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Development of skeletal muscle and adipose tissues in neonatal dairy calves upon a maternal supplementation with essential fatty acids and conjugated linoleic acids

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Abstract

Modern feeding systems of high yielding dairy cows may not provide the animals with sufficient essential fatty acids (EFAs), thereby also reducing the tissue formation of conjugated linoleic acids (CLAs). In addition, the common concentrate-based feed contains a higher *n*-6/*n*-3 EFA ratio than pasture-based feed, of which the effects on the animal are not known yet. During gestation, a higher *n*-6/*n*-3 EFA ratio can have consequences for calf development, too. In this dissertation, the effect of a maternal EFA and CLA supplementation on the cellular development of skeletal muscle and adipose tissues was investigated in neonatal calves. Holstein cows (n = 40) were abomasaly supplemented with coconut oil (control group), or EFAs at an *n*-6/*n*-3 ratio of approximately 3:1, or CLAs or both combined, during the transition period. Calves received their dam's colostrum until slaughter at day 5 of life. Muscle and adipose tissue fatty acid (FA) composition and tissue morphology were analyzed. Markers of muscle metabolism and adipogenesis were detected on a protein and mRNA expression level. The data were analyzed using the MIXED model of SAS® statistical software with EFA and CLA as fixed effects.

Maternal supplementation increased the *n*-3 polyunsaturated FAs and CLAs in the calf tissues. This confirms that changes in maternal EFA and CLA supply during the perinatal period alter the FA composition of the neonatal calf skeletal muscle and adipose tissues. Altered tissue FAs can influence the cellular development. However, the maternal supplementation did not alter the muscle fiber composition of longissimus (MLD) and semitendinosus muscle (MST). In MLD though, CLA supplementation decreased the protein abundance of MYH7 and increased the protein abundance of MYH1. This indicates that the CLAs induced a muscle fiber transition from slow to fast muscle fiber type. While the supplementation did not alter the total adipocyte area in muscle, representing the intramuscular fat (IMF), EFA supplementation decreased the abundance of cells expressing preadipocyte marker DLK-1 in MST and the mRNA abundance of *DLK1* in MLD. This is a hint that the EFAs promoted adipogenesis in the IMF. Similarly, the adipocyte size in intermuscular (INF), subcutaneous (SCF) and perirenal fat (PF) was not affected by supplementation. In SCF, the FA supplementation appeared to increase the percentage of larger cells. In INF, though, the relative abundance of smaller cells appeared to be higher in the EFA groups than in the other groups. This might indicate that the EFA supplement increased preadipocyte recruitment and differentiation in the IMF. At last, no negative effects of variations in maternal *n*-3 EFA and CLA feeding on calf muscle and adipose tissue development or health were observed. Moreover, the organism of the calves might be able to outbalance the induced changes in FA supply.

Zusammenfassung

Die Fütterung von Hochleistungsmilchkühen versorgt die Kühe mit weniger essenziellen Fettsäuren (EFAs) als eine weidebasierte Fütterung, was auch die Bildung von konjugierten Linolsäuren (CLAs) im Tiergewebe reduziert. Insbesondere enthält das häufig maissilagebetonte Futter ein höheres Verhältnis von *n*-6 zu *n*-3 EFAs. Die Folgen für die Tiere sind aktuell nicht vollständig erforscht, insbesondere nicht die Auswirkungen auf die Entwicklung der Nachkommen. In dieser Dissertation soll untersucht werden, wie eine maternale Supplementation mit EFAs und CLAs die Entwicklung von Skelettmuskulatur und Fettgeweben neugeborener Kälber beeinflusst. Während der Transitphase wurden 40 Holstein-Kühe abomasal mit Kokosöl (Kontrollgruppe), EFAs (*n*-6/*n*-3-Verhältnis von etwa 3:1), oder CLAs, oder einer Kombination aus beiden supplementiert. Die Kälber wurden mit dem Kolostrum ihrer Mutterkuh gefüttert und am fünften Lebenstag geschlachtet. Muskeln und Fett wurden auf die Fettsäurezusammensetzung und Gewebemorphologie hin untersucht und die Expression von Muskelmetabolismus- und Adipogenesemarkern gemessen. Die Daten wurden mit der MIXED-Prozedur der Statistiksoftware SAS® untersucht. Dabei wurden EFA und CLA als fixe Effekte eingesetzt.

Die maternale Supplementation erhöhte den Gewebegehalt an mehrfach ungesättigten *n*-3 Fettsäuren und CLAs. Dies bestätigt, dass die Fettsäurezusammensetzung im Muskel- und Fettgewebe des Kalbs durch eine perinatale Supplementation der Kuh beeinflusst werden kann. Die Faserstruktur im *musculus longissimus dorsi* (MLD) und im *musculus semitendinosus* (MST) war nicht beeinflusst, aber im MLD war durch CLAs die Proteinexpression von MYH7 verringert und die von MYH1 erhöht. Dies ist ein Hinweis darauf, dass die CLAs einen Übergang vom langsamen zum schnellen Muskelfasertyp hin induzierten. Auch wenn sich die Adipozytenfläche in den Muskeln beziehungsweise im intramuskulären Fett (IMF) nicht zwischen den Gruppen unterschied, reduzierte die EFA-Supplementation die Häufigkeit DLK-1-exprimierender Adipocytenvorläufer im MST und die mRNA-Expression von *DLK1* im MLD. Dieses Ergebnis impliziert einen proadipogenen Effekt der EFAs im IMF. Ähnlich dazu änderte die Supplementation nicht die durchschnittliche Fettzellgröße in intermuskulärem (INF), subkutanem (SCF) und perirenalem Fett (PF). Sie schien aber den Anteil größerer Fettzellen im SCF zu erhöhen. Im INF hingegen schien die Supplementation mit EFAs den Anteil kleinerer Fettzellen zu erhöhen. Dies könnte ein Hinweis darauf sein, dass die EFAs die Rekrutierung und Differenzierung von Präadipozyten im IMF verstärkten. Letztendlich zeigten sich bei den Kälbern durch den veränderten EFA- und CLA-Gehalt in der maternalen Fütterung keine Negativeffekte auf Muskel- und Fettgewebeentwicklung oder Gesundheit. Möglicherweise ist der Kälberorganismus fähig, solch eine Verschiebung der Fettsäureversorgung zu kompensieren.

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List of abbreviations, acronyms and units

Abbreviation, acronym, unit	Meaning
%	percent
±	plus–minus
°C	degrees Celsius (274.15 Kelvin)
α	alpha (greek)
β	beta (greek)
γ	gamma (greek)
Δ, δ	delta (greek)
µ	micro (10^{-6}) (unit prefix)
a.u.	arbituary unit
aa	amino acid (protein residue)
acc. no.	accession number in NCBI
ADA	adrenic acid
ALA	α-linolenic acid
Aqua dest.	distilled water
ARA	arachidonic acid
ATPase	myofibrillar adenosine 5'-triphosphatase
bp	base pairs
c	centi (10^{-2}) (unit prefix)
cDNA	complementary DNA
CLA	conjugated linoleic acid
CON	control
Cq	quantification cycle
d	day(s) (86,400 sec)
Da	Dalton (1.66×10^{-27} kg) (atomic mass unit)
DHA	docosahexaenoic acid
DHGLA	dihomo-γ-linolenic acid
DNA	deoxyribonucleic acid
DPA	*n*-3 docosapentaenoic acid
dpc	days *post* conception
E	efficiency of amplification
e.g.	for example (latin, '*exempli gratia*')
EDTA	ethylenediaminetetraacetate
EFA	essential fatty acid
EPA	eicosapentaenoic acid
et al.	and others (latin *'et alia"*)
EtOH	ethanol
FA	fatty acid
FAME	fatty acid methyl ester
g	gram (10^{-3} kg)
× g	gravity-force (relative centrifugal force)
g/d	gram/day
GC	gas chromatography
GLA	γ-linolenic acid

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Abbreviation, acronym, unit	Meaning
h	hour(s) (3600 sec)
H/E	Hematoxylin/Eosin (staining)
HA	heneicosanoic acid
HRP	horseradish peroxidase
IHC	immunohistochemistry
IMCL	intramyocellular lipid droplet
IMF	intramuscular fat
INF	intermuscular fat
IR	infrared
Jess SW	Jess Simple Western
k	Kilo (10^3) (unit prefix)
L	liter(s) (10^{-3} m^3)
LA	linoleic acid
LO	linseed oil
LSM	Least Square Means
m	milli (10^{-3}) (unit prefix)
m	meter
M	molar (mol/L)
min	minute(s) (60 sec)
MLD	*musculus longissimus dorsi*
mm^2	square millimeter (10^{-6} m^2)
mRNA	messenger RNA
MST	*musculus semitendinosus*
MUFA	monounsaturated fatty acid
n	nano (10^{-9}) (unit prefix)
NCBI	National Center for Biotechnology Information
NIR	near infrared
NRQ	normalized relative quantities
OA	oleic acid
PA	palmitic acid
PBS	phosphate buffered saline
PBST	phosphate buffered saline-Triton
PCR	polymerase chain reaction
PF	perirenal fat
PN	protein normalisation
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
qPCR	quantitative real-time PCR
R^2	coefficient of correlation
RNA	ribonucleic acid
rpm	revolutions per minute
RQI	RNA quality indicator
RT	room temperature
SA	stearic acid
SBB	Sudan Black B
SC	satellite cell

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Abbreviation, acronym, unit	Meaning
SCD	stearoyl-CoA desaturase
SCF	subcutaneous fat
SDS-PAGE	sodium dodecyl sulfate-polyamide gel electrophoresis
sec	second(s)
SE_{LSM}	Standard Error of LSM
SFA	saturated fatty acid
SO	safflower oil
SPL	Smart Protein Layers
TAE	Tris-acetate-EDTA
UV	ultraviolet
v/v	volume per volume
VA	vaccenic acid
WB	western blot

1 Introduction

Essential fatty acids (EFAs), namely linoleic acid (C18:2 *n*-6, LA) and α-linolenic acid (C18:3 *n*-3, ALA), fulfill important physiologic functions in the mammalian organism (Gurr *et al.*, 2002). In the muscle, they stimulate insulin mediated protein metabolism (Gingras *et al.*, 2007) and have beneficial effects on rodent myoblast development *in vitro* (Lee *et al.*, 2009b; Allen *et al.*, 1985). Conjugated linoleic acids (CLAs) are products of EFA metabolisation (Yurawecz *et al.*, 1999). Off the over 40 known isoforms, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA are characterized best (Yurawecz *et al.*, 1999). They show anti-carcinogenic and anti-arteriosclerotic properties (Yurawecz *et al.*, 1999; Basak and Duttaroy, 2020). Moreover, CLAs, especially the *trans*-10, *cis*-12 isomer, modulate body fat of rodents, pigs and humans (Yamasaki *et al.*, 1999; Clément *et al.*, 2002; Meadus *et al.*, 2002) and increase muscle endurance of rodents (Kim *et al.*, 2012). An insufficient supply of a dam with these fatty acids (FAs) might have consequences for muscle and adipose tissues in the dam and muscle and adipose tissue development in the offspring.

The FAs exert their effects on myogenesis and adipogenesis for example as membrane components (Briolay *et al.*, 2013) or as precursors for longer-chain polyunsaturated FAs (PUFAs) and prostaglandins (Gurr *et al.*, 2002). In addition, CLAs and LA regulate the expression of muscle metabolic marker peroxisome proliferator activated receptor gamma coactivator 1 alpha (*Ppargc1a*) (Kim and Park, 2015), and *n*-6 and *n*-3 PUFAs are ligands for the adipogenesis related nuclear receptors of the PPAR family (Kliewer *et al.*, 1995). On that basis, there is evidence that EFAs and CLAs influence skeletal muscle and adipose tissue development.

Skeletal muscle tissue development occurs mainly during gestation, the number of muscle fibers is fixed after the second trimester and the muscle fibers continue growing by hypertrophy (Bonnet *et al.*, 2010). Adipose tissue development starts during midgestation and continues until after birth, depending on the depot (Bonnet *et al.*, 2010). To regulate these mechanisms prenatally holds the chance to improve offspring growth performance and animal products, and is of interest for livestock industry (Du *et al.*, 2017). The offspring muscle and adipose tissue development depends on placental and milk transfer of nutrients from the maternal nutrition (Raja *et al.*, 2016; MacGhee *et al.*, 2017; Zago *et al.*, 2019). This transfer is regulated in the maternal organism by ruminal biohydrogenation of dietary PUFAs, the transfer restriction in the placenta and, to a lesser extent, in the mammary gland (Noble *et al.*, 1978; Chilliard *et al.*, 2007; Moallem and Zachut, 2012). In addition, other organs of the offspring, such as brain and heart, have higher priority in nutrient supply than skeletal muscle and adipose tissues (Funston *et al.*, 2010; Du *et al.*, 2015).

Therefore, skeletal muscle and adipose tissue development of neonatal calves bred from cows receiving abomasal EFAs and CLAs from late gestation on was investigated. The FA composition of the calves' skeletal muscle and adipose tissues was analyzed to assess the transfer of maternally supplemented FAs. To observe effects of maternal FA on skeletal muscle growth and metabolism, the morphology and composition of muscle fibers were assessed histologically and immunohistochemically. For hints on changes on a molecular plane, muscle metabolic markers were measured on the protein and mRNA level. The impact of maternal supplementation on calf adipose tissue morphology was assessed via measuring the adipocyte area. To assess the ongoing adipogenic process, markers of different stages of adipogenesis were analyzed on the protein and mRNA level.

2 Literature overview

2.1 Dairy cattle feeding

In Germany, high yielding dairy cattle are the most important foundation for dairy production systems and, furthermore, are an important meat source (Deblitz *et al.*, 2008). The animals are commonly fed a total mixed ration consisting of grass and corn silage to ensure high feed efficiency and milk yield (Chilliard *et al.*, 2001; Ponnampalam *et al.*, 2006; Deblitz *et al.*, 2008; Kliem *et al.*, 2008). The proportion of grass respectively corn silage in the feed depends on the availability of the respective feedstuff in the farming area (Deblitz *et al.*, 2008). Lipids of corn silage are rich in linoleic acid (LA), as the main constituent corn grain consists of approximately 60 % LA (of total fatty acids) (Doreau and Chilliard, 1997, cited by Chilliard *et al.*, 2001). While corn silage is richer in LA, grass silage contains more α-linolenic acid (ALA) (Chilliard *et al.*, 2001). Fresh grass in temperate climate zones contains approximately 1–3 % total lipids, 60 % of these are ALA (Kuzdzal-Savoie, 1965; Bauchart *et al.*, 1984; both cited by Chilliard *et al.*, 2001). During ensiling, the content of fatty acids (FAs) in general and ALA in particular are reduced (Doreau and Poncet, 2000; cited by Chilliard *et al.*, 2001), to which extent depends on the quality of the silage (Lough and Anderson, 1973; Dewhurst and King, 1998; both cited by Chilliard *et al.*, 2001). Nevertheless, a concentrate-based diet supplies the animal with less ALA than fresh grass does (Chilliard *et al.*, 2001; Ferlay *et al.*, 2006; Daley *et al.*, 2010). Furthermore, approximately 80 % of dietary polyunsaturated FAs (PUFAs) that reach the rumen are hydrogenated to saturated FAs (SFAs) (Jenkins and Bridges, 2007; Kirchgeßner *et al.*, 2014). Conjugated linoleic acids (CLAs) are the product of incomplete bacterial hydrogenation of essential FAs (EFAs) (Kelly *et al.*, 1998b; Bauman *et al.*, 1999); and products of EFA metabolite vaccenic acid (VA, *trans*-11 C18:1) metabolisation in the mammary gland (Bauman *et al.*, 1999). Upon reduction of dietary ALA, less ALA is taken up in the intestine and is available for the animal. In addition, biosynthesis of CLAs is reduced, further decreasing tissue and milk amounts of CLAs (Kelly *et al.*, 1998b; Bauman *et al.*, 1999).

Linoleic acid and ALA are important for many biological processes such as growth, reproduction and brain development (Innis, 1992; Gurr *et al.*, 2002). For CLAs, among others, anti-carcinogenic effects have been described (Pariza *et al.*, 1999). Although the exact EFA needs for high yielding cows are not known yet, it is possible that feeding high amounts of concentrates and low amounts of grass provides the animal with inadequate amounts of ALA (Palmquist, 2010). This might be true especially during the transition period, from late pregnancy to early lactation, when the energy requirements of the cow increase by approximately 40 % (Bell, 1995). To investigate these consequences, an experiment was conducted at the FBN Dummerstorf, where cows in transition period were fed a total mixed

ration with a low fat content and an elevated *n*-6/*n*-3 EFA ratio. The cows were abomasaly supplemented with EFAs (*n*-6/*n*-3 ratio of approximately $^1/_3$), CLAs or a combination of both; simulating pasture-like EFA and CLA supply compared to concentrate-based feeding (Gnott *et al.*, 2020; Vogel *et al.*, 2020, 2021). The effects on cow performance and body condition, milk performance and FA composition in milk and blood, and the cows' immune and metabolic status were studied (Gnott *et al.*, 2020; Vogel *et al.*, 2020, 2021). The maternal nutrition with FAs not only affects PUFA content of tissue and milk, but also the development and health of the neonate (Danicke *et al.*, 2012; Jacobi and Odle, 2012; Du *et al.*, 2017). Uken *et al.* investigated the calves' plasma FA status and the development of the calves' endocrine and metabolic system (Uken *et al.*, 2021a, 2021b), whereas this dissertation focused on the FA composition, development and metabolism of skeletal muscle and adipose tissues of these calves. First results were published by Dahl *et al.* (2020).

2.2 Essential fatty acids

The importance of adequate LA and ALA intake is widely acknowledged (Gurr *et al.*, 2002; Palmquist, 2010). Most animals, including mammals, cannot synthesize EFAs endogenously, but must consume LA and ALA with the diet (Gurr *et al.*, 2002). As described in detail by Gurr *et al.* (2002), LA can be found in vegetable products made from corn, safflower, sunflower, and rapeseed, while ALA occurs mostly in chloroplasts of green vegetables and grass, or in linseed. Alpha-linolenic acid and LA are precursors for longer-chain *n*-3 respectively *n*-6 PUFAs (Figure 2.1) (Holman, 1986; Abayasekara and Wathes, 1999; Gurr *et al.*, 2002). While the metabolisation of LA and ALA to longer chain PUFAs is catalyzed by the same enzymes, the products have distinct metabolic functions (Sprecher, 2002; Simopoulos, 2008). For instance, as described in detail by Gurr *et al.* (2002) and Simopoulos (2008), 1-series prostaglandins and 2-series prostaglandins are derived from LA metabolites dihomo-γ-linolenic acid (DHGLA, C20:3 *n*-6) and arachidonic acid (ARA, C20:4 *n*-6), respectively. These prostaglandins act pro-inflammatory, whereas the 3-series prostaglandins, that are derived from ALA metabolite eicosapentaenoic acid (EPA, C20:5 *n*-3), act anti-inflammatory (Gurr *et al.*, 2002; Simopoulos, 2008). Apart from being prostaglandin precursors, *n*-3 and *n*-6 PUFAs are incorporated into cell membranes (Andersson *et al.*, 2002; Agatha *et al.*, 2004). The membrane phospholipid fraction not only works as a storage for PUFAs (Abbott *et al.*, 2010; Wathes *et al.*, 2007), membrane PUFAs also affect membrane structure and overall cell integrity (Stubbs and Smith, 1984; Rajamoorthi *et al.*, 2005; Briolay *et al.*, 2013). The membrane abundance of PUFAs is related to the dietary supply (Healy *et al.*, 2000), while at the same time there is also competition between the two PUFA families for incorporation into tissues (Gibson *et al.*, 2011; Philipsen *et al.*, 2018). For example, Philipsen *et al.* (2018) observed higher incorporation of

ALA than of LA into cell membranes *in vitro*. *N*-3 and *n*-6 PUFAs also serve as signaling molecules, e.g. EFAs, longer-chain PUFAs and prostaglandin metabolites bind to nuclear receptors (Kliewer *et al.*, 1995). While the two families of FAs and prostaglandins partly bind the same receptors, their activation capacity differs (Kliewer *et al.*, 1995; Wathes *et al.*, 2007). Due to the overlap in reactivity between *n*-3 and *n*-6 PUFAs, the systemic ratio between the FAs is considered important not only for cattle, but also for other species (Barceló-Coblijn and Murphy, 2009; Tocher *et al.*, 2019; Ponnampalam *et al.*, 2021). In addition to the direct effects, for ruminants the consumption of *n*-3 and *n*-6 PUFAs also determines CLA quantities in tissues and milk (Griinari and Bauman, 1999).

Figure 2.1: *Schematic pathway of* n-*6 and* n-*3 polyunsaturated fatty acid biosynthesis. The involved enzymes are shown in the center. Adapted from Pereira* et al.*, 2012.*

2.3 Conjugated linoleic acids

Conjugated linoleic acids exist in more than forty different isoforms with different double bond configurations, of which *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (Figure 2.2) are the most investigated and best described (Griinari and Bauman, 1999). *Cis*-9, *trans*-11 CLA is the major isoform in ruminant products (Griinari and Bauman, 1999), making up for more than 80 % of milk CLAs, and approximately 75 % of meat CLAs (Chin *et al.*, 1992). Conjugated linoleic acids are by-products of EFA biohydrogenation which escaped from the rumen (Kepler *et al.*, 1966; Griinari and Bauman, 1999). Conjugated linoleic acids are also synthesized in other tissues, mostly in the mammary gland (Bauman *et al.*, 1999; Corl *et al.*, 2001). There, Δ-9 desaturase metabolizes the rumenic EFA metabolite VA to CLAs (Bauman *et al.*, 1999; Corl *et al.*, 2001). The concentration of CLAs in bovine tissues and milk depends on animal and feed (Bauman

et al., 1999). Because of the high ALA content, fresh grass feed increases tissue CLA biosynthesis compared to concentrate-based feed (Precht and Molkentin, 1997; Kelly *et al.*, 1998b). Also, changing the grass silage to corn silage ratio in feed alters the milk abundance of different CLA isomers, which derive from either LA or ALA (Collomb *et al.*, 2004; Roy *et al.*, 2006).

Conjugated linoleic acids have different effects on the metabolism, depending on CLA isomer and animal species (Pariza *et al.*, 2001). They have anti-carcinogenic (Ip *et al.*, 2003) and anti-adipogenic properties (Pariza *et al.*, 1999). Supplementation with *trans*-10, *cis*-12 CLA reduced body fat in mice (Park *et al.*, 1997; DeLany *et al.*, 1999) and in pigs, and increased lean body mass in pigs (Dugan *et al.*, 1997; Ostrowska *et al.*, 1999). In lactating dairy cows, *trans*-10, *cis*-12 CLA reduces milk fat biosynthesis by up to 50 % (Baumgard *et al.*, 2000). Hence, a reduced dietary *n*-6/*n*-3 PUFA ratio also has direct effects on milk fat biosynthesis due to increased formation of *trans*-10, *cis*-12 CLA.

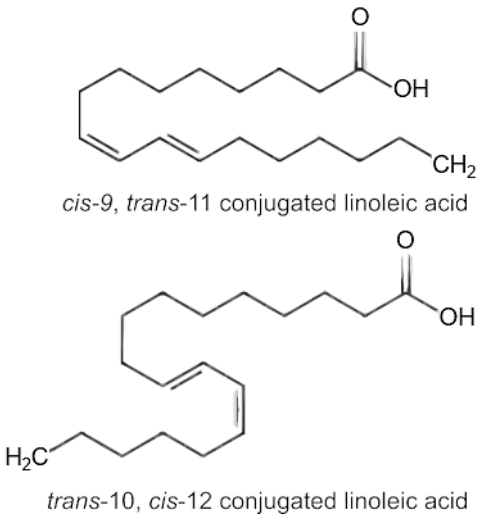

Figure 2.2: *Chemical structure of the two conjugated linoleic acid (CLA) isomers* cis-*9,* trans-*11 CLA and* trans-*10,* cis-*12 CLA.*

2.4 Maternal transfer of polyunsaturated fatty acids

The nutrition with FAs not only affects PUFA content of tissues and milk, but during gestation and lactation also the development of fetus and calf (Bell and Ehrhardt, 2002; Du *et al.*, 2017). Nutrients are transferred from dam to fetus via placenta and, after birth, via milk (Bauman, 2000; Bell *et al.*, 2000). The amount of available FAs is reduced both by maternal ruminal hydrogenation (Bickerstaffe *et al.*, 1972; Bauchart *et al.*, 1990) and by regulation of placental and milk transfer (Bell and Ehrhardt, 2002). Placenta and milk transfer are modulated

differently, meeting the nutrient requirements and metabolizing capacities of fetus and neonate (Noble *et al.*, 1982; Bell and Ehrhardt, 2002).

Upon placental transfer, the fetal FA status does not directly reflect the maternal plasma FA status (Noble *et al.*, 1978, 1982). Lower amounts of EFAs, but higher amounts of longer-chain PUFAs were detected in fetal tissues (Noble *et al.*, 1982; Bell and Ehrhardt, 2002). In sheep, little of the maternally supplemented LA was detected in the fetal plasma (Noble *et al.*, 1978). In contrast, the longer-chain PUFA ARA made up a higher percentage in the fetal plasma than in the maternal plasma (Noble *et al.*, 1978). The fetus appears to be more dependent on longer-chain *n*-6 and *n*-3 PUFAs than on EFAs (Bell and Ehrhardt, 2002), and at the same time cannot effectively desaturate EFAs to longer-chain PUFAs (Bell and Ehrhardt, 2002). Therefore, the longer-chain PUFAs in maternal plasma might be preferentially transferred in comparison to the EFA precursors (Noble *et al.*, 1978, 1982). In addition, plasma EFAs might be desaturated in the placenta and the resulting longer-chain PUFAs are transferred to the fetus (Noble *et al.*, 1978, 1982). The details of placental regulation of nutrient transfer are still under investigation (Bell and Greenwood, 2016).

In comparison to placental transfer, the milk FA profile is strongly affected by supplemented EFAs (Chilliard *et al.*, 2000; Moate *et al.*, 2008; Moallem, 2018). Moate *et al.* (2008) estimated a transfer efficiency of up to 50 % of dietary long-chain FAs into milk fat. Results of Kuzdzal-Savoie (1965) suggest that 1.2 % of grass C18:3 FAs are transferred to milk. Pasture feeding (Precht and Molkentin, 1997; Kelly *et al.*, 1998b; Lahlou *et al.*, 2014) and supplementation with sunflower and linseed oil (Kelly *et al.*, 1998a) increased CLAs in milk fat. Supplementation with rumen protected CLAs increased milk fat concentration of all supplemented isomers (Chouinard *et al.*, 1999; Giesy *et al.*, 2002). When Garcia *et al.* (2016) supplemented pregnant dams with LA, the colostrum contained higher proportions of LA, total *n*-6 PUFAs and total CLAs. The calves' plasma FA composition was unaffected by placental transfer of maternal supplements, though (Garcia *et al.*, 2016). Calves of pregestationally CLA supplemented dams had increased erythrocyte lipid *cis*-9, *trans*-11 CLA content, while maternal erythrocyte lipids were unaltered (Danicke *et al.*, 2012). In calves supplemented with LA before and after birth, plasma LA was elevated (Garcia *et al.*, 2014b). Briefly, maternal nutrition influences calf plasma FA profile, but the extent of placental and milk transfer is not fully described yet. Nevertheless, maternal nutrition affects offspring muscle and fat tissue development (Du *et al.*, 2010a, 2017).

2.5 Development of skeletal muscle tissues

The development of skeletal muscle tissue is described in detail by Picard *et al.* (1994), Bonnet *et al.* (2010) and Chal and Pourquié (2017). During ruminal gestation, fetal skeletal muscle

tissue develops in roughly three stages (Figure 2.3). In the first trimester, myogenic progenitor cells, derived from the peraxial mesoderm, develop into embryonal myoblasts. In several steps, including the elongation and fusion of the embryonal myoblasts, polynuclear primary muscle fibers evolve at between 30 days post conception (dpc) until about 180 dpc. Around the end of the first trimester, more myogenic precursors fuse together or with the primary fibers. They form secondary fibers that surround the primary fibers (Picard *et al.*, 1994; Bonnet *et al.*, 2010; Chal and Pourquié, 2017). After this second stage, the number of muscle fibers is set and muscle fiber growth continues by hypertrophy (Russell and Oteruelo, 1981; Fahey *et al.*, 2005; Du *et al.*, 2017). However, a pool of stem cells, termed satellite cells (SCs), remains located around the muscle fibers (Mauro, 1961; Kuang *et al.*, 2007). For muscle repair, these cells can fuse with the muscle fibers, leading to proliferative muscle growth (Mauro, 1961; Kuang *et al.*, 2007).

Mature muscle fibers within and between muscles differ in their metabolic and contractile behavior, which manifests for example in the three adult muscle fiber types (Picard *et al.*, 2002; Schiaffino and Reggiani, 2011). These fiber types are termed the slow-twitch oxidative (“slow”), the fast-twitch oxido-glycolytic (“intermediate”) and the fast-twitch glycolytic (“fast”) type (Picard *et al.*, 2002; Schiaffino and Reggiani, 2011). The three fibre types are discussed extensively in literature (e.g. Peter *et al.*, 1972; Picard *et al.*, 2002; Schiaffino and Reggiani, 2011). Slow fibers show the slowest contraction speed, and the highest endurance capacity of the three types. They depend largely on oxidative metabolism, and contain more mitochondria than the other two types. Fast-type fibers contract faster than the other two types, but have the least endurance capacity. They depend on glycolysis and contain few mitochondria. The intermediate fibers are fast contracting fibers with intermediate endurance. They depend both on oxidative and glycolytic metabolism, and are rich in mitochondria (Peter *et al.*, 1972; Picard *et al.*, 2002; Schiaffino and Reggiani, 2011). Within a muscle, the fiber type distribution is connected to the muscle function (Peter *et al.*, 1972; Picard *et al.*, 2002; Schiaffino and Reggiani, 2011); e.g. more fast fibers in the leg muscles allow fast running to escape predators, while slow fibers in the back muscles facilitate a continuously upright posture (Schiaffino and Reggiani, 2011). While primary muscle fibers develop mostly into adult slow-twitch oxidative fibers, secondary fibers differentiate into all adult fiber types (Albrecht *et al.*, 2013; Chal and Pourquié, 2017).

Additionally, muscle fiber type conversions have been observed from the second trimester of gestation on until after birth (Pette and Staron, 1997; Wegner *et al.*, 2000; Albrecht *et al.*, 2013). These conversions were associated with moto neuron stimulation (Hämäläinen and Pette, 1996), animal development (Kugelberg, 1976; Rubinstein and Kelly, 1981; Wegner *et al.*, 2000) or hormonal regulation (Bahi *et al.*, 2005). The conversion between fiber types allows adaptation to environmental changes, e.g. changes in nutrition (Peter *et al.*, 1972; Schiaffino

and Reggiani, 2011). Briefly, at the developmental stage of this study's calves, the number of muscle fibers is fixed, but the fibers are still in the process of fiber type formation, and directly after birth fiber type switching is increased.

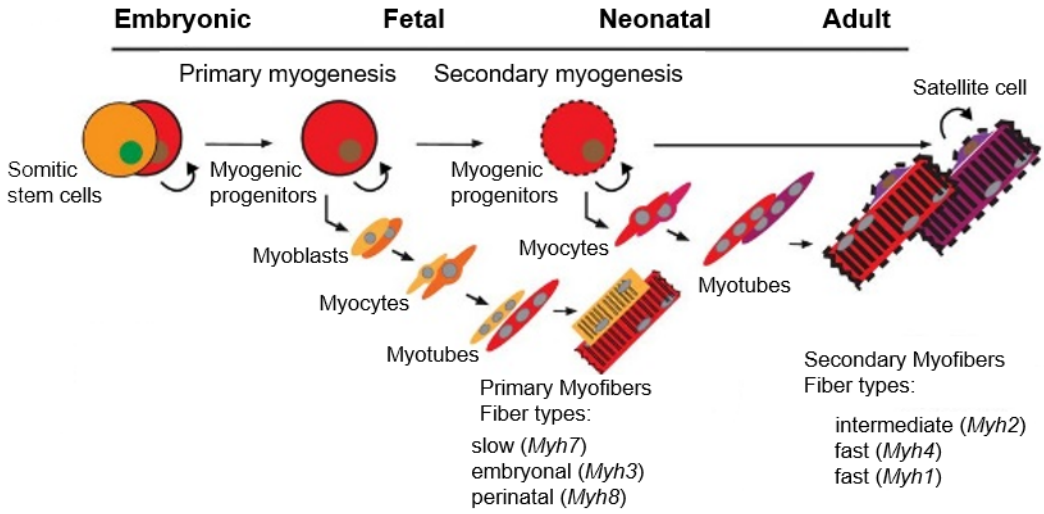

Figure 2.3: *Formation of skeletal muscle fibers during animal development. Primary myogenesis starts in the embryonal state. Myogenic progenitors derive from the mesodermal stem cells (somitic stem cells). The differentiation und fusion of myogenic progenitors leads to formation of primary myofibres of slow, embryonal and perinatal fiber type and the corresponding myosin heavy chain isoforms (*Myh*). Secondary myogenesis starts in the fetal state. More myogenic progenitors differentiate and fuse together or with primary myofibers to form secondary fibers. These develop into slow, but also fast and intermediate fiber types. Remaining progenitors form a stem cell pool of satellite cells, which are able to fuse with the muscle fibers for regenerative purposes. Adapted with permission from Chal and Pourquié, 2017.*

2.6 Development of adipose tissues

Adipose tissue is formed from midgestation on until about 250 d after birth (Robelin, 1986; Gnanalingham *et al.*, 2005; Du *et al.*, 2017). The depots appear in a distinct order: adipocytes were first observed in visceral fat, such as the perirenal fat (PF), at 80 dpc, then subcutaneous (SCF), intermuscular (INF) and intramuscular fat (IMF) (Bonnet *et al.*, 2010; Du *et al.*, 2017). The cellular and molecular events of adipogenesis are described extensively in the literature (Gregoire *et al.*, 1998; Bonnet *et al.*, 2010), but the exact origin of the stem cells and the developmental mechanisms *in vivo* are not well understood yet. Also, they seem to differ between fat depots (Billon *et al.*, 2008; Tang *et al.*, 2008; Bonnet *et al.*, 2010; Rosen and Spiegelman, 2014).

Multipotent mesenchymal stem cells with a fibroblast-like shape are acknowledged as adipocyte precursors (Bosnakovski *et al.*, 2005; Rosen and MacDougald, 2006; Uezumi *et al.*, 2011). These uncommitted adipogenic precursors are induced by specific signals to commit to

the adipogenic lineage (Boone *et al.*, 2000; Urs *et al.*, 2004; Rosen and MacDougald, 2006). The adipogenic precursors start incorporating lipids (Gregoire *et al.*, 1998) and take on a circular shape (Gregoire, 2001). Subsequently, intracellular lipid droplets appear (Gregoire *et al.*, 1998). During that time, the transcription factors CCAAT/enhancer binding protein beta (C/EBPβ) and delta (C/EBPδ) are expressed (Gregoire *et al.*, 1998; Salma *et al.*, 2006). For the final differentiation, C/EBPβ and C/EBPδ induce expression of the transcription factors CCAAT/enhancer binding protein alpha (C/EBPα) and peroxisome proliferator activated receptor gamma (PPARγ) (Salma *et al.*, 2006; Fernyhough *et al.*, 2007; Du *et al.*, 2010b). These two proteins regulate each other in a positive feedback loop to coordinate terminal differentiation of adipocytes (Bosnakovski *et al.*, 2005; Bonnet *et al.*, 2010). They initiate expression of adipogenic genes that are involved for instance in triglyceride uptake, lipogenesis and cell metabolism (Fernyhough *et al.*, 2007; Hausman *et al.*, 2009). Incorporation of lipids further increases the size of the mature adipocyte (Figure 2.4) (Hausman *et al.*, 2018).

Briefly, shortly after calf birth the adipose tissues are in different stages of development. Adipocytes in PF and SCF largely ceased proliferating, perirenal adipocytes grow mainly by hypertrophy, while subcutaneous and intermuscular adipocytes still show differentiation activity. The IMF however is in active formation by hyperplastic and hypertrophic growth.

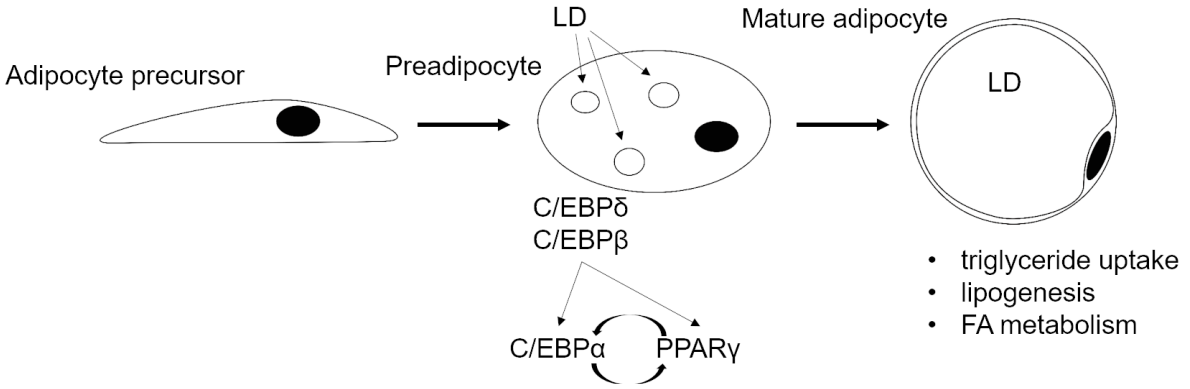

Figure 2.4: *Schematic representation of adipocyte development. CCAAT/enhancer binding protein beta (C/EBPβ) and delta (C/EBPδ) induce expression of CCAAT/enhancer binding protein alpha (C/EBPα), and peroxisome proliferator activated receptor gamma (PPARγ), C/EBPα and PPARγ regulate each other in a positive feedback loop. Fatty acid (FA), Lipid droplet (LD).*

2.7 Effects of maternal nutrition level on skeletal muscle and adipose tissue development

It is of vital interest for the meat producing industry to improve muscle growth, reduce overall fat mass and IMF of the animal, thus increasing meat yield and improving meat palatability

(Bonnet *et al.*, 2010; Du *et al.*, 2017; Hausman *et al.*, 2018). Maternal nutrition works to influence formation and structure of offspring tissues (Bonnet *et al.*, 2010; Underwood *et al.*, 2010; Du *et al.*, 2017). The influence of maternal energy uptake during gestation has been investigated in several studies (e.g. Zhu *et al.*, 2004, 2006; Tong *et al.*, 2008, 2009; Duarte *et al.*, 2014).

In sheep, maternal overnutrition in early gestation promoted adipogenic processes in fetal skeletal muscle (Tong *et al.*, 2008). Overnutrition in pregnant Nellore cows from early to late gestation did not affect myogenic marker and fetal muscle fiber number, but increased expression of fibrogenic and adipogenic markers (Duarte *et al.*, 2014). Maternal overnutrition in mid-term ewes reduced the primary muscle fiber size in fetal muscle (Tong *et al.*, 2009). When sheep were nutrient restricted from early to mid-gestation, the number of muscle fibers tended to be decreased in the offspring and muscle fiber metabolic properties were altered; in addition, perirenal and pelvic fat mass and fat accumulation were increased (Zhu *et al.*, 2006). In another study of nutrient restriction in early-gestating ewes, fetuses had reduced development of muscle and skeleton and less secondary muscle fibers (Zhu *et al.*, 2004).

In addition to the individual tissues, nutrient availability affects the balance between muscle cells and intramuscular adipocytes (Bonnet *et al.*, 2010; Du *et al.*, 2013, 2017). Both derive from the same mesenchymal stem cells, and interdifferentiation between the muscle and adipogenic lineage was induced successfully *in vitro* (Grimaldi *et al.*, 1997; Guillet-Deniau *et al.*, 2004). *In vivo*, nutrient deficiency in late gestation and postnatal reduced the number of intramuscular preadipocytes, and ultimately the formation of IMF (Du *et al.*, 2015). Other studies report mixed effects of maternal nutrition on marbling (Underwood *et al.*, 2010; Du *et al.*, 2015). Greenwood and Café (2007) did not find an effect of maternal nutrition on offspring marbling. Yan *et al.* (2010) observed higher marbling in fetal muscle upon overnutrition of ewes from before conception until late gestation.

Overall, not only the energy level, but also the nutrient composition and nutrient availability of the maternal diet affect offspring tissue development. In conclusion, the consequences of an altered maternal nutritional level depend on the developmental stage of the offspring at the period of nutritional intervention (Du *et al.*, 2017). In early gestation, maternal nutrient restriction leads to less myogenic activity and less hyperplastic growth, while in late gestation, maternal nutrient restriction is more likely to reduce hypertrophic growth of muscle fibers and adipogenic development.

2.8 Effects of essential fatty acids and conjugated linoleic acids on skeletal muscle and adipose tissues

As important nutrients, EFAs and CLAs might influence calf muscle and adipose tissue development. *In vitro*, LA promoted the proliferation of murine myoblasts (Lee *et al.*, 2009b) and increased muscle cell differentiation and nuclei density in rat SC cultures (Allen *et al.*, 1985). Lee *et al.* (2009a) reported that supplementation with *n*-6 PUFAs or *cis*-9, *trans*-11 CLA increased, *n*-3 PUFAs did not change and *trans*-10, *cis*-12 CLA repressed the proliferation of murine myoblasts. In another study, *trans*-10, *cis*-12 CLA inhibited, while *cis*-9, *trans*-11 CLA and LA stimulated the differentiation of rat myoblasts (Hurley *et al.*, 2006).

Leiber *et al.* (2011) reported no change in cow back fat thickness upon dietary supplementation with LA and/or ALA enriched concentrate during dry period and in early lactation. According to Jiang *et al.* (2010), LA and CLA increased the proliferation of porcine subcutaneous preadipocytes *ex vivo*. In mice, *trans*-10, *cis*-12 CLA reduced, while *cis*-9, *trans*-11 CLA did not affect body fat (Park *et al.*, 1999). The *trans*-10, *cis*-12 isomer also increased the expression of lipolytic enzymes in bovine primary adipocytes (Kadegowda *et al.*, 2013). In addition, *trans*-10, *cis*-12 CLA inhibited preadipocyte differentiation, suppressed intracellular triglyceride accumulation and lowered protein levels of adipogenesis regulators C/EBPα and PPARγ (Kang *et al.*, 2003).

Based on the reported effects of EFAs and CLAs on bovine tissues, it is possible that an altered EFA and CLA status of the calves during the perinatal period affects calf muscle and adipose tissue development.

3 Objective

The aim of this study was to determine the impact of maternal supplementation with EFAs and CLAs, during late gestation and postnatally, on the skeletal muscle and adipose tissue development of neonatal calves. Based on literature data, treatment with EFAs and CLAs has fundamental consequences on muscle and fat cells. These data were mostly acquired *in vitro* or by direct supplementation of animals. It needed to be clarified, if the prenatal supplementation via placenta and the postnatal supplementation via colostrum and milk induce similar effects in calves as the described ones. Alterations in calf plasma FA profile imply successful transfer of the maternal FAs (Uken *et al.*, 2021a), which in turn can regulate physiologic processes that will ultimately influence maturation of muscle and adipose tissues.

The main hypothesis was that ALA and/or CLA supplementation in late gestation and shortly after birth improves calf muscle development in an individual or synergistic manner, which will manifest in changes in muscle fiber morphology, muscle metabolism and gene expression of marker genes. In addition, we hypothesized that the variations in maternal FA transfer, induced by maternal FA supplementation, alter the formation of adipose tissues.

Ultimately, the results might add some evidence on how a high *n*-6/*n*-3 PUFA ratio and reduced CLA in the tissues due to maternal corn silage feeding affect neonatal calf development.

4 Materials and methods

4.1 Animals and sampling

The animal experiment was planned and executed by the Institute of Nutritional Physiology "Oskar Kellner" of the Research Institute for Farm Animal Biology (FBN, Dummerstorf, Germany). Animal care and tissue sampling were conducted in compliance with the guidelines of the German Law of Animal Protection. The Animal Care Committee of the State Mecklenburg-Western Pomerania, Germany (State Office for Agriculture, Food Safety and Fishery; LALLF M-V/TSD/7221.3-1-052/15), approved the experimental procedure.

The cow experiment has been described by Gnott *et al.* (2020) and Vogel *et al.* (2020). In total, 40 pregnant Holstein cows were bought from a North German farm (Agrarprodukte Dedelow GmbH, Prenzlau, Germany). The animal experiment was conducted in five time blocks with eight animals each, due to stable capacity. The time blocks were separated by three months. At purchase, all cows were in about the 18th week of their second lactation, with a similar expected milk yield (11,000 kg per 305 d). The cows were housed in a loose-housing freestall stable of the experimental animal facility for cattle at the FBN. The cows were implanted rumen cannulas at about nine weeks before experiment start, and abomasal infusion lines at about three weeks before experiment start, to enable supplementation into the abomasum. The cows were adapted to a standard diet for high yielding dairy cows from 16 weeks before the estimated parturition on. It consisted of a total mixed ration based on corn-silage (*n*-3 fatty acids (FAs): 1.4 g/kg dry matter, *n*-6 FAs: 9.5 g/kg dry matter, *n*-6/*n*-3 ratio: 11 $^{1}/_{9}$). Water was provided *ad libitum*. The supplementation with FAs started 63 days before the estimated parturition. The supplements were given via the fistula into the abomasum twice a day. The control group (CON) received coconut oil (90 % saturated FAs) at 38 gram per day (g/d) during dry period, and 76 g/d during lactation period. The EFA group received linseed oil (LO) and safflower oil (SO) at 41 g/d during dry period (LO: 39 g/d, SO: 2 g/d, *n*-6:*n*-3 polyunsaturated FA ratio: 1:3) and 82 g/d during lactation period (LO: 78 g/d, SO: 4 g/d). The CLA group received Lutalin® (27 % *cis*-9, *trans*-11 CLA, 27 % *trans*-10, *cis*-12 CLA) at 19 g/d during dry period and 38 g/d during lactation period. The EFA+CLA group was supplemented with the rations of both the EFA and the CLA group, consisting of LO, SO and Lutalin® at 60 g/d during dry period and 120 g/d during lactation period. Coconut oil was purchased from Kräuterhaus Sanct Bernhard KG (Bio-Kokosöl #665, Bad Ditzenbach, Germany), LO from Derby Spezialfutter GmbH (DERBY Leinöl #4026921003087, Münster, Germany), SO from Gefro Reformversand Frommlet KG (GEFRO Distelöl, Memmingen, Germany) and Lutalin® from BASF (Ludwigshafen, Germany).

After parturition, the calves were separated and all kept under identical conditions. They received colostrum (first 24 h after calving) and transition milk (day 2 to 5 after calving) from their respective dam. In the first 24 h after birth, the calves were fed in total 10 % of birth weight, 6 % on day 2, and 12 % from day 3 to day 5. Water was available *ad libitum*. The calves were slaughtered at day 5 of life. Details on this study's calves were published by Uken *et al.* (2021a, 2021b).

Samples of *musculus longissimus dorsi* (MLD) and *musculus semitendinosus* (MST) were taken from the left side of the animal for skeletal muscle tissue analysis. Samples of subcutaneous fat (SCF), intermuscular fat (INF) and perirenal fat (PF) were taken for adipose tissue analysis. Subcutaneous fat was prepared from the animal belly. Intermuscular fat was dissected from MLD and MST ("at" MLD/MST), if available. For six samples, the muscle origin of the INF was not recorded. From one calf, no INF could be taken. From five calves, INF was taken only from MLD, from one calf it was only taken from MST. All samples were taken within 30 min after slaughter, frozen and stored at -20 °C (for FA and muscle cross-sectional area analysis) or frozen in liquid nitrogen and stored at -70 °C (all others). Samples of in total 36 calves were collected, due to stillbirth losses and technical reasons. Of the calves, 3 females and 5 males belonged to the CON group, 5 females, 4 males to the EFA group, 7 females, 1 male to the CLA group and 7 females and 4 males to the EFA+CLA group. The sampling of the calves has been published recently (Dahl *et al.*, 2020). Animal experiment and sampling are pictured in Figure 4.1.

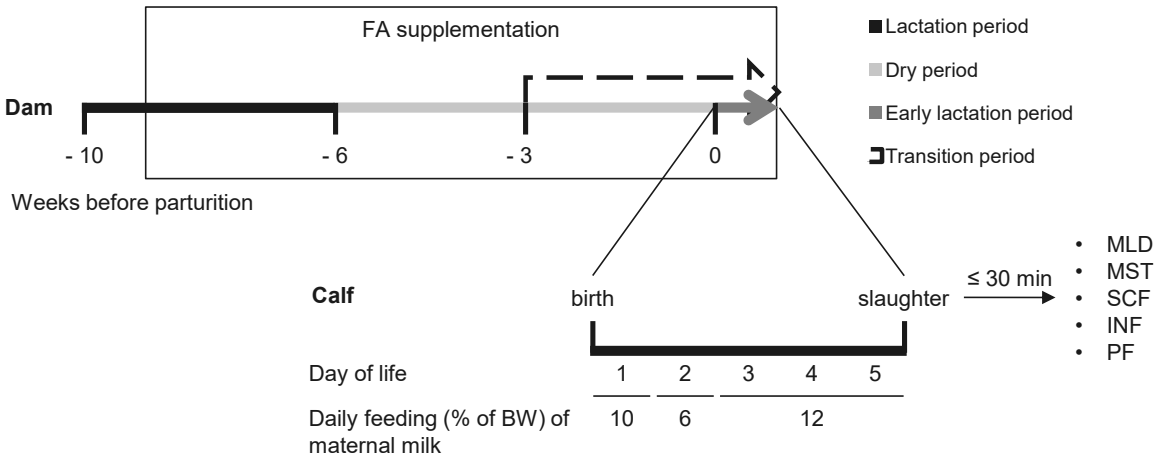

Figure 4.1: *Animal experiment and sampling. In total 40 pregnant Holstein cows, divided into 4 different groups, received FA supplements from about 9 weeks before the estimated parturition on. After parturition, calves were daily given their respective dam's colostral and transitional milk at the indicated percentage of body weight (% of BW). Calves were slaughtered at day 5 of life. Within 30 min after slaughter, samples of* musculus longissimus dorsi *(MLD),* musculus semitendinosus *(MST), subcutaneous fat (SCF), intermuscular fat (INF) from both MLD and MST and perirenal fat (PF) were taken.*

4.2 Tissue fatty acid analysis

The tissue FA composition of both muscles and the three adipose tissues was analyzed as published by Dahl *et al.* (2020). The lipids were extracted as described by Kalbe *et al.* (2019) and analyzed with gas chromatography (GC) as described by Dannenberger *et al.* (2017). Briefly, the frozen skeletal muscle and adipose tissue samples were homogenized with an Ultra Turrax T25 (IKA®-Werke GmbH, Staufen, Germany), together with nonadecanoic acid as internal standard. The total tissue lipids were extracted as duplicates with chloroform/methanol (2:1, v/v). The lipid extracts were stored at -18 °C until FA methyl ester (FAME) preparation. Fatty acid methyl esters were prepared in two steps from an aliquot of 25 mg total tissue lipid sample, by treatment with 0.5 M sodium methoxide and 14 % boron trifluoride in methanol. The FAMEs were extracted three times, in *n*-hexane, and stored at -18 °C until GC analysis. The FAs were analyzed via capillary GC in a CP-Sil 88 CB column (100 m × 0.25 mm; Agilent Technologies, Inc., Santa Clara, CA, USA) in a PerkinElmer CLARUS 680 gas chromatograph (PerkinElmer Instruments, Shelton, CT, USA). It works with a flame ionization detector and split injection. The temperature program ran as follows: 150 °C for 5 min, heating up by 2 °C/min, 200 °C for 10 min, heating up by 1 °C/min, 225 °C for 15 min. The reference standard mixture 'Sigma FAME', the methyl esters of C18:1 *cis*-11, C22:5 *n*-3, C18:2 *cis*-9, *trans*-11 (all Matreya LLC, State College, PA, USA), C22:4 *n*-6 (Sigma-Aldrich, Taufkirchen, Germany) and C18:4 *n*-3 (Larodan, Solna, Sweden) were used for calibration. The range of the five-point calibration for single FAs was 16 to 415 µg/ml. Calibration was checked after GC analysis of five samples. The data were processed using Empower™ 3 chromatography software (Waters GmbH, Eschborn, Germany).

4.3 Morphological description of tissues

4.3.1 Muscle cross-sectional area measurement

The area measurement of muscle cross-sections was described by Dahl *et al.* (2020). For the measurement, approximately 1 cm thick slices of both muscles were taken at slaughter. From MLD, the sample was removed at the twelfth rib; from MST, it was removed at the muscle belly. The slices were thawed before image acquisition. Images of both transverse sides, close to a length scale, were taken with a Nikon Coolpix 8700 camera (Nikon, Düsseldorf, Germany). Picture resolution was 3264 × 2448 pixels. The cross-sectional area was measured with CellˆD image analysis software (OSIS, Münster, Germany). Each image was calibrated to the internal scale and the muscle outline was recreated with the interpolating polygon function in the interactive measurement module. The mean area of both sides (mm^2) and the number of

muscle fibers per mm^2, as measured during muscle fiber type analysis (4.3.4), were used to calculate the apparent total muscle fiber number of MLD and MST.

4.3.2 Histological and histochemical stainings

All histological methods were adapted from standard procedures (Mulisch and Welsch, 2015) and described earlier (Dahl *et al.* 2020)**.** All incubations were performed at room temperature (RT), if not stated otherwise. All chemicals were pursued from Carl Roth GmbH (Karlsruhe, Germany), if not stated otherwise. For histological analysis, frozen tissue samples were cut into serial sections with a Leica CM3050 S cryostate microtome (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany). Muscle sections were cut 12 µm thick; adipose tissue sections were cut 30 µm thick. Until staining, the slides were kept at -70 °C for myofibrillar adenosine 5'-triphosphatase (ATPase) staining or long-term storage, and at -20 °C for short-term storage. The stainings were observed using an Olympus BX43 microscope equipped with a UC30 color camera (both Olympus Corporation, Hamburg, Germany).

4.3.3 Muscle capillarization

To analyze the muscular capillary network, muscle sections were first stained for alkaline phosphatase (Spannhof, 1967), then counterstained with Eosin (Mulisch and Welsch, 2015). With the 12.5× objective, three representative images were taken. Muscle capillary size and density from a total area of over 0.92 mm^2 were analyzed with a macro program in CellˆD image analysis software in a procedure adapted from Zitnan *et al.* (2019). From each image, the red channel was extracted and the contrast enhanced. By threshold method, the capillaries were detected and erroneously detected particles were deleted. Muscle fibers were counted at a higher magnification (40× objective) within one representative part of each region of interest. Capillary area percentage, distance between capillaries and muscle fiber-to-capillary ratio were determined.

4.3.4 Muscle fiber type composition

To analyze the distribution of muscle fiber contraction types, muscle sections were stained for ATPase with alkaline preincubation (Wegner *et al.*, 2000), following standard protocol (Szentkuti and Eggers, 1985). The procedure was published by Dahl *et al.* (2020). Muscle fiber types were analyzed in Hematoxylin/Eosin (H/E) (4.3.6) and in ATPase stained serial sections of MLD and MST, using the muscle fiber measurement module (Material Analytischer Service GmbH, Freiburg, Germany) as described by Albrecht *et al.* (2011). Per sample, at least 300 fibers on three randomly chosen regions were analyzed for muscle fiber size, nuclei and fiber types. With the 40× objective, an area on the H/E stained slide was chosen and an image was taken. The image was desaturated; the outlines of muscle fibers were detected automatically

and corrected manually. On the gray-scale image, the fiber nuclei were detected via threshold and corrected manually. In the ATPase stained serial section, an image of the corresponding region was taken. Referring to this image, the fiber type of each muscle fiber in the H/E image was determined automatically and corrected manually. For each fiber per analyzed region, fiber area and number of nuclei per muscle fiber were acquired from the H/E stained section, the fiber type from the ATPase stained section. Per fiber type, the absolute number and area, the mean fiber area and diameter, the number of nuclei and the percentage of total area were recorded.

4.3.5 Intramuscular fat

To detect intramuscular adipocyte lipid droplets, muscle sections were stained with Oil Red O and the intramuscular adipocyte lipid droplet area was measured as published by Dahl *et al.* (2020). A minimum of three serial sections per sample were examined, with a total area of at least 44 mm^2. To measure the total area of a section, an image was taken with the 1.25× objective and the outline was traced with the interpolating polygon function of the interactive measurement module of CellˆD. With the 20× objective, images of the individual lipid droplets were taken. A macro program allowed for the measurement of adipocyte lipid areas; the green channel was extracted, and the contrast enhanced. The threshold was set to detect the lipid area, and the area of lipids per image was measured. The sum of lipid areas per section was divided by the section area and the result was multiplied by 100 to determine the percentage of lipid area per slide.

4.3.6 Adipose tissue adipocyte size

To detect nuclei, cytoplasm and adipocytes, 12 µm muscle and fat tissue sections were stained with H/E, following standard procedure (Mulisch and Welsch, 2015). The measurement of adipocytes in adipose tissue sections was performed as described by Albrecht *et al.* (2011). In the H/E stained adipose tissue sections, the adipocyte cross-sectional area of at least 300 randomly selected fat cells in one section was measured. With the 40× objective, images of 10 randomly selected regions were taken, and the cells' outlines were traced with the interpolating polygon function of the interactive measurement module of CellˆD image analysis software (OSIS). The cross-sectional area was used as a measure for the size of individual fat cells. In addition, size histograms of the fat cells were created. The cells measured in each sample were distributed into 11 size categories of a 250-$µm^2$ range. This size range allowed observing group differences. Per calf, the relative abundance of adipocytes per size category was calculated from the percentage of adipocytes in a size category from the total adipocyte number. To describe the cell size distribution of each group, also the skewness factor was calculated. For the INF of the two different muscles, not in every sample 300 fat cells could be

observed. Therefore, measurements from INF of both MLD and MST and of unknown muscle were used when available. For the most part, the chronologically first 150 measurements per muscle were included. If less than 150 cells could be measured in INF from one muscle, more measurements from the other muscle's INF were used.

4.4 Detection of myogenic and adipogenic markers in muscle and adipose tissues

Immunohistochemical stainings followed standard procedures (Mulisch and Welsch, 2015). All reagents were purchased from Carl Roth GmbH, if not stated otherwise. For immunohistochemical analysis, frozen tissue samples were cut into serial sections with a Leica CM3050 S cryostat microtome (Leica Mikrosysteme Vertrieb GmbH). Until staining, tissue sections were placed on polylysine-L-covered tissue slides (Carl Roth GmbH) and stored at -70 °C for up to 7 d. All steps were performed at RT, all washing steps were done under constant movement. Nuclei were stained with 1 µg/ml Hoechst 33258 (B2883-100MG, Sigma-Aldrich) in phosphate buffered saline (PBS) buffer. Blocking with normal goat serum (in-house production), antibody incubation and nuclei staining were performed in a humidified chamber. Immunohistochemical stainings were observed using a Nikon Microphot SA fluorescence microscope (Nikon) equipped with a CC-12 high-resolution color camera (OSIS).

4.4.1 Antibodies

The majority of commercially available antibodies are validated for human or murine samples and seldomly for bovine samples. For this study, antibodies were preferentially purchased if target protein or epitope homology between validated target immunogen and bovine amino acid sequence are at least 80 % (Table 4.1). Target sequences of several antibodies have lower sequence identity percentages. The anti-CCAAT/enhancer-binding protein beta (C/EBPβ) antibody sc-150 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was applied in immunohistochemical stainings of bovine samples before (Albrecht *et al.*, 2015). The immunogen of the anti-perilipin-3 (PLIN3) antibody NB110-40765 (Novus Biologicals, Wiesbaden-Nordenstadt, Germany) has an sequence identity of 77 % against the analogous amino acid sequence of the bovine protein. Percent identity between porcine PLIN3 residue 1–100 (UniProt: Q5BLZ2) and bovine PLIN3 residue 1–100 (UniProt: Q3SX32) is 91 % (BLAST®, NCBI; Altschul *et al.*, 1997). As the antibody was applied in porcine samples (Zhao *et al.*, 2021), it was deemed suitable for testing in the calf samples. Anti-perilipin-4 (PLIN4) antibody PAE040Hu01 (Cloud-Clone Corp., Houston, TX, USA) was validated for porcine samples by the manufacturer (http://www.cloud-clone.com/products/PAE040Hu01.html; accessed: September 23th, 2021). When the antibody was tested in the bovine samples, a porcine muscle cross-section was applied as positive control.

Table 4.1: *Antibodies tested in the calf samples. Description (target protein, host, clonality), product number (company), immunogen (Uniprot database identification number[1]), amino acid residues (aa; if published by company), corresponding bovine protein[1] and residues, sequence identity of immunogen sequence with bovine sequence[2].*

Description	Product number	Immunogen[1], residues	Corresponding bovine protein[1], residues	Sequence identity[2]
anti-C/EBPα mouse monoclonal	sc-166258[3]	P49715	O02754	93 %
anti-C/EBPβ rabbit polyclonal	sc-150[3]	P21272	O02755	66 %
anti-DLK-1 rabbit polyclonal	ab21682[4]	P80370, aa350-383	A0A3Q1LYR1, aa350–383	85 %
anti-DLK-1 mouse monoclonal	sc-376755[3]	P80370, aa266-383	A0A3Q1LYR1, aa266–383	91 %
anti-MYH1 chicken polyclonal	ABP-PAB-01616[5]	P12882, aa530-628	Q9BE40, aa530–628	94 %
anti-MYH1 rabbit polyclonal	ab91506[4]	P12882, aa1-100	Q9BE40, aa1–100	93 %
anti-MYH1 rabbit polyclonal	ABIN6570793	P12882	Q9BE40	97 %
anti-MYH2 rabbit monoclonal	ab124937[4]	Q9UKX2, aa50-150	Q9BE41, aa50–150	97 %
anti-MYH2 rabbit polyclonal	ABIN2916107[6]	I6L963, aa1238-1472	Q9BE41, aa1236–1470	97 %
anti-MYH4 rabbit polyclonal	DF12165[7]	Q9Y623	E1BP87	95 %
anti-MYH7 mouse monoclonal	ab11083[4]	P12883	Q9BE39	98 %
anti-MYH7 mouse monoclonal	ABIN3043105[6]	G1TW48	Q9BE39	98 %
anti-PLIN2 rabbit polyclonal	NB110-40878[8]	Q99541, aa150-250	Q9TUM6, aa150–250	87 %
anti-PLIN3 rabbit polyclonal	NB110-40765[8]	Q9DBG5, aa1-100	Q3SX32, aa1–100	77 %
anti-PLIN4 rabbit polyclonal	PAE040Hu01[9]	Q96Q06, aa349-656	F1MNM7, aa201–506	73 %
anti-PLIN5 rabbit polyclonal	NB110-60509[8]	Q00G26	A6QLL0	82 %[1]
anti-ZNF423 rabbit polyclonal	ABIN1385826[6]	Q2M1K9	A6QNQ0	99 %[1]

[1] Universal Protein Resource (UniProt), accession date: 18.08.2021 (Consortium, 2020). [2] Basic Local Alignment Search Tool BLAST®, National Center for Biotechnology Information (Altschul et al., 1997). [3] Santa Cruz Biotechnology, Inc., Dallas, TX, USA. [4] Abcam PLC, Cambridge, UK. [5] Allele Biotechnology, San Diego, CA, USA. [6] Antibodies-online GmbH, Aachen, Germany. [7] Affinity Biosciences, Cincinnati, OH, USA. [8] Novus Biologicals, Wiesbaden-Nordenstadt, Germany. [9] Cloud-Clone Corp., Houston, TX, USA.

Table 4.2: *Primary and secondary antibodies applied in immunohistochemistry (IHC), western blot (WB), WB with quantification via Smart Protein Layers system (WB SPL) or in Jess Simple Western (Jess SW) on samples of longissimus (MLD) and semitendinosus muscle (MST), intermuscular (INF) and subcutaneous fat (SCF). Target protein, product number (company), descriptive section number.*

Primary antibody					Secondary antibody			Section
Target	**Product number[1]**	**Application**	**Tissue**	**Dilution**	**Name**	**Product number**	**Dilution**	
C/EBPα	sc-166258	IHC + C/EBPβ[3]	INF and MLD	1:20	Alexa Fluor 594 goat anti-mouse IgG	A-11005[4]	1:500	4.4.4
C/EBPβ	sc-150	IHC + C/EBPα[3]	INF and MLD	1:100	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:500	4.4.4
C/EBPβ	sc-150	IHC (+ DLK-1[3])	MLD, MST	1:200	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.4
DLK-1	ab21682	WB SPL	MLD, INF	1:3000	Rabbit TrueBlot®	18-8816-33[5]	1:25,000	4.5.2
DLK-1	ab21682	WB	MLD, MST, INF	1:3000	Rabbit TrueBlot®	18-8816-33[5]	1:25,000	4.5.4
DLK-1	ab21682	WB	MLD, INF	1:1000	Rabbit TrueBlot®	18-8816-33[5]	1:25,000	4.5.5
DLK-1	sc-376755	WB	MLD, MST, INF	1:3000	Mouse TrueBlot® ULTRA	18-8817-31[5]	1:5000	4.5.4
DLK-1	sc-376755	IHC (+ C/EBPβ[3])	MLD, MST	1:100	Alexa Fluor 594 goat anti-mouse IgG	A-11005[4]	1:500	4.4.3, 4.4.4
MYH1	ab91506	WB SPL	MST	1:2000	Rabbit TrueBlot®	18-8816-33[5]	1:4000	4.5.2
MYH1	ABIN6570793	Jess SW	MLD	1:50	Anti-Rabbit Secondary NIR Antibody	DM-007[6]	1:20	4.5.3
MYH1	ABP-PAB-01616[2]	WB SPL	MLD	1:2000	Rabbit Anti-Chicken IgY H&L (HRP)	ab6753[7]	1:4000	4.5.2
MYH2	ab124937	IHC + MYH7[3]	MLD	1:300	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.2
MYH2	ab124937	WB SPL	MLD	1:2000	Rabbit TrueBlot®	18-8816-33[5]	1:4000	4.5.2
MYH2	ab124937	WB SPL	MST	1:5000	Rabbit TrueBlot®	18-8816-33[5]	1:25,000	4.5.2
MYH2	ABIN2916107	Jess SW	MLD	1:25	Anti-Rabbit Secondary IR Antibody	DM-008[6]	1:20	4.5.3
MYH4	DF12165	Jess SW	MLD	1:40	Anti-Rabbit Secondary NIR Antibody	DM-007[6]	1:20	4.5.3
MYH7	ab11083	IHC + MYH2[3]	MLD	1:200	Alexa Fluor 594 goat anti-mouse IgG	A-11005[4]	1:1000	4.4.2

Continued on next page

Primary antibody					Secondary antibody			Section
Target	**Product number[1]**	**Application**	**Tissue**	**Dilution**	**Name**	**Product number**	**Dilution**	
MYH7	ab11083	WB SPL	MLD, MST	1:2000	Alexa Fluor 488 goat anti-mouse IgG (H+L)	A-11001[4]	1:25,000 (MLD), 1:4000 (MST)	4.5.2
MYH7	ABIN3043105	Jess SW+ MYH1[3]	MLD	1:100	Anti-Mouse Secondary IR Antibody	DM-010[6]	1:10	4.5.3
MYH7	ABIN3043105	Jess SW+ MYH2 or MYH4[3]	MLD	1:100	Mouse TrueBlot® ULTRA	18-8817-31[5]	1:40	4.5.3
PLIN2	NB110-40878	IHC	MLD	1:100	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.6
PLIN3	NB110-40765	IHC	MLD	1:100	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.6
PLIN4	PAE040Hu01	IHC	MLD	1:50	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.6
PLIN5	NB110-60509	IHC	MLD	1:100	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:1000	4.4.6
ZNF423	ABIN1385826	IHC	MLD, INF	1:50	Alexa Fluor 488 goat anti-rabbit IgG	A-11008[4]	1:500	4.4.6
ZNF423	ABIN1385826	WB	MLD, SCF	1:2000	Rabbit TrueBlot®	18-8816-33[5]	1:25,000	4.5.4

[1] For reference, see Table 4.1. [2] Antibody was discontinued, subsequent use of ab91506. [3] Simultaneous detection of targets. [4] Thermo Fisher Scientific, Schwerte, Germany. [5] Rockland Immunochemicals, Inc., Limerick, PA, USA. [6] Product number of detection module, Bio-Techne, Wiesbaden, Germany. [7] Abcam PLC, Cambridge, UK.

4.4.2 Detection of MYH isoforms in skeletal muscle tissue

To investigate muscle fiber type changes, myosin heavy chain isoforms 2 (MYH2) and 7 (MYH7) were simultaneously detected in an immunohistochemical double staining of MLD sections (Dahl *et al.*, 2020). Muscle sections of 12 µm thickness were fixated with 4 % formaldehyde (in distilled water (Aqua dest.)) and washed twice with PBS. After permeabilization with 0.1 % TritonX-100 (Sigma-Aldrich) in PBS (PBST), free binding sides were blocked with 10 % normal goat serum. Isoform 2 was detected with anti-MYH2 antibody ab124937, isoform 7 was detected with anti-MYH7 antibody ab11083 (both Abcam PLC, Cambridge, UK). The slides were incubated for 1 h with the primary antibody mix of anti-MYH2 and anti-MYH7 antibody in 2 % normal goat serum in PBST at the given dilution (Table 4.2). After washing with PBST, slides were incubated with the secondary antibody mix of Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Table 4.2) in PBST for 45 min. Slides were washed with PBST and PBS, and nuclei were stained with Hoechst 33258 in PBS for 5 min. Finally, slides were washed with PBS and Aqua dest. and mounted with Pro Long Diamond Antifade Mountant (Thermo Fisher Scientific, Schwerte, Germany) and coverslips (Carl Roth GmbH) and kept at 4 °C. The total fluorescence area of each isoform was measured to determine the MYH isoform distribution. With the 10× objective, two images per region of at least three regions per sample were taken. One image was taken in the red channel and one image in the green channel, to detect MYH2 (green signal) and MYH7 (red signal) in the same area. With a macro program in the Cellˆ F image analysis software (OSIS), the area percentage of MYH2 and MYH7 expressing fibers was measured. Included into the macro program, the region of interest was set. The contrast was enhanced, the thresholds for phase analysis were set and the area taken up by each fiber type was measured. As a test, MYH2 and 7 were stained in three samples of MST and showed similar correlation with muscle fiber types as in MLD. Therefore, MYH2 and 7 were not stained in all samples of MST.
In addition, muscle fibers expressing both MYH2 and MYH7 (“hybrid fibers”) were detected to analyze muscle fiber type transition (Dahl *et al.*, 2020). From each sample, at least three images were taken in both red and green channel, using the 20× objective. Within secondary muscle fiber bundles, the number of fibers giving a signal in both green and red channel was counted. For each hybrid fiber, the fluorescent signal was compared to the single expressing fiber in the same channel. The hybrid fibers were allocated to one of three groups, depending on in which channel the hybrid fiber had the stronger signal compared to the single expressing fibers. In case of an equally strong signal in both channels, fibers were allocated to the third group. The number of each fiber group per mm^2 sample area was depicted as percentage of each fiber group of the total hybrid fibers.

To test the colocalization of MYH7 expressing fibers with intramyocellular lipid droplets (IMCLs), a Sudan Black B prestained section (4.4.5) was blocked with goat serum and stained for only MYH7, following the above protocol.

4.4.3 Detection of DLK-1 expressing preadipocytes in skeletal muscle tissue

To quantify delta like non-canonical Notch ligand 1 (DLK-1) expressing adipogenic precursor cells, DLK-1 was detected via immunohistochemistry in calf muscle tissue cross-sections. Sections of 8 µm thickness were stained according to standard protocol (4.4.2), except that slides were incubated with primary antibody sc-376755 (Santa Cruz Biotechnology, Inc.) (Table 4.1 and Table 4.2) for 2 h. Primary and secondary antibodies were diluted as listed (Table 4.2). The number of DLK-1 expressing cells was quantified in a procedure adapted from Albrecht *et al.* (2015). For each sample, images of randomly selected areas were taken. Per MLD sample, at least 25 images were acquired with the 40× objective, per MST sample, at least 10 images with the 20× objective. With the interactive measurement module of CellˆF image analysis software, the image was focused on the DLK-1 positive cells and three images were taken per region, in the red and blue channel and visible light. The background fluorescence was reduced. An overlay image of DLK-1 and nuclei was generated, and the DLK-1 positive areas with corresponding nuclei were counted. At least 1.43 mm^2 per sample were analyzed. From each region of interest and the number of DLK-1 positive cells per sample, the number of DLK-1 positive cells per mm^2 per sample was calculated.

4.4.4 Localization of preadipocytes

For single detection of C/EBPβ in MLD and in combination with CCAAT/enhancer-binding protein alpha (C/EBPα) (in MST and INF) or with DLK-1 (in MLD), 8 µm thick tissue sections were stained according to standard protocol (4.4.2). The simultaneous staining of C/EBPβ and DLK-1 was adapted from Albrecht *et al.* (2015). In the combinatory stainings, the incubation with primary antibody mixes ran for 2 h. For staining of C/EBPβ, with C/EBPα or with DLK-1, antibodies were applied in a primary or secondary antibody mix at the given dilution (Table 4.2). In the simultaneous staining of C/EBPα and C/EBPβ, slides were mounted with ROTI®Mount FluorCare and coverslips (both Carl Roth GmbH). The images of C/EBPβ alone and for colocalization with DLK-1 were acquired with the 20× and the 40× objective. For colocalization of C/EBPα and C/EBPβ, images were taken with the 40× objective. The images were focused on C/EBPβ positive cells (C/EBPβ single staining), C/EBPα respectively DLK-1 positive cells (double staining with C/EBPβ) and taken in the green, red and blue channel and visible light. The background fluorescence was reduced. Overlay images of target proteins and of nuclei with the tissue cross-section were generated. Intramuscular lipid droplets and cells

expressing C/EBPα, C/EBPβ respectively DLK-1 were located to detect cells in different stages of adipogenic differentiation.

4.4.5 Test stainings for detection of intramyocellular lipid droplets

For the detection of IMCLs and for colocalization of IMCLs with MYH7 or perilipin-2 (PLIN2), a staining with Sudan Black B (SBB) was tested in two different samples. The protocol was adapted from Mulisch and Welsch (2015). Nile Blue was tested as a stain for the detection of IMCLs in eight samples as well. The protocol was adapted from Bastes and Marques (1972). For Nile Blue staining or SBB prestaining with subsequent PLIN2 or MYH7 detection, the tissue sections were placed on polylysine-L-covered tissue slides (Carl Roth GmbH). For single staining of SBB, muscle cross-sections of 12 µm were placed on uncoated tissue slides. For Nile Blue staining, slides were incubated in filtered 0.005 % Nile Blue (Chroma Gesellschaft Schmid GmbH) in Aqua dest. for 30 min. The slides were washed and covered with IS Mounting Medium (DIANOVA GmbH, Hamburg, Germany) and cover slips (Carl Roth GmbH). For SBB staining, slides were incubated in 0.1 % SBB (Chroma Gesellschaft Schmid GmbH) in 70 % EtOH (in Aqua dest.) for 30 min. When subsequently stained for MYH7 or PLIN2, the slides were washed with PBS before blocking with goat serum, see following paragraph. For direct observation, slides were washed and covered with glycerol gelatin and cover slips (both Carl Roth GmbH). For staining of MYH7 after preincubation with SBB, and for testing of anti-PLIN2 antibody NB110-40878 (Novus Biologicals), the same protocol was applied as described (4.4.2), with antibody dilutions given in Table 4.2. The anti-PLIN2 antibody was tested in 12 µm thick MLD cross-sections in single staining and after SBB prestaining. Instead of PBST, PBS buffer was used. The slides were observed using the green (PLIN2), red (MYH7) or blue channel (Nile Blue) and visible light (SBB). Because the staining of PLIN2 did not yield a specific signal, the antibody was not applied further. Of the other stainings, images were taken with the 40× objective (SBB or Nile Blue single staining), and the 20× objective (MYH7 staining with SBB prestaining). From the image showing MYH7 staining, the background fluorescence was reduced. In the overlay image of SBB and MYH7 staining, the location of IMCLs and MYH7 expressing fibers was observed.

4.4.6 Test stainings for detection of preadipocytes and lipid droplets

For detection of adipogenic markers in muscle and fat tissues, several antibodies were tested, but not applied further. Anti-zinc finger protein 423 (ZNF423) antibody ABIN1385826 (Antibodies-online GmbH, Aachen, Germany) was tested in 8 µm thick INF and MLD cross-sections. The procedure was conducted as described (4.4.2); primary antibody incubation ran for 2 h. The antibody dilutions are displayed in Table 4.2. Antibodies against PLIN3 (NB110-40765, Novus Biologicals), PLIN4 (PAE040Hu01, Cloud-Clone Corp.) and perilipin-5 (PLIN5)

(NB110-60509, Novus Biologicals) were tested in 12 µm thick MLD cross-sections; PBS buffer was applied instead of PBST. Representative images of the stained cross-sections were taken in the green (perilipins, ZNF423) and blue (nuclei) channel, using the 20× objective (PLIN2, 3 and 5, ZNF423) and the 40× objective (PLIN4). From each image, the background fluorescence was reduced and intensity and location of fluorescence signal assessed.

4.5 Protein expression of myogenic and adipogenic markers

4.5.1 Protein isolation

Protein samples were isolated from frozen muscle and adipose tissues as described by Schering *et al.* (2017). From the muscle tissues, 90 to 230 mg sample were homogenized twice, using a Xiril Dispomix® (Xiril AG, Hombrechtikon, Switzerland); from the adipose tissues, 100 to 320 mg sample were homogenized using a Polytron PT 1200 E homogenizer (Kinematica AG, Malters, Schweiz), both with CelLytic™ MT Cell Lysis Reagent with protease inhibitor (both Sigma-Aldrich), according to manufacturer's instructions. The tissue lysates were spun down at 20,817 × g for 15 min (Centrifuge 5417 R, Eppendorf AG, Hamburg, Germany) and protein concentration of the isolated supernatant was measured via Nanodrop Spectrometer (Protein A280 module; ND-1000, PEQLAB Biotechnologie GmbH, Erlangen, Germany). Protein samples were diluted to 1 mg/ml for MYH detection via traditional western blot with the Smart Protein Layers system (WB SPL) (4.6), or via the Jess Simple Western (Jess SW) system (4.6.1). Samples for protein quantification of DLK-1 in INF via WB SPL (4.6) and for ZNF423 antibody testing (4.6.2) were diluted to 2 mg/ml. Protein samples for the DLK-1 antibody test and for protein quantification of DLK-1 in MLD via WB SPL (4.6) were diluted to 4 mg/ml. The diluted samples were either applied in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) immediately or stored at -70 °C. For western blot on protein fractions, protein samples of MLD and INF were processed with the CNMCS compartmental extraction kit (K3013010, Biochain Institute Inc., Newark, CA, USA), according to manufacturer's instructions. From MLD and INF, 110 mg were homogenized as described above, with lysis buffer and protease inhibitor (both provided with the kit). After isolation of cytoplasmic and nuclear protein fractions, protein concentration was measured via Nanodrop Spectrometer (Protein A280 module; ND-1000). From the same tissue samples, total protein was isolated as described in the above paragraph. All protein samples were diluted to 2 mg/ml and stored at -70 °C.

4.6 Protein quantification of MYH isoforms and DLK-1 via western blot and the Smart Protein Layers system

The procedures of protein quantification via traditional western blot coupled with the SPL system (NH DyeAgnostics, Halle, Germany) are described by Dahl *et al.* (2020) and Zhao *et al.* (2020). Protein abundance of MYH isoforms and DLK-1 was measured in different muscle and fat tissues. All chemicals were purchased from Carl Roth GmbH, if not stated otherwise. The protein samples were blotted in a standard procedure for western blot. The relative protein abundance was normalized and quantified with the SPL Red Kit (PR926, NH DyeAgnostics). The reagents were prepared as described by Faden *et al.* (2016). The protein samples were mixed with loading buffer, denatured at 95 °C for 5 min and separated via SDS-PAGE. Per lane, 2 µg protein were used for detection of MYH isoforms in muscle. Due to high signal intensity, 1 µg protein was used for detection of MYH2 in MST. For DLK-1 quantification in MLD, 20 µg protein, and for quantification in INF, 10 µg protein were used per lane. Protein samples were separated on a 4–15 % Criterion™ TGX Stain-Free™ Protein Gel (26 lanes; Bio-Rad, Munich, Germany) in 1× Towbin buffer with 0.1 % SDS. SERVA Triple Color Protein Standard II and Standard III (SERVA Electrophoresis GmbH, Heidelberg, Germany) served as markers for molecular weight. In the Trans-Blot® semi-dry blotter (Bio-Rad), total proteins were blotted with the Trans-Blot® Turbo™ RTA Midi 0.2 µm PVDF Transfer Kit (1704275, Bio-Rad) on a 0.45 µm low fluorescence polyvinylidene difluoride (PVDF) membrane (included in the kit). Antibodies were bound using the iBind™ Flex Solution Kit (Thermo Fisher Scientific). The primary and secondary antibodies and applied dilutions are listed in Table 4.1 and Table 4.2. Proteins on gels and blots were detected with a Chemocam HR-16 imager system (Intas Science Imaging Instruments GmbH, Göttingen, Germany). The signal of horseradish peroxidase (HRP) labeled secondary antibodies Mouse TrueBlot® ULTRA, Rabbit TrueBlot® (both Rockland Immunochemicals, Limerick, PA, USA) and Rabbit Anti-Chicken IgY H&L (HRP) (Abcam PLC) was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific). For quantification via SPL, the pictures of gel and blot were processed with the LabImage 1D software 4.2.3 (Kapelan Bio-Imaging GmbH, Leipzig, Germany), following manufacturer's instructions. As the protein detection of MYH isoforms in MLD was run twice for each sample, the arithmetical mean protein volume between the two runs was calculated. For each sample, the relative target protein volume was normalized against the total protein volume and the total mean.

4.6.1 Protein quantification of MYH isoforms via Jess Simple Western

Myosin heavy chain proteins (isoform 1, 2, 4 and 7) in MLD were quantified with a Jess Simple Western (Jess SW) system (ProteinSimple, San Jose, CA, USA) following the manufacturer's

instructions. The procedure was described by Zhao *et al.* (2020). First, the protein samples, diluted to 0.5 µg/µl, were mixed 1:1 with 0.1× sample buffer, then 1:5 with 5× Mastermix (both ProteinSimple). The sample mix was incubated for 5 min at 95 °C, vortexed, spun down and placed on ice. Primary and secondary antibody dilutions were prepared with milk-free antibody diluent (Bio-Techne, Wiesbaden, Germany). The respective primary and secondary antibodies are listed in Table 4.1 and Table 4.2. The sample mix, antibody dilutions, Luminol-S/Peroxide mix (if necessary, both Bio-Techne), buffers, protein ladder and protein normalization reagent (all ProteinSimple) were loaded onto the plates. The incubation times followed the standard protocol. Target signals were recorded in the system's NIR, IR and chemiluminescence channels, total protein signals were recorded in the PN (protein normalization) channel. Compass for SW software version 4.1.0 (ProteinSimple) was used to analyze the results. The relative protein abundance was calculated from the target protein band volume divided by the total protein volume, resulting in the relative protein abundance.

4.6.2 Antibody tests for detection of adipogenic markers

Antibodies against adipogenesis marker DLK-1 were tested in a western blot following standard procedures (Schering *et al.*, 2017; Liu *et al.*, 2018). Anti-DLK-1 antibodies ab21682 (Abcam PLC) and sc-376755 (Santa Cruz Biotechnology, Inc.) were applied in protein samples of calf MLD and MST, INF (at MLD) and adult bovine MLD (control). The application of antibody ab21682 (Abcam PLC) in bovine samples was published by Albrecht *et al.* (2015). The specificity of the antibody ab21682 (Abcam PLC) was additionally tested by preincubation with 3× blocking peptide (ab23442, Abcam PLC). Per lane, 20 µg protein were prepared, separated and blotted as described (4.6). The SERVA Triple Color Protein standard III served as molecular weight marker.

The anti-ZNF423 antibody ABIN1385826 (Antibodies-online GmbH) was tested in protein samples of calf, adult bovine and mouse muscle and adipose tissue and in piglet muscle, following standard procedures. The protein samples were subjected to western blot as described above. Per lane, 20 µg protein were separated on a 10 % separating gel, with the SERVA Triple Color Protein Standard III as molecular weight marker. Total proteins were blotted on a 0.2 µm ROTI®PVDF membrane (Carl Roth GmbH).

In both tests, the membranes were stained with Coomassie Blue (3862.1, Carl Roth GmbH) according to standard protocol, to confirm even protein transfer. The membranes were washed, blocked with 10 % ROTI®Block (Carl Roth GmbH) in Tris-buffered saline (TBS) for 1 h at RT and incubated with the primary antibodies overnight at 4 °C. After washing, the membranes were incubated with the secondary antibodies for 90 min at RT. Specifics about the primary and secondary antibodies and dilutions are listed in Table 4.1 and Table 4.2. In

both western blots, the antibody label was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate in the Chemocam HR-16 imager system.

4.6.3 Detection of DLK-1 in cell fractions

To verify the signal specificity of anti-DLK-1 antibody ab21682 (Abcam PLC), it was tested via western blot in cytoplasmic and nuclear protein fractions, compared to total protein, isolated from MLD and INF following standard procedure (4.6).
After preparation of protein samples, 20 µg protein of all three fractions from muscle, cytoplasm fraction and total protein of INF and 30 µg protein from the INF nuclear fraction were separated on a 12 % separating gel, together with SERVA Triple Color Protein Standard III. Total proteins were blotted on a 0.2 µm ROTI®PVDF membrane, stained with Coomassie Blue and incubated with primary and secondary antibodies as described above (4.6.2). Specifics about the primary and secondary antibodies and dilutions are listed in Table 4.1 and Table 4.2. The antibody label was detected with SuperSignal™ West Femto Maximum Sensitivity Substrate in the Chemocam HR-16 imager system.

4.7 MRNA expression of myogenic and adipogenic markers

4.7.1 RNA isolation

The applied methods of ribonucleic acid (RNA) isolation followed the description of Schering *et al.* (2017) and were conducted according to manufacturer's instructions. All chemicals were purchased from Carl Roth GmbH, if not stated otherwise. From the frozen muscle tissue samples, 90–240 mg were homogenized with QIAzol reagent (QIAGEN, Hilden, Germany), using the Xiril Dispomix®, and precipitated with chloroform and isopropanol. The sample RNA was purified with the NucleoSpin® Gel & PCR Clean-up kit (740609.50, Macherey-Nagel, Düren, Germany), according to manufacturer's instructions. For RNA isolation from INF, 120–340 mg sample tissue in QIAzol lysis reagent (QIAGEN) were homogenized with the Polytron PT 1200 E. The sample RNA was then isolated using the miRNeasy Mini Kit (217004, QIAGEN), according to manufacturer's instructions. The RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (RNA-40 module), and the RNA samples were stored permanently at -70 °C. In parallel to the RNA concentration, the RNA quality was determined based on the sample absorbance ratio at 260 and 280 nanometer (260/280) and 260 and 230 nanometer (260/230). The measured 260/280 ratio ranged from 1.87 to 2.19, the 260/230 ratio ranged from 1.55 to 2.23. Quality and integrity of selected RNA samples from MLD and MST were additionally analyzed with the Experion™ Automated Electrophoresis System (Bio-Rad), using the Experion RNA StdSens Analysis Kit (7007103, Bio-Rad). An RNA

quality indicator (RQI) of ≥ 8.6 was determined for all samples, which is within the acceptable range of 7–10 according to the manufacturer (Denisov *et al.*, 2008).

4.7.2 Reverse transcription

The isolated RNA samples were transcribed to complementary deoxyribonucleic acid (cDNA), using the iScript cDNA synthesis kit (1708891, Bio-Rad). Ribonucleic acid samples with a concentration higher than 500 ng/µl were diluted to 100 ng/µl. The diluted RNA was used for cDNA synthesis directly, and then stored permanently at -70 °C. When the RNA concentration was lower than 500 ng/µl, the RNA was directly used for cDNA synthesis. For cDNA synthesis, a reaction master mix was prepared. For the master mix, 5× iScript mix and iScript Reverse Transcriptase (both in the synthesis kit) were mixed 5:1. Into each cDNA sample tube, 5 µl DNAse free water (from synthesis kit) and 5 µl of the master mix were added. From the prediluted RNA, 10 µl (corresponds to 1000 ng) were added into the reaction tube. From the undiluted RNA, a volume corresponding to 1000 ng and water for a total reaction volume of 20 µl were added. The tubes were directly subjected to the reverse transcription reaction in a peqSTAR 96 Universal thermocycler (PEQLAB Biotechnology GmbH). The incubation ran at 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. The cDNA samples were permanently stored at -20 °C. Based on the inserted RNA amount of 1000 ng and the total volume of 20 µl, the concentration was presumed to be 50 ng/µl. For primer amplification and for primer efficiency tests, representative samples were pooled and stored permanently at -20 °C. Sample dilutions of 10 ng/µl were prepared for gene expression measurements in individual samples, and stored permanently at -20 °C.
For the first reference gene tests (4.7.6), RNA from eight samples (two samples per group) was transcribed differently, also following standard protocol. The RNA samples were diluted to 10 ng/µl, and inserted into the reaction mix at 10 µl volume, corresponding to 100 ng. This protocol was discontinued in favor of the protocol above with a higher cDNA yield.

4.7.3 Primer design

Primers were designed in reference to the mRNA sequence of the gene of interest, published on NCBI database (Nucleotide. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004). Either the mRNA sequence was processed with Primer3web (version 4.1.0; https://primer3.ut.ee/; Untergasser *et al.*, 2012), or with the NCBI Primer-Blast tool (Ye *et al.*, 2012). The primers were synthesized by Sigma-Aldrich (cartridge purification) and delivered in a dry state. After delivery, primers were either processed immediately, stored at 4 °C for up to four days or at -20 °C for longer time before processing. Lyophilized primers were dissolved in nuclease-free water (QIAGEN) to the final concentration of 100 µM. From the stock solution, 10 µM aliquots were prepared with nuclease-free water

(QIAGEN). Both stock solution and aliquots were permanently stored at -20 °C. The primer amplification was tested in a qualitative polymerase chain reaction (PCR). Primer data of measured genes are listed in Table 9.11. Primer data of tested, but not used, reference genes are listed in Table 4.3.

4.7.4 Polymerase chain reaction

The amplification of primers in the target tissues were tested via PCR, using the PCR Master Mix kit (2X) (K0171, Thermo Fisher Scientific) and following standard protocol. For a total reaction volume of 10 µl, a 9 µl reaction master mix of 2 µM forward and reverse primer, 1× PCR Mix and nuclease-free water (both included in the kit) was prepared. From the cDNA, 1 µl of 10 ng/µl was inserted. A no-template control containing nuclease-free water (in the kit) served as negative control. The reaction tubes were spun down at 500 rpm (15 × g) for 30 sec (Gusto® High-Speed Mini Centrifuge, Heathrow Scientific, Nottingham, UK), before they were subjected to the thermocycler (peqSTAR 96 Universal, PEQLAB Biotechnology GmbH) program of 1 cycle of 94 °C for 4 min, 40 cycles of 94 °C for 30 sec, 60 °C for 45 sec, 72 °C for 45 sec and 1 cycle of 72 °C for 7 min. After the PCR reaction, samples were either stored at 4 °C or directly subjected to gel electrophoresis. For the gel electrophoresis, PCR products together with FastRuler™ Low Range DNA Ladder (Thermo Fisher Scientific) were separated on a 3 % agarose (Biodeal Handelsvertretung Edelmann e.K., Markkleeberg, Germany) gel in 1× Tris acetate EDTA (TAE) buffer, stained with ROTI®GelStain (Carl Roth GmbH). The product bands were detected under UV light with the QUANTUM gel documentation system model 1199 ‘Super-Bright’ (Peqlab, VWR International GmbH, Darmstadt, Germany). Gel images were printed with a Mitsubishi P95DE printer (Mitsubishi Electric Europe B.V., Ratingen, Germany).

4.7.5 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed on a total reaction volume of 10 µl, using the FastStart Essential DNA Green Master kit (06402712001, Roche, Basel, Switzerland), as described by Zhao *et al.* (2021). For each primer pair, a reaction master mix, with a volume of 9 µl per reaction, was prepared from 1× FastStart Essential DNA Green Master (included in the kit), 2 µM forward and reverse primer (Table 9.11) and PCR grade water (included in the kit). From the cDNA, 1 µl was inserted in duplicates. The amount of 10 ng was chosen as in a basic dilution series targeting two reference gene candidates and two target genes, this amount yielded the most similar quantification cycle (Cq) values between reference and target genes and lower replicate errors between duplicates than other cDNA samples (20 ng and 5 ng cDNA). The PCR grade water served as negative (no-template) control. The reaction was run in a 96 Well Lightcycler Plate, sealed with adhesive foil (both SARSTEDT AG, Nürnbrecht,

Germany). The sealed plate was spun down at 500 × g (Mini Plate Spinner MPS 1000™, Labnet Internation, Inc., Edison, USA). The qPCR was set up with the LightCycler® application version 1.1.0.1320 and performed with a LightCycler® 96 real-time qPCR system (both Roche). The reaction temperature profile was as follows: a preincubation at 95 °C for 10 min, 45 amplication cycles of 95 °C for 10 sec, 60 °C for 10 sec, 72 °C for 25 sec, and a melting step of 95 °C for 10 sec, 65 °C for 60 sec and 97 °C for 1 sec. The acquired data were processed with the Roche LightCycler® application, and the raw data exported into Microsoft® Excel 2016 (Microsoft Corporation). The amplification efficiency of primer pairs was calculated from a standard curve acquired in a serial dilution series by the formula $Efficiency = 10^{-\frac{1}{\text{slope}}}$. The efficiencies for applied primer pairs were within the range 1.8 to 2.0, the coefficient of correlation (R^2) ranged from 0.97 to 1.0 (Table 9.11). The expression level of RNA was calculated as normalized relative quantities (NRQ; Hellemans *et al.*, 2007) in relation to the two reference genes *UXT* and *PPIA*.

4.7.6 Selection of reference genes

The relative gene expression of target genes was normalized against the relative gene expression of two reference genes. Suitable reference gene Cq values were required to be close to the target gene Cq and to have an inter-sample variance as low as possible. For selection of reference genes, candidate genes were chosen based on established reference genes and literature research. Several different gene candidates were measured in a serial dilution series and in the single samples of cDNA from MLD and MST. Sequences, efficiency and R^2 values for tested and excluded primers are listed in Table 4.3. DNA topoisomerase II beta (*TOP2B*) and beta-2 microglobulin (*B2M*) were used as reference genes in bovine MLD (Schering *et al.*, 2017). In this study, they were first tested as reference genes together with the two target genes platelet derived growth factor receptor alpha (*PDGFRA*) and *ZNF423* in cDNA of two MLD samples (1× CON, 1× EFA group). The cDNA was inserted at a concentration of 20 ng/µl, 10 ng/µl and 5 ng/µl. The primers were provided by L. Schering (Schering *et al.*, 2017), the 100 µM stock solutions were freshly aliquoted before usage. Beta-2 microglobulin was discarded because of high errors between replicates (Cq error ≥ 1.77) and high Cq difference to target gene Cq (≥ 5 cycles). DNA topoisomerase II beta was chosen as a preliminary reference gene. As a second reference gene, 18S ribosomal RNA (*18S rRNA*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were tested. Both primers pairs were also provided by L. Schering. This time, primers were applied as unicates in a dilution series in parallel with the *TOP2B* primer pair. Because the Cq number was lower than the target gene Cq number by 17 cycles, *18S rRNA* was discarded as reference gene. Glyceraldehyde-3-phosphate dehydrogenase, together with *TOP2B*, was further applied in

efficiency testing. In a dilution series together with target genes *PDGFRA* and *ZNF423*, efficiency of *TOP2B* was tested as 1.61, with an R^2 value of 0.98. The efficiency of the *GAPDH* primers was tested as 1.57, and R^2 was 0.87. For the target genes *PDGFRA* and *ZNF423* primer efficiencies of 1.52, respectively 1.51, were yielded. Because efficiency of *TOP2B* and *GAPDH* was similar to the target gene efficiency, *TOP2B* and *GAPDH* were maintained as reference genes. In the measurement of *TOP2B* relative expression in the single samples though, the Cq value of *TOP2B* varied between duplicates (Cq error > 0.5) and between samples (up to 14.58 cycles). The dilution series with the *TOP2B* primer pair was repeated, the measured efficiency was 1.83, and R^2 was 0.98. Consequently, expression of *TOP2B* was considered too unstable for application as a reference gene. Similarly, the relative mRNA expression of *GAPDH* was measured in all samples and the Cq variation was ≤ 16.67 cycles. In addition, replicate error between duplicates was higher than 0.5. In a repetitive dilution series, the *GAPDH* primer pair yielded an efficiency of 1.84, R^2 was 1.00. Due to the high inter-sample variance, *TOP2B* and *GAPDH* were excluded as reference genes. Based on the review of Pérez *et al.* (2008), eukaryotic translation elongation factor 1 alpha 2 (*EEF1A2*), β-actin (*ACTB*), splicing factor 3 subunit 1 (*SF3A1*) and hydroxymethylbilane synthase (*HMBS*) were chosen. One primer pair for each gene was tested in a dilution series. Based on the results, *EEF1A2* and *HMBS* were chosen as reference genes and measured in the single samples. The calculated Cq values for *EEF1A2* and *HMBS* ranged over 13 cycles, and both genes were excluded as reference genes due to high inter-sample variance and variance between duplicates. Ubiquitously-expressed transcript (*UXT*) (Schering *et al.*, 2017), lipoprotein receptor-related protein 10 (*LRP10*) and emerin (*EMD*) (Saremi *et al.*, 2012) were tested in a serial dilution of cDNA isolated from MST. Based on the low efficiency values, *EMD* and *LRP10* were excluded as reference genes. After single sample testing, *UXT* was chosen as a reference gene. For the second reference gene, primer pairs for peptidylprolyl isomerase A (*PPIA*) and RNA terminal phosphate cyclase like 1 (*RCL1*) (Jennings *et al.*, 2016) were tested in a serial dilution. RNA terminal phosphate cyclase like 1 was excluded, but *PPIA* was chosen as a reference gene (Table 9.11).

Table 4.3: *Primer sequences of tested reference genes. Gene (accession number[1]), forward and reverse primer sequence, source of primer sequence, amplicon size, efficiency (E) and correlation coefficient (R^2).*

Gene (acc. no.)	Primer sequence (5'–3') forward/reverse	Source	Amplicon size (bp)	E	R^2
18S rRNA (NR_036642.1)	GCCGTTCTTAGTTGGTGGAG/ CGCTGAGCCAGTCAGTGTAG	L. Schering unpublished	217	Not determined	
ACTB (NM_173979.3)	CTGGACTTCGAGCAGGAGAT/ TAGTTTCGTGAATGCCGCAG	Primer3web[2]	172	1.71	1.00
B2M (NM_173893)	CAGCTGCTGCAAGGATGG/ ATTTCAATCTGGGGTGGATG	Schering *et al.*, 2017	184	Not determined	
EEF1A2 (NM_001037464.2)	AACATAGTGGTCATCGGCCA/ TCAGCTTGTCCAGTACCCAG	Primer3web[2]	166	1.88	0.99
EMD (NM_203361.1)	CTCGGACTTAGATTCGGCGT/ CAGGTTCCCCGTAAGTCCTG	NCBI[3]	143	1.64	0.99
GAPDH (NM_001034034.1)	CAGGTTGTCTCCTGCGACTT/ GGTCCAGGGACCTTACTCCT	L. Schering unpublished	183	1.84	0.99
HMBS (NM_001046207.1)	GGAAGTTCGAGCCAAAGACC/ ATGGTGGTTTGCATGGTGTC	Primer3web[2]	231	1.99	1.00
LRP10 (Bc149232)	CCAGAGGATGAGGACGATGT/ ATAGGGTTGCTGTCCCTGTG	Saremi *et al.*, 2012	139	1.72	0.98
RCL1 (NM_001077131.1[4])	ACTTGTGTTGGCATTGGCTTCTCC/ ACGGGAAGTTGGAAAGTCCTGGAT	Jennings *et al.*, 2016	147	1.42	0.94
SF3A1 (NM_001081510.1)	AAGATCCCTGCCAGCAAGAT/ CCAAAGATGTCTGTACGCCG	Primer3web[2]	179	1.88	1.00
TOP2B (XM_002698750)	AAGAAAACAGCACCGAAAGG/ GAGGTCTGAGGGGAAGAGGT	Schering *et al.*, 2017	174	1.83	0.97

[1] Accession number on NCBI database (Nucleotide. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004). [2] Untergasser *et al.*, 2012. [3] NCBI Primer-Blast tool (Ye *et al.*, 2012). [4] The gene accession number was not listed in the source. NCBI blast of primer sequences yields this gene accession number as target for primers.

4.8 Statistical analysis

Microsoft® Excel 2016 (Microsoft Corporation) was used for basic data processing. For data analysis, the MIXED model in SAS® statistical software (SAS 9.4, SAS Institute, Cary, NC, USA) was applied. Fixed effects were the supplemented FAs (EFA, CLA) and their interactions, the levels were “yes” (if supplemented) or “no” (not supplemented). Other fixed effects were the calf sex and its interaction with the supplemented FAs. The experimental blocks were introduced as random factors. The gestation length (days) was included in the model as a covariate. When it had no significant effect ($p > 0.05$), it was removed from the model. The denominator degrees of freedom was calculated by Kenward-Roger estimation. The Least Square Means (LSMs) were compared by the PDIFF statement and adjusted with a Tukey-Kramer post-hoc test. The presented data are depicted as LSM ± Standard Error of LSM (SE_{LSM}). All data that were to be correlated were tested for normality distribution with the Shapiro-Wilk test in the UNIVARIATE procedure. Pearson and Spearman correlation were calculated with the CORR procedure. In the calculation of DLK-1 expressing cells per mm^2 of MLD muscle sample, the number of the immunohistochemistry run (1–3) had a significant effect ($P < 0.05$), therefore it was included as random factor. For the fat cell size histograms, the skewness factors were analyzed with the MEANS procedure.

5 Results

5.1 Effects of maternal supplementation on offspring body weight

In this study, the calves were exposed to the effects of their dam's supplementation prenatally, during the last 63 days of gestation, and postnatally, during the first five days of life. To address the impact of maternal supplementation on calf development, birth and slaughter weight of the calves were analyzed. The birth weight of all 36 calves ranged from 28 to 50.5 kg, with an LSM ± SE_{LSM} of 42.01 ± 0.80 kg. The weight at slaughter varied between 33 and 53 kg, the LSM ± SE_{LSM} was 44.12 ± 0.84 kg. There was no difference between the supplementation groups in neither birth nor slaughter weight ($P > 0.75$).

5.2 Effects of maternal supplementation on longissimus and semitendinosus muscle

5.2.1 Fatty acid composition of muscle tissues

The FAs were transferred from the dam to the calf, according to the results on plasma fatty acids (FAs) reported by Uken *et al.* (2021a). Their incorporation into the skeletal muscle tissues is a prerequisite for effects on tissue development. To verify a change in muscle FAs upon maternal supplementation, the FA profiles of *musculus longissimus dorsi* (MLD) and *musculus semitendinosus* (MST) were measured. Fatty acid concentration and composition of all measured FAs are shown in the supplement (Table 9.1–Table 9.4). Table 5.1 and Table 5.2 list the FA concentration (mg/100 g tissue) in MLD and MST respectively, focusing on the supplemented FAs, longer-chain and sum of *n*-3 and *n*-6 polyunsaturated FAs (PUFAs) and the sum of PUFAs, as well as selected and sum of monounsaturated FAs (MUFAs) and saturated FAs (SFAs), and total fat content (%).

Supplementation with essential fatty acids (EFAs) affected α-linolenic acid (ALA), longer-chain *n*-3 PUFAs and, to a small extent, longer-chain *n*-6 PUFAs, C18:1 *cis*-9 (oleic acid, OA) and the sum of MUFAs. The EFA+CLA supplementation further altered the content of ALA in both muscles and of the sum of *n*-3 PUFAs in MST. Supplementation with conjugated linoleic acid (CLAs) affected *cis*-9, *trans*-11 CLA and C21:0 (heneicosanoic acid, HA, including the signal for *trans*-10, *cis*-12 CLA); also C18:1 *trans*-11 (vaccenic acid, VA) in MST and C18:0 (stearic acid, SA) in both muscles were affected.

In detail, the EFA supplementation increased ALA in both muscles, compared to CON and CLA ($P < 0.01$). The sum of *n*-3 PUFAs and in particular longer chain *n*-3 PUFAs C20:5 *n*-3 (eicosapentaenoic acid, EPA), C22:5 *n*-3 (*n*-3 docosapentaenoic acid, DPA) and C22:6 *n*-3 (docosahexaenoic acid, DHA) were increased ($P < 0.05$). The EFA+CLA increased the EFA

effect on ALA in both muscles, compared to the other three groups ($P < 0.05$). Similarly, the sum of *n*-3 PUFAs was increased in MST of EFA+CLA supplemented animals, compared to the other three supplementation groups ($P < 0.05$). The EFA supplementation did not affect linoleic acid (LA) in MLD and the sum of *n*-6 PUFAs in both muscles ($P > 0.213$). Longer chain *n*-6 PUFA C20:4 *n*-6 (arachidonic acid, ARA) was decreased in the EFA group's MLD, compared to the CLA group ($P = 0.03$). The longer chain *n*-6 PUFA C22:4 *n*-6 (adrenic acid, ADA) was decreased in both muscles of the two EFA groups, compared to CON and CLA ($P < 0.05$). In MST, but not in MLD, the CLA supplementation further enhanced the increasing effect of EFA on the sum of PUFAs, compared to CON ($P = 0.005$). Supplementation with EFA decreased the tissue concentration of OA and the sum of MUFAs in MLD, compared to the CLA group ($P < 0.05$).

The CLA supplementation increased *cis*-9, *trans*-11 CLA in both muscles ($P < 0.01$). It also had an increasing effect on muscle LA, but without significant differences between individual groups (CLA effect in MLD: $P = 0.048$ and in MST: $P = 0.065$). The concentration of HA, which includes the signal for *trans*-10, *cis*-12 CLA, was affected by CLA supplementation. In MLD, the concentration was increased (CLA effect: $P = 0.021$), but there was no significant difference among individual groups. In MST, the concentration of HA was higher in the two CLA groups compared to EFA and CON ($P < 0.01$). Supplementation with CLAs increased the concentration of VA and SA in the MST of the EFA+CLA group, compared to CON and EFA ($P < 0.05$). Similarly, the sum of SFAs was increased compared to EFA ($P = 0.049$) in the MST of the EFA+CLA group.

Supplementation with EFA tended to increase LA in MST of the EFA+CLA group, compared to CON ($P > 0.056$). Also in MST, EFA supplementation tended to decrease ARA compared to CON and CLA ($P > 0.063$). In MLD, there was a trend toward increased SA in the EFA+CLA group, compared to the EFA group ($P = 0.067$). Unaffected by the supplementation were the sum of SFAs in MLD, and C16:0 (palmitic acid, PA) and total fat content (%) in both muscles ($P > 0.118$).

Table 5.1: *Fatty acid concentration in longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}. Results for all measured fatty acids are listed in the supplement.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11 (CLA)	1.10 ± 0.10^{b}	0.85 ± 0.09^{b}	1.85 ± 0.14^{a}	1.65 ± 0.08^{a}	0.036	<.001	0.815
C18:3 *n*-3 (ALA)	2.03 ± 1.04^{c}	11.22 ± 0.91^{b}	1.99 ± 1.47^{c}	15.19 ± 0.86^{a}	<.001	0.073	0.066
C20:5 *n*-3 (EPA)	1.89 ± 0.78^{b}	8.73 ± 0.69^{a}	1.67 ± 1.08^{b}	10.24 ± 0.66^{a}	<.001	0.409	0.265
C22:5 *n*-3 (DPA)	8.72 ± 1.07^{b}	16.55 ± 0.98^{a}	8.64 ± 1.54^{b}	19.32 ± 0.91^{a}	<.001	0.250	0.232
C22:6 *n*-3 (DHA)	5.79 ± 0.95^{b}	9.44 ± 0.86^{a}	4.96 ± 1.27^{b}	9.65 ± 0.82^{a}	<.001	0.718	0.552
Sum of *n*-3 PUFAs[1]	18.55 ± 3.14^{b}	46.15 ± 2.78^{a}	17.44 ± 4.45^{b}	54.97 ± 2.62^{a}	<.001	0.233	0.128
C18:2 *n*-6 (LA)	63.30 ± 6.43	63.51 ± 5.70	75.05 ± 9.11	78.58 ± 5.39	0.781	0.048	0.798
C20:4 *n*-6 (ARA)	67.29 ± 4.20^{ab}	57.23 ± 3.80^{b}	75.42 ± 5.59^{a}	62.91 ± 3.64^{ab}	0.008	0.077	0.749
C22:4 *n*-6 (ADA)	19.58 ± 1.33^{a}	13.53 ± 1.20^{b}	22.27 ± 1.79^{a}	15.07 ± 1.14^{b}	<.001	0.093	0.642
Sum of *n*-6 PUFAs[2]	181.82 ± 12.68	163.08 ± 11.31	204.08 ± 17.71	188.74 ± 10.71	0.196	0.066	0.893
Sum of PUFAs[3]	202.77 ± 14.83	211.39 ± 13.21	225.11 ± 20.81	246.61 ± 12.49	0.329	0.062	0.666
C18:1 *cis*-9 (OA)	270.83 ± 12.51^{ab}	240.65 ± 11.59^{b}	297.36 ± 15.71^{a}	247.38 ± 11.24^{b}	0.001	0.099	0.321
C18:1 *trans*-11 (VA)	0.45 ± 0.15	0.32 ± 0.13	0.48 ± 0.21	0.39 ± 0.13	0.474	0.749	0.892
Sum of MUFAs[4]	349.86 ± 15.98^{ab}	313.24 ± 14.79^{b}	383.29 ± 20.10^{a}	320.03 ± 14.34^{b}	0.001	0.120	0.299
C16:0 (PA)	147.14 ± 9.41	128.36 ± 8.81	148.85 ± 11.51	134.83 ± 8.59	0.029	0.552	0.731
C18:0 (SA)	93.25 ± 5.49	87.85 ± 4.89	104.17 ± 7.64	104.22 ± 4.64	0.633	0.017	0.616
C21:0 (HA)[5]	0.20 ± 0.06	0.11 ± 0.05	0.33 ± 0.07	0.28 ± 0.05	0.249	0.021	0.730
Sum of SFAs[6]	257.48 ± 14.36	230.10 ± 13.26	269.91 ± 18.16	255.19 ± 12.85	0.086	0.111	0.585
Total fat content (%)	0.81 ± 0.04	0.76 ± 0.04	0.87 ± 0.05	0.82 ± 0.04	0.143	0.066	0.997

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0. [a-c] Different superscripts indicate significant differences among groups ($P < 0.05$).

Table 5.2: *Fatty acid concentration in semitendinosus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}. Results for all measured fatty acids are listed in the supplement.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11 (CLA)	0.91 ± 0.12^{b}	0.72 ± 0.11^{b}	1.67 ± 0.17^{a}	1.66 ± 0.10^{a}	0.447	<.001	0.494
C18:3 *n*-3 (ALA)	1.94 ± 0.71^{c}	10.02 ± 0.63^{b}	2.04 ± 0.98^{c}	13.00 ± 0.60^{a}	<.001	0.032	0.043
C20:5 *n*-3 (EPA)	1.60 ± 0.35^{b}	5.73 ± 0.31^{a}	1.34 ± 0.47^{b}	6.49 ± 0.29^{a}	<.001	0.458	0.133
C22:5 *n*-3 (DPA)	8.55 ± 0.68^{b}	15.72 ± 0.62^{a}	7.80 ± 0.97^{b}	17.60 ± 0.57^{a}	<.001	0.441	0.085
C22:6 *n*-3 (DHA)	4.53 ± 0.57^{b}	7.03 ± 0.50^{a}	3.88 ± 0.81^{b}	7.23 ± 0.47^{a}	<.001	0.698	0.467
Sum of *n*-3 PUFAs[1]	17.29 ± 1.46^{c}	39.34 ± 1.34^{b}	15.62 ± 2.10^{c}	45.44 ± 1.23^{a}	<.001	0.165	0.021
C18:2 *n*-6 (LA)	55.58 ± 4.78	59.89 ± 4.20	63.15 ± 6.89	71.24 ± 3.97	0.232	0.065	0.701
C20:4 *n*-6 (ARA)	55.19 ± 2.03	48.13 ± 1.81	56.52 ± 2.88	51.44 ± 1.71	0.008	0.268	0.632
C22:4 *n*-6 (ADA)	15.20 ± 0.76^{a}	11.23 ± 0.67^{b}	15.73 ± 1.04^{a}	11.99 ± 0.64^{b}	<.001	0.380	0.876
Sum of *n*-6 PUFAs[2]	161.34 ± 7.51	153.88 ± 6.89	167.99 ± 10.82	171.39 ± 6.34	0.804	0.144	0.512
Sum of PUFAs[3]	184.63 ± 7.95^{b}	198.97 ± 7.29^{ab}	191.22 ± 11.45^{ab}	223.20 ± 6.71^{a}	0.012	0.081	0.317
C18:1 *cis*-9 (OA)	228.54 ± 11.02	215.81 ± 10.43	241.22 ± 13.10	216.98 ± 10.22	0.022	0.349	0.439
C18:1 *trans*-11 (VA)	1.32 ± 0.10^{b}	1.20 ± 0.09^{b}	1.51 ± 0.14^{ab}	1.67 ± 0.08^{a}	0.864	0.003	0.195
Sum of MUFAs[4]	293.08 ± 13.41	279.61 ± 12.58	309.84 ± 16.33	279.85 ± 12.28	0.038	0.381	0.398
C16:0 (PA)	129.97 ± 9.09	118.68 ± 8.50	122.80 ± 11.16	136.86 ± 8.28	0.842	0.414	0.070
C18:0 (SA)	84.00 ± 4.81^{b}	86.67 ± 4.54^{b}	92.63 ± 5.79^{ab}	98.49 ± 4.43^{a}	0.224	0.005	0.635
C21:0 (HA)[5]	0.36 ± 0.05^{b}	0.34 ± 0.05^{b}	0.69 ± 0.07^{a}	0.77 ± 0.04^{a}	0.643	<.001	0.386
Sum of SFAs[6]	236.24 ± 15.15^{ab}	224.48 ± 14.23^{b}	237.33 ± 18.39^{ab}	259.29 ± 13.89^{a}	0.649	0.105	0.130
Total fat content (%)	0.71 ± 0.03	0.70 ± 0.03	0.74 ± 0.04	0.76 ± 0.03	0.785	0.091	0.487

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0. [a-c] Different superscripts indicate significant differences among groups (P < 0.05).

5.2.2 Capillarization of muscle tissues

To elucidate whether the observed changes in muscle FA composition were accompanied by changes in blood supply, capillarization of MLD and MST was investigated. Muscle cross-sections were stained for alkaline phosphatase, an established marker for capillaries (Mulisch and Welsch, 2015). The muscles differed in their capillarization (Figure 5.1), but neither capillarization as percent of sample area occupied by capillaries, nor intercapillary distance, nor muscle fiber to capillary ratio differed among groups ($P > 0.183$). Capillarization was greater in MLD than in MST (LSM ± SE_{LSM}: 4.61 ± 0.37 % compared to 3.31 ± 0.37 %; $P = 0.001$) (Figure 5.1 b). Accordingly, the distance between capillaries was 28.28 ± 0.61 µm in MST, compared to 24.52 ± 0.61 µm in MLD (LSM ± SE_{LSM}; $P < 0.0001$) (Figure 5.1 c). In addition, one capillary in MST supplied more muscle fibers than in MLD (LSM ± SE_{LSM}: 4.88 ± 0.64 fibers/capillary compared to 3.54 ± 0.64 fibers/capillary; $P = 0.040$) (Figure 5.1 d).

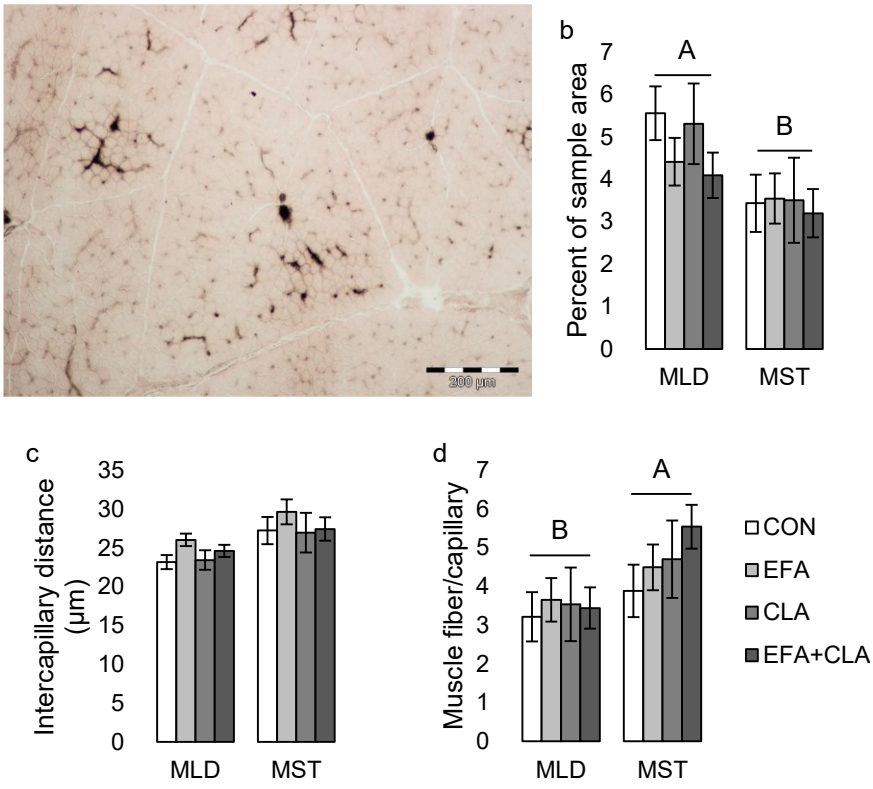

Figure 5.1: *Capillarization in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). Capillaries were detected in tissue cross-sections stained for alkaline phosphatase. (a) Representative image of cross-section with capillaries stained in black. Scale bar = 200 µm. (b) Percentage of capillary area of total sample area. (c) Distance between capillaries (µm). (d) Number of muscle fibers per capillary. Data are given as LSM ± SE_{LSM}. ^{A,B} Different superscripts indicate significant differences between muscles ($P < 0.05$).*

5.2.3 Total muscle fiber number and fiber type composition

To assess the effect of maternal fatty acid supplementation on muscle fiber growth, the skeletal muscle size of MLD and MST was analyzed. Skeletal muscle size is defined both by the total muscle fiber number and by the size of the individual fiber. The apparent total muscle fiber number was determined from the muscle cross-sectional area and the number of muscle fibers per area unit, quantified during muscle fiber measurements (Figure 5.3 c). The investigated muscles differed in their cross-sectional area and, consequently, the apparent total muscle fiber number was greater in MLD than in MST (LSM ± SE_{LSM}: 6.06 ± 0.16 × 10^6 fibers compared to 3.68 ± 0.16 × 10^6 fibers; P < 0.0001). In both MLD and MST, the supplementation did not influence the apparent total muscle fiber number (P > 0.847) (Figure 5.2). The individual fibers were examined in the following section.

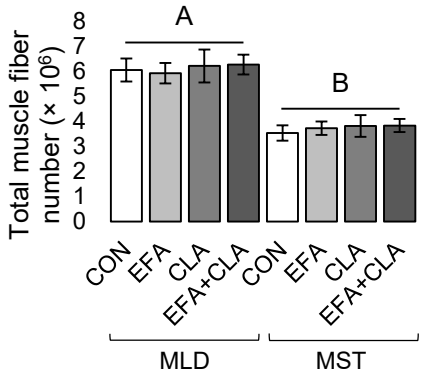

Figure 5.2: *Apparent total muscle fiber number (× 10^6) in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). Total muscle fiber number was extrapolated from the muscle cross-section area (mm²) and the number of muscle fibers per area unit. Data are given as LSM ± SE_{LSM}. ^{A,B} Different superscripts indicate significant differences between muscles (P < 0.05).*

Compared to the muscle fiber number, the metabolic properties of offspring skeletal muscles are more responsive to changes in maternal nutrition during the late gestation and after birth (Wegner *et al.*, 2000; Du *et al.*, 2010a). Skeletal muscle fiber types are established markers for muscle metabolic properties (Astruc, 2014). Therefore, the calf muscle fiber type composition was examined. From the H/E respectively ATPase stained serial sections, three muscle fiber types and fiber nuclei could be identified (Figure 5.3 a and b). The three fiber types had a similar cross-sectional area, area percentage and number of nuclei per fiber in all four groups (P > 0.072) (Figure 5.3 c–e). The fiber composition differed between the muscles, though. Fibers in MST had a bigger cross-sectional area than in MLD (LSM ± SE_{LSM}: 485.99 ± 9.22 µm² compared to 315.51 ± 9.22 µm²; P < 0.0001). Fast fibers had the largest muscle fiber cross-sectional area in both muscles, compared to intermediate and slow fibers (LSM ± SE_{LSM}:

557.40 ± 11.29 µm^2 compared to 334.58 ± 11.29 µm^2, respectively 310.28 ± 11.29 µm^2; P < 0.0001). Fast fibers had a higher area percentage in MLD than in MST (LSM ± SE_{LSM}: 55.05 ± 0.97 % compared to 47.29 ± 0.97 %; P < 0.0001). Intermediate fibers had a higher area percentage in MST than in MLD (LSM ± SE_{LSM}: 37.41 ± 0.82 % compared to 27.88 ± 0.82 %; P > 0.0001). The area percentage of slow fibers was similar between the muscles (P = 0.177). More nuclei per fiber were detected in MLD than in MST (LSM ± SE_{LSM}: 1.17 ± 0.04 nuclei/fiber compared to 0.49 ± 0.04 nuclei/fiber; P < 0.0001). When comparing the number of nuclei per fiber type in MLD, there was a trend toward more nuclei per fast fiber than per intermediate fiber (LSM ± SE_{LSM}: 1.28 ± 0.06 nuclei/fiber compared to 1.02 ± 0.06 nuclei/fiber; P = 0.069), but no difference to slow fibers (P > 0.366). In MST, the fiber types did not differ in the number of nuclei per fiber (P > 0.562).

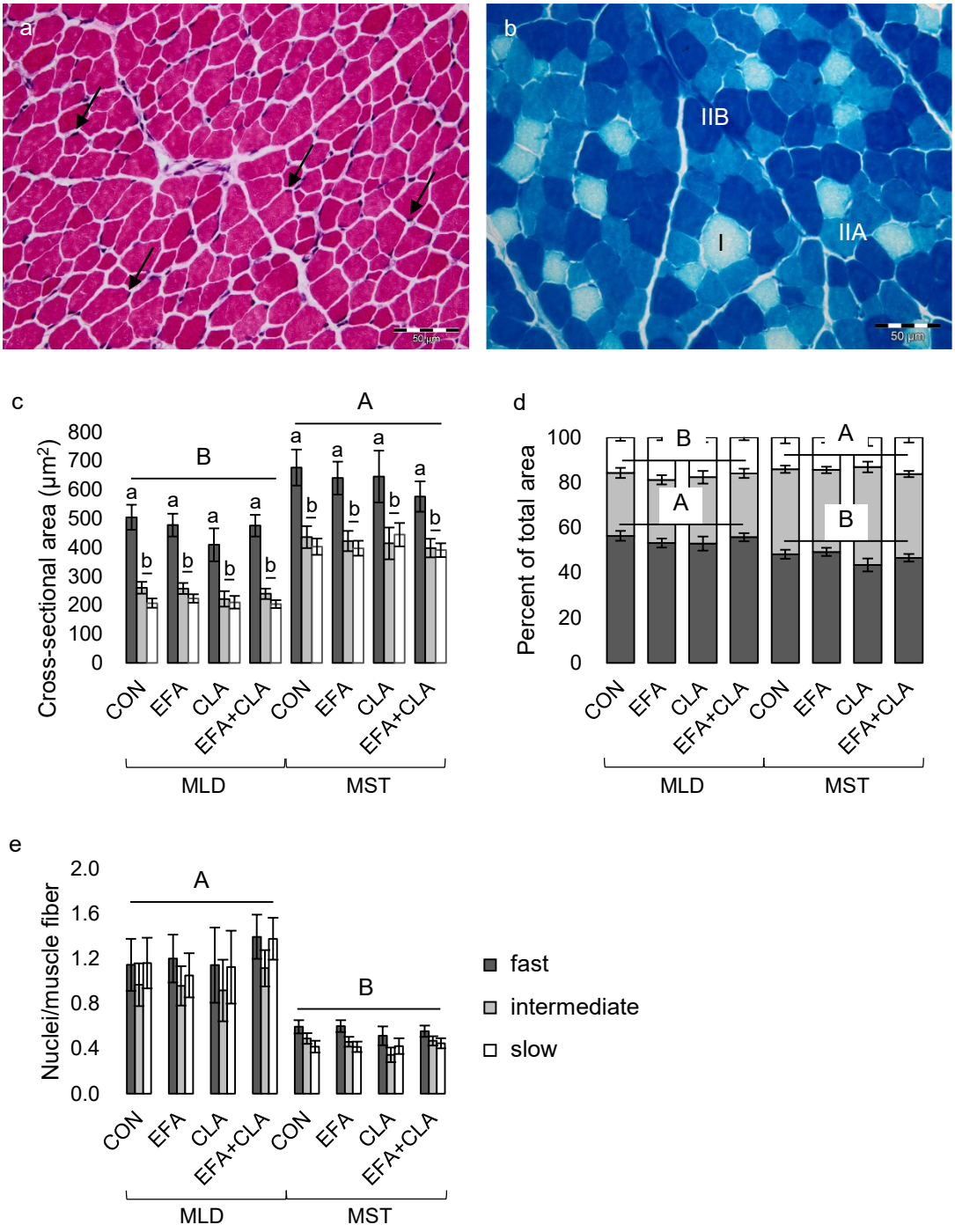

Figure 5.3: *Distribution of muscle fiber types in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). (a) Image of muscle cross-section stained with H/E. Arrows indicate exemplary muscle fiber nuclei. (b) Image of muscle cross-section stained for myofibrillar adenosine 5'-triphosphatase with alkaline preincubation. Exemplary fibers of the three fiber types are indicated as slow fiber (I), intermediate fiber (IIA) and fast fiber (IIB). Scale bars = 50 µm. (c) Muscle fibre cross-sectional area (µm²). (d) Area percentage per fiber type. (e) Number of nuclei per muscle fiber of each fiber type. Data are given as LSM ± SE_{LSM}. ^{A,B} Different superscripts indicate significant differences between muscles (P < 0.05). ^{a,b} Different superscripts indicate significant differences among fiber types (P < 0.05).*

5.2.4 Transition of muscle fiber types

The results on muscle fiber type composition reflect the state at day five of calf life, but in the early postnatal period fiber type switches still occur (Wegner *et al.*, 2000). The ATPase staining does not allow identification of these transitional fibres. In order to examine fiber type transitions, two different myosin heavy chain isoforms, isoform 2 (MYH2) and isoform 7 (MYH7), were detected via immunohistochemical staining of muscle cross-sections from MLD (Figure 5.4 a and b, respectively). Myosin heavy chain isoform 2 is mostly expressed in fast and intermediate fibres, MYH7 is mostly expressed in slow muscle fibres (Schiaffino and Reggiani, 2011). In MLD, the area percentage of fast and intermediate fibers correlated with the area percentage of MYH2 (Spearman correlation coefficient: 0.55, $P = 0.001$). Correspondingly, the area percentage of slow muscle fibers correlated with the area percentage of MYH7 (Spearman correlation coefficient: 0.71, $P < 0.0001$). The area percentage of MYH2 and MYH7 expressing fibers was similar between the supplementation groups ($P > 0.51$) (Figure 5.4 d). In MST, three samples were stained for MYH2 and MYH7 in the same manner as in MLD. The three samples showed similar correlation between fiber types and MYH isoform percentage as observed in MLD (data not shown). In the overlay picture of the MYH2 and MYH7 stained MLD cross-sections, muscle fibers expressing both MYH2 and 7 were detected (Figure 5.4 c). These fibers were quantified and categorized according to their fluorescence intensity (stronger signal for MYH2 than MYH7, reverse or equal signal intensity). The apparent higher number of hybrid fibers giving stronger signal for MYH2, as observed in the EFA, CLA and EFA+CLA group, was not significant compared to CON ($P > 0.460$) (Figure 5.4 d). The total number of hybrid fibers per mm^2 (no./mm^2) was similar among groups (LSM ± SE_{LSM}: 100 ± 20 no./mm^2 in the CON group, 72 ± 18 no./mm^2 in the EFA group, 81 ± 27 no./mm^2 in the CLA group and 91 ± 18 no./mm^2 in the EFA+CLA group; $P > 0.695$).

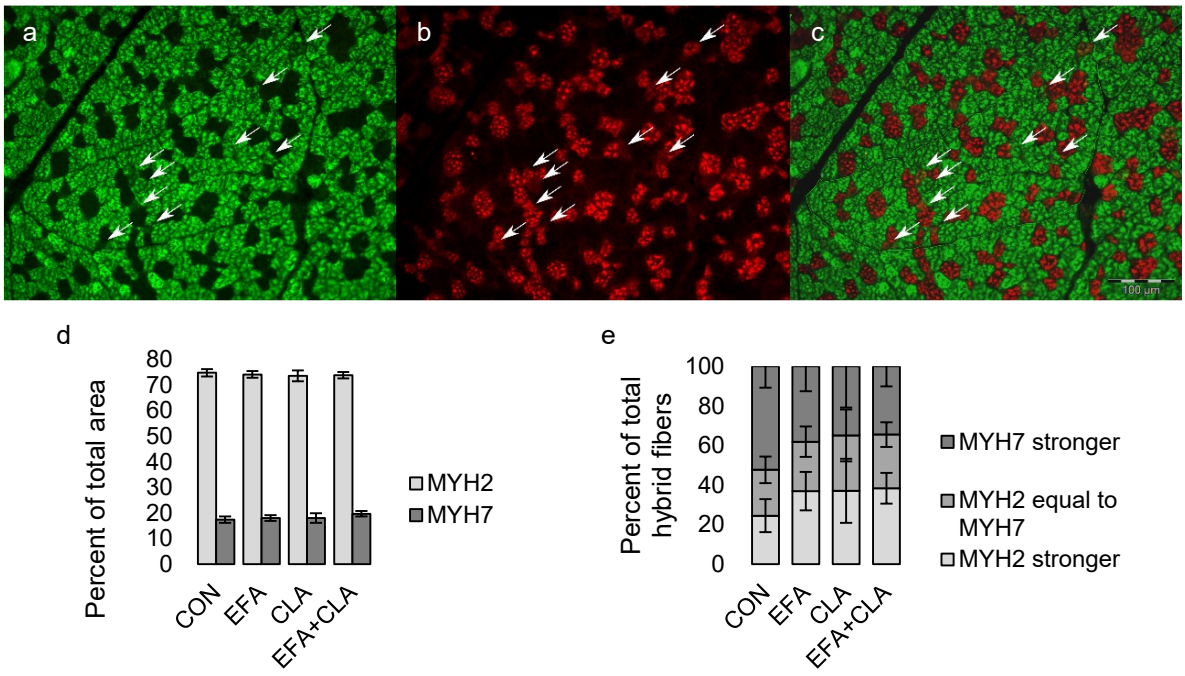

Figure 5.4: *Detection of hybrid fibers in longissimus muscle of calves of four different supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). (a–c) Representative immunofluorescence images of muscle cross-sections stained for (a) MYH2, (b) MYH7 and (c) these merged. Images were taken in the (a) green and (b) red fluorescence channel. In the three images, arrows indicate hybrid fibers. Scale bar = 100 µm. (d) Area percentage of muscle fibers expressing MYH2 and MYH7. (e) Hybrid fibers were categorized depending on signal intensity. The graph shows the percentage of each hybrid fiber group of the total hybrid fibers. Data are given as LSM ± SE_{LSM}.*

5.2.5 Protein abundance of muscle fiber MYH isoforms

In addition to immunohistochemical localization, the protein abundance of different myosin heavy chain isoforms was measured in samples of MLD and MST. In both muscles, MYH2, MYH7 and MYH1 were detected with western blot and quantified using the SPL system (Faden *et al.*, 2016) (Figure 5.5 a). In MLD, MYH1 was increased in the CLA group, compared to the EFA group (P = 0.032). The relative protein abundance of MYH1 in MST and of MYH2 and MYH7 in both MLD and MST was similar between groups (P > 0.20) (Figure 5.5 b).

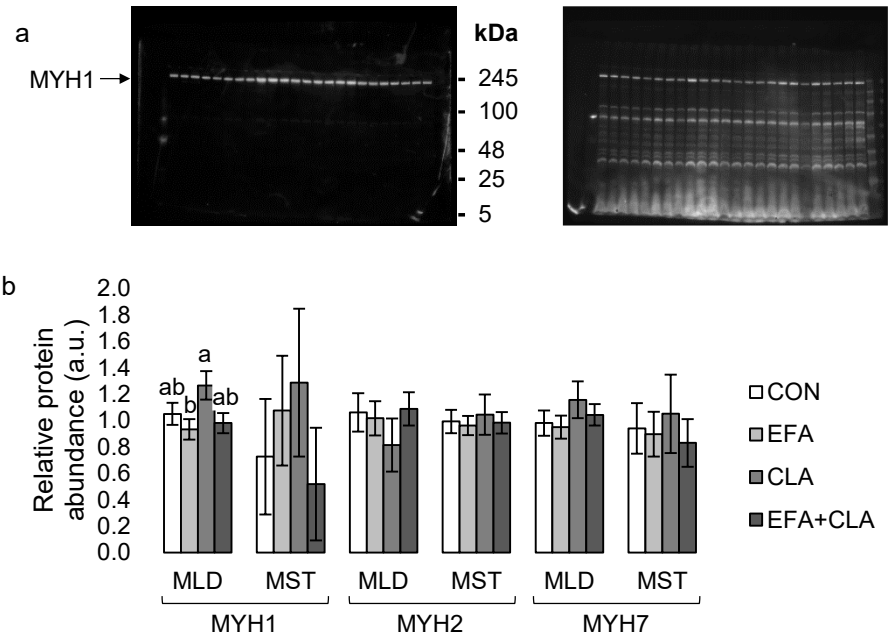

Figure 5.5: *Protein quantification of myosin heavy chain isoform 1 (MYH1), 2 (MYH2) and 7 (MYH7) in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8 (MLD), n = 5 (MST)), supplementation with essential fatty acids (EFA, n = 9 (MLD), n = 7 (MST)), with conjugated linoleic acids (CLA, n = 8 (MLD), n = 5 (MST)), with EFA and CLA combined (EFA+CLA n = 11 (MLD), n = 7 (MST)), via western blot combined with the SPL system. (a) Representative western blot images of MYH1 detection in MLD, with target protein (left, indicated by arrow) and total protein (right). Protein size standard (kDa). (b) The relative protein abundance was normalized against the total protein abundance. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.). MYH2 and MYH7: Representative blot images of target and total protein are displayed in Figure 9.1.* ^{a,b} *Different superscripts indicate significant differences among groups (P < 0.05).*

In order to compare two protein quantification systems, the protein abundance of MYH1, MYH2, MYH7 and MYH4 (fast fiber marker; Schiaffino and Reggiani, 2011) in MLD was additionally measured with Jess Simple Western (Jess SW) (Figure 5.6). There was no group difference in the relative protein abundance of MYH1, MYH2 and MYH4 (P > 0.122). In each run, MYH7 was detected alongside with one of the other isoforms, resulting in three MYH7 measurements per sample. The repeated measurement of MYH7 enabled detection of lower amounts of MYH7 in the MLD of EFA+CLA calves than of CON calves (P = 0.013). Because the quantification and normalization procedure was optimized for each isoform individually and two different detection modules, chemiluminescence or fluorescence, were applied, a comparison of the abundance of different isoforms is not possible.

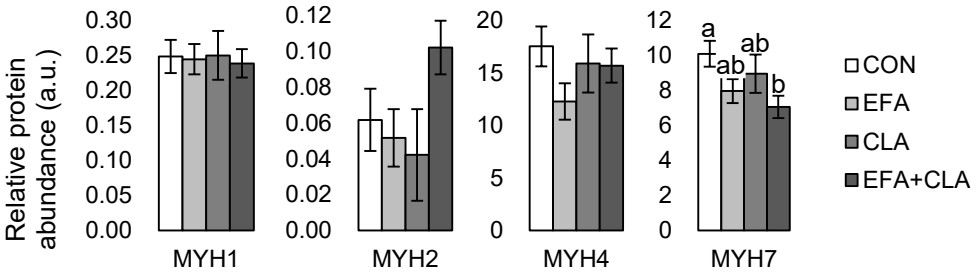

Figure 5.6: *Protein quantification of myosin heavy chain isoform 1 (MYH1), 2 (MYH2), 4 (MYH4) and 7 (MYH7) in longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11), with Jess SW. For each sample, the relative protein abundance was normalized against the total protein abundance. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).* ^{a,b} *Different superscripts indicate significant differences among groups (P < 0.05).*

5.2.6 Relative mRNA abundance of muscle metabolic markers

Effects of FA supplementation on muscle metabolism that did not manifest on a morphological or protein level might be detectable on the mRNA level. To detect early changes in muscle metabolism, the mRNA abundance of muscle metabolic marker genes peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*) and prospero homeobox 1 (*PROX1*) was measured. Their relative mRNA abundance in MLD and MST showed strong intergroup variation, but did not differ between the groups (P > 0.171) (Figure 5.7). When compared to muscle composition characteristics, *PPARGC1A* mRNA abundance correlated positively with the apparent total muscle fiber number in MLD. Spearman correlation coefficient was 0.373 (P = 0.025). In MST, this effect was not observed. There was no correlation between *PROX1* mRNA abundance and muscle fiber types analyzed with ATPase staining, neither with hybrid fiber abundance.

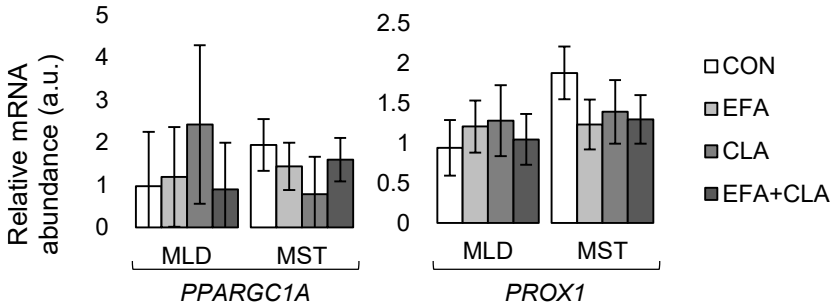

Figure 5.7: *Relative mRNA abundance of muscle metabolic markers peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PPARGC1A*) and prospero-related homeobox 1 (*PROX1*) in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Quantification cycle values of target genes* PPARGC1A *and* PROX1 *were normalized against reference genes* PPIA *and* UXT *via geometrical mean. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).*

5.2.7 Abundance of intramuscular lipid droplets

Apart from effects on skeletal muscle cells, supplementation with *n*-3 PUFAs or CLAs was shown to influence intramuscular adipogenesis in cattle (Cooke *et al.*, 2011; Zhang *et al.*, 2016). In the muscle tissues of the 5-day-old calves, intramuscular adipocytes (respectively intramuscular fat, IMF) were scarce. The low overall number of adipocytes did not allow for individual measurement of a representative number of adipocytes, as it was applied in the adipose tissues. Instead, the intramuscular adipocyte lipids were detected by staining muscle cross-sections with Oil Red O, and the adipocyte lipid area was measured. Figure 5.8 a shows an image of the sample with the greatest lipid droplet area among all samples (not representative). The percentage of lipid droplet area of the total sample area did not differ between muscle tissues (Figure 5.8 b), with 0.026 ± 0.011 % in MLD and 0.035 ± 0.011 % in MST (LSM ± SE_{LSM}) (P = 0.557). In both muscles, approximately 23 of 36 samples had an adipocyte area of less than 0.01 %, approximately 10 samples had an adipocyte area of 0.01 to 0.1 % and three samples had an adipocyte area of more than 0.1 % per total sample area. In MST, the apparently greater lipid droplet area percentage of the EFA+CLA group was similar to the other groups (P > 0.2347).

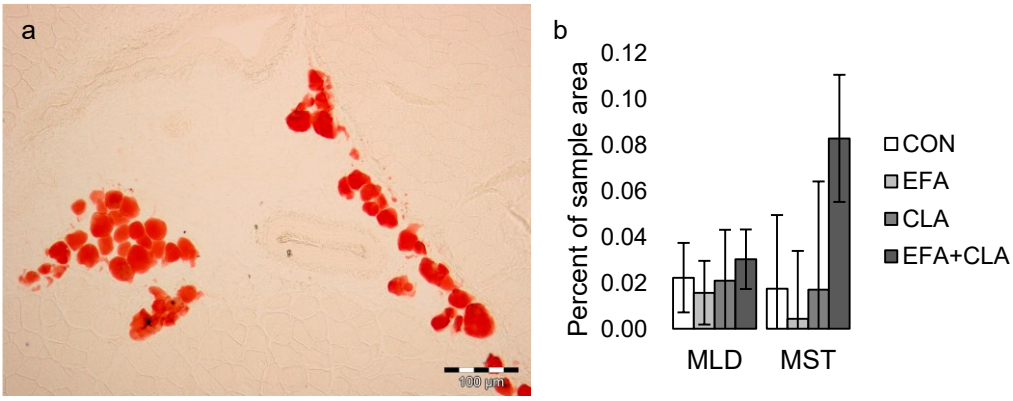

Figure 5.8: *Intramuscular adipocytes in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA n = 11). (a) Cross-section stained with Oil Red O, image of the sample with the greatest measured lipid droplet area. Lipid droplets are stained red. Scale bar = 100 µm. (b) Intramuscular adipocyte lipid droplet abundance as percentage of lipid area per total sample area in the Oil Red O stained muscle cross-sections. Data are given as LSM ± SE_{LSM}.*

In addition, the effect of FA supplementation on the incorporation of lipid droplets into the muscle fibers was to be assessed. These intramyocellular lipid droplets (IMCLs) could be observed in muscle sections stained with Oil Red O, Sudan Black B and Nile Blue staining (Figure 5.9 a–c). To test a connection between lipid droplets and fiber type, an MLD cross-section of one calf sample was prestained with Sudan Black B before immunohistochemistry

staining of MYH7. It could be observed that fibers with visible lipid droplets overlapped with slow fibers (Figure 5.9 d–f).

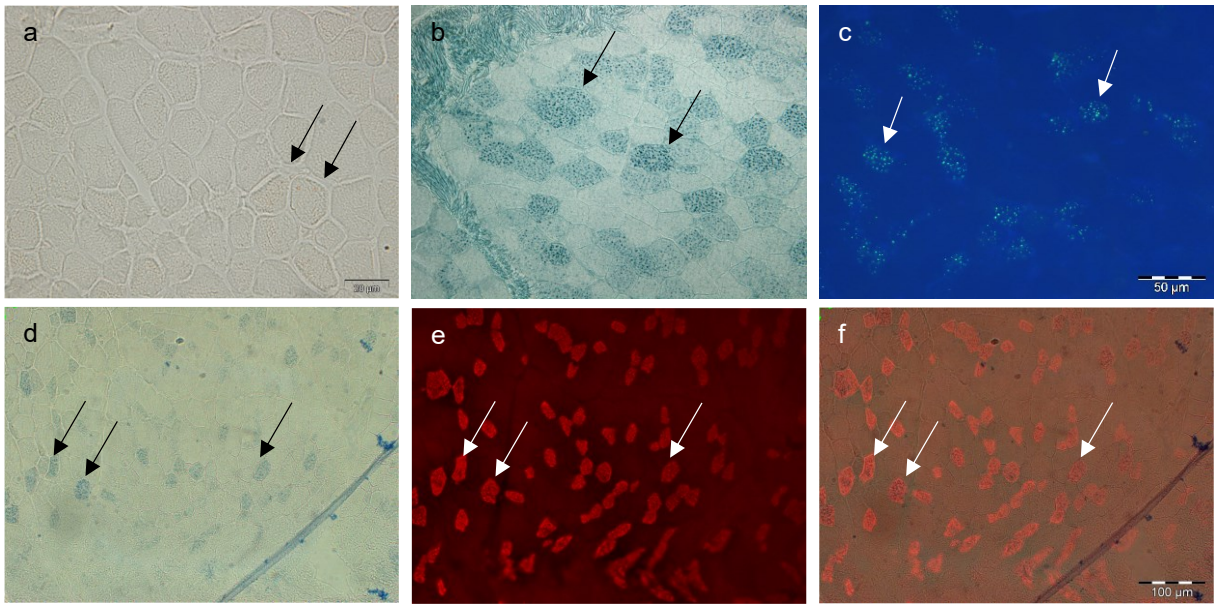

Figure 5.9: *Histological staining of intramyocellular lipid droplets in skeletal muscle. (a–c) Cross-sections of (a and c) longissimus and (b) semitendinosus muscle were stained with (a) Oil Red O, (b) Sudan Black B and (c) Nile Blue. The Images were taken with (a and b) visible light (bright field image) and (c) blue fluorescence channel. (a) Scale bar = 20 µm, (b and c) Scale bar = 50 µm. Arrows indicate exemplary fibers with intramyocellular lipid droplets. (d–f) Overlap of intramyocellular lipid droplets with myosin heavy chain 7 (MYH7) expression in longissimus muscle fibers. (d) Image of a cross-section prestained with Sudan Black B before (e) immunohistochemical staining of MYH7, (f) overlap of the two images. Images were taken with (a) visible light (bright field image) and (b) red fluorescence channel. Scale bar = 100 µm. Arrows indicate exemplary fibers with intramyocellular lipid droplets and expression of MYH7.*

5.2.8 Intramuscular adipocyte precursors

Oil Red O staining allows to detect mature adipocytes with lipid droplets. To examine the effect of maternal supplementation on earlier stages of adipogenesis, several markers were detected that represent different stages in the adipogenic process. As preadipocyte markers, delta like non-canonical Notch ligand 1 (DLK-1) and platelet-derived growth factor receptor alpha (PDGFRα) were chosen. As early differentiation markers, CCAAT/enhancer binding protein beta (C/EBPβ) and zinc finger protein 423 (ZNF423), and as late differentiation markers CCAAT/enhancer-binding protein alpha (C/EBPα), peroxisome proliferator activated receptor gamma isoform 2 (PPARγ2) and lipoprotein lipase (LPL) were selected. Via immunohistochemistry staining, cells expressing preadipocyte marker DLK-1 could be identified in MLD and MST muscle cross-sections (Figure 5.10 a). The cells expressing DLK-1 had a fibroblast-like shape, were located in the connective tissue between muscle fibers, and,

at higher abundance, close to blood vessels. Within the cells, the DLK-1 signal was detected in the cytoplasm. In MLD, the FA supplementation did not affect the number of DLK-1 expressing cells per mm^2 of cross-sectional area ($P > 0.418$). In MST though, FA supplementation reduced the number of DLK-1 expressing cells in the EFA group ($P = 0.028$), and, with a trend, in the CLA group ($P = 0.098$) and the EFA+CLA group ($P = 0.071$), compared to CON (Figure 5.10 b).

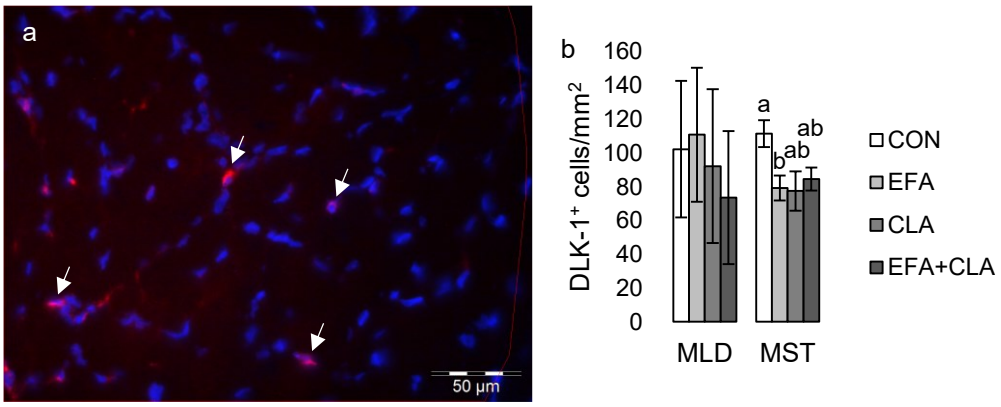

Figure 5.10: *Localization and quantification of delta like non-canonical Notch ligand 1 (DLK-1) expressing cells in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA n = 11). (a) Representative overlay image of immunohistochemical staining of DLK-1 and nuclear staining with Hoechst 33258, acquired in the red (DLK-1) and blue (nuclei) fluorescence channel. Arrows indicate exemplary DLK-1 expressing cells. Scale bar = 50 µm. (b) Number of DLK-1 expressing cells per mm^2 of muscle cross-sectional area in MLD and MST. Data are given as LSM ± SE_{LSM}. ^{a,b} Different superscripts indicate significant differences among groups ($P < 0.05$).*

Nuclei expressing early marker C/EBPβ could be detected via immunohistochemical staining of MLD in eight samples. It could be observed that these nuclei were located between muscle fibers and within the connective tissue (Figure 5.11 a–d). However, the nuclei within the connective tissue, within developing adipocyte clusters, and of cells with small lipid droplets had a higher signal intensity than the nuclei in the surrounding muscle tissue. To test the relation between cells expressing C/EBPβ and DLK-1, the two proteins were simultaneously stained via immunohistochemistry of MLD muscle cross-sections of eight samples (Figure 5.11 e and f). As expected, cells were positive for either nuclear C/EBPβ or cytoplasmic DLK-1. The majority of cells was located within the connective tissue rather than between muscle fibers.

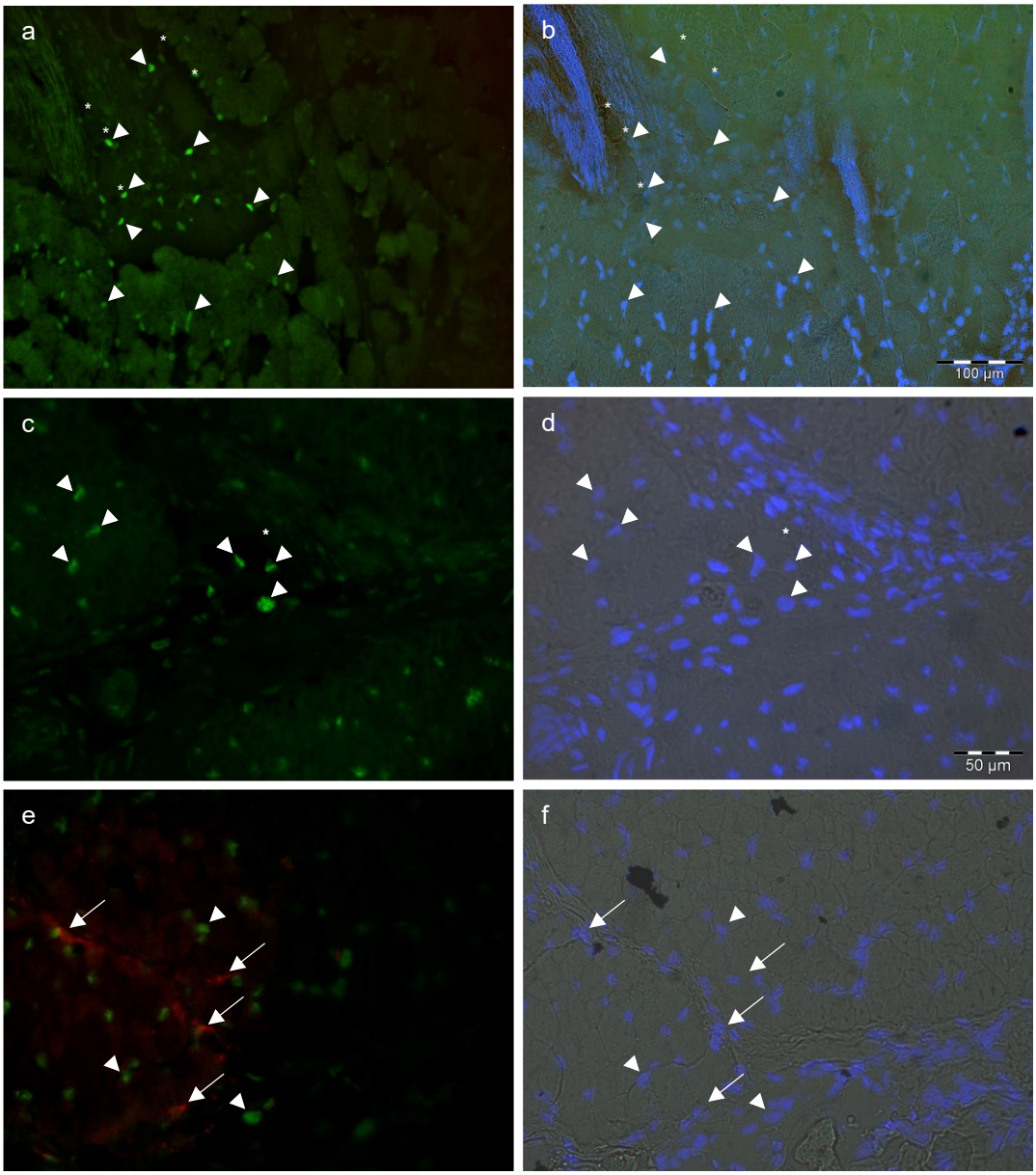

Figure 5.11: *Detection of CCAAT/enhancer-binding protein beta (C/EBPβ) and colocalization with delta like non-canonical Notch ligand 1 (DLK-1) in longissimus muscle via immunohistochemical staining. (a and c) Representative images of CEBPβ staining, acquired in the green fluorescence channel. Arrowheads indicate C/EBPβ positive nuclei. Asterisks indicate lipid droplets. (e and f) Simultaneous staining of C/EBPβ and DLK-1. (e) Representative overlay image, acquired in the green (C/EBPβ) and red (DLK-1) fluorescence channel. Arrows indicate DLK-1 expressing cells; arrowheads indicate C/EBPβ positive nuclei. (b, d and f) Corresponding overlay images of nuclear staining (with Hoechst 33258) and muscle cross-section, acquired in the blue fluorescence channel (nuclei) and with visible light (bright field image). (a and b) Scale bar = 100 µm. (c–f) Scale bar = 50 µm.*

In addition to the localization of DLK-1 protein, the protein abundance was determined via western blot and quantified via the Smart Protein Layers system (WB SPL). At first, the anti-DLK-1 rabbit polyclonal antibody ab21682 (Abcam PLC) and the anti-DLK-1 mouse monoclonal antibody sc-376755 (Santa Cruz Biotechnology, Inc.) were tested in western blot (Figure 9.2 a). The polyclonal antibody produced a band at approximately 55 kDa in both muscle and fat samples, which was strongly reduced by antibody preincubation with blocking

peptide ab23442 (Abcam PLC). In a test incubation of protein fractions from cytosol and nucleus, the signal could be assigned to the cytoplasmic cell fraction, confirming specificity of the band (Figure 9.2 b). The monoclonal antibody did not yield a specific signal. Based on these tests, the relative protein abundance of DLK-1 in MLD was quantified with the rabbit polyclonal antibody via SPL, The relative protein abundance was similar between the groups ($P > 0.763$) (Figure 5.12). Representative blot images of target and total protein are displayed in the supplement (Figure 9.2 c)

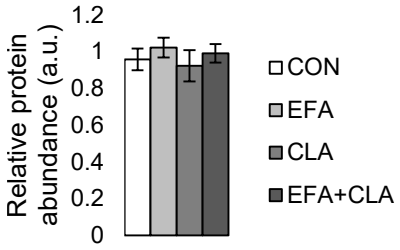

Figure 5.12: *Protein quantification of delta like non-canonical Notch ligand 1 (DLK-1) via WB SPL in longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). The relative protein abundance was normalized against the total protein abundance. Representative blot images of target and total protein are displayed in the supplement. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).*

To detect supplementation effects not yet manifested on the morphological or protein level, the relative mRNA abundance of adipogenic markers was measured (Figure 5.13). Supplementation of EFA reduced the mRNA abundance of *DLK1* in MLD in the two EFA groups, compared to the CLA group ($P < 0.05$). Markers CCAAT enhancer-binding protein beta (*CEBPB*), platelet derived growth factor receptor alpha (*PDGFRA*) and lipoprotein lipase (*LPL*) in MLD and *DLK1* and zinc finger protein 423 (*ZNF423*) in MST did not differ among groups ($P > 0.257$). For several genes, amplification efficiency (E) and linearity of standard curve (R^2) of at least one primer pair were tested in both muscles. Due to low efficiency and/or R^2 value, primer pairs were not used to measure mRNA abundance in individual samples. In MLD, this was the case for *CEBPA* (E: 46 %, R^2: 0.93), and *ZNF423* (E: 71 %, R^2: 0.99). In MST, this was the case for *CEBPA* (E: 51 %, R^2: 0.91), *CEBPB* (E: 35 %, R^2: 0.74) and peroxisome proliferator activated receptor gamma isoform 2 (*PPARG2*) (E: 73 %, R^2: 0.99). Because no group differences were detected in MLD, forkhead box O1 (*FOXO1*), *LPL* and *PDGFRA* mRNA abundance was not measured in individual samples of MST. The primer pair against *PPARG2* was tested in a representative cDNA sample pool from MLD, via qPCR and PCR. It did not yield an amplification product. All tested primer pairs are listed in the supplement (Table 9.11).

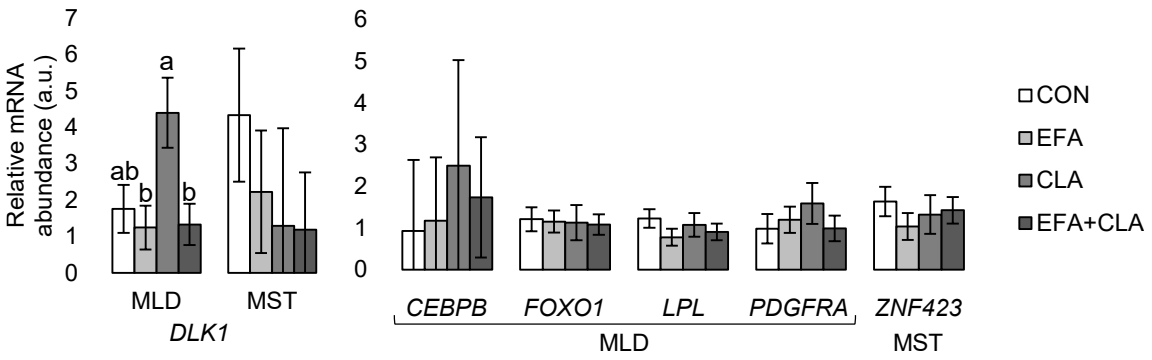

Figure 5.13: *Relative mRNA abundance of adipogenic markers in longissimus (MLD) and semitendinosus muscle (MST) of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 11). The quantification cycle values of target genes delta like non-canonical Notch ligand 1 (*DLK1*), CCAAT enhancer binding protein beta (*CEBPB*), forkhead box O1 (*FOXO1*), lipoprotein lipase (*LPL*), platelet derived growth factor receptor alpha (*PDGFRA*), and zinc finger protein 423 (*ZNF423*) normalized against reference genes* PPIA *and* UXT *via geometrical mean. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).* ^{a,b} *Different superscripts indicate significant differences among groups (P < 0.05).*

In summary, the maternal FA supplementation altered the FA composition in the calf muscle tissues, and especially increased the tissue abundance of *n*-3 PUFAs and CLAs. While it did not affect the tissue morphology, the CLA supplementation decreased the protein abundance of MYH7 and increased the protein abundance of MYH1. In the intramuscular fat (IMF) from MST, the supplementation with EFAs decreased the abundance of cells expressing preadipocyte marker DLK-1. Similarly, it decreased the mRNA abundance of *DLK1* in IMF from MLD.

5.3 Effects of maternal supplementation on adipose tissues

Apart from the skeletal muscle tissues, the ability to influence offspring adipose tissues via maternal supplementation is of great interest for cattle industry (Du *et al.*, 2017). How supplementation influences individual adipose tissues is also connected to their developmental stage. In this study, the calf intermuscular fat (INF) as a late developing adipose tissue was analyzed in comparison to subcutaneous fat (SCF) and the early developing perirenal fat (PF).

5.3.1 Fatty acid composition of adipose tissues

As in the muscle tissues, the supplemented FAs must be incorporated into the adipose tissues in order to induce specific changes. To verify transfer into the adipose tissues, the tissue FA profiles of INF (at MLD), SCF and PF were determined. Over all of the five-day-old calves, the total fat percentage ranged from 0.81 to 45.33 % in the INF (at MLD), and from 3.8 to 42.1 % in the SCF, showing that these fat depots were not fully developed in all calves. Due to the high variation between samples, the percentage of total FAs in INF and SCF is presented, instead of the absolute fat content (mg/100 g tissue) (Table 5.3 and Table 5.4, respectively). For PF, the FA concentration (mg/100 g tissue) is shown (Table 5.5). The tables list the supplemented FAs, longer-chain and sum of *n*-3 and *n*-6 PUFAs, selected MUFAs and SFAs, the total sum of PUFAs, MUFAs, SFAs and total fat content (%). Fatty acid concentration and composition of all measured FAs are shown in the Supplement (Table 9.5–Table 9.10).

The EFA supplementation affected ALA and longer-chain *n*-3 PUFA concentration in all three tissues, and SA in SCF. Supplementation with CLAs affected *cis*-9, *trans*-11 CLA in all three tissues, HA (including the signal for *trans*-10, *cis*-12 CLA) in SCF and PF, but not in INF, and C18:3 *n*-6 (γ-linolenic acid, GLA) in SCF.

In the INF (at MLD), EFA supplementation increased ALA in the EFA group in comparison to the CLA group ($P = 0.040$). Longer-chain *n*-3 PUFA EPA was increased by EFA supplementation, but without significant differences between individual groups ($P > 0.061$). Simultaneous supplementation of CLAs with EFAs further increased ALA in the EFA+CLA group, compared to CON and CLA ($P < 0.05$), and the sum of *n*-3 PUFAs, compared to the CLA group ($P = 0.044$). Supplementation with CLAs increased *cis*-9, *trans*-11 CLA in the EFA+CLA group, compared to the EFA group ($P = 0.029$). Of the saturated FAs, supplementation with EFA+CLA increased SA ($P = 0.033$) and tended to increase the sum of SFAs ($P = 0.095$), compared to the CON group. Longer-chain *n*-3 PUFAs SDA, DPA and DHA, LA and longer-chain *n*-6 PUFAs GLA, dihomo-γ-linolenic acid (DHGLA), ARA and ADA, the sum of *n*-6 PUFAs and the sum of PUFAs were unaffected by supplementation ($P > 0.114$). Monounsaturated FAs OA, VA, saturated FAs PA and HA (including signal for *trans*-10, *cis*-12

CLA) and total fat content (%) were unaffected by supplementation as well (P > 0.216). The percentage of longer-chain *n*-3 PUFA SDA was too low to be quantified in 14 of 36 samples.
In the SCF, the EFA supplement increased ALA in both EFA groups, compared to CON and CLA (P < 0.01). In addition, the sum of *n*-3 PUFAs was increased in both EFA groups, compared to CON and CLA (P < 0.005). Longer chain *n*-3 PUFA EPA was increased in both EFA groups, compared to CON (P < 0.05), while longer chain PUFA DPA was increased in the single EFA group, compared to CON (P = 0.039). Supplementation with CLA increased *cis*-9, *trans*-11 CLA (P < 0.05) and HA (including *trans*-10, *cis*-12 CLA) (P < 0.005) in both CLA and EFA+CLA group, compared to CON and EFA. Of the *n*-6 PUFAs, GLA was increased by CLA supplementation, compared to the other three groups (P < 0.0001). This effect was not observed for the other *n*-6 PUFAs (P > 0.151). Supplementation with EFA+CLA led to reduced percentage of longer chain *n*-6 PUFA ADA, compared to CON (P = 0.04). The combined supplementation also increased SA, compared to CON (P = 0.006). The maternal supplementation did not affect the percentage of *n*-3 PUFA DHA, *n*-6 PUFAs LA, DHGLA, ARA, the sum of *n*-6 PUFAs and the sum of PUFAs, the sum of MUFAs, OA and VA, the sum of SFAs and SFA PA as well as total fat content (g/100g tissue) (P > 0.112). The percentage of longer-chain *n*-3 PUFAs EPA, DPA and DHA was too low to be quantified in 9, 2 and 7 of 35 samples, respectively.
In the PF, CLA supplementation increased *cis*-9, *trans*-11 CLA in both CLA groups, compared to the EFA group (P < 0.05). Supplementation with CLA also increased the tissue concentration of HA (including *trans*-10, *cis*-12 CLA), which was attenuated by the EFA supplement in the EFA+CLA group, compared to CON and EFA (P < 0.01). Supplementation with EFA increased ALA in both EFA groups, compared to CON (P < 0.05); double supplementation with EFA+CLA further increased ALA compared to CLA (P = 0.001). The sum of *n*-3 PUFAs, and in particular longer chain *n*-3 PUFAs EPA and DPA were elevated in the EFA groups (P < 0.005). Double supplementation tended to increase the sum of PUFAs compared to the CON group (P = 0.079). The FA supplementation did not change the tissue concentration of longer-chain *n*-3 PUFAs DHA, LA and longer chain *n*-6 PUFAs GLA, DHGLA, ARA, ADA, the sum of *n*-6 PUFAs, monounsaturated FAs OA, VA, the sum of MUFAs, saturated FAs PA, SA and the sum of SFAs as well as total fat content (%) (P > 0.108).

Table 5.3: *Fatty acid composition in intermuscular fat of calves of four maternal supplementation groups: control group (CON, n = 7), essential fatty acids (EFA, n = 8), conjugated linoleic acids (CLA, n = 5) and EFA and CLA combined (EFA+CLA, n = 7). Data are given as LSM ± SE_{LSM}. Results for all measured fatty acids are listed in the supplement.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11 (CLA)	0.11 ± 0.02^{ab}	0.08 ± 0.02^{b}	0.13 ± 0.03^{ab}	0.16 ± 0.02^{a}	0.897	0.023	0.191
C18:3 *n*-3 (ALA)	0.14 ± 0.07^{bc}	0.40 ± 0.06^{ab}	0.06 ± 0.10^{c}	0.48 ± 0.07^{a}	<.001	0.951	0.313
C18:4 *n*-3 (SDA)	0.02 ± 0.01	0.03 ± 0.01	NA	0.02 ± 0.01	0.190	0.084	0.343
C20:5 *n*-3 (EPA)	0.02 ± 0.02	0.07 ± 0.02	0.00 ± 0.02	0.06 ± 0.02	0.005	0.293	0.643
C22:5 *n*-3 (DPA)	0.21 ± 0.08	0.25 ± 0.07	0.05 ± 0.10	0.24 ± 0.08	0.132	0.221	0.295
C22:6 *n*-3 (DHA)	0.05 ± 0.02	0.06 ± 0.02	0.01 ± 0.03	0.05 ± 0.02	0.247	0.245	0.372
Sum of *n*-3 PUFAs[1]	0.48 ± 0.16^{ab}	0.81 ± 0.14^{ab}	0.16 ± 0.23^{b}	0.88 ± 0.15^{a}	0.006	0.460	0.243
C18:2 *n*-6 (LA)	2.08 ± 0.32	1.86 ± 0.28	1.57 ± 0.43	2.13 ± 0.30	0.583	0.703	0.205
C18:3 *n*-6 (GLA)	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.981	0.539	0.142
C20:3 *n*-6 (DHGLA)	0.47 ± 0.08	0.30 ± 0.07	0.18 ± 0.11	0.30 ± 0.08	0.753	0.072	0.082
C20:4 *n*-6 (ARA)	1.55 ± 0.36	0.77 ± 0.32	0.43 ± 0.48	0.77 ± 0.34	0.529	0.114	0.111
C22:4 *n*-6 (ADA)	0.51 ± 0.11	0.20 ± 0.09	0.16 ± 0.16	0.25 ± 0.10	0.343	0.180	0.087
Sum of *n*-6 PUFAs[2]	4.74 ± 0.81	3.26 ± 0.72	2.46 ± 1.09	3.59 ± 0.78	0.825	0.214	0.098
Sum of PUFAs[3]	5.20 ± 0.95	4.06 ± 0.84	2.61 ± 1.28	4.46 ± 0.91	0.702	0.233	0.107
C18:1 *cis*-9 (OA)	34.46 ± 0.94	33.35 ± 0.77	34.45 ± 1.34	32.84 ± 0.87	0.182	0.780	0.782
C18:1 *trans*-11 (VA)	0.22 ± 0.03	0.16 ± 0.03	0.12 ± 0.04	0.18 ± 0.03	0.993	0.298	0.137
Sum of MUFAs[4]	41.59 ± 1.01	40.54 ± 0.84	41.38 ± 1.42	38.53 ± 0.94	0.076	0.278	0.359
C16:0 (PA)	34.16 ± 1.34	35.76 ± 1.19	36.52 ± 1.78	35.66 ± 1.29	0.769	0.369	0.321
C18:0 (SA)	12.78 ± 0.60^{b}	13.54 ± 0.54^{ab}	13.46 ± 0.76^{ab}	14.70 ± 0.58^{a}	0.068	0.083	0.633
C21:0 (HA)[5]	0.09 ± 0.03	0.02 ± 0.02	0.05 ± 0.04	0.07 ± 0.03	0.318	0.976	0.116
Sum of SFAs[6]	53.12 ± 1.26	55.20 ± 1.08	55.93 ± 1.71	56.78 ± 1.20	0.249	0.082	0.595
Total fat content (%)	10.74 ± 3.94	9.78 ± 3.89	13.36 ± 5.63	6.79 ± 3.57	0.399	0.966	0.528

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. (Continued on next page)

[3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 +C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a-c] Different superscripts indicate significant differences among groups ($P < 0.05$).
NA: Not calculated by SAS software due to small sample size.

Table 5.4: *Fatty acid composition in subcutaneous fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 10). Data are given as LSM ± SE_{LSM}. Results for all measured fatty acids are listed in the supplement.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11 (CLA)	0.12 ± 0.02^{b}	0.09 ± 0.01^{b}	0.20 ± 0.02^{a}	0.17 ± 0.01^{a}	0.102	<.001	0.958
C18:3 *n*-3 (ALA)	0.10 ± 0.05^{b}	0.35 ± 0.04^{a}	0.06 ± 0.07^{b}	0.47 ± 0.04^{a}	<.001	0.484	0.167
C20:5 *n*-3 (EPA)	0.01 ± 0.01^{b}	0.03 ± 0.00^{a}	NA	0.03 ± 0.00^{a}	<.001	0.898	0.716
C22:5 *n*-3 (DPA)	0.08 ± 0.01^{b}	0.14 ± 0.01^{a}	NA	0.13 ± 0.01^{ab}	<.001	0.339	0.642
C22:6 *n*-3 (DHA)	0.03 ± 0.01	0.04 ± 0.00	NA	0.02 ± 0.00	0.067	0.049	0.686
Sum of *n*-3 PUFAs[1]	0.22 ± 0.07^{b}	0.57 ± 0.06^{a}	0.10 ± 0.09^{b}	0.68 ± 0.06^{a}	<.001	0.986	0.121
C18:2 *n*-6 (LA)	1.64 ± 0.15	1.78 ± 0.14	1.68 ± 0.22	1.78 ± 0.13	0.491	0.910	0.915
C18:3 *n*-6 (GLA)	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.11 ± 0.01^{a}	0.03 ± 0.00^{b}	<.001	<.001	<.001
C20:3 *n*-6 (DHGLA)	0.17 ± 0.01	0.17 ± 0.01	0.13 ± 0.02	0.15 ± 0.01	0.706	0.081	0.579
C20:4 *n*-6 (ARA)	0.30 ± 0.03	0.25 ± 0.03	0.24 ± 0.04	0.21 ± 0.03	0.218	0.164	0.795
C22:4 *n*-6 (ADA)	0.11 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.09 ± 0.02^{ab}	0.07 ± 0.01^{b}	0.031	0.240	0.659
Sum of *n*-6 PUFAs[2]	2.32 ± 0.19	2.37 ± 0.17	2.33 ± 0.27	2.30 ± 0.16	0.971	0.898	0.849
Sum of PUFAs[3]	2.71 ± 0.25	3.07 ± 0.23	2.79 ± 0.36	3.19 ± 0.22	0.169	0.705	0.938
C18:1 *cis*-9 (OA)	35.71 ± 0.90	33.82 ± 0.82	34.88 ± 1.29	33.97 ± 0.78	0.161	0.731	0.622
C18:1 *trans*-11 (VA)	0.17 ± 0.03	0.14 ± 0.03	0.21 ± 0.04	0.18 ± 0.03	0.425	0.184	0.973
Sum of MUFAs[4]	42.57 ± 0.93	40.72 ± 0.85	41.72 ± 1.34	40.27 ± 0.81	0.113	0.523	0.844
C16:0 (PA)	36.94 ± 0.80	38.01 ± 0.73	37.05 ± 1.14	37.35 ± 0.69	0.434	0.747	0.663
C18:0 (SA)	12.23 ± 0.34^{b}	13.08 ± 0.30^{ab}	12.77 ± 0.44^{ab}	13.56 ± 0.29^{a}	0.013	0.096	0.901
C21:0 (HA)[5]	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.05 ± 0.01^{a}	0.05 ± 0.00^{a}	0.947	<.001	0.838
Sum of SFAs[6]	54.72 ± 0.99	56.21 ± 0.91	55.49 ± 1.43	56.53 ± 0.86	0.248	0.614	0.839
Total fat content (g/100g tissue)	16.09 ± 3.99	16.21 ± 3.52	16.31 ± 5.70	19.95 ± 3.40	0.659	0.633	0.666

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. (Continued on next page)

[4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 +C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a,b] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

Table 5.5: *Fatty acid concentration in perirenal fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}. Results for all measured fatty acids are listed in the supplement.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11 (CLA)	49.92 ± 6.30^{ab}	35.58 ± 5.78^{b}	68.13 ± 9.07^{a}	62.58 ± 5.32^{a}	0.154	0.002	0.527
C18:3 *n*-3 (ALA)	37.86 ± 21.49^{c}	120.27 ± 19.72^{ab}	24.25 ± 30.95^{bc}	179.10 ± 18.15^{a}	<.001	0.334	0.133
C20:5 *n*-3 (EPA)	3.47 ± 0.82^{b}	9.08 ± 0.73^{a}	2.21 ± 1.17^{b}	10.68 ± 0.69^{a}	<.001	0.842	0.100
C22:5 *n*-3 (DPA)	24.36 ± 2.50^{b}	38.88 ± 2.30^{a}	15.36 ± 3.61^{b}	45.30 ± 2.11^{a}	<.001	0.635	0.009
C22:6 *n*-3 (DHA)	13.54 ± 2.18	19.78 ± 1.92	12.59 ± 3.20	20.00 ± 1.81	0.008	0.874	0.798
Sum of *n*-3 PUFAs[1]	84.62 ± 23.39^{b}	200.15 ± 21.46^{a}	58.14 ± 33.69^{b}	270.20 ± 19.75^{a}	<.001	0.391	0.069
C18:2 *n*-6 (LA)	571.82 ± 56.81	594.53 ± 52.12	549.65 ± 81.81	663.59 ± 47.97	0.274	0.702	0.467
C18:3 *n*-6 (GLA)	12.49 ± 1.47	12.14 ± 1.34	8.11 ± 2.11	10.90 ± 1.24	0.448	0.084	0.335
C20:3 *n*-6 (DHGLA)	112.74 ± 12.08	113.85 ± 11.08	105.10 ± 17.40	117.79 ± 10.20	0.600	0.887	0.663
C20:4 *n*-6 (ARA)	113.92 ± 8.34	99.50 ± 7.65	118.28 ± 12.01	107.55 ± 7.04	0.173	0.492	0.841
C22:4 *n*-6 (ADA)	45.67 ± 4.96	38.90 ± 4.55	49.34 ± 7.14	44.30 ± 4.19	0.279	0.400	0.874
Sum of *n*-6 PUFAs[2]	884.34 ± 71.73	890.02 ± 65.81	862.91 ± 103.30	978.00 ± 60.57	0.441	0.668	0.490
Sum of PUFAs[3]	1035 ± 88	1142 ± 81	1006 ± 127	1326 ± 75	0.033	0.420	0.277
C18:1 *cis*-9 (OA)	22614 ± 957	23365 ± 839	22018 ± 1374	23662 ± 795	0.246	0.880	0.648
C18:1 *trans*-11 (VA)	58.18 ± 8.34	44.99 ± 7.66	50.03 ± 12.02	57.02 ± 7.05	0.733	0.829	0.277
Sum of MUFAs[4]	29499 ± 1161	30750 ± 1028	28989 ± 1621	29717 ± 977	0.408	0.504	0.820
C16:0 (PA)	20678 ± 917	19976 ± 829	19867 ± 1211	20328 ± 796	0.886	0.778	0.479
C18:0 (SA)	6301 ± 485	6071 ± 442	6591 ± 628	6821 ± 426	1.000	0.214	0.580
C21:0 (HA)[5]	2.49 ± 3.11^{b}	2.11 ± 2.67^{b}	15.27 ± 4.59^{ab}	16.28 ± 2.55^{a}	0.927	<.001	0.827
Sum of SFAs[6]	29546 ± 1294	28193 ± 1194	28950 ± 1632	29329 ± 1158	0.649	0.793	0.406
Total fat content (%)	60.32 ± 1.87	60.09 ± 1.72	59.06 ± 2.36	60.46 ± 1.67	0.707	0.764	0.587

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3+ C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13 + C24:1 *cis*-15. (Continued on next page)

[5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0.
[a-c] Different superscripts indicate significant differences among groups ($P < 0.05$).

5.3.2 Adipocyte size in different adipose tissues

In the INF (at MLD), SCF and PF, the FA profile differed between the supplementation groups. To detect differences in adipocyte formation induced by incorporated FAs, the individual adipocytes were measured in H/E-stained tissue sections. The mean adipocyte cross-sectional area did not differ between the groups ($P > 0.1981$) (Figure 5.14 a). However, it differed between the tissues. In INF, the adipocyte area was 515.1 ± 30.6 µm^2, compared to 819.82 ± 30.2 µm^2 in SCF and 879.33 ± 30.2 µm^2 in PF (LSM ± SE_{LSM}) ($P < 0.001$). The mean cross-sectional area does not give information about group effects on individual fat cells. Therefore, the distribution of adipocyte size within supplementation groups was assessed.

The FA supplementation induced some minor changes on the size distribution of adipocytes. The relative frequency of INF cells in the smallest size category (1–251 µm^2) appeared to be greater in the EFA group (LSM ± SE_{LSM}: 36.1 ± 5.8 %) than in the other three groups (LSM ± SE_{LSM} of CON: 28.5 ± 6.6 %, CLA: 23.42 ± 9.9 % and EFA+CLA: 30.7 ± 5.7 %), but there was no difference induced by supplementation ($P > 0.708$) (Figure 5.14 b). Similarly, the skewness coefficients of the distribution were greater in the EFA groups, with 1.33 (EFA) and 1.32 (EFA+CLA), compared to 1.08 (CON) and 0.69 (CLA). The EFA supplementation tended to reduce the relative frequency of cells with a cross-sectional area between 1001 and 1251 µm^2 (EFA effect: $P = 0.098$). However, the relative cell frequency was similar between individual groups in this size category ($P > 0.356$), as well as in the other size categories ($P > 0.282$). Nevertheless, the INF adipocyte area was negatively correlated with the percentage of ALA and the sum of *n*-3 PUFAs of the total FAs in INF. Spearman correlation coefficients were -0.425 ($P = 0.027$) and -0.440 ($P = 0.022$), respectively.

In the SCF, the relative frequency of adipocytes in the smallest size category (1–251 µm^2) was greater in the CON group (LSM ± SE_{LSM}: 22.6 ± 4.6 %) than in the other three groups (LSM ± SE_{LSM} of EFA: 14.3 ± 4.2 %, CLA: 9.3 ± 6.7 % and EFA+CLA: 12.5 ± 3.9 %) (Figure 5.14 c). In accordance, the distribution was shifted towards the smaller size categories in the CON group (skewness coefficient: 0.56), compared to the EFA (0.19), CLA (-0.01) and EFA+CLA group (0.35). However, the relative abundance of adipocytes in the smallest size category was similar among groups ($P > 0.348$). In the size category 501 to 751 µm^2, the EFA supplementation increased the relative frequency of cells (EFA effect: $P = 0.036$). As a result, the percentage of cells in the EFA+CLA group tended to be greater than in the CLA group ($P = 0.099$). In the size category 1001 to 1251 µm^2, the CLA supplementation tended to increase the relative frequency of cells (CLA effect: $P = 0.054$). In addition, there tended to be a synergistic effect of EFA and CLA supplementation (EFA×CLA effect: $P = 0.088$). The effects did not lead to differences between the individual groups, though ($P > 0.119$).

In size category 1501 to 1751 µm^2, the EFA supplementation tended to reduce the relative frequency of cells (EFA effect: P = 0.071). Supplementation with CLA tended to increase the relative cell abundance (CLA effect: P = 0.078), which showed in a tendency towards more cells in the CLA group than in the CON and the EFA+CLA group (P < 0.1). On this basis, an EFA×CLA effect was observed (P = 0.039). In the other size categories, the relative cell abundance was similar among groups (P > 0.129). Besides, in SCF the adipocyte area was negatively correlated with the percentage of the sum of *n*-3 PUFAs of the total fatty acids (Spearman correlation coefficient: -0.40, P = 0.017) and positively correlated with the total fat content (g/100 g tissue) (Pearson correlation coefficient: 0.37, P = 0.029).

In the PF, the CLA supplementation increased the relative frequency of cells with an area between 751 and 1001 µm^2 (CLA effect: P = 0.036), which did not show in differences between individual groups (P > 0.117) (Figure 5.14 d). In the size category 1251 to 1501 µm^2, the supplementation reduced the relative cell abundance (EFA×CLA effect: P = 0.013), which tended to be lower in the EFA and the CLA group, compared to the CON group (P < 0.071). In the other size categories, the relative cell abundance was similar among groups (P > 0.141). For the PF, no correlation between adipocyte area and tissue FA percentage was found.

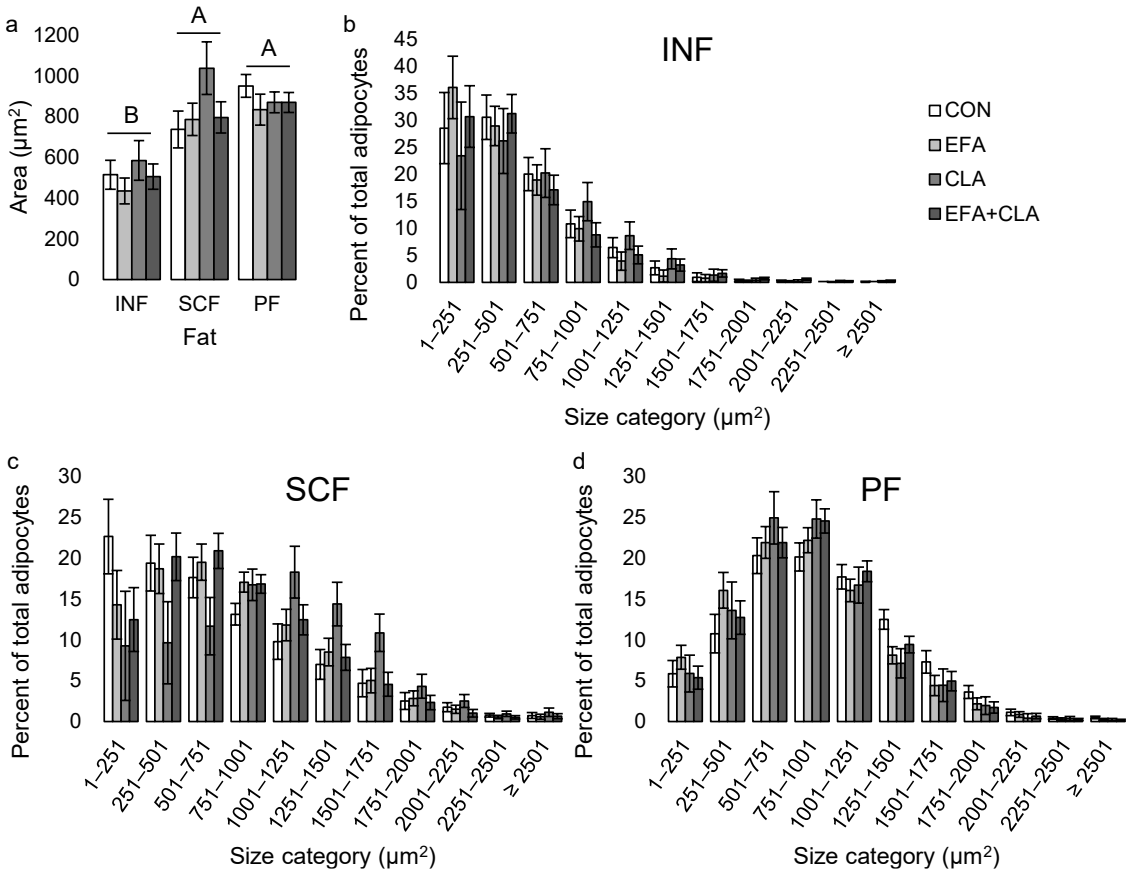

Figure 5.14: *Fat cell size distribution in intermuscular (INF), subcutaneous (SCF), and perirenal (PF) fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8), EFA+CLA (n = 10 (INF), n = 11 (SCF, PF)). (a) Fat cell area (µm²).* ^{A,B} *Different superscripts indicate significant differences among adipose tissues (P < 0.05). (b–d) Relative frequency: Percentage of adipocytes per size category in (b) INF, (c) SCF and (d) PF. Data are given as LSM ± SE$_{LSM}$.*

5.3.3 Detection of adipogenic precursors in adipose tissues

For a more detailed analysis of the adipogenic development in INF, the adipogenic markers C/EBPβ and DLK-1 were simultaneously stained via immunohistochemistry of INF cross-sections of eight samples (Figure 5.15). Cells with C/EBPβ positive nuclei had relatively small lipid droplets and the nuclei had a circular shape compared to the nuclei of adipocytes with bigger lipid droplets. The number of DLK-1 expressing cells was relatively scarce, and the cells were mostly located close to smaller adipocytes or at the border of fat cell clusters. To quantify DLK-1 expression on a protein level, the relative protein abundance was measured in samples of INF (at MLD and at MST). The maternal supplementation did not alter the relative protein abundance of DLK-1 (Figure 5.16). Representative blot images of target and total protein are displayed in the supplement (Figure 9.2 c).

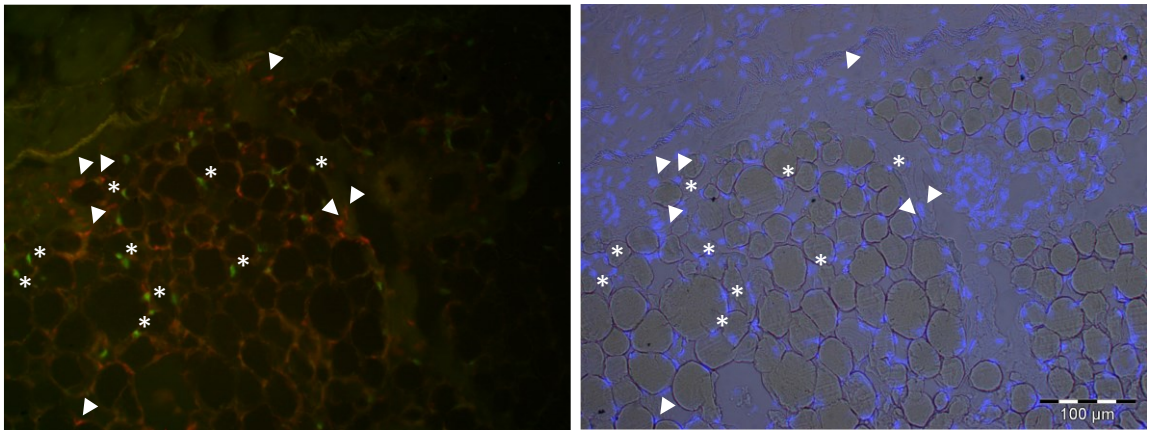

Figure 5.15: *Detection of CCAAT/enhancer-binding protein beta (C/EBPβ) and delta like non-canonical Notch ligand 1 (DLK-1) in intermuscular fat (from longissimus muscle) via simultaneous immunohistochemical staining. (a) Representative overlay image, acquired in the green (C/EBPβ) and red (DLK-1) fluorescence channel. (b) Corresponding overlay image of nuclear staining (with Hoechst 33258) and tissue cross-section, acquired in the blue fluorescence channel (nuclei) and with visible light (bright field image). Arrowheads indicate position of exemplary DLK-1 expressing cells, asterisks indicate exemplary C/EBPβ positive nuclei. Scale bar = 100 µm.*

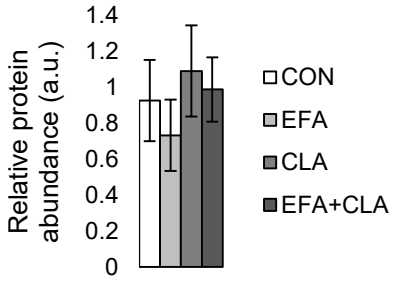

Figure 5.16: *Protein quantification of delta like non-canonical Notch ligand 1 (DLK-1) via WB SPL in intermuscular fat isolated from longissimus and semitendinosus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 8), with conjugated linoleic acids (CLA, n = 10), with EFA and CLA combined (EFA+CLA, n = 10). The relative protein abundance was normalized against the total protein abundance. Representative blot images of target and total protein are displayed in the supplement. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).*

Apart from the protein abundance of preadipogenic marker *DLK1*, also the relative mRNA abundance of *DLK1*, of preadipogenic marker nuclear receptor subfamily 2 group C member 1 (*NR2C1*), of early marker adipogenesis associated Mth938 domain containing (*AAMDC*) and *CEBPA, CEBPB*, *FOXO1*, *PPARG2* and *ZNF423* was measured (Figure 5.17). Supplementation with EFA reduced the mRNA level of *ZNF423* (EFA effect: P = 0.031), but did not lead to significant differences among groups (P > 0.122). Furthermore, supplementation with EFA tended to reduce *CEBPA* and *NR2C1* (EFA effect: P < 0.1), without differences among groups as well (P > 0.244). The supplementation with FAs did not affect

expression levels of *AAMDC, CEBPB*, *DLK1*, *FOXO1*, and *PPARG2* (P > 0.236). The expression levels did not correlate with the area or diameter of INF adipocytes.

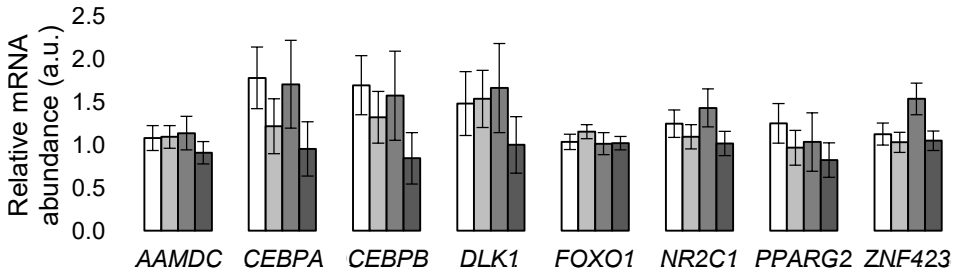

Figure 5.17: *Relative mRNA abundance of adipogenic markers in intermuscular fat from longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), supplementation with essential fatty acids (EFA, n = 9), with conjugated linoleic acids (CLA, n = 8), with EFA and CLA combined (EFA+CLA, n = 10). Quantification cycle values of target genes adipogenesis associated Mth938 domain containing (*AAMDC*), CCAAT enhancer binding protein alpha (*CEBPA*), CCAAT enhancer binding protein beta (*CEBPB*), delta like non-canonical Notch ligand 1 (*DLK1*), forkhead box O1 (*FOXO1*), nuclear receptor subfamily 2 group C member 1 (*NR2C1*), peroxisome proliferator activated receptor gamma isoform 2 (*PPARG2*) and zinc finger protein 423 (*ZNF423*) were normalized against reference genes* PPIA *and* UXT *via geometrical mean. Data are given as LSM ± SE_{LSM} in arbitrary unit (a.u.).*

In summary, the maternal supplementation altered calf INF, SCF and PF fatty acid composition to a differing extent. Especially the tissue content of supplemented FA ALA, some longer-chain *n*-3 PUFAs and *cis*-9, *trans*-11 CLA were elevated in the respective supplementation groups. The supplementation exerted some minor effects on the adipocyte size distribution. In addition, the adipocyte area in the INF was negatively correlated with the tissue percentage of ALA and the total *n*-3 PUFAs (% of total fatty acid). In the SCF, the adipocyte area was positively correlated with the total fat content (g/100 g tissue).

5.4 Testing of antibodies against adipogenesis markers

Antibodies against several adipogenic markers were tested in immunohistochemistry and western blot. In a simultaneous immunohistochemical staining, the adipogenic markers C/EBPα and C/EBPβ could be detected in cell nuclei of MLD and INF cross-sections (Figure 5.18). In the muscle samples, the anti-C/EBPα antibody yielded a signal only in the connective tissue, the major site of adipocyte formation (Figure 5.18 a). The anti-C/EBPβ antibody gave a ubiquitous signal in the muscle fiber nuclei, but the stronger signal was observed in nuclei of the connective tissue (Figure 5.18 b), where there were also nuclei positive for both C/EBPα and C/EBPβ. In INF, both C/EBPα and C/EBPβ expressing cells were small adipocytes located in a cluster of cells at different stages of adipogenesis (Figure 5.18 d–f). Western blot detection of C/EBPα and C/EBPβ yielded weak or no specific bands and high background noise, therefore protein quantification was not possible.

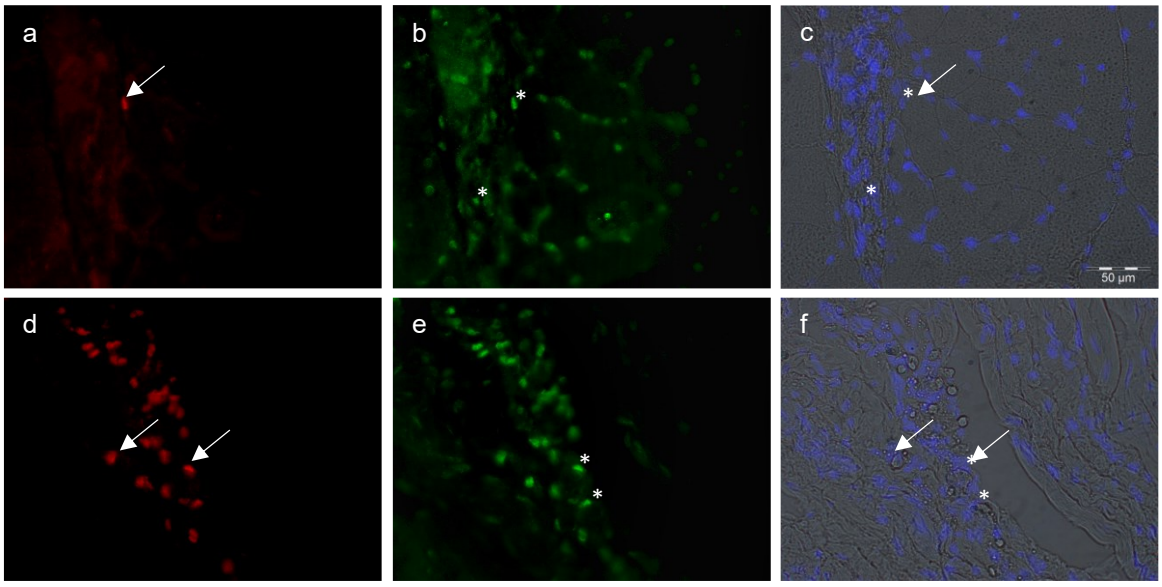

Figure 5.18: *Localization of (a and d) CCAAT/enhancer-binding protein alpha (C/EBPα) and (b and e) beta (C/EBPβ), in tissue cross-sections of (a–c) longissimus muscle and (d–f) intermuscular fat. (c and f) Overlay of nuclear staining (with Hoechst 33258) and bright field image. Arrows indicate exemplary nuclei expressing C/EBPα. Asterisks indicate exemplary nuclei expressing C/EBPβ. Images were taken in the (a and d) red, (b and e) green, and (c and f) blue (nuclei) fluorescence channel and with visible light (bright field image). Scale bar = 50 µm.*

The antibody against zinc finger protein 423 gave unspecific signals in cell nuclei when tested in IHC on cross-sections of muscle and fat tissue (Figure 5.19). When applied in western blot, the antibody gave no specific signal in the calf samples as well (Figure 9.3). Therefore, protein expression of ZNF423 was not analyzed further.

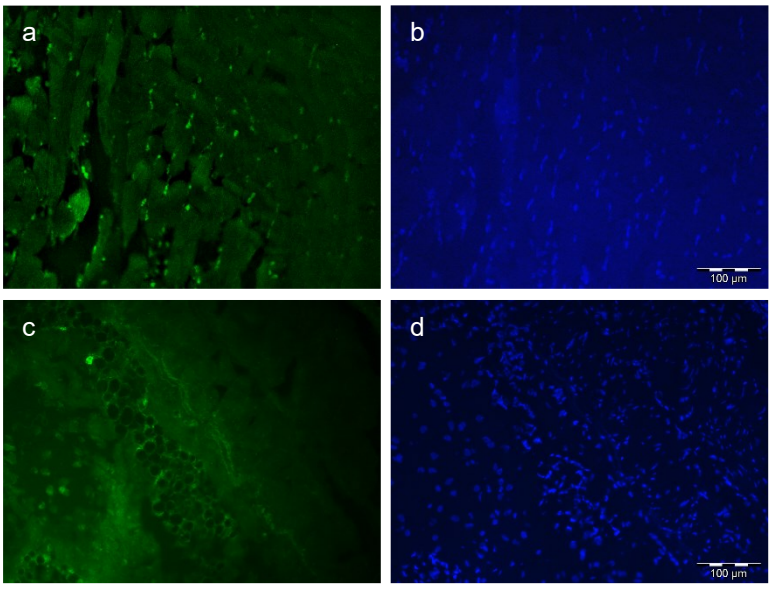

Figure 5.19: *Antibody test for immunohistochemical staining of (a and c) ZNF423 on cross-sections of (a and b) skeletal muscle and (c and d) intermuscular fat. (b and d) Nuclear staining with Hoechst 33258. Images were taken in the (a and c) green and (b and d) blue fluorescence channel. Scale bar = 100 µm.*

Antibodies against lipid droplet associated proteins PLIN2, PLIN3, PLIN4 and PLIN5 were tested for immunohistochemical staining of muscle cross-sections. The antibody against PLIN2 did not give the signal expected to indicate intramyocellular or preadipocyte multilocular lipid droplets (Figure 5.20 a and b). The antibody against PLIN3 did not yield a specific signal (Figure 5.20 c and d). The antibody against PLIN4 gave a signal in the muscle fiber membranes (Figure 5.20 e and f). The antibody against PLIN5 gave a ubiquitous signal in nuclei of cells between muscle fibers and in connective tissue (Figure 5.20 g and h). Specific binding of antibodies could not be confirmed; therefore, the antibodies against perilipins were not applied in individual samples.

Briefly, the maternal FA supplementation altered the FA composition in the calf muscle and adipose tissues. While it did not affect tissue morphology in muscle tissues, the CLA supplementation decreased the protein abundance of MYH7 and increased the protein abundance of MYH1. In the IMF, the supplementation with EFAs decreased the abundance of cells expressing preadipocyte marker DLK-1 and the mRNA abundance of DLK1. In the INF, the content of ALA and the sum of *n*-3 PUFAs (% of total FAs) were negatively correlated with the adipocyte area, but there was no clear EFA effect on the size distribution of adipocytes. In the SCF, the adipocyte area was negatively correlated with the content of sum of *n*-3 PUFAs (% of total FAs) and positively correlated with total fat content (g/100 g tissue). There were some tendencies towards more bigger adipocytes in the FA supplementation groups, but no clear effects.

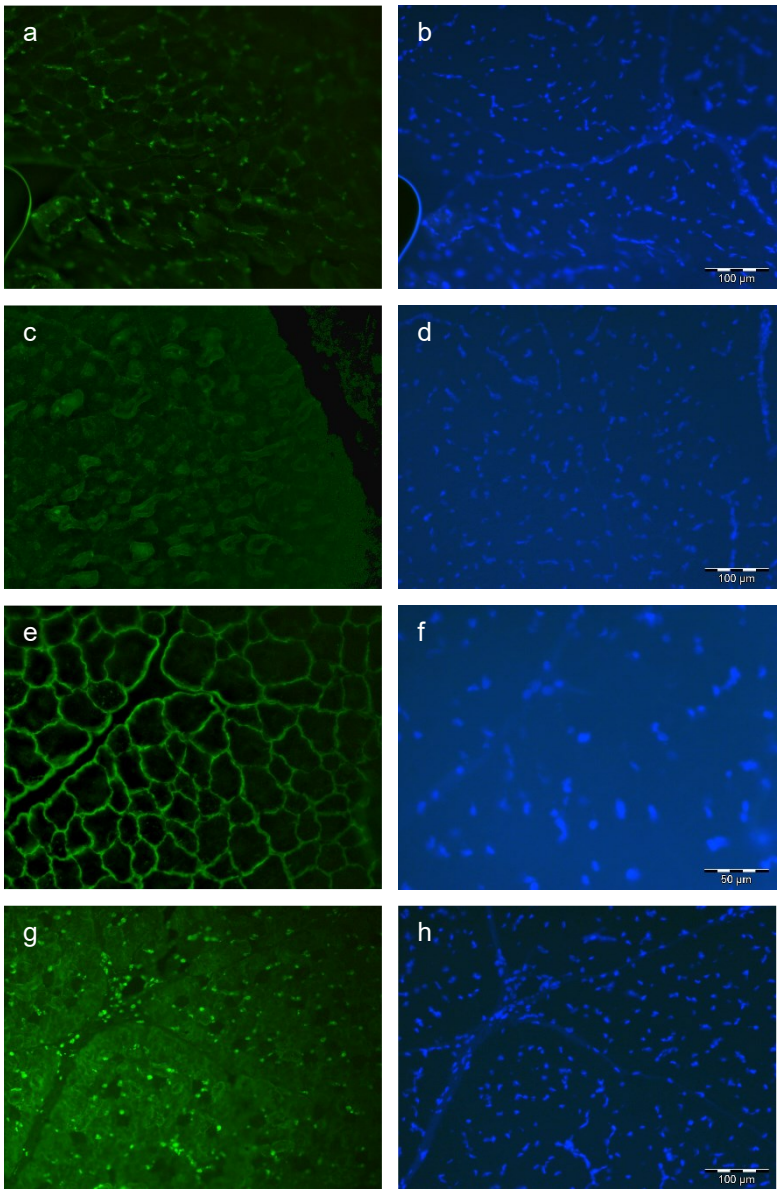

Figure 5.20: *Antibody test for immunohistochemical staining of (a) intramyocellular lipid droplet marker perilipin-2, (b–e) lipid droplet-related proteins (c) perilipin-3, (e) perilipin-4 and (g) perilipin-5, in skeletal muscle cross-sections. (b, d, f and h) Respective nuclear staining with Hoechst 33258. Images were taken in the (a–d, g and h) green and (b, d, f and h) blue fluorescence channel. (a–d, g and h) Scale bar = 100 µm. (e and f) Scale bar = 50 µm.*

6 Discussion

In dairy industry, high yielding cows are mainly fed with concentrate-based feeding, which contains less of the *n*-3 essential fatty acid (EFA) α-linolenic acid (ALA), and more of the *n*-6 EFA linoleic acid (LA) than fresh grass (French *et al.*, 2000; Chilliard *et al.*, 2001). This diet reduces *n*-3 polyunsaturated fatty acids (PUFAs) and conjugated linoleic acids (CLAs) in the animal's tissues (Chilliard *et al.*, 2001; Dannenberger *et al.*, 2005). Even though the animal's energy requirements are met (Khan *et al.*, 2015), the consequences of an ALA reduction on cow health and performance are not fully understood yet. In addition, the reduced ALA and CLA supply might have consequences on offspring pre- and postnatal development, especially calf skeletal muscle and adipose tissue growth (Du *et al.*, 2017), which is the subject of the presented study.

6.1 Effect of maternal FA supplementation on FA and metabolic status of dams and offspring

Vogel *et al.* (2020) reported that the CLA supplementation reduced milk fat and improved the metabolic condition in the cows of this study. Treatment with EFAs had only minor effects, observed in metabolism and milk yield of the cows (Vogel *et al.*, 2020). Gestation length tended to be increased by the EFA supplementation (Uken *et al.*, 2021a). A higher gestation length may increase maturity of the fetus, thereby influencing characteristics of muscle and adipose tissues. Therefore, the gestation length was included in the statistical model when a significant effect was observed. Between cow and calf, maternal fatty acids (FAs) were transferred via placenta or milk to different extents and calf plasma FAs were altered in correspondence to the maternal supplementation (Uken *et al.*, 2021a). Calves receiving maternal EFA supplement had an elevated nutrient transfer via placenta and a better energy status (Uken *et al.*, 2021a). An altered nutrient transfer might have additional effects on muscle and fat tissues (Du *et al.*, 2017). However, Uken *et al.* (2021a) observed that maternal supplementation had no effect on calf performance, such as birth weight or slaughter weight. They concordantly reported that the single organ weight did not differ among the supplementation groups (Uken *et al.*, 2021b). Supplementation of late gestational ewes with the longer-chain *n*-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), compared to saturated fatty acids (SFAs), did not affect postnatal lamb weight (Coleman *et al.*, 2018). Correspondingly, supplementation of cows with LA, compared to SFA supplementation, from 8 weeks prepartum on did not affect neonatal body weight in a study of Garcia *et al.* (2014a). In this study's calves, the individual body weight of the calves was not affected as well. Nevertheless, adaptations of the calves'

skeletal muscle and adipose tissue structure to changes in maternal *n*-6/*n*-3 PUFA and CLA ratios were to be observed.

6.2 Effect of maternal supplementation on the calf skeletal muscle tissues

6.2.1 Fatty acid composition of skeletal muscle tissues

The FA composition of the calf muscle tissues was analyzed to verify that FAs were altered in response to maternal FA supplementation. At the observed calf age of five days, the calves' rumen is not fully developed (Tamate *et al.*, 1962) and the calves' digestive system works similar to that of a monogastric animal (Huber, 1969). As such, the supplemented FAs were expected to be incorporated into the calf tissues, instead of being hydrogenated in the rumen. In both investigated muscle tissues, EFA supplementation increased concentration of ALA and longer-chain *n*-3 PUFAs EPA, docosapentaenoic acid (DPA) and DHA. According to Uken *et al.* (2021a), colostrum feeding, but not *de novo* biosynthesis, was the main contributor to the elevation of longer-chain *n*-3 PUFAs in the plasma of these calves. Hence, ALA and longer-chain *n*-3 PUFAs were transferred mostly after birth.

No changes in LA levels were observed. The EFA supplementation group had reduced muscle tissue levels of longer-chain *n*-6 PUFAs arachidonic acid (ARA) and adrenic acid (ADA). Similarly, in the calf plasma, LA did not differ between the groups and longer-chain PUFA ARA was decreased in the EFA groups (Uken *et al.*, 2021a). Literature states that *n*-3 and *n*-6 PUFAs compete for incorporation into the tissue (Wathes *et al.*, 2007; Gibson *et al.*, 2011). At high *n*-6/*n*-3 ratios, as in the CON or the CLA group, the affinity is higher for LA; upon ALA supplementation, more *n*-3 PUFAs are taken up (Gibson *et al.*, 2011; Wood *et al.*, 2014). Considering the low *n*-6/*n*-3 PUFA ratio in the supplement and reaching the calf plasma, ALA was perhaps preferentially incorporated into the skeletal muscle. In addition, ALA might have been preferentially metabolized to longer-chain *n*-3 PUFAs (Innis, 1992; Soriguer *et al.*, 2000; Gibson *et al.*, 2011), while formation of longer-chain *n*-6 PUFAs ARA and ADA was reduced in the EFA groups. Angulo *et al.* (2012) described that diets with a high *n*-6/*n*-3 ratio, irrespective of the absolute dietary LA, did not significantly change Holstein cow muscle LA. A high amount of ALA and a low *n*-6/*n*-3 ratio in the diet significantly increased muscle ALA and reduced muscle *n*-6/*n*-3 ratio (Angulo *et al.*, 2012). Nevertheless, Angulo *et al.* (2012) observed adult animals with a mature rumen, in which PUFAs are largely hydrogenated. This lessens the transferability of results.

The supplementation with EFAs reduced the concentration of oleic acid (OA) and total monounsaturated FAs (MUFAs) in MLD of both EFA supplementation groups, compared to the single CLA group. Oleic acid and other MUFAs are formed by desaturation of their SFA counterparts by stearoyl-CoA desaturase (SCD) (Enoch *et al.*, 1976; Paton and Ntambi, 2009).

N-3 and *n*-6 PUFAs have been reported to reduce mRNA level and protein activity of *SCD* in muscle, respectively intramuscular fat (IMF), thereby reducing formation of MUFAs from SFAs (Herdmann *et al.*, 2010; Hiller *et al.*, 2012). On the other hand, the group differences in the percentages of MUFAs and SFAs were similar between muscle tissues and calf plasma at day five (Uken *et al.*, 2021a) and might be the direct result of FA transfer from plasma to muscle tissues.

In calf MLD and MST, *cis*-9, *trans*-11 CLA was elevated in the CLA groups, in concordance with increased plasma values (Uken *et al.*, 2021a). The second supplemented isomer *trans*-10, *cis*-12 CLA is included in the signal given by heneicosanoic acid (HA) for technical reasons (peak overlays in the used GC method). In both muscles, concentration of HA was also elevated in both CLA supplementation groups, in concordance with the plasma values at day five of life (Uken *et al.*, 2021a). It is likely that the CLA effect in HA + *trans*-10, *cis*-12 CLA is caused by an elevation of *trans*-10, *cis*-12 CLA after transfer from plasma to muscle. In the plasma, only minor group differences with respect to the CLAs were observed at the day of birth, but became apparent at day five (Uken *et al.*, 2021a). Also, total plasma CLA percentage increased mainly after birth (Uken *et al.*, 2021a). The amount of CLAs incorporated into the muscle before birth was not available, but probably the larger part accumulated during the five days after birth. This would leave a period of five days to influence tissue development. In the muscle tissue, the mean concentration of *cis*-9, *trans*-11 CLA was more than double as much as the concentration of HA (and *trans*-10, *cis*-12 CLA). In the calf plasma, similar differences in FA percentage were observed (Uken *et al.*, 2021a). Both CLA isomers were supplemented to the dams at equal amounts. Possibly the two isomers are incorporated into the muscle at different rates in accordance with Sébédio *et al.* (2001), who described that dietary *cis*-9, *trans*-11 CLA was incorporated into rat adipose tissue at twice the extent of dietary *trans*-10, *cis*-12 CLA. Thus, the difference in tissue concentration might be an effect of both different plasma percentages and differences in tissue incorporation.

It can be deduced that the maternal supplementation with CLAs and ALA increased the skeletal muscle concentration of CLAs, ALA and longer-chain *n*-3 PUFAs either by maternal transfer or, but to a lesser extent, by *de novo* biosynthesis of longer-chain PUFAs. It is possible that CLAs and *n*-3 PUFAs affected the composition and metabolic properties of the calves skeletal muscle tissues.

6.2.2 Capillarization in the muscle tissues

Blood plasma-derived FAs are an important energy source for skeletal muscle (Dyck *et al.*, 2006; Noland, 2015). Blood vessels within the muscle transfer the FAs to the adjacent muscle fibers (van Hall, 2015). In this study, the results indicated no direct effect of supplementation on capillarization of both investigated muscles. According to literature, prostaglandins derived

from *n*-6 PUFAs are proangiogenic (Pozzi *et al.*, 2005; Cheranov *et al.*, 2008; Szymczak *et al.*, 2008), while prostaglandins derived from *n*-3 PUFAs inhibit angiogenesis *in vivo* (Zhang *et al.*, 2013) and reduce endothelial cell migration *in vitro* (Szymczak *et al.*, 2008; Zhang *et al.*, 2013). In the cited studies, the prostaglandins derived from either *n*-6 or *n*-3 EFAs, which were applied separately. The effect of an altered *n*-6/*n*-3 ratio was not described. As the prostaglandins from the two families are similar in reactivity, but differ in activation capacity (Faust *et al.*, 1989; Kiriyama *et al.*, 1997; Anderson *et al.*, 1999), the lower *n*-6/*n*-3 PUFA ratio in the muscle of ALA supplemented calves might affect angiogenic processes via altered prostaglandin biosynthesis and signaling.

For CLAs, Basak and Duttaroy (2013) described that *cis*-9, *trans*-11 CLA stimulated capillarization in placental trophoblast cells, while *trans*-10, *cis*-12 CLA had no such effect. Masso-Welch *et al.* (2002, 2004) reported antiangiogenic effects of both *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA *in vivo* and *in vitro*. Anyway, the effects were observed in mice or in cell culture. In this study's calves, the variation in tissue *n*-6/*n*-3 PUFA ratio and overall FAs might have been too small to cause an effect on tissue morphology.

6.2.3 Structure of muscle fibers

At the starting point of maternal FA supplementation, the cows were in the middle of the last trimester of gestation. At that time, the majority of offspring fetal muscle fibers is already formed (Russell and Oteruelo, 1981; Picard *et al.*, 1995). Several studies investigated the influence of nutrient restriction during this period on muscle fiber formation and observed that maternal undernutrition reduced muscle fiber growth, but not number, in ovine fetuses (Greenwood *et al.*, 1999). Therefore, the total muscle fiber number was not expected to be greatly affected by FA supplementation.

The altered FAs incorporated into the muscle changed neither the apparent total muscle fiber number nor the size of the individual fibers. Hypertrophic growth of fetal muscle fibers occurs mostly in the last trimester of gestation (Meara, 1947; Brameld *et al.*, 2000; Wegner *et al.*, 2000) and is the main contributor to the increase in fetal body weight (O'Rourke *et al.*, 1991; Mao *et al.*, 2008). Correspondingly, neither the muscle fiber number and size, nor the calves' body weight were affected by the supplementation.

The priority of skeletal muscle in nutrient partitioning is low, compared to tissues such as the brain or heart (Funston *et al.*, 2010). Therefore, muscle tissue development is highly responsive to malnutrition (Funston *et al.*, 2010; Du *et al.*, 2015). In this study, no model of maternal undernutrition was applied (Vogel *et al.*, 2020). With the silage-based feeding, the animals were sufficiently supplemented with LA. Apparently, when nutrient requirements are met, a reduction in ALA has only minor effects on growth. Possibly, the development of the fetal muscle was too far in progression to be affected by the FA variation. The physiologic

PUFA requirements of the animal muscle could be met also in the CON group, and excess *n*-3 PUFAs and CLAs were probably directed to other tissues to exert beneficial effects.

6.2.4 Distribution of muscle fiber types

Supplemented CLAs and *n*-3 PUFAs were mostly transferred via milk, affecting the calves after birth. At this time, muscle fiber contractile properties and metabolism can still change after birth (Rubinstein and Kelly, 1981; Wegner *et al.*, 2000). In this study, three different fiber types could be detected via staining of myofibrillar adenosine 5'-triphosphatase (ATPase) in the muscle tissues. The distribution of fiber types was similar to other studies (Calkins *et al.*, 1981), with the majority comprised of fast glycolytic fibers, with the biggest cross-sectional area, less and smaller intermediate glycolytic fibers and a minority of small slow oxidative fibers. Fibers of all types were smaller in MLD than in MST, indicating differences in maturity between muscles.
In the animal, the two muscles fulfill different functions; they might have a different developmental stage at birth (Berg and Butterfield, 1976). As precocial animals, neonatal cattle need to be able to escape from predators. For this task, the neonate's leg muscle MST, in contrast to the back muscle MLD, needs to be well developed (Berg and Butterfield, 1976). This might be indicated by the bigger muscle fibers.
In this study's calves, no effects of the FA supplementation on the distribution of fiber types were observed. In literature, no reports were available on effects of the supplemented FA, but on effects of feed ingredients on muscle fiber type composition. Shibata *et al.* (2009) reported an increased protein content of slow fiber type myosin heavy chain (MYH) and reduced protein content of fast fiber type MYH in grazing cattle compared to grain-fed cattle. Similarly, they observed increased slow *MYH* mRNA levels to the expense of fast *MYH* in longissimus lumborum muscle of grazed cattle, compared to concentrate fed animals, while the MST was not affected (Shibata *et al.*, 2014). This indicates that PUFA enriched feeding increases slow fiber type markers. However, in these studies the animals were older and observed for a longer period than the calves in this study. The supplementation period and the FA dose might not have been sufficient to induce changes in fiber types.

6.2.5 Nuclei number of muscle fibers

While the number of muscle fibers is fixed after the second trimester, the muscle retains some proliferative capacity in form of satellite cells (SCs) (Mauro, 1961; Kuang *et al.*, 2007). These are stem cells located between basal lamina and the muscle fibers (Mauro, 1961; Kuang *et al.*, 2007). Their number and fusion with existing muscle fibers are crucial for postnatal muscle growth capacities (Kuang *et al.*, 2007). The percentage of SC nuclei of total nuclei is approximately 32 % in neonatal mice (Allbrook *et al.*, 1971; Cardasis and Cooper, 1975). Campion *et al.* (1979) observed approximately 15 % SC nuclei of total nuclei in 1-day-old

piglets, while Mesires and Doumit identified up to 60 % of nuclei as SC nuclei in 1-week-old piglets (Mesires and Doumit, 2002). Upon these differing numbers, no information on the percentage of SC nuclei in neonatal calf muscle tissue could be found. The number of nuclei surrounding the muscle fiber was higher in MLD than in MST, but was not affected by supplementation.

Maternal undernutrition during late gestation can reduce the number of precursor cells (Woo *et al.*, 2011). Treatment with ALA promoted myogenic differentiation, thereby reducing SC number in a hamster model of muscle dystrophy (Fiaccavento *et al.*, 2010). The higher number of nuclei per fiber in MLD than in MST might be the result of the inverse relationship between nuclear density and muscle fiber size (Acevedo and Rivero, 2006). Alternatively, as the nuclei number decreases with animal development (Allbrook *et al.*, 1971; Cardasis and Cooper, 1975), the higher nuclei density in MLD might be the result of a lower developmental stage of MLD than of MST, as discussed above (6.2.4). Still, the full effect of PUFA supplementation on SCs is not fully known yet (Isesele and Mazurak, 2021).

6.2.6 Transition of muscle fiber types

The detection of MYH isoforms in skeletal muscle adds additional information on muscle fiber characteristics (Acevedo and Rivero, 2006; Choi and Kim, 2009), which the ATPase staining cannot provide. The majority of muscle fibers expresses only one MYH isoform (Acevedo and Rivero, 2006), and the simultaneous expression of two isoforms by a fiber indicates that the fiber is in transition between fiber types (Staron and Pette, 1987; Picard *et al.*, 1998; Acevedo and Rivero, 2006).

In the presented study, a correlation between area percentage of fibers expressing MYH2 and MYH7 and area percentage of fast and intermediate, respectively slow fibers detected via ATPase staining was observed. This correlation and the low overall abundance of detected hybrid fibers indicate a high specificity of the used antibodies. As observed hybrid fibers were unlikely to be measurement artifacts, they were subjected to quantification. Moreover, the distribution of fibers according to the dominantly expressed isoform was investigated.

Fiber transition follows the sequential order of fast to intermediate to slow fiber type or vice versa (Pette and Staron, 2001). Hybrid fibers show characteristics of fibers expressing only one of the two isoforms (Pette and Staron, 2000). A distribution based on the two isoforms is a method of classifying hybrid fibers (Pette and Staron, 2000). The supplementation with FAs had no effect on the number per mm^2 and the distribution of transitional fibers. There was no clear indication for an influence of maternal FA supplementation in our project. As a limiting factor, fast and intermediate fibers cannot be distinguished clearly in immunohistochemistry (IHC), because the available anti-MYH1 and anti-MYH2 antibodies show cross-reactivity and signal overlap. However, the duration of FA supplementation might have been too short, the

FA concentration not sufficient, or the calves too young to see any effects. Moreover, alterations might have been visible upon sampling at two different points in time during calf life, which was not possible in this project.

In order to detect changes that manifest later in calf life, the relative protein amounts of isoforms MYH2, 7 and the fast fiber markers MYH1 and 4 were quantified. An increased protein abundance of MYH1 in MLD of CLA calves compared to EFA calves was observed. This suggests that CLA stimulates growth and development of fast fibers, corresponding to the decreased abundance of MYH7 in the EFA+CLA group compared to the CON calves.

In contradiction to this result, Men *et al.* (2013) observed increased *MYH7* mRNA levels in CLA supplemented pigs. Similarly, Shibata *et al.* (2009, 2014) indicated a switch from fast to slow fiber type *MYH* gene expression upon pasture feeding of cattle, compared to grain feeding, as discussed before (6.2.4). As pasture feeding increases CLA formation, this might be an effect of both EFAs and CLAs.

In this project, the observed CLA effect on fast fiber formation might be related to animal maturity. In calves of different breeds, Wegner *et al.* (2000) described a general increase of fast fiber percentage on cost of intermediate fibers during the first two months of age. Conclusively, both animal age and FA supplementation are reported to affect muscle fiber type transition, but the exact mechanisms need to be studied further.

6.2.7 MRNA expression of muscle metabolic markers

The altered FA status did not visibly modify the structure and contraction behavior of MLD and MST. With histological and immunohistochemical methods, the situation could only be observed as it was at day five of calf life. Protein quantification of MYHs yielded no clear perspective on changes in calf muscle development. For hints on future muscle development, the relative mRNA abundance of peroxisome proliferator activated receptor gamma coactivator 1 alpha (*PPARGC1A*) and prospero homeobox 1 (*PROX1*), two genes connected to muscle metabolism, was investigated.

Peroxisome proliferator-activated receptor γ coactivator 1-α is involved in mitochondrial biosynthesis, for example by activating energy homeostasis and metabolism related genes (Wu *et al.*, 1999; Knutti *et al.*, 2000). Vaughan *et al.* (2012) treated human muscle cells with the *n*-3 PUFAs EPA and DHA, which temporarily increased *PPARGC1A* mRNA abundance, ultimately stimulating oxidative metabolism, glycolytic capacity and metabolic rate. Treatment with EPA and DHA or with CLA also increased mitochondrial content (Vaughan *et al.*, 2012).

Kivelä *et al.* (2016) detected prospero homeobox protein 1 (Prox1) in human skeletal muscle fibers expressing MYH7, and in SCs. Induction of *Prox1* expression in murine fast skeletal muscle fibers promoted expression of *Myh7*. In murine and primary human myoblasts, *PROX1* expression was increased during differentiation and silencing of *PROX1* inhibited myoblast

differentiation (Kivelä *et al.*, 2016). In pigs, Dong *et al.* (2019) reported a higher *PROX1* mRNA abundance in the soleus muscle, which contains more slow muscle fibers than in the biceps femoris, which contains more fast type fibers. The mRNA abundance of *PROX1* was negatively correlated with the *MYH4* to *MYH7* mRNA abundance ratio (Dong *et al.*, 2019). In human neuronal cells, the supplementation with DHA in combination with Wnt3a increased mRNA abundance of *PROX1* (Zhao *et al.*, 2019).

It appears that both proteins are connected to the slow muscle fiber type and treatment with *n*-3 PUFAs or CLA increases their expression (Vaughan *et al.*, 2012; Zhao *et al.*, 2019). In this study's calf muscle tissues, the mRNA abundance of both *PPARGC1A* and *PROX1* was similar among the supplementation groups. For both genes, the high intra-group variance or the high activity of other postnatally induced genes might mask effects of the supplementation.

6.2.8 Incorporation of fatty acids into the muscle tissues

The overall fat content in the skeletal muscles was low, as expected in calves of this age (Wegner *et al.*, 2000), compared to mature Holstein cattle (Albrecht *et al.*, 2011). When FAs reach the muscle tissue, they are incorporated into the muscle fibers, e.g. the fiber membranes (van Hall, 2015), or into intramyocellular lipid droplets (IMCLs) (Sacchetti *et al.*, 2002; Coen and Goodpaster, 2012). The majority though is incorporated into intramuscular adipocytes ("marbling") (de Smet *et al.*, 2004).

In the muscle fibers, IMCLs could be observed with different staining techniques, but were too small and too scant for quantification. In a study on five-day old piglets, conducted by Zhao *et al.* (2020), IMCLs were bigger and more numerous than in this study's calves' muscle samples. With increasing age of the piglets, the number and size of IMCLs decreased, while the area of intramuscular adipocytes increased (Hauser *et al.*, 1997; Zhao *et al.*, 2020). The higher abundance of IMCLs in the five-day old piglets shows their muscle tissue's lower maturity compared to the calves. Intracellular lipid droplets store lipids, mainly in triglycerol form (Murphy and Vance, 1999; Walther and Farese Jr., 2012). The lipid content provides membrane components (Walther and Farese Jr., 2012) and acts as an energy source for muscle cell contraction (Carlson *et al.*, 1971; Walther and Farese Jr., 2012). Myoblasts incorporate FAs mainly into triacylglycerol, while myotubes incorporate FAs mainly into phospholipids, for example within membranes (Sauro and Strickland, 1987). During myogenic differentiation, a 50 % reduction of triacylglycerol was observed (Pediconi *et al.*, 1992). This suggests that IMCLs as FA storage are important for the energy supply during muscle fiber development. During myogenic development, the first emerging adult fibers are of the slow fiber type (Albrecht *et al.*, 2013; Chal and Pourquié, 2017). Slow fibers contain and utilize more lipids than the other two fiber types (Ashmore *et al.*, 1972; Picard *et al.*, 2002; Hwang *et al.*, 2010), which might align with the role of lipids for muscle fiber development. In accordance

with this observation, IMCLs were predominantly observed in slow muscle fibers in a test staining of calf muscle cross-sections from our study.

Apart from lipid droplets within muscle fibers, intramuscular adipocytes were also detected. The area of intramuscular adipocyte lipids was as low as expected in dairy calves of this age (Robelin, 1986). In the samples with high area percentage of lipid droplets, the adipocytes were found as fat islands, while in samples with a low area percentage, the adipocytes were scattered over the sectional area. The location of adipocytes in the cross-section of a sample was similar across several sections. The observed single or small groups of adipocytes are probably the origin of new adipocyte clusters (Albrecht *et al.*, 2006). In the early stage of IMF formation, these cells are thought to recruit and promote differentiation of preadipocytes, leading to hyperplastic tissue growth (Albrecht *et al.*, 2006; Hausman *et al.*, 2009). Skeletal muscle cells and adipocytes derive from the same mesenchymal precursors (Joe *et al.*, 2010; Uezumi *et al.*, 2010; Du *et al.*, 2013). Fatty acid supplementation in this study might have influenced the commitment of stem cells to the myogenic, adipogenic or fibrogenic lineage. Transdifferentiation of committed cells from the myogenic to the adipogenic lineage was induced *in vitro* (Grimaldi *et al.*, 1997; Guillet-Deniau *et al.*, 2004). Transdifferentiation is, among others, mediated by peroxisome proliferator activated receptor gamma (PPARγ) (Grimaldi *et al.*, 1997; Aguiari *et al.*, 2008). Polyunsaturated FAs and their prostaglandin metabolites are potent ligands for PPARγ (Forman *et al.*, 1995; Kliewer *et al.*, 1995; Belury and Vanden Heuvel, 1999; Bell-Parikh *et al.*, 2003). Linoleic acid activated PPARγ isoform 2 in mouse adipose tissues (Tontonoz *et al.*, 1994) and mixed CLA isomers induced PPARγ in a dose dependent manner in the adipose tissue of rats (Houseknecht *et al.*, 1998). Supplementation of pigs with CLA increased IMF content but not adipocyte diameter, when compared to LA feeding (Dugan *et al.*, 1999; Jiang *et al.*, 2010). In Holstein bulls supplementary linseed or CLA did not affect the size and area of intramuscular adipocytes, but linseed feeding increased the number of marbling flecks (Albertí *et al.*, 2013). Holló *et al.* (2008) observed that not the linseed supplementation, but the muscle origin affected IMF content in bulls. The literature results indicate effects of the FA supplements, but the studies were conducted in animals older than this study's calves. The hyperplastic development of IMF was still in process at the time of slaughter, and probably the calves were too young to observe supplementation effects.

6.2.9 Adipogenic precursors in the muscle tissues

Via Oilred O staining, the adipogenic cells with lipid droplets could be detected, but not precursor cells without lipid droplets. Several early and late markers of adipogenesis were measured in order to investigate preadipocyte development. Delta like non-canonical Notch ligand 1 (*DLK1*) is a marker for a specific lineage of adipogenic precursors, its protein and

mRNA abundance were measured in MLD and MST. The shape and location of DLK-1 expressing cells, as well as the signal location within the cell, confirmed earlier studies (Vuocolo *et al.*, 2003; Albrecht *et al.*, 2015; Zwierzina *et al.*, 2015). For the staining, animals of each group had been evenly distributed among the IHC runs. In MLD, there was a statistical effect of the IHC run on the number of counted DLK-1 expressing cells. Therefore, the IHC number was included into the statistical model as a random effect. This effect is in part responsible for the high intra-group variation. The number of DLK-1 expressing cells and the relative protein abundance of DLK-1 in MLD were not affected by supplementation. In MST, EFA supplementation reduced the number of DLK-1 expressing cells compared to CON. On an mRNA level, EFA supplementation reduced the expression of *DLK1* in MLD, compared to the supplementation with CLA. In MST, *DLK1* mRNA abundance was unchanged.

As observed in murine preadipocytes, DLK-1 inhibits the expression of CCAAT enhancer binding protein beta (*Cebpb*), whereby the cells remain in a predifferentiation state prior to lipid droplet incorporation (Wang and Sul, 2009). As reported for mice, *Cebpb* transcription factor expression is activated upon *Dlk1* downregulation, the cells undergo terminal differentiation and lipid droplets are formed (Hudak *et al.*, 2014). According to Wang and Sul (2009), mRNA expression of *Dlk1* in murine preadipocytes declined by 50 % within 12 h after differentiation was induced, and by another 85 % until day two. In parallel, *Cebpb* mRNA expression increased by 16 times within one day after induction (Wang and Sul, 2009).

Lee *et al.* (2003) observed that transgenic expression of *Dlk1* in mice reduced adipocyte volume and fat mass (Lee *et al.*, 2003). Moreover, *Dlk1*$^{-/-}$ mice were found to have more proliferative adipocyte precursors than the wild-type littermates (Mortensen *et al.*, 2012). In mature cattle, the expression of *DLK1* negatively correlated with IMF content (Albrecht *et al.*, 2015) and intermuscular fat (INF) adipocyte diameter (Pickworth *et al.*, 2011). The reduction of *DLK1* mRNA hints at lower number of adipocyte precursors without lipid droplets in the MLD of EFA supplemented animals, compared to CLA supplemented animals. This implies increased formation of IMF in EFA supplemented animals of this study. The effect might manifest later in calf life, upon IMF maturation.

To date, no literature describing the effect of EFA supplementation on *DLK1* has been available available. Kanosky *et al.* (2013) observed no effect of CLA supplementation on mRNA expression of *Dlk1* in murine skeletal muscle. The same was reported for skeletal muscle of CLA supplemented pigs (Barnes *et al.*, 2012). This indicates that CLAs do not affect *DLK1* expression.

As DLK-1 exerts its antiadipogenic effects via inhibition of C/EBPβ, the transcription factor was localized via immunohistochemical staining of muscle cross-sections. The location of C/EBPβ expressing cells was similar to that reported by Albrecht *et al.* (2015), in the connective tissue between muscle fibers and at sides of adipocyte formation. For technical reasons, C/EBPβ

positive nuclei could not be quantified. In addition, DLK-1 and C/EBPβ were localized in a simultaneous immunohistochemical staining of muscle cross-sections. Again, the location was similar to as reported (Albrecht *et al.*, 2015). Apart from the study conducted by Albrecht *et al.* (2015), no literature about the colocalization of DLK-1 and C/EBPβ was available. Schering *et al.* (2017) observed downregulation of *Dlk1* mRNA levels and steady *Cebpb* mRNA levels during differentiation of murine preadipocytes.

The mRNA abundance of adipogenesis markers *CEBPB*, platelet derived growth factor receptor alpha (*PDGFRA*), lipoprotein lipase (*LPL*) and zinc finger protein 423 (*ZNF423*) was not affected by supplementation. The mRNA of *PPARG2* could not be detected and mRNA abundance of the markers *DLK1*, *ZNF423* and *CEBPB* in the muscle tissues was low, indicated by high mean Cq numbers (> 28.87). For these genes, the relative amount of RNA was limited due to the small number of expressing cells, as detected via immunohistochemistry. Moreover, in calves of this age, the genes regulating muscle fiber growth show high activity, which can further obscure the low expression of adipogenic genes.

In summary, the investigation of the calves' MLD and MST yielded a change of muscle FA composition due to maternal supplementation. This FA modifications did not alter the overall number and fiber type composition of the muscle tissues, but protein quantification of the MYH isoforms indicated a shift from slow toward fast fibers induced by CLA supplementation. The second target side of FAs in the muscle, the IMF, was not affected in terms of lipid droplet area. The analysis of preadipocyte markers implied proadipogenic effects of EFA supplementation in both muscles. Nevertheless, the organism probably adjusted to the differential FA supply, without short-term changes in muscle and IMF development. This does not exclude long-term effects, but these could not be explored in the current investigation.

6.3 Effect of maternal supplementation on the calf adipose tissue development

6.3.1 Fatty acid composition of adipose tissues

The investigated fat depots were differentially developed in the five-day old calves; the total fat percentage in INF and subcutaneous fat (SCF) showed high variation between the samples. This implies that these tissues were less mature than the perirenal fat (PF). Adipose tissue growth starts at about mid-gestation, overlapping with secondary myogenesis (Du *et al.*, 2010a), and the different depots develop in a distinct order (Bonnet *et al.*, 2010; Du *et al.*, 2017). At the time of supplementation, INF and SCF were in active formation by hyperplasia and hypertrophy (Vernon, 1986, as cited in Bonnet *et al.*, 2010) and the incorporated FAs were expected to influence adipocyte abundance and size. The PF is one of the first adipose tissues to emerge (Vernon, 1986; as cited in Bonnet *et al.*, 2010). At the start of supplementation, its hyperplasic growth was in decline and the cells were mainly in hypertrophic growth (Vernon, 1986, as cited in Bonnet *et al.*, 2010). Thus, the effects of FA supplementation on PF were expected to be small.

In all fat tissues, the elevation of ALA and some longer-chain *n*-3 PUFAs was probably an effect of ALA supplementation. This is supported by the low content of several *n*-3 PUFAs in the INF and SCF of the CLA group. In all three adipose tissues, supplementation with CLAs increased *cis*-9, *trans*-11 CLA and HA (+ *trans*-10, *cis*-12 CLA) to a differing extent.

In SCF, GLA was increased in the CLA supplementation group. In the plasma fat of the five-day old calves, the percentage of GLA was higher in the CLA group than in the EFA+CLA group but not different from CON and EFA (Uken *et al.*, 2021a). In SCF, longer-chain *n*-6 PUFA ADA was significantly lower in the EFA+CLA group, compared to CON. Adrenic acid was lowered by EFA supplementation in the plasma of five-day old calves (Uken *et al.*, 2021a). *Trans*-10, *cis*-12 CLA suppresses desaturation of *n*-6 PUFAs in the mammary tissues (Banni *et al.*, 1999; Loor and Herbein, 2003). In this study's calves, the effect might suppress desaturation of GLA and reduce the formation of ADA. As a result, GLA accumulates and the formation of ADA from ARA is reduced.

The elevated stearic acid (SA) in INF and SCF of the EFA+CLA group, compared to CON, might be an effect of SCD suppression, as discussed in section 6.2.1 and by literature (Herdmann *et al.*, 2010; Hiller *et al.*, 2012). The supplementation might reduce desaturation of SA to OA, leading to accumulation of SA. Consequently, OA and the sum of MUFAs would have been reduced, which is not the case here.

Overall, it can be deduced that the FAs did reach the three adipose tissues. The low tissue FA content of INF and SCF was expected due to the young age of the calves. The high variation between samples was most likely not a result of supplementation as the total fat content in INF and SCF did not differ between groups. While INF und SCF FA composition changed similarly

to the muscle tissues, the levels of FA in PF were less affected as the number of altered FAs was lower than in the other adipose tissues. The PF was more developed at the onset of supplementation and therefore, it might have to a more lipid storage-based role (compare to Muhlhausler *et al.*, 2007). That is a possible reason for the reduced responsiveness to variations in FA supply. In INF and SCF, the high variation within groups might conceal group differences in single FA percentage. With higher age of the calves, supplementation effects might become more evident.

6.3.2 Adipose tissue cellularity

Fatty acids incorporated into the adipose tissues did not affect the mean size of individual fat cells, but induced minor changes in adipocyte size distribution. In the INF, the shift in skewness factors hints that maternal supplementation with EFAs induced a shift towards more smaller cells in the two EFA supplementation groups. Anyway, the supplementation did not change the size distribution of adipocytes. However, the adipocyte area in INF was negatively correlated with the percentage of ALA and the sum of *n*-3 PUFAs of the total FAs in INF. This implies an effect of the *n-3* PUFAs, which was not observed within the different size categories. For comparison, in the SCF, the distribution skewness hints that EFA and CLA supplementation increased the percentage of bigger cells compared to CON. Moreover, the cross-sectional area of SCF adipocytes was negatively correlated with the percentage of the sum of *n*-3 PUFAs of the total FAs, similar to the INF. This might be connected to a lower relative frequency of cells in the higher size categories, compared to the CLA and the EFA+CLA group. In addition, the area of SCF adipocytes positively correlated with the total fat content in the SCF. In this case, supplemented PUFAs might increase the incorporation of FAs and promote hypertrophic growth of adipocytes.

In the PF, the supplementation only had minor effects on adipocyte size distribution. The cell size distribution resembled a standard curve, compared to the other two tissues. The EFA or CLA supplementation appeared to have slightly reducing effects on the abundance of bigger adipocytes. The low reactivity of the PF was expected as the FA composition was also hardly affected by supplementation.

Alpha-linolenic acid stimulation of bovine *ex vivo* SCF and IMF increased adipocyte volume by uptake and *de novo* biosynthesis of FAs (Choi *et al.*, 2014). This result indicates that ALA increased adipocyte hypertrophy and FA incorporation, confirming the observation in SCF, but in contradiction to the increased abundance of smaller cells in the INF. Still, the experimental model and the usage of tissue explants from adult bovine animals can account for the differences.

Schoonmaker *et al.* (2004) observed thicker SCF and a larger subcutaneous adipocyte diameter in steers fed high concentrate feed, compared to high forage feed. Adipocyte number

in SCF was lowest in the concentrate fed animals. Perirenal fat and IMF did not differ between the diet groups (Schoonmaker *et al.*, 2004). Similarly, stimulation with LA increased adipocyte volume and lipid content of bovine INF and SCF *in vitro* (Yanting *et al.*, 2018), and porcine adipocytes from SCF incorporated more lipids after treatment with LA compared to palmitic acid (PA) or OA (Yu *et al.*, 2017). Applied to our project, this indicates that the control group feeding, with a high *n*-6/*n*-3 PUFA ratio, promoted hypertrophic growth of adipocytes. This could not be confirmed in this study's calves.

Akter *et al.* (2011) observed reduced adipocyte cross-sectional areas in different subcutaneous and visceral fat depots of dairy cows by supplementation with mixed CLA. Meadus *et al.* (2002) found that dietary supplementation of pigs with mixed CLA, compared to sunflower oil, reduced SCF but increased lean muscle and INF percentage per kg of body weight. Dugan *et al.* (1997) observed reduced SCF (g/kg of tissue cut) in mixed CLA supplemented pigs, when compared to LA supplemented pigs. *Trans*-10, *cis*-12 CLA reduced the incorporation of lipids into murine preadipocytes (den Hartigh *et al.*, 2013) and human adipocytes (Brown *et al.*, 2003). These findings confirm the anti-adipogenic effect of *trans*-10, *cis*-12 CLA, which was not specifically observed in the adipose tissues in our study.

There are also studies reporting no effect of the applied fatty acids. Wachira *et al.* (2002) supplemented sheep with linseed and/or fish oil, with no consequences on SCF and INF abundance. In lambs supplemented with sunflower oil, the tissue percentage of INF and SCF was unaffected, such as the total mass of PF (Manso *et al.*, 2009). In Holstein bulls supplemented with linseed or rumen-protected CLAs or both combined, the size, area and diameter distribution of subcutaneous adipocytes were not affected (Albertí *et al.*, 2013). According to these studies, ALA, longer-chain *n*-3 PUFAs or CLA supplementation do not alter adipose tissue cellularity, such as in this study only minor effects were observed.

In the INF, the EFA supplementation probably stimulated the recruitment of premature adipocytes, and their terminal adipogenic differentiation in INF. These cells might mature and undergo hypertrophy in a later growth phase (Kaplan *et al.*, 1980) than the cells in the other supplementation groups, ultimately leading to increased INF content. In the SCF, supplementation with EFAs and CLAs might have provided more substrate for FA incorporation of adipocytes, which increased hypertrophic growth.

The effects of supplementation with LA, ALA or CLAs were mostly observed *in vitro*, but not *in vivo*. In addition, the studies often report the tissue percentage of adipose depots, but little is shown regarding the adipocyte size distribution (compare to Albertí *et al.*, 2013). Furthermore, the varying experimental models, animal species, animal maturity, period of supplementation and ruminal biohydrogenation of supplemented PUFAs differ from this study. In this study's calves, the low maturity of the calves' SCF and INF increased the variance between the samples. As a result, the group variation in adipocyte size was high also within the

supplementation groups. This variation might have masked effects of the supplementation. In addition, the different fat depots have different metabolic properties (Kempster, 1981; Dodson *et al.*, 2010; Hausman and Dodson, 2013) and respond differently to the supplemented FAs (Urrutia *et al.*, 2020). The exact mechanisms on how to regulate adipocyte formation via FA supplementation are not clear yet (Hausman *et al.*, 2009; Urrutia *et al.*, 2020).

6.3.3 Adipogenic precursors in the intermuscular adipose tissue

Investigating adipogenic precursors in the INF gives an idea of the adipose tissue future development. Cells expressing C/EBPβ or DLK-1 were detected via immunohistochemical staining, as in skeletal muscle tissue. Cell shape and localization were similar to what was observed in the muscle tissues (6.2.9). Based on the size of their lipid droplets and the shape of their nuclei, cells positive for C/EBPβ were differentiating preadipocytes, as expected. Preadipocytes expressing DLK-1 mostly appeared within the adipocyte clusters rather than in the connective tissue between clusters. This might indicate that the cells give rise to new cells within the same cluster, rather than to a new cluster of cells.

In contrast to the effects observed in the muscles, the protein and mRNA abundance of *DLK1* in INF were unaffected by supplementation. The EFA supplementation reduced INF mRNA abundance of *ZNF423* and tended to reduce mRNA abundance of CCAAT enhancer binding protein alpha (*CEBPA*) and nuclear receptor subfamily 2 group C member 1 (*NR2C1*) to a small extent, but expression levels of *CEBPB*, *PPARG*, forkhead box O1 (*FOXO1*), *PDGFRA* and adipogenesis associated Mth938 domain containing (*AAMDC*) were not affected. Nuclear receptor *NR2C1* mRNA expression tended to be reduced in the EFA animals. According to Gupta *et al.* (2007), NR2C1 is necessary for murine preadipocyte proliferation and the knockdown of *Nr2c1* inhibited proliferation of and promoted lipid incorporation by preadipocytes. In this case, a reduction of *NR2C1* mRNA levels in the EFA animals might not lead to a higher fat incorporation, but possibly to a higher abundance of small preadipocytes in terminal differentiation. On the other hand, the reduction of *NR2C1* might not be sufficient to cause any effects in the EFA group. The relative mRNA abundance of *ZNF423* was lower in INF of EFA supplemented animals. Transcription factor *ZNF423* is expressed in committed adipocytes (Huang *et al.*, 2012) and induces gene expression of *PPARG2* (Gupta *et al.*, 2010; Shao *et al.*, 2017). Thereby, ZNF423 promotes terminal differentiation of preadipocytes. A low level of *ZNF423* implies that more adipocytes remain in an early state of differentiation with smaller lipid droplets in EFA supplemented animals. This fits to the higher percentage of smaller adipocytes in the INF of EFA animals. The direct link between supplemented FAs and *ZNF423* expression though might be content of further investigation. In addition, how reduced *ZNF423* led to a reduced adipocyte size is not clear, as the target *PPARG2* mRNA level was

unaltered in the EFA animals. The expression of *PPARG2* interaction partner *CEBPA* tended to be reduced in EFA supplemented animals, also implying that EFA supplementation increased the preadipocyte abundance in accordance with the cell size distribution (Shao *et al.*, 2017).

In summary, the investigation of calf adipose tissues indicated successful transfer of maternally supplemented FAs to INF, SCF and PF. There, they serve as signaling molecules in adipogenesis, influencing recruitment, proliferation and differentiation of preadipocytes. Moreover, they are, in the form of triacylglycerols, incorporated into adipocyte lipid droplets and increase the adipocyte volume. As such, the FAs promote hypertrophic growth of adipose tissues. The supplementation did not affect the mean adipocyte area, but had minor effects on the cell size distribution. In SCF, the relative abundance of bigger adipocytes appeared to be increased in the supplementation groups. A positive effect of FA supplementation on adipocyte hypertrophy might be the cause. In INF, the supplementation with EFAs apparently increased the relative abundance of smaller adipocytes, which was supported by reduction of *ZNF423* mRNA expression. The supplementation might have augmented the recruitment and proliferation of adipogenic precursors, and promoted their terminal differentiation.

7 Conclusion

This study showed for the first time that CLA and *n*-3 PUFA composition of calf muscle and adipose tissues can be manipulated via maternal supplementation with EFAs and CLAs during late gestation and especially after birth, via colostrum and transition milk.

In the skeletal muscle tissues, tissue morphology and fiber contraction behavior were not affected by the altered tissue FA content. There was evidence though, that CLA supplementation induced a muscle fiber type switch towards fast fibers in MLD. Intramuscular adipocyte area was unaffected by supplementation, but when adipogenic precursor cells were observed, EFA supplementation appeared to act proadipogenic. In INF and SCF, the supplementation with EFAs and CLAs induced minor morphological changes. In INF, *n*-3 PUFAs appeared to increase the abundance and differentiation of immature adipocytes, while in SCF, PUFA supplementation apparently increased adipocyte hypertrophy.

The reduced EFAs and CLAs in maternal corn silage-based feeding in the perinatal period, as applied in the control group, did not have apparent detrimental effects on offspring health. Moreover, the calves' organism appeared to compensate for the induced *n*-3 PUFA reduction over the time of supplementation and observation. An increase of supplementation and observation time will give further insight into the longer-term development of the calves, and might be conducted in future supplementation studies.

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9 Supplement

9.1 Fatty acid content in skeletal muscle and adipose tissues

9.1.1 Fatty acid content in *musculus longissimus dorsi*

Table 9.1: *Fatty acid concentration in longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	1.10 ± 0.10^{b}	0.85 ± 0.09^{b}	1.85 ± 0.14^{a}	1.65 ± 0.08^{a}	0.036	<.001	0.815
C18:3 *n*-3	2.03 ± 1.04^{c}	11.22 ± 0.91^{b}	1.99 ± 1.47^{c}	15.19 ± 0.86^{a}	<.001	0.073	0.066
C20:5 *n*-3	1.89 ± 0.78^{b}	8.73 ± 0.69^{a}	1.67 ± 1.08^{b}	10.24 ± 0.66^{a}	<.001	0.409	0.265
C22:5 *n*-3	8.72 ± 1.07^{b}	16.55 ± 0.98^{a}	8.64 ± 1.54^{b}	19.32 ± 0.91^{a}	<.001	0.250	0.232
C22:6 *n*-3	5.79 ± 0.95^{b}	9.44 ± 0.86^{a}	4.96 ± 1.27^{b}	9.65 ± 0.82^{a}	<.001	0.718	0.552
Sum of *n*-3 PUFAs[1]	18.55 ± 3.14^{b}	46.15 ± 2.78^{a}	17.44 ± 4.45^{b}	54.97 ± 2.62^{a}	<.001	0.233	0.128
C18:2 *n*-6	63.30 ± 6.43	63.51 ± 5.70	75.05 ± 9.11	78.58 ± 5.39	0.781	0.048	0.798
C18:3 *n*-6	1.21 ± 0.15	1.18 ± 0.14	1.12 ± 0.21	1.28 ± 0.13	0.680	0.969	0.514
C20:3 *n*-6	27.80 ± 2.22	25.87 ± 2.04	26.34 ± 3.20	28.50 ± 1.88	0.962	0.806	0.406
C20:4 *n*-6	67.29 ± 4.20^{ab}	57.23 ± 3.80^{b}	75.42 ± 5.59^{a}	62.91 ± 3.64^{ab}	0.008	0.077	0.749
C22:4 *n*-6	19.58 ± 1.33^{a}	13.53 ± 1.20^{b}	22.27 ± 1.79^{a}	15.07 ± 1.14^{b}	<.001	0.093	0.642
C22:5 *n*-6	1.55 ± 0.25^{a}	0.65 ± 0.23^{b}	1.87 ± 0.28^{a}	0.71 ± 0.26^{b}	<.001	0.270	0.424
Sum of *n*-6 PUFAs[2]	181.82 ± 12.68	163.08 ± 11.31	204.08 ± 17.71	188.74 ± 10.71	0.196	0.066	0.893
C18:2 *trans*-9, *trans*-12	1.28 ± 0.15	1.37 ± 0.14	1.51 ± 0.18	1.14 ± 0.14	0.178	0.988	0.031
C20:3 *n*-3	NA	0.26 ± 0.07^{b}	NA	0.58 ± 0.05^{a}	0.964	0.002	NA
C20:2 *n*-6	1.20 ± 0.17^{ab}	1.12 ± 0.16^{b}	1.54 ± 0.25^{ab}	1.75 ± 0.15^{a}	0.724	0.015	0.445
Sum of PUFAs[3]	202.77 ± 14.83	211.39 ± 13.21	225.11 ± 20.81	246.61 ± 12.49	0.329	0.062	0.666

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Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C16:1 *cis*-9	27.77 ± 1.74[ab]	24.59 ± 1.57[ab]	31.26 ± 2.34[a]	22.97 ± 1.50[b]	0.002	0.559	0.120
C17:1 *cis*-9	7.88 ± 0.82	7.22 ± 0.74	8.71 ± 1.09	7.36 ± 0.70	0.199	0.512	0.647
C18:1 *cis*-9	270.83 ± 12.51[ab]	240.65 ± 11.59[b]	297.36 ± 15.71[a]	247.38 ± 11.24[b]	0.001	0.099	0.321
C18:1 *cis*-11	39.78 ± 2.38	37.77 ± 2.14	42.42 ± 3.22	39.32 ± 2.04	0.272	0.349	0.808
C18:1 *trans*-9	1.08 ± 0.09[ab]	0.90 ± 0.08[b]	1.42 ± 0.12[a]	0.99 ± 0.07[b]	0.001	0.014	0.127
C18:1 *trans*-11	0.45 ± 0.15	0.32 ± 0.13	0.48 ± 0.21	0.39 ± 0.13	0.474	0.749	0.892
C20:1 *cis*-11	1.89 ± 0.12	1.61 ± 0.11	1.90 ± 0.18	1.80 ± 0.10	0.174	0.457	0.508
C22:1 *cis*-13	0.14 ± 0.02	NA	0.19 ± 0.03	0.16 ± 0.04	0.541	0.209	NA
Sum of MUFAs[4]	349.86 ± 15.98[ab]	313.24 ± 14.79[b]	383.29 ± 20.10[a]	320.03 ± 14.34[b]	0.001	0.120	0.299
C10:0	0.49 ± 0.07	0.55 ± 0.07	0.61 ± 0.08	0.50 ± 0.07	0.465	0.328	0.013
C12:0	0.44 ± 0.08	0.50 ± 0.07	0.47 ± 0.11	0.55 ± 0.07	0.370	0.655	0.876
C14:0	7.54 ± 0.81[a]	4.54 ± 0.72[b]	6.80 ± 1.12[ab]	6.26 ± 0.68[ab]	0.038	0.534	0.128
C15:0	1.28 ± 0.09	1.06 ± 0.08	1.34 ± 0.12	1.26 ± 0.07	0.085	0.120	0.401
C16:0	147.14 ± 9.41	128.36 ± 8.81	148.85 ± 11.51	134.83 ± 8.59	0.029	0.552	0.731
C17:0	5.64 ± 0.32	5.22 ± 0.30	5.89 ± 0.47	6.11 ± 0.27	0.781	0.113	0.381
C18:0	93.25 ± 5.49	87.85 ± 4.89	104.17 ± 7.64	104.22 ± 4.64	0.633	0.017	0.616
C20:0	0.83 ± 0.08	0.78 ± 0.07	1.09 ± 0.11	0.84 ± 0.07	0.047	0.026	0.172
C21:0[5]	0.20 ± 0.06	0.11 ± 0.05	0.33 ± 0.07	0.28 ± 0.05	0.249	0.021	0.730
Sum of SFAs[6]	257.48 ± 14.36	230.10 ± 13.26	269.91 ± 18.16	255.19 ± 12.85	0.086	0.111	0.585

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0. [a–c] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

Table 9.2: *Fatty acid composition in longissimus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.14 ± 0.01[b]	0.11 ± 0.01[b]	0.21 ± 0.01[a]	0.20 ± 0.01[a]	0.146	<.001	0.430
C18:3 *n*-3	0.25 ± 0.11[c]	1.45 ± 0.10[b]	0.23 ± 0.16[c]	1.84 ± 0.09[a]	<.001	0.114	0.078
C20:5 *n*-3	0.22 ± 0.08[b]	1.15 ± 0.08[a]	0.18 ± 0.11[b]	1.23 ± 0.07[a]	<.001	0.765	0.416
C22:5 *n*-3	1.07 ± 0.11[b]	2.18 ± 0.10[a]	0.97 ± 0.15[b]	2.34 ± 0.10[a]	<.001	0.768	0.180
C22:6 *n*-3	0.71 ± 0.11[b]	1.24 ± 0.10[a]	0.57 ± 0.14[b]	1.17 ± 0.10[a]	<.001	0.264	0.706
Sum of *n*-3 PUFAs[1]	2.24 ± 0.30[b]	6.05 ± 0.27[a]	1.94 ± 0.39[b]	6.64 ± 0.26[a]	<.001	0.582	0.103
C18:2 *n*-6	7.73 ± 0.51	8.36 ± 0.47	8.39 ± 0.73	9.51 ± 0.43	0.121	0.106	0.654
C18:3 *n*-6	0.15 ± 0.02	0.15 ± 0.01	0.13 ± 0.02	0.15 ± 0.01	0.297	0.501	0.500
C20:3 *n*-6	3.39 ± 0.25	3.46 ± 0.22	2.93 ± 0.33	3.44 ± 0.21	0.219	0.285	0.332
C20:4 *n*-6	8.29 ± 0.31	7.57 ± 0.28	8.57 ± 0.39	7.66 ± 0.27	0.004	0.461	0.715
C22:4 *n*-6	2.39 ± 0.09[a]	1.81 ± 0.08[b]	2.53 ± 0.13[a]	1.83 ± 0.08[b]	<.001	0.432	0.551
C22:5 *n*-6	0.19 ± 0.03[a]	0.08 ± 0.03[b]	0.21 ± 0.03[a]	0.08 ± 0.03[b]	<.001	0.675	0.765
Sum of *n*-6 PUFAs[2]	22.36 ± 0.74	21.55 ± 0.68	23.21 ± 1.07	22.81 ± 0.62	0.459	0.193	0.800
C18:2 *trans*-9, *trans*-12	0.16 ± 0.02	0.18 ± 0.02	0.18 ± 0.02	0.14 ± 0.02	0.490	0.314	0.048
C20:3 *n*-3	NA	0.03 ± 0.01[b]	NA	0.07 ± 0.01[a]	0.940	0.001	NA
C20:2 *n*-6	0.15 ± 0.02[b]	0.15 ± 0.02[ab]	0.17 ± 0.03[ab]	0.21 ± 0.02[a]	0.333	0.032	0.372
Sum of PUFAs[3]	24.95 ± 0.92[b]	27.90 ± 0.81[ab]	25.61 ± 1.35[b]	29.77 ± 0.76[a]	0.001	0.206	0.536
C16:1 *cis*-9	3.44 ± 0.19	3.28 ± 0.17	3.58 ± 0.27	2.81 ± 0.16	0.029	0.424	0.150
C17:1 *cis*-9	0.96 ± 0.08	0.96 ± 0.08	1.00 ± 0.11	0.89 ± 0.07	0.434	0.782	0.469
C18:1 *cis*-9	33.83 ± 0.56[a]	31.96 ± 0.52[ab]	34.03 ± 0.81[a]	30.31 ± 0.47[b]	<.001	0.241	0.144
C18:1 *cis*-11	4.88 ± 0.18	5.00 ± 0.17	4.88 ± 0.26	4.78 ± 0.15	0.964	0.570	0.583
C18:1 *trans*-9	0.14 ± 0.01	0.12 ± 0.01	0.16 ± 0.01	0.12 ± 0.01	0.010	0.173	0.202
C18:1 *trans*-11	0.06 ± 0.02	0.05 ± 0.02	0.05 ± 0.02	0.05 ± 0.01	0.674	0.886	0.920

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Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C20:1 *cis*-11	0.24 ± 0.02	0.22 ± 0.01	0.22 ± 0.02	0.22 ± 0.01	0.513	0.561	0.552
C22:1 *cis*-13	0.02 ± 0.00	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.016	0.251	0.836
Sum of MUFAs[4]	43.57 ± 0.72[a]	41.58 ± 0.66[ab]	43.90 ± 1.04[a]	39.19 ± 0.61[b]	<.001	0.194	0.094
C10:0	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.786	0.819	0.018
C12:0	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.352	1.000	0.845
C14:0	0.93 ± 0.09[a]	0.60 ± 0.08[b]	0.77 ± 0.12[ab]	0.77 ± 0.07[ab]	0.067	0.967	0.061
C15:0	0.16 ± 0.01	0.14 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.177	0.443	0.190
C16:0	18.02 ± 0.74	17.01 ± 0.66	16.96 ± 1.01	16.44 ± 0.63	0.298	0.254	0.726
C17:0	0.70 ± 0.03	0.69 ± 0.03	0.68 ± 0.04	0.74 ± 0.03	0.361	0.727	0.224
C18:0	11.56 ± 0.23[b]	11.67 ± 0.21[b]	11.76 ± 0.33[ab]	12.59 ± 0.20[a]	0.072	0.032	0.164
C20:0	0.11 ± 0.01	0.10 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.152	0.274	0.288
C21:0[5]	0.02 ± 0.01	0.02 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.470	0.068	0.762
Sum of SFAs[6]	31.78 ± 0.71	30.47 ± 0.65	30.85 ± 0.94	31.08 ± 0.62	0.405	0.799	0.228
Total fat content (%)	0.81 ± 0.04	0.76 ± 0.04	0.87 ± 0.05	0.82 ± 0.04	0.143	0.066	0.997

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0. [a–c] Different superscripts indicate significant differences among groups (P < 0.05). NA: Not calculated by SAS software due to small sample size.

9.1.2 Fatty acid content in *musculus semitendinosus*

Table 9.3: *Fatty acid concentration in semitendinosus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.91 ± 0.12^{b}	0.72 ± 0.11^{b}	1.67 ± 0.17^{a}	1.66 ± 0.10^{a}	0.447	<.001	0.494
C18:3 *n*-3	1.94 ± 0.71^{c}	10.02 ± 0.63^{b}	2.04 ± 0.98^{c}	13.00 ± 0.60^{a}	<.001	0.032	0.043
C18:4 *n*-3	0.33 ± 0.03	0.32 ± 0.03	0.35 ± 0.04	0.32 ± 0.03	0.486	0.802	0.846
C20:5 *n*-3	1.60 ± 0.35^{b}	5.73 ± 0.31^{a}	1.34 ± 0.47^{b}	6.49 ± 0.29^{a}	<.001	0.458	0.133
C22:5 *n*-3	8.55 ± 0.68^{b}	15.72 ± 0.62^{a}	7.80 ± 0.97^{b}	17.60 ± 0.57^{a}	<.001	0.441	0.085
C22:6 *n*-3	4.53 ± 0.57^{b}	7.03 ± 0.50^{a}	3.88 ± 0.81^{b}	7.23 ± 0.47^{a}	<.001	0.698	0.467
Sum of *n*-3 PUFAs[1]	17.29 ± 1.46^{c}	39.34 ± 1.34^{b}	15.62 ± 2.10^{c}	45.44 ± 1.23^{a}	<.001	0.165	0.021
C18:2 *n*-6	55.58 ± 4.78	59.89 ± 4.20	63.15 ± 6.89	71.24 ± 3.97	0.232	0.065	0.701
C18:3 *n*-6	1.31 ± 0.10	1.27 ± 0.09	1.17 ± 0.15	1.28 ± 0.09	0.725	0.537	0.510
C20:3 *n*-6	29.66 ± 2.13	29.59 ± 1.95	26.54 ± 3.07	30.38 ± 1.80	0.418	0.613	0.406
C20:4 *n*-6	55.19 ± 2.03	48.13 ± 1.81	56.52 ± 2.88	51.44 ± 1.71	0.008	0.268	0.632
C22:4 *n*-6	15.20 ± 0.76^{a}	11.23 ± 0.67^{b}	15.73 ± 1.04^{a}	11.99 ± 0.64^{b}	<.001	0.380	0.876
C22:5 *n*-6	2.81 ± 0.19	2.37 ± 0.17	2.86 ± 0.26	2.34 ± 0.17	0.016	0.976	0.816
Sum of *n*-6 PUFAs[2]	161.34 ± 7.51	153.88 ± 6.89	167.99 ± 10.82	171.39 ± 6.34	0.804	0.144	0.512
C18:2 *trans*-9, *trans*-12	1.65 ± 0.17	1.56 ± 0.16	1.96 ± 0.24	1.57 ± 0.14	0.197	0.403	0.417
C20:3 *n*-3	NA	0.53 ± 0.05^{b}	NA	0.85 ± 0.05^{a}	<.001	<.001	NA
C20:2 *n*-6	1.65 ± 0.20^{ab}	1.57 ± 0.18^{b}	1.82 ± 0.27^{ab}	2.19 ± 0.17^{a}	0.478	0.044	0.244
C20:3 *n*-9	3.44 ± 0.34	3.46 ± 0.31	3.99 ± 0.48	3.15 ± 0.28	0.270	0.749	0.247
Sum of PUFAs[3]	184.63 ± 7.95^{b}	198.97 ± 7.29^{ab}	191.22 ± 11.45^{ab}	223.20 ± 6.71^{a}	0.012	0.081	0.317
C16:1 *cis*-9	23.65 ± 1.61	23.41 ± 1.48	26.69 ± 2.32	21.23 ± 1.36	0.113	0.805	0.149
C17:1 *cis*-9	2.41 ± 0.28	1.67 ± 0.26	2.36 ± 0.39	1.68 ± 0.24	0.015	0.940	0.906

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Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *cis*-9	228.54 ± 11.02	215.81 ± 10.43	241.22 ± 13.10	216.98 ± 10.22	0.022	0.349	0.439
C18:1 *cis*-11	33.22 ± 1.42	34.08 ± 1.25	33.80 ± 2.00	34.78 ± 1.18	0.533	0.655	0.967
C18:1 *trans*-9	1.14 ± 0.11^{ab}	1.06 ± 0.09^{b}	1.55 ± 0.14^{a}	1.24 ± 0.09^{ab}	0.074	0.006	0.259
C18:1 *trans*-11	1.32 ± 0.10^{b}	1.20 ± 0.09^{b}	1.51 ± 0.14^{ab}	1.67 ± 0.08^{a}	0.864	0.003	0.195
C20:1 *cis*-11	2.13 ± 0.16	1.92 ± 0.14	1.97 ± 0.20	2.10 ± 0.14	0.748	0.911	0.203
C22:1 *cis*-13	0.43 ± 0.03	0.40 ± 0.02	0.42 ± 0.04	0.41 ± 0.02	0.414	0.892	0.680
Sum of MUFAs[4]	293.08 ± 13.41	279.61 ± 12.58	309.84 ± 16.33	279.85 ± 12.28	0.038	0.381	0.398
C10:0	0.81 ± 0.07	0.82 ± 0.07	0.82 ± 0.10	0.74 ± 0.06	0.567	0.624	0.476
C12:0	1.05 ± 0.10^{b}	0.65 ± 0.09^{a}	0.76 ± 0.14^{ab}	0.92 ± 0.09^{ab}	0.227	0.936	0.007
C14:0	7.67 ± 1.06	5.47 ± 0.97	6.57 ± 1.36	7.94 ± 0.94	0.655	0.445	0.054
C15:0	1.35 ± 0.12	1.36 ± 0.10	1.37 ± 0.15	1.51 ± 0.10	0.491	0.407	0.554
C16:0	129.97 ± 9.09	118.68 ± 8.50	122.80 ± 11.16	136.86 ± 8.28	0.842	0.414	0.070
C17:0	7.99 ± 0.55	7.66 ± 0.51	7.89 ± 0.69	8.90 ± 0.49	0.452	0.192	0.129
C18:0	84.00 ± 4.81^{b}	86.67 ± 4.54^{b}	92.63 ± 5.79^{ab}	98.49 ± 4.43^{a}	0.224	0.005	0.635
C20:0	1.15 ± 0.06^{b}	1.25 ± 0.06^{ab}	1.35 ± 0.09^{ab}	1.37 ± 0.05^{a}	0.361	0.016	0.546
C21:0[5]	0.36 ± 0.05^{b}	0.34 ± 0.05^{b}	0.69 ± 0.07^{a}	0.77 ± 0.04^{a}	0.643	<.001	0.386
C22:0	1.05 ± 0.10	0.93 ± 0.09	1.23 ± 0.13	1.06 ± 0.08	0.126	0.091	0.806
C23:0	0.67 ± 0.08	0.67 ± 0.07	0.97 ± 0.11	0.73 ± 0.06	0.141	0.027	0.132
Sum of SFAs[6]	236.24 ± 15.15^{ab}	224.48 ± 14.23^{b}	237.33 ± 18.39^{ab}	259.29 ± 13.89^{a}	0.649	0.105	0.130

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0. [a–c] Different superscripts indicate significant differences among groups (P < 0.05). NA: Not calculated by SAS software due to small sample size.

Table 9.4: *Fatty acid composition in semitendinosus muscle of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.13 ± 0.01[b]	0.10 ± 0.01[b]	0.23 ± 0.02[a]	0.22 ± 0.01[a]	0.177	<.001	0.670
C18:3 *n*-3	0.26 ± 0.10[b]	1.43 ± 0.09[a]	0.27 ± 0.14[b]	1.71 ± 0.09[a]	<.001	0.154	0.181
C18:4 *n*-3	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.436	0.648	0.694
C20:5 *n*-3	0.21 ± 0.06[b]	0.82 ± 0.05[a]	0.17 ± 0.08[b]	0.86 ± 0.05[a]	<.001	0.881	0.431
C22:5 *n*-3	1.21 ± 0.11[b]	2.24 ± 0.10[a]	1.06 ± 0.15[b]	2.33 ± 0.09[a]	<.001	0.799	0.264
C22:6 *n*-3	0.64 ± 0.09[b]	1.01 ± 0.08[a]	0.50 ± 0.12[b]	0.97 ± 0.08[a]	<.001	0.230	0.540
Sum of *n*-3 PUFAs[1]	2.36 ± 0.28[b]	5.63 ± 0.25[a]	2.04 ± 0.36[b]	6.00 ± 0.25[a]	<.001	0.918	0.159
C18:2 *n*-6	7.76 ± 0.49	8.53 ± 0.45	8.71 ± 0.70	9.36 ± 0.41	0.188	0.100	0.908
C18:3 *n*-6	0.18 ± 0.01	0.18 ± 0.01	0.16 ± 0.02	0.17 ± 0.01	0.788	0.177	0.726
C20:3 *n*-6	4.19 ± 0.27	4.22 ± 0.24	3.60 ± 0.40	4.00 ± 0.23	0.464	0.163	0.507
C20:4 *n*-6	7.81 ± 0.27[a]	6.92 ± 0.25[b]	7.63 ± 0.34[ab]	6.80 ± 0.25[b]	0.001	0.491	0.897
C22:4 *n*-6	2.15 ± 0.09[a]	1.61 ± 0.08[b]	2.17 ± 0.13[a]	1.58 ± 0.07[b]	<.001	0.960	0.787
C22:5 *n*-6	0.40 ± 0.03[a]	0.34 ± 0.03[ab]	0.39 ± 0.04[ab]	0.31 ± 0.03[b]	0.017	0.492	0.659
Sum of *n*-6 PUFAs[2]	22.76 ± 0.76	22.02 ± 0.70	23.16 ± 1.09	22.50 ± 0.64	0.402	0.590	0.960
C18:2 *trans*-9, *trans*-12	0.24 ± 0.03	0.23 ± 0.02	0.27 ± 0.04	0.21 ± 0.02	0.207	0.818	0.319
C20:3 *n*-3	NA	0.08 ± 0.01[b]	NA	0.11 ± 0.01[a]	<.001	0.001	NA
C20:2 *n*-6	0.23 ± 0.02	0.22 ± 0.02	0.24 ± 0.03	0.28 ± 0.02	0.331	0.110	0.303
C20:3 *n*-9	0.50 ± 0.05	0.50 ± 0.05	0.55 ± 0.08	0.41 ± 0.05	0.254	0.796	0.228
Sum of PUFAs[3]	25.96 ± 0.97[b]	28.51 ± 0.86[ab]	26.14 ± 1.37[ab]	29.32 ± 0.81[a]	0.008	0.615	0.748
C16:1 *cis*-9	3.37 ± 0.22	3.36 ± 0.20	3.69 ± 0.31	2.76 ± 0.18	0.054	0.558	0.061
C17:1 *cis*-9	0.33 ± 0.03[a]	0.24 ± 0.03[ab]	0.33 ± 0.04[ab]	0.22 ± 0.03[b]	0.004	0.728	0.732
C18:1 *cis*-9	32.39 ± 0.57[a]	30.54 ± 0.52[ab]	32.96 ± 0.81[a]	28.71 ± 0.48[b]	<.001	0.304	0.062
C18:1 *cis*-11	4.70 ± 0.12	4.87 ± 0.11	4.67 ± 0.17	4.55 ± 0.10	0.846	0.186	0.296

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Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *trans*-9	0.16 ± 0.01^{ab}	0.15 ± 0.01^{ab}	0.22 ± 0.02^{a}	0.16 ± 0.01^{ab}	0.032	0.031	0.119
C18:1 *trans*-11	0.18 ± 0.01^{ab}	0.17 ± 0.01^{b}	0.21 ± 0.02^{ab}	0.22 ± 0.01^{a}	0.680	0.006	0.504
C20:1 *cis*-11	0.30 ± 0.02	0.27 ± 0.01	0.26 ± 0.02	0.27 ± 0.01	0.641	0.252	0.227
C22:1 *cis*-13	0.06 ± 0.00	0.06 ± 0.00	0.06 ± 0.00	0.05 ± 0.00	0.198	0.248	0.862
Sum of MUFAs[4]	41.50 ± 0.75^{a}	39.65 ± 0.69^{a}	42.41 ± 1.08^{a}	36.95 ± 0.63^{b}	<.001	0.272	0.035
C10:0	0.12 ± 0.01	0.12 ± 0.01	0.11 ± 0.01	0.10 ± 0.01	0.470	0.263	0.381
C12:0	0.14 ± 0.01^{a}	0.09 ± 0.01^{b}	0.10 ± 0.01^{ab}	0.12 ± 0.01^{ab}	0.117	0.407	0.002
C14:0	1.03 ± 0.10	0.77 ± 0.09	0.85 ± 0.13	1.00 ± 0.09	0.556	0.769	0.034
C15:0	0.18 ± 0.01	0.19 ± 0.01	0.18 ± 0.02	0.20 ± 0.01	0.349	0.998	0.894
C16:0	18.01 ± 0.58	16.77 ± 0.53	16.46 ± 0.73	17.85 ± 0.51	0.885	0.613	0.010
C17:0	1.11 ± 0.04	1.08 ± 0.04	1.06 ± 0.05	1.16 ± 0.03	0.280	0.687	0.068
C18:0	11.77 ± 0.19^{b}	12.29 ± 0.17^{b}	12.49 ± 0.25^{ab}	12.93 ± 0.16^{a}	0.011	<.001	0.808
C20:0	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.581	0.295	0.323
C21:0[5]	0.05 ± 0.01^{b}	0.05 ± 0.01^{b}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.911	<.001	0.700
C22:0	0.15 ± 0.01	0.13 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.087	0.195	0.594
C23:0	0.10 ± 0.01	0.10 ± 0.01	0.13 ± 0.02	0.10 ± 0.01	0.114	0.083	0.081
Sum of SFAs[6]	32.85 ± 0.73^{ab}	31.76 ± 0.68^{b}	31.93 ± 0.91^{ab}	33.88 ± 0.66^{a}	0.459	0.290	0.012
Total fat content (%)	0.71 ± 0.03	0.70 ± 0.03	0.74 ± 0.04	0.76 ± 0.03	0.785	0.091	0.487

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6 + C22:5 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0. [a,b] Different superscripts indicate significant differences among groups (P < 0.05). NA: Not calculated by SAS software due to small sample size.

9.1.3 Fatty acid content in intermuscular fat

Table 9.5: *Fatty acid concentration in intermuscular fat (at longissimus muscle) of calves of four maternal supplementation groups: control group (CON, n = 7), essential fatty acids (EFA, n = 8), conjugated linoleic acids (CLA, n = 5) and EFA and CLA combined (EFA+CLA, n = 7). Data are given as LSM ± SE_{LSM}.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	10.04 ± 3.82	4.32 ± 3.77	17.79 ± 5.46	11.08 ± 3.46	0.159	0.100	0.908
C18:3 *n*-3	15.52 ± 9.36	15.66 ± 11.86	20.15 ± 8.71	37.64 ± 8.59	0.114	0.263	0.261
C18:4 *n*-3	0.80 ± 0.24	0.55 ± 0.25	NA	0.97 ± 0.20	0.835	0.291	0.358
C20:5 *n*-3	1.49 ± 0.46[b]	3.15 ± 0.45[ab]	1.12 ± 0.65[b]	3.50 ± 0.41[a]	0.001	0.987	0.494
C22:5 *n*-3	8.54 ± 2.05	12.48 ± 1.64	7.05 ± 3.02	12.95 ± 1.62	0.037	0.804	0.598
C22:6 *n*-3	1.62 ± 0.74	3.53 ± 0.69	1.72 ± 0.92	2.72 ± 0.69	0.029	0.544	0.428
Sum of *n*-3 PUFAs[1]	28.14 ± 11.55	41.41 ± 10.61	26.14 ± 15.01	60.23 ± 10.42	0.037	0.410	0.301
C18:2 *n*-6	151.31 ± 50.11	126.05 ± 44.38	190.95 ± 68.04	156.43 ± 43.32	0.552	0.462	0.919
C18:3 *n*-6	3.26 ± 1.30	2.36 ± 1.18	3.25 ± 1.69	3.04 ± 1.16	0.645	0.766	0.757
C20:3 *n*-6	23.06 ± 5.23	20.52 ± 5.16	20.31 ± 7.47	16.69 ± 4.73	0.601	0.572	0.926
C20:4 *n*-6	46.88 ± 5.68	39.38 ± 5.14	43.42 ± 8.04	37.76 ± 4.89	0.285	0.663	0.870
C22:4 *n*-6	16.05 ± 2.51	11.87 ± 2.28	15.62 ± 3.45	11.34 ± 2.19	0.113	0.845	0.984
Sum of *n*-6 PUFAs[2]	244.64 ± 65.14	205.80 ± 56.67	278.34 ± 90.54	231.83 ± 55.11	0.528	0.637	0.950
C18:2 *trans*-9, *trans*-12	2.12 ± 0.75	1.84 ± 0.68	1.81 ± 0.93	1.26 ± 0.60	0.586	0.566	0.853
C20:3 *n*-3	1.58 ± 0.86	1.77 ± 0.65	NA	2.76 ± 0.69	0.450	0.216	0.760
C20:2 *n*-6	5.65 ± 1.96	5.15 ± 1.77	7.33 ± 2.58	6.27 ± 1.74	0.675	0.429	0.870
C22:2 *n*-6	0.42 ± 0.13	0.27 ± 0.11	0.56 ± 0.12	0.50 ± 0.10	0.409	0.152	0.703
C20:3 *n*-9	4.25 ± 1.11	3.10 ± 1.10	4.04 ± 1.59	2.84 ± 1.01	0.354	0.851	0.985
Sum of PUFAs[3]	287.43 ± 79.26	255.76 ± 69.67	326.08 ± 108.65	306.71 ± 67.89	0.751	0.555	0.933
C14:1 *cis*-9	61.62 ± 30.09	61.83 ± 26.70	68.44 ± 45.05	29.49 ± 25.29	0.568	0.693	0.523
C16:1 *cis*-9	515.82 ± 228.55	546.16 ± 225.51	601.46 ± 326.45	215.06 ± 206.71	0.490	0.629	0.421

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Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C17:1 *cis*-9	15.52 ± 6.09	10.24 ± 5.93	17.97 ± 6.82	14.79 ± 5.90	0.227	0.296	0.744
C18:1 *cis*-9	3784 ± 1390	3331 ± 1371	4671 ± 1985	2175 ± 1257	0.350	0.931	0.515
C18:1 *cis*-11	196.32 ± 77.27	178.27 ± 76.24	257.56 ± 110.37	121.98 ± 69.88	0.381	0.977	0.500
C18:1 *trans*-9	16.02 ± 5.30	8.84 ± 5.23	14.60 ± 7.56	10.69 ± 4.79	0.356	0.970	0.783
C18:1 *trans*-11	13.94 ± 5.65	10.83 ± 5.20	18.62 ± 7.31	14.46 ± 5.12	0.481	0.400	0.913
C20:1 *cis*-11	16.66 ± 7.25	15.30 ± 6.50	22.84 ± 10.37	11.51 ± 6.18	0.420	0.872	0.491
C22:1 *cis*-13	1.44 ± 0.32	1.45 ± 0.29	1.49 ± 0.45	1.37 ± 0.28	0.872	0.975	0.835
Sum of MUFAs[4]	4619 ± 1734	4159 ± 1711	5667 ± 2477	2590 ± 1568	0.369	0.892	0.504
C10:0	9.73 ± 3.09	7.13 ± 2.84	10.77 ± 4.02	8.28 ± 2.79	0.375	0.685	0.983
C12:0	30.49 ± 11.55	17.19 ± 10.09	31.80 ± 16.26	26.53 ± 9.75	0.447	0.642	0.715
C13:0	1.89 ± 0.76	1.29 ± 0.64	2.12 ± 1.13	1.68 ± 0.62	0.533	0.695	0.913
C14:0	389.98 ± 139.72	313.79 ± 137.86	500.14 ± 199.57	299.64 ± 126.37	0.383	0.757	0.692
C15:0	23.33 ± 10.18	15.66 ± 8.36	29.60 ± 15.23	21.84 ± 8.16	0.494	0.552	0.996
C16:0	3927 ± 1503	3626 ± 1483	4836 ± 2147	2467 ± 1360	0.433	0.940	0.542
C17:0	124.16 ± 42.01	95.20 ± 41.45	155.80 ± 60.00	106.59 ± 37.99	0.411	0.645	0.830
C18:0	1339 ± 559	1294 ± 482	1797 ± 838	962 ± 462	0.483	0.915	0.475
C20:0	10.69 ± 2.75	9.36 ± 2.52	11.36 ± 3.94	7.98 ± 2.38	0.433	0.901	0.716
C21:0[5]	2.17 ± 0.90	1.25 ± 0.89	5.07 ± 1.29	3.54 ± 0.81	0.233	0.017	0.759
C22:0	2.77 ± 0.51	2.13 ± 0.37	2.35 ± 0.76	2.29 ± 0.38	0.529	0.794	0.497
C23:0	1.75 ± 1.17	2.47 ± 0.10	3.19 ± 1.44	1.42 ± 0.91	0.656	0.867	0.300
C24:0	0.93 ± 0.19	0.73 ± 0.15	1.07 ± 0.24	0.71 ± 0.14	0.170	0.746	0.643
Sum of SFAs[6]	5857 ± 2158	5375 ± 2129	7386 ± 3082	3903 ± 1951	0.417	0.990	0.537

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a,b] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

Table 9.6: *Fatty acid composition in intermuscular fat (at longissimus muscle) of calves of four maternal supplementation groups: control group (CON, n = 7), essential fatty acids (EFA, n = 8), conjugated linoleic acids (CLA, n = 5) and EFA and CLA combined (EFA+CLA, n = 7). Data are given as LSM ± SE_{LSM}.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.11 ± 0.02^{ab}	0.08 ± 0.02^{b}	0.13 ± 0.03^{ab}	0.16 ± 0.02^{a}	0.897	0.023	0.191
C18:3 *n*-3	0.14 ± 0.07^{bc}	0.40 ± 0.06^{ab}	0.06 ± 0.10^{c}	0.48 ± 0.07^{a}	<.001	0.951	0.313
C18:4 *n*-3	0.02 ± 0.01	0.03 ± 0.01	NA	0.02 ± 0.01	0.190	0.084	0.343
C20:5 *n*-3	0.02 ± 0.02	0.07 ± 0.02	0.00 ± 0.02	0.06 ± 0.02	0.005	0.293	0.643
C22:5 *n*-3	0.21 ± 0.08	0.25 ± 0.07	0.05 ± 0.10	0.24 ± 0.08	0.132	0.221	0.295
C22:6 *n*-3	0.05 ± 0.02	0.06 ± 0.02	0.01 ± 0.03	0.05 ± 0.02	0.247	0.245	0.372
Sum of *n*-3 PUFAs[1]	0.48 ± 0.16^{ab}	0.81 ± 0.14^{ab}	0.16 ± 0.23^{b}	0.88 ± 0.15^{c}	0.006	0.460	0.243
C18:2 *n*-6	2.08 ± 0.32	1.86 ± 0.28	1.57 ± 0.43	2.13 ± 0.30	0.583	0.703	0.205
C18:3 *n*-6	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.981	0.539	0.142
C20:3 *n*-6	0.47 ± 0.08	0.30 ± 0.07	0.18 ± 0.11	0.30 ± 0.08	0.753	0.072	0.082
C20:4 *n*-6	1.55 ± 0.36	0.77 ± 0.32	0.43 ± 0.48	0.77 ± 0.34	0.529	0.114	0.111
C22:4 *n*-6	0.51 ± 0.11	0.20 ± 0.09	0.16 ± 0.16	0.25 ± 0.10	0.343	0.180	0.087
Sum of *n*-6 PUFAs[2]	4.74 ± 0.81	3.26 ± 0.72	2.46 ± 1.09	3.59 ± 0.78	0.825	0.214	0.098
C18:2 *trans*-9, *trans*-12	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.02	0.03 ± 0.01	0.673	0.873	0.628
C20:3 *n*-3	0.01 ± 0.01^{b}	0.03 ± 0.00^{ab}	NA	0.03 ± 0.01^{a}	0.023	0.486	0.870
C20:2 *n*-6	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.08 ± 0.01	0.548	0.887	0.186
C22:2 *n*-6	NA	NA	0.01 ± 0.01	NA	0.453	0.116	NA
C20:3 *n*-9	0.09 ± 0.02	0.05 ± 0.01	0.04 ± 0.02	0.05 ± 0.02	0.464	0.168	0.133
Sum of PUFAs[3]	5.20 ± 0.95	4.06 ± 0.84	2.61 ± 1.28	4.46 ± 0.91	0.702	0.233	0.107
C14:1 *cis*-9	0.46 ± 0.08	0.49 ± 0.07	0.43 ± 0.11	0.33 ± 0.08	0.657	0.237	0.402
C16:1 *cis*-9	3.68 ± 0.49	4.15 ± 0.43	4.07 ± 0.70	2.79 ± 0.47	0.448	0.345	0.095
C17:1 *cis*-9	0.33 ± 0.14	0.17 ± 0.12	0.13 ± 0.20	0.18 ± 0.13	0.695	0.521	0.466
C18:1 *cis*-9	34.46 ± 0.94	33.35 ± 0.77	34.45 ± 1.34	32.84 ± 0.87	0.182	0.780	0.782

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Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *cis*-11	2.03 ± 0.13	1.86 ± 0.11	1.91 ± 0.17	1.84 ± 0.12	0.365	0.582	0.694
C18:1 *trans*-9	0.16 ± 0.02	0.13 ± 0.02	0.12 ± 0.03	0.16 ± 0.02	0.696	0.686	0.166
C18:1 *trans*-11	0.22 ± 0.03	0.16 ± 0.03	0.12 ± 0.04	0.18 ± 0.03	0.993	0.298	0.137
C20:1 *cis*-11	0.24 ± 0.03	0.18 ± 0.03	0.16 ± 0.05	0.19 ± 0.03	0.634	0.356	0.184
C22:1 *cis*-13	0.05 ± 0.01	0.02 ± 0.01	0.01 ± 0.02	0.03 ± 0.01	0.633	0.206	0.117
Sum of MUFAs[4]	41.59 ± 1.01	40.54 ± 0.84	41.38 ± 1.42	38.53 ± 0.94	0.076	0.278	0.359
C10:0	0.12 ± 0.02	0.09 ± 0.02	0.08 ± 0.03	0.10 ± 0.02	0.801	0.426	0.229
C12:0	0.35 ± 0.06	0.31 ± 0.06	0.22 ± 0.09	0.32 ± 0.06	0.624	0.353	0.312
C13:0	0.03 ± 0.01	0.02 ± 0.00	0.02 ± 0.01	0.02 ± 0.00	0.605	0.311	0.168
C14:0	3.69 ± 0.45	3.77 ± 0.39	3.79 ± 0.59	3.86 ± 0.43	0.855	0.823	0.992
C15:0	0.27 ± 0.04	0.26 ± 0.03	0.25 ± 0.05	0.29 ± 0.03	0.721	0.953	0.391
C16:0	34.16 ± 1.34	35.76 ± 1.19	36.52 ± 1.78	35.66 ± 1.29	0.769	0.369	0.321
C17:0	1.25 ± 0.12	1.22 ± 0.10	1.31 ± 0.16	1.47 ± 0.11	0.579	0.182	0.394
C18:0	12.78 ± 0.60[b]	13.54 ± 0.54[ab]	13.46 ± 0.76[ab]	14.70 ± 0.58[a]	0.068	0.083	0.633
C20:0	0.19 ± 0.03	0.12 ± 0.03	0.09 ± 0.05	0.14 ± 0.03	0.826	0.268	0.111
C21:0[5]	0.09 ± 0.03	0.02 ± 0.02	0.05 ± 0.04	0.07 ± 0.03	0.318	0.976	0.116
C22:0	0.10 ± 0.03	0.04 ± 0.02	0.03 ± 0.04	0.05 ± 0.03	0.385	0.245	0.129
C23:0	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.874	0.285	0.349
C24:0	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.02 ± 0.00	0.878	0.423	0.287
Sum of SFAs[6]	53.12 ± 1.26	55.20 ± 1.08	55.93 ± 1.71	56.78 ± 1.20	0.249	0.082	0.595
Total fat content (%)	10.74 ± 3.94	9.78 ± 3.89	13.36 ± 5.63	6.79 ± 3.57	0.399	0.966	0.528

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a–c] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

9.1.4 Fatty acid content in subcutaneous fat

Table 9.7: *Fatty acid concentration in subcutaneous fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 10). Data are given as LSM ± SE_{LSM}.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	18.83 ± 6.61	12.88 ± 5.85	31.46 ± 9.54	32.87 ± 5.63	0.750	0.026	0.594
C18:3 *n*-3	12.72 ± 8.92^{c}	50.47 ± 8.17^{b}	10.06 ± 12.84^{bc}	88.81 ± 7.75^{a}	<.001	0.074	0.045
C18:4 *n*-3	0.87 ± 0.20	0.65 ± 0.23	NA	0.94 ± 0.18	0.619	0.358	0.705
C20:5 *n*-3	1.23 ± 0.63^{b}	4.48 ± 0.56^{a}	NA	6.32 ± 0.53^{a}	<.001	0.105	0.383
C22:5 *n*-3	11.43 ± 2.45^{b}	17.37 ± 2.29^{ab}	NA	24.56 ± 2.07^{a}	0.003	0.060	0.499
C22:6 *n*-3	2.84 ± 0.55^{b}	3.62 ± 0.46^{ab}	NA	4.74 ± 0.41^{a}	0.015	0.324	0.312
Sum of *n*-3 PUFAs[1]	27.87 ± 10.80^{c}	77.45 ± 9.89^{b}	17.27 ± 15.54^{c}	130.93 ± 9.38^{a}	<.001	0.076	0.012
C18:2 *n*-6	255.58 ± 58.39	249.99 ± 51.18	268.45 ± 85.43	347.39 ± 49.38	0.567	0.381	0.489
C18:3 *n*-6	6.26 ± 1.41^{b}	5.03 ± 1.23^{b}	15.03 ± 2.08^{a}	6.34 ± 1.19^{b}	0.003	0.003	0.017
C20:3 *n*-6	25.61 ± 6.09	26.14 ± 5.58	22.49 ± 8.76	29.82 ± 5.29	0.556	0.966	0.614
C20:4 *n*-6	41.55 ± 6.41	34.28 ± 5.68	36.22 ± 8.88	41.21 ± 5.49	0.861	0.900	0.333
C22:4 *n*-6	16.04 ± 2.56	10.61 ± 2.28	13.80 ± 3.45	13.45 ± 2.22	0.252	0.900	0.293
Sum of *n*-6 PUFAs[2]	353.57 ± 75.42	334.70 ± 66.18	367.86 ± 109.45	450.88 ± 63.84	0.695	0.417	0.515
C18:2 *trans*-9, *trans*-12	2.25 ± 0.46^{b}	2.13 ± 0.43^{b}	14.28 ± 0.67^{a}	1.99 ± 0.43^{b}	<.001	<.001	<.001
C20:3 *n*-3	0.76 ± 1.15^{b}	3.59 ± 0.64^{b}	NA	5.95 ± 0.61^{a}	0.005	0.096	0.914
C20:2 *n*-6	8.46 ± 2.30	8.47 ± 2.01	11.46 ± 3.34	12.45 ± 1.94	0.842	0.161	0.838
C20:3 *n*-9	5.50 ± 0.80	5.44 ± 0.71	5.28 ± 1.12	5.59 ± 0.69	0.881	0.966	0.815
Sum of PUFAs[3]	408.04 ± 88.86	432.08 ± 77.96	436.65 ± 129.39	622.42 ± 75.20	0.284	0.253	0.384
C14:1 *cis*-9	95.83 ± 26.17	74.54 ± 23.03	79.55 ± 38.45	91.59 ± 22.18	0.872	0.989	0.546
C16:1 *cis*-9	647.02 ± 178.86	691.33 ± 156.95	657.74 ± 262.91	751.76 ± 151.35	0.726	0.854	0.894
C17:1 *cis*-9	4.90 ± 2.61	4.76 ± 2.34	4.62 ± 3.52	3.73 ± 2.27	0.839	0.788	0.878

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Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *cis*-9	5632 ± 1261	5442 ± 1113	5455 ± 1778	6711 ± 1074	0.686	0.671	0.569
C18:1 *cis*-11	291.86 ± 58.70	286.54 ± 51.58	314.18 ± 85.89	343.91 ± 49.72	0.850	0.528	0.775
C18:1 *trans*-9	25.60 ± 5.82	22.01 ± 5.10	20.51 ± 8.62	31.86 ± 4.92	0.549	0.707	0.230
C18:1 *trans*-11	27.48 ± 9.79	21.57 ± 8.65	33.13 ± 13.93	34.59 ± 8.34	0.830	0.360	0.712
C20:1 *cis*-11	21.01 ± 4.02	19.46 ± 3.69	20.54 ± 5.79	25.63 ± 3.50	0.688	0.515	0.457
Sum of MUFAs[4]	6753 ± 1535	6563 ± 1354	6589 ± 2176	7993 ± 1306	0.708	0.688	0.608
C10:0	15.37 ± 4.95	14.53 ± 4.36	15.01 ± 6.92	19.83 ± 4.22	0.697	0.619	0.565
C12:0	61.99 ± 17.36	41.21 ± 15.28	50.37 ± 24.62	58.82 ± 14.75	0.736	0.866	0.408
C13:0	3.54 ± 0.98	2.64 ± 0.86	3.66 ± 1.38	3.39 ± 0.83	0.569	0.656	0.746
C14:0	685.40 ± 205.65	607.06 ± 181.41	697.97 ± 290.95	800.06 ± 175.08	0.956	0.626	0.664
C15:0	41.53 ± 11.38	36.58 ± 10.43	54.21 ± 16.38	57.55 ± 9.89	0.948	0.180	0.741
C16:0	6055 ± 1638	6269 ± 1443	6250 ± 2354	7527 ± 1391	0.672	0.672	0.753
C17:0	104.08 ± 22.18	91.85 ± 20.32	114.05 ± 31.91	131.96 ± 19.26	0.907	0.302	0.539
C18:0	1946 ± 515	2125 ± 454	2093 ± 742	2719 ± 438	0.470	0.495	0.674
C20:0	11.87 ± 2.71	11.32 ± 2.39	12.74 ± 3.88	15.04 ± 2.30	0.762	0.418	0.608
C21:0[5]	1.86 ± 1.12[b]	1.20 ± 1.02[b]	7.82 ± 1.61[a]	9.75 ± 0.97[a]	0.604	<.001	0.298
C22:0	2.45 ± 0.34	2.91 ± 0.31	NA	2.75 ± 0.29	0.398	0.967	0.601
C23:0	1.57 ± 0.72	1.66 ± 0.58	2.39 ± 0.69	1.91 ± 0.61	0.761	0.451	0.665
C24:0	2.53 ± 0.54	3.28 ± 0.51	NA	2.69 ± 0.47	0.310	0.513	0.544
Sum of SFAs[6]	8930 ± 2399	9210 ± 2114	9299 ± 3443	11344 ± 2038	0.651	0.618	0.720
Total fat content (g/100g tissue)	16.09 ± 3.99	16.21 ± 3.52	16.31 ± 5.70	19.95 ± 3.40	0.659	0.633	0.666

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a–c] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

Table 9.8: *Fatty acid composition in subcutaneous fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 10). Data are given as LSM ± SE_{LSM}.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.12 ± 0.02^{b}	0.09 ± 0.01^{b}	0.20 ± 0.02^{a}	0.17 ± 0.01^{a}	0.102	<.001	0.958
C18:3 *n*-3	0.10 ± 0.05^{b}	0.35 ± 0.04^{a}	0.06 ± 0.07^{b}	0.47 ± 0.04^{a}	<.001	0.484	0.167
C20:5 *n*-3	0.01 ± 0.01^{b}	0.03 ± 0.00^{a}	NA	0.03 ± 0.00^{a}	<.001	0.898	0.716
C22:5 *n*-3	0.08 ± 0.01^{b}	0.14 ± 0.01^{a}	NA	0.13 ± 0.01^{ab}	0.001	0.339	0.642
C22:6 *n*-3	0.03 ± 0.01	0.04 ± 0.00	NA	0.02 ± 0.00	0.067	0.049	0.686
Sum of *n*-3 PUFAs[1]	0.22 ± 0.07^{b}	0.57 ± 0.06^{a}	0.10 ± 0.09^{b}	0.68 ± 0.06^{a}	<.001	0.986	0.121
C18:2 *n*-6	1.64 ± 0.15	1.78 ± 0.14	1.68 ± 0.22	1.78 ± 0.13	0.491	0.910	0.915
C18:3 *n*-6	0.04 ± 0.00^{b}	0.04 ± 0.00^{b}	0.11 ± 0.01^{a}	0.03 ± 0.00^{b}	<.001	<.001	<.001
C20:3 *n*-6	0.17 ± 0.01	0.17 ± 0.01	0.13 ± 0.02	0.15 ± 0.01	0.706	0.081	0.579
C20:4 *n*-6	0.30 ± 0.03	0.25 ± 0.03	0.24 ± 0.04	0.21 ± 0.03	0.218	0.164	0.795
C22:4 *n*-6	0.11 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.09 ± 0.02^{ab}	0.07 ± 0.01^{b}	0.031	0.240	0.659
Sum of *n*-6 PUFAs[2]	2.32 ± 0.19	2.37 ± 0.17	2.33 ± 0.27	2.30 ± 0.16	0.971	0.898	0.849
C18:2 *trans*-9, *trans*-12	0.01 ± 0.00^{b}	0.02 ± 0.00^{b}	0.11 ± 0.01^{a}	0.01 ± 0.00^{b}	<.001	<.001	<.001
C20:3 *n*-3	NA	0.02 ± 0.00^{b}	NA	0.03 ± 0.00^{a}	0.003	0.048	NA
C20:2 *n*-6	0.06 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.608	0.117	0.365
C20:3 *n*-9	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.242	0.265	0.659
Sum of PUFAs[3]	2.71 ± 0.25	3.07 ± 0.23	2.79 ± 0.36	3.19 ± 0.22	0.169	0.705	0.938
C14:1 *cis*-9	0.54 ± 0.04	0.45 ± 0.04	0.41 ± 0.06	0.45 ± 0.04	0.590	0.166	0.188
C16:1 *cis*-9	3.90 ± 0.23	4.13 ± 0.21	3.85 ± 0.33	3.64 ± 0.20	0.966	0.286	0.391
C17:1 *cis*-9	0.05 ± 0.03	0.03 ± 0.02	0.05 ± 0.03	0.02 ± 0.02	0.380	0.895	0.792
C18:1 *cis*-9	35.71 ± 0.90	33.82 ± 0.82	34.88 ± 1.29	33.97 ± 0.78	0.161	0.731	0.622
C18:1 *cis*-11	1.92 ± 0.11	1.87 ± 0.10	2.06 ± 0.15	1.73 ± 0.10	0.092	0.965	0.181
C18:1 *trans*-9	0.16 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.16 ± 0.01	0.788	0.850	0.157

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Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *trans*-11	0.17 ± 0.03	0.14 ± 0.03	0.21 ± 0.04	0.18 ± 0.03	0.425	0.184	0.973
C20:1 *cis*-11	0.14 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.450	0.717	0.628
Sum of MUFAs[4]	42.57 ± 0.93	40.72 ± 0.85	41.72 ± 1.34	40.27 ± 0.81	0.113	0.523	0.844
C10:0	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.02	0.10 ± 0.01	0.346	0.743	0.523
C12:0	0.34 ± 0.05	0.28 ± 0.04	0.30 ± 0.07	0.32 ± 0.04	0.634	0.947	0.407
C13:0	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.207	0.582	0.704
C14:0	3.93 ± 0.34	3.75 ± 0.30	4.02 ± 0.49	4.06 ± 0.29	0.847	0.563	0.748
C15:0	0.26 ± 0.03	0.25 ± 0.03	0.34 ± 0.04	0.29 ± 0.03	0.351	0.066	0.418
C16:0	36.94 ± 0.80	38.01 ± 0.73	37.05 ± 1.14	37.35 ± 0.69	0.434	0.747	0.663
C17:0	0.66 ± 0.03	0.63 ± 0.03	0.71 ± 0.05	0.66 ± 0.03	0.281	0.257	0.802
C18:0	12.23 ± 0.34^{b}	13.08 ± 0.30^{ab}	12.77 ± 0.44^{ab}	13.56 ± 0.29^{a}	0.013	0.096	0.901
C20:0	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.01	0.08 ± 0.00	0.468	0.497	0.694
C21:0	0.01 ± 0.01^{b}	0.01 ± 0.01^{b}	0.05 ± 0.01^{a}	0.05 ± 0.00^{a}	0.947	<.001	0.838
C22:0	0.02 ± 0.00	0.02 ± 0.00	NA	0.01 ± 0.00	0.842	0.202	0.717
C23:0	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.066	0.778	0.952
C24:0	0.03 ± 0.01	0.03 ± 0.01	NA	0.01 ± 0.01	0.795	0.305	0.486
Sum of SFAs[5]	54.72 ± 0.99	56.21 ± 0.91	55.49 ± 1.43	56.53 ± 0.86	0.248	0.614	0.839

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C12:0 +C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a,b] Different superscripts indicate significant differences among groups (P < 0.05). NA: Not calculated by SAS software due to small sample size.

9.1.5 Fatty acid content in perirenal fat

Table 9.9: *Fatty acid concentration in perirenal fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	49.92 ± 6.30^{ab}	35.58 ± 5.78^{b}	68.13 ± 9.07^{a}	62.58 ± 5.32^{a}	0.154	0.002	0.527
C18:3 *n*-3	37.86 ± 21.49^{c}	120.27 ± 19.72^{ab}	24.25 ± 30.95^{ac}	179.10 ± 18.15^{a}	<.001	0.334	0.133
C18:4 *n*-3	2.48 ± 0.27^{a}	1.51 ± 0.25^{ab}	1.58 ± 0.39^{ab}	1.31 ± 0.23^{b}	0.044	0.070	0.244
C20:5 *n*-3	3.47 ± 0.82^{b}	9.08 ± 0.73^{a}	2.21 ± 1.17^{b}	10.68 ± 0.69^{a}	<.001	0.842	0.100
C22:5 *n*-3	24.36 ± 2.50^{b}	38.88 ± 2.30^{a}	15.36 ± 3.61^{b}	45.30 ± 2.11^{a}	<.001	0.635	0.009
C22:6 *n*-3	13.54 ± 2.18	19.78 ± 1.92	12.59 ± 3.20	20.00 ± 1.81	0.008	0.874	0.798
Sum of *n*-3 PUFAs[1]	84.62 ± 23.39^{b}	200.15 ± 21.46^{a}	58.14 ± 33.69^{b}	270.20 ± 19.75^{a}	<.001	0.391	0.069
C18:2 *n*-6	571.82 ± 56.81	594.53 ± 52.12	549.65 ± 81.81	663.59 ± 47.97	0.274	0.702	0.467
C18:3 *n*-6	12.49 ± 1.47	12.14 ± 1.34	8.11 ± 2.11	10.90 ± 1.24	0.448	0.084	0.335
C20:3 *n*-6	112.74 ± 12.08	113.85 ± 11.08	105.10 ± 17.40	117.79 ± 10.20	0.600	0.887	0.663
C20:4 *n*-6	113.92 ± 8.34	99.50 ± 7.65	118.28 ± 12.01	107.55 ± 7.04	0.173	0.492	0.841
C22:4 *n*-6	45.67 ± 4.96	38.90 ± 4.55	49.34 ± 7.14	44.30 ± 4.19	0.279	0.400	0.874
Sum of *n*-6 PUFAs[2]	884.34 ± 71.73	890.02 ± 65.81	862.91 ± 103.30	978.00 ± 60.57	0.441	0.668	0.490
C18:2 *trans*-9, *trans*-12	5.74 ± 0.94	5.31 ± 0.83	6.26 ± 1.30	6.17 ± 0.79	0.786	0.455	0.852
C20:3 *n*-3	2.92 ± 1.25^{b}	10.66 ± 1.15^{a}	1.93 ± 1.81^{b}	13.83 ± 1.06^{a}	<.001	0.425	0.140
C20:2 *n*-6	27.69 ± 4.11	30.23 ± 3.63	30.59 ± 5.84	33.01 ± 3.43	0.566	0.499	0.988
C22:2 *n*-6	0.38 ± 0.40^{b}	0.78 ± 0.37^{b}	2.75 ± 0.58^{a}	0.98 ± 0.34^{ab}	0.122	0.006	0.019
C20:3 *n*-9	10.64 ± 0.92	10.87 ± 0.85	10.25 ± 1.33	9.14 ± 0.78	0.659	0.294	0.509
Sum of PUFAs[3]	1035 ± 88	1142 ± 81	1006 ± 127	1326 ± 75	0.033	0.420	0.277
C14:1 *cis*-9	609.73 ± 62.79	507.47 ± 57.61	491.40 ± 90.42	425.66 ± 53.02	0.225	0.147	0.792
C16:1 *cis*-9	4549 ± 415	4998 ± 364	4546 ± 602	3816 ± 343	0.754	0.181	0.178

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Fatty acid (mg/100 g tissue)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C17:1 *cis*-9	7.37 ± 1.88	7.66 ± 1.80	7.27 ± 2.16	7.70 ± 1.77	0.758	0.976	0.950
C18:1 *cis*-9	22,614 ± 957	23,365 ± 839	22,018 ± 1374	23,662 ± 795	0.246	0.880	0.648
C18:1 *cis*-11	1341 ± 124	1614 ± 108	1619 ± 182	1472 ± 102	0.640	0.609	0.115
C18:1 *trans*-9	63.01 ± 11.34	54.98 ± 10.89	58.17 ± 13.02	54.56 ± 10.72	0.400	0.691	0.741
C18:1 *trans*-11	58.18 ± 8.34	44.99 ± 7.66	50.03 ± 12.02	57.02 ± 7.05	0.733	0.829	0.277
C20:1 *cis*-11	121.80 ± 24.60	147.01 ± 22.57	166.61 ± 35.42	163.20 ± 20.77	0.684	0.256	0.598
C22:1 *cis*-13	5.56 ± 0.80	5.87 ± 0.73	6.75 ± 1.15	6.21 ± 0.67	0.893	0.376	0.631
C24:1 *cis*-15	3.13 ± 0.52	3.27 ± 0.45	3.31 ± 0.75	2.97 ± 0.43	0.861	0.913	0.657
Sum of MUFAs[4]	29,499 ± 1161	30,750 ± 1028	28,989 ± 1621	29,717 ± 977	0.408	0.504	0.820
C10:0	30.31 ± 3.22	26.24 ± 2.85	26.50 ± 4.50	25.81 ± 2.70	0.473	0.509	0.596
C11:0	3.60 ± 0.40	3.21 ± 0.37	2.94 ± 0.58	2.81 ± 0.34	0.561	0.230	0.767
C12:0	93.59 ± 10.64	58.82 ± 9.77	76.81 ± 15.33	67.08 ± 8.99	0.063	0.711	0.290
C13:0	7.49 ± 0.72	5.58 ± 0.66	6.01 ± 1.03	5.22 ± 0.61	0.093	0.243	0.481
C14:0	1802 ± 136	1490 ± 125	1703 ± 196	1519 ± 115	0.101	0.810	0.670
C15:0	63.19 ± 6.63	48.50 ± 5.81	57.89 ± 9.75	53.56 ± 5.48	0.198	0.987	0.459
C16:0	20,678 ± 917	19,976 ± 829	19,867 ± 1211	20,328 ± 796	0.886	0.778	0.479
C17:0	499.36 ± 29.44	417.29 ± 27.01	442.17 ± 42.40	448.49 ± 24.86	0.243	0.683	0.179
C18:0	6301 ± 485	6071 ± 442	6591 ± 628	6821 ± 426	1.000	0.214	0.580
C20:0	56.94 ± 7.10	44.38 ± 6.29	51.89 ± 9.99	53.73 ± 5.96	0.468	0.764	0.317
C21:0[5]	2.49 ± 3.11[b]	2.11 ± 2.67[b]	15.27 ± 4.59[ab]	16.28 ± 2.55[a]	0.927	<.001	0.827
C22:0	3.60 ± 0.47	3.51 ± 0.44	3.98 ± 0.58	3.74 ± 0.43	0.660	0.394	0.836
C23:0	14.25 ± 2.92	16.65 ± 2.60	18.60 ± 4.06	15.59 ± 2.47	0.919	0.567	0.351
C24:0	4.09 ± 0.83	4.03 ± 0.74	4.91 ± 1.19	4.57 ± 0.70	0.822	0.432	0.871
Sum of SFAs[6]	29,546 ± 1294	28,193 ± 1194	28,950 ± 1632	29,329 ± 1158	0.649	0.793	0.406

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. (Continued on next page)

[4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13 + C24:1 *cis*-15. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a–c] Different superscripts indicate significant differences among groups ($P < 0.05$).

Table 9.10: *Fatty acid composition in perirenal fat of calves of four maternal supplementation groups: control group (CON, n = 8), essential fatty acids (EFA, n = 9), conjugated linoleic acids (CLA, n = 8) and EFA and CLA combined (EFA+CLA, n = 11). Data are given as LSM ± SE_{LSM}.*

Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:2 *cis*-9, *trans*-11	0.07 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.08 ± 0.01	0.191	0.940	0.075
C18:3 *n*-3	0.11 ± 0.07	0.24 ± 0.06	0.18 ± 0.09	0.08 ± 0.06	0.831	0.397	0.056
C20:5 *n*-3	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.610	0.764	0.079
C22:5 *n*-3	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.944	0.588	0.174
C22:6 *n*-3	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.862	0.937	0.300
Sum of *n*-3 PUFAs[1]	0.21 ± 0.08	0.37 ± 0.08	0.29 ± 0.11	0.17 ± 0.07	0.845	0.403	0.044
C18:2 *n*-6	0.90 ± 0.12	1.11 ± 0.11	1.06 ± 0.17	0.89 ± 0.10	0.867	0.810	0.118
C18:3 *n*-6	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.043	0.488	0.178
C20:3 *n*-6	0.18 ± 0.02	0.20 ± 0.02	0.20 ± 0.03	0.19 ± 0.02	0.833	0.874	0.553
C20:4 *n*-6	0.16 ± 0.01	0.18 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.323	0.529	0.517
C22:4 *n*-6	0.06 ± 0.01	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.097	0.986	0.949
Sum of *n*-6 PUFAs[2]	1.38 ± 0.15	1.65 ± 0.13	1.59 ± 0.21	1.42 ± 0.12	0.763	0.938	0.147
C18:2 *trans*-9, *trans*-12	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.285	0.975	0.457
C20:3 *n*-3	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.998	0.471	0.040
C20:2 *n*-6	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.828	0.707	0.220
C20:3 *n*-9	0.01 ± 0.00[b]	0.02 ± 0.00[a]	0.02 ± 0.00[ab]	0.02 ± 0.00[ab]	0.008	0.777	0.044
Sum of PUFAs[3]	1.68 ± 0.21	2.15 ± 0.19	2.01 ± 0.29	1.69 ± 0.18	0.713	0.743	0.055
C14:1 *cis*-9	0.84 ± 0.12	0.80 ± 0.11	1.04 ± 0.18	0.90 ± 0.10	0.476	0.251	0.685
C16:1 *cis*-9	7.34 ± 0.78	7.06 ± 0.69	7.96 ± 1.14	7.75 ± 0.65	0.772	0.436	0.970
C17:1 *cis*-9	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.993	0.631	0.933
C18:1 *cis*-9	37.61 ± 1.01	38.07 ± 0.93	37.49 ± 1.45	39.14 ± 0.85	0.341	0.662	0.592
C18:1 *cis*-11	2.32 ± 0.19	2.51 ± 0.17	2.25 ± 0.26	2.78 ± 0.16	0.066	0.565	0.348
C18:1 *trans*-9	0.11 ± 0.01	0.09 ± 0.01	0.10 ± 0.02	0.08 ± 0.01	0.268	0.394	0.920

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Fatty acid (% of total fatty acids)	Supplementation group				Effect (P-value)		
	CON	EFA	CLA	EFA+CLA	EFA	CLA	EFA×CLA
C18:1 *trans*-11	0.08 ± 0.01	0.11 ± 0.01	0.08 ± 0.02	0.08 ± 0.01	0.207	0.452	0.192
C20:1 *cis*-11	0.23 ± 0.04	0.26 ± 0.04	0.18 ± 0.05	0.29 ± 0.03	0.093	0.693	0.262
C22:1 *cis*-13	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.038	0.641	0.824
C24:1 *cis*-15	0.01 ± 0.00	0.01 ± 0.00	NA	0.01 ± 0.00	0.898	0.993	0.954
Sum of MUFAs[4]	48.51 ± 1.14	48.96 ± 1.04	49.09 ± 1.64	51.00 ± 0.96	0.342	0.291	0.558
C10:0	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.962	0.542	0.423
C11:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.820	0.488	0.731
C12:0	0.10 ± 0.02	0.16 ± 0.02	0.12 ± 0.03	0.12 ± 0.02	0.096	0.644	0.135
C13:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.311	0.582	0.230
C14:0	2.59 ± 0.22	2.93 ± 0.20	2.87 ± 0.31	2.61 ± 0.18	0.867	0.947	0.214
C15:0	0.08 ± 0.01[b]	0.12 ± 0.01[a]	0.10 ± 0.02[ab]	0.09 ± 0.01[ab]	0.190	0.713	0.031
C16:0	35.02 ± 0.69	33.51 ± 0.63	34.38 ± 0.99	33.47 ± 0.58	0.113	0.646	0.696
C17:0	0.68 ± 0.05	0.84 ± 0.05	0.84 ± 0.07	0.72 ± 0.04	0.714	0.743	0.017
C18:0	11.08 ± 0.70	11.13 ± 0.64	10.29 ± 1.00	10.06 ± 0.59	0.904	0.222	0.857
C20:0	0.08 ± 0.01	0.10 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.203	0.416	0.876
C21:0[5]	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.01 ± 0.01	0.668	0.708	0.200
C22:0	0.01 ± 0.00	0.01 ± 0.00	NA	0.01 ± 0.00	0.873	0.092	0.693
C23:0	0.03 ± 0.00	0.02 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.960	0.428	0.226
C24:0	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.354	0.249	0.819
Sum of SFAs[6]	49.75 ± 1.11	48.91 ± 1.02	48.80 ± 1.60	47.26 ± 0.94	0.329	0.284	0.774
Total fat content (%)	60.32 ± 1.87	60.09 ± 1.72	59.06 ± 2.36	60.46 ± 1.67	0.707	0.764	0.587

[1] Sum of *n*-3 PUFAs: C18:3 *n*-3 + C18:4 *n*-3 + C20:3 *n*-3 + C20:5 *n*-3 + C22:5 *n*-3 + C22:6 *n*-3. [2] Sum of *n*-6 PUFAs: C18:2 *n*-6 + C18:3 *n*-6 + C20:2 *n*-6 + C20:3 *n*-6 + C20:4 *n*-6 + C22:2 *n*-6 + C22:4 *n*-6. [3] Sum of PUFAs: C18:2 *cis*-9, *trans*-11 + C18:2 *trans*-9, *trans*-12 + C20:3 *n*-9 + Sum of *n*-3 PUFAs + Sum of *n*-6 PUFAs. [4] Sum of MUFAs: C14:1 *cis*-9 + C16:1 *cis*-9 + C17:1 *cis*-9 + C18:1 *cis*-9 + C18:1 *cis*-11 + C18:1 *trans*-9 + C18:1 *trans*-11 + C20:1 *cis*-11 + C22:1 *cis*-13 + C24:1 *cis*-15. [5] C21:0 includes *trans*-10, *cis*-12 CLA. [6] Sum of SFAs: C10:0 + C11:0 + C12:0 + C13:0 + C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0. [a,b] Different superscripts indicate significant differences among groups ($P < 0.05$). NA: Not calculated by SAS software due to small sample size.

9.2 Protein quantification of myosin heavy chain isoforms

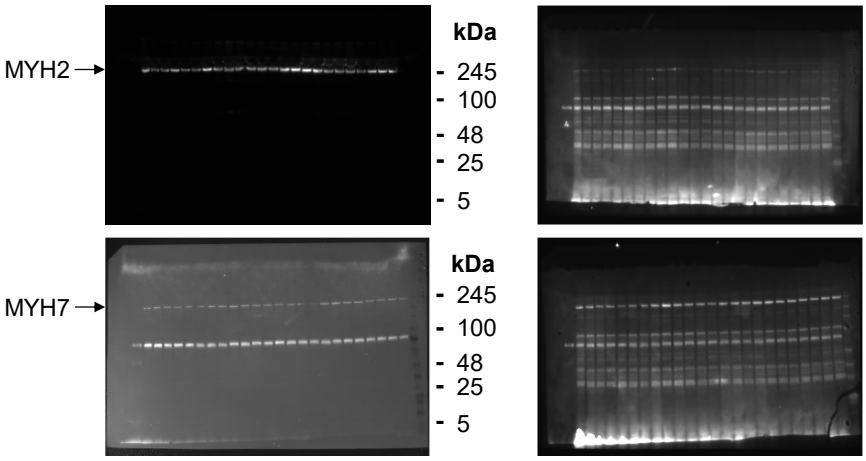

Figure 9.1: *Representative western blot images of MYH2 and MYH7 in skeletal muscle. Left column: arrows indicate target protein band, right column: total protein. Protein size standard (kDa).*

9.3 Protein quantification of DLK-1

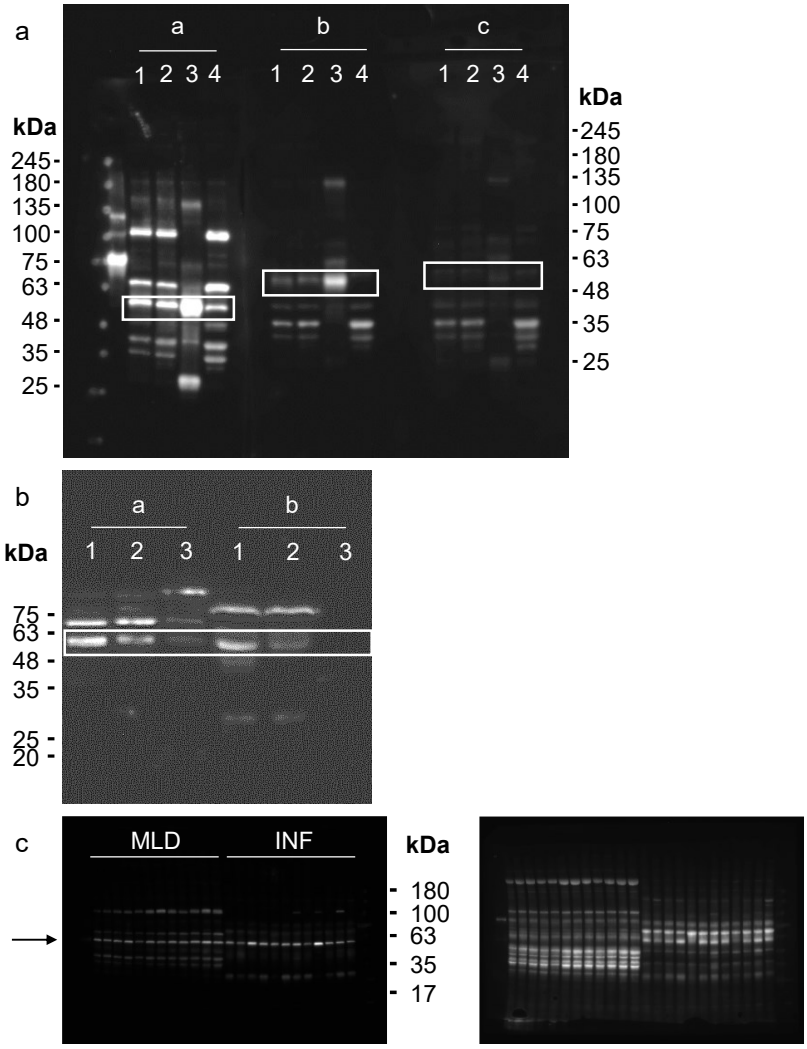

Figure 9.2: *Protein quantification of DLK-1 via western blot. (a) Detection of DLK-1 in calf muscle and adipose tissue. Anti-DLK-1 antibody ab21682 (a), preincubated with 3x blocking peptide (b), and anti-DLK-1 antibody sc-376755 (c) were tested in protein samples of calf longissimus (1) and semitendinosus muscle (2), calf intermuscular fat (3) and adult bovine longissimus muscle (4). (b) Detection of DLK-1 protein isolated from different cellular fractions of longissimus muscle (a, MLD) and intermuscular fat (b, INF): total cellular protein (1), cytoplasmic protein (2) and nuclear protein (NP) (3). (a and b) White frames indicate specific bands. (c) Representative western blot images of DLK-1 in MLD and INF. Left column: arrow indicates target protein band, right column: total protein. Protein size standard (kDa).*

9.4 Antibody test for protein quantification of ZNF423

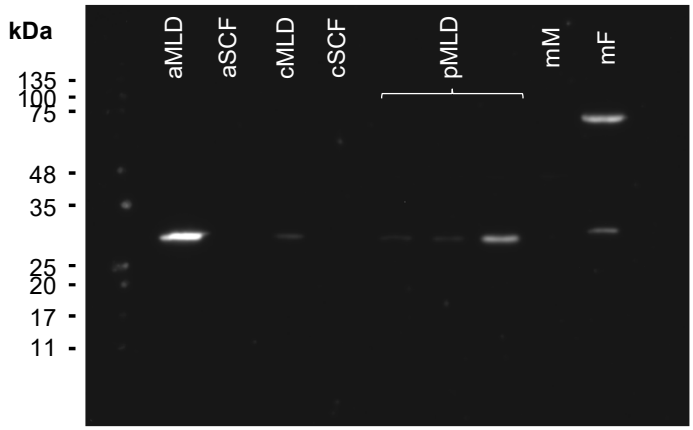

Figure 9.3: *Antibody test for protein quantification of ZNF423 via Western Blot. Test run of anti-ZNF423 antibody ABIN1385826 in samples of longissimus muscle (MLD) and subcutaneous fat (SCF) from adult cattle (aMLD and aSCF) and calf (cMLD and cSCF), porcine MLD (pMLD), murine muscle (mM) and murine fat (mF). Protein size standard (kDa).*

9.5 Primers

Table 9.11: *Primers used in quantitative real-time PCR. Gene accession number, forward and reverse primer sequence, source of primer pair, amplicon size, coefficient of correlation (R^2) and amplification efficiency (E) for target and reference genes in longissimus muscle (MLD), semitendinosus muscle (MST) and intermuscular fat from MLD (INF). Reference genes:* PPIA, UXT.

Gene (acc. no.)	Primer sequence (5'-3') forward/reverse	Source	Amplicon size (bp)	MLD (E, R^2)	MST (E, R^2)	INF (E, R^2)
PPIA (NM_178320.2[1])	CATGCCCTCTTTCACCTTGCCAAA/ AGCATACAGGTCCTGGCATCTTGT	Jennings *et al.*, 2016	137	1.89, 1.00	1.83, 0.99	1.85, 0.99
UXT (NM_001037471.2)	TGTGGCCCTTGGATATGGTT/ AAGCCCCTCTAGCAACATGT	Primer3web[2]	157	1.85, 0.99	1.80, 0.98	1.79, 0.99
AAMDC (NM_001037473.2)	CGGGATTGGGATTGGAGAGA/ ACTCTTTCACTGCCTGCTCT	Primer3web[2]	211	not determined	not determined	1.83, 1.00
CEBPA (NM_176784.2)	TGGACAAGAACAGCAACGAGT/ AGTTCGCGGCTCAGTTGTTC	NCBI[3]	172	1.46, 0.93	1.51, 0.91	1.80, 0.97
CEBPB (NM_176788.1)	Primer pair used in muscle: AGCACAGCGACGAGTACAAG/ CGTCTTGAACAAGTTCCGCA	NCBI[3]	194	1.80, 0.98	1.35, 0.74	not determined
	Primer pair used in INF: AAGAAGACGGTGGACAAGCA/ CGTCTTGAACAAGTTCCGCAG		210	not determined	not determined	2.06, 0.99
DLK1 (NM_174037.2)	GACCTAGACATCCGGGCTTG/ GGCCCATCCATTTCCTGACA	NCBI[3]	134	1.85, 0.99	2.03, 0.99	1.88, 0.98
FOXO1 (XM_025000053.1)	GGACGGAGACACGTTGGATT/ AAGTGTAGCCTGCTTGCTCG	NCBI[3]	131	1.88, 1.00	not determined	1.85, 0.99
LPL (NM_001075120.1)	CCCCGGCTTTGATATTGGGA/ GCTTTGCCAAGTTTCAGCCA	NCBI[3]	173	1.83, 0.99	not determined	not determined
NR2C1 (NM_001077904.1)	AGGGCATGGAAGGAAACGTA/ GGCAGACTCCCCAATGTAGT	Primer3web[2]	161	not determined	not determined	1.98, 0.98
PDGFRA (NM_001192345.3)	AGACACAGCTCACAGACCTC/ GAAGCTGTCCTCCACCAGAT	Primer3web[2]	150	2.00, 0.97	not determined	not determined

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Gene (acc. no.)	Primer sequence (5'-3') forward/reverse	Source	Amplicon size (bp)	MLD (E, R^2)	MST (E, R^2)	INF (E, R^2)
PPARGC1A (NM_177945.3)	GAAGACCAGCCTCTTTGCCC/ CTGCACCACTTGAGTCCACC	NCBI[3]	104	1.86, 0.97	2.00, 0.98	not determined
PPARG2 (NM_181024.2)	ATTGACCCAGAGAGTGAGCC/ AAAGGCATGGGAGTGGTCAT	Primer3web[2]	153	no amplification product	1.73, 0.99	1.96, 0.97
PROX1 (NM_001193232.1)	ATCAAATCCGAGTGTGGCGA/ GTTGGAGCTGGGATAACGGG	NCBI[3]	141	1.85, 1.00	2.01, 0.99	not determined
ZNF423 (NM_001101893.1)	CAAGCTCCTCTGTCACCTCA/ GAACTTCTGTGGGCACTGTG	Primer3web[2]	172	1.71, 0.99	1.91, 0.99	1.85, 0.98,

[1] Gene accession number was not listed in the source. NCBI blast of primer sequences yields this gene accession number as target template. [2] Untergasser *et al.*, 2012. [3] Ye *et al.*, 2012.

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