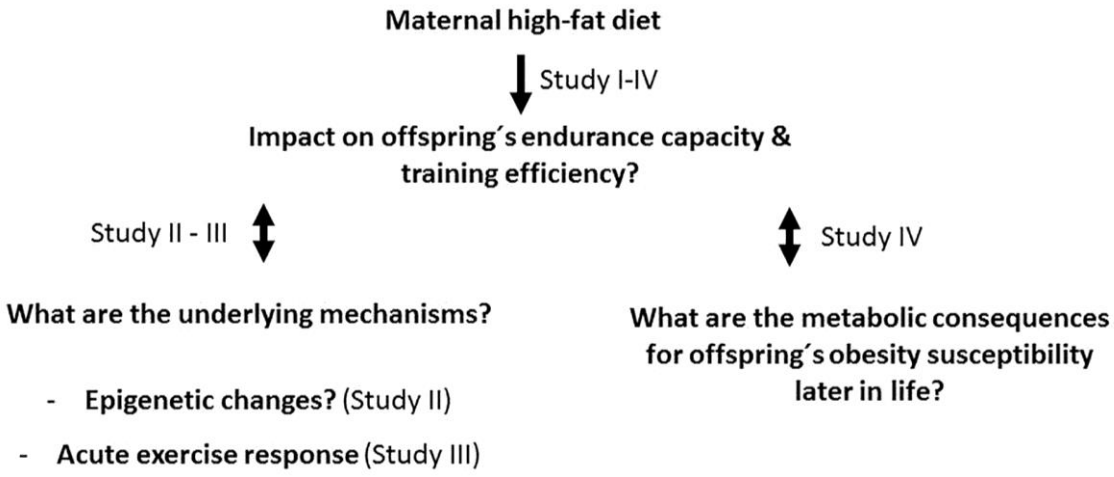

Figure 3: Objectives
The Aim of this PhD thesis is to investigate the impact of maternal high-fat diet on offspring skeletal muscle energy metabolism and endurance performance. In order to investigate the underlying mechanisms and metabolic consequences for the offspring of the possibly impaired endurance performance four studies were conducted.

2 MATERIAL AND METHODS

2.1 Animal experiments

The animal experiments of Study I, II and IV (Figure 4) were designed and performed by Isabel Kanzleiter. During my PhD thesis, I analyzed the corresponding data and conducted all molecular assays. To investigate the underlying mechanisms in more detail, I designed, performed and analyzed study III on my own (Figure 4).

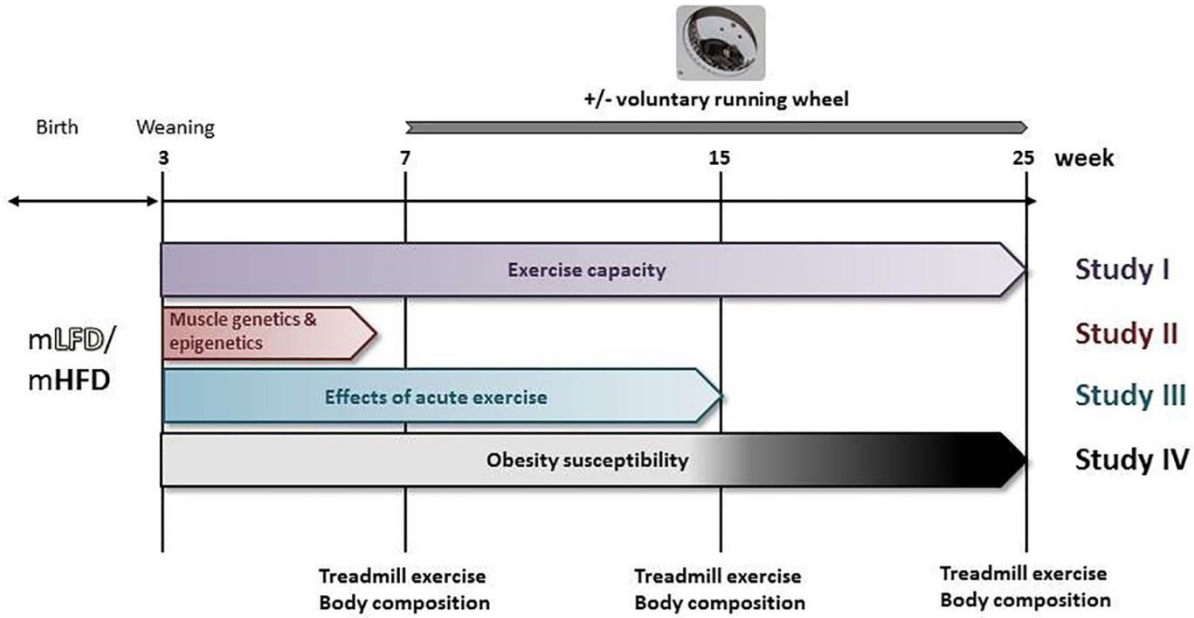

Figure 4: Overview of the conducted studies

All studies had the same prerequisites. Female C57Bl/6J mice (8 weeks old) received a low-fat diet (LFD; 10% energy from fat, 23% energy from protein, 67% energy from carbohydrate; Research Diets Services) or high-fat diet (HFD; 40% energy from fat, 23% energy from protein and 37% energy from carbohydrate; Research Diets Services) three days before mating throughout pregnancy and lactation ad libitum. Two virgin females were mated with one male for one week (wk) and subsequently individually caged. Litter size was adjusted to 5-8 pubs. All mice were kept in a temperature- and light-controlled facility with a 12 h light and 12 h dark cycle at 22 °C.

Since the various studies focused on different issues, the offspring underwent different treatments during the different studies (Figure 4), which will be explained in more detail in the following paragraphs.

2.1.1 Study I: Exercise capacity

Study I (Figure 5) focused on the impact of maternal overnutrition on offspring exercise capacity.

Male offspring were single caged after weaning and received a LFD until an age of 25 wks. At an age of seven wks, half of maternal HFD (mHFD) and maternal LFD (mLFD) offspring got access to a running wheel (RW; TSE Systems, Bad Homburg, Germany) as voluntary exercise training, which resulted in these respective groups: mLFD –RW LFD, mLFD +RW LFD, mHFD –RW LFD and mHFD +RW LFD. Body composition (section 2.2) and treadmill exercise capacity (section 2.3) were determined at an age of seven, 15 and 25 wks. All mice were sacrificed 24 h after the last exercise bout and after 2 h fasting with an age of 25 wks using isoflurane and cardiac puncture (Figure 5). The blood was collected in an EDTA-coated tube and centrifuged (10 min, 9000 x g, 4°C) to receive the plasma. Tissues were removed, weighed, frozen in liquid nitrogen and stored at -80 °C.

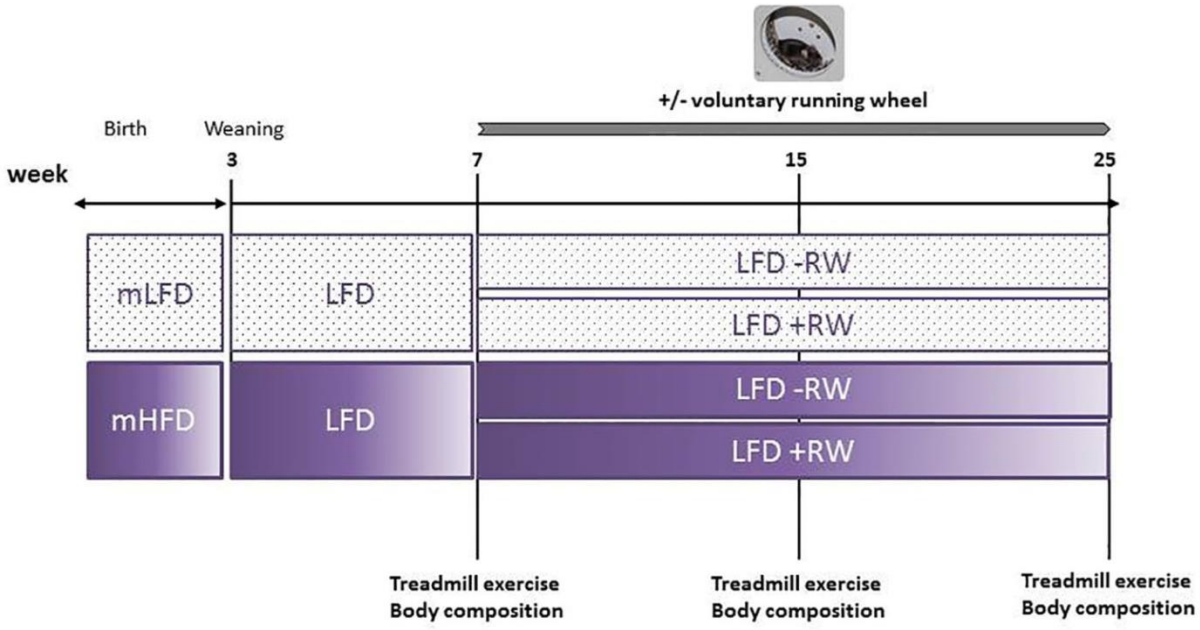

Figure 5: Study I: Impact of maternal high-fat diet on offspring exercise performance.
Dams were fed a low-fat (mLFD) or a high-fat diet (mHFD) during pregnancy and lactation. Maternal low-fat diet or maternal high-fat diet offspring received a LFD after weaning until an age of 25 wks. Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. Treadmill exercise and body composition were measured at an age of seven, 15 and 25 wks.

2.1.2 Study II: Early muscle development

To determine the effects of maternal overnutrition on offspring genetics and epigenetics study II was conducted (Figure 6). It focused on epigenetic changes in offspring skeletal muscle caused by maternal malnutrition.

Male offspring were single caged and received LFD after weaning. At an age of six wks mice were sacrificed by the same procedure as mentioned before (section 2.1.1) without any further intervention. Muscle tissue was removed, weighed, frozen in liquid nitrogen and stored at -80 °C. Microarray analysis (section 2.9.5) was performed in *M. quadriceps* in collaboration with Jaap Keijer (Wageningen University & Research, the Netherlands). This transcriptome analysis represents the basis for epigenetic analysis using bisulfite and Pyrosequencing in collaboration with Annette Schürmann (German Institut of Human Nutrition, Germany).

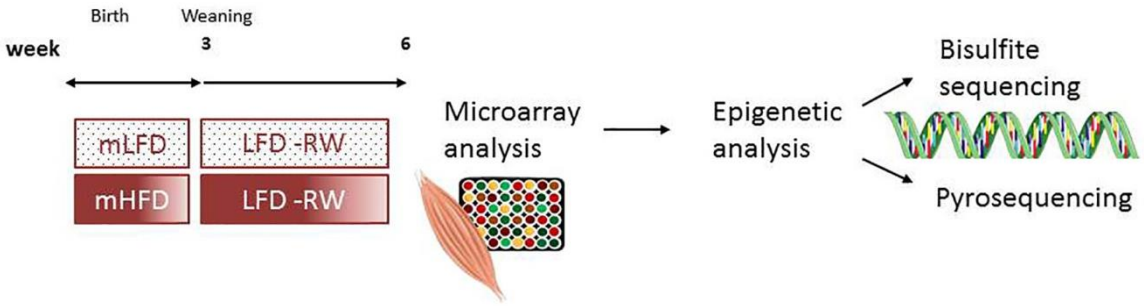

Figure 6: Study II: Impact of maternal high-fat diet on offspring early skeletal muscle development.
Dams were fed a low-fat (mLFD) or a high-fat diet (mHFD) during pregnancy and lactation. Maternal low-fat diet or maternal high-fat diet offspring received a LFD after weaning until an age of six wks without any prior intervention. A microarray and epigenetic analysis were performed in offspring *M. quadriceps*. This figure was created using Servier Medical Art (http://www.servier.com).

2.1.3 Study III: Effects of an acute exercise bout

Study III (Figure 7) aimed to clarify offspring metabolic response during an endurance exercise bout. Single caged post-weaning male offspring got access to a LFD ad libitum until an age of 15 wks. As described before (section 2.1.1) half of each group received a RW as voluntary training. Endurance capacity (section 2.3) was determined at wk seven, eleven and 15 and body composition (section 2.2) at four, seven and 15 wks of age. The alterations in blood glucose concentrations during the exercise bout at wk 15 were determined by measuring tail vein blood glucose

levels using the ContourXt glucose sensor (Bayer AG, Leverkusen, Germany). To investigate the metabolic consequences of maternal overnutrition on offspring energy metabolism in response to an acute exercise bout, mice were sacrificed at an age of 15 wks at three different time points: Mice were sacrificed at a basal level without any treadmill exercise intervention, immediately after the treadmill test, or 3 h after the test. The sacrifice procedure was the same as described in section 2.1.1.

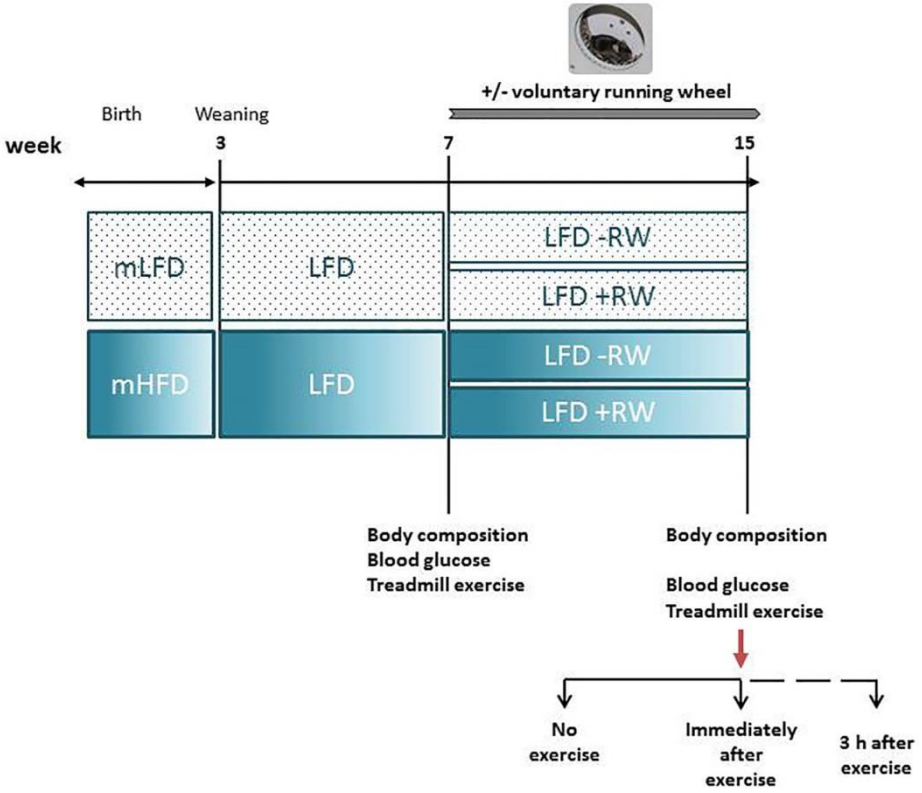

Figure 7: Study III: Impact of maternal high-fat diet on offspring response to an acute exercise bout.
Dams were either fed a low-fat (mLFD) or a high-fat diet (mHFD) during pregnancy and lactation. Maternal low-fat diet or maternal high-fat diet offspring received a LFD after weaning until an age of 15 wks. Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. Treadmill exercise, body composition, and blood glucose were measured at an age of seven and 15 wks. Mice were sacrificed at an age of 15 wks at three different time points, before, immediately after and 3 h after an acute exercise bout.

2.1.4 Study IV: Obesity susceptibility

Study IV (Figure 8) was performed to investigate the impact of maternal overnutrition during pregnancy and lactation on offspring obesity susceptibility.
Male offspring were single-caged after weaning and received a LFD until an age of 15 wks. At an age of seven wks half of mHFD and mLFD offspring got access to a RW as voluntary exercise training. At an age of 15 wks, all mice received a HFD

until the end of the study (25 wks), which resulted in the four respective groups: mLFD –RW HFD, mLFD +RW HFD, mHFD –RW HFD and mHFD +RW HFD. Body composition (section 2.2) and treadmill exercise capacity (section 2.3) were measured at an age of seven, 15 and 25 wks. An oral glucose tolerance test (section 2.5) was performed at an age of 22 wks and indirect calorimetry was measured with 24 wks of age. All mice were sacrificed 24 h after the last exercise bout and after 2 h fasting with an age of 25 wks, as described before (section 2.1.1).

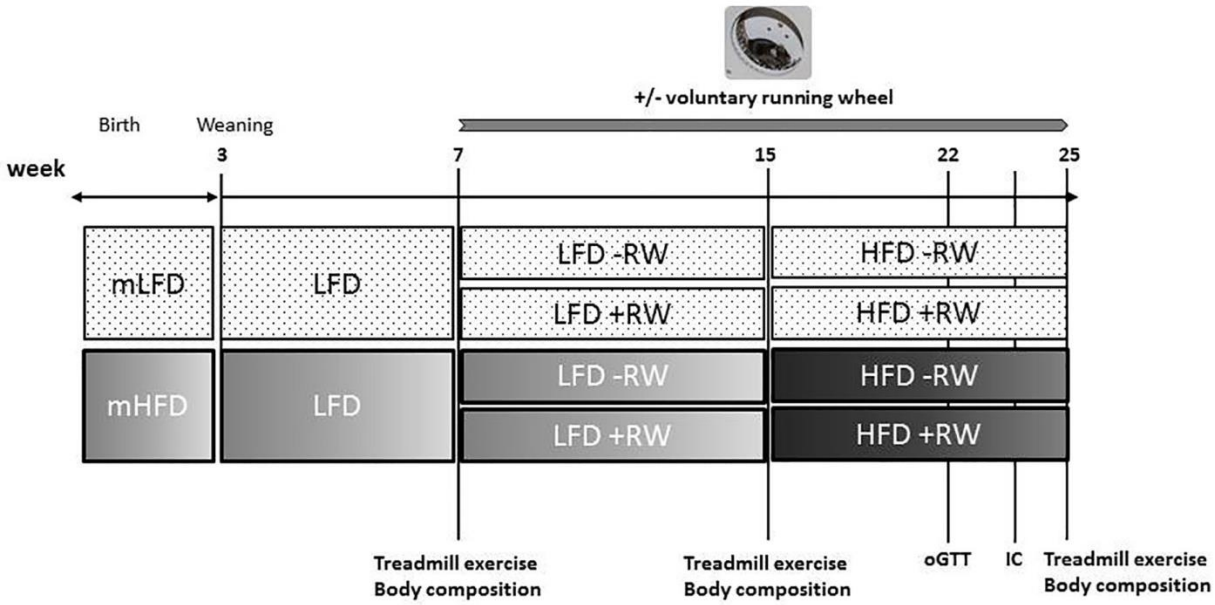

Figure 8: Study IV: Impact of maternal high-fat diet on offspring obesity susceptibility.
Dams were fed a low-fat (mLFD) or a high-fat diet (mHFD) during pregnancy and lactation. Maternal low-fat diet or maternal high-fat diet offspring received a LFD after weaning until an age of 15 wks. From an age of 15 wks on both groups received a HFD for ten wks until the end of the study (25 wks) Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. Treadmill exercise and body composition were measured at an age of seven, 15 and 25 wks, an oral glucose tolerance test (oGTT) was performed at an age of 22 wks and indirect calorimetry (IC) was measured with 24 wks of age.

2.2 Body composition

Body composition was measured by quantitative nuclear magnetic resonance spectroscopy (NMR; Minispec MQ10 NMR Analysis Bruker, Karlsruhe, Germany). This noninvasive method is used to determine the fat mass and fat-free mass in mice. Body composition was determined at an age of four, seven, 15 and 25 wks. First, a self-test was performed as well as a control measurement of a defined volume of oil. Mice were then put in a synthetic pipe to measure fat mass. By subtracting body fat mass from body mass, the lean mass was calculated.

2.3 Endurance exercise capacity

Endurance exercise capacity was determined at an age of seven, eleven, 15 and 25 wks depending on the respective study on a six-lane treadmill (Columbus Instruments, Columbus, USA). The starting speed (5 m/min) was increased every 5 min until a maximum speed of 28 m/min (Table 1). Mice were forced to run until exhaustion or a maximum of 100 min. Exhaustion was the inability of mice to resume running, which was defined by continuous contact (> 5 sec) with the electrical shock grid at the end of each running lane. Running time and speed of the treadmill were measured to calculate the distance as the product of time and speed. The difference in distance between the first, second and/or third treadmill test is termed as training efficiency.

Table 1: Endurance treadmill protocol

Time [min]	10	15	20	25	30	35	40	45	50	55	60	100
Speed [m/min]	0	5	9	12	14	16	18	20	22	24	26	28

To get used to the treadmill, mice were put on the treadmill 10 min prior starting the endurance test. The speed was increased every 5 min until a maximum of 28 m/min.

2.4 Indirect calorimetry

Indirect calorimetry is used to determine the energy expenditure. For the measurement, the PhenoMaster System (TSE Systems, Bad Homburg, Germany) was used. This system consists of air-sealable respiratory cages, an analyzer, a climate control cabinet and a computer that records the data. To minimize measuring errors, mice were familiarized with the system for three days. Afterwards, indirect calorimetry was measured for 23 h and energy expenditure and RQ were calculated [78].

2.5 Oral glucose tolerance test

Oral glucose tolerance tests (oGTT) are performed to examine the individual glucose tolerance and insulin sensitivity. Glucose is applied orally and a time course of the

blood glucose concentration is determined. Furthermore, plasma insulin levels are measured to investigate the insulin sensitivity.

Mice were fasted for 16 h and a four-hour oGTT was performed at an age of 22 wks. Glucose concentrations were measured in whole blood of the tail vein by ContourXt glucose sensor (Bayer AG, Leverkusen, Germany) before glucose application and 15, 30, 60, 120 and 240 min after oral glucose application by gavage (2 mg glucose/g body weight). Plasma insulin was measured before (at baseline), 15 min and 30 min after glucose application by Insulin Mouse Ultrasensitive ELISA (Alpco Diagnostics, Salem, USA).

2.6 Histology

Histological staining is used to visualize the cytoplasmic and nuclear structures of tissues. *M. quadriceps* tissue was removed from the mice, immediately fixed in 4% formaldehyde solution and embedded in paraffin. 2 µm sections were produced and a Hematoxylin-eosin (H&E) staining was performed. For the analysis of the muscle fiber number, slides were imaged on the microscope (Eclipse E800, Nikon GmbH, Germany).

2.7 Biochemical methods

2.7.1 Triglyceride analysis

The triglyceride (TG) content was measured in plasma, liver and *M. quadriceps*. The tissues (40 mg) were either homogenized in 800 µL (liver) or 400 µL (muscle) of 10 mM sodium hydrogen phosphate buffer (pH 7.4, 1mM EDTA, 1% polyoxyetylene-10-tridecyl ether) for 3 min at 50 Hz (TissueLyser LT; Qiagen GmbH, Hilden, Germany). The homogenate was centrifuged at 4°C and 23100 x g for 30 min. The supernatant was incubated at 70 °C at 600 rpm for 5 min in the thermoshaker, stored on ice for 5 min and centrifuged again at 4°C and 23100 x g for 30 min.

The TG content was measured in the plasma or supernatants using Triglyceride Determination Kit (Sigma Aldrich Chemie GmbH, Steinheim, Germany), which consists of Reagent A, a free glycerol reagent and Reagent B, a triglyceride reagent. The photometric measurement was performed in 96-well plate-format. The sample

(5µL) was transferred to a 96-well plate in triplicates and mixed with 100 µL Reagent A. Afterwards the plate was incubated for 5 min at 37°C and the initial absorbance at 540 nm was measured to determine free glycerol (Eon; Biotek Instruments, Vermont, USA). Subsequently, 25 µL of Reagent B were added to the mixture and again incubated for 15 min at 37°C. Finally, the second absorbance measurement was performed at a wavelength of 540 nm to determine the TG content. For TG concentration calculation the initial absorbance of free glycerol was subtracted from the second absorbance to obtain the absorbance of the TG. A standard curve of glycerol standard solution (c = 2.5 mg/mL) from 0.075 µg/µL to 2.5 µg/µL was then used to determine the final TG concentration in the samples.

2.7.2 Glycogen analysis

The glycogen concentration was measured in liver and *M. quadriceps*. Liver (35 mg) and muscle (50 mg) were homogenized for 90 sec at 50 Hz in 750 µL (liver) or 1000 µL (muscle) of 1 N NaOH. The resulting supernatant was incubated at 70°C and 1000 rpm for 45 min in a thermoshaker. Afterwards, the samples were mixed gently, stored on ice for 5 min and centrifuged at 4°C and 12400 x g for 45 min. The supernatant was centrifuged (at 4 °C, 12400 x g, 30 min) again. This process was repeated until the supernatant was clear. For the analysis of the glycogen concentration, 150 µL of the clear supernatant are used.

For glycogen measurement (R-Biopharm AG, Germany) in the samples, two approaches were necessary, one to analyze the glycogen glucose (A+) in the samples and one to analyze the free glucose (B-). Fifty µL of each sample were mixed with 1 µL acetic acid and either 100 µL amyloglucosidase (solution 1) for A+ or 100 µL distilled water for B-. The mixture was gently mixed and incubated at 60°C for 15 min. Subsequently, the samples were centrifuged at room temperature (23100 x g, 10 min).

The measurement was carried out in triplicates using a 96-well plate. In each well 30 µL supernatant was mixed with 100 µL distilled water, 100 µL NADP/ATP (solution 2), shortly centrifuged and shaken for 3 min at room temperature. The first absorbance was measured at 340 nm. Subsequently, 12 µL Hexokinase/Glucose-6-phosphat-dehydrogenase (solution 3) were added to each well and the 96-well plate was shaken for further 15 min at room temperature. Afterwards, a second

measurement of the absorption was performed at 340 nm. For glycogen concentration calculation the initial absorbance of plate A+ and B- was subtracted from the second absorbance of each plate. Additionally, the resulted absorbance of plate B- was subtracted from the absorbance of A+ to obtain the absorbance of glycogen in the tissues. The standard curve of glycogen standard solution (c = 0.2 µg/µL) from 0.01 µg/µL to 3 µg/µL was then used to calculate the glycogen concentration in the samples.

2.7.3 Plasma analysis

Free fatty acid (FFA) concentration was determined in murine plasma samples. Five µL plasma sample were mixed with 100 µL color reagent A (WAKO Chemicals GmbH, Neuss, Germany) in a 96-well plate-format and shaken at 37 °C for 5 min. Subsequently, the first absorbance was measured at 550 nm. Afterwards, 50 µL color reagent B (WAKO Chemicals GmbH, Neuss, Germany) was added to each well and incubated for further 5 min at 37 °C. The second absorbance measurement also took place at 550 nm. The calculation of the final absorbance was performed like described before (section 2.7.1). To calculate then the FFA concentration in plasma a standard curve of oleic acid (28.2 mg/dL) from 0.05 to 1 was used.

2.7.4 Pancreas insulin

Pancreatic insulin content was measured as an indicator of beta cell-mediated insulin production. Pancreas tissue was placed in 1 mL ice-cold acid-ethanol (0.18 M HCl in 70% (v/v) ethanol) and homogenized for 5 min at 50 Hz. The homogenate was stored over night at 4°C. On the next day, the homogenate was centrifuged at 4°C and 5000 x g for 15 min. Pancreatic insulin concentration was determined in the supernatant using the Insulin Mouse Ultrasensitive ELISA (Alpco Diagnostics, Salem, USA).

2.8 Protein biochemical methods

2.8.1 RIPA protein extraction

Fifty mg tissue was homogenized in 1000 µL lysis buffer (RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na-Desoxycholate and 1% Triton X-100, protease inhibitor, phosphatase inhibitor, 2 mM sodium vanadate) for 3 min at 50 Hz. The homogenates were stored on ice for 30 min and subsequently centrifuged (4°C, 21000 x g, 30 min). The supernatant was again centrifuged at 4°C and 23000 x g for 30 min until it was clear. For protein concentration measurements (section 2.8.3) and Western blot analyses (section 2.8.4.3), the supernatant was stored at -20°C.

2.8.2 Plasma membrane isolation

The plasma membrane isolation was performed according to the published protocol of Nushiumi and Ashida [79]. *M. gastrocnemius* (50 mg) was homogenized in 200 µL buffer A (50 mM Tris (pH 8.0), 0.5 mM Dithiothreitol, 0.1% Triton X-100). The homogenate was sheared by passing it five times through a 23-gauge needle. After centrifugation 1 (at 4°C, 1000 x g, 10 min), the precipitate 1 was suspended in 150 µL buffer B (50 mM Tris (pH 8.0), 0.5 mM Dithiothreitol) and incubated for 10 min on ice (occasional mixing), while the supernatant 1 is stored on ice. The suspended precipitate 1 was centrifuged at 4°C and 1000 x g for 10 min. Subsequently, supernatant 2 was gathered with supernatant 1, while precipitate 2 was washed with buffer B and centrifuged again (4°C, 1000 x g, 10 min). This step was repeated twice before precipitate 2 was suspended in 100 µL buffer C (50 mM Tris (pH 8.0), 0.5 mM Dithiothreitol, 1 % Triton X-100), centrifuged at 4°C and incubated on ice for 1 h (with occasional mixing). Afterwards, the suspended precipitate 2 and supernatant 1/2 were centrifuged at 4°C and 16000 x g for 20 min. Consequently, supernatant 3 is characterized as post-plasma membrane fraction, while supernatant 4 make up the plasma membrane fraction (Figure 9).

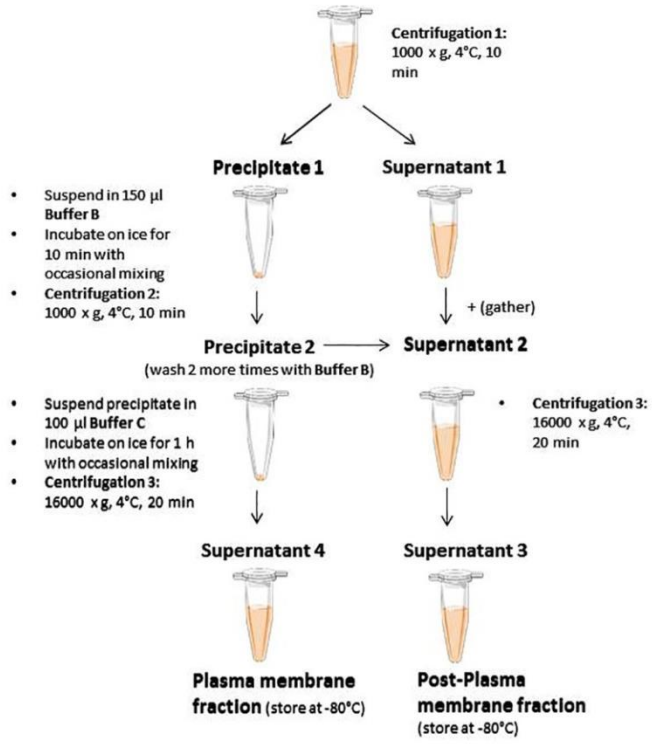

Figure 9: Protocol of the plasma membrane isolation
The figure was created using Servier Medical Art (http://www.servier.com).

2.8.3 Lowry protein assay

For the analyses of tissue protein concentrations, the "Bio-Rad DC Protein Assay Kit" (Bio-Rad Laboratories GmbH, Munich, Germany) was used. It consists of reagent A, S and B. Fifteen µL diluted samples (1:5 for muscle) were mixed with 25 µL of a mixture of reagent A and S in the dilution 1:50 in a 96-well plate. Samples were measured in triplicates. The plate was mixed gently and centrifuged. Subsequently, 200 µL of reagent B were added to each well. After 15 min incubation, absorbance was measured at 750 nm. To calculate the protein concentration of the samples a standard curve of BSA (c = 2 mg/mL; GBiosciences, St. Louis, USA) from 0.1 µg/µL to 1 µg/µL was performed.

2.8.4 Semi-dry western blot analysis

2.8.4.1 Sample preparation

For western blot analysis, the samples were diluted with 4x Laemmli (0.25 M Tris-HCl, 8 % (w/v) SDS, 40 % (v/v) Glycerol, 0.04 % (w/v) Bromphenol blue, pH 6.8) containing 10 % (w/v) 1 M Dithiothreitol (DTT) to destroy the disulfide bonds and complex structure of proteins. The protein samples (100 µL) were mixed with 33.33 µL of this mixture and incubated at 95 °C for 5 min in a thermoshaker.

2.8.4.2 SDS-PAGE

Separation and stacking gel were prepared according to Table 2. After polymerization, 20 µg of protein from the prepared Laemmli dilutions and the protein marker BPS (Pro Sieve® QuadColor™ Protein marker 4.6 – 300 kDa; Biozym, Hess. Oldendorf, Germany) were loaded on a gel. For gel electrophoresis, 1x SDS buffer served as running buffer (10x TGS Buffer, BioRad Laboratories, Germany). The SDS-PAGE was first started for 30 min at 60 V until the samples were visible at the boundary between stacking and separation gel. This was followed by the separation of the proteins at 120 V for further 90 to 120 min.

Table 2: Composition of separation and stacking gel

		Separation gel		**Stacking gel**
Content		**10%**	**12%**	**4%**
$Aqua_{dest}$	mL	2.94	2.64	1.6
10% w/v SDS	µL	60	60	25
4x Separation gel buffer	mL	1.5	1.5	-
4x Stacking gel buffer	mL	-	-	0.625
Acrylamide/Bis	mL	1.5	1.8	0.25
APS 10%	µL	30	30	15
TEMED	µL	4.5	4.5	2.5

2.8.4.3 Western Blot

For semi-dry western blotting, eight filter papers and one PVDF membrane were prepared for each gel. The filter papers were equilibrated in transfer buffer (48 mM Tris, 1.3 mM SDS, 20 % (v/v) Methanol, pH 9.2), while the membrane was activated with methanol for 1 min and subsequently equilibrated in transfer buffer. The transfer of the proteins to the membrane was performed by using the Trans Blot Turbo™ system (BioRad Laboratories, USA) for 10 min at 2.5 A and 25 V. To check whether the transfer to the membrane was successful a Ponceau staining (0.4 % (w/v) Ponceau-S, 40 % (v/v) Ethanol, 15 % (v/v) Acetic-acid) was performed. After washing the membrane with 1 % TBS-T (1x TBS Buffer (10x TBS Buffer: 200 mM Tris, 1.37 M Sodium chloride), 0.1 % (v/v) Tween20) the membrane was subsequently incubated in a 5 % milk solution for 1 h to block the free binding sites. Afterwards, the membrane was washed with TBS-T three times and incubated with the primary antibody at 4°C overnight with agitation. In Table 3 the used antibodies and dilutions are listed. Next day the membranes were washed three times with TBS-T and incubated with the secondary antibody (Table 3) for 1 h at room temperature. The secondary antibody is coupled to horseradish peroxidase, which allows the detection of the proteins by a chemiluminescens reaction (advansta, Biozym, Germany). For detection Fusion SL Vilber Lourmat (Peqlab, Erlangen, Germany) was used and the protein expression was calculated using the BioID software (Peqlab, Erlangen, Germany). The calculated expression of the target protein was normalized to the expression of a reference protein. To analyze more than one protein, the membrane was stripped with stripping buffer (200 mM Glycine, 0.1 % SDS, 0.1 % Tween20, pH 2.2), blocked for 1 h with 5 % milk solution and incubated overnight at 4°C with another primary antibody, like described above.

Table 3: List of antibodies

Primary antibody	**Manufacturer**	**Order ID**	**Size (kDa)**	**Dilution**	
GLUT4	Cell Signaling	#2213	45	1:1000	Milk (5%)
OXPHOS	Acris (Mito Science)	MS604-300		1:2000	BSA (5%)
PGC1α+β	abcam	ab71130	91	1:1000	BSA (5%)
Porin	Cell Signaling	#4886	32	1:2000	BSA (5%)
Secondary antibody					
mouse	Cell signaling	#7076		1:20000	Milk (5%)
rabbit	Cell signaling	#7074		1:20000	Milk (5%)

BSA, bovine serum albumin

2.8.5 ELISA

To determine the content of the Vascular endothelial growth factor ß (VEGFß) and Interleukin 6 (IL6) in skeletal muscle an ELISA was performed. For the Vegfß ELISA (Mouse Vascular Endothelial Cell Growth Factor B ELISA Kit, BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany), 50 mg *M. gastrocnemius* were homogenized in 500 µL 1x PBS (10x PBS in distilled water (v/v)) and centrifuged at 4°C and 5000 x g for 5 min. The supernatant was removed to a new reaction tube and 100 µL of each sample was used for the ELISA measurement according to manufacturer´s protocol.

For IL6 ELISA the Mouse IL-6 Quantikine ELISA Kit by R&D Systems (R&D Systems Inc, Minneapolis, USA) was used. For sample preparation 40 mg of *M. quadriceps* were homogenized in 120 µL hypotonic buffer (10 mM Tris, pH 7.4; 0.02 % (v/v) Triton X-100; 1x EDTA-free protease inhibitor). The homogenates were incubated for 10 min on ice and centrifuged for 10 min at maximum speed and 4°C. The supernatant was removed to a new reaction tube and 50 µL of each sample was used for the ELISA measurement, which was performed according to manufacturer´s protocol.

2.8.6 Cytokine-Assay

Since some cytokines are also released during exercise, a cytokine assay (Bio-Plex Pro™ Mouse Cytokine 23-plex Assay, Bio-Rad Laboratories GmbH, Munich, Germany) was performed in collaboration with Stefan Lehr (Deutsches Diabetes Zentrum (DDZ), Düsseldorf, Germany). Plasma samples were analyzed according to manufacturer´s protocol at the DDZ.

2.9 Molecular biological methods

2.9.1 RNA isolation

To analyze the gene expression in muscle, liver and heart RNA was isolated using peqGOLD TriFast™ (Peqlab, Erlangen, Germany). The tissues were ground and 20 mg were used for isolation. Tissues were mixed with 1 mL TriFast reagent and

homogenized for 3 min at 50 Hz and subsequently incubated for 5 min at room temperature. After a short centrifugation, the homogenate was gently mixed with 300 µL chloroform, incubated for 10 min at room temperature and centrifuged at 4°C and 18400 x g for 20 min. For RNA precipitation the upper phase was mixed with 500 µL isopropyl alcohol in an RNase-free reaction tube. After inversion, the samples were incubated on ice for 10 min and again centrifuged at 4°C and 18400 x g for 60 min. The pellet was washed three times with 75 % (v/v) ethanol, air dried and solved in 30 µL – 50 µL Diethyl pyrocarbonate (DEPC) water (MP Biomedicals LLC, Ohio, USA), depending on the pellet size. The RNA samples were incubated at 60 °C for 5 min to increase their solubility. The RNA concentration was measured using the Take3 plate (BIOTEK, Gene5 2.05).

2.9.2 DNase digestion, test for genomic contamination and RNA agarose gel

Since even traces of genomic DNA in the RNA samples can function as a template for the primer and real-time analysis a genomic DNA digestion was performed. Eight µg RNA were mixed with DEPC water to receive a total volume of 25 µL. This was subsequently mixed with 5 µL reaction mix (3 µL TurboDNase buffer, 0.75 µL RNase inhibitor, 1 µL DNase and 0.25 µL DEPC; Ambion TURBO DNA-free Kit, Thermo Fisher Scientific, Massachusetts, USA). The samples were incubated at 37°C for 20 min. To stop the reaction, 3 µL DNase inactivation reagent (Ambion TURBO DNA-free Kit, Thermo Fisher Scientific, Massachusetts, USA) were added. This was incubated at room temperature for 5 min, centrifuged at room temperature and 10000 x g for 90 sec and the supernatant transferred to a new reaction tube.
To verify whether the digestion was successful a test for genomic contamination was performed. Therefore 1 µL digested RNA was diluted to a final concentration of 5 ng/µL with DEPC. 1 µL of each sample was transferred in triplicates to a 384-well plate and mixed with 4 µL master mix (0.5 µL 18S forward and reverse primer (c = 3 mM), 0.5 µL 18S probe (c = 2 mM) and 2.5 µL TaqMan® Universal Mastermix (Power TaqMan gene expression master mix, Applied Biosystems, California, USA)). The used primers are specific for the ribosomal 18S-RNA section of the DNA.
Furthermore, a horizontal agarose gel electrophoresis was performed. Therefore, a 1 % RNA denaturing agarose gel was prepared. Three gram RNase-free agarose (Biozym, Hess. Oldendorf, Germany) were boiled in 261 mL distilled water. The mixture was cooled and 30 mL 10x 3-(N-morpholino propane sulfonic acid (MOPS)

buffer (0.2 M MOPS, 0.05 M Sodium acetate, 0.01 M EDTA, pH 6.0) and 9 mL formaldehyde were added. Subsequently, the gel was cast. Two µL of RNA samples were mixed with 2 µL 2x loading dye (Fermentas Life Science, Missouri, USA), incubated at 70°C for 10 min and stored on ice for 5 min before the whole samples were transferred to the gel and the electrophoresis was started for 30 min at 100 V. The bands of the RNA were visualized by a gel documentation system (Biometra, Jena, Germany).

2.9.3 Reverse transcription into cDNA

For PCR reactions DNA is used as a template and so the digested RNA has to be converted to cDNA to analyze the expression of target genes. To synthesize the cDNA
1 µg digested RNA was transferred to PCR reaction tubes and filled to a total volume of 10 µL with DEPC. Subsequently, 1 µL Random primer (RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Massachusetts, USA) was added and the mix incubated at 70°C for 5 min to destroy the secondary structure of the RNA. After cooling, 4 µL of reaction mix (5x buffer, 1 µL RNase inhibitor, 2 µL dNTPs (RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Fisher Scientific, Massachusetts, USA, 1 µL DEPC) were added and incubated for 5 min at room temperature. Afterwards, 1 µL reverse transcriptase was added and the reverse transcription was performed (10 min at 25 °C, 60 min at 42 °C and 10 min at 70 °C). Finally, the concentration of cDNA was adjusted to 5 ng/µL.

2.9.4 Quantitative real-time PCR

To measure the expression of target genes a qPCR was performed, which monitors the amplification of target genes during the PCR in contrast to the conventional PCR, which monitors the amplification at the end of the run. As described before (section 2.9.2) 1 µL cDNA (c = 5 ng/µL) were mixed with 4 µL TaqMan® (0.5 µL forward and reverse primer (c = 3 mM), 0.5 µL probe (c = 2 mM) and 2.5 µL TaqMan® Universal Mastermix (Power TaqMan gene expression master mix, Applied Biosystems, California, USA)) or SYBR®Green (Applied Biosystems, California, USA) master mix in a 384-well plate. For the SYBR®Green master mix,

0.5 µL DEPC were added to the mix instead of 0.5 µL probe. To calculate the gene expression level the ViiA7 Software v1.2 (Applied Biosystems, California, USA) was used. The used primers are listed below (Table 4). At first, a threshold was determined for each gene in the exponential phase of the logarithmic amplification curve. For the calculation, the dCt- and ddCt-method was used. Here, the Ct- values of the target gene are related to the Ct-values of an unregulated reference gene (dCt). Afterwards, the calculated means are normalized to a defined control group (ddCt).

Table 4: Primer sequences

Gene	Gene ID	Primer
60 S ribosomal protein L13a	Rpl13a	f GTTCGGCTGAAGCCTACCAG r TTCCGTAACCTCAAGATCTGCT
Activating transcription factor 3/ cAMP dependent transcription factor ATF3	Atf3	f GCTGCCAAGTGTCGAAACAAG r CAGTTTTCCAATGGCTTCAGG
Activating transcription factor 4/ cAMP dependent transcription factor ATF4	Atf4	f GGAATGGCCGGCTATGG r TCCCGGAAAAGGCATCCT
Activating transcription factor 6/ cAMP dependent transcription factor ATF6	Atf6	f CTTCCTCCAGTTGCTCCATC r CAACTCCTCAGGAACGTGCT r TCTCACAATGCCCTTGAAGGT
B-cell lymphoma 6	Bcl6	f CTGCAGATGGAGCATGTTGT r TTAAGTGCAGGGGCCATTT
Cardiotrophin 1	Ctf1	f AGAGGGAGGGAAGTCTGGAAGA r CGGATCTTGGCCTCCAAATG
Carnitine Palmitoyltransferase 1b	Cpt1b	f GAAGAGATCAAGCCGGTCAT r CTCCATCTGGTAGGAGCACA
Cluster of differentiation 36	Cd36	f CCAAGCTATTGCGACATGAT r ACAGCGTAGATAGACCTGCAAA
Cytochrome c	Cyt c	f ATAGGGGCATGTCACCTCAAAC r GTGGTTAGCCATGACCTGAAAG
DNA Damage-Inducible Transcript 3	Chop	f AGAGTGGTCAGTGCGCAGC r CTCATTCTCCTGCTCCTTCTCC
Dual Specificity Tyrosine Phosphorylation-Regulated Kinase 2	Dyrk2	f CACTACAGCCCACAGATTG r GCTGCTGAACCTGGATCTGT
Estrogen-related receptor α	Esrrα	f AACCTGAGAAGCTGTACGCC r AGTGAGGAGAAGCCTGGGAT
Fatty acid transport protein 3	Fatp3/Slc27a3	f AGGACAAGCTGCTGAAGGAT r TGTCTCCAGTACGATCGTGG
Fatty acid transport protein 4	Fatp4/Slc27a4	f CTCAGCAGGAAACATCGTGG r ACAGTCATGCCGTGGAGTAA
Fibronectin Type III Domain Containing 5	Fndc5	f ATGAAGGAGATGGGGAGGAA r GCGGCAGAAGAGAGCTATAACA
Glucose transporter 1	Glut1	f CGGGTATCAATGCTGTGTTC r GTCCAGCTCGCTCTACAACA
Glucose transporter 4	Glut4	f CTATGCTGGCCAACAATGTC r CCCTGATGTTAGCCCTGAGT

Gene	Gene ID	Primer
Growth Arrest And DNA Damage-Inducible protein 34	Gadd34	f GTCCATTTCCTTGCTGTCTG r AAGGCGTGTCCATGCTCTGG
Hypoxia inducible factor α	Hif1a	f GAAATGGCCCAGTGAGAAAA r TATCGAGGCTGTGTCGACTG
Interleukin 15	Il15	f CTC AGA GAG GTC AGG AAA GAA TCC r GAC CAT GAA GAG GCA GTG CTT
Interleukin 6	Il6	f TAGTCCTTCCTACCCCAATTTCC r TTGGTCCTTAGCCACTCCTTC
Mitochondrial ATP Synthetase, Beta Subunit	Atp5b	f TCATTGGAGAACCTATTGATGAGA r CCAGAATCTCCTGCTCAACAC
Monocarboxylate transporter 1	Mct1	f TGTTAGTCGGAGCCTTCATTTC r CACTGGTCGTTGCACTGAATA
Monocarboxylate transporter 4	Mct4	f CTGAGGCACGAGCAAGAGTA r GGCTGCTTTCACCAAGAACTG
Myosin heavy chain I	Mhc I	f CCAAGAGCCGGGACATTG r TTGGAGCTGGGTAGCACAAGA
Myosin heavy chain IIa	Mhc IIa	f GTCTGCGCAAACACGAGAGA r CCAAATCCTGAAGCCTGAGAATAT
Myosin heavy chain IIb	Mhc IIb	f AACAGAAGCGCAACATCGAA r TTTAGTCTGTAGTTTGTCCACCAAGTC
Myosin heavy chain IIx	Mhc IIx	f CAGATCGGGAGAACCAGTCT r CCTGCATTTTGCCAGAAGTT
NADH: Ubiquinone Oxidoreductase Subunit A5	Ndufa5	f AGGGTGGTGAAGTGGAAGAG r TGGCCACTTCCACTGGTTA
Nuclear receptor coactivator 1	Ncoa1	f GCTCTCGTCCACTGACCTTC r CTGAACCTGCTGCACCTGTA
Nuclear Receptor Subfamily 4 Group A Member 1	Nr4a1	f AGCTTGGGTGTTGATGTTCC r AATGCGATTCTGCAGCTCTT
Osteocrin	Ostn	f CCATGGATCGGATTGGTAGA r TCTGTGCCATCTCACACAAGT
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Pgc1α	f CTACAGACACCGCACACACC r GCGCTCTTCAATTGCTTTCT
Popeye Domain Containing 3	Popdc3	f AAGGCCGGGAAGGAAATAGC r GGGTTCAGCCCCAGTTTCTT
Salt Inducible Kinase 1	Sik1	f GACGGAGAGCGTCTGATACC r GGTCCTCGCATTTTTCCTC
TNF Receptor Superfamily Member 1A	Tnfrsf1a	f CACCGTGACAATCCCCTGTAA r TTTGCAAGCGGAGGAGGTAG
Transcription Factor A, Mitochondrial	Tfam	f AAGACCTCGTTCAGCATATAACATT r TTTTCCAAGCCTCATTTACAAGC
Vascular endothelial growth factor receptor 1	Flt1	f GTCCTATCGGCTGTCCATGA r GCATCCTCGGTTGTCACATC
Vascular endothelial growth factor ß	Vegfß	f TATCTCCCAGAGCTGCCATCTA r AGCCAGAAGATGCTCACTTGAC
Vascular endothelial growth factor α	Vegfα	f CTGTAACGATGAAGCCCTGGAG r TGGTGAGGTTTGATCCGCAT

2.9.5 Microarray

Microarrays allow a screening of several thousand candidate genes, which were scanned for differences in their expression in a few biological samples.
Agilent 8 x 60 k whole-genome mouse microarrays (G4852A, Agilent Technologies Inc., Santa Clara, USA) were used. Amplification, labeling, and microarray hybridization of individual *M. quadriceps* samples of 6 wk old mice (mLFD, mHFD; n = 10; study II) were performed in collaboration with Jaap Keijer (Wageningen University & Research, the Netherlands).

2.9.6 Methylation measurements

Epigenetic changes in the DNA methylation pattern could lead to changes in gene expression. Here, a methyl group is transferred to a cytosine which occurs more frequently in promoter regions of a gene and causes a modified function of it.
These DNA methylations in the promoter region of a gene can be detected by bisulfite sequencing and pyrosequencing.
The bisulfite sequencing of a large region (-2000 through 1000 bp around the transcriptional start site (TSS)) gives first indications of possible methylation sites, whereas the exact methylation status was verified by pyrosequencing.

2.9.6.1 Genomic DNA extraction

For DNA extraction the Invisorb® Genomic DNA Kit II (Stratec Biomedical AG, Birkenfeld, Germany) was used according to manufacturer´s instructions. Briefly, 50 mg frozen and ground *M. quadriceps* were mixed with 300 µL Lysis Buffer G and 20 µL Proteinase K and incubated for 2–3 h at 60°C with continuous shaking on a thermomixer. Subsequently, the samples were centrifuged, mixed with Binding Buffer, centrifuged again and the pellet used for the washing steps until elution. The DNA-containing supernatant was transferred to a new reaction tube. The DNA concentration was calculated by Take3 as described before (section 2.9.1).

2.9.6.2 Bisulfite sequencing

Bisulfite conversion

To measure DNA methylation, the genomic DNA was bisulfite converted. Here, cytosines are converted to uracils, while 5-methylcytosines are unaffected. This converted DNA consists only of methylated cytosines and so the methylation status of specific genes can be determined (Figure 10).

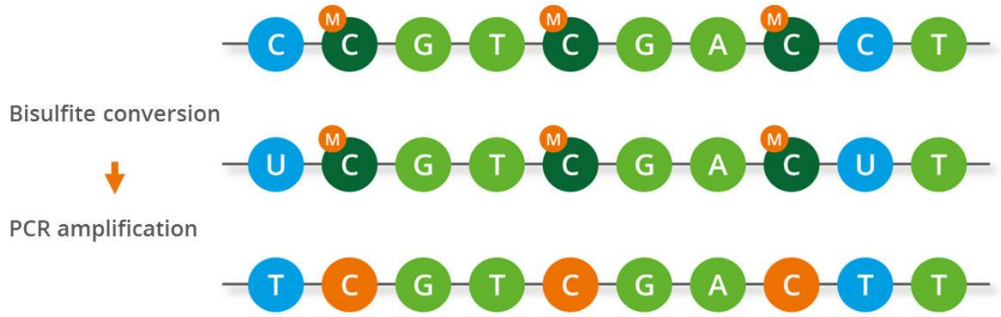

Figure 10: Bisulfite conversion
By bisulfite conversion, unmethylated cytosines are converted to uracils, while 5´-methylcytosines remain unaffected. (https://www.gatc-biotech.com/en/expertise/epigenetics/bisulfite-profiling.html, Access: 12.05.2017)

For bisulfite conversion, the EpiTect Fast Bisulfite Kit (Qiagen GmbH, Hilden, Germany) was used. Genomic DNA (500 ng) was adjusted to 20 µl with RNase-free water and mixed with 120 µL master mix (85 µL Bisulfite mix, 35 µL DNA Protect Buffer). After mixing and a short centrifugation, bisulfite conversion was performed using a thermal cycler and the following program: 95 °C for 5 min, 60 °C for 10 min, 95 °C for 5 min and 60 °C for 10 min. Afterwards, the purification was performed according to manufacturer´s protocol.

To investigate the methylation status of the gene of interest, a PCR using Takara Epi Taq HS (Takara Clontech, Otsu, Japan) was performed. In detail, 2.5 µL bisulfite converted DNA were mixed with 22.5 µL Takara Epi Taq HS master mix (13.375 µL aqua dest., 2.5 µL 10x Buffer, 3 µL dNTPs (2.5 mM), 2.5 µL 25 mM $MgCl_2$, 0.5 µL 10 µM forward and reverse primer and 0.125 µL Takara Epi Taq HS (5 U/µL)). The used primers for Nr4a1 were designed using the online tool MethPrimer for fragments with the size of approximately 400 bp (Figure 11; Table 5). The PCR amplification was performed according to the temperature profile listed in Table 6.

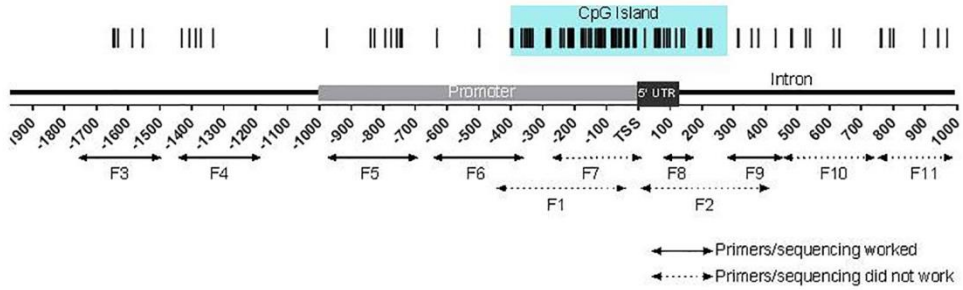

Figure 11: Whole genome methylation of NR4A1

Table 5: Primer sequences for bisulfite sequencing

Gene	Position	Sequence
Nr4a1	F3	f TGTGTAAGGGTTTAAGGAAAGAAAG
		r TATAACCTAAAATCCATCCCATCAC
	F4	f TGATGGGATGGATTTTAGGTTATAA
		r TCCATCCCAATCTAAACTAAAAAAA
	F5	f ATTGGGATTAGGGAATTGATAGAA
		r AAAATTCCTTCCCAAAAAACATAAC
	F6	f TTTGTATATTGTTAAGGTTGAGGTA
		r TAACTTCCAAACCCACTAAACTCAC
	F8	f TAGGGGAGTTTTAGTGTAGGAGGTT
		r CTCTTTCATACACTATCCCTAAAAACAA
	F9	f TAGTTTTTGGAGTAGGGTGAGTTAG
		r CTTATACTTAAATAAATCCCAACAAC

f, forward; r, reverse primer

Table 6: PCR Programm

Step	Temperature [°C]	Time	
Initial denaturation	95	30 sec	45 cycles
Denaturation	98	10 sec	
Primer hybridization	55	30 sec	
Polymerization	72	30 sec	
Finale polymerization	72	7 min	
"Hold"	4	undefined	

Quality control

To verify the PCR quality, an agarose gel electrophoresis was performed. Therefore, 3 µL of PCR product were mixed with 5 µL DNA loading dye (Thermo Fisher Scientific, Germany). For the preparation of a 1.5 % agarose gel, 4.5 mg agarose pills (Thermo Fisher Scientific, Massachusetts, USA) were filled with 1x TAE buffer (20 mM Tris, 10 mM Acetate, 0.5 M EDTA, pH 7.4) until a total weight of 300 mg and boiled until the agarose was completely dissolved. Then 15 µL ethidium bromide was added to the solution and the gel was cast. The whole samples and the DNA ladder (O´Gene Ruler 50 bp DNA Ladder, Fermentas, USA) were transferred to the gel and the electrophoresis was performed for 30 min at 150 V. The PCR products were visualized by a gel documentation system (Biometra, Germany).

Sequencing

When the PCR was specific, the PCR product was purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to manufacturer´s instructions.
For the sequencing, 7.5 µL DNA (40 – 80 ng/µL) was mixed with 2.5 µL forward or reverse primer (10 µM). Sequencing was performed by GATC-Biotech AG (Konstanz, Germany) by Sanger sequencing. All sequences were analyzed for methylation differences using the Codon Code Aligner software. The bisulfite sequencing was a first screening to detect differentially methylated CpG islands in the region 2000 bp before and 1000 bp after the transcription start site. This region was screened by various overlapping fragments with an approximate size of 400 bp. The differentially methylated areas are further analyzed by pyrosequencing (section 2.9.6.3).

2.9.6.3 Pyrosequencing

Using Pyrosequencing, single nucleotide polymorphisms or methylation patterns in a defined DNA sequence could be detected. It is based on the “sequencing by synthesis” principle for the quantitative analysis of DNA sequences.
After hybridizing the sequencing primer to the single-stranded DNA template and incubating it with enzymes and substrates, one of the four nucleotides was added to the reaction at a time. If the nucleotide matches the DNA, the DNA polymerase

incorporates it and simultaneously releases pyrophosphate (Figure 12). The ATP sulfurylase converts the pyrophosphate quantitatively to ATP, which drives the conversion of luciferin to oxyluciferin. The resulting light signal is proportional to the amounts of ATP and is detected as a peak in the Pyrogram®. The light signals are proportional to a number of incorporated nucleotides in the DNA strand. Unincorporated nucleotides and ATP are degraded by the Apyrase. After completing the degradation the next nucleotide is added to the reaction. This process is repeated until all nucleotides are incorporated and the complementary sequence is completed. The final nucleotide sequence then can be determined from the peaks in the Pyrogram®.

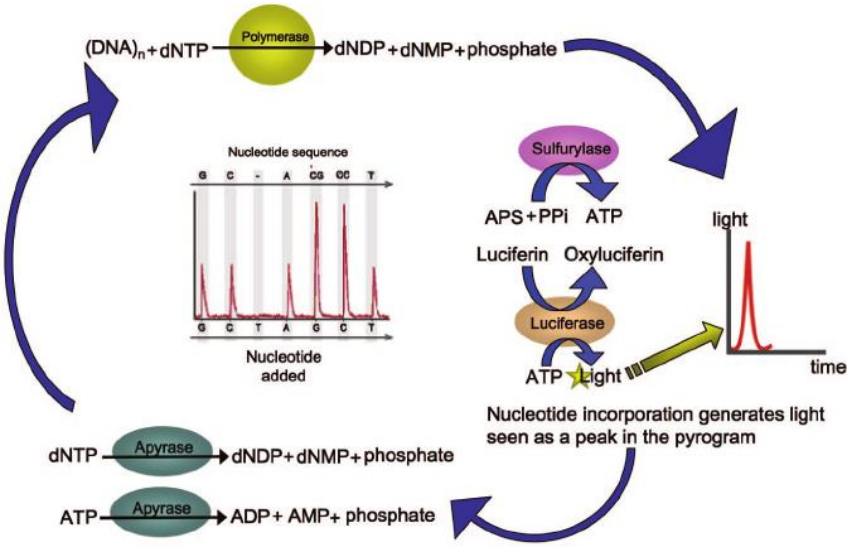

Figure 12: Principle of the pyrosequencing
Pyrosequencing is based on the detection of light emission in the presence of luciferase by the release of a pyrophosphate [80].

For CpGs that were differentially methylated, HPLC purified, biotinylated forward, reverse and sequencing primer were designed using the PyroMark Assay Design Software (Table 7). The amplicon size was between 100 and 200 bp. In order to carry out the pyrosequencing, the DNA was first bisulfite converted as described in section 2.9.6.2.

For pyrosequencing implementation, PyroMark Gold Q24 Reagents (Qiagen GmbH, Hilden, Germany) were used according to manufacturer´s protocol. Briefly, 10 µL – 20 µL of each bisulfite converted PCR product were mixed with a binding mix and placed on a 24-well plate (PyroMark Q24 Plate low, Qiagen GmbH, Hilden, Germany). For binding the PCR product to the beads the plate was shaken for at

least 10 min before the annealing primer mix (0.75 µL sequencing primer (10 mM), 24.25 µL PyroMark Annealing Buffer) was added to each sample. Then the measurement was started.

Table 7: Primer sequences for Pyrosequencing

Gene	Position	Sequence
Nr4a1	F4	f GGGATAGTGATTAAAAAGAAATAGTAGGT
		r CCCCTAATTCCCCATTTCTACTACCA
		S GAGTTAAGTTTTTAGTTTATTTTTA
	F5	f TGTATTTATTTTGAGAGGGGTTAAGAGG
		r ACTAAACCCCTACCTCAACCTTAACAATAT
		S AGAGGTTATAGTTGAGAAATTAGTA

f, forward; r, reverse; S, sequencing primer

2.10 Enzymatic assays

2.10.1 Citrate synthase activity

The citrate synthase reaction is the first step of the tricarbon acid cycle in mitochondria. Therefore citrate synthase activity was used to calculate the mitochondrial amount of the tissues. To measure citrate synthase activity, *M. quadriceps* samples were homogenized in 19 volume portions of 50 nM Tris, 1 mM EDTA (pH 7.4) and 0.1 % (v/v) Triton X-100 for 3 min using a tissue lyser, followed by a centrifugation (4 °C, 13000 x g, 10 min). The supernatant was transferred to a new reaction tube and a 1:6 dilution with distilled water was prepared. The photometer was preheated to 37°C and the reaction buffer (100 mM Tris, 1 mM MgCl2, 1 mM EDTA (pH 8.2), and 0.1 M DTNB), 3.6 mM acetyl-CoA and 3 mM oxaloacetate were freshly prepared. Ten µL sample were mixed in triplicates with 25 µL acetyl-CoA and 215 µL reaction buffer in a 96-well plate. To start the reaction 50 µL oxaloacetate were added to each well and the absorbance change was measured for 5 min at 412 nm. To calculate the citrate synthase activity the absorbance difference between 2 min and 4 min was used.

2.10.2 Lactate dehydrogenase activity

The lactate dehydrogenase catalyzes the conversion of pyruvate to lactate and vice versa in the absence of oxygen. Thereby NADH is either consumed or produced. Lactate dehydrogenase activity was determined in *M. quadriceps*, liver and heart muscle according to a published protocol [81]. Either 30 mg of liver and skeletal muscle tissue or 10 mg of heart tissue were homogenized in 600 µL of 890 mM 2-Amino-2-methyl-1-propanol for pyruvate determination (Equation 1) or 50 mM Potassium phosphate buffer for lactate determination (Equation 2). For pyruvate determination 720 mM L-(+) lactic acid and 55 mM NAD+ were used as substrates, while 1.5 mM Sodium pyruvate and 2.0 mM b-NADH were used for the lactate measurement. Enzyme activity was measured at a wavelength of 340 nm using Power Waver 340 (Biotek Instruments, Vermont, USA).

Equation 1

$$\text{Lactate} + NAD^+ \longrightarrow \text{Pyruvate} + NADH + H^+$$

Equation 2

$$\text{Pyruvate} + NADH + H^+ \longrightarrow \text{Lactate} + NAD^+$$

2.11 Statistical analysis

For statistical analysis, SPSS (version 20; IBM SPSS Inc.) and GraphPad Prism 6 (GraphPad Software Inc.) were used. Normal distribution was tested by performing Kolmogorov-Smirnov test. Normally distributed data were analyzed either by two-way ANOVA with Bonferroni post hoc test or an unpaired t-test with Welch´s correction. If the data were not normally distributed, a Kruskal-Wallis test with Dunn´s multiple comparison test was performed. Data were defined as statistically significant with a p-value < 0.05 (*$p < 0.05$; **$p < 0.01$; ***$p<0.001$).

3 RESULTS

3.1 Impact of high-fat diet intervention on pregnant dams

In order to investigate the impact of maternal nutrition on offspring energy metabolism female C57Bl/6J mice were either fed a low-fat (LFD) or high-fat diet (HFD) three days before mating throughout gestation and lactation. Body weight and body composition of the dams were determined before mating and after weaning to evaluate the impact of high-fat diet feeding (Figure 13). LFD and HFD fed dams did not differ in body weight and lean mass at mating and weaning, while HFD dams displayed a significantly higher fat mass after weaning. Thus, without becoming obese, HFD intervention during pregnancy and lactation resulted in an increased fat mass in the dams. Nevertheless, it was assumed that the obtained results on offspring metabolism can be mainly attributed to the effects of maternal nutrition and not to maternal obesity during pregnancy and lactation. Thus, we were aiming to evaluate if the maternal perinatal consumption of HFD alone leads to the detrimental effects on offspring energy metabolism.

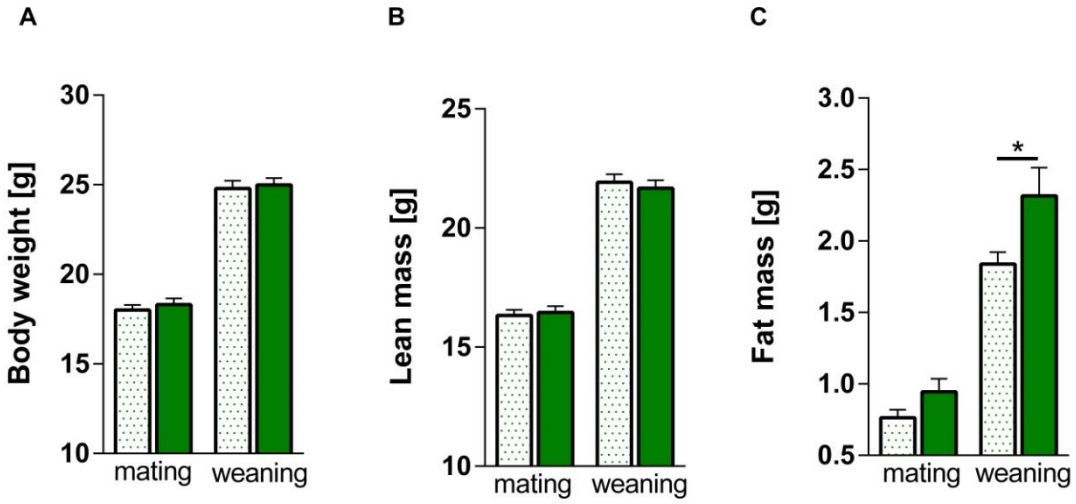

Figure 13: Impact of high-fat diet feeding on dams (A) body weight and (B/C) body composition.
Female C57Bl/6J dams were either fed a low-fat (LFD; green dotted bars) or a high-fat diet (HFD; green bars) three days prior mating throughout pregnancy and lactation. Body weight and composition were determined before mating and after weaning. Data are mean + SEM; n = 13-19. Data were analyzed by using an unpaired t-test and Welch´s correction.

3.2 Maternal high-fat diet elevated offspring body weight and body fat

To evaluate the impact of maternal overnutrition on the offspring, body weight and body composition were determined in all studies (study I – III) (Figure 14). While at an age of four weeks (wks) mHFD offspring had a significantly higher body weight compared to mLFD offspring (Figure 14A), this was not seen anymore in six (Figure 14B) or seven wk old offspring (Figure 14A/C). However, mHFD offspring tended to have a higher body weight compared to mLFD offspring at an age of 15 but not 25 wks (Figure 14C). To determine whether the -in tendency- elevated body weight of mHFD offspring was due to an increase in fat mass offspring body composition was determined. mHFD offspring at four wks had a significantly increased fat mass, as well as lean mass (Figure 14D). The increased fat mass in mHFD offspring was at least in tendency also detectable at six wks of age (Figure 14E), while there were no differences at seven wks anymore (Figure 14D/F). Here, mHFD offspring showed a higher lean mass compared to mLFD offspring but no effect on fat mass. This might be due to an increase in body length, i.e. accelerated somatic growth. At an age of seven wks half of the offspring got access to a running wheel (RW) as voluntary training. Thus, the training effect on body composition could also be measured in 15 and 25 wk old offspring. 15 wk old mHFD offspring displayed a higher fat mass independently of RW training, while the voluntary training led to a significant reduction of fat mass in both offspring groups (Figure 14D/F). With 25 wks of age, the RW training effect on the fat mass was not detectable anymore (Figure 14F). Thus mLFD offspring with and without RW intervention showed a significantly lower fat mass weight. To underline the voluntary training effect on fat mass weight, fat mass gain from wk seven until wk 15 and wk 25 was calculated as well. The results show clearly a reduction in fat mass from wk seven until wk 15 in both offspring groups, while it was much more pronounced in mLFD offspring (Figure 14G/H). At 25 wks of age, RW intervention reduced the fat mass gain from wk seven until wk 25 only in mLFD but not in mHFD offspring (Figure 14H). Thus, voluntary running wheel intervention had no positive effect on the fat mass gain in mHFD offspring.

Taken together, maternal high-fat diet intervention resulted in an increased fat mass gain in the offspring, which could not be prevented by voluntary training.

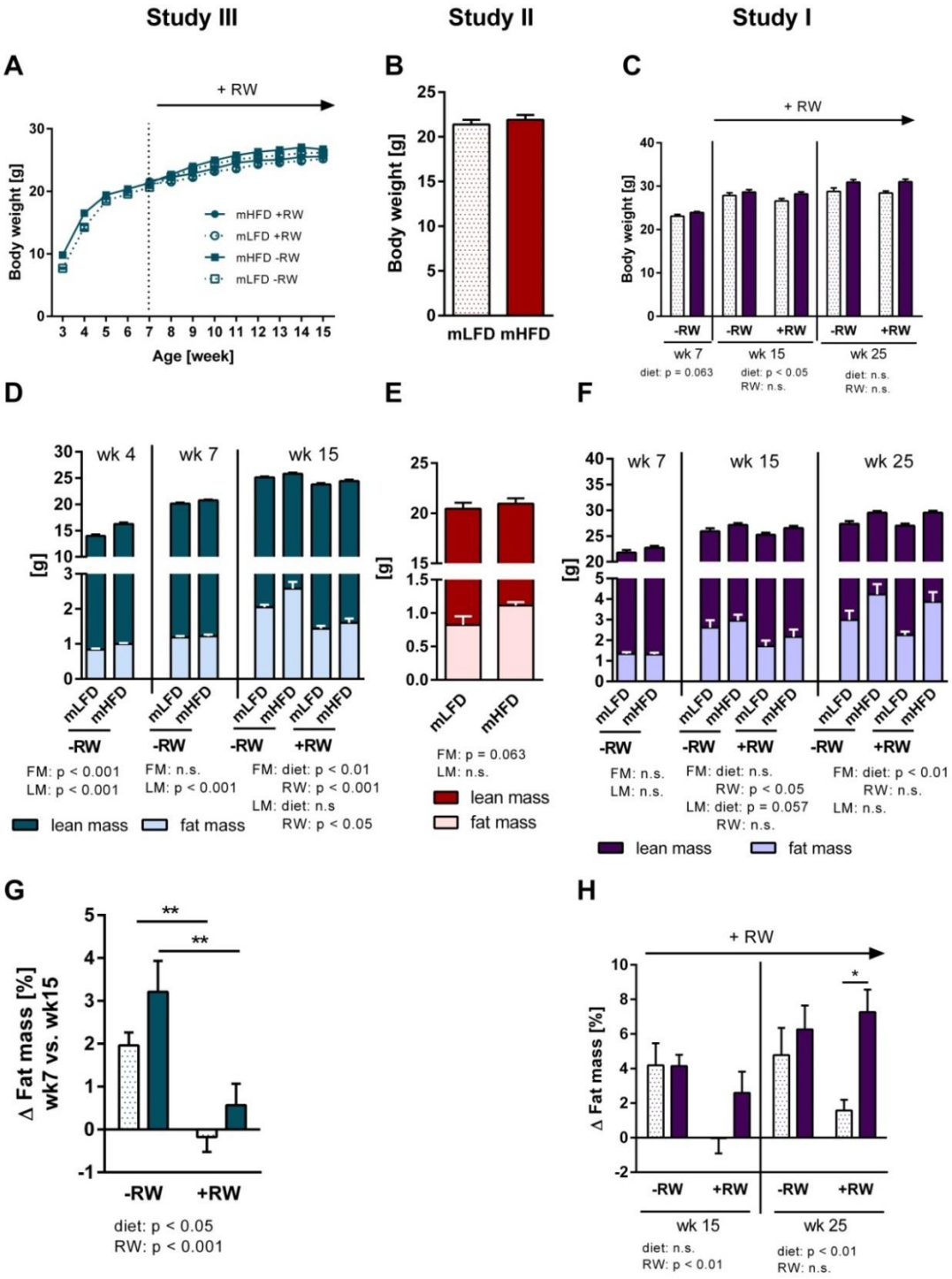

Figure 14: Impact of maternal high-fat diet on offspring body weight, body composition, and fat mass gain.
Dams were fed a low-fat (LFD) or a high-fat diet (HFD) during pregnancy and lactation. Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; filled bars) offspring received a LFD after weaning until the end of the study. Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. (A-C) Body weight development from wk four until wk 25 (A, Study III; B, Study II; C, Study I), (D-F) Body composition (D, Study III; E, Study II; F, Study I) and (G/H) Fat mass gain from wk 7 until wk 15 and from wk 7 until wk 25 (G, Study III; H, Study I) is shown. Data are mean + SEM; (A) n=51-65; (B/E) n= 10; (C) n = 42-53; (D) wk4-7: n = 51-65, wk 15: n = 26-35; (F) wk 7: n = 42-53, wk 15-25: n = 12-13; (G) n = 19-25 and (H) n = 9-13. Data were analyzed by using two-way ANOVA (Bonferroni post hoc test) or an unpaired t-Test with Welch´s correction (B/E).

3.3 Exercise capacity and muscle physiology

Since mHFD offspring displayed a higher fat mass weight over the whole time and RW training could not completely prevent fat mass gain, the question arises, whether this has also an impact on the endurance capacity and training efficiency of the offspring. To investigate this, an endurance treadmill test was performed at an age of seven, eleven, 15 and 25 wks. In Figure 15 the change in endurance capacity from wk seven until wk eleven, 15 or 25 is displayed (Figure 15A Study III; Figure 15C Study I). Voluntary running wheel training led to an induction of the exercise capacity in both maternal groups over the whole period, while this was much more pronounced in mLFD offspring at eleven (Figure 15A) and 15 wks (Figure 15A/C). At 25 wks exercise capacity of mLFD and mHFD offspring with running wheel training was not different anymore (Figure 15C). Hence, mHFD offspring seem to have a delayed training response.

To see whether this effect is due to a changed muscle physiology, *M. quadriceps* weight (Figure 15B Study III; Figure 15D Study I), as well as muscle fiber number and size (Figure 15G Study I), were analyzed revealing no differences between the groups.

Furthermore, the fiber type composition was analyzed by gene expression measurements of myosin heavy chains (Mhc) (Figure 15E) since endurance training can result in a fiber type switch from glycolytic to oxidative muscle fibers. Skeletal muscle contains red and white muscle fibers, due to differences in the myoglobin content. Based on histochemical staining one can distinguish between slow-twitch muscle fibers (stained in red, oxidative) and fast-twitch muscle fibers (pale stained, glycolytic). For endurance athletes is known that skeletal muscle contains relatively more slow-twitch muscle fibers, while sprinters show more fast-twitch muscle fibers in their musculature [43]. The most prominent marker for the fiber type distribution in skeletal muscle is the myosin heavy chains (MHC). There are four major types characterized as MHCI (slow-twitch), MHCIIa, MHCIIx and MHCIIb (fast-twitch) [82]. To clarify whether the variation in exercise performance can be explained by the heterogeneity of the fiber type composition in skeletal muscle, gene expression was determined in two different muscle types, *M. quadriceps* (a fast-twitch, glycolytic muscle with a mixed fiber type composition) and *M. soleus* (a slow-twitch, oxidative muscle). In *M. quadriceps,* there were no differences between the groups at the age of six wks (Figure 15E Study II). With 15 wks of age MhcIIa and MhcIIx

gene expression was increased in both groups with RW intervention, while mLFD and mHFD offspring did not differ between the groups (Figure 15E Study III). The highest expression level was detectable for MhcIIb, which did also not differ between the groups. For the mice with an age of 25 wks (Figure 15E Study I), the highest gene expression level was detected for the glycolytic MhcIIx and MhcIIb fibers, which also fits the fact that *M. quadriceps* is more glycolytic than oxidative. Furthermore, mHFD offspring showed a higher expression of these fibers compared to mLFD offspring, independently of voluntary running wheel intervention. The more oxidative muscle fibers MhcIIa are expressed to a lesser extent, but also higher in mHFD offspring, while gene expression of MhcI fibers was not detectable (Figure 15E Study I). In *M. soleus* of 25 wk old mice gene expression of MhcIIa fibers was the highest, but there were no differences between the groups (Figure 15F Study I). Voluntary running led to a reduction in expression level in both groups. Furthermore, the oxidative MhcI fibers were higher expressed in mLFD than in mHFD offspring. This gene expression pattern is in line with the fact that *M. soleus* is an oxidative muscle. The glycolytic muscle fibers MhcIIx and MhcIIb were expressed to a lesser extent than MhcIIa fibers in *M. soleus*.

In summary, RW training had no effect on the fiber type number but caused significant differences in fiber type expression. However, these differences are so small that the biological relevance is questionable.

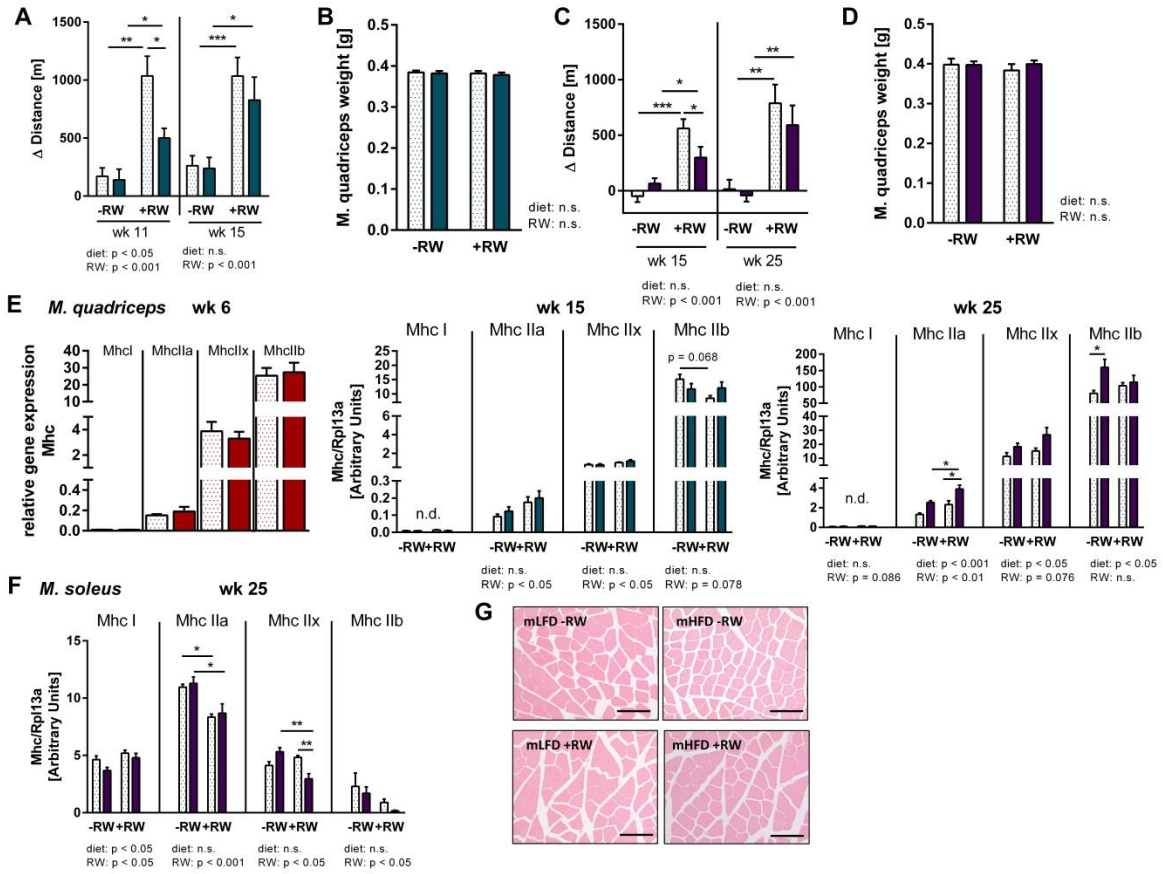

Figure 15: **Impact of maternal overnutrition on offspring endurance capacity and skeletal muscle morphology/physiology.**
Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; filled bars) offspring received a LFD after weaning until the end of each study (Study I – III). Half of the groups got access to a running wheel (RW) as voluntary training (Study I/III). (A/C) Endurance capacity from wk seven until wk eleven and 15 (A, Study III) and from wk seven until wk 15 and 25 (C, Study I), (B) *M. quadriceps* weight of 15 wk old mice (Study III) and (D) of 25 wk old mice (Study I), (E) gene expression (dCt) of myosin heavy chain (Mhc) isoforms in *M. quadriceps* (6 wk, Study II; 15 wk, Study III, 25 wk, Study I) and (F) *M. soleus* (Study I) and (G) Light microscopy pictures of *M. quadriceps* (H&E staining) of 25 wk old mice (Study I). Data are mean + SEM; (A) n = 6-10; (B) n = 25-30; (C) wk 15: n = 20-28, wk 25: n = 9-13; (D) n = 9-11; (E) wk 6: n = 10, wk 15/25: n = 6 and (F) n = 6. Data were analyzed by unpaired t-Test and Welch´s correction (A/C and E (6 wks)) and two-way ANOVA (Bonferroni post hoc test) (A-F).

3.4 Impact of maternal overnutrition on offspring muscle genetics and epigenetics

The offspring of LFD or HFD fed dams received a LFD after weaning and were sacrificed at an age of six wks without any prior intervention (Study II). A microarray analysis in *M. quadriceps* was performed to elucidate the question whether maternal high-fat consumption results in early transcriptional changes of offspring skeletal muscle.

3.4.1 Transcriptional modifications of offspring skeletal muscle by maternal high-fat diet

In total 706 genes showed a significant differential expression. For further analysis, only genes with a fold change higher than +/- 1.2 were included, which resulted in 317 genes, from which 160 were upregulated and 157 were downregulated. The TOP-40 candidates are listed in Figure 16A.

The most strongly upregulated genes in mHFD offspring include B- cell CLL lymphoma 6 (Bcl6), Dual specificity tyrosine-phosphorylation-regulated kinase 2 (Dyrk2), Nuclear receptor coactivator (Ncoa1), Nuclear receptor subfamily 4 group A member 1 (Nr4a1), Popeye domain-containing protein 3 (Popdc3) and Serine/threonine-protein kinase 1 (Sik1), which were verified by qPCR (Figure 16B). However, the upregulation could only be validated for Sik1 and Nra41. From the downregulated genes, Anoctamin1 (Ano1), Cardiotrophin1 (Ctf1) and Tumor necrosis factor receptor superfamily member 1a (Tnfrs1a) (Figure 16B) were verified by qPCR, but only the expression of Ctf1 could be confirmed.

A

	mLFD										mHFD											
GeneName	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	FC	p-value
Bcl6	11,29	10,95	10,71	10,83	11,00	10,65	10,97	11,34	11,26	10,85	11,65	11,22	11,41	11,21	11,24	11,54	11,65	11,58	11,56	11,44	1,38	0,0001
Abtb2	8,23	8,15	7,95	8,00	8,03	7,87	7,85	8,18	7,96	8,07	8,04	8,15	8,24	8,34	8,22	8,36	8,35	8,36	8,47	8,29	1,19	0,0003
Pdia6	6,75	6,78	6,76	6,79	6,71	6,72	6,75	6,62	6,76	6,68	7,10	6,85	7,03	7,08	6,88	6,96	6,89	6,78	6,71	6,88	1,14	0,0004
Ccdc50	6,89	6,91	6,81	6,52	6,82	6,36	6,76	7,05	6,77	7,19	7,43	7,72	7,32	7,40	7,10	6,92	7,37	6,97	7,65	6,97	1,39	0,0007
Ints13	7,51	7,57	7,41	7,42	7,47	7,30	7,47	7,42	7,56	7,22	7,44	7,65	7,71	7,59	7,61	7,50	7,61	7,67	7,62	7,69	1,13	0,0008
Tmem55a	8,97	9,10	8,92	8,95	8,98	8,80	8,76	9,09	8,82	8,94	9,11	8,97	9,20	9,12	8,98	9,13	9,13	9,13	9,28	9,06	1,13	0,0012
Med14	6,30	6,40	6,52	6,54	6,52	6,52	6,25	6,21	6,37	6,47	6,54	6,85	6,54	6,54	6,63	6,44	6,59	6,66	6,61	6,68	1,15	0,0015
Slc38a2	9,73	9,67	9,34	9,20	9,57	9,50	9,98	9,90	9,85	9,53	10,32	9,79	9,92	9,77	9,87	10,23	9,92	10,51	9,90	10,08	1,32	0,0019
3830612M24	6,84	6,64	6,76	6,70	6,28	6,53	6,55	6,53	6,64	6,71	6,89	6,93	6,95	6,79	6,78	6,84	6,84	6,85	7,57	6,87	1,24	0,0021
Ncoa1	8,01	8,19	7,84	7,91	7,90	7,71	7,70	8,04	7,95	7,85	8,19	7,95	8,07	8,16	8,10	8,22	8,11	8,49	8,11	8,06	1,18	0,0021
Dyrk2	7,31	7,64	7,20	7,52	7,31	7,12	7,41	7,45	7,37	7,36	7,80	7,90	7,49	7,45	7,54	7,59	7,73	7,78	7,41	7,52	1,19	0,0025
Sik1	6,03	6,71	6,44	6,04	5,78	5,84	6,29	6,77	6,36	6,63	7,77	6,93	6,84	7,04	6,24	6,47	7,14	7,04	6,99	6,53	1,53	0,0026
Popdc3	7,93	8,07	7,63	7,79	7,96	7,56	7,70	7,90	7,71	7,56	8,26	8,05	7,96	7,97	8,04	8,06	7,92	7,93	7,95	7,91	1,17	0,0030
Skil	6,61	6,47	6,81	6,74	6,53	6,52	6,84	6,91	6,75	6,34	6,87	6,87	6,90	6,72	6,85	7,02	6,93	6,74	7,05	6,87	1,17	0,0032
Esr1	7,05	7,00	6,78	6,84	6,95	6,63	6,83	6,83	6,61	7,05	6,93	7,35	7,02	7,03	7,22	7,26	7,02	7,01	6,99	7,00	1,17	0,0034
Nr4a1	12,43	12,16	11,86	12,00	11,88	12,10	11,88	12,51	12,80	12,24	13,52	12,81	12,21	12,71	12,67	12,54	12,83	13,00	12,35	12,40	1,43	0,0035
Slc2a4rg-ps	7,05	7,33	7,04	7,25	7,15	7,39	7,13	7,32	7,16	7,40	7,72	7,52	7,25	7,43	7,46	7,27	7,38	7,42	7,30	7,67	1,17	0,0036
Egfr	7,85	8,15	8,20	7,99	7,61	7,50	7,77	8,05	8,29	7,99	8,52	8,40	8,27	8,41	8,32	8,13	8,06	8,10	8,48	8,03	1,26	0,0037
Mob1a	7,31	7,56	7,40	7,55	7,37	7,16	7,56	7,55	7,40	7,56	7,50	7,64	7,61	7,46	7,62	7,58	7,62	7,78	7,69	7,64	1,13	0,0040
Ctbp2	7,82	7,66	7,77	8,06	7,95	7,82	7,66	7,78	7,74	7,81	8,13	7,82	8,20	7,76	7,91	8,03	7,91	8,12	8,06	8,11	1,15	0,0041
Scube2	8,03	8,11	8,24	8,13	8,09	8,34	8,06	8,37	8,22	8,22	7,77	7,76	7,89	8,10	7,89	8,12	7,90	7,94	8,04	7,86	-1,19	0,0002
Hmbs	7,64	7,65	7,63	7,72	7,76	7,64	7,70	7,81	7,79	7,74	7,55	7,45	7,51	7,61	7,48	7,59	7,61	7,66	7,56	7,71	-1,10	0,0005
Tle6	7,73	7,51	7,67	7,51	7,59	7,72	7,81	7,60	7,86	7,61	7,64	7,52	7,55	7,34	7,51	7,40	7,29	7,40	7,27	7,49	-1,16	0,0007
Tmem80	7,49	7,52	7,46	7,49	7,42	7,39	7,67	7,73	7,50	7,70	7,23	7,21	7,41	7,39	7,35	7,42	7,41	7,40	7,38	7,42	-1,13	0,0011
Pkn1	8,57	8,50	8,50	8,60	8,44	8,63	8,24	8,49	8,55	8,33	8,38	8,15	8,17	8,14	8,25	8,35	8,31	8,45	8,27	8,38	-1,15	0,0011
Gba	7,44	7,73	7,41	7,62	7,67	7,56	7,62	7,74	7,75	7,69	7,55	7,40	7,28	7,23	7,35	7,35	7,49	7,58	7,53	7,48	-1,15	0,0015
Sox4	7,29	7,28	7,18	7,74	7,40	7,84	7,84	7,18	7,37	7,34	7,00	7,12	6,99	7,19	7,17	7,39	7,22	6,99	7,00	6,85	-1,28	0,0016
Slc50a1	7,90	8,05	7,88	7,97	8,13	8,11	7,85	7,75	7,98	7,94	7,69	7,89	7,74	7,69	7,87	7,74	7,87	7,82	7,58	7,88	-1,13	0,0023
Ctf1	7,26	6,93	7,16	7,21	7,01	7,31	7,49	7,26	7,49	7,18	6,82	6,88	7,30	6,91	7,08	7,01	6,85	6,93	6,91	7,05	-1,19	0,0024
Ano1	8,07	7,74	7,79	8,03	8,09	8,36	8,36	7,39	7,68	7,89	7,01	7,44	7,43	7,49	7,62	8,12	7,31	7,38	7,45	7,57	-1,37	0,0025
Sfxn5	7,08	7,30	7,49	7,50	7,33	7,07	7,28	7,77	7,25	7,31	7,03	6,97	7,07	7,10	7,15	6,93	7,02	7,24	7,27	7,03	-1,20	0,0027
Pex11c	8,26	7,90	7,79	8,11	8,04	8,16	7,95	8,20	8,20	8,09	7,89	7,80	7,84	7,76	8,07	7,91	7,84	7,69	7,88	8,00	-1,15	0,0028
Tst	8,95	8,68	8,48	8,78	8,92	9,07	8,76	8,72	8,71	8,87	8,53	8,21	8,39	8,24	8,65	8,70	8,67	8,47	8,48	8,80	-1,21	0,0031
Art5	8,50	8,12	8,14	8,53	8,49	8,51	7,84	7,73	8,01	7,60	7,66	7,80	7,46	7,68	7,96	7,81	7,80	7,89	7,30	7,75	-1,35	0,0031
Acvr2b	9,97	9,24	9,72	9,34	9,90	10,08	9,69	9,61	9,38	9,56	9,42	8,99	9,00	9,12	9,47	9,66	8,95	9,13	8,62	9,54	-1,37	0,0033
Aldh16a1	9,00	8,83	8,77	9,09	8,95	9,11	8,73	9,04	9,08	8,83	8,83	8,76	8,65	8,72	8,89	8,90	8,67	8,59	8,73	8,82	-1,14	0,0034
Zbtb7b	7,60	7,45	7,56	7,51	7,53	7,66	7,62	7,61	7,64	7,77	7,27	7,41	7,60	7,49	7,40	7,45	7,44	7,45	7,53	7,54	-1,10	0,0035
Ubfd1	7,42	7,62	7,53	7,48	7,49	7,38	7,44	7,52	7,68	7,73	7,43	7,46	7,39	7,43	7,22	7,29	7,37	7,27	7,54	7,31	-1,12	0,0036
Tnfrsf1a	8,79	8,32	8,85	8,43	8,70	8,91	9,07	8,87	8,65	9,09	8,65	8,21	8,14	8,23	8,60	8,56	8,02	8,41	8,08	8,83	-1,31	0,0037
Cd52	7,12	7,04	7,05	7,32	7,17	7,44	7,75	7,21	7,35	7,84	6,92	7,18	7,22	7,07	7,18	6,94	6,71	6,94	6,76	6,95	-1,27	0,0040

B

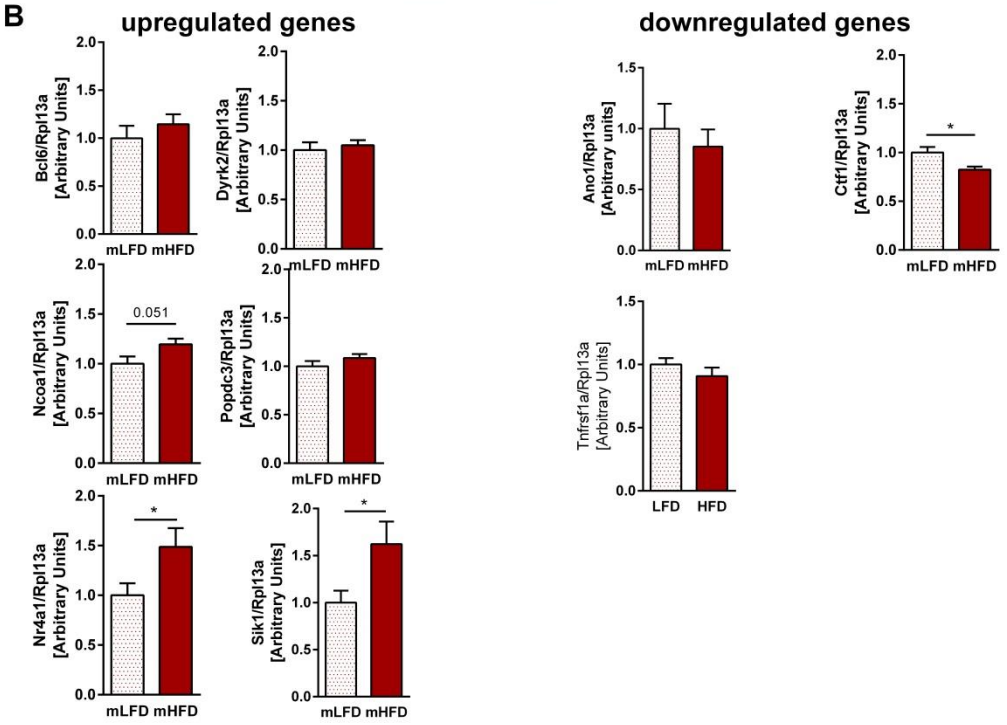

Figure 16: Transcriptional modifications of offspring skeletal muscle by maternal high-fat diet.

(A) Heat map (green – down-regulated, red – up-regulated) of the TOP 40 differentially regulated genes identified by microarray analysis in *M. quadriceps* of 6 wk old offspring of low-fat (mLFD, dotted bars) or high-fat diet (mHFD, red bars) fed dams, p-value sorted. (B) Microarray verification by qPCR analysis; upregulated genes with a fold change > 1.2, downregulated genes with a fold change <-1.2. Data are mean + SEM; n = 10. Data were analyzed by unpaired t-Test and Welch´s correction; Bcl6, B- cell CLL lymphoma 6 ; Dyrk2, Dual specificity tyrosine-phosphorylation-regulated kinase 2; Ncoa1, Nuclear receptor coactivator; Nr4a1, Nuclear receptor subfamily 4 group A member 1; Popdc3, Popeye domain-containing protein 3; Sik1, Serine/threonine-protein kinase 1; Ano1, Anoctamin1; Ctf1, Cardiotrophin1 and Tnfrsf1α, Tumor necrosis factor receptor superfamily member 1α

3.4.2 Maternal high-fat consumption results in a hypomethylation in fragments of the Nr4a1 gene

The aim of the microarray analysis was to identify candidate genes whose expression was influenced by maternal high-fat diet during early muscle development. Since maternal nutrition can cause epigenetic changes in offspring DNA such as DNA methylation, DNA methylation analyses in the two candidate genes Nr4a1 and Sik1 were performed. Therefore, DNA of *M. quadriceps* was bisulfite converted and analyzed by Sanger sequencing and later pyrosequencing. At first, the promotor region of the gene (-1000 bp – 2000 bp around the transcription start side) was screened for differentially regulated CpGs. Since there were no significant differences in the methylation pattern of mHFD vs. mLFD offspring for the Sik1 gene, only differentially regulated CpGs in the Nr4a1 gene were further verified by pyrosequencing. Nr4a1 is a transcription factor, which is discussed to induce the expression of genes involved in glucose utilization [83] and is increased with exercise [84, 85]. Here, for all analyzed CpGs the same methylation pattern could be detected (Figure 17A). mHFD offspring showed a hypomethylation, which reaches significance for the CpG at position 1408 and at positions 758 and 747. A trend was also detectable at position 1372. Furthermore, the gene expression of Nr4a1 and the delta methylation of the CpGs at position 758, 747 and 741 correlated negatively (Figure 17B).

Consequently, the hypermethylation of CpGs in the promotor region of the Nr4a1 gene in mLFD offspring resulted in a significantly lower gene expression compared to mHFD offspring. This indicates a modified function of the Nr4a1 gene in mLFD offspring.

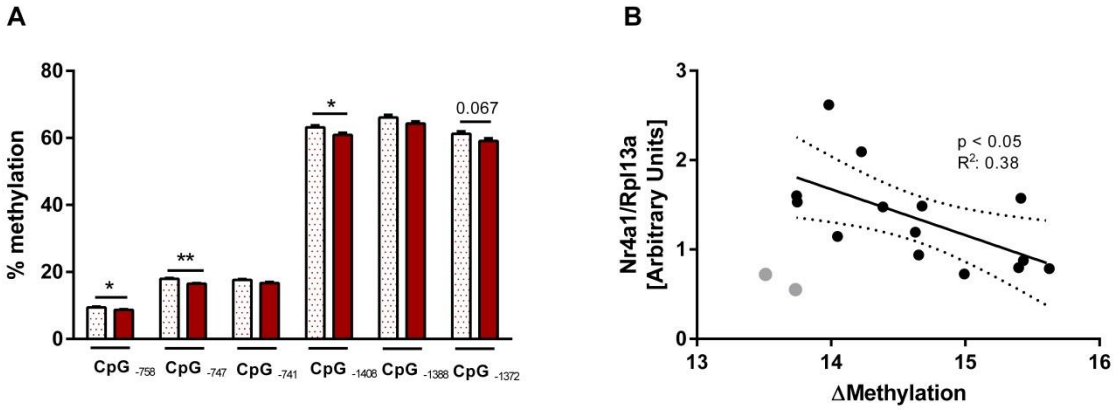

Figure 17: Hypomethylation in the Nr4a1 gene of mHFD offspring.
Pyrosequencing in *M. quadriceps* of 6 wk old maternal LFD (mLFD, dotted bars) and maternal HFD (mHFD, red bars) offspring (A) and correlation of methylation data with the corresponding Nr4a1 gene expression level (B) (Spearman correlation analyses), the gray dots are excluded values. Data are mean + SEM; n = 8. Data were analyzed by unpaired t-Test and Welch´s correction.

3.5 Impact of maternal malnutrition on the effects of an acute exercise bout

In order to investigate the underlying mechanisms of the impaired endurance capacity of mHFD offspring, male offspring were sacrificed at an age of 15 wks at three different time points. Mice were sacrificed before the treadmill endurance test to obtain basal levels, immediately after the test, to investigate the acute effect of an exercise bout, and 3h after the test, to investigate the recovery after an acute exercise bout in skeletal muscle (Study III).

3.5.1 Acute exercise response: Impact of maternal high-energy diet on mitochondrial biogenesis

To clarify the reason of the reduced endurance capacity of mHFD offspring we focused on skeletal muscle metabolism immediately after the endurance treadmill test. Since exercise induces the mitochondrial biogenesis in skeletal muscle, we firstly investigated the impact of the acute exercise bout on skeletal muscle mitochondria.

Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) is known to be induced by exercise and is the major player in mitochondrial biogenesis [64]. Thus, we focused on the expression of PGC1α and its downstream targets as a marker for mitochondrial activity and density (Figure 18). Gene expression of Pgc1α was

significantly reduced in mHFD offspring, which suggests a diminished mitochondrial biogenesis. However, this result could not be confirmed on protein expression level. There were no differences in PGC1α protein expression between the groups (Figure 18A/B).

To investigate the mitochondrial activity the expression of genes of complex V (Mitochondrial ATP Synthetase Beta Subunit; Atp5b), complex IV (Cytochrome c; Cytc) and complex I (NADH: Ubiquinone Oxidoreductase Subunit A5; Ndufa5) of the mitochondrial respiratory chain (Table 8) as well as protein expression of the oxidative phosphorylation (Figure 18C) were measured revealing no differences. Furthermore, gene expression of Carnitine palmitoyltransferase 1b, which is the rate-limiting step of mitochondrial fatty acid oxidation, and citrate synthase activity was measured as a marker for mitochondrial density. Cpt1b gene expression was significantly reduced in mHFD offspring, while citrate synthase activity did not differ between the groups (

Table 8; Figure 18D). This suggests that a reduced mitochondrial biogenesis is not the reason of the diminished endurance capacity of mHFD offspring.

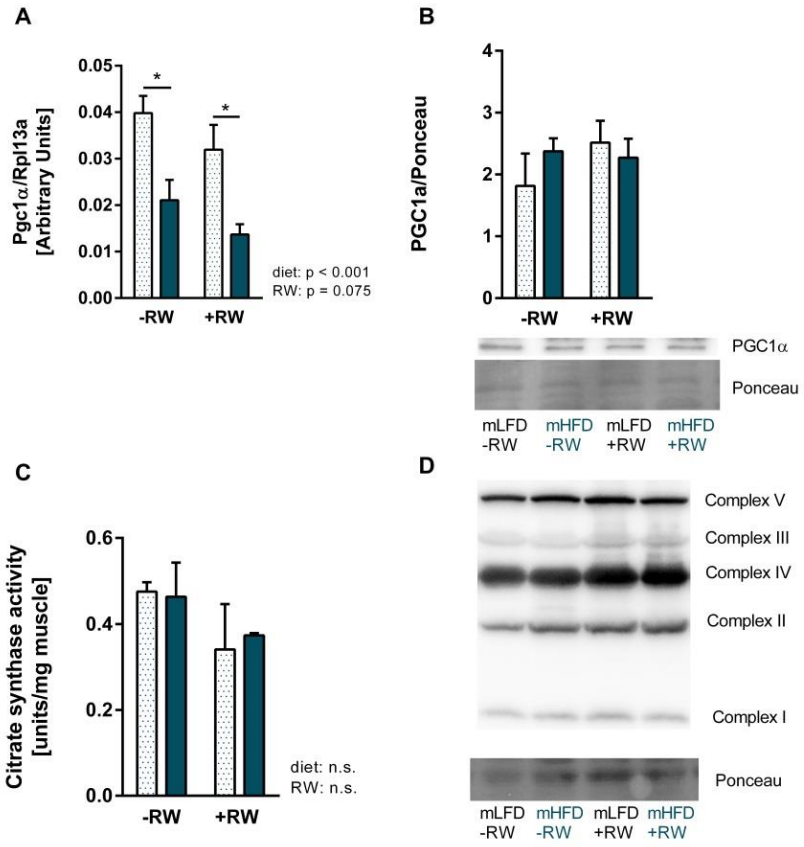

Figure 18: Impact of maternal high-energy diet on mitochondrial biogenesis immediately after an exercise bout.
Gene and protein expression of Peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) in *M. quadriceps* (A/B); Citrate synthase activity (C) and protein expression of the oxidative phosphorylation (OXPHOS); mLFD, dotted bars, mHFD, turquoise bars. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data are mean + SEM; (A) n = 6; (B) n = 7 and (C) n = 3-4. Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

Table 8: Gene expression (dCt) of mitochondrial proteins

	no exercise (-RW)		exercise (+RW)		p-Value	
Gene	**mLFD**	**mHFD**	**mLFD**	**mHFD**	**mDiet**	**RW**
Atp5b	0.827 ± 0.054	0.816 ± 0.069	0.936 ± 0.044	0.754 ± 0.083	n.s.	n.s.
Cpt1b	0.012 ± 0.001	0.007 ± 0.001	0.1 ± 0.001	0.005 ± 0.001	< 0.001	< 0.05
Cytc	0.084 ± 0.004	0.088 ± 0.006	0.088 ± 0.005	0.093 ± 0.006	n.s.	p = 0.094
Ndufa5	0.132 ± 0.009	0.121 ± 0.012	0.134 ± 0.015	0.165 ± 0.015	n.s.	n.s.
Tfam	0.023 ± 0.003	0.016 ± 0.002	0.028 ± 0.003	0.017 ± 0.001	< 0.01	n.s.

Atp5, Mitochondrial ATP Synthetase, Beta Subunit; Cpt1b, Carnitine Palmitoyltransferase 1b; Cytc, Cytochrome c; Ndufa5, NADH: Ubiquinone Oxidoreductase Subunit A; Tfam, Transcription Factor A, Mitochondrial. Data are mean ±SEM; n = 6. Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

3.5.2 Acute exercise response:
Impaired myokine expression in skeletal muscle of mHFD offspring

For the crosstalk with other organs such as liver or adipose tissue, myokines are released from skeletal muscle in response to exercise intervention. Thus, myokines are muscle-derived secretory factors which communicate an energy demand of the active muscle to other organs. Therefore, myokines can act in an endocrine or auto-/paracrine manner. The first cytokine termed as a myokine was Interleukin 6 (IL6) [86]. Here (Study III), IL6 gene expression, plasma concentration and protein concentration in *M. quadriceps* were measured immediately after the last exercise bout (Figure 19A). Gene expression was not different between mLFD and mHFD offspring, while there was a lower expression level in offspring with voluntary running wheel intervention. Plasma concentrations and skeletal muscle protein concentrations also did not differ between the groups. Taken together, IL6 release from muscle to the periphery was not different between the groups after exercise.
Endurance training also causes the release of Interleukin 15 (Il15), Osteocrin (Ostn, Musclin), Fibronectin type III containing-domain protein 5 (Fndc5) and Vascular endothelial growth factor ß (Vegfß) [69]. Thus, gene expression of these candidates was measured in skeletal muscle to get a first impression about myokine production in response to the acute endurance intervention (Figure 19B-E). All candidates showed reduced expression levels in mHFD offspring.

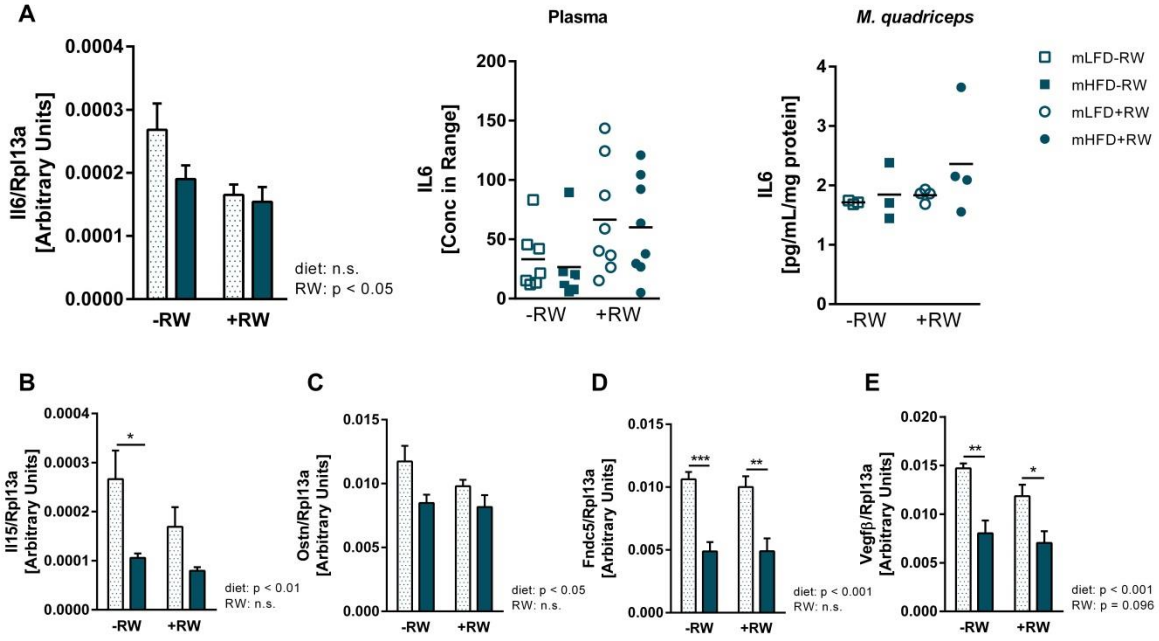

Figure 19: Myokine expression in response to an acute exercise bout.
Myokine expression in skeletal muscle and plasma was measured immediately after the final endurance treadmill test. (A) Interleukin 6 (IL6) gene and protein expression in *M. quadriceps* and plasma concentration; gene expression of Interleukin 15 (Il15) (B); Osteocrin (Ostn) (C); Fibronectin type III domain-containing protein 5 (Fndc5) (D) and vascular endothelial growth factor ß (Vegfß) (E). Data are mean + SEM, n = 6 (gene expression); n = 6-8 (IL6 protein concentration in *M. quadriceps*); n = 3–4 (plasma concentration of IL6); mLFD, dotted bars, mHFD, turquoise bars. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

Because of its outstanding role in angiogenesis and fatty acid trafficking, Vegfß was investigated in more detail. To characterize angiogenesis, gene expression of Estrogen-related receptor α (Esrrα) and Hypoxia-inducible factor 1α (Hif1α) were determined (Figure 20A), because the expression of both genes is induced by exercise and thus, contribute to the induction of angiogenesis in skeletal muscle [87]. Both genes showed the same expression pattern as Vegfß. Usually, the function of Vegfß is poorly angiogenic in most tissues whereas vascular endothelial growth factor α (Vegfα) is induced by hypoxia and required for angiogenesis [87-89]. Here, gene expression of Vegfα did not differ between the groups (Figure S1). Hence, it can be assumed that Vegfß might have also an impact on angiogenesis. Furthermore, there are studies attributing Vegfß a role in the targeting of lipids to peripheral tissues, due to the transcriptional regulation of fatty acid transport proteins. Vegfß controls the endothelial uptake of fatty acids by the regulation of the VEGF receptor, Flt1 [90, 91]. Gene expression of Flt1 and the fatty acid transporters Fatp3, Fatp4 and Cluster of differentiation 36 (Cd36) were investigated (Figure 20B), as well as

plasma analysis performed (Table S1). The receptor, as well as the fatty acid transporters, did not show the same expression pattern as Vegfß, accept Fatp3. Furthermore, there were no differences in plasma triglycerides and free fatty acid concentration between the groups (Table S1). Thus, Vegfß might not play a role in fatty acid trafficking in response to an acute exercise bout in this study.

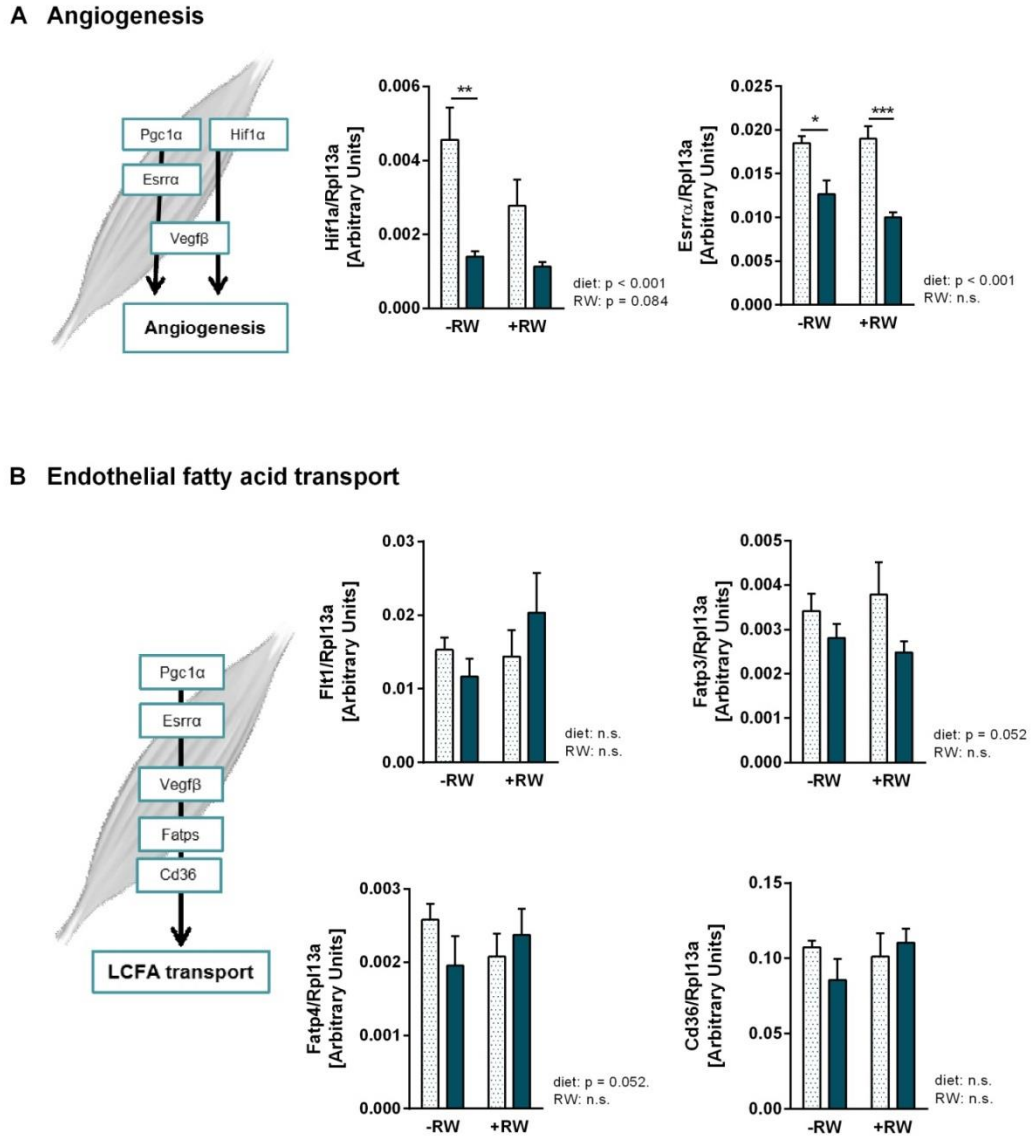

Figure 20: The role of Vegfβ in angiogenesis (A) and endothelial fatty acid transport (B). Gene expression of (A) Hypoxia-inducible factor 1α (Hif1α); Estrogen-related receptor α (Esrrα); (B) Vascular endothelial growth factor receptor 1 (Flt1); Fatty acid transport protein 3 (Fatp3); Fatty acid transport protein 4 (Fatp4) and Cluster of differentiation (Cd36). Data are mean + SEM, n = 6; mLFD, dotted bars, mHFD, turquoise bars. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data were analyzed by two-way ANOVA and Bonferroni posthoc test. LCFA, Long chain fatty acid.

3.5.3 Acute exercise response: Maternal high-fat diet impairs skeletal muscle glucose utilization

To investigate offspring´s glucose homeostasis we focused on the cross talk between skeletal muscle and liver. By breakdown of muscle glycogen, ATP is provided for muscular activity. This ATP has to be constantly replenished during muscular activity, which is guaranteed by feeding pyruvate into the tricarboxylic acid cycle when oxygen supply is sufficient as a substrate in the respiratory chain. During prolonged, intense muscular activity such as endurance exercise, the oxygen supply becomes limiting. Thus, energy has to be provided by anaerobic metabolism which is characterized by the production of lactate out of pyruvate. During lactic acid fermentation lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate into lactate (Figure 21), which results in ATP production. Lactate reaches the periphery and is taken up by the liver and initiates gluconeogenesis. Here, lactate is converted into pyruvate by LDH and finally into glucose, which is supplied to skeletal muscle through the bloodstream [56].

Muscle glucose metabolism was investigated by measuring glycogen at a basal level, immediately after, and 3 h after the endurance treadmill test (Figure 22A). Glycogen content was decreased in response to the acute exercise bout while the stores were refilled within 3 h after the endurance test. Furthermore, LDH activity in *M. gastrocnemius* was measured (Figure 22B). An increased LDH activity in mHFD offspring was detectable, which suggests a higher lactate production in skeletal muscle. Additionally, gene expression of Monocarboxylate transporter 1 (Mct1) and 4 (Mct4) was measured in *M. quadriceps* to investigate whether the transport of lactate into the bloodstream was altered (Figure 22C/D). Here, a decreased expression in mHFD offspring was observed indicating an enrichment of lactate in the skeletal muscle, which might cause muscle fatigue.

Consequently, also LDH activity in liver was measured to investigate the conversion from lactate into pyruvate (Figure 22I), since it is important for the energy metabolism of skeletal muscle that the liver is able to provide glucose to maintain energy homeostasis. LDH activity tended to be decreased in mHFD offspring, which might be due to the decreased Mct1 and 4 expressions and, therefore, decreased lactate levels in plasma of mHFD offspring. Furthermore, also glycogen content in liver was determined (Figure 22J). This is reduced in response to the acute exercise bout, showing that liver provides glucose to skeletal muscle in response to physical

activity. This is also shown by an increase in blood glucose level immediately after the endurance test (Figure 22H). Here, mHFD offspring displayed a significantly higher level compared to mLFD offspring. This might be due to an impaired glucose transport or uptake in skeletal muscle. Thus, gene expression of Glucose transporter 1 (Glut 1) and 4 (Glut 4) in *M. quadriceps* (Figure 22E-F) was measured. Gene expression of both glucose transporters was decreased in mHFD offspring. The reduction in Glut 4 gene expression of mHFD offspring could also be confirmed on protein expression level in a plasma membrane fraction of *M. gastrocnemius* (Figure 22G).

In summary, maternal overnutrition during pregnancy and lactation might result in an impaired glucose utilization of skeletal muscle which could be the cause of offspring reduced exercise capacity.

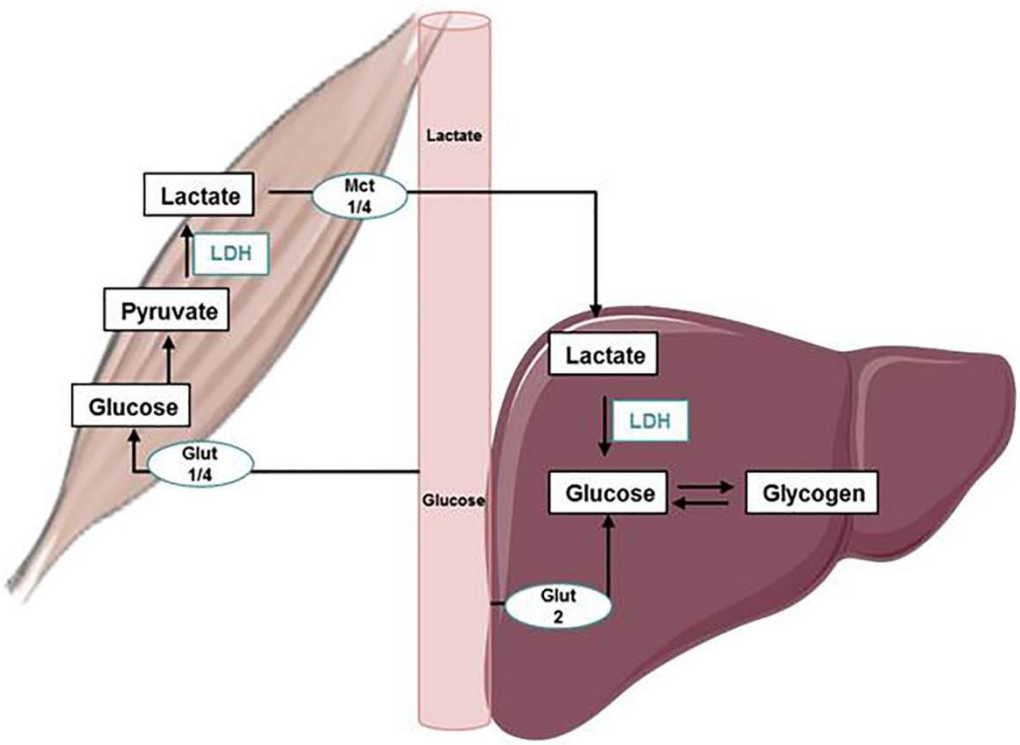

Figure 21: Glucose homeostasis in skeletal muscle and liver.
LDH, Lactate dehydrogenase; Mct1/4, Monocarboxylate transporter 1/4; Glut1/4/2, Glucose transporter 1/4/2. This figure was created using Servier Medical Art (http://www.servier.com).

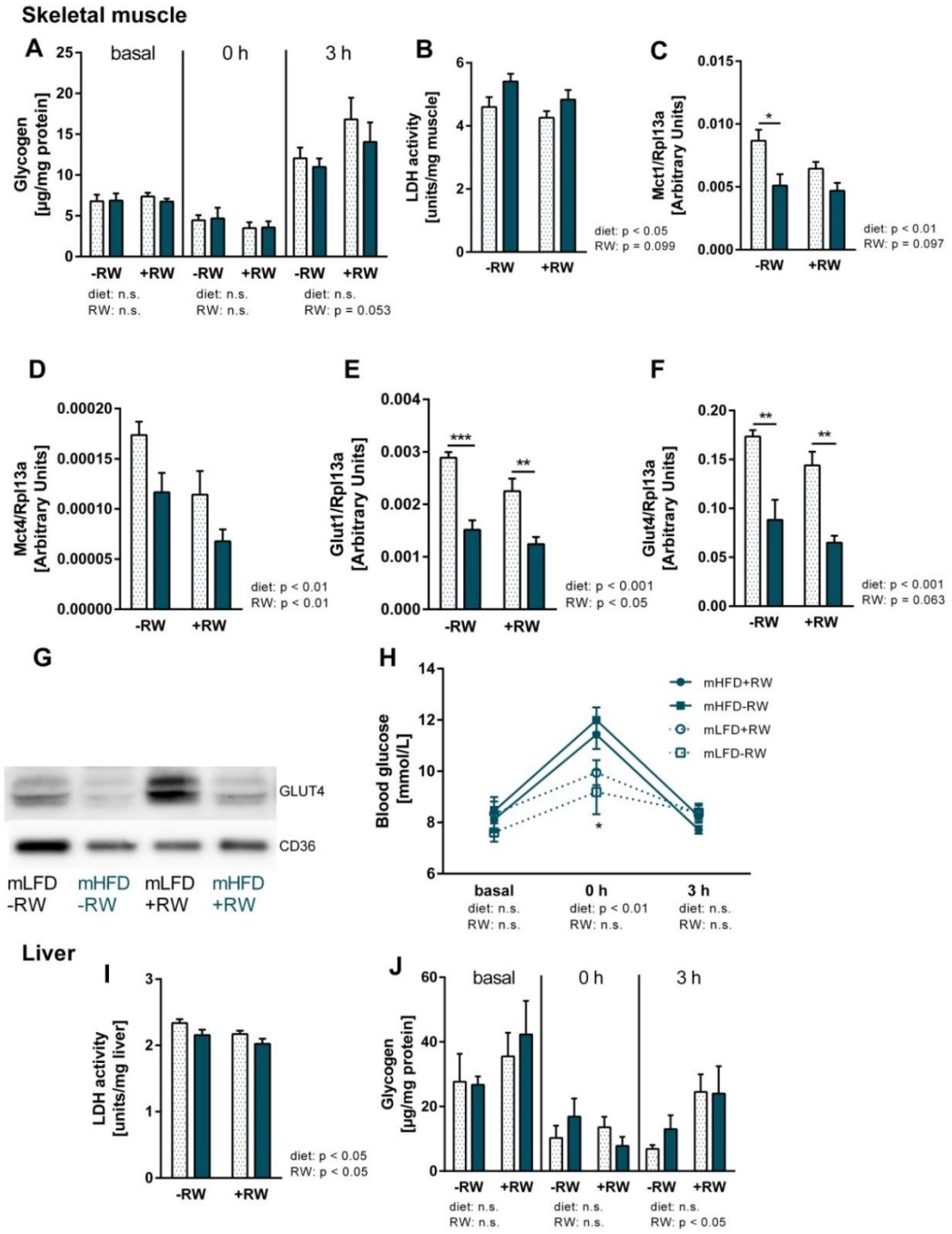

Figure 22: Impaired glucose homeostasis in mHFD offspring in response to an acute exercise bout.

The glucose homeostasis in skeletal muscle (A-H) and liver (I-K) were analyzed immediately after an acute exercise bout. (A) Glycogen content in *M. quadriceps*; (B) Lactate dehydrogenase (LDH) activity was analyzed in *M. gastrocnemius*, (C-E) gene expression of Monocarboxylate transporter 1 and 4 (Mct1, Mct4), Glucose transporter 1 (Glut1) in *M. quadriceps* and gene- and (G) plasma membrane protein expression of Glucose transporter 4 (Glut 4) in *M. quadriceps* (F) and *M. gastrocnemius*. (H) Blood glucose concentration at basal level, immediately after an exercise bout and 3 h after the endurance test. (I) Hepatic LDH activity immediately after exercise, and (J) glycogen content. Data are mean ± SEM, (A) n = 6-10; (B) n = 8-10 (C-F) n = 6; (H) n = 7-10; (I) n = 7-9 and (J) n = 6-9; mLFD, dotted bars, mHFD, filled bars. Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

3.5.4 Recovery state: Impact of maternal high-fat diet on offspring skeletal muscle recovery

Since it is published that exercise also causes endoplasmatic reticulum (ER) stress by an increased production of reactive oxygen species [69, 92], ER stress marker were measured at a basal level and 3 h after the acute exercise bout to see whether there are differences in skeletal muscle recovery between the groups (Figure 23). For the Activating transcription factor 3 (Atf3) a striking increase in gene expression 3 h after exercise was detected in both groups without voluntary running wheel intervention (Figure 23A). The groups had a 20-fold higher gene expression level compared to their trained littermates. Also gene expression of the Activating transcription factor 4 (Atf4), Growth arrest and DNA damage-inducible protein 34 (Gadd34), DNA damage-inducible transcript 3 (Chop), Cytochrome c (Cytc) and the Activating transcription factor 6 (Atf6) showed an increase 3 h after the exercise bout compared to basal level, but there were no differences between the sedentary and the trained groups (Figure 23B-F). For Atf6 mHFD offspring showed a lower gene expression level compared to mLFD offspring and a significant correlation with the gene expression of Pgc1α 3 h after the exercise bout (Figure 23G).

Together, gene expression data did not show consistent results. Thus, we speculate that voluntary wheel running might lead to a reduction in skeletal stress response due to an adaptation process. Hence, the recovery process 3 h after an acute exercise bout is influenced by skeletal muscle adaptation due to both, voluntary RW training and maternal nutrition. Since an induction by RW is evident for Atf3 gene expression, but a maternal effect is seen for Atf6, regarding these data it is not clear if and which cascade of the unfolded protein response (UPR) play a major role 3 h after the acute exercise bout.

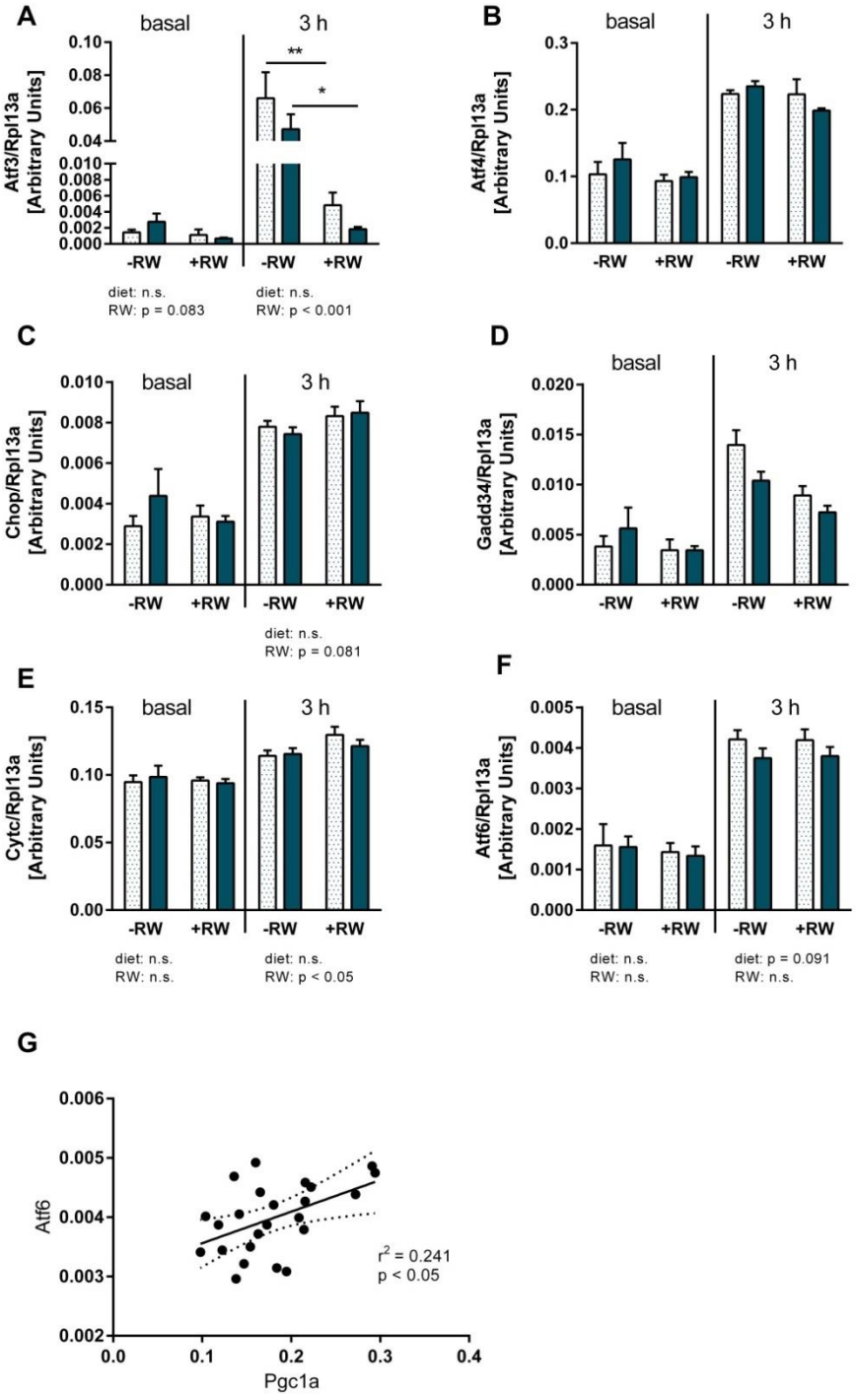

Figure 23: Impact of maternal high-fat diet on offspring skeletal muscle recovery 3h after an acute exercise bout.

Gene expression of (A) Activating transcription factor 3 (Atf3), (B) Activating transcription factor 4 (Atf4), , (C) DNA damage-inducible transcript 3 (Chop), (D) Growth arrest and DNA damage-inducible protein 34 (Gadd34), (E) Cytochrome c (Cytc) and (F) Activating transcription factor 6 (Atf6) in *M. quadriceps* at a basal level (before the endurance treadmill test) and 3 h after the exercise bout to clarify the impact of maternal high-energy diet on offspring muscular recovery. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data are mean + SEM, n = 6; mLFD, dotted bars, mHFD, turquoise bars. Data were analyzed by two-way ANOVA (Bonferroni posthoc test). The correlation between the gene expression of Atf6 and Pgc1α was performed using Spearman correlation analyses (G).

3.6 Impact of maternal overnutrition on offspring obesity susceptibility

To investigate if the reduced endurance capacity of mHFD offspring has an impact on offspring obesity susceptibility later in life, 15 wk old male offspring underwent a HFD intervention for ten wks. Briefly, after weaning male offspring received a LFD until an age of 15 wks and afterwards the ten-wk HFD intervention started. Mice were sacrificed at an age of 25 wks, 24 h after the last treadmill endurance test [93].

3.6.1 Maternal high-fat diet attenuates the positive effects of exercise on offspring fat mass gain

Body weight was comparable between the groups at the age of seven, 15, and 25 wks (Figure 24A). Fat mass gain from wk seven until wk 15 did not differ between offspring without voluntary running, while daily voluntary running prevented this fat mass gain in mLFD but not in mHFD offspring (Figure 24B). This effect was gone with ten wks of high-fat feeding of the offspring (25 wks). Voluntary running had no further impact on fat mass gain (wk seven until wk 25), but mHFD offspring tended to have a higher fat mass gain in both intervention groups (Table 9). There were no differences in lean mass, plasma parameters, energy expenditure and RQ between the groups. Thus, maternal diet or offspring´s daily voluntary running had no impact on these parameters.

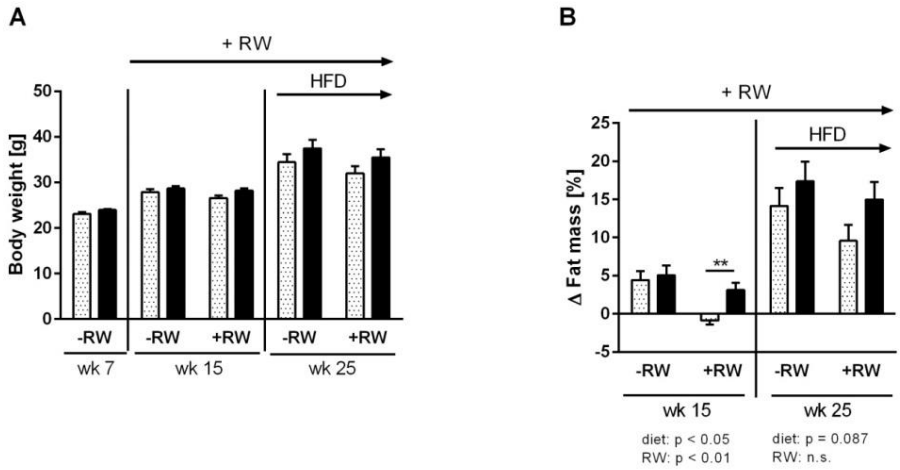

Figure 24: Impact of maternal overnutrition on offspring body weight development (A) and fat mass gain (B).
Dams were fed a low-fat (LFD) or a high-fat diet (HFD) during pregnancy and lactation. Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; black bars) offspring received a LFD after weaning until an age of 15 wks. Afterwards, offspring were fed a HFD throughout the end of the study (25 wks). Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. (B) Fat mass gain in relation to body weight from wk 7 until wk 15 and from wk 7 until wk 25. Data are mean + SEM; (A) wk 7: n = 42-53 wk 15: n = 20-28, wk 25: n = 10-14 and (B) n = 10-15. Data were analyzed by using two-way ANOVA (Bonferroni post hoc test).

Table 9: Offspring biometric data, energy metabolism, and plasma parameters

		no exercise (-RW)		exercise (+RW)		p-Value	
	Con	mLFD	mHFD	mLFD	mHFD	mDiet	RW
Biometric data							
Lean mass at wk 7 [g]	20.35 ± 0.75	21.74 ± 0.81	21.57 ± 0.41	20.76 ± 0.79	22.05 ± 0.52	n.s.	n.s.
Fat mass at wk 7 [g]	1.20 ± 0.10	1.10 ± 0.08	1.35 ± 0.09	1.41 ± 0.11	1.27 ± 0.08	n.s.	n.s.
Lean mass at wk 15 [g]	23.30 ± 0.59	24.66 ± 0.62	24.40 ± 0.45	23.87 ± 0.75	24.57 ± 0.46	n.s.	n.s.
Fat mass at wk 15 [g]	2.62 ± 0.35	2.68 ± 0.44	3.23 ± 0.49	1.42 ± 0.17	2.46 ± 0.33	0.057	<0.05
Lean mass at wk 25 [g]	24.37 ± 0.54	26.30 ± 0.75	26.44 ± 0.65	25.40 ± 0.83	26.43 ± 0.64	n.s.	n.s.
Fat mass at wk 25 [g]	2.98 ± 0.45	6.80 ± 1.14	9.67 ± 1.22	5.26 ± 0.82	7.65 ± 1.24	<0.05	n.s.
Energy metabolism							
EE [kJ/d]	55.5 ± 0.9	56.8 ± 1.7	57.8 ± 2.0	55.6 ± 2.3	56.4 ± 1.6	n.s.	n.s.
RQ	0.95 ± 0.01	0.84 ± 0.01	0.83 ± 0.01	0.86 ± 0.01	0.86 ± 0.01	n.s.	n.s.
Plasma parameters							
TG [mmol/L]	0.421 ± 0.054	0.402 ± 0.094	0.286 ± 0.063	0.460 ± 0.086	0.411 ± 0.082	n.s.	n.s.
FFA [mmol/L]	0.662 ± 0.035	0.568 ± 0.084	0.669 ± 0.072	0.616 ± 0.065	0.562 ± 0.053	n.s.	n.s.
Chol [mg/dL]	200.4 ± 16.8	212.6 ± 21.5	227.1 ± 20.6	217.4 ± 31.5	200.2 ± 21.6	n.s.	n.s.

mLFD or mHFD were fed a LFD after weaning until an age of 15 wks. Afterwards they received a HFD for 10 wks. As control group (Con) mLFD offspring without voluntary RW intervention on LFD were defined. Data are mean ± SEM; n = 6-12. Indirect calorimetry for the determination of energy expenditure (EE) and RQ was performed in wk 24. Data was analyzed by two-way ANOVA (Bonferroni post hoc test). TG, Triglycerides; FFA, Free fatty acids; Chol, Cholesterol.

3.6.2 Impact of maternal overnutrition on liver physiology

Since it is known that a high energy diet during pregnancy and lactation can result in offspring fatty liver disease [26, 94], liver triglyceride and glycogen content were determined (Figure 25). mHFD offspring showed an increased liver mass, but no differences in liver triglycerides between the groups (Figure 25A/B). There was also no correlation between liver weight and triglyceride content (Figure 25C), which indicates that the higher liver weight is not due to higher hepatic triglyceride levels. Glycogen level in liver showed a trend to be higher in mHFD offspring and correlate with liver weight (Figure 25D/E). In summary, the increase in liver mass might be explained by a higher hepatic glycogen level.

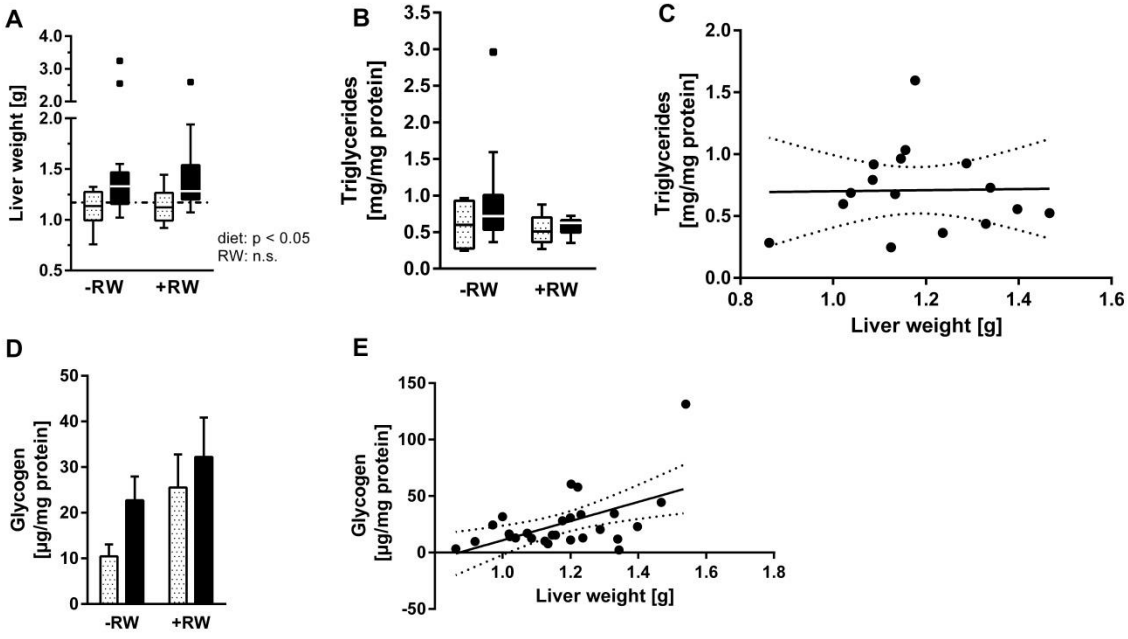

Figure 25: Impact of maternal overnutrition on liver physiology.
Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; black bars) offspring received a LFD after weaning until 15 wks of age. Afterwards, offspring were fed a HFD throughout the end of the study (25 wks). Half of the group got access to a running wheel (RW) as voluntary training. (A) offspring liver weight, (B/C) hepatic triglyceride concentration and the correlation with liver weight, (D/E) hepatic glycogen content and its correlation with liver weight. Data are shown as median with interquartile range (A/B) or mean +SEM (D), (A) n=10-15; (B) n=6-12; (D) n=4-11. The dotted lines (A/B/D) are the mean value of the control group (mLFD-RW of Study I). Data were analyzed by Kruskal-Wallis (Dunn´s test) (A/B) or two-way ANOVA (Bonferroni post hoc test) (D). For –RW animals Spearman correlation analyses was performed (C/E).

3.6.3 Enhanced exercise capacity in mLFD, but not mHFD offspring by daily voluntary running

Voluntary running resulted in a 160 % increase of endurance capacity of mLFD offspring from wk seven until wk 15, while mHFD offspring only increased their capacity by about 70 % (Figure 26A). While mLFD offspring increased their capacity from wk seven until wk 25 further on (200 %), there was no further increase in exercise performance of mHFD offspring. *M. quadriceps* glycogen and triglyceride content did not differ between the groups (Figure 26B/C). To clarify whether ten wks on HFD had an impact on offspring fiber type composition in skeletal muscle, MhcI-IIb gene expression was determined in *M. quadriceps* and *M. soleus* (Figure 26D/E). The analysis showed for *M. quadriceps* more glycolytic (MhcIIx, MhcIIb) muscle fibers in all groups, while *M. soleus* composed of more oxidative (MhcI, MhcIIa) fibers, which also did not differ between the groups.

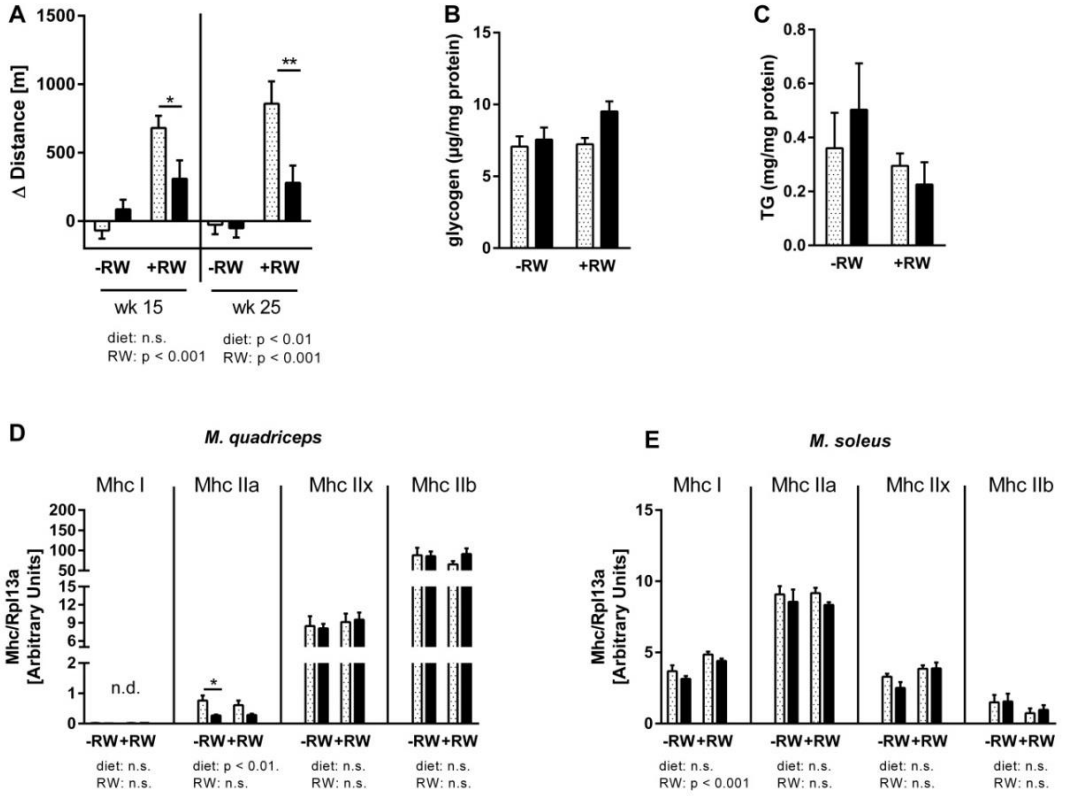

Figure 26: Effect of maternal high-fat diet on offspring exercise capacity and skeletal muscle physiology.
Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; black bars) offspring received a LFD after weaning until 15 wks of age. Afterwards, offspring were fed a HFD throughout the end of the study (25 wks). Half of the groups got access to a running wheel (RW) as voluntary training. (A) endurance capacity from wk 7 until wk 15 and from wk 7 until wk 25, (B) glycogen content in *M. quadriceps*, (C) triglyceride content in *M. quadriceps*, (D) gene expression of myosin heavy chain (Mhc) isoforms in *M. quadriceps* and (E) *M. soleus*. Data are mean + SEM; (A) n = 10-15; (B) n = 4-11; (C) n = 6-12 and (D/E) n = 5-8. Data were analyzed by unpaired t-Test and Welch´s correction (A) and by two-way ANOVA (Bonferroni post hoc test) (D/E).

There were no differences in Pgc1α gene expression between the groups in *M. quadriceps* (Figure 27A) although it is known, that it is higher expressed with exercise intervention [92]. Furthermore, mitochondrial density and activity are influenced by exercise. Therefore citrate synthase activity and oxidative phosphorylation (OXPHOS) were measured in *M. quadriceps* (Figure 27B/C). Citrate synthase activity as a marker of mitochondrial density was significantly increased by voluntary running wheel exposure while maternal diet had no effect. OXPHOS protein expression was significantly higher in mLFD offspring for complex I and a similar trend was also seen for complex III-V.

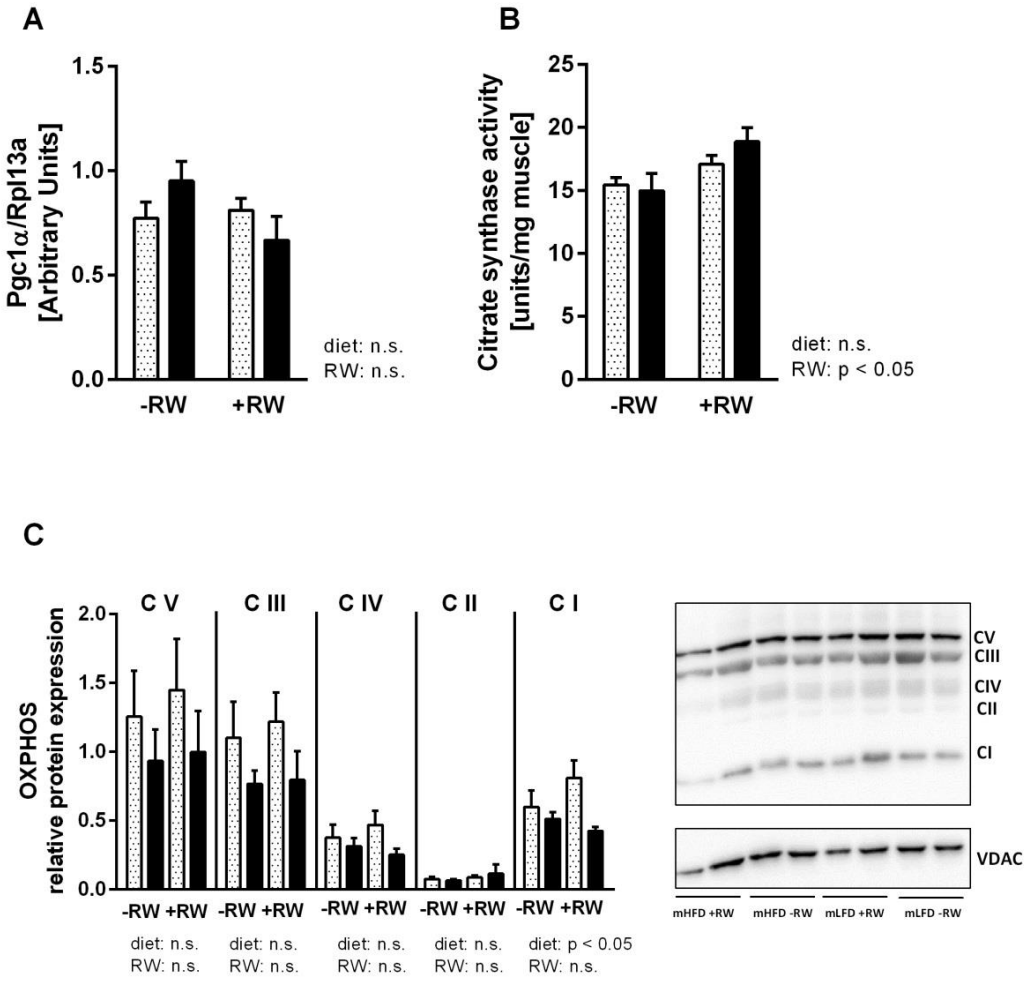

Figure 27: Impact of maternal overnutrition on offspring mitochondrial biogenesis in *M. quadriceps*.
Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; black bars) offspring received a LFD after weaning until 15 wks of age. Afterwards, offspring were fed a HFD throughout the end of the study (25 wks). Half of the groups got access to a running wheel (RW) as voluntary training. (A) relative gene expression (ddCt; as control group mLFD-RW of study I was used) of *Pgc1α*, (B) citrate synthase activity and (C) protein expression of OXPHOS, normalized to VDAC. Data are mean +SEM; (A) n=6-8; (B) n=6-7; (C) n=5-6. Data were analyzed by two-way ANOVA (Bonferroni post hoc test).

3.6.4 Maternal high-fat diet impairs the positive effect of exercise intervention on offspring insulin sensitivity

In order to investigate the impact of maternal overnutrition and the effect of voluntary running wheel training on offspring glucose homeostasis, an oGTT was performed at an age of 22 wks (Figure 28A/B). Blood glucose levels did not differ between the groups, but plasma insulin was decreased 15 min after glucose gavage in mLFD offspring with voluntary running wheel intervention compared to mHFD offspring. Additionally, mLFD offspring showed reduced pancreas insulin levels

independently of running wheel training, while blood glucose levels at an age of 25 wks did not differ between the groups (Figure 28C/D). Summarizing these data, the beneficial effects of voluntary RW intervention on insulin resistance were reduced by mHFD.

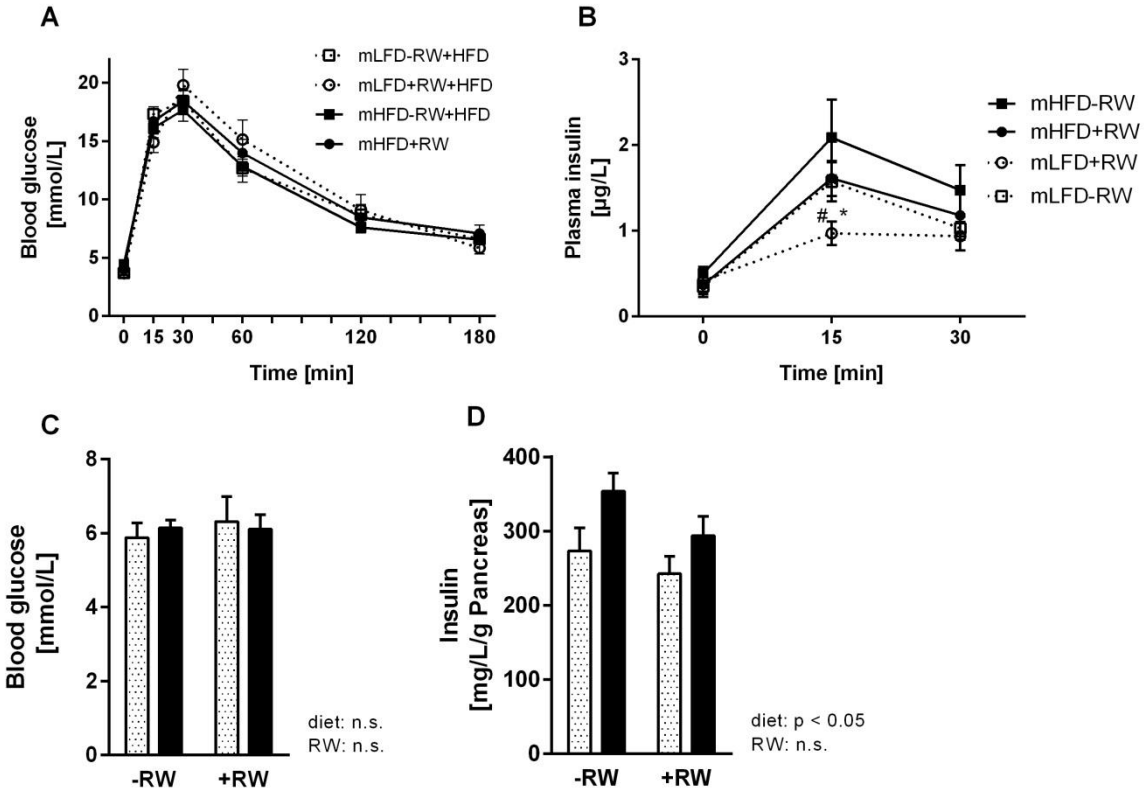

Figure 28: Impact of maternal high-fat consumption on offspring insulin sensitivity.
Maternal low-fat diet (mLFD; dotted bars) or maternal high-fat diet (mHFD; black bars) offspring received a LFD after weaning until 15 wks of age. Afterwards, offspring were fed a HFD throughout the end of the study (25 wks). Half of the groups got access to a running wheel (RW) as voluntary training. (A) glucose tolerance, (B) plasma insulin, (C) pancreas insulin content (25 wks) and (D) basal blood glucose concentration (25 wks). Data are mean ±SEM; (A) n=8-15; (B) n=10-15; (C) n=5-6; (D) n=6-12. Data were analyzed by two-way ANOVA (Bonferroni post hoc test). * mLFD vs. mHFD, whereas # -RW vs. +RW.

4 DISCUSSION

Maternal obesity has detrimental effects on the offspring´s development. Consequently, offspring born to obese mothers have a higher risk to develop type 2 diabetes, obesity, hypertension or coronary heart disease due to metabolic alterations such as insulin resistance in the liver, adipose tissue and skeletal muscle, leptin resistance in the brain or atherosclerosis [11, 19].

This PhD thesis aimed to investigate the impact of perinatal maternal high-fat diet consumption on offspring skeletal muscle energy metabolism and voluntary running wheel training efficiency. To elucidate this, four studies were conducted: Study I focused on the impact of mHFD on offspring training response and endurance capacity and indicated an impaired training response in offspring from HFD-fed dams. Study II and III were conducted to investigate the reasons for this effect. While study II focused on genetic/epigenetic changes in offspring skeletal muscle of young mice (6 wks old), Study III was performed to investigate the underlying molecular mechanisms during/after an acute exercise bout in 15 wk old offspring. In order to investigate the consequences of mHFD and regular voluntary exercise training on offspring obesity susceptibility later in life, study IV was conducted.

4.1 Impact of maternal high-fat diet during pregnancy and lactation on offspring phenotype

In order to investigate the impact of maternal HFD (without being obese) on offspring phenotype, offspring body weight and composition, as well as their endurance exercise capacity were measured. While the body weight of the dams was not different between the groups, mHFD offspring displayed a slightly higher weight at an age of three and four wks, in accordance with a higher lean and fat mass at four wks of age. For C3H mice Walter and Klaus also showed a significantly higher body weight in three wk old mHFD offspring, which was not detectable anymore at an age of seven to twelve wks [73]. Similar results were obtained for C57Bl/6J offspring in the present PhD thesis. From an age of seven wks on until an age of 25 wks mHFD offspring did not differ in body weight compared to mLFD offspring since mLFD reach the same body weight like mHFD offspring with age. This is partly in contrast to studies focusing on the effect of maternal obesity on the

offspring. Hence, it was shown an induction in offspring adiposity in adult life, which occurs earliest after weaning, but rather at an age of ten wks and maintains throughout life [95-97]. One explanation for the difference might be that dams of the present studies did not develop obesity by HFD feeding three days prior mating throughout pregnancy and lactation.

While body weight between mLFD and mHFD offspring did not differ from seven wks on, fat mass gain in mLFD offspring was completely prevented by RW training, in contrast to mHFD offspring at an age of 15 wks. Since daily RW intervention (Figure S3) was not different between the groups, this suggests that mHFD did not result in a "lazy" phenotype.

At an age of 25 wks voluntary running only reduced fat mass gain of mLFD but not mHFD offspring. Hence, mHFD feeding attenuates the positive effects of voluntary training on offspring body fat. This is in contrast to the data of Rajia et al., who showed that voluntary running in chow-fed female rat offspring reversed the metabolic effects of maternal obesity [72]. Since mice in the present study received a LFD after weaning, which is comparable to chow diet, an improved fat mass gain in mHFD offspring would be expected. As Rajia and colleagues performed this animal experiment in female rats, these differences might be due to different species or sexes.

For the further characterization of skeletal muscle physiology of offspring born to mHFD dams, an endurance treadmill test was performed to investigate the impact of maternal overnutrition on offspring endurance capacity. Confirming previous data of our group [73], voluntary RW training resulted in an increased offspring endurance capacity. This increase was significantly higher in eleven wk old mLFD compared to mHFD offspring, and at least in tendency higher in 15 wk old mLFD offspring, while there was no difference in endurance capacity in 25 wk old offspring anymore. Hence, it can be concluded that mHFD offspring display a delayed training response with age.

Since endurance training results in skeletal muscle adaptation processes such as a fiber type switch towards more oxidative fibers to counteract skeletal muscle fatigue [42], gene expression of Myosin heavy chain (Mhc) isoforms was measured in *M. quadriceps* (glycolytic, type II, fast-twitch) in six, 15 and 25 wk old offspring and in *M. soleus* (oxidative, Type I, slow-twitch) in 25 wk old offspring. Since there were no meaningful effects of maternal diet on gene expression of Mhcs in *M. quadriceps* and *M. soleus*, nor in skeletal muscle weight or fiber number and size of 15 and

25 wk old offspring, the impaired training efficiency and endurance capacity of mHFD offspring could not be explained by an altered skeletal muscle physiology. This might be due to the voluntary training intervention using a RW, since skeletal muscle adaptations resulting in an altered skeletal muscle fiber type phenotype mainly occur with a distinct training protocol [43].
Consequently, to elucidate the underlying mechanisms of the maternal impact on offspring endurance capacity, epigenetic changes and the response of skeletal muscle energy metabolism to an acute exercise bout was investigated in more detail.

4.2 Epigenetic changes in offspring skeletal muscle in response to maternal high-fat diet feeding

The occurrence of diseases, such as diabetes or coronary heart disease, is in part due to genetic predisposition. However, genome-wide association studies could not fully clarify the increasing incidence of these non-communicable diseases by genetic variants that explain the considerable proportion of disease risk [98, 99]. This suggests that environmental factors, such as diet and the physical activity level, play also an essential role in the development of these diseases.
Above all, the early life environment plays an important role in the disease development potentially due to a changed epigenetic regulation of genes. Epigenetic changes of the DNA include histone modifications, non-coding RNAs, and DNA methylation. High levels of DNA methylation (hypermethylation) within the promotor regions are associated with inhibited binding of transcription factors, leading to transcriptional silencing [24].
Hence, we hypothesized that the diminished endurance exercise capacity of mHFD offspring might be due to alterations in DNA methylation caused by maternal high-fat diet intake during pregnancy and lactation. To identify candidate genes, whose expression was influenced by maternal diet, male mice offspring were sacrificed at an age of six wks and a microarray analysis was performed in *M. quadriceps*. This resulted in 317 differentially regulated genes (mHFD vs. mLFD offspring), with 160 up-regulated and 157 down-regulated genes. Verification of the microarray data was performed by gene expression analysis. Interestingly, a significant up-regulation of gene expression in mHFD offspring was detected for Nuclear receptor subfamily 4 group a member 1 (Nr4a1). Nr4a1 is known as a transcription factor that is a regulator of genes involved in muscular glucose utilization. It has further been

demonstrated that Nr4a1 is one of the TOP candidates that are increased after exercise in humans and mice [83-85]. Hence, this candidate was further analyzed for DNA methylation. Pyrosequencing revealed a hypomethylation at CpG position 758, 747 and 1408 in the promotor region of Nr4a1 of mHFD offspring. The hypomethylation is associated with an increased gene expression of Nr4a1. As a result, more Nr4a1 can be formed and thus act as a transcription factor. This suggests that the transcriptional activity of Nr4a1 is more pronounced in mHFD than mLFD offspring.

Nr4a1 is important in the regulation of muscle growth during prenatal and postnatal myogenesis [100]. It is published, that it mainly occurs in glycolytic than in oxidative skeletal muscle fibers [83]. Consequently, a higher expression of type II muscle fibers was assumed in mHFD offspring. Since there were no differences in fiber type expression between six wk old mLFD and mHFD offspring, this might not be the cause for the increased gene expression of Nr4a1. Moreover, Tontonoz and co-workers could show that the global and muscle-specific deficiency of Nr4a1 in mice resulted in the reduction of muscle mass and myofiber size [101]. This reduction already occurred in the embryonic state (E18.5) and became more pronounced with three wks of age [100]. Besides the regulation of myogenesis, Nr4a1 also functions as a modulator of glycolysis, glycogenolysis, oxidative metabolism in skeletal muscle and energy expenditure in exercise [83-85, 101] as well as wound healing [102].

Since the main function of Nr4a1 during exercise is the increased glucose utilization by the induction of Glut4 expression [83], an up-regulation of Nr4a1 gene expression in mLFD compared to mHFD offspring was expected. An increasing glucose supply during endurance exercise would explain pronounced exercise capacity and training efficiency in mLFD offspring. However, our data showed an up-regulation of Nr4a1 in *M. quadriceps* of six wk old mHFD offspring, which did not have any exercise intervention and tests. For 25 wk old offspring (Figure S2) an increased gene expression in mHFD offspring without RW was also detectable, while this effect was gone with RW intervention. Here, both groups displayed the same expression level of Nr4a1. While Tontonoz et al. showed only positive effects of Nr4a1 overexpression in skeletal muscle, a negative association of a higher expression of Nr4a1 in white adipose tissue (WAT) was published by Pérez-Sieira et al. They showed that HFD feeding resulted in increased Nr4a1 mRNA level in WAT of DIO rats, which was also seen in morbid obese patients [103, 104]. Since the NR4A family also plays a role as key factors in inflammation, Veum and colleagues hypothesized that the increased

expression of Nr4a1 in obese patients might be due to stress response and inflammation. Nr4a1 seems to mediate a role in low-grade inflammation in WAT [104]. So far, this has not been shown for skeletal muscle, but it could be an explanation for the increased gene expression level in mHFD offspring. Furthermore, Pérez-Sieira and co-workers found that in rats also food-restriction results in an increased expression of Nr4a1 in WAT [103]. This was also shown for liver and skeletal muscle [105]. Since the six wk old mHFD offspring of the present study (Study II) received a LFD after weaning, offspring could also display an energy demand comparable to food restriction. Thus, the higher Nr4a1 gene expression might be due to the role of Nr4a1 in stress response.

However, to fully understand the role of Nr4a1 in the present study, further analysis will be necessary. Thus, the energy expenditure and energy metabolism in skeletal muscle of six wk old offspring have to be investigated in more detail by measuring energy expenditure, protein expression of NR4A1, oxidative metabolism, GLUT4 translocation as well as stress response markers in skeletal muscle. This will give more detailed insights into the role of Nr4a1 in mHFD offspring.

4.3 Impact of maternal high-fat diet on the offspring's response to an acute exercise bout

In addition to the occurrence of metabolic disorders, epigenetic changes due to the maternal diet could also be the reason for the failure of individuals to improve their metabolic response to exercise. Since the epigenetic studies were carried out in six wk old offspring without any further intervention, thus not allowing any conclusions about the cause of offspring diminished exercise performance later in life, this study aimed to clarify the underlying mechanisms of an impaired training response by investigating skeletal muscle energy metabolism in 15 wk old offspring.

Therefore, offspring of LFD and HFD fed dams were sacrificed at an age of 15 wks at three different time points, before an exercise intervention, immediately after a treadmill exercise test and 3 h after the test, to investigate the impact of maternal nutrition on the metabolic response to an acute exercise bout.

4.3.1 Impact of maternal high-fat diet on mitochondrial biogenesis

Repeated bouts of contractile activity, induced by regular exercise interventions, result in metabolic and morphological adaptations of skeletal muscle. Hence, chronic contractile activity leads to changes in the expression of a variety of genes, causing an altered skeletal muscle phenotype with improved fatigue resistance, an increased mitochondrial density, and enzyme activity [53, 64]. This increase in mitochondrial enzyme activity and density due to improved exercise endurance is referred to as "mitochondrial biogenesis" [106]. Skeletal muscle mitochondrial biogenesis as a possible cause for the diminished endurance capacity in offspring was analyzed at the time point immediately after the endurance test since this time point reflects the metabolically alterations in skeletal muscle during the acute exercise bout.

Although the improved mitochondrial biogenesis during physical activity has been discussed in many studies [107-109], the detailed underlying mechanisms still remain unclear. A key player in mitochondrial biogenesis is PGC1α which co-activates a variety of mitochondrial transcription factors to regulate skeletal muscle mitochondria content [106, 110]. Pgc1α gene expression was reduced in mHFD offspring independent of RW intervention immediately after the acute exercise bout. Consequently, we hypothesized that the reduced gene expression of Pgc1α impaired mitochondrial biogenesis by an impaired transcriptional regulation, which resulted in the reduced endurance capacity of mHFD offspring. PGC1α induces several transcription factors such as mitochondrial transcription factor A (Tfam), which is important for the transcription and replication of mitochondrial DNA [111], and the estrogen-related receptor α (Esrrα), which plays also a role in the maintenance of energy homeostasis, glucose metabolism and thus, mitochondrial biogenesis [112]. Gene expression of both transcription factors was significantly reduced in mHFD offspring in response to the acute exercise bout supporting the hypothesis of an impaired endurance capacity of mHFD offspring as a result of an impaired mitochondrial biogenesis during an acute exercise bout (Figure 29). To proof, if alterations in gene expression also led to altered protein levels, Western Blot analysis of PGC1α was performed in *M. quadriceps*. There were no differences between the groups in protein expression of PGC1α, measured immediately after the endurance treadmill test. These contradictory data could be explained by studies showing that changes in protein level regarding adaptation processes in skeletal muscle due to exercise training only occur within months, while early adaptation

alterations can be detected on mRNA level already [113]. According to this, alterations in skeletal muscle protein expression are hardly detectable immediately after acute exercise. Therefore, alterations in gene expression have a stronger significance for the detection of alterations in response to an acute exercise bout. Hence, the missing differences in protein expression of PGC1α do not show that Pgc1α does not play a role in maintaining energy homeostasis during an acute exercise bout. A study of the Washington School of Medicine in Missouri showed that mitochondrial biogenesis occurred already before the expression of PGC1α [110]. They hypothesized that the initial phase of skeletal muscle adaptation caused the increase of mitochondrial density and only at a second step the up-regulation of PGC1α expression, which then resulted in a continuing increase in mitochondrial density [110].

To determine whether the mitochondrial density is reduced in mHFD mice, citrate synthase activity was measured in *M. quadriceps*. Since citrate synthase activity did not differ between the groups, there is no indication of an impaired mitochondrial function regarding alterations in mitochondrial density.

Additionally, gene expression of Carnitine palmitoyltransferase 1b (Cpt1b) was measured as the time-limiting step of mitochondrial beta-oxidation. Gene expression of Cpt1b was significantly lower in mHFD compared to mLFD offspring, suggesting an impaired transport of long-chain fatty acids from cytosol into mitochondria.

To further investigate mitochondrial activity, oxidative phosphorylation was measured on gene and protein expression level. There were neither differences in gene expression nor on protein expression level. Böhm and colleagues showed that an increased Transforming growth factor beta (TGFß) signaling attenuates the training-induced mitochondrial fuel oxidation in men with a high risk of developing type 2 diabetes. Transcriptomes of men who did not respond to the training intervention displayed an activation of TGFß and its target genes with a downregulation in PGC1α, AMPKα2, TFAM, and mitochondrial enzymes such as CPT1b and ADP5 [114]. Since gene expression of Cpt1b, Tfam and Pgc1a was reduced in mHFD offspring, we hypothesized that these offspring behave like the nonresponders mentioned in the publication of Böhm et al. However, gene and protein expression of mitochondrial oxidative phosphorylation was not different, which is in contrast to Böhm et al. Thus, regarding these data, it seems that mHFD does not impair mitochondrial fuel supply by enhancing TGFß signaling.

In summary, the data on mitochondrial biogenesis are not conclusive, and therefore no clear statement about its role in the response to an acute exercise bout can be made. Further investigations such as the analysis of Cytochrome C oxidase activity or direct measurements of mitochondrial respiratory activity are necessary.
Since PGC1α is a key regulator in response to exercise, it can also affect other metabolic pathways. Its influence on mitochondrial biogenesis seems to be of minor importance in this context.

4.3.2 Impact of maternal high-fat diet on offspring myokine expression and angiogenesis in *M. quadriceps*

Myokines are defined as muscle-derived secretory factors which communicate an energy demand of the active muscle to other organs such as liver or adipose tissue. Myokines induced by exercise or endurance training are Interleukin 15 (IL15), Osteocrin (OSTN), Interleukin 6 (IL6), and the Vascular endothelial growth factor (VEGF) of which VEGF and IL6 can be also induced by PGC1α. OSTN and IL15, on the other hand, are induced by Akt phosphorylation or AMPK activation [69]. Gene expression of these myokines displayed a reduced expression pattern in mHFD offspring for all myokines except Il6. Although it is published that plasma IL6 concentration can increase up to 100-fold in response to exercise and circulating Il6 level are correlated with the intensity and duration of exercise [115], there was neither a difference in gene expression nor in plasma concentration and skeletal muscle protein level between the groups detectable.
While the effect of maternal high-fat diet on gene expression of Ostn was only marginal, the reduction in the expression of Il15, Fndc5, and Vegfß in mHFD offspring was obvious. Especially Ostn, Fndc5, and Il15 are still controversially discussed in their role as myokines during exercise performance. Thus, Fndc5 encodes the precursor of irisin, which is expressed in rodent muscles but not in humans, which makes its biological relevance doubtful [116].
IL15 is known to have positive effects on obesity and type 2 diabetes and its circulation levels are highly regulated with treadmill training [117, 118]. However, it is not yet clear if the circulating level is due to muscular secretion [69]. In the mHFD animals, only a reduced gene expression was detected and plasma levels were not

determined. Measuring plasma IL15 concentrations would be interesting in order to elucidate the role of Il15 in the impaired endurance capacity of the offspring.

Above all, Ostn is known as a secretory factor in the reaction to nutritional changes [119]. Recently, however, the relationship with exercise has also been established. Ostn seems to improve the endurance capacity of mice by increasing mitochondrial biogenesis [120]. However, this is in contradiction with its role in insulin resistance and hypertension [69]. Consequently, it is considered that these myokines have no major role in the adaptation process of skeletal muscle to exercise performance.

Since the capillary density and functionality has a great influence on skeletal muscle metabolism [110], the regulation of skeletal muscle angiogenesis is also of high importance in the adaptation to exercise. It is known that VEGF plays a major role in the regulation of angiogenesis during exercise and ischaemia [87, 121, 122] and that the angiogenesis might be also regulated by PGC1α. Consequently, trained PGC1α knock-out mice displayed an impaired angiogenesis in comparison with trained wild-type mice [123].

As Pgc1α gene expression is reduced in mHFD offspring, we hypothesized that this reduction results in an impaired angiogenesis in skeletal muscle of mHFD offspring. To further investigate this hypothesis gene expression of Vegfα and ß was determined in *M. quadriceps* immediately after the acute exercise bout. While Vegfα gene expression did not show the same expression pattern as Pgc1α (Figure S1), Vegfß gene expression was significantly lower in mHFD compared to mLFD offspring. VEGF plays a major role in the regulation of angiogenesis during exercise and ischaemia [87, 121, 122]. Arany et al. published that PGC1α regulates the expression of VEGF and angiogenesis *in vivo* and *in vitro* by co-activating EsRRα rather than activating the Hypoxia-inducible factor 1α (HIF1α). Hence, they show that the major regulators of mitochondrial function, PGC1α, and EsRRα, also play a role in regulating angiogenesis in response to exercise [87, 124]. Since gene expression patterns of Pgc1α, Esrrα, and Vegfß are similar in mHFD offspring, the reduced endurance capacity of mHFD mice could be due to an impaired angiogenesis in these offspring mediated via Vegfß. Moreover, also gene expression of Hif1α was significantly reduced in mHFD offspring immediately after the endurance treadmill test suggesting a reduced oxygen supply due to less vascularization during the endurance test.

However, VEGFß is known to be poorly angiogenic in most tissues, and its biological role is poorly understood. Hagberg and co-workers suggested a role of VEGFß in the

control of the endothelial fatty acid uptake and transport into skeletal and heart muscle [90]. VEGFß regulates the transcription of vascular fatty acid transport proteins and thus, the endothelial uptake of long-chain fatty acids, which is regulated by the VEGF receptor 1 (FLT1) and neuropilin 1. Consequently, Vegfß knock-out mice showed a reduced lipid uptake and accumulation in skeletal muscle, heart and brown adipose tissue [90]. In rodent models with type 2 diabetes, a deletion of Vegfß restored insulin sensitivity and improved glucose tolerance in skeletal muscle [91]. To investigate the endothelial fatty acid uptake during an acute exercise bout, gene expression of Flt1, Fatty acid transport protein (Fatp) 3 and 4, and Cluster of differentiation 36/Fatty acid translocase (Cd36/Fat) was determined immediately after the endurance test. There were no differences between mHFD and mLFD offspring in gene expression, except for Fatp3. Here, mHFD offspring displayed a significantly reduced gene expression level. Since a reduced endothelial fatty acid uptake resulted in lipid shunting to white adipose tissue [90], a higher white adipose tissue mass was expected in mHFD offspring. However, there were no differences in tissue weight of subcutaneous white adipose tissue between mHFD and mLFD offspring (Table S1). In addition to a reduced fatty acid uptake an increased free fatty acid concentration in the circulation (plasma) was expected, but mice did not differ in free fatty acid concentration in plasma immediately after the acute exercise bout (Table S1). Resulting from these data, Vegfß expression is not associated with an altered fatty acid uptake in skeletal muscle. Furthermore, bioinformatic analysis showed a co-expression of Vegfß with nuclear-encoded mitochondrial genes such as Cytochrome c (Cytc) and NADH:Ubiquinone oxidoreductase subunit 5a (Ndufa5), which might be due to the same regulation by PGC1α and EsRRα [90]. Additionally, gene expression of Ndufa5 and Cytc was determined but showed no differences as well. This suggests that Pgc1α, Esrrα, and Vegfß are not involved in mitochondrial beta-oxidation in response to an acute exercise bout but rather play a major role in angiogenesis.

In consideration of all available data, no impact of maternal high-fat diet on the mitochondrial beta-oxidation can be inferred. Maternal diet rather impacts skeletal muscle angiogenesis by an impaired gene expression of Pgc1α, Esrrα, and Vegfß, which might consequently result in a decreased oxygen supply during the exercise bout. Hence, the reduced endurance capacity of mHFD offspring could be partly explained by an impaired angiogenesis (Figure 29).

4.3.3 Maternal high-fat diet impairs skeletal muscle glucose utilization

Skeletal muscle is able to generate glucose by glycogenolysis of the internal glycogen stores. Within an acute training performance, however, muscle glycogen stores are rapidly exhausted. Therefore, glucose must be obtained from the liver for ATP production [59]. To clarify whether maternal high-fat consumption during pregnancy and lactation has an impact on skeletal muscle glucose utilization during an acute exercise bout, which results in offspring impaired exercise capacity, the cross-talk between skeletal muscle and liver was further investigated.

The glucose uptake into skeletal muscle is mediated by two glucose transport proteins, GLUT1 and GLUT4. Under resting conditions, the glucose transport is the rate-limiting step due to a low GLUT1 expression and the storage of GLUT4 in intracellular vesicles. With exercise, GLUT4 translocates to the sarcolemma and thus increases skeletal muscle glucose uptake [125]. Gene expression of Glut1 and Glut4 was decreased in mHFD offspring with and without voluntary RW intervention in response to the acute exercise bout, suggesting limited glucose utilization in skeletal muscle. To underline these results protein expression of GLUT4 in the plasma membrane fraction of *M. quadriceps* was determined in samples taken immediately after the exercise bout. Here, also a decreased expression of GLUT4 was detectable in mHFD mice, supporting the gene expression data.

The question arises whether the liver provides less glucose to skeletal muscle or whether the provided glucose was not used by skeletal muscle cells during the acute exercise bout. To investigate this, blood glucose concentrations were measured before, immediately after and 3 h after the acute exercise bout. Immediately after the exercise bout, blood glucose concentrations of both mHFD offspring, with and without RW training, displayed significantly higher levels compared to mLFD offspring. This suggests that the glucose provision from the liver to skeletal muscle is not impaired in mHFD offspring. Furthermore, liver glycogen concentrations were reduced in all groups immediately after the endurance capacity test, supporting the suggestion of an intact glucose provision to skeletal muscle. Consequently, glucose utilization in skeletal muscle was further investigated by measuring the lactate dehydrogenase (LDH) activity in *M. gastrocnemius*. During prolonged, intense muscular activity, such as endurance exercise, the oxygen supply to skeletal muscle becomes insufficient and energy (ATP) has to be provided by anaerobic glycolysis.

Here, ATP is generated by the production of lactate out of pyruvate, catalyzed by LDH. Lactate reaches the periphery and is taken up by the liver, initiating gluconeogenesis. Hence, lactate is converted in the liver by LDH into pyruvate and finally glucose, which is supplied to skeletal muscle through the bloodstream [56]. LDH activity in *M. gastrocnemius* was significantly higher in mHFD offspring, suggesting an increased lactate production in skeletal muscle of mHFD offspring (Figure 29). This is in line with the reduced angiogenesis, resulting in a reduced oxygen supply to skeletal muscle, which contributes to an increased anaerobic metabolism. Moreover, these results support the treadmill exercise data, showing a reduced endurance capacity of these mice. Summermatter and colleagues showed that PGC1α prevented the blood lactate concentration to rise in response to exercise as an adaptation process due to regular exercise training. The induction of PGC1α due to exercise resulted in a reduced expression of the LDH muscle subunit LDH A and an increased induction of LDH B, which catalyzes the conversion of lactate into pyruvate in an EsRRα dependent manner. PGC1α could thus, enhance exercise performance [126]. These data are in line with the aforementioned data regarding skeletal muscle LDH activity and Pgc1α gene expression. Since gene expression of Pgc1α is reduced in mHFD offspring, an increased LDH activity of these offspring was expected as confirmed by these data. In contrast, LDH activity in liver was significantly lower in mHFD offspring. Due to technical problems, lactate concentration in blood and plasma could not be analyzed. However, higher lactate concentrations in blood would be expected, which should lead to a higher LDH activity in liver. Since LDH activity in liver was reduced in mHFD offspring we hypothesized that the transport of lactate from skeletal muscle into the blood stream might be impaired in mHFD offspring. Thus, gene expression of the monocarboxylate transporter (Mct) 1 and 4 in *M. quadriceps* was measured and indeed found to be reduced in mHFD compared to mLFD offspring. This reduced expression might cause a reduced transport of lactate into the bloodstream, resulting in a reduced lactate concentration in the liver and thus a diminished LDH activity. Additionally, GLUT4 expression is also reduced in mHFD offspring, leading to an accumulation of glucose in the bloodstream (Figure 29). Thus, glucose provided by the liver is not available for skeletal muscle ATP production. Consequently, skeletal muscle of mHFD offspring displayed an impaired glucose utilization during an acute exercise bout, which is a likely explanation for the reduced exercise capacity of mHFD offspring.

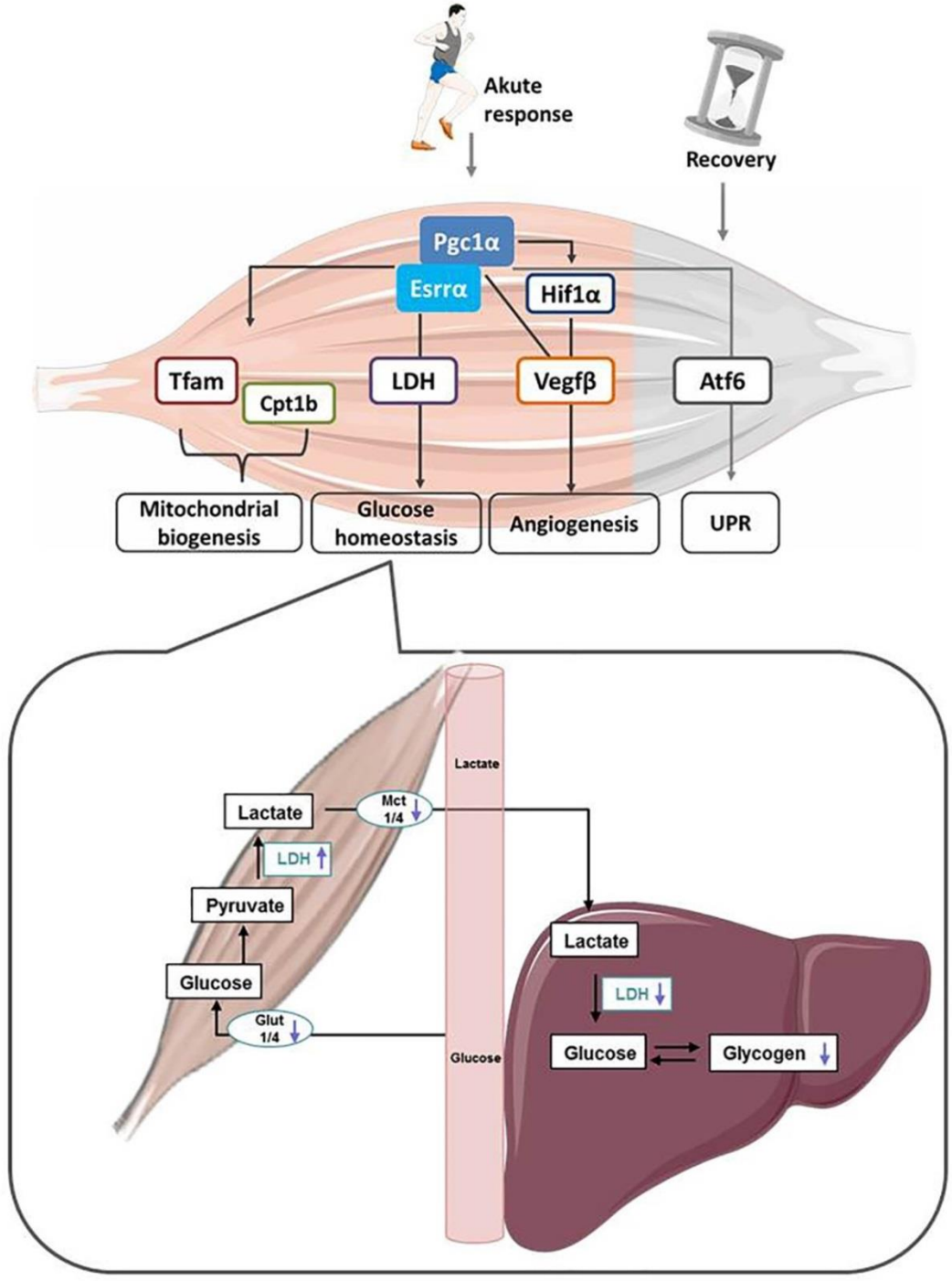

Figure 29: Impact of maternal high-fat diet feeding during pregnancy and lactation on offspring response to an acute exercise bout.
Maternal high-fat diet offspring fail to adapt to an acute exercise bout by improving mitochondrial biogenesis, glucose homeostasis, angiogenesis (during the acute exercise bout) and unfolded protein response (UPR, at the recovery phase, 3 h after the bout). This is due to a reduced gene expression Pgc1α, which co-activates Esrrα and is responsible for the regulation of skeletal muscle energy metabolism in response to exercise.
Atf6, Activating transcription factor 6; Cpt1b, Carnitine palmitoyl transferase 1b; Esrrα, Estrogen-related receptor α, Glut, Glucose transporter; Hif1α, Hypoxia-inducible factor 1α, LDH, Lactate dehydrogenase, Mct, Monocarboxylate transporter, Pgc1α, Peroxisome proliferator-activated receptor gamma coactivator 1α, Tfam, Mitochondrial transcription factor A. This figure was created using Servier Medical Art (http://www.servier.com).

4.3.4 Impact of maternal high-fat diet on offspring skeletal muscle unfolded protein response in the recovery state

As already mentioned, exercise performance results in many adaptations within the skeletal muscle such as an improved mitochondrial biogenesis, angiogenesis and also an unfolded protein response to maintain the endoplasmic reticulum (ER) homeostasis [64, 67, 92]. The ER has several functions such as protein synthesis, folding, assembly, and transport. In the ER lumen, newly synthesized proteins are folded into their proper conformation by molecular chaperones. Under conditions of nutrient deprivation or Ca^{2+} depletion, the protein folding is disturbed and unfolded/misfolded proteins accumulate. To counter the disturbance of protein folding, the expression of chaperones is induced by signal transduction cascades, which are termed as unfolded protein response (UPR) [70]. Thus, the activation of the UPR is of major importance in the adaptive response to ER stress [71]. In the case of ER stress and the occurrence of misfolded proteins, the sensors Protein kinase RNA-like endoplasmic reticulum kinase (PERK), Inositol-requiring enzyme1α (IRE1α) and Activating transcription factor 6 (ATF6) are activated [69]. Since it is known that skeletal muscle physiology is influenced by hypoxia, glucose deprivation, and imbalances in calcium homeostasis which trigger UPR [127, 128], we hypothesized that UPR also has an impact on skeletal muscle function and regeneration/recovery in response to an acute exercise bout.

In order to investigate this hypothesis, the expression of UPR marker genes such as Atf3, Atf4, Chop, Gadd 34, Cytc and Atf6 was determined 3h after an acute exercise bout. In response to ER stress PERK activation induces the expression of Atf4, which as a result induces Atf3, Gadd34 and Chop expression [69]. Gene expression of Atf4 and Chop was not different between the groups, while Atf3 expression showed a significant increase in mLFD and mHFD offspring with voluntary RW training. The same trend was observed for Gadd34 gene expression. Wu and colleagues showed an increase in gene expression of Atf4, Gadd34, Atf3 and Chop after acute exercise in treadmill-trained mice compared to sedentary mice [92]. They depicted an increase in Atf3 gene expression in trained mice 5 h after an acute exercise bout which is in line with our gene expression data of Atf3 3 h after an acute exercise bout in voluntarily trained mice. Thus, also voluntary running wheel training might induce the expression of UPR marker genes. However, this effect was only detectable for Atf3 and not for the other candidate genes. Besides PERK, ATF6 is another sensor of

UPR, which is important in the cellular adaptation to chronic ER stress [129]. Gene expression of Atf6 tended to be lower in mHFD offspring compared to mLFD offspring. Thus, the Atf6 cascade seemed not to be induced by adaptation signals to voluntary RW training such as Atf3, but by maternal nutrition. Wu et al. postulated a connection between PGC1α expression and the induction of ATF6. Consequently, they showed that PGC1α co-activates ATF6 in skeletal muscle, which might be, at least partly, an explanation for the exercise intolerance of PGC1α knock-out mice [92]. However, the underlying mechanism is not known yet. Since Pgc1α gene expression is also reduced in mHFD mice 3h after the acute exercise bout (Figure S4) and gene expression of Pgc1α correlates significantly with Atf6 expression, the conclusion of Wu et al. might also be a possible explanation for the impaired endurance capacity of mHFD offspring (Figure 29). Probably, mHFD mice adapt to a lesser extend to voluntary exercise due to the induction of Pgc1α/Atf6 and consequently to the activation of UPR. This might lead to a higher ER stress level, which could result in an impaired training efficiency and endurance capacity of these offspring.

4.4 Impact of maternal high-fat diet on offspring obesity susceptibility

In order to investigate whether the impaired exercise capacity and perinatal maternal HFD consumption per se have long-term effects on the development of offspring diet-induced obesity, offspring of LFD and HFD-fed dams received HFD from wk15 wk onwards. The impact on the beneficial effects of exercise on body composition and glucose metabolism were determined by body composition measurements at wk seven, 15 and 25 and an oral glucose tolerance test (oGTT) at an age of 22 wks.

4.4.1 Maternal high-fat diet attenuates the positive effects of exercise on offspring fat mass gain and promotes offspring obesity.

After ten wks of HFD feeding, RW training could not prevent fat mass gain in both groups anymore, but mHFD offspring displayed a higher fat mass gain (in tendency). Rajia and co-workers also described that voluntary training in female rats was not sufficient to completely reverse the negative effects of HFD feeding [72].

Additionally, Linden et al. showed that a more efficient regular treadmill exercise training only leads to mild effects on HFD-induced body weight and fat mass gain [130].

Furthermore, maternal HFD intervention increased offspring liver weight, which is in line with a growing body of literature focusing on the connection between maternal obesity and offspring fatty liver [26, 94, 131, 132]. However, this effect was not due to an increase in liver triglycerides. As published previously in our group, liver glycogen content of mHFD offspring correlated significantly with liver weight [73]. Nevertheless, this is not in accordance with other studies publishing a connection between an increased liver weight and hepatic lipid accumulation [26, 133]. One possible explanation for the absence of the effects on hepatic triglyceride content might be the use of different dietary fat composition or contents. The semisynthetic HFD used in the present study contains 40 % kcal from fat, which was composed of 12 % flaxseed oil, 18 % coconut oil and 70 % sunflower oil. In contrast, HFDs used in most studies contain 60 % kcal from fat, while the Western-style diet used by Pruis et al. only contains 45 % kcal from fat, which is comparable to our diet, but mainly consists of lard [26]. Thus, it was mainly composed of saturated fatty acids, while the fats used in our study contained more unsaturated fatty acids. According to Tsuduki and colleagues maternal cholesterol intake also plays a role in the development of a fatty liver by stimulating offspring hepatic lipid accumulation [134]. The HFD used in our studies did not contain cholesterol, which might explain the lack of increased hepatic triglycerides in mHFD offspring.

Taken together, mHFD attenuates the positive effects of voluntary RW training on offspring fat mass gain and caused an increased liver weight in the offspring, which was independent of voluntary training.

4.4.2 Enhanced exercise capacity in mLFD, but not mHFD offspring by daily voluntary running

Along with Study I and sStudy III mHFD feeding resulted in impaired offspring endurance capacity and training efficiency in Study IV. mLFD offspring increased their endurance capacity after ten wks of HFD intervention to a much higher extent compared to mHFD offspring. mHFD offspring were not able to increase their endurance capacity from wk 15 until wk 25 anymore. Furthermore, the increase in training efficiency of mHFD offspring from wk seven until wk 15 was much lower

compared to mLFD offspring. Hence, mHFD feeding displayed negative long-term effects on offspring training efficiency.

As in the other studies, skeletal muscle gene expression of myosin heavy chains (Mhc) was determined also in Study IV. *M. quadriceps* and *M. soleus* were analyzed in more detail since endurance training results in pronounced changes in the metabolic properties of skeletal muscle by influencing the fiber type conversion [43]. Characteristically for a glycolytic, fast-twitch, type II muscle such as *M. quadriceps*, the abundance of type II muscle fibers was much higher than that of type I fibers. In *M. soleus*, an oxidative, slow-twitch, type I muscle where the oxidative fiber types MhcI and MchIIa showed the highest gene expression levels. Since oxidative muscle fibers (Mhc I; Mhc IIa) are more pronounced in endurance athletes than in sprinters [43], and at least in tendency increased in mLFD offspring after ten wks on HFD, this could be one explanation for the pronounced training efficiency of these offspring.

While PGC1α plays an essential role in skeletal muscle adaptation processes during exercise [67], there were neither differences in Pgc1α expression between the dietary groups nor had RW training an impact on the expression level in Study IV, while gene expression of Pgc1α in 15 wk old mHFD offspring was reduced immediately after the acute exercise bout (Study III), independently of RW access. Since exercise leads to the induction of mitochondrial oxidative capacity [135], the citrate synthase activity was determined as a marker of mitochondrial density. In accordance with the literature, citrate synthase activity was increased with voluntary training. However, in accordance with study III, there were no differences between mLFD and mHFD offspring. Furthermore, mHFD seems to impair the ability of the electron transport chain to generate a proton gradient for ATP production. In contrast to 15 wk old mHFD offspring, OXPHOS protein expression displayed a significant reduction in complex I and in tendency also in complex III-V in 25 wk old mHFD offspring after ten wks on HFD. This might at least partly explain the impaired endurance capacity of mHFD offspring.

4.4.3 Maternal high-fat diet impairs the positive effect of exercise intervention on offspring insulin sensitivity

Many studies show that maternal obesity is linked to offspring hyperinsulinemia [72, 96], while exercise interventions are known to improve skeletal muscle insulin sensitivity in animals [136] and humans [137]. The data of the oGTT at an age of 22 wks revealed reduced plasma insulin levels 15 min after glucose gavage in mLFD offspring with RW training, while this was not the case for mHFD offspring. Hence, RW intervention prevented the negative effects of HFD-induced obesity in mLFD, but not in mHFD offspring. Furthermore, pancreas insulin content was also increased in mHFD offspring, while blood glucose levels were not altered, suggesting the need for an increased beta-cell insulin production to maintain a normal blood glucose level. Gregorio et al. mentioned an increased insulin production in mHFD offspring due to an altered pancreas morphology [138]. This is also interesting to analyze in further studies.
In summary, the data revealed an increased pancreatic insulin production in mHFD offspring to counteract insulin resistance. The development of insulin resistance is prevented by voluntary training in mLFD offspring only. This indicates that mHFD feeding during pregnancy and lactation impairs the beneficial effects of RW training on offspring skeletal muscle energy metabolism.

4.5 Limitations

In this PhD thesis, the impact of maternal high-fat diet feeding on offspring endurance performance and skeletal muscle energy metabolism was investigated by feeding female C57Bl/6J mice either a LFD or HFD three days prior mating throughout pregnancy and lactation. To investigate offspring skeletal muscle energy metabolism, analysis in *M. quadriceps* and *M. gastrocnemius* were mainly performed. Both skeletal muscle types contribute to the mixed fiber type muscles, containing more glycolytic (type II) than oxidative (type I) muscle fibers. For the investigations of skeletal muscle fiber switch towards a more oxidative fiber type analysis of more glycolytic muscle types such as *M. tibialis anterior* or EDL would be useful. Furthermore, a large part of the present results is gene expression data. It is necessary to confirm these data on protein expression level by performing ELISA, Western Blot or immune histological staining. For the analysis of transcription

factors, a fractionation in the nuclear and cytoplasm fraction of the muscle tissue could be particularly helpful. However, for fractionations, fresh tissue is needed and thus, the experiment should be repeated for detailed analyses of the transcription factors. The collection of the blood/plasma samples is also a limitation of the present experiments. Ideally, they should be centrifuged and aliquoted immediately after collection in order to prevent the eruption of erythrocytes and other cell ingredients and thus to ensure measurements such as lactate concentration or circulating myokines such as IL15. In our studies, blood samples were collected and only centrifuged at the end of each preparation day. Furthermore, some plasma samples were hemolytic, which can also disturb ELISA measurements.

5 CONCLUSION AND PERSPECTIVES

This PhD thesis provides insights into the role of maternal high-fat diet (mHFD) consumption on offspring skeletal muscle energy metabolism and endurance performance. Male offspring of mHFD fed C57Bl/6J dams displayed an impaired training efficiency and endurance capacity compared to maternal low-fat diet (mLFD) offspring, despite voluntary running wheel (RW) intervention. This diminished endurance capacity is less attributable to an altered skeletal muscle morphology but appears to be the consequence of a disturbed skeletal muscle substrate metabolism during an acute exercise bout (Figure 30). In this connection adaptation processes of the skeletal muscle regarding exercise performance play a major role. Thus, the Pgc1α-Esrrα complex has been shown to play an essential role in skeletal muscle mitochondrial biogenesis, angiogenesis, glucose homeostasis, and unfolded protein response. For most of the key players of all these mechanisms a reduced gene expression in mHFD offspring skeletal muscle was detectable. Thus, maternal high-fat diet feeding resulted in a reduced adaptation of offspring skeletal muscles to exercise and consequently, represents a possible explanation for the impaired endurance capacity of the offspring.
As a further adaptation process of mHFD offspring regarding the impaired skeletal muscle glucose homeostasis, a hypomethylation in the Nr4a1 gene was detectable. Moreover, mHFD offspring showed a higher incidence of obesity and decreased insulin sensitivity, when fed a high-fat diet for ten wks later in life. Hence, maternal nutrition also plays a decisive role in offspring obesity susceptibility.

This PhD thesis mainly focused on the impact of maternal nutrition on offspring exercise performance and skeletal muscle energy metabolism in LFD fed offspring to examine exclusively the effect of maternal nutrition on the offspring. Since there are first indications that offspring HFD feeding results in an obese phenotype it would be interesting to investigate also the impact of maternal overnutrition on skeletal muscle energy metabolism in response to an acute exercise bout in HFD fed offspring. This would provide further insights whether mLFD offspring are protected from the detrimental effects of high-fat consumption in adulthood or whether the HFD intervention completely overrides the positive effects of the mLFD. Since the HFD intervention of the offspring only took place at 15 wks of age for a period of

ten wks, it would also be interesting to investigate the influence of HFD feeding after weaning and beyond an age of 25 wks on the metabolism of the offspring. Furthermore, primary cell culture experiments could be performed to clarify the underlying mechanism of the hypomethylation in the Nr4a1 gene of mHFD offspring. Since gene expression of 25 wk old mHFD offspring without voluntary running intervention was also increased, it is interesting to investigate also DNA methylation in these offspring.

Even though these studies are only fundamental research, they can help to explain why some individuals fail to improve their endurance capacity and energy metabolism despite regular exercise training. Since it is already known for women born to obese mothers displaying insulin resistance and impaired muscle glucose uptake, our studies can give mechanistic insights regarding the altered energy metabolism.

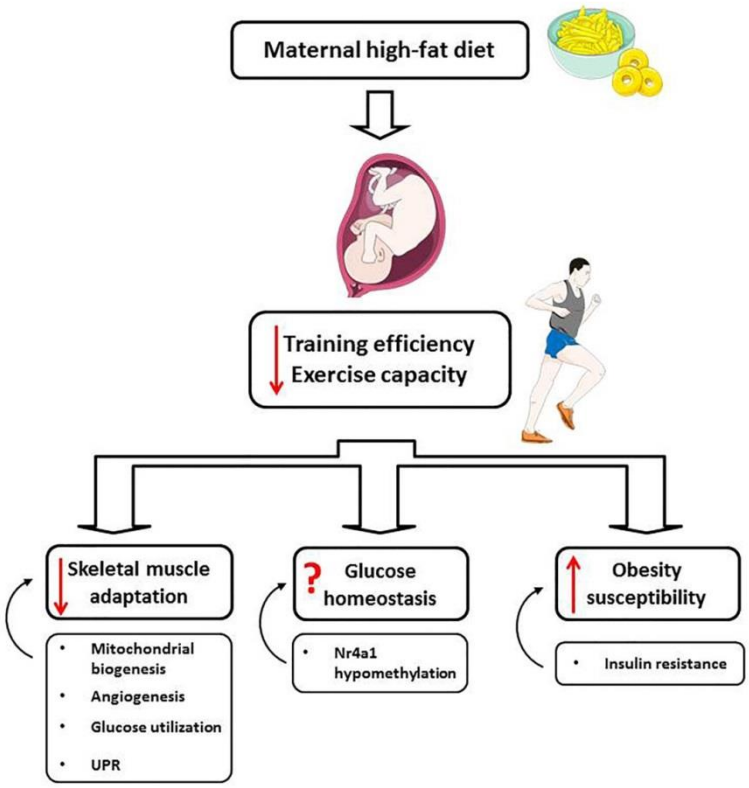

Figure 30: Conclusion
A perinatal high-fat diet results in an impaired training efficiency and endurance capacity of the offspring, which is due to an impaired skeletal muscle adaptation due to an impaired mitochondrial biogenesis, angiogenesis and glucose utilization as well as an increased risk of obesity susceptibility and the risk to develop insulin resistance. To compensate the impaired glucose utilization in skeletal muscle, maternal high-fat diet offspring improve their expression of the transcription factor Nr4a1.
UPR, unfolded protein response; Nr4a1, Nuclear receptor subfamily 4 group a number 1. This figure was created using Servier Medical Art (http://www.servier.com).

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SUPPLEMENTS

Table of contents

Table S1: Offspring phenotypic characteristics

		no exercise (-RW)		exercise (+RW)		p-Value	
	time	**mLFD**	**mHFD**	**mLFD**	**mHFD**	**mDiet**	**RW**
Skeletal muscle							
Weight [g]		0.384 ± 0.005	0.382 ± 0.006	0.382 ± 0.006	0.378 ± 0.006	< 0.05	p = 0.075
Triglycerides [mg/mg protein]	basal	0.51 ± 0.04	0.63 ± 0.06	0.47 ± 0.04	0.58 ± 0.06	< 0.05	n.s.
	0h	0.96 ± 0.13	0.75 ± 0.09	0.54 ± 0.06	0.42 ± 0.08	p = 0.077	< 0.001
	3h	0.74 ± 0.12	1.06 ± 0.24	0.78 ± 0.13	0.59 ± 0.05	n.s.	n.s.
Glycogen [µg/mg protein]	basal	6.78 ± 0.81	6.86 ± 0.87	7.36 ± 0.46	6.74 ± 0.36	n.s.	n.s.
	0h	4.45 ± 0.63	5.34 ± 1.41	3.48 ± 0.72	3.56 ± 0.75	n.s.	n.s.
	3h	12.05 ± 1.32	10.99 ± 1.04	16.83 ± 2.63	14.06 ± 2.40	n.s.	p = 0.053
Liver							
Weight [g]		0.944 ± 0.025	1.013 ± 0.024	0.995 ± 0.022	1.005 ± 0.031	n.s.	n.s.
Triglycerides [mg/mg protein]	basal	0.61 ± 0.06	0.65 ± 0.03	0.49 ± 0.03	0.53 ± 0.03	n.s.	< 0.05
	0h	0.65 ± 0.04	0.68 ± 0.06	0.73 ± 0.06	0.70 ± 0.09	n.s.	n.s.
	3h	0.71 ± 0.08	0.80 ± 0.09	0.63 ± 0.09	0.55 ± 0.10	n.s.	n.s.
Glycogen [µg/mg protein]	basal	27.68 ± 8.64	26.72 ± 2.60	35.50 ± 7.30	42.32 ± 10.33	n.s.	n.s.
	0h	10.26 ± 3.79	16.89 ± 5.95	13.56 ± 3.26	7.82 ± 2.83	n.s.	n.s.
	3h	6.86 ± 1.22	13.00 ± 4.25	24.51 ± 5.47	24.03 ± 8.44	n.s.	< 0.05
sWAT							
Weight [g]		0.312 ± 0.009	0.346 ± 0.016	0.279 ± 0.009	0.282 ± 0.012	n.s.	< 0.001
Plasma							
Triglycerides [mmol/L]	basal	0.436 ± 0.052	0.447 ± 0.039	0.440 ± 0.067	0.566 ± 0.100	n.s.	n.s.
	0h	0.146 ± 0.033	0.114 ± 0.022	0.193 ± 0.025	0.183 ± 0.026	n.s.	< 0.05
	3h	0.163 ± 0.025	0.252 ± 0.034	0.278 ± 0.036	0.296 ± 0.044	n.s.	< 0.05
Free Fatty Acids [mmol/L]	basal	0.564 ± 0.030	0.548 ± 0.041	0.514 ± 0.039	0.490 ± 0.039	n.s.	n.s.
	0h	0.741 ± 0.083	0.538 ± 0.048	0.698 ± 0.062	0.756 ± 0.094	n.s.	n.s.
	3h	0.490 ± 0.062	0.565 ± 0.073	0.414 ± 0.055	0.431 ± 0.044	n.s.	p = 0.093
Cholesterol [mg/dL]	basal	77.1 ± 7.4	102.9 ± 2.4	101.8 ± 13.4	100.0 ± 4.2	n.s.	n.s.
	0h	131.2 ± 6.9	133.3 ± 16.8	123.7 ± 13.8	104.8 ± 11.1	n.s.	n.s.
	3h	94.3 ± 16.1	159.6 ± 11.1	145.7 ± 7.5	129.8 ± 10.0	p = 0.066	n.s.

Maternal low-fat diet (mLFD) or maternal high-fat diet (mHFD) offspring received a LFD after weaning until an age of 15 wks. Half of mLFD and mHFD offspring got access to a running wheel (RW) as voluntary training. Mice were sacrificed at an age of 15 wks at three different time points, before, immediately after and 3 h after an acute exercise bout. Data are mean ± SEM; basal: n = 9-11, 0 h: n = 8-11, 3 h: n = 7 - 9. Data were analyzed by two-way ANOVA (Bonferroni post hoc test). sWAT, subcutaneous white adipose tissue.

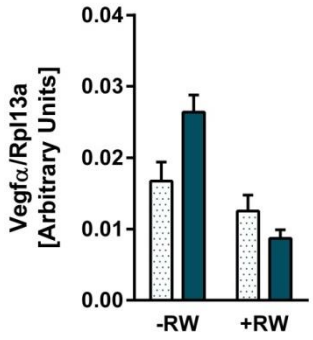

Figure S1: Gene expression of Vegfα in 15 wk old offspring immediately after an acute exercise bout (Study III).
Gene expression was measured in *M. quadriceps*. Data are mean + SEM, n = 6. mLFD, dotted bars, mHFD, turquoise bars. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

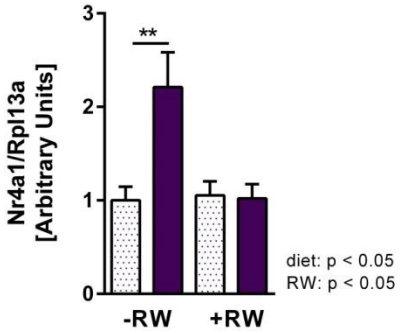

Figure S2: Gene expression of Nr4a1 in 25 wk old offspring (Study I).
Gene expression was measured in *M. quadriceps*. Data are mean + SEM, n = 6. mLFD, dotted bars, mHFD, purple bars. Gene expression data was analyzed by using the ddCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a) (dCt) and further of the control group mLFD-RW (ddCt). Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

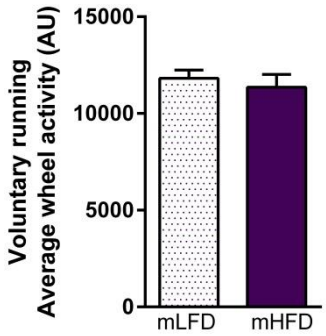

Figure S3: Voluntary running wheel usage of 9 wk old offspring (Study I).
Data are mean + SD

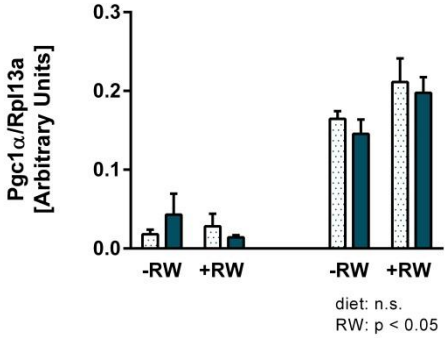

Figure S4: Gene expression of Pgc1α in 15 wk old offspring at a basal level and 3 h after an acute exercise bout (Study III).
Gene expression was measured in *M. quadriceps*. Data are mean + SEM, n = 6. mLFD, dotted bars, mHFD, turquoise bars. Gene expression data was analyzed by using the dCt method, normalizing the Ct-value of the target gene with the Ct-value of the reference gene Ribosomal protein L13 (Rpl13a). Data were analyzed by two-way ANOVA and Bonferroni posthoc test.

Table S2: Significantly up-regulated genes in the microarray of 6 wk old offspring (Study II)

GeneName	SystematicName	fold change (mLFD/mHFD)	p-value
Bcl6	NM_009744	1,38	0,00011
Ccdc50	NM_026202	1,39	0,00074
A_55_P2034320	A_55_P2034320	1,22	0,00115
Slc38a2	NM_175121	1,32	0,00191
3830612M24	AK028406	1,24	0,00206
5031434C07Rik	AK030322	1,41	0,00251
Sik1	NM_010831	1,53	0,00262
Nr4a1	NM_010444	1,43	0,00354
Egfr	NM_207655	1,26	0,00370
Gm8801	NR_028278	1,25	0,00507
Atf3	NM_007498	1,67	0,00538
1810011O10Rik	NM_026931	1,35	0,00545
Mup20	NM_001012323	2,35	0,00617
Etl4	NM_001177630	1,23	0,00708
1110046J04Rik	NR_040707	1,22	0,00718
Shisa2	NM_145463	1,30	0,00749
NAP005045-002	NAP005045-002	1,22	0,00816
Id1	NM_010495	1,31	0,00874
Arglu1	NM_176849	1,21	0,00900
Ccrn4l	NM_009834	1,34	0,00945
Pde4d	NM_011056	1,31	0,00989
Rbp7	NM_022020	1,28	0,01015
Optn	NM_181848	1,24	0,01081
9630033F20Rik	NM_177003	1,21	0,01108
Slc20a1	NM_015747	1,26	0,01187
Abi3	NM_025659	1,22	0,01192
Ucp3	NM_009464	1,29	0,01243
Fabp1	NM_017399	1,98	0,01390
Serpina1e	NM_009247	2,70	0,01423
Maff	NM_010755	1,30	0,01427
Kcna1	NM_010595	1,21	0,01473
Flcn	NM_146018	1,24	0,01558
Ppp1r12b	ENSMUST00000141419	1,39	0,01567
Cpeb1	NM_001252525	1,20	0,01592
Vegfa	NM_001025257	1,21	0,01597
Ppargc1a	NM_008904	1,29	0,01763
Serpina1a	NM_001252569	2,70	0,01770
Apoa2	NM_013474	1,83	0,01777
Acrbp	NM_016845	1,27	0,01787
Ramp3	NM_019511	1,25	0,01792
Ap1g1	NM_009677	1,25	0,01825
Serpina1c	NM_009245	2,75	0,01848
Alb	NM_009654	1,95	0,01875

GeneName	SystematicName	fold change (mLFD/mHFD)	p-value
Slc9a3r2	NM_023055	1,20	0,01898
Serpina1e	NM_009247	1,81	0,01938
Ppargc1a	NM_008904	1,36	0,01959
Serpina3k	NM_011458	1,88	0,01971
Ccdc50	NM_026202	1,21	0,02142
A430107O13Rik	NM_001081351	1,22	0,02399
Tnfrsf12a	NM_013749	1,32	0,02419
Catsper4	NM_177866	1,22	0,02440
Serpina1d	NM_009246	1,71	0,02575
Nr4a2	NM_013613	1,38	0,02614
Nup153	NM_175749	1,33	0,02714
1810011O10Rik	NM_026931	1,22	0,02721
Ahsg	NM_013465	2,10	0,02730
A_55_P2046709	A_55_P2046709	2,54	0,02834
Bhmt	NM_016668	1,51	0,02878
Serpina1a	NM_001252569	1,64	0,02974
Irs2	NM_001081212	1,49	0,03016
Ambp	NM_007443	1,70	0,03071
Cebpb	NM_009883	1,22	0,03082
Pde4d	NM_011056	1,23	0,03096
Pde7a	NM_008802	1,20	0,03097
Tmem100	NM_026433	1,22	0,03179
Gc	NM_008096	1,32	0,03243
Fgg	NM_133862	1,74	0,03266
Piga	NM_011081	1,22	0,03314
2010109K11Rik	NM_001162903	1,21	0,03460
Nfil3	NM_017373	1,48	0,03583
Gc	NM_008096	1,76	0,03761
Srxn1	NM_029688	1,35	0,03845
Qk	NM_021881	1,30	0,03955
NAP000123-003	NAP000123-003	1,25	0,03957
Tra2a	NM_198102	1,22	0,03978
Rgs4	NM_009062	1,25	0,04011
ENSMUST00000156693	ENSMUST00000156693	1,51	0,04053
Zbtb43	NM_027947	1,25	0,04097
Gm16062	NR_045686	1,28	0,04360
ENSMUST00000153865	ENSMUST00000153865	1,41	0,04368
Fos	NM_010234	1,23	0,04422
Actg2	NM_009610	1,26	0,04462
Zbtb43	NM_027947	1,21	0,04484
Fbp1	NM_019395	1,50	0,04565
Aldob	NM_144903	1,59	0,04604
Bhmt	NM_016668	1,48	0,04607
A_55_P2144090	A_55_P2144090	1,30	0,04614

GeneName	SystematicName	fold change (mLFD/mHFD)	p-value
D19Wsu162e	NM_001177812	1,22	0,04633
TC1627089	TC1627089	1,31	0,04697
Cyp2d26	NM_029562	1,40	0,04720
Ccbp2	NM_021609	1,20	0,04888

Significantly up-regulated genes are listed with a fold change > +1.2.

Table S3: Significantly down-regulated genes in the microarray of 6 wk old offspring (Study II)

GeneName	SystematicName	fold change (mLFD/mHFD)	p-value
Sox4	ENSMUST00000067230	-1,28	0,00164
Ano1	NM_178642	-1,37	0,00255
Tst	NM_009437	-1,21	0,00308
Art5	NM_007491	-1,35	0,00310
Acvr2b	NM_007397	-1,37	0,00326
Tnfrsf1a	NM_011609	-1,31	0,00368
Cd52	NM_013706	-1,27	0,00399
Nfatc2	NM_001136073	-1,31	0,00417
Ctf1	NM_007795	-1,22	0,00418
E2f2	NM_177733	-1,32	0,00443
Exoc7	NM_016857	-1,20	0,00571
Art5	NM_007491	-1,27	0,00600
Dlk1	NM_010052	-1,22	0,00615
Igtp	NM_018738	-1,25	0,00621
Sin3b	NM_001113248	-1,20	0,00659
Igtp	NM_018738	-1,25	0,00853
Scarb1	NM_016741	-1,23	0,00876
Mpp1	NM_008621	-1,24	0,00892
A_55_P2125643	A_55_P2125643	-1,26	0,00923
2310047D07Rik	XR_104959	-1,25	0,01004
Apoe	NM_009696	-1,22	0,01058
Cry2	NM_009963	-1,34	0,01087
Tnfaip2	NM_009396	-1,28	0,01134
2610029G23Rik	ENSMUST00000119477	-1,21	0,01166
Neat1	NR_003513	-1,52	0,01279
Dyrk1b	NM_001037957	-1,27	0,01303
Gm6548	NR_003363	-1,20	0,01332
Pop7	NM_028753	-1,31	0,01376
Cetn3	NM_007684	-1,26	0,01385
Mfap2	NM_008546	-1,29	0,01406
Gm16525	XR_105602	-1,24	0,01414
Rps19-ps13	ENSMUST00000091925	-1,23	0,01528
Sep 04	NM_011129	-1,21	0,01565

Vwf	NM_011708	-1,21	0,01583

GeneName	**SystematicName**	**fold change (mLFD/mHFD)**	**p-value**
2310015K22Rik	AK009366	-1,26	0,01700
Nnt	NM_008710	-1,21	0,01707
1700052K11Rik	NR_027956	-1,23	0,01862
Serpinb1a	NM_025429	-1,24	0,01953
NAP111303-1	NAP111303-1	-1,27	0,01956
XM_003085019	XM_003085019	-1,20	0,02031
Scrn1	NM_027268	-1,21	0,02241
CF550882	CF550882	-1,21	0,02250
Agpat5	NM_026792	-1,24	0,02272
1700056E22Rik	NM_028516	-1,24	0,02353
Clu	NM_013492	-1,26	0,02515
Col1a1	NM_007742	-1,32	0,02584
Gm10406	NM_001164727	-1,22	0,02613
Gm4841	NM_001034859	-1,63	0,02625
F830016B08Rik	NM_001101475	-1,23	0,02659
Wif1	NM_011915	-1,26	0,02674
1200009I06Rik	NM_028807	-1,27	0,02704
Slc1a5	NM_009201	-1,35	0,02715
ENSMUST00000091375	ENSMUST00000091375	-1,25	0,02754
Maged2	NM_030700	-1,22	0,02804
Usp20	NM_028846	-1,21	0,02852
Cygb	NM_030206	-1,29	0,02899
Txnrd3	NM_001178058	-1,23	0,02974
Fbln5	NM_011812	-1,20	0,03092
Tnfaip2	NM_009396	-1,24	0,03247
1110054M08Rik	NR_037954	-1,21	0,03270
Tph1	NM_009414	-1,27	0,03427
Apol10b	NM_177820	-1,20	0,03521
Atp5l	NM_013795	-1,30	0,03551
Lin52	NM_173756	-1,21	0,03565
Mamstr	NM_172418	-1,22	0,03603
H19	NR_001592	-1,41	0,03628
Prkag1	NM_016781	-1,24	0,03715
Gamt	NM_010255	-1,38	0,03747
Gm5506	NM_001025388	-1,22	0,03912
Odz4	NM_011858	-1,22	0,03920
Snrpa1	NM_021336	-1,26	0,03958
Apoe	NM_009696	-1,21	0,04052
Apoe	NM_009696	-1,32	0,04151
Gpsm3	NM_134116	-1,22	0,04228
H2-Q2	NM_010392	-1,20	0,04474
Mmaa	NM_133823	-1,35	0,04497
Gadd45b	NM_008655	-1,28	0,04499

2310002L09Rik	NM_027104	-1,28	0,04509
GeneName	**SystematicName**	**fold change (mLFD/mHFD)**	**p-value**
Aldh1l1	NM_027406	-1,31	0,04554
Gm8709	NR_033633	-1,24	0,04625
H2-K1	NM_001001892	-1,21	0,04640
Psme1	NM_011189	-1,26	0,04749
Sypl2	NM_008596	-1,23	0,04791
Col16a1	NM_028266	-1,28	0,04832
Clpb	NM_009191	-1,23	0,04915
Kdm5b	NM_152895	-1,21	0,04971

Significantly down-regulated genes are listed with a fold change < -1.2.

PUBLICATIONS

Reviews:

Ost M., Coleman V., **Kasch J.**, Klaus S. (2016) *Regulation of myokine expression: Role of exercise and cellular stress.* Free Radic Biol Med. 98:78-89.

Research articles:

Kasch J., Schumann S., Schreiber S., Klaus S., Kanzleiter I. (2017) *Beneficial effects of exercise on offspring obesity and insulin resistance are reduced by maternal high-fat diet*. PLoS ONE. 12 (2): e0173076.

Kasch, J., Kanzleiter, I., Saussenthaler, S., Schürmann, A., Keijer, J., van Schothorst, E., Klaus, S., Schumann, S. (2018) *Insulin sensitivity linked skeletal muscle Nr4a1 DNA methylation is programmed by the maternal diet and modulated by voluntary exercise in mice.* The Journal of Nutritional Biochemistry.

CONTRIBUTION TO CONFERENCES

Oral presentations:

Kasch J., Schumann S., Kanzleiter I., Klaus S. (2015) *Impact of maternal nutrition on muscle energy metabolism of offspring – Effects on obesity susceptibility?*. 3rd Helmholtz-Nature Medicine Diabetes Conference 2015, September 18-21, 2015, Munich, Germany (Round table session)

Kasch J., Schumann S., Kanzleiter I., Klaus S. (2014) *A maternal high-fat diet impairs endurance capacity in high-fat fed offspring*. 3rd Doctoral Workshop on Molecular Nutrition, June 26-27, 2014, Tarragona, Spain

Kasch J., Schumann S., Kanzleiter I., Klaus S. (2014) *A maternal high-fat diet impairs endurance capacity in high-fat fed offspring*. 3rd International Advanced Course on Epigenesis and Epigenetics –Physiological consequences of perinatal nutritional programming-, May 21-23, 2014, Wageningen, The Netherlands

Poster presentations:

Kasch J., Schumann S., Haase K., Lasik I., Klaus S. (2017) *Impact of maternal nutrition on offspring exercise performance and skeletal muscle energy metabolism*. Cell Symposia-Exercise metabolism, May 21-23, 2017, Gothenburg, Sweden

Kasch J., Schumann S., Haase K., Klaus S. (2016) *Impact of maternal nutrition on offspring muscle energy metabolism after an acute exercise bout*. 36. Blankenese Konferenz: "Metabolic Control – Gut Microbiome – Cellular Circuits – Synthetic Signaling Cascades -" May 28. – June 01, 2016, Hamburg Blankenese, Germany

Kasch J., Schumann S., Klaus S. (2015) *Consequences of maternal nutrition on muscle energy metabolism of offspring - Effects on obesity susceptibility?*. 31. Jahrestagung der Deutschen Adipositas Gesellschaft, October 15-17, 2015, Berlin, Germany

Kasch J., Schumann S., Kanzleiter I., Klaus S. (2015) *Consequences of maternal nutrition on muscle physiology and energy metabolism of offspring*. Keystone Symposia: "The Crossroads of Lipid Metabolism and Diabetes", April 20-23, 2015, Kopenhagen, Denmark

Kasch J., Schumann S., Kanzleiter I., Klaus S. (2014) *Einfluss einer maternalen Hochfett-Diät auf die Ausdauerleistung und Adipositasprädisposition der Nachkommen*. 8. Diabetes Herbsttagung / 30. Adipositas Jahrestagung, November 21-22, 2014, Leipzig, Germany

DECLARATION OF ACADEMIC HONESTY

I hereby confirm that my thesis entitled “Impact of maternal high-fat consumption on offspring exercise performance, skeletal muscle energy metabolism and obesity susceptibility” is my own original work and has not previously, in part or in its entirety, been submitted at any university for a degree. Information derived from published work of others has been stated in the text and a list of references is given in the bibliography.

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Juliane Kasch