DISS. ETH NO. 25867

Investigating fatty acid metabolism in mammals, with specific emphasis on the female reproductive organs

A thesis submitted to attain the degree of

DOCTOR OF SCIENCES of ETH ZURICH
(Dr. sc. ETH Zurich)

presented by

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2019

“The true statesman is the one who is willing to take risks.”

— Charles de Gaulle

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List of Abbreviations

AA	Arachidonic acid
ACACA	Acetyl-CoA carboxylase alpha
ACO1	Aconitase 1
ACOX	Acyl-CoA oxidase 1
ACTB	Actin beta
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
B2M	Beta-2-microglobulin
BOEC	Bovine oviduct epithelial cells
BP	Blood plasma
C16:0	PA; Palmitic acid
C18:0	SA; Stearic acid
C18:1*cis*9	OA; Oleic acid
C18:1*trans*11	Vaccenic acid
C18:2*n*6	LA; Linoleic acid
C18:3*n*3	ALA; Alpha-linolenic acid
C20:4*n*6	Arachidonic acid
C20:5*n*3	Eicosapentaenoic acid
C22:5*n*3	DPA; Docosapentaenoic acid
C22:6*n*3	Docosahexaenoic acid
CAT	Catalase
CD36	CD36 molecule
CL	*Corpus luteum*
CLA	Conjugated linoleic acid
CH	*Corpus hemmoragicum*
COX1	Prostaglandin-endoperoxide synthase 1
CP	Crude protein
CPT1A	Carnitine palmitoyltransferase 1A
CTSL	Cathepsin L
DAG	Diacylglycerol
DAGLA	Diacylglycerol lipase alpha
DGLA	Dihomo-γ-linolenic acid
DHA	Docosahexaenoic acid
DM	Dry matter
E2	Estradiol
EHHADH	Enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase
ELISA	Enzyme-linked immunosorbent assay
EOEC	Equine oviduct epithelial cells
EPA	Eicosapentaenoic acid
ERRFI1	ERBB receptor feedback inhibitor 1
ESR1	Estrogen receptor 1
ESR2	Estrogen receptor 2
FA	Fatty acid(s)
FABP4	Fatty acid binding protein 4
FAS	Fatty acid synthase
FATP	Long-chain fatty acid transport protein 1
FFAR4	Free fatty acid receptor 4
FGF9	Fibroblast growth factor 9
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
FSH	Follicle-stimulating hormone
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GJA1	Gap junction protein alpha 1
GLUT4	Glucose transporter type 4
GnRH	Gonadotropin-releasing hormone
GPT	Glutamic-pyruvic transaminase
GPX1	Glutathione peroxidase 1
GPX4	Glutathione peroxidase 4
GSR	Glutathione-disulfide reductase

H3F3A	H3 Histone family member 3A
HHIP	Hedgehog interacting protein
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
HSPA5	Heat shock protein family A (Hsp70) member 5
HSPB8	Heat shock protein family B (small) member 8
ICSI	Intracytoplasmic sperm injection
IFI6	Interferon alpha inducible protein 6
IFN-tau	Interferon-tau
INSR	Insulin receptor
IVC	*In vitro* culture
IVM	*In vitro* maturation
LEP	Leptin
LEPR	Leptin receptor
LH	Luteinizing hormone
LIPC	Lipase C, hepatic type
LIPE	Lipase E, hormone sensitive type
LPL	Lipoprotein lipase
MUFA	Monounsaturated fatty acid(s)
*n*3 / ω3	Omega−3 fatty acid(s)
*n*6 / ω6	Omega−6 fatty acid(s)
NADPH	Nicotinamide adenine dinucleotide phosphate
Nrf2	Nuclear factor, Erythroid 2 Like 2
OF	Oviduct fluid
OXT	Oxytocin
OXTR	Oxytocin receptor
P19	P19 lipocalin
P4	Progesterone
PAQR5	Progestin and adipoQ receptor family member 5
PG	Prostaglandin(s)
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2α
PGFS	Prostaglandin F synthase
PLA2G2A	Phospholipase A2 group IIA
PLCG1	Phospholipase C gamma 1
PGC-1α	Peroxisome proliferator activated receptor gamma coactivator 1 alpha
PPAR	Peroxisome proliferator activated receptor
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1
PTGER	Prostaglandin E receptor
PTGS2	Prostaglandin-endoperoxide synthase 2
PUFA	Polyunsaturated fatty acid(s)
RPL19	Ribosomal protein L19
S100A6	S100 calcium binding protein A6
SCD	Stearoyl-CoA desaturase
SFA	Saturated fatty acid(s)
SLC03A1	Solute carrier family 3 member 1
SLC27A1	Solute carrier family 27 member 1
SLC2A1/GLUT1	Solute carrier family 2 member 1
SLC36A2	Solute carrier family 36 member 2
SOCS3	Suppressor of cytokine signaling 3
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
SPINK7	Serine peptidase inhibitor, Kazal type 7
SREBP-1c	Sterol regulatory element binding transcription factor 1
STK11	Serine/Threonine kinase 11
SYT5	Synaptotagmin 5
TEP	Total extractable polyphenols
TG	Triglyceride(s)
TP	Total protein
UBB	Ubiquitin B
UCP	Uncoupling protein(s)
UF	Uterine fluid
VEGFA	Vascular endothelial growth factor A
YWHAG	Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase activation protein gamma
YWHAZ	Tyrosine 3-monooxygenase/Tryptophan 5-monooxygenase activation protein zeta

Summary

Modern animal husbandry and management account for intensive use of animals for obtaining high quality products. This extreme pressure for maximal performance is often accompanied by metabolic challenges, which can consequently manifest in different health issues and suboptimal reproductive performance. Early embryo loss is of particular interest since it is still one of the major drawbacks in achieving high fertility rates and thereby improving particular breeding programs. The environment for fertilization and early embryo development within the reproductive tract is commonly known as histotroph and it is characterized by the oviduct (OF) and the uterine (UF) fluid. Thus, knowledge about their composition is of foremost importance. Fatty acids (FA) as an integral part of the histotroph serve as one of the principal energy supplies for the growing embryo. The main goal of the doctoral thesis was to obtain information about the FA composition and metabolism of the female reproductive organs and fluids in order to understand their specific characteristics and needs as one basis for improving fertility in domestic animals.

In a first study, we investigated the FA profile in plasma and reproductive fluids (OF and UF) in ten mares, using gas chromatography combined with mass spectrophotometry (GC-MS). Overall, the FA composition of the three matrices was specific, whereas we observed differences between early and late luteal stage only in the OF, with increased concentrations of particular FA known for their beneficial impact on embryo development evident during the early luteal phase. Therefore, besides transudation from plasma, we suggest specific FA synthesis pathways or selective transport mechanisms or both in the oviduct and the uterus. This is the first time that the mare histotroph was described with regard to FA composition and the obtained results provide a basis for future research.

The impact of environmental factors on the onset of specific biological and molecular mechanisms, same as the effect of different nutritional strategies, in reproductive tissues and histotroph was seldom studied until now. To address this aspect, we investigated in a second study, involving 24 ewes, the influence of dietary supply with feed additives rich in phenolic compounds (i.e. grape seed and *Acacia mearnsii* extract; 13 g/kg diet; either alone or in combination) on the FA profiles in the reproductive tissues, fluids and plasma, using GC and GC-MS. The FA profile of uterus and oviduct lipids remained widely unaffected by the diet, which was different from liver, muscle, and adipose tissue lipids. Still, the FA profiles of the lipids of the reproductive fluids were affected by the diet, and this effect was more pronounced in the UF compared to OF. Moreover, the observed high proportions of specific FA emphasize

their importance for the growing embryo and its mother during the periconceptional period that includes the phase of early embryo development. The gene expression of the respective tissues was investigated in relation to possible differences caused by the dietary supplementation with phenolic compounds. Effects were very limited which might have been related to the observed non-significant differences in plasma total phenol concentrations. These findings suggest a limited effect of phenol-rich plant extracts on the FA composition of the reproductive tissues and fluids, but the observed changes are still encouraging in showing that maternal nutrition can modify the environment of the developing embryo to some extent.

Finally, in a third study, we investigated the effects of a hormonal challenge (i.e. leptin and oxytocin) in an equine oviduct epithelial cell culture model (EOEC, 48h). High amounts of circulating leptin are linked to obesity, but it was also shown that leptin expression in adipocyte cells might change as consequence of polyphenol extract supplementation. We hypothesized that leptin might as well affect the prostanoid signalling pathway in EOEC, thereby affecting muscular contraction or relaxation in relation to embryo transport. The expression of a broad panel of 27 genes was tested, and for the first time we identified leptin receptor mRNA transcripts in EOEC, using the Fluidigm platform. Our model might serve as an approach for future studies, because of the attained explant morphology and a stable expression of genes related to cell functionality. In addition, we confirmed the prostaglandin E_2 and prostaglandin $F_{2\alpha}$ synthesis in EOEC using enzyme-linked immunosorbent assay (ELISA), but did not observe the hypothesized increase in their synthesis due to leptin stimulation. Expression of genes was affected by stimulation time, and lower expression of certain genes was observed. These results could be explained by the sampled material, and more specifically by the cycle stage of mares. So far, an effect of leptin as an obesity-related hormone on EOEC could not be confirmed by the present study, and an optimization of the protocol is necessary.

In conclusion, we were able to describe the characteristic FA profile of the reproductive fluids in both the horse and the sheep, and of reproductive organs in ewes, thereby contributing to research and animal husbandry practices by filling the gap of knowledge about species-specific FA profiles. Moreover, the observed changes in the OF and the UF composition are in accordance with the suggested dynamic modulation of the histotroph. Together, the results are indicative for a possible improvement of the environment for the oocyte and the growing embryo, both in *in vivo* and *in vitro* conditions. In addition, the tested diet supplementation with phenolic compounds in sheep proved as a promising strategy to modify the FA composition of important tissues and fluids, thereby encouraging scientific efforts to improve

the reproductive parameters via dietary interventions. Finally, the EOEC culture proved to be a promising model for a range of experiments concerning the exploration of different biological mechanism in the oviduct of mares. The influence of obesity via high leptin concentrations on the oviduct muscle contraction and relaxation remains to be demonstrated in the horse. The results of this doctoral project serve as a comprehensive source of information concerning the histotroph composition in general and the effects of dietary supplementation with phenolic compounds in particular. Ultimately, the results represent the frame for further studies within the field of reproductive physiology and nutrition of domestic female animals in the periconceptional period.

Zusammenfassung

Moderne Tierhaltung und modernes Tiermanagement führen zu einer intensiven Nutzung der Tiere zur Gewinnung qualitativ hochwertiger Produkte. Die geforderte maximale Leistung wird oft von metabolischen Herausforderungen begleitet, die zu verschiedenen gesundheitlichen Problemen und suboptimalen Fortpflanzungsleistungen führen können. Der frühe Embryonenverlust ist von besonderer Bedeutung, da er immer noch einer der größten Hindernisse für die Erreichung hoher Fruchtbarkeitsraten ist und damit der Optimierung spezifischer Zuchtprogramme entgegenwirkt. Das Milieu, in dem sowohl die Befruchtung als auch die frühe Embryonalentwicklung im Reproduktionstrakt stattfinden, wird auch als Histiotrophe bezeichnet und durch von Eileiter (OF) und Uterus (UF) sezernierte Flüssigkeiten gekennzeichnet. Daher ist die Kenntnis über die Zusammensetzung dieser Flüssigkeiten von grösster Bedeutung. Fettsäuren (FS) als integraler Bestandteil der Histiotrophe dienen als einer der Hauptenergielieferanten für den sich entwickelnden Embryo. Das Hauptziel der vorliegenden Doktorarbeit bestand darin, Informationen über die FS-Zusammensetzung und den Stoffwechsel der Reproduktionsorgane und -flüssigkeiten zu erhalten, um deren spezifische Bedürfnisse bzw. Merkmale als Grundlage für die Verbesserung der Fertilität von Haustieren zu verstehen.

In der ersten Studie untersuchten wir das FS-Profil im Plasma und den Reproduktionsflüssigkeiten (OF und UF) von zehn Stuten unter Verwendung von Gaschromatographie kombiniert mit Massenspektrometrie (GC-MS). Insgesamt zeigte sich eine spezifische FS-Zusammensetzung der drei untersuchten Flüssigkeiten, wobei wir Änderungen der FS-Zusammensetzung zwischen der frühen und späten Lutealphase nur in der OF beobachten konnten. Dabei zeigten sich während der frühen Lutealphase erhöhte Konzentrationen spezifischer FS, welche bekannt dafür sind, sich positiv auf die Embryonalentwicklung auszuwirken. Daher nehmen wir an, dass es in Eileiter und Uterus spezifische FS Synthesewege und/oder selektive Transportmechanismen gibt. Diese Studie beschreibt zum ersten Mal die Histiotrophe der Stute in Bezug auf seine FS-Zusammensetzung und die daraus resultierenden Ergebnisse bieten somit eine Basis für die zukünftige Forschung.

Die Beeinflussung spezifische biologischer und molekularer Mechanismen durch Umweltfaktoren und damit auch durch verschiedene Fütterungsstrategien in Reproduktionsgeweben und Histiotrophe wurde bisher nur selten untersucht. Um diesem Aspekt zu begegnen untersuchten wir in einer zweiten Studie an 24 Mutterschafen den Einfluss von phenolreichen Futtermittelzusatzstoffen (Extrakte aus Traubenkernen und *Akazie*

mearnsii; 13 g/kg Futter, entweder allein oder in Kombination) auf die FS-Profile in Reproduktionsgeweben, Flüssigkeiten und Plasma unter Verwendung von GC und GC-MS. Die FS-Profile der Lipide von Uterus und Eileiter blieben weitestgehend unbeeinflusst von der Diät, aber die FS-Zusammensetzung von Leber, Muskel und Fettgewebe war unterschiedlich. Die FS-Profile der Histiotrophen wurden durch die Ernährung beeinflusst wobei dieser Effekt in der UF stärker ausgeprägt war als in der OF. Darüber hinaus verdeutlichen die beobachteten hohen Anteile spezifischer FS ihre Bedeutung für den wachsenden Embryo und dessen Mutter während der perikonzeptionellen Periode, die auch die Phase der frühen Embryonalentwicklung umfasst. Die Genexpression der jeweiligen Gewebe wurde im Hinblick auf mögliche Änderungen durch die phenolischen Substanzen untersucht. Die Effekte waren sehr begrenzt, was sich möglicherweise durch fehlende signifikante Unterschiede in den Gesamtphenolkonzentrationen im Plasma begründen lässt. Diese Ergebnisse deuten auf eine sehr begrenzte Wirkung von phenolreichen Pflanzenextrakten auf die FS-Zusammensetzung von Reproduktionsgeweben und deren Flüssigkeiten hin. Die beobachteten Veränderungen sind jedoch immer noch vielversprechend, da sie zeigen, dass die Ernährung der Mutter die Umgebung des sich entwickelnden Embryos bis zu einem gewissen Grad beeinflussen kann.

Schließlich untersuchten wir in einer dritten Studie die Auswirkungen einer Hormonstimulation (mit Leptin und Oxytocin) auf ein Zellkulturmodell für Eileiterepithelzellen vom Pferd (EOEC, 48h). Hohe Mengen an zirkulierendem Leptin werden mit Fettleibigkeit assoziiert, aber es wurde ebenso gezeigt, dass sich die Leptinexpression als Folge einer Polyphenol-Supplementierung ändern kann. Wir stellten die Hypothese auf, dass Leptin auch den Prostanoid-Signalweg in EOEC beeinflussen könnte, wodurch die für den Embryonentransport benötigte Muskelkontraktion oder -entspannung beeinflusst wird. Die Expression eines breiten Spektrums von 27 Genen wurde getestet. Zum ersten Mal identifizierten wir Leptinrezeptor-mRNA-Transkripte in EOEC mithilfe der Fluidigm Plattform. Unser Modell könnte aufgrund der erzielten Explantationsmorphologie und der stabilen Expression von Genen im Zusammenhang mit der Zellfunktionalität als Ansatz für zukünftige Studien dienen. Darüber hinaus konnten wir die Prostaglandin E_2 und Prostaglandin $F_{2\alpha}$ Synthese in EOEC mittels Enzyme-linked Immunosorbent Assay (ELISA) zwar bestätigen, den vermuteten Anstieg ihrer Synthese aufgrund der Stimulation mit Leptin konnten wir allerdings nicht beobachten. Die Expression der Gene wurde aber durch die Dauer der Stimulation beeinflusst und es wurde eine verminderte Expression verschiedener Gene beobachtet. Diese Ergebnisse könnten durch das gewonnene Probenmaterial und insbesondere durch die Zyklusphase der Stute erklärt werden. In der vorliegenden Studie konnte eine

Wirkung von Leptin auf EOEC nicht bestätigt werden. Eine Optimierung des Protokolls ist notwendig.

Zusammenfassend lässt sich sagen, dass wir das charakteristische FS-Profil der Histiotrophe beim Pferd und beim Schaf, sowie auch das der Reproduktionsorgane beim Schaf beschreiben konnten. Diese Ergebnisse leisten durch das Schliessen der Wissenslücke in Bezug auf speziesspezifische Unterschiede im FS-Profil einen Beitrag sowohl für die Forschung als auch für die Tierhaltungspraxis. Darüber hinaus bestätigen die beobachteten Änderungen der OF- und UF-Zusammensetzung die vermutete dynamische Modulation der Histiotrophen. Insgesamt sind die Ergebnisse ein Indikator für eine mögliche Milieu-Optimierung von Eizelle und wachsendem Embryo sowohl *in vivo* als auch *in vitro*. Außerdem erwies sich die getestete Supplementation mit phenolreichen Extrakten bei Schafen als vielversprechende Strategie zur Modifikation der Lipidzusammensetzung wichtiger Gewebe und Flüssigkeiten, was eine weiterführende Forschung in Hinblick auf eine mögliche Verbesserung von Reproduktionsparametern durch Fütterungsinterventionen bestärkt. Die EOEC-Kultur stellt ein vielversprechendes Modell für eine Reihe von Experimenten zur Erforschung verschiedener biologischer Mechanismen im Eileiter von Stuten dar. Der potentielle Einfluss einer erhöhten Leptinkonzentration auf Muskelkontraktion oder -entspannung im Eileiters bei Stuten muss erfordert weitere Untersuchungen. Die Ergebnisse der vorliegenden Doktorarbeit dienen als umfassende Informationsquelle über die Zusammensetzung von Histiotrophe im Allgemeinen und die Auswirkungen der Nahrungsergänzung mit phenolischen Substanzen im Speziellen. Letztendlich bilden die Ergebnisse den Rahmen für weitere Studien auf dem Gebiet der Reproduktionsphysiologie und der Ernährung von weiblichen Haustieren in der perikonzeptionalen Periode.

Résumé

L’élevage moderne repose sur l’utilisation intensive des animaux visant à l’obtention de produits de haute qualité. Cette pression extrême en faveur d'une performance maximale s'accompagne souvent de défis métaboliques, qui peuvent se manifester par différents problèmes de santé et des performances reproductives qui ne sont pas optimales. L’avortement précoce doit être étudié en particulier, car il s’agit de l’une des principales entraves à l'obtention de taux élevés de fécondité, et donc à l’amélioration de programmes particuliers de sélection. L'environnement pour la fertilisation et le développement embryonnaire précoce dans le tractus génital, communément connu sous le nom d’histotrophe, est caractérisé par les fluides oviducte (FO) et utérin (FU). C’est pourquoi la connaissance de leur composition est de la plus haute importance. Les acides gras (AG) en tant que partie intégrante de l'histotrophe constituent l'une des principales sources d'énergie pour l'embryon en croissance. L'objectif principal de la thèse de doctorat était donc d'obtenir des informations sur la composition en AG des tissus et des fluides reproductifs et sur leur métabolisme ; ceci afin de comprendre leurs caractéristiques et leurs besoins spécifiques pour tenter d’améliorer la fertilité des animaux domestiques.

Dans une première étude, nous avons analysé le profil en AG du plasma et des fluides reproductifs (FO et FU) de dix juments, par chromatographie en phase gazeuse couplée à de la spectrométrie de masse (GC-MS). Globalement, la composition en AG était spécifique à chacune des trois matrices. Seuls des changements entre la période lutéale précoce et tardive ont pu être observés, avec, en début de période lutéale, une concentration accrue d’AG connus pour leur impact bénéfique sur le développement embryonnaire. Par conséquent, nous suggérons l’existence, dans l'oviducte et l’utérus, soit de voies particulières de synthèse des AG, soit de mécanismes de transport sélectif, soit les deux. Il s’agit de la première description de la composition en AG de l’histotrophe de la jument, et les résultats obtenus fournissent une base pour la recherche future.

Dans les tissus reproductifs et l'histotrophe, l’impact des facteurs environnementaux sur l'apparition de mécanismes biologiques et moléculaires spécifiques n’a été que rarement étudié jusqu’à présent, tout comme celui de stratégies nutritionnelles différentes. Afin de traiter cet aspect, nous avons cherché, dans une deuxième étude portant sur 24 brebis, l’influence de la supplémentation avec des additifs riches en composés phénoliques (de l’extrait de pépins de raisin et d’*Acacia mearnsii*; 13 g/kg d’aliments; seuls ou combinés) sur les profils en AG dans les tissus et fluides reproductifs, et le plasma, en utilisant les GC et GC-MS. Le régime alimentaire n’a pas modifié le profil en AG des lipides de l’utérus et de l’oviducte,

contrairement à celui des lipides du foie, des muscles et des tissus adipeux. Le profil en AG des lipides des fluides reproductifs a quant à lui été affecté par le régime, et cet effet a été plus prononcé dans le FU que dans le FO. De plus, les proportions accrues d'AG spécifiques observées soulignent l'importance de ces derniers pour la croissance de l'embryon et pour sa mère pendant la période périconceptionnelle, incluant la phase de développement embryonnaire précoce. L'expression des gènes des tissus respectifs a ensuite été étudiée en relation avec d'éventuels changements causés par les extraits phénoliques. Les effets ont été très limités, ce qui pourrait être lié aux changements non significatifs observés dans les concentrations plasmatiques totales de phénols. Ces résultats suggèrent un effet limité des extraits de plantes riches en phénols sur la composition en AG des tissus et fluides reproductifs, mais les changements observés sont encourageants et suggèrent que la nutrition maternelle peut modifier, dans une certaine mesure, l'environnement de l'embryon pendant sa croissance.

Enfin, dans un troisième essai, nous avons étudié les effets d'un défi hormonal (leptine et ocytocine), à l'aide d'un modèle de culture de cellules épithéliales d'oviducte équin (EOEC, 48h). L'obésité est habituellement liée à de hauts niveaux de leptine en circulation, mais il a également été démontré que l'expression de la leptine pouvait changer en fonction de la supplémentation en polyphénol. Nous avons émis l'hypothèse que la leptine pourrait tout aussi bien affecter la voie de signalisation des prostanoïdes dans les EOEC, affectant ainsi la contraction / relaxation musculaire en relation avec le transport des embryons. L'expression d'un large panel de 27 gènes a été testée et, pour la première fois, nous avons identifié les transcrits d'ARNm du récepteur de la leptine dans l'EOEC, à l'aide de la plateforme Fluidigm. Notre modèle pourrait servir d'approche pour de futures études, en raison du maintien de la morphologie de l'explants et d'une expression stable des gènes liés à la fonctionnalité cellulaire. En outre, nous avons confirmé la synthèse de Prostaglandine E_2 et de Prostaglandine $F_{2\alpha}$ dans l'EOEC en utilisant la méthode immuno-enzymatique (ELISA), mais n'avons pas observé l'augmentation hypothétique de leur synthèse due à la stimulation de la leptine. L'expression des gènes était influencée par le temps de stimulation et une diminution de la régulation de certains gènes a été observée au cours du temps. Ces résultats pourraient être expliqués par le matériel échantillonné, et plus précisément par la phase de cycle des juments. A ce jour, un effet de la leptine comme une hormone associée à l'obésité sur EOEC n'a pas pu être confirmé par l'étude, et une optimisation du protocole est nécessaire.

En conclusion, nous avons pu décrire le profil caractéristique des AG des fluides reproductifs du cheval et du mouton, ainsi que celui des AG des organes reproductifs chez les ovins, contribuant à la recherche et au développement des pratiques d'élevage en comblant le

manque de connaissances sur les valeurs de cette gamme d'espèces spécifique. De plus, les changements observés dans la composition du FO et du FU sont conformes à la modulation dynamique suggérée de l'histotrophe. Dans leur ensemble, les résultats sont indicatifs pour l'optimisation de l'environnement pour l'ovocyte et l'embryon en croissance, à la fois dans des conditions *in vivo* et *in vitro*. En outre, le régime testant la supplémentation avec des extraits phénoliques chez le mouton semble une stratégie prometteuse pour la modification du stockage des AG des tissus importants et des fluides, favorisant ainsi les efforts scientifiques visant à améliorer les paramètres de reproduction par des interventions alimentaires. Enfin, la culture EOEC s'est avérée être un modèle prometteur pour une série d'expériences concernant l'exploration des différents mécanismes biologiques dans l'oviducte des juments. L'influence de l'obésité sur la contraction et relaxation des muscles de l'oviducte, habituellement associée à des concentrations en leptine élevées, reste à démontrer chez le cheval. Les résultats de ce travail de doctorat servent donc de source d'informations, d'une part sur la composition de l'histotrophe en général, et, d'autre part, sur les effets de la supplémentation alimentaire avec des extraits phénoliques en particulier. Finalement, ces résultats pourront venir soutenir de futures études en rapport avec la physiologie de la reproduction et de la nutrition des animaux domestiques, particulièrement celle des femelles en période périconceptionnelle.

Chapter 1

General introduction

Taxonomy and digestive physiology of the horse and the sheep

The horse and the sheep are both classified as *Mammalia* (mammals), whereas the first mentioned belongs to the order of *Perissodactyla* (odd-toed ungulates) and the latter to *Artiodactyla* (even-toed ungulates). Apart from the number of toes, another important difference between the horse and the sheep concerns the digestive system. No enzyme produced by mammals can break down cellulose to simple sugars, imposing differing strategies to ingest and process feeds that contain different amounts of cellulose and other dietary fibre. Both the horse and the sheep perform fibre digestion via gut microbes but the location in their bodies, where fermentation takes place, differs. In the sheep, this happens in the 'foregut', also called rumen, which is therefore a pre-gastric microbial fermentation. The horse has a relatively simple non-compartmented stomach, so fermentation takes place in the hindgut, i.e. in the voluminous caecum and colon [1] (Figure 1). For example, in the sheep the stomach-caecum-large intestine ratio is 67-3-10 % of the digestive tract, respectively. On the other hand, in the horse this ratio is 9-16-45 %, clearly indicating the different locations where digestion of fibre mainly takes place. Interestingly, the number and variability of microorganisms in the caeco-colon complex are comparable between the horse and the ruminant species [1]. Still, ruminants have a competitive advantage compared to non-ruminant herbivores in fibre digestion because of the greater breakdown of feed particles caused by the process of rumination. That said, horses digest fibrous material less effectively and make less use of legumes than sheep, but they evolved to possess a greater intake capacity of roughages than ruminants and spend considerably more time grazing (i.e. up to 70 % of the daylight time) [2]. Thereby, they are less efficient but can process more feed in a given time if conditions are optimal. In addition, equids are known as strongly selective grazers (e.g. they prefer grass with better nutritional quality) meaning that they can adjust their grazing behaviour in order to meet their nutritional requirements (e.g. increase grazing time to up to 19 hours per day when grass availability is decreased). As already mentioned, the colon and the ceacum are the main sites of fermentation in the horse, whereas carbohydrates and proteins are digested in the small intestine without loss, as compared to ruminants that arguably lose a part of nutritive substrates from the feed due to the process of microbial digestion and the resulting gas formation. However, the microbial protein produced by the gut microbes can be absorbed as amino acids in the small intestine in the ruminant, but this is not possible in the hindgut. Lipids are not really nutrients

for microbes and they even have developed a protective mechanism against the particularly detrimental unsaturated fatty acids (FA), the process of hydrogenation that converts these FA via saturation of double bonds to saturated FA (primarily palmitic and stearic acid). Polyunsaturated FA (PUFA) are even more susceptible to this biohydrogenation in the rumen than monounsaturated FA (MUFA) [3]. The most common pathway of microbial FA modification is the transformation of α-linolenic acid (C18:3*cis*9,*cis*12) and linoleic acid (C18:2*cis*9,*cis*12) first to biohydrogenation intermediates like vaccenic acid (C18:1*trans*11) and conjugated linoleic acid (C18:2*cis*9,*trans*11) and finally stearic acid (C18:0) is generated. Still, some protozoa incorporate PUFA and release them during intestinal digestion, thereby avoiding biohydrogenation, and serving as the main source of PUFA in ruminants. Ruminal protection of PUFA however is more efficiently achieved by feed processing [4]. In the intestinal cells, the FA are re-esterified with glycerol after absorption and coated with protein to form chylomicrons. These enter the blood system via lymph vessels and the contained FA are directly used by all tissues after liberation by the enzyme lipoprotein lipase (LPL) without a preliminary processing by the liver.

The microbial digestive processes in ruminants might have another evolutionary advantage, since foregut fermentation of dietary fibre is also a way to detoxify plant secondary compounds (e.g. tannins and other polyphenols). Brooker et al. [5] suggested that the action of tannins may actually have a more profound effect on intestinal rather than ruminal function and that microbial interactions in the rumen may reduce but not eliminate tannin toxicity. Still, the main advantage of fermentation in ruminants is the breakdown of cellulose by microorganisms to yield high concentrations of volatile FA, namely acetic, propionic and butyric acid (on average in a ratio 75:15:10, respectively), that are rapidly absorbed or metabolized as readily available main source of energy. Methane (CH_4) and carbon dioxide (CO_2), as the remaining by-products of rumen fermentation, are released by eructation. Methanogens and acetogens are two important H_2 disposal microbial groups in the guts of herbivores, which play important roles in reducing CO_2 into methane or acetate, the latter providing significant energy benefits to host animals [6]. In the horse, as non-ruminant herbivore, it is the acetogenesis that takes place as the main microbial process in the fermentation compartment namely hindgut. The effect of immune secretions, such as antimicrobial peptides, immunoglobulins, innate lymphoid cells and mucin, as caused by excessive gas production during fermentation is to exclude methanogens from the caecum and create opportunities for acetogenic microbes to thrive [7]. Animals benefit by eliminating the damage to the gut tissues from excessive gas, while maintaining mechanisms for efficient digestion of fibrous feed.

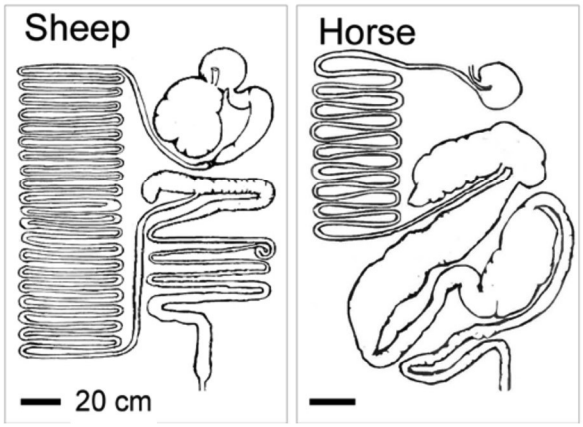

Figure 1. Comparison of the digestive tract anatomy. The sheep, a ruminant foregut fermenter, has a large sacculated stomach, whereas the hindgut fermenter, the horse, has a capacious, multicompartment large bowel [8].

Traditionally, research on the microbiome of the hindgut in the horse was based on information about the microbial physiology and ecology of the rumen. It is only since around two decades that the microflora of the equine large intestine has been studied more profoundly with insights suggesting that it accommodates many novel bacteria species, and is variable between digestive sites and individual horses [9, 10]. Thus, our understanding of the equine digestive system and microbial physiology has to be re-evaluated and appraised.

Lipid metabolism in the horse and the sheep

As already described in the first subchapter *Taxonomy and digestive physiology of the horse and the sheep*, dietary FA in horses are absorbed in the small intestine and triglyceride (TG) are combined with protein to form chylomicrons to reach the lymphatic system. From there, the FA can reach the blood stream and the FA constituents can be taken up by different tissues via LPL [11]. Still, the liver plays a central role in lipid metabolism, encompassing several pathways such as *de novo* lipogenesis, very-low density lipoprotein (VLDL) production, and β-fatty acid oxidation of the FA taken up from the bloodstream. The hepatic β-fatty acid oxidation happening in the mitochondria is responsible for the oxidation of short-, medium- and long-chain FA for energy production. In ruminants, where the major oxidative fuels are the volatile FA, namely acetate, propionate and butyrate, originating from the rumen microbial carbohydrate fermentation, *de novo* lipogenesis occurs predominantly in the adipose tissue [12] and only in modest amounts in the liver [13]. The volatile FA, and thereby mostly acetate, are transformed into acetyl-CoA, the latter being indispensable for the synthesis of long-chain FA and subsequent TG formation. In addition, propionyl-CoA can also be used instead of acetyl-CoA as a molecule for FA synthesis, giving rise to the odd-numbered FA

found as a product of biohydrogenation [12]. The TG are the most commonly stored and circulating forms of energy in the organism [13]. The accumulation of TG in the liver happens by mobilization and conversion of non-esterified fatty acids (NEFA) released by the adipose tissue (Figure 2). The NEFA enter hepatocytes by transport proteins (e.g. long-chain FA transport protein 1 (FATP) and CD36 molecule (CD36) and their supply depends on the rate of lipolysis in the adipose tissue. Circulating NEFA levels should remain low and this is achieved by their assimilation into lipids. Any alterations in NEFA levels or β-fatty acid oxidation could lead to accumulation of lipids in hepatocytes and cause metabolic problems such as hepatic lipidosis [14]. However, this problem is seldom reported in horses (only in miniature ponies) [15], but a situation involving negative energy balance and a frequent feeding of high-carbohydrate diets could substantially contribute to the risk of developing hyperlipidemias.

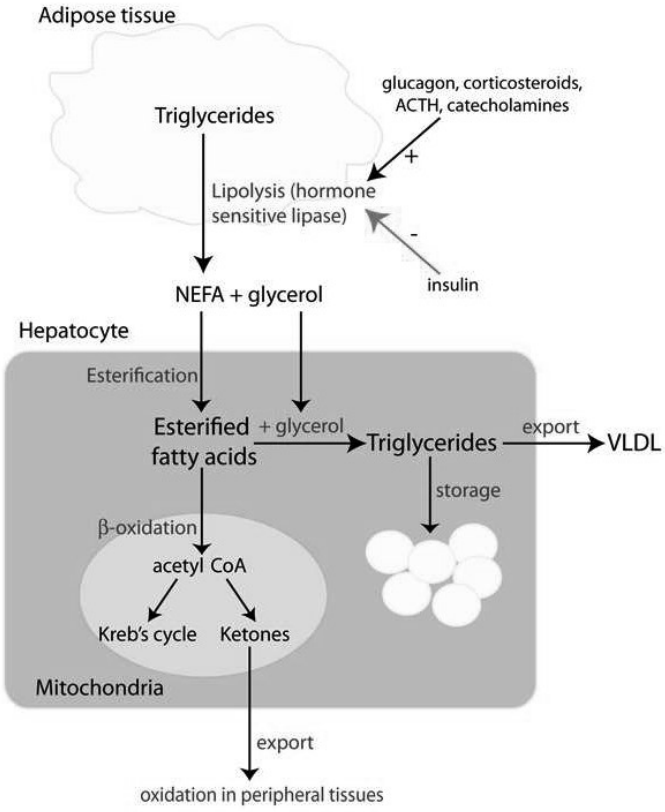

Figure 2. Non-esterified fatty acids (NEFA) metabolism [16].

Due to a lack in acyl-CoA synthases, mitochondria cannot activate FA containing 14 or more carbon atoms. Therefore, the transport of these long-chain FA into mitochondria is directed by the enzyme carnitine palmitoyltransferase 1 (CPT1A), which is highly sensitive to inhibition by (methyl) malonyl-CoA [13] in ruminants, acting to prevent simultaneous oxidation and synthesis of FA in hepatocytes, as a potential futile cycle (Figure 3).

Dietary lipids in general, but also specific FA, can control the overall lipid metabolism by binding and activating nuclear receptors that modulate the gene transcription. For example, Jump [17] described that feeding mice high fat diets for a short time increases the transcription and nuclear content of sterol regulatory element-binding protein 1 (SREBP-1c). This gene in turn induces FA synthesis, *de novo* TG synthesis and storage. However, practicing such a regime for a longer period has the opposite effect and leads to constantly elevated leptin concentration in the circulation. High circulating leptin consequently activates the AMP-activated protein kinase (AMPK) as the key process in regulating cell energy status, which targets acetyl-CoA carboxylase (ACC) phosphorylation, supressing its activity and malonyl-CoA synthesis, respectively (Figure 3). Considering that the FA synthase consumes mitochondrial malonyl-CoA and limits its accumulation, the CPT1A expression can consequently increase, and two distinct pathways, i.e. β-fatty acid oxidation and lipogenesis, encompassing FA synthesis, can simultaneously occur and create futile cycles [18].

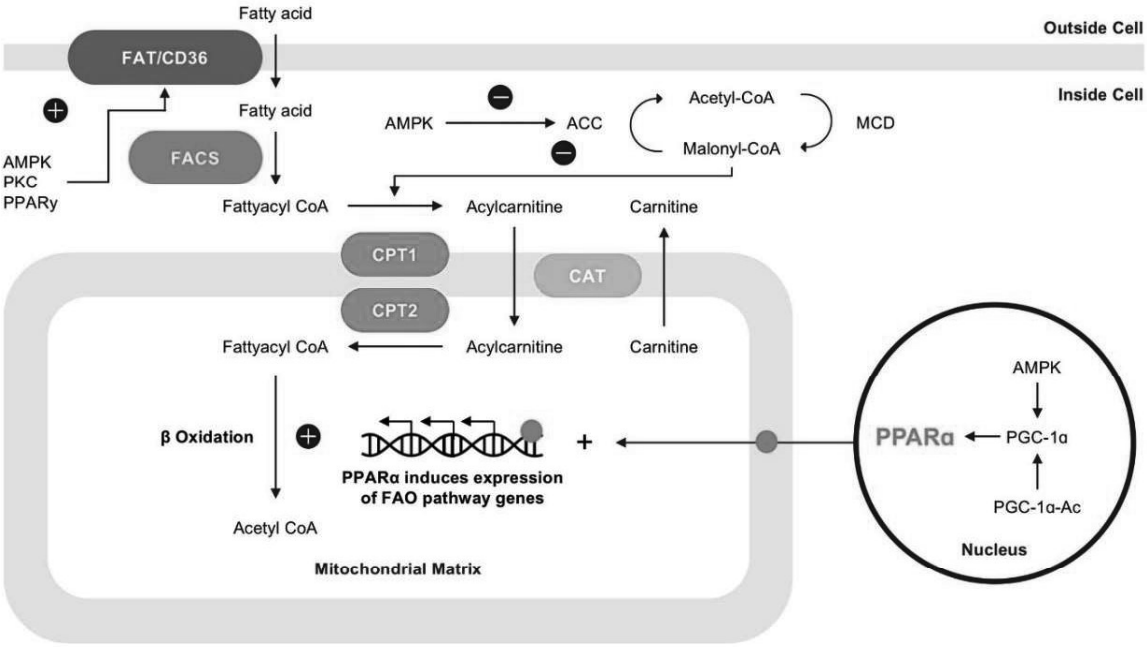

Figure 3. β-fatty acid oxidation (FAO) in cells [19].

Physiology and endocrinology of reproduction of the horse and the sheep

The oviduct and the uterus, as parts of the female reproductive tract, host the periconceptional events, namely oocyte maturation, fertilization, embryogenesis, pre- and implantation of the embryo, eventually leading to establishment of pregnancy. Thus, the oviduct and the uterine milieu that nurture the oocyte, sperm and the embryo during this period, play a decisive role in reproductive success in mammals. The periconceptional period has been a rather neglected and understudied stage of early embryo development, but has attracted

greater attention in the recent years with intensified employment of assisted reproductive technologies [20]. The onset of the periconceptional period is marked by gametogenesis, in females particularly referring to the final maturation and transport of oocytes to the oviduct. The oviduct is the organ that connects the ovary to the uterine horn and is the place where the fertilization, the first cellular divisions and the early embryo development happen. It is divided into five functional and morphologically different parts: the infundibulum, the ampulla, the ampullary-isthmic junction, the isthmus, and the utero-tubal junction. In ruminants, the embryo spends its first 4 days, or in the mare 5 to 6 days, in the ampulla and passes to the isthmus, reaching the uterus in a short time afterwards, usually within 24 hours [21]. This happens because of two coordinated actions: the contraction and relaxation of the oviduct muscular tissue, notably at ampullary-isthmic junction, and the ciliary beats of the oviduct epithelial ciliary cells along the ampulla and isthmus [22]. Apart from the mentioned ciliary cells, the oviduct is also characterized by the secretory cells, that secret the fluid which plays a major role by contributing to adequate development of the early embryo [23]. The oviduct fluid (OF) presents a mix of secretions from the secretory cells and transudation from plasma. Drews et al. [24], for example, characterized the fatty acid (FA) composition in the OF in mares, and found a different concentration of certain FA, namely oleic (OA) and palmitic acid, that were present at a higher concentration in early compared to late luteal phase (see details in Chapter 2). This suggested that the oviduct environment is preparing for the presence and the specific nutritional needs of an embryo (Figure 5), considering the beneficial role of OA for embryo development [25]. In equines and pigs it was revealed on a transcriptional level and using proteomics, that the presence of an embryo affects both the oviduct epithelial cells and the oviduct fluid, respectively [26, 27]. Velasquez [28] described that the nutrient concentrations in the reproductive tract are not necessarily reflected in blood levels, probably due to the unknown maternal adaptations that aim at keeping the nutritional microenvironment favourable for embryo formation and development. However, a study of Allen and Wilsher [29] confirmed still high rates of early pregnancy loss in Thoroughbred mares that remain undiagnosed. Moreover, Hinrichs [30] described that the embryos at earliest of seven days old are able to develop into a successful pregnancy when transferred into uterus of recipient mares, compared to earlier retrieved embryos. This leaves a gap in understanding the environmental conditions necessary for an optimal embryo growth during earlier stages when it is still located in the oviduct. In addition, O'Connell et al. [31] as well highlighted the importance of the uterine environment and the oocyte quality for a successful pregnancy establishment, based on a finding that most of the early embryo losses occur within the first 14 days of gestation in the

ewe. In the horse, early embryonic loss between fertilization and approximately day 40 ranges from 10 – 20 % in young and up to until 70 % in aged mares [32]. Nevertheless, age is not the major subfertility cause, but rather alterations of the uterine environment and function (e.g. endometritis and bacterial infections) play an important role for elevated subfertility rates in mares.

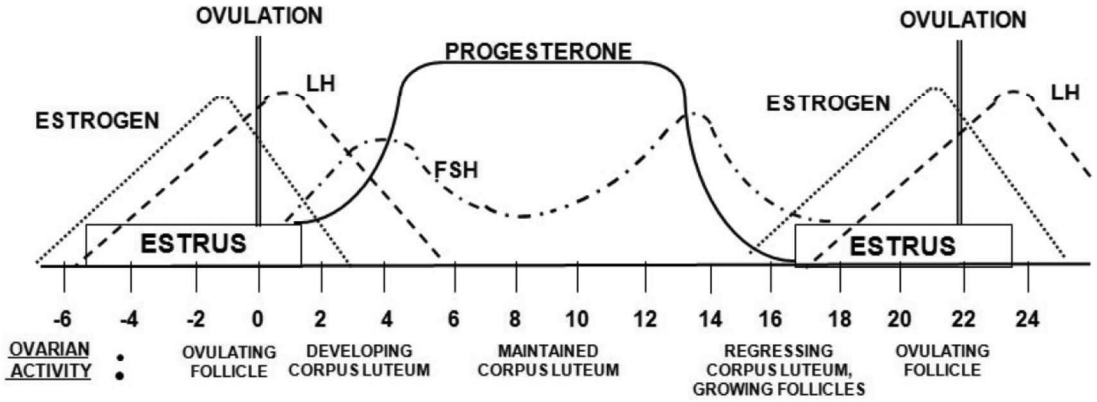

Figure 4. Hormone levels and corresponding ovarian activity in the estrous period in the mare; FSH, gonadotropin follicle-stimulating; LH, luteinizing hormone [33].

Both the horse and the sheep are characterized with a polyestrous and seasonal cyclic activity. Horses are considered long-day breeders as they exert sexual activity during the long days (from April to October in the Northern Hemisphere) [34]. On the contrary, short days are acting stimulatory on the sheep, which are in the Northern Hemisphere sexually active from around October until December, with various breeds expressing variability in seasonality traits. In order to control the seasonal reproduction and advance the breeding season in particular to ensure offspring each year, photoperiodic treatments were introduced and are intensively applied [35, 36]. Evolutionary, the birth season is placed within the period of year when forages in the nature are ample, which means that the offspring is born in spring in both species, and the pregnancy lasting 11 and 5 months, in the horse and the sheep, respectively. Ewes are becoming mature between six and eight months of age, with a moderate variation between breeds. Mares can reach puberty already around twelve months, but it is advisable not to breed them prior to maturity at two or three years of age. The estrous cycle in sheep lasts 17 days, as compared to 21 days in mares (Figure 4), and is governed by the hormones of hypothalamus, pituitary and pineal gland, ovaries and endometrium [37]. In particular, the pineal gland is responsible for secretion of melatonin (in dark hours) and its absence is a trigger for cyclic activity via regulating the gonadotropin-releasing hormone (GnRH) secretion. The hypothalamus secrets GnRH to induce the secretion of gonadotropin follicle-stimulating (FSH) and luteinizing hormone (LH) in the anterior pituitary. This is ultimately a stimulation for the onset of cycle and ovulation [38]. The hormone FSH in particular promotes follicular growth,

while follicular maturation and ovulation occur because of prominent LH levels. Interestingly, in the horse, the LH peak happens a few days after ovulation, which is in contrast to the sheep. The hormone FSH also acts on the ovary to stimulate the estradiol production, which is thereupon responsible for estrous behaviour and ovulation. *Corpus hemmoragicum* and *corpus luteum* are formed in the ovary, respectively, from the ruptured follicle and start to produce the hormone progesterone (P4). This hormone is indispensable to support a potential pregnancy and early embryo development in the uterus [39]. The P4 notably blocks the secretion of GnRH in the hypothalamus and by this the onset of a new estrous cycle. Marinone et al. [32] suggested low plasmatic P4 concentration as a cause of early embryo loss in mares and 4 ng/mL plasma as the minimal level necessary for pregnancy maintenance. If fertilization does not occur, the hypothalamus produces the hormone oxytocin that reaches the endometrium and stimulates the secretion of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), leading to luteolysis and the onset of a new estrous cycle. One of the major differences in pregnancy establishment in the horse and the sheep are the tau interferons (IFN-tau), known for their role as pregnancy recognition signals. The pregnancy recognition happens in both species around days 12 to 15 after fertilization, when signalling factors are secreted by the embryo and act on the endometrium. The IFN-tau is active in ruminants, while in the horse the signal for maternal recognition of pregnancy remains unidentified [40]. In the sheep, the trophoblast cells of the conceptus secrete the IFN-tau that acts on the uterus to supress the transcription of oxytocin receptor, blocking the previously described luteolytic mechanism and $PGF_{2\alpha}$ secretion, to establish pregnancy [41].

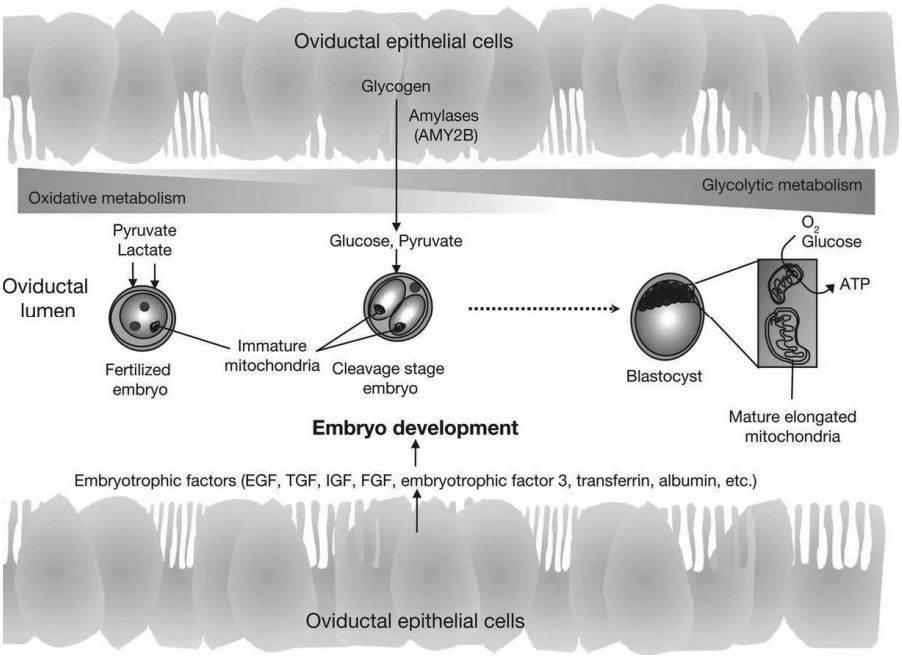

Figure 5. The influence of the oviduct on embryo development [42].

Lipids and fatty acids in relation to reproductive status in farm animals

It is well known that lipid supplementation positively influences reproduction in ruminants, including increased numbers of ovarian follicles, increased size of the ovulatory follicle, increased plasma concentration of progesterone (P4), reduced secretion of prostaglandin (PG) metabolites, increased lifespan of the *corpus luteum* (CL), and improved fertility rates [43]. In particular, the major benefit of dietary lipids is attributed to specific fatty acids (FA). In various tissues, FA have been recognized as major regulators of biological processes. They are the main component of cell membranes, and their composition affects cell membrane functions [44]. The FA from the embryo environment are an energy source for the embryo and a precursor for the embryonic FA synthesis, with the ability for the exogenous uptake of FA by the embryo demonstrated in multiple studies of various species [24]. Velasquez [28] suggested that unsaturated FA supplementation, as compared to saturated FA, could improve reproductive performance in cows. Similar studies in equines, relating FA supplementation and effect on reproduction are limited. Still, it must be noted that in horses the majority of dietary fat is absorbed before it can be fermented in the large intestine. The study of Duvaux-Ponter et al. [45] diet-supplemented mares with extruded linseed (rich in alpha-linolenic acid; ALA) and rapeseed (high in OA), and observed changes in ALA and OA in mammary secretions, present in lower and higher percentage in extruded rapeseed treatment, respectively. This confirms the possibility to modify the profile of FA in mares, at least in mammary secretions, by diet. Dietary consumption of FA can as well modify the FA composition of cell membranes in tissues of the reproductive tract [46, 47]. Special attention lies on the ratio of *n*6 to *n*3 PUFA as one of the central factors in tissue functionality. Due to high *n*6 FA contents of concentrates, this ratio in intensively farmed livestock is generally >10:1 whereas it should be closer to 1:1 [48]. Reduced *n*6/*n*3 ratio was shown to influence the follicular status and increase the cleavage rate of oocytes [44, 49]. Ciepiela et al. [50] demonstrated that elevated concentrations of arachidonic (AA) and linoleic (LA) acid derivates (*n*6 FA) in follicular fluid at the time of human oocyte retrieval significantly decrease the ability of oocytes to form pronuclei after intracytoplasmic sperm injection (ICSI), although the levels could not be associated with subsequent embryo quality or pregnancy rate. Moreover, Wathes et al. [48] suggested that both *n*6 and *n*3 PUFA can influence reproductive processes through a variety of mechanisms, such as decreased availability of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) due to an increased competition of *n*3 FA with its precursor AA for binding to prostaglandin H synthase (PTGS2), and inhibition of PG synthesis and activity. As suggested, change in PG production by eicosapentaenoic acid (EPA; *n*3 FA) via altering the preferential incorporation of AA in uterine endometrium to

produce PG of series 2, may affect many reproductive processes [51]. Mattos et al. [52] suggested that *n*3 FA reduce ovarian and endometrial synthesis of $PGF_{2\alpha}$, decrease ovulation rate in rats and delay parturition in sheep and humans. Still, Moussavi et al. [53] could not confirm an apparent effect of supplemented *n*3 FA on endometrial PTGS2 or $PGF_{2\alpha}$ production in response to oxytocin challenge, but did observe modified uterine *n*3 FA concentrations when cows fed fishmeal or *n*3 FA. Still, there seems to be a threshold of effectiveness in case of FA deficit and negative effects on fertilization in case of supplementing already FA-rich oocytes and embryos [28]. Supplementation with specific dietary FA to enhance efficiency of reproductive management and fertility remains to be further explored.

The adipokine leptin in relation to nutrition and reproductive performance

Leptin is an adipocyte-derived hormone that acts as a major regulator for food intake and energy homeostasis. It has a molecular mass of 16 kDa, consist of 167 amino acids and is coded by the obese (ob) gene [54]. Leptin secretion has a circadian and pulsatile rhythm. It is primarily secreted by white adipose tissue and therefore acts as an endocrine indicator of energy stores to the hypothalamus and other tissues in the coordination of appetite and metabolism with nutrient availability [55]. Waller et al. [56] reported that the circulating concentration of leptin in mares varies directly with the percentage of body fat. Leptin concentration is not only linked to the body condition but as well to gender and age. Unger and Zhou [57] suggested that an important function of leptin is to confine the storage of TG to the adipocytes, while limiting TG storage in other cells, thus protecting them from the non-oxidative metabolic products of long-chain FA during periods of overnutrition. By that, the β-fatty acid oxidation of surplus FA is increased, thus reducing lipogenesis. Leptin deficiency causes obesity but it seems unlikely that prevention of obesity is its primary physiologic role [58]. Adipose tissue as an endocrine organ and more specifically leptin as a hormone produced by adipocytes are suggested to be important for normal reproductive function [59]. In mammals, the function of the reproductive system is dependent on the availability of energy [60]. Undernutrition delays the onset of sexual maturation and in sexually mature animals it disrupts the estrous cycle and delays the postpartum return to estrous. In addition, defence mechanisms against the bacteria that usually infect the endometrium of postpartum cattle are impaired under such metabolic stress, leading to the onset of endometritis and infertility [61]. Leptin provides a metabolic information for the brain to determine whether the body's energy stores are sufficient for reproduction, with reproductive dysfunctions due to energy imbalance at both ends linked to leptin [62], and accordingly it needs to be within a threshold to maintain normal reproductive function [60]. An excessive energy supply and storage interfere as well

with the reproductive axis. In a study by Kumari et al. [54] it was demonstrated that plasma leptin levels are significantly higher in obese subjects with unexplained infertility compared to non-obese subjects from a fertile group. Although leptin clearly influences reproduction, it is not completely elucidated where leptin acts to exert its effects. Much data supports the notion that the reproductive actions of leptin involve actions in the brain and, more specifically, functions driven by the hypothalamic-pituitary-gonadal axis, as previously described. Odle et al. [63] proposed that the leptin receptivity of both the hypothalamus and the pituitary are vital for the body's ability to delay or slow reproduction during periods of low nutrition. In addition, it has been suggested that the influence of leptin on reproduction might also involve actions outside the brain.

Balogh et al. [64] described that leptin affects reproductive processes both centrally and peripherally with regard to uterine receptivity and embryo development. In addition to reproductive and adipose tissue, leptin expression was confirmed in many other tissues. Leptin receptors are described in different equine tissues [65] and they support the autocrine and paracrine actions of leptin. In equine CL, leptin supports luteal establishment in a dose-dependent manner [66]. Mitchell et al. [67] reviewed leptin and its receptor expression in the oviduct and the endometrium of humans, rodents and pigs, suggesting a role for the developing embryo. In rats, leptin inhibited P4 and estradiol (E2) production, leading to a reduced ovulation rate and reproductive dysfunctions [60]. Leptin receptor signalling in peripheral tissues is not well understood but certainly involves various transcription factors [67]. Ceddia [68] proposed the effect of leptin on muscular lipid metabolism by leptin favouring lipid oxidation through the activation of the AMPK pathway. This was already described in the second chapter *Lipid metabolism in the horse and the sheep*. Long et al. [69] described that the modification of synthases, transporters, and FA metabolism enzymes such as ACC, FAS, CD36, FATP and glucose transporter type 4 (GLUT4) in offspring is related to maternal obesity in sheep. Moreover, maternal obesity was shown to be responsible for programing the sheep offspring for metabolic syndrome characteristics [69]. Exposure of porcine embryo to leptin increased their development *in vitro*, suggesting the involvement of formerly mentioned AMPK pathway [67]. The local influence of leptin on the early embryo was further shown to increase the cell numbers and blastocyst formation in ovine embryos, but the contrary was shown in horses [64]. Balogh et al. [64] confirmed a variable regulation of leptin receptor within canine uterine epithelial cells, suggesting its role in early embryo-maternal cross talk. Interestingly, Kawwass et al. [70] observed in mice and humans, that leptin may directly affect peripheral reproductive tissues, but only indirectly and modest affect the reproductive

performance. However, the effects of leptin signalling on the reproductive tissues remain scarcely investigated. In addition, interspecies comparisons of leptin influence on the reproductive system must be carefully considered, as of conflicting results of its enhancing or impairing effect on reproduction.

Phenols in animal nutrition

Phenols, a group of plant secondary metabolites, gained high research interest in the last decades. They are a heterogeneous group of phytochemicals with around 8000 structurally different metabolites ranging from simple phenolic acids up to large polymeric macromolecules [71], that can be classified according to their chemical composition (i.e. number of phenol rings and structural elements binding rings to one another). Phenols naturally occur with sugar residues linked to hydroxyl groups, but other compounds as well, such as organic acids, lipids, or amines. Shikimic acid is the precursor for all the phenolic compounds. As classified by Hardman [72], the main phenol classes include flavonoids, stilbenes, lignans, and phenolic acids (Figure 6). The latter are divided into hydroxybenzoic and hydroxycinnamic acids whereas flavonoids are further classified as flavonols, flavones, isoflavones, flavanones, flavanols and anthocyanidins. Such structural diversity is responsible for a wide range of phenolic compounds that occurs in nature [73]. For example, the extract from *Acacia mearnsii* bark is rich in proanthocyanidins, mostly represented by gallocetechnis, catecatechins with fisetinidol and robinetinidol as extender units [74]. Grape seed extract is rich in flavonoids such as gallic acid, epi-, gallo-, and catechin procyanidin dimers and trimers [75]. There is a great variety of forage plants naturally rich in phenols. This group especially includes plant species growing in tropical and subtropical areas [76]. The application of phenols in animal nutrition has been particularly practiced via extract supplementation especially because phenol concentrations can be better standardised; attempts to manipulate forages in order to introduce polyphenolics into commercial varieties has proved difficult [77].

Phenols are known for their anti-oxidative, -inflammatory, -carcinogenic, chemo- and neuroprotective activities, but as well for their ability to regulate apoptosis [78, 79]. In addition, Ikarashi [80] showed that phenols also exert strong anti-obesity and -diabetic effects. As such, they are contributing to health and activate defence mechanisms in the organism. The digestive processes in ruminants do not inactivate the antioxidant properties of plant extracts rich in phenols *in vivo* and are even beneficial by improving the biological effect of polymeric proanthocyanidins [81]. After ingestion and during passage through the digestive tract, the rumen and gut bacteria can transform phenolic substances before a part of them, especially

those with lower molecular weight (LMW), are absorbed and enter the circulation. The advantage of LMW over high molecular weight polyphenolic fraction in increasing the concentration of various plasma metabolites, including epicatechin, was also described in rats by Hamuzu and Nakamura [82]. Consequently, the biological availability and activity of these smaller metabolites in the organism can change upon digestion. Ozdal [83] reviewed this modulation of phenolic compounds and confirmed that the activity of the gut microbiota significantly affects their structure. For example, catechins from tea are substantially transformed by the gut microbiota, followed by their absorption into bloodstream or excretion with faeces. Some of these phenols reach the liver as modified smaller or persistent larger compounds, and as such interplay in different metabolic pathways. Further, it is known that plant phenols can protect proteins and lipids from ruminal degradation and biohydrogenation, respectively, and improve the efficiency of their conversion to high quality animal products [77].

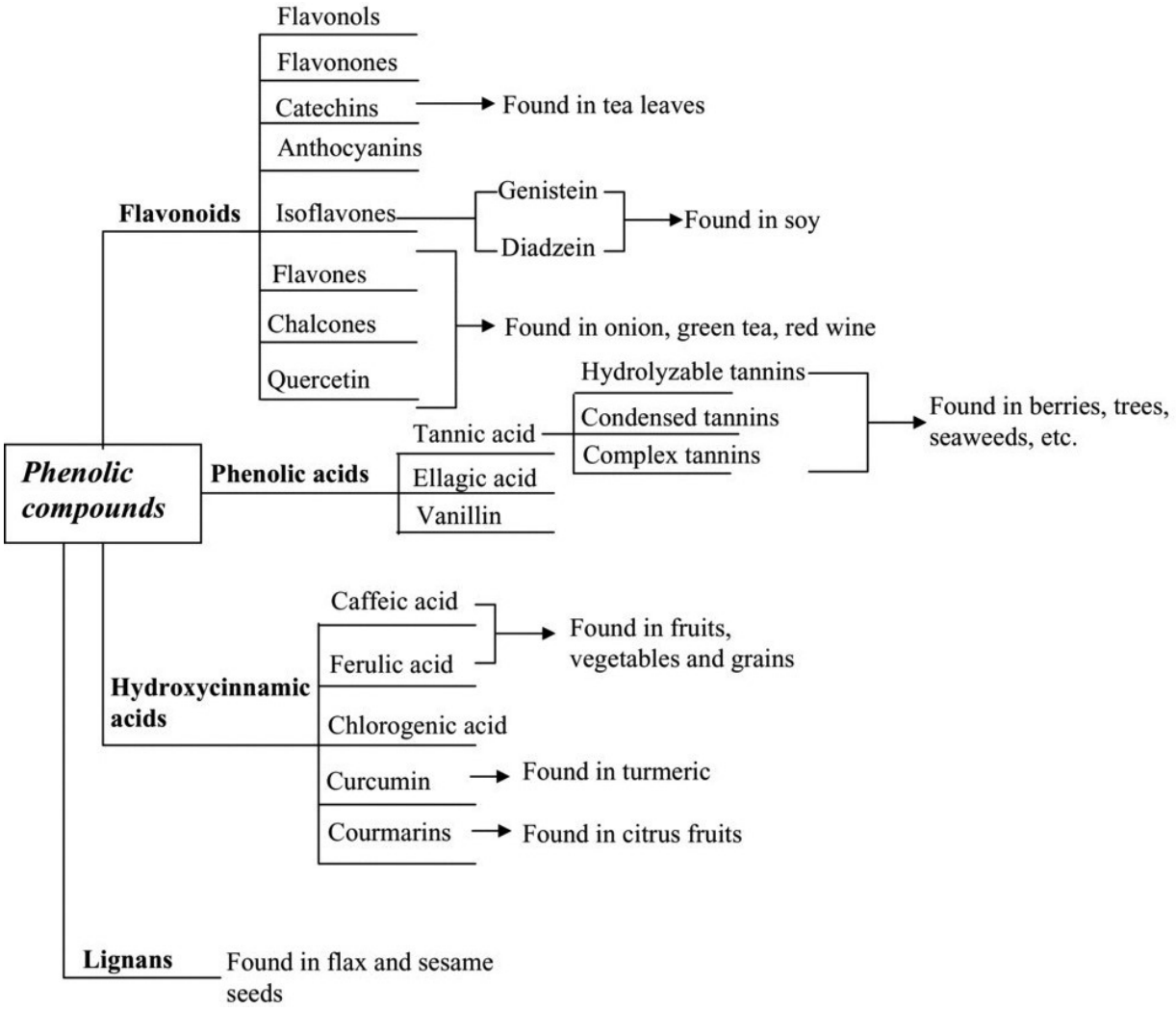

Figure 6. Classification of phenolic compounds [84].

There are almost no studies in the area of using naturally occurring phenols in horses. Wein and Wolffram [85] tested the absorption of the flavonol quercetin from the gastrointestinal tract in horses, and found total plasma flavonol concentrations comparable with those found in pigs, dogs, or humans. In an *in vitro* study, using blood mononuclear cells from aged horses, it was shown that the polyphenol compounds curcuminoids, resveratrol, quercetin, pterostilbene, and hydroxypterostilbene, significantly decreased inflammation without becoming cytotoxic. Thus, future research on biological effect of these compounds in horses is warranted.

Extensive research has proven that feeding plants or plant extracts rich in phenols can contribute to animal health, but has ambiguous effects on nutrient digestibility and growth performance [86]. Therefore, and based on available studies, both positive and negative effects on animal metabolism and physiology have been associated with phenol ingestion, and drawing conclusions regarding the potential benefits or adverse effects of phenols have to be drawn with caution. Finally, this must be even further appraised in ruminants, with regard to the effects on lipid metabolism.

Effects of phenols on lipid metabolism

Phenols may modulate lipid metabolism by regulation of signalling pathways via gene expression changes (Figure 7), particularly the activation of hepatic AMPK, as has been shown in numerous studies [87]. Fatty acid synthesis is inhibited by AMPK via 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) phosphorylation and the respective gene expression is downregulated in studies using dietary phenol supplementation [88-91]. In addition, Pan [92] demonstrated on mRNA and protein level that the phenolic group of theaflavins significantly reduces the expression of FAS and thereby the biosynthesis of FA, TG, and cholesterol. Phenols may also activate sirtuin-1 (SIRT1), which is an upstream regulator of AMPK and is directly involved in the deacetylation of the peroxisome proliferator activated receptor (PPAR) gamma coactivator 1 alpha (PGC-1α) [87]. Activated PGC-1α is needed to modulate the transcription of further genes that control energy expenditure. An increase in PGC-1α mRNA expression, together with uncoupling proteins (UCPs) and PPARα (transactivator for the expression of UCPs), is responsible for an increased β-fatty acid oxidation and lipolysis.

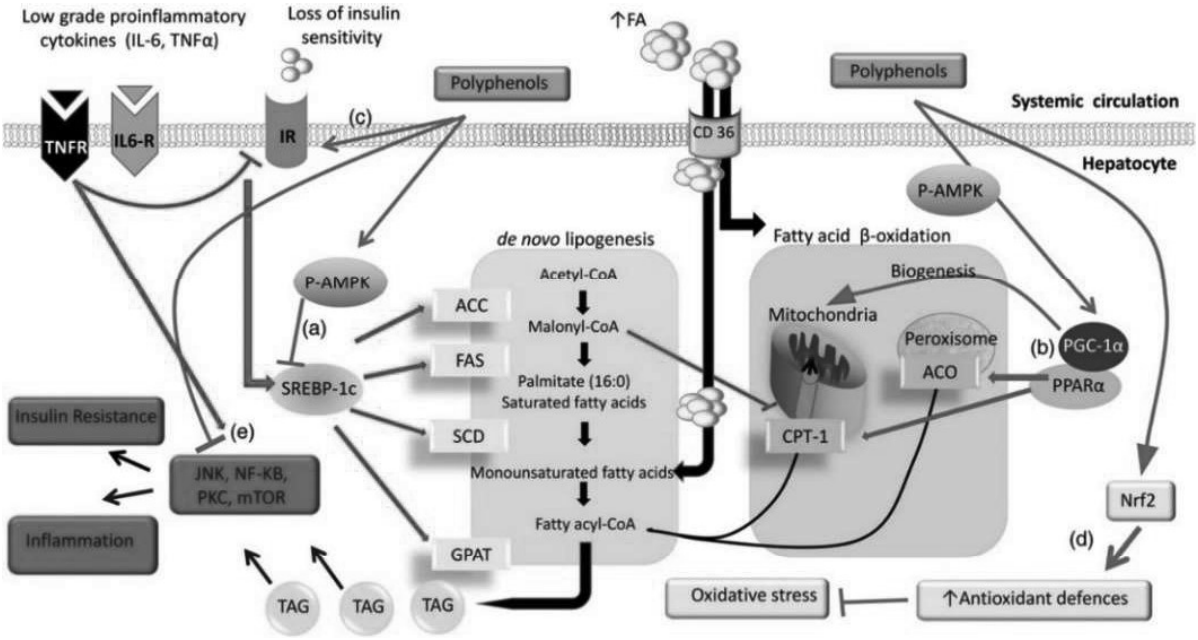

Figure 7. Possible mechanisms underlying the effect of phenols in hepatocytes through different mechanism of action including: (a) reducing lipogenesis through SREBP-1c downregulation, (b) increasing β-fatty acid oxidation by PPARα up-regulation, (d) reducing oxidative stress through increasing the antioxidant defence levels via nuclear factor, Erythroid 2 Like 2 (Nrf2), (e) attenuating the inflammatory pathways [93].

In mice, Yeh [88] demonstrated the role of the ethyl acetate fraction of chinese olive fruit extract, rich in phenols, which increased mRNA and protein expression of β-fatty acid oxidation related genes such as AMPK, acyl-CoA oxidase and PPARα, respectively downregulating the expression of SREBP-1c, FAS and CD36. Thereby, a specific action of phenols on different lipid metabolic pathways was suggested. Among the modified genes, PPARα has an important role for β-fatty acid oxidation by inducing the expression of pathway-related enzymes such as FA binding protein [13]. Inactivation of FAS by phenols causes reduction in malonyl-CoA levels and increases β-fatty acid oxidation in the liver [94]. Treatment with phenolic compounds was further shown to have an ameliorating effect on obesity markers and disorders by inhibiting lipogenesis and promoting β-fatty acid oxidation in different animal studies [95]. Wang et al. [95] demonstrated in mice and rats that dietary phenols reduce the proliferation of preadipocytes and viability of adipocytes, suppress the adipocyte differentiation and TG accumulation, stimulate lipolysis and β-fatty acid oxidation, and eventually reduce inflammation.

Effects of phenols on reproduction

The major intention of feeding phenols is minimizing the adverse effects of lipid peroxidation and improving antioxidant status of animals [86]. Moreover, they are gaining attention because of their potential role in reproduction [96], especially in relation to their property of reducing oxidative stress. Antioxidant agents were shown to improve the oocyte fertilization rate by protecting cells from apoptosis (i.e. through upregulation of SIRT1), but as well increase the blastocyst formation rate and decrease the reactive oxygen species levels, in a study using heat-shocked embryos [97, 98]. Lapointe and Bilodeau [99] described a heterogeneous expression of antioxidants such as glutathione peroxidase (GPX; GPX-1,-2), on contrary to superoxide dismutase (SOD1), along the oviduct and during the estrous cycle, suggesting a possible physiological role in the events leading to successful fertilization and implantation *in vivo*. In a study using condensed tannins in diet of ewes, a greater proportion of multiple ovulations compared to single ovulation was observed, suggesting a beneficial role in reproductive efficiency [96]. Wang et al. [100] further reported that green tea phenols, added during *in vitro* maturation and *in vitro* culture, improved pregnancy rates and decreased apoptosis in bovine blastocysts. These authors associated this with the increased transcript abundances of antioxidant enzyme genes SOD1, catalase (CAT) and GPX.

On contrary, there was no effect on the number of resorptions, implantations, litter size, or postnatal survival, when mice were consuming high amounts of epicatechin, catechin, and rutin during gestation and lactation [101]. Moreover, the phenolic extract used in the mouse adipocyte cell culture, significantly decreased leptin expression (90 %) and respectively regulated its synthesis [102], suggesting an effect of phenolic compounds on regulation of leptin levels. Treatment with the phenol curcumin downregulated the serum leptin levels and increased the levels of leptin receptors in testis tissue, in rats fed a high-fat diet, affirming that phenolic compounds may reduce the reproductive dysfunction caused by increased leptin signalling [103].

Outline of the doctoral thesis

The present doctoral thesis aims at investigating the fatty acid (FA) profile of the reproductive organs and fluids including a potential nutritional and hormonal modulation thereof. One of the major obstacles to animal husbandry on a global scale is the challenge of achieving optimised reproductive management. In this context, an understanding of the environment required for optimal embryo development during the critical phase of preimplantation is of crucial importance to improve the reproductive success of animals. Not only hormone signalling but also maternal nutrition is known to affect the preimplantation embryo environment. A special focus lies on oviduct and uterine FA as they serve as an essential energy source for the growing embryo. However, FA profiles and their variability in the reproductive organs and fluids in different species are still largely unknown. Thus, the project targeted the description of FA profile in selected reproductive tissues and fluids and their comparison to metabolically highly active organs (i.e. liver, muscle, adipose) and blood plasma, respectively, taking into account a potential modification by maternal dietary and hormonal signals, e.g. via differential gene expression. By this, the project contributes to fundamental and applied research.

In detail, this doctoral project aimed at answering the following research questions:

(i) The FA profile of the reproductive fluids in the horse (Chapter 2)

- Do the FA profiles of the reproductive fluids (i.e. oviduct and uterine fluid) reflect the blood plasma FA profile?
- Do the FA profiles of the reproductive fluids depend on the cycle stage, notably early and late luteal phase, corresponding to the presence of the embryo in the oviduct and uterus?

(ii) The FA profile of the reproductive tissues and fluids in the sheep (ewes) (Chapter 3)

- Are the FA profiles of the reproductive tissues (i.e. oviduct and uterus) and respective fluids comparable to those of blood plasma and metabolically highly active organs (i.e. liver, muscle, adipose)?
- Does dietary supplementation with phenolic compounds (i.e. grape seed and *Acacia mearnsii* bark extract) modify the FA profile of selected tissues and fluids?

- Is the expression of genes related to lipid metabolism in selected tissues affected by dietary supplementation with phenolic compounds?

(iii) The effect of the adipokine leptin on prostanoid synthesis in equine oviduct epithelial cell (EOEC) culture model (Chapter 4)

- Does the hormonal stimulation with leptin or oxytocin affect prostanoid (i.e. prostaglandin E_2 and prostaglandin $F_{2\alpha}$) synthesis in the EOEC culture *in vitro*?
- Does the hormonal stimulation with leptin or oxytocin affect gene expression in the EOEC culture *in vitro*?

The project was carried out in two species, the horse and the sheep, and combined *in vitro* and *in vivo* models, respectively. In the first experiment, the characterization of the reproductive fluids and blood plasma was performed by gas chromatography combined with mass spectrometry (GC-MS) using slaughterhouse material from healthy cyclic mares that were in defined cycle stages. Ovarian structures and hormonal profile were considered in characterizing the early and late luteal phase. In the second experiment, organs (i.e. oviduct, uterus, liver, muscle and adipose) and fluids (blood plasma, oviduct and uterine fluid) of ewes supplemented with different phenolic compounds were likewise subjected to FA profiling by GC and GC-MS. The associated gene expression was analysed using a high throughput gene expression platform. For the third experiment, a short-term (48h) cell culture model was established using EOEC collected *post mortem* from cyclic mares to investigate the impact of hormonal stimulation on gene expression using the above-mentioned technique. In addition, prostanoid synthesis was quantified in the cell culture supernatant using enzyme-linked immunosorbent assay analysis (ELISA).

Chapter 2

Fatty acid profile of blood plasma and oviduct and uterine fluid during early and late luteal phase in the horse

Based on:

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Published in:

Theriogenology 114 (2018) 258-265

https://doi.org/10.1016/j.theriogenology.2018.04.003

Abstract

During early pregnancy, the secretome of both oviduct and uterus serves as exchange medium for signalling factors between embryo and mother and provides the embryo with nutrients. The preimplantation embryo can utilize the fatty acids (FA) therein via direct incorporation into cell membrane lipid bilayers and for energy production via β-fatty acid oxidation. The FA concentration and composition of the oviduct (OF) and uterine fluid (UF) might be regulated by ovarian hormones to meet the changing needs of the growing embryo. In our study, we analysed the FA profile of blood plasma (BP) and reproductive fluid samples obtained *post mortem* from slaughtered mares by gas chromatography combined with mass spectrometry. Cycle stage was determined by visual evaluation of the ovary and measurement of plasma progesterone. No major effect of cycle stage on the FA profile was observed. However, the composition of FA was different between BP and both OF and UF. While linoleic, stearic, oleic and palmitic acid were the four most prevalent FA in both BP and reproductive fluids, the latter contained higher concentrations of arachidonic, eicosapentaenoic and dihomo-γ-linolenic acid. The finding suggests selective endometrial transport mechanisms from plasma into the reproductive fluids or increased endometrial synthesis of selected FA.

Introduction

During the preimplantation period, the embryo is supported by maternal secretions from the oviduct and the uterus. These secretions form a complex milieu containing e.g. proteins, amino acids, pyruvate, lactate, carbohydrates and fatty acids (FA) [104-109]. The oviduct supports gamete transportation and maturation, sperm capacitation, fertilization and development of the early embryo. Its fluid derives from blood filtrates, epithelial secretions, and, in part, follicular fluid [110]. The uterine fluid provides ongoing nutrition of the preimplantation embryo and enables the exchange of signalling factors vital for continuous embryonic growth and maternal recognition of pregnancy. It is composed of blood filtrates and secretions of endometrial luminal and glandular epithelial cells [104]. The relative quantitative contribution of FA via direct transudation from BP, uterine gland secretions and endometrial *de novo* synthesis are unknown. Transport of FA across membranes involves two components, passive diffusion [111] and selective protein mediated transfer [112-114]. In bovine, genes related to FA uptake are upregulated in the embryonic trophectoderm during elongation, suggesting that FA from the UF are utilized by the embryo [115].

The composition of the reproductive fluids is regulated by endocrine and local hormones related to the ovarian cycle and pregnancy status [107, 116-121]. The total protein content of uterine fluid in bovine [120, 122], pig [123] and horse [124, 125], for example, is the highest in the luteal phase of the estrous cycle. Concomitantly, a higher content of free amino acids has been reported in luteal reproductive fluids of cows [126], sows [109, 127] and mares [128], compared to estrous.

In contrast, cyclic effects for classical energy substrates such as lactate, pyruvate and glucose were neither evident in pig [109] nor in bovine [129]. To our knowledge, cyclic effects on the FA composition in reproductive fluids have not been investigated so far. Based on the fact that the key enzyme for FA production, FA synthase, is regulated by progesterone [130, 131], a cycle effect on FA seems plausible.

The multifaceted role of FA in reproduction has only recently gained increasing attention. Fatty acids are a potent source of energy. Their catabolism via β-fatty acid oxidation yields net amounts of ATP 3-times higher than the oxidation of glucose [132]. Although carbohydrates are considered as the classical nutrients for the early embryo [133-136], latest studies have shown that the oocyte as well as the early embryo can contain considerable amounts of lipids providing a potential endogenous energy reserve [137]. This particularly applies to species with a long preimplantation period such as the dog, pig, bovine and horse [138-141], as well as to species exhibiting delayed implantation (diapause) such as many marsupials, phocidae,

mustelids, dasypodidae (armadillo) and one representative of the cervidae, the roe deer [142-144]. Interestingly, the endometrial glands of armadillo and roe deer contain lipid droplets that seem to be released at the time of reactivation from diapause [142, 145] and might thereby serve as a direct energy substrate for the growing embryo and/or as a precursor for the embryonic FA synthesis.

The ability for the exogenous uptake of FA by the embryo has been demonstrated in multiple studies of various species. The rabbit embryo can absorb FA already at the zygote stage [146]. Similar observations have been made in the mouse embryo, where the rate of β-fatty acid oxidation is constant from the zygote to the eight-cell stage and then increases to the blastocyst stage [147]. In the bovine *in vitro* embryo culture, the addition of the β-fatty acid oxidation co-factor L-carnitine supports development to the morula stage [148], most likely due to the more effective use of FA as an energy substrate. Likewise, studies in human preimplantation embryos confirmed the stage specific uptake of different FA [149]. Furthermore, the lipid profile of the oocyte and the embryo show a high plasticity dependent on the environment in which they develop [150-152]. This finding suggests that direct integration of external FA in the embryonic phospholipid layer takes place.

Apart from their role as energy substrates and membrane compounds, FA can act as signalling factors by directly altering gene expression or by mediating inflammatory responses [153-155]. By this way, maternally derived FA can influence embryonic development. These influences might not only have an immediate effect but are also thought to induce epigenetic changes relevant for the adaption of the postnatal phenotype to its environment [156].

The equine embryo has an extraordinary long preimplantation period of 40 days and is supported by the maternal secretome for an extended period of time. Up to now, comprehensive data on the nature of the FA composition of the equine secretome is lacking and a possible impact of the ovarian cycle has not been investigated yet.

In our study, we collected the fluid of oviduct and uterus and corresponding plasma samples *post mortem* from slaughtered mares. The analyses focused on the characterization of the FA profile of maternal plasma and fluid of the oviduct and uterus during the early and late luteal phase. The early luteal phase overlaps with the oviduct period of the embryo (day 0 to day 6 after ovulation) and its arrival in the uterus (reviewed by Betteridge et al. [157]). The late luteal phase corresponds to the uterine period of the embryo during which pregnancy recognition must take place at the latest.

Materials & Methods

Animals

All samples were collected from ten healthy cyclic adult thoroughbred and warmblood mares after slaughtering (including five Franche-Montagnes breed mares that are described as "heavy warmblood") at a commercial abattoir in Switzerland between June and October 2016. The animals were aged between 5 and 22 (13.3 ± 6.3, n = 10 mares) years. The reproductive tract was collected immediately after slaughter and transported on crushed ice. The time span from the slaughter of mares to the sample freezing at -80°C until further analysis was on average 4 hours. The blood was collected from the jugular vein at the moment of slaughter into tubes containing EDTA, transported on ice and centrifuged (1500 x g for 10 min). Blood plasma was frozen at -80°C until further analysis (n = 8 animals, 4 per group).

Collection of oviduct and uterine fluid and endometrial samples

The uterus was trimmed of excess connective tissue and the cervix was sealed off with a cable binder. The oviducts were clipped at the uterotubal junction and a blunt cannula connected to a syringe was introduced into the uterine horn contralateral to the ovary with the functional *corpus luteum* (CL). The uterus was flushed with 10 ml of phosphate-buffered saline (PBS) solution which was collected by gravitation from the ipsilateral horn into a petri dish. Attention was taken to strictly avoid blood contamination. The collected UF was centrifuged (800 x g for 10 min) and the supernatant was frozen at -80°C until further analysis. The OF was collected from the ipsilateral oviduct. The oviduct and infundibulum were dissected free from connective tissue. Using a stereo microscope, a rinsing cannula (0.6 x 35 mm, Provet, Switzerland) was introduced into the infundibulum and the oviduct was flushed with 0.8 – 1.5 ml of PBS and the retrieved volume was recorded. The collected OF was centrifuged (800 x g for 10 min) and the supernatant was frozen at -80°C until further analysis. The UF and OF were collected from 10 animals (5 animals per group). For endometrial tissue collection, the uterine body and the uterine horns were opened longitudinally. With a scissor, endometrial stripes were carefully cut out and immediately transferred into cryo-tubes. Endometrial samples (n = 5 mares) were frozen in liquid nitrogen and subsequently stored at -80°C for later progesterone analysis.

Analysis of total protein

The bicinchoninic acid commercial assay (BCA assay) (ThermoFisher Scientific, USA) was used to determine the total protein (TP) content in the OF and UF, according to the provided protocol. It was assumed that the protein concentration of the retrieved fluid was dependent on the inserted volume but not on the recollected volume. Due to the different volumes used for flushing the oviduct, the TP content of the OF was corrected according to the inserted PBS volume.

Measurement of plasma and endometrial progesterone

Progesterone (P4) concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) according to Prakash et al. [158]. The lower detection limit for P4 was 0.1 ng/ml. The average intra- and inter-assay coefficients of variation (CV) were < 10 %. Endometrial P4 was measured after tissue extraction according to Luttgenau et al. [159].

Evaluation of cycle stage based on ovarian functional bodies and progesterone concentrations

The functional bodies of the ovaries were morphologically evaluated according to their size, texture and colour (Figure 8). The ovaries were cut open and the size of the CL was recorded. The "early luteal phase" was defined by a large CL (4 cm, n = 5 mares), sometimes with a central lacuna and reddish in colour, which was accompanied by smaller follicles (< 2.5 cm). The presence of numerous medium sized follicles and a regressing CL, indicated by its relatively small size (2.1 ± 0.1 cm, n = 5 mares) and yellowish colour, were characteristic for the "late luteal phase".

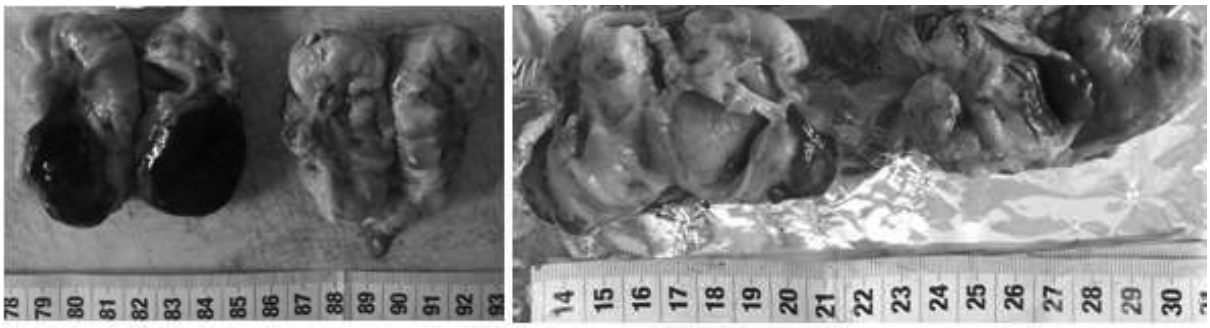

Figure 8. Functional bodies of sampled ovaries. Early (left) and late luteal phase (right).

Analysis of fatty acids

The fatty acid (FA) profile was determined as the FA methyl esters (FAME) by gas chromatography followed by mass spectrometry (GC-MS). Before derivatization to FAME, BP samples were pre-processed by precipitation of protein and DNA/RNA and lipid extraction. Therefore, 25 µl of each BP sample was placed into a 1.5 ml polypropylene plastic tube. Subsequently, 500 µl methanol:MTBE:CHCl3 (MMC) 1.33:1:1 v/v/v were added. After vortexing, the mix was incubated at +23°C/0.5 h/650 rpm, vortexed again and then centrifuged (10 min/13.2 krpm). From each of the clear supernatants of OF and UF, 0.5 ml and 1 ml, respectively, were transferred to Duran GL14 screw capped test tubes and evaporated to dryness in a sample concentrator at +40°C under a gentle stream of nitrogen. The evaporation residues of BP, OF and UF samples were methylated by adding 0.2 ml toluene, 1.5 ml MeOH and 0.3 ml 8 % (w/v) HCl (in 85 % v/v MeOH / 15 % v/v H2O, final HCl content of reaction mixture was 1.2 % w/v = 0.39 M), vortexing and incubating overnight at +45°C. Then, 1 ml hexane and 1 ml H_2O were added and the reaction mixes were vortexed 10x for 3 s each. After centrifugation for 60 s at 2000 rpm, 850 µl of the light upper hexane phases were isolated with a graduated glass syringe. From the hexane extracts resulting from BP samples, 1 µl was directly subjected to the GC-MS. Hexane extracts from OF and UF were transferred to 2 ml glass MS vials. The FAME extracts were evaporated to dryness in a sample concentrator at +40°C under a gentle stream of N_2. Each residue was redissolved in 0.2125 ml toluene and 1 µl of each extract was directly subjected to GC-MS.

The quantification was performed on the QuanLynx modul of the Waters software MassLynx 4.1. The lower limit of quantification (LLOQ) was determined taking into account the FAME standard at 12.5 ng/µl. The limit of detection was determined for each FA individually, taking into account any specific area corresponding to the respective FAME of the standard (Supelco 37 Component FAME Mix) at 100 ng/µl.

It was assumed that the FA concentration of the retrieved OF and UF was dependent on the inserted volume but not on the recollected volume. Due to the different volumes used for flushing the oviducts, the FA content of the OF was corrected according to the inserted PBS volume.

Statistical analysis

The SPSS program package (version 22, SPSS GmbH Software, Munich, Germany) was used for statistical analysis. Normal distribution was tested by Kolmogorov-Smirnov and Shapiro-Wilk tests. In case of normal distribution, one-way-ANOVA analysis was performed

to test for differences between the random factor (BP, OF and UF) and proportion of FA as a fixed factor. In case of non-normal distribution, Kruskal-Wallis-Test and Mann-Whitney-U-Test were used. Student's *t* test was employed to test for differences in FA concentrations and P4 values between early and late luteal phase. All results are presented as means ± SEM. P-values < 0.05 were considered statistically significant.

Results

Correlation of plasma and endometrial progesterone concentrations and morphological ovarian characteristics

The plasma P4 concentrations showed a statistically significant difference between early and late luteal phase groups (41.4 ± 9.6 vs 14.2 ± 2.2 ng/ml, $P \leq 0.05$), which corresponded to the grouping of mares based on morphological evaluation of the ovarian functional bodies. Accordingly, endometrial tissue P4 values showed significant differences between the early luteal phase and late luteal phase group (10.4 ± 2.3 vs 2.6 ± 0.4 ng/g, $P \leq 0.05$). Plasma and endometrial P4 levels exhibited a moderate coefficient of correlation ($r^2 = 0.65$).

Total protein

The amount of TP in OF and UF differed considerably between animals and failed to show a significant correlation. Due to the relative difference of oviduct and uterus size and PBS used for flushing, the UF contained in average 3-fold less TP than the OF. There was no statistical difference observed in the TP content between early and late luteal phase of each matrix (Figure 9).

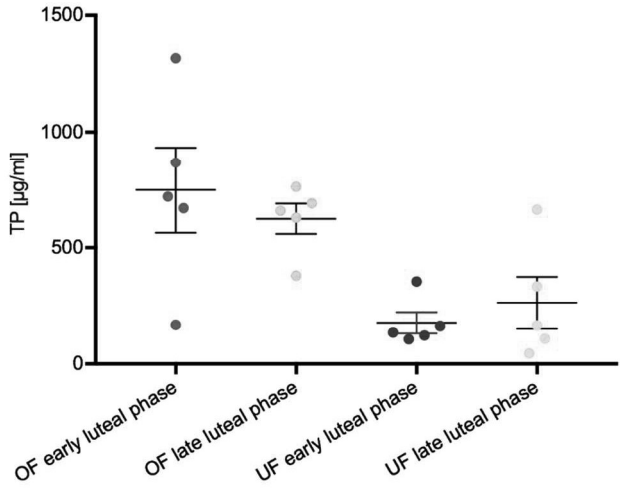

Figure 9. Total protein content (TP) in early and late luteal phase oviduct (OF) and uterine (UF) fluid (n = 5 mares per group, mean + SEM values).

Fatty acids

The composition of the FA pattern in BP, OF and UF was compared between the cycle stages (Figure 10). In addition, the relative amount of relevant FA expressed as percent of total FA was compared between BP, OF and UF (Figure 11).

Fatty acids in blood plasma

In BP, 17 different FA were identified in total, of which 5 were below the LLOQ (Table 1). The major FA were linoleic, stearic, oleic and palmitic acid (243.5 ± 61.8 ng/µl, 46.9 ± 0.6 ng/µl, 34.6 ± 8.1 ng/µl and 33.4 ± 6.5 ng/µl). In lower abundance, linolelaidic (7.2 ± 1.6 ng/µl), α-linolenic (4.1 ± 1.8 ng/µl), elaidic (1.7 ± 0.57 ng/µl), arachidonic (1.5 ± 0.8 ng/µl), eicosapentaenoic (1.2 ± 0.4 ng/µl) and dihomo-γ-linolenic acid (0.8 ± 0.4 ng/µl) were detected. There was no significant effect of the cycle stage on the FA composition in BP (Figure 10a).

Fatty acids in the oviduct fluid

In the OF, 20 FA were identified, of which 14 were quantifiable in at least one of the samples (Table 1). The order of the FA sorted in descending concentration was stearic (0.3 ± 0.3 ng/µl), linoleic (0.2 ± 0.3 ng/µl) and oleic acid (0.1 ± 0.3 ng/µl), followed by the less abundant arachidonic (0.07 ± 0.05 ng/µl), palmitic (0.05 ± 0.05 ng/µl) and eicosapentaenoic (0.02 ± 0.02 ng/µl) acid (Figure 10b). There was a significant difference ($P < 0.05$) between early versus late luteal phase in oleic (0.21 ± 0.13 versus 0.04 ± 0.01 ng/µl) and palmitic (0.10 ± 0.01 versus 0.01 ± 0.01 ng/µl) acid concentrations in the OF. In the early luteal phase group, the total concentration of FA was numerically higher compared to late luteal phase (1.33 ± 0.94 ng/µl and 0.36 ± 0.12 ng/µl), although this was not statistically significant.

Fatty acids in the uterine fluid

In the UF, 28 FA were identified and quantified in at least one of the samples (Table 1). Consistent with BP and OF, linoleic (8.5 ± 0.44 ng/µl) and stearic acid (4.4 ± 0.3 ng/µl) were the most abundant FA, but in UF they were followed by arachidonic (3.3 ± 1.0 ng/µl) and oleic acid (3.6 ± 0.6 ng/µl) that were equally abundant, and palmitic acid (1.6 ± 0.4 ng/µl) as the fifth most abundant FA (Figure 10c). There was no significant effect of early versus late luteal phase on FA composition in the UF.

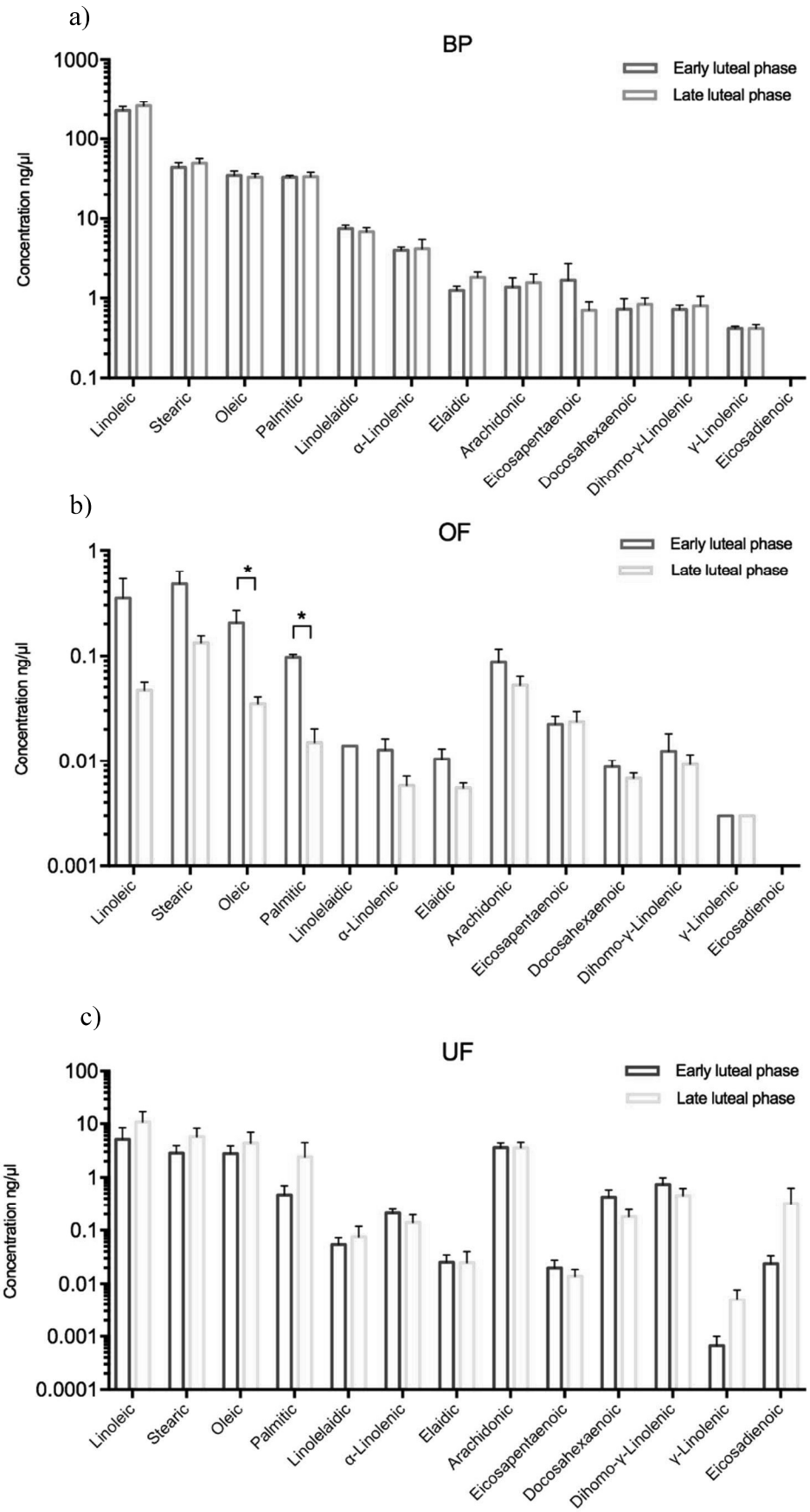

Figure 10. Fatty acid concentration in blood plasma (BP), oviduct (OF) and uterine (UF) fluid in early and late luteal phase (mean + SEM values).

Comparison of the fatty acid profile in blood plasma, oviduct and uterine fluid

The relative ratio of saturated (SFA) versus unsaturated FA (mono- and polyunsaturated, MUFA and PUFA, respectively) was significantly lower in BP (P = 0.013) and UF (P = 0.006) than in OF (Figure 12), mainly attributed to the high concentration of stearic acid in the OF (Figure 10). There was a significantly lower concentration of ω6 FA in the OF and UF than in BP whereas OF had significantly higher concentrations of ω3 FA than BP (Figure 12). The same trend for higher ω3 FA was observed in UF compared to BP (P = 0.052).

In BP, linoleic acid was by far the most prominent ω6 FA, while the most abundant ω3 FA in OF and UF were α-linolenic, eicosapentaenoic and docosahexaenoic acid. In OF and UF, derivates of linoleic acid (γ-linolenic, dihomo-γ-linolenic, arachidonic acid) as prostanoid precursors were more abundant than in BP (Figure 11).

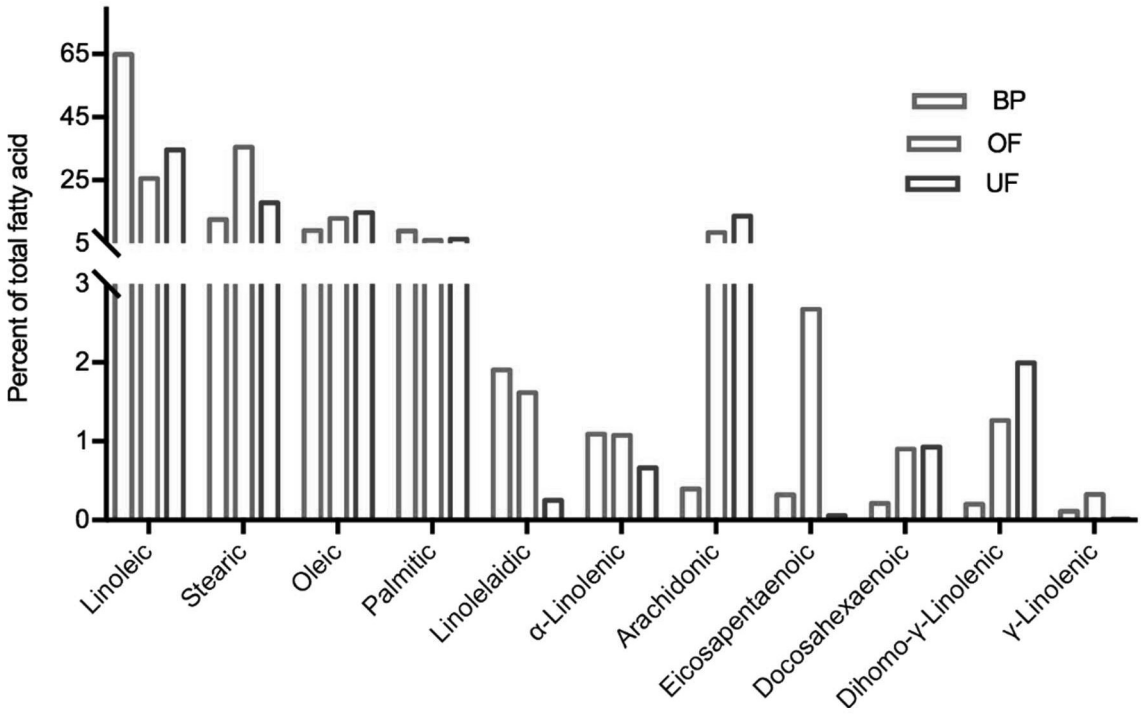

Figure 11. Proportions of select fatty acids (FA) in percent of total FA in blood plasma (BP), oviduct (OF) and uterine (UF) fluid.

Table 1. Fatty acid composition in blood plasma (BP), oviduct (OF) and uterine (UF) fluid in mares (n = 10 animals). The values are the average of all measurements, irrespective of luteal phase (LLOQ – lower limit of quantification; SEM – standard error of mean; ND – not detected).

Fatty acid	Formula	BP [ng/µl]			UF [ng/µl]			OF [ng/µl]		
		Conc.	SEM	LLOQ	Conc.	SEM	LLOQ	Conc.	SEM	LLOQ
Myristic	n14:0	< LLOQ	-	1.90	0.00	-	0.00	ND	-	0.01
Pentadecanoic	n15:0	< LLOQ	-	0.57	0.01	-	0.00	ND	-	0.00
Palmitoleic	n16:1-9c	< LLOQ	-	5.60	0.09	-	0.01	ND	-	0.03
Palmitic	n16:0	33.41	2.29	1.33	1.43	1.03	0.00	0.05	0.01	0.01
cis-10-Heptadecenoic	n17:1-10c	ND	-	5.69	0.02	0.01	0.01	ND	-	0.03
Heptadecanoic	n17:0	< LLOQ	-	1.35	0.07	0.05	0.00	< LLOQ	-	0.01
γ-Linolenic acid	n18:3-6,9,12c	0.26	0.08	0.35	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic	n18:2-9,12c	243.48	21.84	2.23	7.92	3.45	0.00	0.22	0.12	0.01
α-Linolenic	n18:3-9,12,15c	4.09	0.63	0.14	0.17	0.03	0.00	0.01	0.00	0.00
Oleic	n18:1-9c	33.92	2.85	1.32	3.58	1.36	0.00	0.11	0.04	0.01
Linolelaidic	n18:2-9,12t	7.14	0.57	1.35	0.06	0.02	0.00	0.01	-	0.01
Elaidic	n18:1-9t	1.55	0.20	0.45	0.03	0.01	0.00	0.01	0.00	0.00
Stearic	n18:0	46.88	4.59	0.29	4.32	1.36	0.00	0.30	0.09	0.00
Arachidonic	n20:4-5,8,11,14c	1.48	0.29	0.03	3.58	0.59	0.00	0.07	0.02	0.00
Eicosapentaenoic (EPA)	n20:5-5,8,11,14,17c	1.20	0.52	0.07	0.02	0.00	0.00	0.02	0.00	0.00
Dihomo-γ-linolenic (DGLA)	n20:3-8,11,14c	0.76	0.13	0.34	0.60	0.15	0.00	0.01	0.00	0.00
Eicosadienoic	n20:2-11,14c	ND	-	2.95	0.08	0.06	0.00	ND	-	0.01
Eicosenoic	n20:1-11c	ND	-	1.17	0.07	0.05	0.00	< LLOQ	-	0.01
Eicosatrienoic	n20:3-11,14,17c	ND	-	2.59	0.02	0.01	0.00	ND	-	0.01
Arachidic	n20:0	< LLOQ	-	0.61	0.21	0.13	0.00	0.02	0.00	0.00
Heneicosanoic	n21:0	ND	-	0.70	0.00	0.00	0.00	< LLOQ	-	0.00
Docosahexaenoic (DHA)	n22:6-4,7,10,13,16,19c	0.79	0.14	0.25	0.30	0.09	0.00	0.01	0.00	0.00
Docosadienoic	n22:2-13,16c	ND	-	3.45	0.01	-	0.00	ND	-	0.02
Erucic	n22:1-13c	ND	-	1.20	0.01	0.01	0.00	ND	-	0.01
Behenic acid	n22:0	ND	-	0.86	0.20	0.13	0.00	0.01	0.00	0.00
Tricosylic acid	n23:0	ND	-	1.04	0.03	0.02	0.00	< LLOQ	-	0.01
Nervonic acid	n24:1-15c	ND	-	3.24	0.45	0.30	0.00	< LLOQ	-	0.02
Lignoceric acid	n24:0	ND	-	1.53	0.67	0.47	0.00	< LLOQ	-	0.01

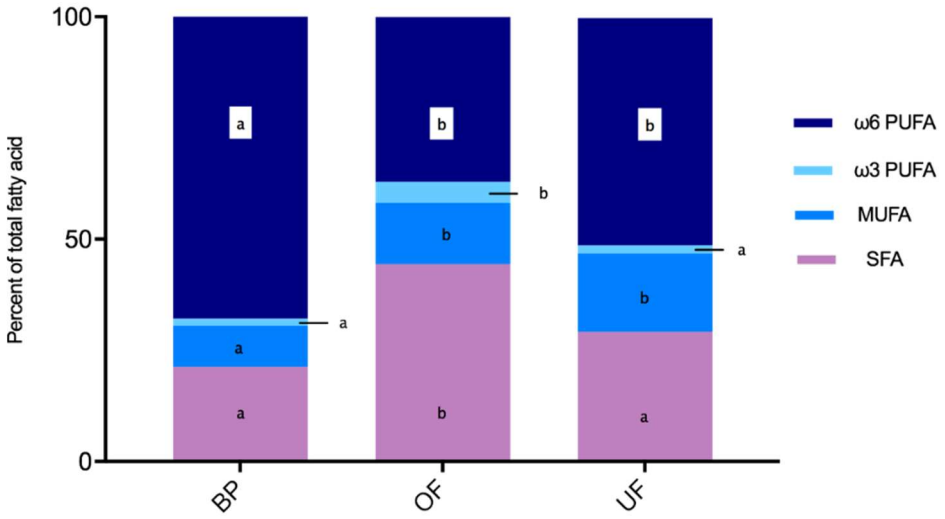

Figure 12. Comparison of the relative abundance of saturated (SFA), ω3- and ω6-polyunsaturated FA (PUFA) and monounsaturated FA (MUFA) in blood plasma (BP), oviduct (OF) and uterine (UF) fluid. Different superscripts a, b, and c indicate statistical differences between the three matrices.

Discussion

Gamete transport, oocyte maturation, sperm capacitation, fertilization and nutrition during early development involve FA as membrane components, energy substrates and mediators of embryo-maternal communication. Various studies have documented that supplementation of dietary ω3 and ω6 PUFA leads to an increase of the supplemented FA in BP and subsequently, also in reproductive tissues [52, 160-162] and embryonic tissue [160]. During early pregnancy prior implantation, the embryo has no direct contact to the maternal endometrium and nutritional components as well as signalling factors are transmitted via the OF and UF. Therefore, if the FA composition of the BP is reflected in the embryonic tissue it can only be mediated by the reproductive fluids. Fatty acids not derived from BP might derive from endometrial *de novo* synthesis or glandular secretions.

Studies investigating the FA content of reproductive fluids are scarce and incomplete [128, 141, 163-165]. To our knowledge, this is the first study that characterizes the FA profile of the equine OF and UF with respect to BP.

In our study, the relative concentration of SFA was significantly lower in BP than in OF and in UF by trend. Saturated FA are not known to play a major role in embryo-maternal communication but serve as energy substrates via β-fatty acid oxidation and as components of biological membranes. In bovine, sheep and pig, the high abundance of palmitic, oleic and stearic acid in the reproductive fluids [141] is reflected in the lipid composition of the blastocysts, where these FA are the most prominent ones [166]. Cell and embryo culture

experiments indicate that FA from the medium are incorporated into the cell phospholipid membranes [167]. This finding is supported by an *in vitro* study of Sata et al. [152], where bovine blastocysts cultured in serum displayed a different FA profile than those cultured in serum free medium. In the study of Ferreira et al. [168], the lipid profile of single embryos and oocytes of human, bovine, sheep, fish and insects has been characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Differences in the fatty acyl residue composition of the membrane phospholipids allowed for differentiation between the species under investigation. The FA composition of the equine reproductive fluids might therefore be indicative for the FA profile of the horse oocyte and early embryo. In this respect, the relatively higher amount of SFA in the oviduct and uterus needs to be considered in the context that SFA are less prone to lipid peroxidation than unsaturated FA.

The work of del Corrado et al. [169] shows that the total amount of lipids in the oocyte and embryo is also influenced by the surrounding environment. A surplus of FA availability might result in the accumulation of excess intracellular lipid droplets, decreasing the resilience towards freezing and thawing. In bovine for example, the exogenous supply of linoleic acid via diet [170, 171] as well as direct addition of linoleic acid to culture medium [172] had negative effects on oocyte maturation, embryo development and cryotolerance. The equine embryo contains numerous lipid droplets [173] which, on top of the embryo's large size and abundant blastocoelic fluid, diminishes its suitability for cryopreservation.

In the horse, of all FA determined, the five most abundant FA in both OF and UF respectively were stearic (36 and 18 % of total FA), linoleic (26 and 35 % of total FA), oleic (13 and 15 % of total FA) and arachidonic acid (8 and 14 % of total FA) followed by palmitic acid (6 and 6 % of total FA). In the bovine and pig reproductive fluids, oleic acid was the most abundant (22 – 36 % of total FA) followed by palmitic (19 – 32 % of total FA) and stearic acid (10 – 20 % of total FA) [141]. For the development of adequate equine *in vitro* culture media, knowledge about the physiological FA levels is of importance, especially if cryopreservation is a goal. Compared to bovine, equine reproductive fluid seems to contain a relatively higher amount of linoleic acid, which on top of the high intracellular lipid content probably contributes to the cryosensitivity of equine oocytes and embryos [171]. The addition or depletion of certain FA to/from culture medium may therefore influence equine oocyte and embryo tolerance to freezing and thawing [174].

In our study, all three matrices (BP, OF, UF) contained ω6 and ω3 PUFA. The relative proportion of ω6 FA was significantly lower in OF and UF than in BP. Compared to BP, the relative proportion of linoleic acid was reduced in OF and UF. Since the ω6 arachidonic acid is synthesized from linoleic acid, this finding could be attributed to a higher synthesis of arachidonic acid in the oviduct and, most prominently, in the uterus. Arachidonic acid is the precursor of several prostanoids, of which prostaglandins can bind to receptors in the subendometrial myometrium and stimulate muscle contractions [175-177]. In the horse, the preimplantation embryo is moved across the entire length of the uterine lumen [178] and its mobility is highest between day 11 and 14 [179]. The migration of the embryo is caused by local myometrial contractions that are thought to be initiated by embryonic or endometrial prostaglandin production [180]. Since artificial constraint of embryo mobility results in luteolysis and subsequent pregnancy failure [181], these uterine contractions seem to warrant embryo-maternal interaction over a large part of the endometrium for pregnancy recognition. Arachidonic acid is also present in the uterine fluid of bovine, pig [141] and rabbit [163, 165], although in these species its relative abundance is lower than in the horse. γ-Linolenic acid, an intermediate product in the synthesis of arachidonic acid from linoleic acid, is present in lower concentration in the equine uterine fluid than in bovine and pig [141], which suggest a greater turnover from linolenic acid (and/or dihomo-γ-linolenic acid) to arachidonic acid in the horse. In the OF, the ω3 eicosapentaenoic acid was present in prominent concentrations. Eicosapentaenoic acid (together with docosahexaenoic acid) mediates important aspects of the immune response. Direct anti-inflammatory effects involve the reduction of adhesion molecules and chemotaxis of immune cells. Furthermore, eicosanoids derived from ω3 FA promote the synthesis of anti-inflammatory resolvins and protectins (reviewed by Calder [182]). The different prostaglandins can therefore contribute to a local anti-inflammatory environment, enabling the tolerance of the paternal major histocompatibility complex (MHC) class I antigens of sperm and embryo [183, 184].

Although not significant, we found a higher absolute FA concentration in the OF in early (1.1 ng/µl) than in late luteal phase (0.3 ng/µl). Generally, lower progesterone levels are associated with higher fluid secretion by the oviduct epithelium [109, 185-189]. The amount of secreted fluid can dilute the concentration of components such as FA, which is in agreement with the observation of a lower protein content in OF under the influence of low progesterone levels during estrous in bovine [190]. In contrast, Alavi-Shoushtari et al. [191] reported a higher protein content at estrous in bovine. In our study, we could not confirm a dilution effect

concerning the TP content of neither oviduct nor uterine fluid in early versus late luteal phase. The observed trend for a higher absolute FA concentration in the early luteal phase in the OF might therefore indicate an increased transport of FA from BP into the OF or for FA synthesis and release of lipid droplets by the oviduct epithelium. The presence of distinct lipid droplets in the epithelium of oviduct and endometrium has been reported for a number of species. The distribution of cells containing lipids within the oviduct is hereby dependent on the hormonal status [192-194] and localization within the different compartments of the oviduct. According to Wordinger et al. [195], the lipid content of bovine ampullary epithelium is higher at diestrous and prestrous compared to estrous and metestrous. A lower cellular lipid content could be attributed to the release of lipid droplets into the oviduct lumen. These droplets contain neutral lipids that can be enzymatically cleaved into glycerin and FA and thereby be used by the developing embryo. Via this mechanism, cells of the reproductive tract could actively influence the lipid composition of the histotroph independently from transudation, as observed in our study.

Although we could only detect minor differences in the FA composition between the different cycle stages, a possible general impact of ovarian hormones cannot be ruled out. External influences that affected reproductive fluids such as maternal nutritional and metabolic status [196-198] were not taken into consideration in our study and might conceal a potential cycle effect. In addition, due to the unavailability of samples from mares at estrous, this important phase for gamete maturation remains to be characterized.

Conclusion

Our observation that FA composition in equine reproductive fluids differs from plasma suggests that the FA composition is not merely dependent on linear transudation from BP but also obviously modulated by secretions from the endometrial glands and oviduct epithelium. Further research is needed to explore even minor cycle effects on the FA composition of the oviductal and uterine secretome. This is particularly important with respect to the role of FA in providing energy, influencing cell membrane properties and serving as signalling molecules that contribute to decisively supporting oocyte and embryo development.

Chapter 3

Partitioning of fatty acids into tissues and fluids from reproductive organs of ewes as affected by dietary phenolic extracts

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In preparation for publication in Theriogenology

Abstract

Evaluation of dietary interventions with regard to the fertility problems often encountered by ruminant livestock is of global interest. Supplementation with polyphenols has well described effects on meat characteristics, but the impact on reproductive tissues and fluids remains scarcely investigated. Polyphenols possess antioxidant properties that may protect dietary unsaturated fatty acids (FA) from oxidation and thus saturation. In addition, transcriptional modification of genes involved in FA metabolism by polyphenols has been described in different species. As certain FA are reported to be crucial for fertility and the embryo, especially during the preimplantation phase, we characterized for the first time the FA profiles of reproductive tissues and fluids and investigated their potential modification by different dietary polyphenols in 22 cyclic ewes. These were randomly divided into four groups and fed meadow hay and one of four concentrates types either non- (control) or supplemented with grape seed extract, *Acacia mearnsii* bark extract (13 g/kg DM each) or a combination of both extracts (26 g/kg DM). After 10 weeks of feeding, animals were slaughtered and samples of reproductive (oviduct, uterus) and metabolically differently active tissues (liver, muscle, adipose), as well as of plasma and oviduct and uterine fluid were collected. The FA profiles were analysed in all samples. Expression of lipid metabolic and antioxidant genes was analysed in all tissues except the adipose tissue. Both FA profiles in the tissues and fluids, as well as gene expression in tissues, largely differed between the different matrices. In contrast, only a few diet effects were observed. These were limited to nine FA in the tissues and seven FA in fluids investigated. Interestingly, the FA profile of the uterus was the only one not at all affected by diet. Interactions were observed for only four FA in the tissues and two FA in fluids. Unexpectedly, total plasma FA concentration was significantly increased in all treatment groups compared to control. The mRNA expression was not affected by diet for any of the genes investigated, which might in part be explained by the similar polyphenol plasma concentrations at slaughter. Overall, our findings contribute to an improved understanding of the characteristic FA composition of reproductive tissues and fluids in sheep and the effect of maternal polyphenol intake on the preimplantation embryo environment in both the oviduct and uterus.

Introduction

Reproductive performance is one of the most important factors for successful livestock production systems. More specifically, the early embryo loss still represents a major concern for the breeding industry. Within the first 30 days of gestation more than 30 % of embryos are lost [31], which highlights the importance of both the oviduct and uterine environment for the embryo to successfully develop, leading up to and including pregnancy recognition. There are different strategies to mitigate these challenges, with dietary interventions representing promising means to eventually improve fertility [196].

One key aspect is the fatty acid (FA) profile of reproductive organs and fluids. Dietary lipid supplementation has repeatedly been suggested to improve fertility in cattle and sheep [52, 199]. In addition, Santos et al. [162] confirmed that in particular dietary *n*6 and *n*3 FA affect reproductive performance of cattle, including embryo quality and maintenance of pregnancy. Drews et al. [24] were the first to describe the characteristic FA profile of reproductive fluids in mares, but did not find a major influence of the cycle stage on the FA profile. Nevertheless, the differences in the FA composition between the fluids secreted from reproductive tissues and blood plasma suggested a selective enrichment of certain FA in the reproductive fluids, supporting the idea of their involvement as energy substrates or immune modulators or both in the presence of an embryo.

Modification of the FA composition of body tissues and fluids is possible by strategic dietary FA supplementation. Dietary *n*3 and *n*6 polyunsaturated FA (PUFA) were found to be efficiently incorporated into muscles and the endometrium of cattle when using rumen-protected oils as feed supplements [4, 47]. Supplementation of pregnant heifers with *n*3 FA also increased the proportion of the *n*3 FA eicosapentaenoic acid (EPA, C20:5) in the uterine fluid [200]. Another strategy to affect the FA profile of body tissues is the supply of plant secondary metabolites, which affect ruminal biohydrogenation of unsaturated FA [201]. This approach preserves dietary essential FA [202] and generates biohydrogenation intermediates of nutritional importance that may also act in metabolism upon absorption. For example, in dairy ewes, the use of crude phenolic olive pomace increased the proportion of PUFA in the milk fat [203], as did rolled linseed in intramuscular lipids of the *Longissimus dorsi muscle* in lambs [204]. Moreover, feeding of ensiled red grape pomace to lambs increased the proportions of *n*3 FA, whereas supplementation of goats with a polyphenol extract from olive mill wastewaters increased the ratio of unsaturated to saturated FA in meat [205, 206]. However, the possibility to modify and improve FA profile and metabolism in the reproductive organs and fluids by dietary means and especially by polyphenol supplementation remains scarcely explored.

The plasma lipid content is predominantly influenced by the interplay of liver, skeletal muscle and adipose tissues [207], and thus not only plasma lipids but also the metabolic status of these tissues is important for the FA supply of the reproductive organs. The liver plays a central role in regulating whole-body energy homeostasis and is also the main site of phase II metabolism and thereby conjugation of polyphenols absorbed from the intestine [208]. The expression of genes regulating FA metabolism in the liver tissue can hence provide important information about why a polyphenol-rich diet modulates the blood lipid profile [209]. Wu et al. [210] suggested that the effect of plant polyphenols is mainly due to reduction in the FA synthesis, increase in FA oxidation, and reduction in oxidative stress and inflammation via effects on the respective involved enzymes. Ingestion of polyphenols was indeed shown to regulate the expression of genes involved in lipid metabolism in different animal models (reviewed by [211]). For example, polyphenols of Chinese olive fruit extract were shown to activate AMPK pathway phosphorylation, notably suppressing the mRNA levels of FA transporter genes (CD36 and FABP) and lipogenesis genes (SREBP-1c, FAS and ACC), but upregulating genes that govern lipid oxidation (PPARα, CPT1A and ACOX) [88]. Sinz et al. [212] have shown that phenolic extracts, such as those from grape seed and *Acacia mearnsii* bark, are generally bioavailable in sheep to a certain degree as can be seen from increased plasma phenol concentrations [213]. Therefore, we aimed to investigate if this absorption of polyphenols has an impact on tissues that play a role in lipid metabolism and reproduction.

In our study, we investigated the FA proportions of reproductive tissues and fluids in relation to that of liver, muscle and adipose tissue, likewise blood plasma, in female lambs supplemented with two different extracts rich in phenols and a combination thereof. This increased the generalisation of the phenol effects, if any. In detail, the following hypotheses were tested: (1) The reproductive tissues and fluids have a distinct and different FA profile, which deviates from that of other tissues and plasma due to a selective functional partitioning. (2) Dietary phenols modify the FA profile of tissues and fluids, but distinctively in different tissues and fluids. For this purpose, tissues of metabolically differently active organs (liver, muscle, adipose) and reproductive tissues (oviduct, uterus and endometrium), same as reproductive fluids (uterine and oviduct) and plasma were collected at slaughter from the female subset of lambs used in the experiment by Sinz et al. [212]. Apart from the detailed FA profiles, the expression of genes involved in lipid metabolism and antioxidant defence was analysed to determine potential regulations caused by polyphenols.

Material & methods

Animals, housing and experimental design

Twenty-four healthy female lambs (18 Black Mountain Sheep and 6 Black Mountain Sheep × Texel crossbred) were housed at the ETH research station Chamau (Hünenberg, Switzerland). The ewes had an initial average age of 4.8 ± 1.0 months and a body weight (BW) of 29.7 ± 5.1 kg (average ± standard deviation). The animals were kept on straw in separate pens (size 2 × 2 m) in pairs. The animal experiment was approved by the respective cantonal authority (license number ZG 87/16). The animals were divided into four diet groups of six animals each. Two animals, one from grape and the other from grape + acacia group, were excluded for sampling of reproductive tissues and fluids, as of poorly developed reproductive organs. Initially, an adaption period of 2 weeks was introduced to allow animals to gradually adapt to the high concentrate amount. From then on, they received for 10 weeks the experimental diet twice a day (6:00 h and 17:00 h). The daily feed amount (kg dry matter (DM)/day) was restricted to BW (kg) × 0.04, following the recommendations of Agroscope [214]. The feed amounts were adjusted weekly after weighing. Concentrate was only fed after they finished at least half of the hay. At the end of the experiment, the animals had an average BW of 50.8 ± 2.6 kg.

Experimental diets

The basal diet consisted of concentrate and meadow hay in a ratio of 2:1. The hay was from a first cut with 600 g grasses/kg (dominated by Italian ryegrass) and 400 g legumes and herbs/kg. The ingredients of the concentrate (g/kg as fed) were soybean meal, 334.2; wheat, 300; maize, 117; barley, 100; mill by-product mix, 35; soybean oil, 25; molasses, 25; urea, 16.5; $CaCO_3$, 15.6; NaCl, 7.9; $Ca(H_2PO_4)_2$, 7.8; vitamin-trace element mix, 5, sulphur premix, 1. The concentrates differed in proportions of extracts replacing wheat. No extract was included in the control diet (8.1 g total extractable phenols (TEP) analysed per kg of diet DM). The other diets were supplemented with 20 g/kg DM of grape seed extract (‘grape’) (16.6 g TEP/kg DM) or *Acacia mearnsii* bark extract (‘acacia’) (14.3 g TEP/kg DM) or both extracts together (‘grape + acacia’) at 20 g/kg DM each (25.4 g TEP/kg DM). The grape seed extract (OmniVin 10R, S.A. Ajinomoto OmniChem, Wetteren, Belgium) contained, as analysed, 707 g TEP/kg DM (thereof 658 g total tannins (TT)/kg DM). The *Acacia mearnsii* extract (Weibull Black, CDM GmbH, Hamburg, Germany) and contained 513 g TEP and 465 g TT/kg DM. All experimental diets were rich in crude protein (CP) with about 230 g/kg DM in order to additionally test the ability of the extracts to bind excessive rumen-degradable nitrogen and reduce the metabolic N load (Sinz et al. [212]; see reference for more experimental details). The composition of nutrients and phenol fractions in the hay and concentrates is given in Supplementary Table S1.

Sample collection

At slaughter, carried out in the abattoir of the University of Zurich (Zurich, Switzerland) after stunning using a captive bolt gun, the lambs had an average BW of 50.8 ± 2.6 kg. Blood was collected directly at slaughter into tubes containing EDTA, shortly stored on ice and centrifuged at 1500 × *g* for 10 min. The plasma was stored at -20°C for later analysis. Immediately after slaughter, samples of uterus, endometrium, oviducts (including ampulla and isthmus), liver, muscle (*longissimus thoracis*) and subcutaneous adipose tissue were obtained. For the sampling of reproductive tissues, the reproductive tract was removed from the carcass and the excess tissue was removed. The oviducts were separated from the uterus and rinsed from the outside with phosphate-buffered saline (PBS). Then the ovaries were removed taking care to keep the infundibulum as a part of the oviduct. A rinsing cannula (0.6 × 35 mm, Provet, Switzerland) was introduced through the infundibulum into the ampulla. The entrance of the oviduct was secured with forceps and the oviduct held in vertical position to allow collection of the fluid in a clean Petri dish. The oviduct was then carefully flushed with 1.5 ml sterile PBS and the collected liquid was centrifuged at 800 × *g* for 10 min to remove any cellular debris. The supernatant was pipetted into sterile 1.5 ml Eppendorf tubes (Eppendorf AG, Germany) and frozen at -80°C until further analysis. The uterus was flushed in a similar manner with the cervix sealed using a cable binder. The rinsing cannula was inserted into one of the uterine horns that was subsequently sealed with forceps. Then, 10 ml of sterile PBS were introduced. The uterine fluid was collected through the opposite uterine horn via gravitation into a clean Petri dish. The liquid was processed like that from the oviduct. Finally, the uterine body was opened longitudinally and whole uterine wall and endometrium only samples were collected. Oviducts were kept intact. After collection, tissue samples were immediately snap-frozen in liquid nitrogen and stored at -80°C for later analysis.

Total phenols in blood plasma

In blood plasma, total phenols were analysed by the method developed by Serafini et al. [215] and the absorbance was determined at 765 nm using a spectrophotometer (VWR UV-6300, VWR international, Radnor, Pennsylvania), as described by Leparmarai et al. [213].

Fatty acid analysis in experimental feeds and tissues

Fatty acids (FA) in the experimental diets and the tissues were analysed as described by Wolf et al. [4] and Ineichen et al. [216]. Lipids were extracted with hexane:isopropanol (HIP) in a ratio of 3:2 (vol/vol) using an accelerated solvent extractor (ASE 200, Dionex Corporation, Sunnyvale, CA, USA). Lipid extracts were evaporated under nitrogen at 50°C until being almost dry. Then, 2 ml of internal standard (C11:0 triglyceride; Fluka Chemie, Buchs, Switzerland) was added and

evaporated again under the same conditions as before. The internal standard and HIP (2 and 15 ml) were added to 500 mg of homogenized tissue. Sunflower oil was used as the external standard to calculate the response factor for the experimental diets. The mixture was dispersed (Polytron® model PT 6000, Kinematica AG, Luzern, Switzerland), stored for 1 h, then vortexed, and centrifuged at 1000 × *g* for 6 min. The hexane phase was evaporated under nitrogen at 50°C. Residues of FA were concentrated by flushing with dichloromethane followed by evaporation. Methylation of extracts to FA methyl esters (FAME) was performed in two steps: by adding 2 ml methanolic NaOH, boiling for 3 min and adding 3 ml BF_3, boiling for 4 min [217]. The reaction was stopped with 7 ml NaCl, and 4 ml hexane were added, followed by vortexing and centrifugation. The hexane layer was transferred into GC vials. The FAME were separated on a gas chromatograph (HP 6890, Agilent Technologies, Inc., Wilmington, PA, USA) using a CP7421 column (wall-coated open tubular fused silica 200 m × 0.25 mm; Varian Inc., Lake Forest, CA 92630-8810, USA) suitable to separate C18 *cis*- and *trans*-isomers. The extracts were injected at an amount of 1 µl and with a split ratio of 1:20. Hydrogen, used as a carrier gas, was provided with a flow rate of 1.7 ml/min. The initial oven temperature was 170°C for 60 min, followed by an increase by 5°C/min up to 230°C, isotherm at 230°C for 32 min, increase by 5°C/min up to 250°C, and isotherm at 250°C for 15 min. The detector temperature was 270°C. Peaks were identified by comparing chromatograms with those of a Supelco 37-component FAME standard (Supelco Inc., Bellefonte PA, USA). The FAME present on the chromatograms were quantified by integrating the peak areas with the HP ChemStation® software (Agilent, Palo Alto, CA, USA). Peak identification was further confirmed using chromatograms from Collomb and Bühler [218]. Proportions were expressed as percentages of the total area of injected FAME. Total FA contents of the diets were computed by multiplying these proportions with the content of ether extract. Fatty acids with a mean value of < 0.2 g/100 g were considered as traces and are not shown in tables.

Fatty acid analysis in the fluids

Owing to the small FA contents/the small sample sizes, the FA profiles of the oviduct (OF) and uterine (UF) fluid, as well as of plasma, were determined by a different method that was described in Drews et al. [24] with slight modifications. Gas chromatography (7890A GC System, Agilent Technologies, Wilmington, DE, USA), followed by mass spectrometry (GCT Premier, Waters, Milford, MA, USA) was applied (GC-MS). Before derivatisation to FAME, plasma samples were purified by precipitation of protein and DNA/RNA, and lipids were extracted. For that, 25 µl of each plasma sample was placed into a 1.5 ml polypropylene plastic tube. Subsequently, 0.5 ml methanol:MTBE:$CHCl_3$ (MMC) 1.33:1:1 v/v/v were added. After

vortexing, the mix was incubated at 23°C/0.5 h/650 rpm, vortexed again and then centrifuged for 10 min at 13 200 rpm). Subsequently, 0.2 ml of each clear supernatant were transferred to Duran GL 14 screw capped glass test tubes and evaporated to dryness in a sample concentrator at 40°C under a gentle stream of nitrogen. From each oviduct and uterine flushing sample, 0.5 ml were transferred directly to Duran GL14 screw capped glass test tubes and evaporated to dryness (+40°C/N2). The evaporation residues of plasma, OF and UF samples were methylated by adding 0.2 ml toluene, 1.5 ml MeOH and 0.3 ml 8 % (w/v) HCl (in 85 % v/v MeOH/15 % v/v H_2O with a final HCl content of reaction mixture of 1.2 % w/v = 0.39 M), vortexing and incubating overnight at 45°C. Then, 1 ml hexane and 1 ml H_2O were added, and the reaction mixes were vortexed 10 times for three seconds. After centrifugation for 60 s at 448 × *g*, 0.85 ml of the light upper hexane phase was isolated with a graduated glass syringe, transferred to 2 ml glass mass spectrometry vials and evaporated to dryness (40°C/N2). The evaporation residues of plasma extracts and those of the OF and UF were redissolved in 0.2125 and 0.17 ml toluene, respectively. Finally, 1 µl of each redissolved extract was directly subjected to GC-MS analysis (column, Rxi-5Sil MS wall coated open tubular fused silica 30 m × 0.25 mmID, 0.25 µm df, Restek, Bellefonte, PA, USA). It was assumed that the FA concentration of the retrieved OF and UF was dependent on the inserted volume but not on the recollected volume. The quantification of the individual FA was performed using the QuanLynx modul of the Waters MassLynx 4.1 software. The lower limit of quantification was determined for each FA individually by taking into account the results obtained with the FAME standard (Supelco 37 Component FAME Mix) at 12.5 ng/ml. Only FA in concentrations above this limit were considered for further analysis.

Gene expression analysis

The frozen tissues were cut into pieces of 50 to 100 mg and immediately processed according to the manufacturer's instructions of the respective kit used. The homogenisation of endometrium and oviduct samples was done in lysis buffer (Buffer RLT; Qiagen, Venlo, The Netherlands) and β-mercaptoethanol (Sigma-Aldrich, Germany) using MagNA Lyser Green Beads (Roche, Switzerland) and MagNA Lyser Instrument (Roche, Germany) at 7000 × *g* for 30 s. The RNA from the oviduct and endometrium was extracted using the RNeasy® Mini kit (Qiagen) according to the manufacturer's protocol, whereas from the liver and muscle samples it was extracted using the AllPrep DNA/RNA Mini and the RNeasy® Fibrous Tissue Mini Kit (both Qiagen), respectively. Ethanol was used in different concentrations ranging from 50 to 100 % depending on the tissue type. The quantity of isolated RNA was checked for all samples using a NanoDrop One (Thermo Fisher Scientific, USA) and the quality control was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Only samples with distinctive 18S to 28S ribosomal

bands, optimal 260/280 and 260/230 ratios, and RNA integration number (RIN) above 6 were considered for further analysis (RIN range for liver 6.1-9.5; endometrium 7.8-9.9; oviduct 7.7-10; muscle 6.6-9.9). The cDNA synthesis was performed using 180 ng of RNA of each sample using a Transcriptor cDNA Synthesis Kit (Roche, Switzerland) according to the manufacturer's protocol, on the Eppendorf MasterCycler X50s (Eppendorf, Germany).

Specific primers for genes of interest were designed using NCBI Primer3 and BLAST software that is publicly available. Fatty Acid Metabolism RT² Profiler PCR Array from Qiagen (publicly available) was consulted in scope of selecting target genes, same as papers from the PubMed database containing keywords: 'fatty acid', 'polyphenols', and 'gene expression'. Target genes were tested against the NCBI *Ovis aries* (taxid: 9940) sequences found in NCBI GenBank database (http://www.ncbi.nlm.nih.gov/, accessed April – July 2018) and purchased from Microsynth (Balgach, Switzerland). Primer sequences, NCBI accession numbers and amplicon sizes are summarized in Supplementary Table S2. All amplicons were tested for reliability via RT-PCR (using melt-curve analysis) and confirmed on a 10 % agarose gel (gel electrophoresis) in order to demonstrate the amplification of a single product of the expected size. Ultra-pure distilled water free of DNase/RNase was used for the preparation of the stock and the working solutions (Invitrogen, Life Sciences Limited, UK). The primer efficiency was confirmed using LinRegPCR 2016 (Amsterdam, the Netherlands). The synthesized cDNA was preamplified using the TaqManPreAmp® Master Mix (Fluidigm, USA). Gene expression was measured using a high throughput gene expression platform based on Dynamic Array™ microfluidic chips – 48.48 Dynamic Array IFCs (Integrated Fluidic Circuits) (Fluidigm) and EvaGreen® DNA binding dye (Fluidigm) following the manufacturer's protocol that involves performing specific target amplification. The unprocessed mRNA data was obtained and handled with the Fluidigm Real-Time PCR Analysis Software 4.5.1. (Fluidigm). A linearity range of 7 to 25 cycle thresholds (Ct) was selected for further analysis. The geometric mean of all reference genes' (UBB, B2M, RPL19, YWHAZ) Ct values was confirmed to be the most stable reference using the GeNorm option in the GenEx Pro software (MultiD Analyses AB, Sweden). Therefore, normalised expression delta Ct (ΔCt) values were calculated as difference between the Ct value of the respective target genes and the geometric mean of reference genes. The ΔCt values were used for statistical analysis of differentially expressed genes. Fold changes were calculated according to the ΔΔCt method.

Statistical analysis

For statistical analysis, the SPSS software package (SPSS, IBM Corp. Released 2013. IBM SPSS Statistics for Windows, version 24.0, Armonk, NY:IBM Corp.) was used. For analysis of the FA and gene expression data, three different models were used. The first analysed the effect

of diet, the second the effect of tissue (uterus (for FA analysis), endometrium (for gene expression analysis), liver, muscle and adipose tissue) or fluid (plasma, uterine and oviduct fluid) and the third considered diet, tissue/liquid and the interaction of both as fixed effects. Slaughter date was set as random effect for all models. Furthermore, animal was set as repeated random statement. Unstandardized residuals of subjects were tested for normal distribution using Shapiro-Wilk test and visual inspection of Q-Q plots. In case of non-normal distribution, data was transformed into log values and normality test repeated. Normalised datasets were submitted to one-way analysis of variance (Linear Mixed Model). Bonferroni and LSD Post Hoc tests were used in pairwise comparisons and all the results calculated as means ± standard error of means (SEM).

Results

Total extractable phenols in plasma

The total extractable phenols (TEP) measured from blood plasma collected at slaughter (mg/l) were lowest in the control (259 ± 10.5, mean ± SEM; n = 6), followed by grape (254 ± 10.6; n = 5). Acacia alone and grape + acacia (n = 4 per group) had plasma TEP values of 284 ± 20.9 and 299 ± 25, respectively. Despite these numerically higher plasma TEP values observed in acacia and grape + acacia as compared to control and grape group, the difference was not statistically significant (Figure 13).

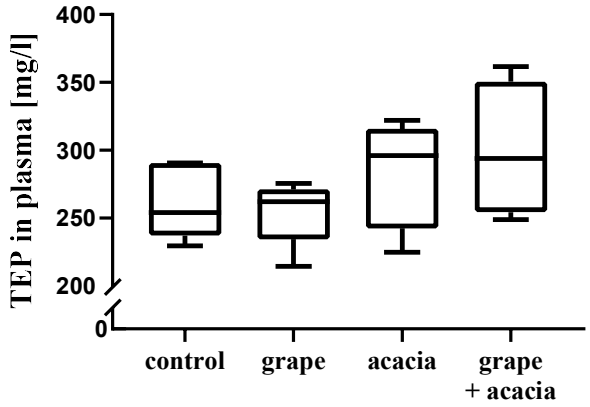

Figure 13. Total extractable phenols (TEP) measured from blood plasma collected at slaughter (mg/l): control (259 ± 10.5, mean ± SEM; n = 6), grape (254 ± 10.6; n = 5); acacia (284 ± 20.9; n =4); grape + acacia (299 ± 25; n = 4)

Fatty acid profile of the experimental feeds

Proportions of FA measured in the lipids of hay and concentrates are shown in Table 2. In total, 16 FA were identified in proportions > 0.2 g/100 g total FA analysed. The differences among the concentrates were small compared to those in hay. The supplements added to always the same ingredients were likely almost free of lipids. The hay had a higher proportion of SFA, PUFA and *n*3 FA than the concentrates, the lipids of which contained more MUFA and *n*6 FA.

Table 2. Proportions of fatty acids (FA; g/100 g total FA analysed) in the experimental feeds offered to the ewes (n = 3 per item).

		Experimental concentrate			
Fatty acid	Hay	Control	Grape	Acacia	Grape + acacia
C12:0	0.307	0.048	0.041	0.020	0.016
C14:0	0.656	0.130	0.132	0.038	0.003
C16:0	24.8	16.8	17.7	16.8	15.2
C18:0	2.21	5.46	5.86	5.54	5.00
C20:0	0.889	0.524	0.573	0.545	0.492
C22:0	1.40	0.495	0.536	0.506	0.456
C24:0	1.18	0.295	0.314	0.303	0.277
C12:1	0.255	0.005	0.004	0.004	0.006
C16:1*n*9	0.326	0.121	0.124	0.116	0.109
C18:1*n*9	3.45	26.6	28.2	26.8	24.8
C18:1*cis*11	0.483	1.45	1.52	1.48	1.37
C20:1*n*9	0.122	0.374	0.384	0.378	0.348
C18:2*n*6	18.4	42.4	40.4	42.9	46.5
C18:3*n*6	0.287	0.032	0.025	0.028	0.030
C18:3*n*3	41.1	4.21	3.18	3.52	4.29
C22:2*n*6	0.284	0.086	0.095	0.086	0.075
Σ SFA	34.3	24.2	25.7	24.3	22.0
Σ MUFA	5.2	28.9	30.5	29.1	27.0
Σ PUFA	60.5	46.9	43.8	46.6	51.0
Σ *n*3	41.4	4.27	3.26	3.59	4.34
Σ *n*6	18.8	42.5	40.5	43.0	46.6
*n*6/*n*3 ratio	0.460	10.1	12.4	12.0	10.7

SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA

Fatty acid profile of the tissues

There were large differences ($P < 0.001$, except C18:0 in acacia treatment ($P < 0.05$) between the five tissues in the five selected individual SFA occurring in proportions of > 0.2 g/100 g total FA analysed (Table 3). Tissues of the reproductive organs (uterus and oviduct) were lower ($P < 0.001$) in C14:0 and C16:0 proportions compared to muscle and adipose tissues with the liver resembling more the former organs. The proportion of C15:0 was higher ($P < 0.001$) in adipose and lower in muscle tissue compared to reproductive tissues, and C18:0 proportion was the highest ($P < 0.001$) in liver compared to all other tissues. Diet effects on individual SFA ($P < 0.05$) were found only in C16:0 and C17:0 proportions of the adipose tissue, and the C17:0 proportions in the oviduct and muscle tissue. Interactions of diet and tissue were observed for C16:0 ($P < 0.05$) and C17:0 ($P < 0.01$), and there were trends for interactions ($P < 0.10$) with C14:0 and C15:0 proportions.

Almost each of the selected individual MUFA exhibited tissue differences ($P < 0.001$) in any dietary treatment (Table 4). An exception were C18:1*cis*12 and C18:1*trans*10. For C18:1*cis*12, the proportion in tissue lipids in control differed only at $P < 0.05$ and in acacia at $P < 0.01$, whereas no difference was observed in grape and grape + acacia group. For C18:1*trans*10, in acacia group only a trend ($P < 0.10$) was observed. The lipids of uterus and oviduct tissues very often contained

less ($P < 0.001$) C16:1*n*7, C16:1*n*9, C17:1, C18:1*n*9, and C18:1*trans*11 compared to lipids of muscle, adipose and liver tissue (except C17:1 and C18:1*n*9): Liver mostly contained more ($P < 0.001$) C18:1*cis*11. Diet effects ($P < 0.05$) were quite rare and occurred in proportions of C16:1*n*7 (oviduct, muscle and adipose tissue), C16:1*n*9 (adipose tissue), C17:1 (liver tissue) and C18:1*cis*12 (muscle tissue). Only C16:1*n*7 showed an interaction ($P < 0.01$), and there was a trend towards and interaction ($P < 0.10$) in C17:1.

Table 3. Effect of diet and tissue on proportions of selected SFA (g/100 g analysed FA)[1].

		Dietary treatment					
Fatty acid[2]	Tissue	Control n = 6	Grape n = 6	Acacia n = 5	Grape + acacia n = 5	SEM[3]	P-value
C14:0	Uterus	0.55^{a}	0.63^{a}	0.57^{a}	0.58^{a}	0.067	0.489
†	Oviduct	0.58^{a}	0.57^{a}	0.47^{a}	0.55^{a}	0.051	0.228
	Liver	0.81^{a}	0.83^{a}	0.72^{a}	0.67^{a}	0.098	0.287
	Muscle	2.29^{b,z}	1.92^{b,yz}	1.69^{b,y}	2.03^{b,yz}	0.136	0.110
	Adipose	2.48^{b}	2.17^{b}	2.23^{c}	2.25^{b}	0.169	0.271
	SEM[3]	0.098	0.163	0.136	0.169		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C15:0	Uterus	0.53^{a}	0.53^{b}	0.58^{cd}	0.51^{b}	0.069	0.604
†	Oviduct	0.50^{a}	0.43^{ab}	0.46^{bc}	0.43^{b}	0.062	0.363
	Liver	0.36^{a}	0.33^{ab}	0.33^{ab}	0.31^{ab}	0.024	0.133
	Muscle	0.29^{a}	0.25^{a}	0.22^{a}	0.22^{a}	0.024	0.054
	Adipose	0.98^{b}	1.06^{c}	0.68^{d}	0.90^{c}	0.139	0.080
	SEM[3]	0.139	0.094	0.075	0.082		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C16:0	Uterus	16.5^{ab}	17.0^{ab}	16.5^{a}	16.7^{b}	0.73	0.567
*	Oviduct	17.4^{b}	18.0^{bc}	16.5^{a}	17.7^{b}	0.55	0.092
	Liver	14.5^{a}	15.1^{a}	14.8^{a}	13.8^{a}	0.74	0.309
	Muscle	23.7^{d}	23.2^{d}	23.0^{b}	23.7^{d}	0.52	0.542
	Adipose	21.4^{c,yz}	19.6^{c,y}	22.2^{b,z}	20.3^{c,yz}	0.77	0.035
	SEM[3]	0.74	0.77	0.75	0.59		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C17:0	Uterus	0.51^{a}	0.49^{a}	0.51^{a}	0.47^{a}	0.021	0.375
**	Oviduct	0.55^{a,z}	0.50^{a,yz}	0.50^{a,yz}	0.47^{a,y}	0.019	0.025
	Liver	1.07^{a}	1.01^{a}	0.97^{a}	0.97^{a}	0.044	0.133
	Muscle	0.86^{a,z}	0.78^{a,yz}	0.71^{a,y}	0.73^{a,y}	0.049	0.044
	Adipose	3.55^{b,z}	3.75^{b,z}	2.49^{b,y}	3.45^{b,z}	0.403	0.049
	SEM[3]	0.403	0.274	0.258	0.311		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:0	Uterus	17.8^{a}	17.3^{a}	17.6^{a}	18.5^{ab}	0.68	0.158
	Oviduct	16.8^{a}	16.5^{a}	17.0^{a}	16.6^{a}	0.33	0.408
	Liver	20.8^{b}	20.5^{b}	20.3^{b}	22.7^{c}	0.93	0.060
	Muscle	16.7^{a}	16.7^{a}	17.3^{a}	16.9^{ab}	0.72	0.914
	Adipose	17.6^{a}	18.5^{ab}	19.1^{ab}	20.1^{bc}	1.33	0.362
	SEM[3]	1.11	0.75	0.93	1.33		
	P-value	< 0.001	< 0.001	0.026	< 0.001		

[1]Fatty acids not occurring in proportions of > 0.2 g/100 g total fatty acids in any of the tissues are not displayed.
[2]Interaction Tissue × Dietary treatment: **: $P < 0.01$; *: $P < 0.05$; †: $P < 0.10$.
[3]Maximal SEM.
[a,b,c,d]Within a column, means without a common superscript differ at $P < 0.05$.
[y,z]Within a row, means without a common superscript differ at $P < 0.05$.

Table 4. Effect of diet and tissue on proportions of selected MUFA (g/100 g analysed FA)[1].

		Dietary treatment					
Fatty acid[2]	Tissue	Control	Grape	Acacia	Grape + acacia	SEM[3]	P-value
C16:1*n*7	Uterus	0.28[a]	0.32[a]	0.30[a]	0.29[a]	0.041	0.523
**	Oviduct	0.26[a,z]	0.24[a,yz]	0.21[a,y]	0.24[a,yz]	0.013	0.014
	Liver	0.65[b]	0.62[b]	0.63[b]	0.58[b]	0.039	0.202
	Muscle	0.48[ab,z]	0.46[ab,z]	0.35[a,y]	0.40[ab,yz]	0.043	0.043
	Adipose	1.41[c,yz]	1.57[c,z]	1.06[c,y]	1.34[c,yz]	0.140	0.047
	SEM[3]	0.039	0.140	0.091	0.119		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C16:1*n*9	Uterus	0.58[a]	0.65[a]	0.64[a]	0.60[a]	0.058	0.396
	Oviduct	0.49[a]	0.53[a]	0.44[a]	0.47[a]	0.044	0.206
	Liver	1.23[b]	1.28[b]	1.29[b]	1.06[b]	0.085	0.124
	Muscle	1.61[c]	1.57[c]	1.46[b]	1.56[c]	0.102	0.640
	Adipose	1.45[bc,z]	1.38[bc,yz]	1.39[b,yz]	1.24[b,y]	0.045	0.011
	SEM[3]	0.085	0.075	0.102	0.078		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C17:1	Uterus	0.20[a]	0.20[ab]	0.21[a]	0.16[a]	0.022	0.265
†	Oviduct	0.19[a]	0.18[a]	0.23[ab]	0.18[a]	0.022	0.124
	Liver	0.43[a,z]	0.43[c,z]	0.43[c,z]	0.33[a,y]	0.037	0.042
	Muscle	0.43[a]	0.40[bc]	0.37[bc]	0.37[a]	0.029	0.157
	Adipose	1.21[b]	1.16[d]	0.84[d]	1.00[b]	0.166	0.175
	SEM[3]	0.166	0.104	0.074	0.127		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:1*n*9	Uterus	21.5[a]	20.6[a]	21.9[a]	21.0[a]	0.79	0.377
	Oviduct	21.2[a]	20.2[a]	20.4[a]	20.3[a]	0.64	0.566
	Liver	19.6[a]	20.0[a]	20.9[a]	18.7[a]	0.80	0.133
	Muscle	39.8[b]	39.7[c]	40.1[c]	40.5[c]	1.16	0.886
	Adipose	37.4[b]	36.2[b]	36.6 [b]	36.5[b]	1.19	0.768
	SEM[3]	0.80	0.85	0.66	1.19		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:1*cis*11	Uterus	2.54[a]	2.61[a]	2.69[c]	2.46[c]	0.200	0.544
	Oviduct	4.10[c]	4.35[d]	4.34[d]	4.12[d]	0.283	0.663
	Liver	1.34[b]	1.39[c]	1.43[b]	1.15[b]	0.155	0.388
	Muscle	0.96[b]	1.01[bc]	1.05[ab]	0.82[ab]	0.109	0.106
	Adipose	0.74[b]	0.77[b]	0.77[a]	0.67[a]	0.039	0.060
	SEM[3]	0.283	0.155	0.200	0.133		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:1*cis*12	Uterus	0.86[ab]	0.81	0.52[ab]	0.66	0.206	0.455
	Oviduct	0.57[a]	0.48	0.51[ab]	0.52	0.068	0.560
	Liver	1.37[b]	1.11	0.76[b]	0.94	0.370	0.324
	Muscle	0.75[a,yz]	1.40[z]	0.36[a,y]	1.01[yz]	0.315	0.023
	Adipose	0.49[a]	0.99	0.56[ab]	0.63	0.415	0.407
	SEM[3]	0.370	0.415	0.092	0.253		
	P-value	0.043	0.209	0.008	0.256		
C18:1*trans*10	Uterus	0.30[a]	0.41[a]	0.40	0.16[a]	0.217	0.600
	Oviduct	0.30[a]	0.32[a]	0.40	0.15[a]	0.154	0.595
	Liver	1.12[ab]	1.46[b]	1.63	0.54[b]	0.708	0.485
	Muscle	0.69[ab]	0.79[ab]	0.80	0.46[b]	0.332	0.771
	Adipose	1.43[b]	1.71[b]	1.90	0.92[c]	0.670	0.513
	SEM[3]	0.523	0.708	0.670	0.052		
	P-value	0.045	0.036	0.065	< 0.001		
C18:1*trans*11	Uterus	0.49[ab]	0.49[a]	0.42[a]	0.51[a]	0.064	0.638
	Oviduct	0.47[a]	0.44[a]	0.43[a]	0.44[a]	0.043	0.803
	Liver	1.41[bc]	1.44[c]	1.29[c]	1.50[c]	0.162	0.635
	Muscle	0.95[b]	0.88[b]	0.84[b]	0.92[b]	0.082	0.729
	Adipose	1.72[c]	1.89[d]	1.74[d]	1.97[d]	0.156	0.461
	SEM[3]	0.162	0.126	0.107	0.135		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		

[1]Fatty acids not occurring in proportions of > 0.2 g/100 g total fatty acids in any of the tissues are not displayed.
[2]Interaction Tissue × Dietary treatment: **: P < 0.01; †: P < 0.10.
[3]Maximal SEM.
[a,b,c,d]Within a column, means without a common superscript differ at P < 0.05.
[y,z]Within a row, means without a common superscript differ at P < 0.05.

Table 5. Effect of diet and tissue on proportions of selected PUFA (g/100g analysed FA)[1].

		Dietary treatment					
Fatty acid[2]	Tissue	Control	Grape	Acacia	Grape + acacia	SEM[3]	P-value
C18:2*n*6	Uterus	6.37^{c}	6.19^{b}	5.64^{b}	6.57^{b}	0.604	0.398
†	Oviduct	8.40^{d}	8.99^{c}	8.00^{c}	10.03^{c}	0.811	0.120
	Liver	10.09^{e}	10.28^{c}	10.30^{d}	11.19^{c}	0.675	0.199
	Muscle	4.07^{b,y}	4.47^{ab,yz}	5.19^{b,z}	4.24^{a,yz}	0.463	0.034
	Adipose	2.43^{a,y}	3.04^{a,z}	3.05^{a,z}	3.01^{a,yz}	0.203	0.024
	SEM[3]	0.327	0.780	0.649	0.811		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:2*cis*9,	Uterus	0.25^{a}	0.25^{ab}	0.24^{a}	0.21^{a}	0.064	0.917
*trans*11	Oviduct	0.22^{a}	0.23^{a}	0.25^{a}	0.26^{a}	0.028	0.486
	Liver	0.82^{c}	0.78^{d}	0.81^{c}	0.80^{c}	0.067	0.964
	Muscle	0.50^{b}	0.42^{bc}	0.41^{ab}	0.46^{b}	0.039	0.341
	Adipose	0.58^{b}	0.59^{c}	0.52^{b}	0.58^{b}	0.047	0.464
	SEM[3]	0.067	0.064	0.056	0.043		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:3*n*3	Uterus	0.16^{a}	0.20^{a}	0.19^{a}	0.12^{a}	0.070	0.563
	Oviduct	0.18^{a}	0.17^{a}	0.24^{a}	0.18^{a}	0.026	0.166
	Liver	1.06^{d}	1.06^{c}	1.03^{c}	1.03^{c}	0.068	0.955
	Muscle	0.71^{c}	0.64^{b}	0.63^{b}	0.60^{b}	0.047	0.242
	Adipose	0.49^{b}	0.53^{b}	0.51^{b}	0.52^{b}	0.040	0.746
	SEM[3]	0.068	0.072	0.070	0.061		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C20:4*n*6	Uterus	13.95^{d}	14.52^{d}	14.15^{d}	15.33^{d}	0.709	0.275
†	Oviduct	11.25^{c}	11.27^{c}	11.84^{c}	10.97^{c}	0.483	0.325
	Liver	7.68^{b,y}	8.14^{b,yz}	7.82^{b,y}	9.06^{b,z}	0.402	0.017
	Muscle	0.99^{a,y}	1.17^{a,yz}	1.35^{a,z}	0.97^{a,y}	0.139	0.017
	Adipose	0.12^{a}	0.14^{a}	0.13^{a}	0.12^{a}	0.012	0.140
	SEM[3]	0.420	0.624	0.709	0.402		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C20:5*n*3	Uterus	0.21^{b}	0.20^{b}	0.18^{ab}	0.18^{b}	0.020	0.490
**	Oviduct	0.17^{ab}	0.14^{ab}	0.14^{ab}	0.14^{b}	0.016	0.137
	Liver	1.19^{c,z}	1.00^{c,yz}	0.91^{c,y}	1.02^{c,yz}	0.078	0.042
	Muscle	0.25^{b}	0.28^{b}	0.28^{b}	0.23^{b}	0.030	0.210
	Adipose	0.02^{a}	0.02^{a}	0.02^{a}	0.02^{a}	0.003	0.691
	SEM[3]	0.073	0.074	0.078	0.041		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C22:4*n*6	Uterus	2.61^{c}	2.91^{c}	2.93^{c}	2.96^{c}	0.158	0.160
	Oviduct	2.71^{c}	3.03^{c}	3.23^{c}	2.91^{c}	0.324	0.486
	Liver	0.96^{b}	1.27^{b}	1.30^{b}	1.30^{b}	0.110	0.062
	Muscle	0.06^{a}	0.07^{a}	0.07^{a}	0.05^{a}	0.018	0.513
	Adipose	0.03^{a}	0.03^{a}	0.04^{a}	0.03^{a}	0.007	0.271
	SEM[3]	0.261	0.086	0.283	0.324		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C22:5*n*3	Uterus	1.73^{b}	1.77^{b}	1.71^{c}	1.77^{c}	0.069	0.671
	Oviduct	2.14^{b}	2.26^{b}	2.03^{c}	2.14^{c}	0.199	0.798
	Liver	3.41^{c}	3.27^{c}	3.10^{d}	3.45^{d}	0.234	0.438
	Muscle	0.37^{a}	0.42^{a}	0.44^{b}	0.33^{b}	0.051	0.092
	Adipose	0.09^{a}	0.10^{a}	0.10^{a}	0.09^{a}	0.007	0.554
	SEM[3]	0.234	0.199	0.141	0.171		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C22:6*n*3	Uterus	2.85^{b}	2.51^{b}	2.65^{b}	2.11^{b}	0.521	0.290
	Oviduct	2.48^{b}	2.10^{b}	2.35^{b}	1.78^{b}	0.220	0.062
	Liver	2.42^{b}	1.80^{b}	1.97^{b}	2.05^{b}	0.300	0.148
	Muscle	0.10^{a}	0.09^{a}	0.11^{a}	0.08^{a}	0.015	0.080
	Adipose	0.03^{a}	0.03^{a}	0.03^{a}	0.02^{a}	0.006	0.330
	SEM[3]	0.521	0.362	0.444	0.110		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		

[1]Fatty acids not occurring in proportions of > 0.3 g/100 g total fatty acids in any of the tissues are not displayed.
[2]Interaction Tissue × Dietary treatment: **: P < 0.01; †: P < 0.10.
[3]Maximal SEM.
[a,b,c,d,e]Within a column, means without a common superscript differ at P < 0.05.
[y,z]Within a row, means without a common superscript differ at P < 0.05.

Table 6. Effect of diet and tissue on proportions of groups of FA (g/100 g analysed FA).

		Dietary treatment					
Groups of FA	Tissue	Control	Grape	Acacia	Grape + acacia	SEM[1]	P-value
Σ saturated	Uterus	37.8[a]	38.1[a]	37.8[a]	38.5[a]	0.42	0.235
	Oviduct	37.2[a]	37.3[a]	36.4[a]	37.2[a]	0.45	0.239
	Liver	39.0[a]	39.1[a]	38.5[a]	39.7[a]	0.81	0.639
	Muscle	44.8[b]	43.7[b]	43.8[b]	44.5[b]	1.03	0.724
	Adipose	47.3[c]	46.5[b]	47.7[c]	48.2[c]	1.18	0.619
	SEM[1]	0.81	1.15	0.86	1.18		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ monounsaturated	Uterus	29.2[a]	28.5[a]	29.5[a]	28.0[a]	1.07	0.414
	Oviduct	29.9[a]	28.9[a]	29.5[a]	28.9[a]	0.72	0.665
	Liver	29.9[a]	30.2[a]	31.0[a]	27.3[a]	1.52	0.071
	Muscle	47.4[b]	48.0[b]	47.0[b]	47.9[b]	1.12	0.710
	Adipose	48.5[b]	48.6[b]	47.4[b]	46.9[b]	1.34	0.563
	SEM[1]	0.84	1.11	1.52	1.34		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ polyunsaturated	Uterus	32.2[c]	32.7[d]	32.0[c]	32.9[c]	1.06	0.761
	Oviduct	32.3[c]	33.2[d]	33.6[c]	33.4[c]	0.81	0.445
	Liver	30.1[c]	29.9[c]	29.8[c]	32.2[c]	0.90	0.089
	Muscle	7.6[b,y]	8.1[b,yz]	9.0[b,z]	7.4[b,y]	0.69	0.049
	Adipose	4.1[a]	4.8[a]	4.7[a]	4.6[a]	0.28	0.126
	SEM[1]	0.87	1.06	0.90	0.81		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ C18:1*cis*	Uterus	25.5[ab]	24.7[a]	25.7[a]	24.6[a]	1.09	0.574
	Oviduct	26.4[b]	25.5[a]	25.9[a]	25.6[a]	0.70	0.751
	Liver	23.2[a]	23.3[a]	23.9[a]	21.6[a]	0.86	0.114
	Muscle	42.1[c]	42.7[c]	42.0[c]	43.0[c]	1.15	0.785
	Adipose	39.5[c]	38.9[b]	38.7[b]	38.6[b]	1.22	0.885
	SEM[1]	0.79	1.09	0.86	1.22		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ C18:1*trans*	Uterus	1.32[a]	1.47[a]	1.39[a]	1.20[a]	0.280	0.764
	Oviduct	1.26[a]	1.20[a]	1.34[a]	1.06[a]	0.188	0.596
	Liver	3.82[bc]	4.14[b]	4.22[bc]	3.33[c]	0.780	0.717
	Muscle	2.29[ab]	2.34[a]	2.31[ab]	2.11[b]	0.388	0.949
	Adipose	4.37[c]	4.96[b]	4.86[c]	4.19[d]	0.791	
	SEM[1]	0.608	0.771	0.791	0.239		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ *n*3	Uterus	5.19[b]	4.90[b]	4.97[b]	4.37[c]	0.514	0.237
	Oviduct	5.37[b]	5.03[b]	5.15[b]	4.60[c]	0.284	0.113
	Liver	8.34[c]	7.40[c]	7.25[c]	7.77[d]	0.483	0.117
	Muscle	1.58[a]	1.67[a]	1.90[a]	1.55[b]	0.366	0.701
	Adipose	0.63[a]	0.68[a]	0.66[a]	0.66[a]	0.045	0.739
	SEM[1]	0.514	0.318	0.408	0.242		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ *n*6	Uterus	26.62[d]	27.40[c]	26.64[d]	28.25[d]	1.187	0.413
	Oviduct	26.69[d]	27.97[c]	28.17[d]	28.58[d]	0.738	0.096
	Liver	20.79[c,y]	21.57[b,yz]	21.52[c,yz]	23.56[c,z]	0.864	0.023
	Muscle	5.37[b,y]	5.87[a,yz]	6.62[b,z]	5.33[b,y]	0.464	0.040
	Adipose	2.71[a,y]	3.37[a,z]	3.39[a,z]	3.28[a,z]	0.216	0.032
	SEM[1]	0.494	1.187	0.864	0.738		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
*n*6/*n*3 ratio	Uterus	5.39[c]	5.75[c]	5.54[b]	6.50[c]	0.540	0.166
	Oviduct	5.01[c,y]	5.60[c.yz]	5.54[b,yz]	6.23[c,z]	0.330	0.016
	Liver	2.52[a,y]	2.95[a,yz]	3.00[a,yz]	3.03[a,z]	0.100	0.027
	Muscle	3.50[ab]	3.79[ab]	4.00[ab]	3.70[a]	0.300	0.855
	Adipose	4.28[bc,y]	4.95[bc,z]	5.14[b,z]	4.99[b,z]	0.130	0.002
	SEM[1]	0.529	0.509	0.581	0.406		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		

[1]Maximal SEM.

[a,b,c,d] Within a column, means without a common superscript differ at P < 0.05.

[y,z] Within a row, means without a common superscript differ at P < 0.05.

The proportions of each of the eight selected PUFA exhibited tissues differences ($P < 0.001$) in all four dietary treatment groups (Table 5). The lipids of the uterus and oviduct were higher in C20:4*n*6, C22:4*n*6 and C22:6*n*3 than those of muscle and adipose tissue. The C18:3*n*3 was the only *n*3 FA were lower in proportion in uterus and oviduct tissue lipids compared to the muscle. The adipose tissue was low in proportions of all FA larger than C18, and therefore in all individual PUFA, compared to the uterus and oviduct. Lipids in the liver varied in PUFA proportions in relation to the other organs, resembling in some cases those of the reproductive organs, in others the muscle tissue. Diet effects ($P < 0.05$) only occurred in C18:2*n*6 (muscle and adipose tissue), C20:4*n*6 (liver and muscle tissue) and C20:5*n*3 (liver tissue). An interaction ($P < 0.01$) was found for the proportion of C20:5*n*3 and trends ($P < 0.10$) for C18:2*n*6 and C20:4*n*6.

Proportions of groups of FA and the *n*6/*n*3 ratio were always different ($P < 0.001$) among tissues in any of the dietary treatments (Table 6). Uterus, oviduct and liver tissue lipids were richest in PUFA at cost of SFA and MUFA, and contained more ($P < 0.001$) *n*6 than muscle and adipose tissue lipids. The liver tissue lipids contained most ($P < 0.001$) *n*3 FA proportion, muscle and adipose tissue least ($P < 0.001$), and values for uterus and oviduct tissue were intermediate. The *n*6/*n*3 ratio was lowest for the liver and muscle, followed by adipose tissue and the highest was in uterus and oviduct tissue ($P < 0.001$). Lipids of uterus, oviduct and liver tissue were lower ($P < 0.001$) in C18:1*cis* than those of muscle and adipose tissue. The C18:1*trans* was highest in liver and adipose while it was lowest in the uterus and oviduct tissue lipids ($P < 0.001$). Diet effects ($P < 0.05$) were rare and restricted to proportions of PUFA (muscle tissue), *n*6 FA (liver, muscle and adipose tissue) and *n*6/*n*3 ratio (oviduct, liver and adipose tissue). There were no interactions between diet and tissue for any group of FA.

Fatty acid profile of the fluids

All six SFA selected, as well as total SFA were different ($P < 0.05$) in proportions between plasma, UF and OF (Table 7). Exceptions were C22:0 in the treatment groups control and grape ($P < 0.10$) and C24:0 in control group ($P = 0.05$). Blood plasma lipids were richest (mostly $P < 0.001$) in C16:0, C17:0 and C18:0, compared to OF (not C16:0 in control) and UF. This order was reversed for C20:0 ($P < 0.05$), where plasma contained the lowest proportions, but without a significant difference in control and grape + acacia treatments between plasma and OF. The proportion of C22:0 in the fluid of the acacia and grape + acacia groups was most prominent in UF and differed ($P < 0.001$) to plasma and OF. The same was valid with the proportion of C24:0 in the grape + acacia group, whereas plasma contained the lowest proportion compared to UF and OF in the grape group. In the acacia treatment group, all three fluids contained different ($P < 0.001$) proportions of C24:0. These FA, however, did not contributed a lot to total SFA. There

were diet effects ($P < 0.05$) in plasma on some SFA (C17:0, C20:0 C22:0 and C24:0) and, in addition on C18:0 in UF. Only for C24:0 there was a trend ($P < 0.10$) for an interaction of fluid and diet.

The proportions of the two selected, same as total MUFA differed between fluids and plasma values were much lower ($P < 0.05$) than those of OF and UF. The only exception was C24:1*n*9 that showed no significant difference between fluids in the grape + acacia group. There was no diet effect and no interaction in MUFA proportions. The method applied for the fluids did not allow differentiating between *cis* and *trans* isomers of MUFA.

Fluid differences (often $P < 0.001$) were found in proportions of all individual, sums of *n*3 and *n*6 FA, as well as total PUFA (Table 8). Most PUFA did not occur at proportions > 0.2 g/100 g in one of the fluids. In these cases, only the two remaining matrices were compared. In general, the lipids of either the OF or UF or both were richer ($P < 0.05$) in PUFA than those of the plasma. An exception was the proportion of C22:6*n*3, which was the highest ($P < 0.001$) in plasma. Proportions of PUFA often also differed between the OF and UF, but these differences were not systematic. In total PUFA and *n*6 FA proportions, the UF was higher ($P \leq 0.001$) than the other two fluids, and the *n*6/*n*3 ratio was highest accordingly. Most *n*3 FA were found in plasma and UF lipids, whereas the OF lipids contained only a few *n*3 FA ($P \leq 0.001$). Diet effects ($P < 0.05$) were found for C20:3*n*6 (OF) and C22:6*n*3 (UF), whereas in the first mentioned FA also an interaction ($P < 0.01$) occurred.

The total FA contents of the plasma (ng/µl) were highest ($P < 0.05$) with acacia group (781 ± 79.1), followed by grape + acacia (695 ± 39.4) and grape (603 ± 22.5; $P < 0.05$ compared to acacia), and were much lower ($P < 0.05$ to grape, acacia, grape + acacia) in control group (427 ± 33.6). The total FA contents of the OF and UF were not different between treatments.

Gene expression

The relative expression of 24 genes was analysed in endometrium, oviduct, liver and muscle tissue, and differential expression between tissues were observed for most of the genes (Table 9 and Supplementary Table S3). An exception were GSR and SREBP-1c for all tissues, same as PTGS2 and PGC-1α, which showed no significant difference between tissues for acacia and grape + acacia (PTGS2), and grape (PGC-1α) group. The FAS showed a differential expression between the reproductive tissues as compared to the muscle and liver tissue, whereas for PRKAA1 and ACACA, the expression in the reproductive tissues and liver was different as compared to muscle tissue. The gene expression was not affected by diet, with the exception of FABP4 that was less expressed ($P < 0.05$) in the control compared to grape group. Similarly, there were no interactions between the tissue and diet.

Table 7. Effect of diet and fluid on proportions of selected SFA and MUFA (g/100 g analysed FA)[1].

		Dietary treatment					
Fatty acid[2]	Fluid[3]	Control	Grape	Acacia	Grape + acacia	SEM[4]	P-value
C16:0	Plasma	20.4^{b}	21.6^{b}	22.6^{b}	22.2^{b}	1.14	0.243
	Uterine	11.9^{a}	11.1^{a}	11.8^{a}	9.1^{a}	4.23	0.584
	Oviduct	16.0^{ab}	11.7^{a}	14.3^{a}	11.1^{a}	1.56	0.108
	SEM[4]	1.36	1.57	1.50	1.78		
	P-value	0.016	< 0.001	< 0.001	< 0.001		
C17:0	Plasma	1.42^{b,z}	1.20^{b,y}	1.14^{b,y}	1.14^{c,y}	0.105	0.042
	Uterine	0.46^{a}	0.43^{a}	0.52^{a}	0.43^{a}	0.131	0.585
	Oviduct	0.72^{a}	0.53^{a}	0.58^{a}	0.58^{b}	0.205	0.187
	SEM[4]	0.154	0.108	0.094	0.098		
	P-value	0.002	< 0.001	< 0.001	< 0.001		
C18:0	Plasma	52.0^{b}	50.3^{b}	52.2^{b}	53.1^{b}	1.72	0.615
	Uterine	20.1^{a,y}	21.6^{a,y}	21.3^{a,y}	25.5^{a,z}	1.84	0.025
	Oviduct	23.2^{a}	22.8^{a}	23.0^{a}	25.5^{a}	4.28	0.828
	SEM[4]	5.08	4.02	4.54	4.00		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C20:0	Plasma	0.18^{a,z}	0.11^{a,y}	0.12^{a,y}	0.12^{a,y}	0.008	< 0.001
	Uterine	0.43^{b}	0.34^{b}	0.32^{b}	0.44^{b}	0.135	0.527
	Oviduct	0.29^{ab}	0.35^{b}	0.28^{b}	0.36^{ab}	0.093	0.641
	SEM[4]	0.040	0.038	0.028	0.054		
	P-value	0.011	0.001	< 0.001	0.008		
C22:0	Plasma	0.41^{z}	0.31^{yz}	0.27^{a,y}	0.29^{a,y}	0.037	0.003
	Uterine	1.31	1.35	1.12^{b}	2.12^{b}	0.789	0.436
	Oviduct	0.52	0.81	0.46^{a}	0.90^{a}	0.341	0.517
	SEM[4]	0.170	0.191	0.111	0.267		
	P-value	0.107	0.065	< 0.001	0.003		
C24:0	Plasma	0.46^{z}	0.30^{a,y}	0.28^{a,y}	0.36^{a,yz}	0.027	0.001
†	Uterine	2.41	1.97^{b}	1.97^{c}	3.49^{b}	1.428	0.216
	Oviduct	1.01	1.55^{b}	0.89^{b}	1.40^{a}	0.425	0.447
	SEM[4]	0.333	0.252	0.210	0.424		
	P-value	0.052	0.002	< 0.001	< 0.001		
Σ saturated	Plasma	77.7^{b}	76.1^{b}	78.7^{b}	79.5^{b}	1.96	0.501
	Uterine	36.6^{a}	36.7^{a}	37.1^{a}	41.0^{a}	1.82	0.177
	Oviduct	41.7^{a}	37.7^{a}	39.5^{a}	39.6^{a}	4.00	0.815
	SEM[4]	6.41	5.52	6.06	5.63		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C18:1*n*9	Plasma	1.61^{a}	4.41^{a}	2.85^{a}	2.00^{a}	1.78	0.315
	Uterine	30.0^{b}	30.5^{b}	30.7^{b}	25.5^{b}	2.73	0.162
	Oviduct	41.5^{c}	40.6^{c}	41.0^{c}	39.4^{c}	3.57	0.937
	SEM[4]	5.73	4.62	5.20	4.68		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C24:1*n*9	Plasma	0.29^{a}	0.24^{a}	0.24^{a}	0.26	0.097	0.845
	Uterine	1.03^{b}	0.84^{ab}	0.74^{b}	1.47	0.372	0.312
	Oviduct	1.25^{b}	2.06^{b}	0.91^{b}	1.44	0.629	0.450
	SEM[4]	0.156	0.287	0.096	0.295		
	P-value	0.005	0.012	< 0.001	0.208		
Σ mono-unsaturated	Plasma	1.91^{a}	4.65^{a}	3.09^{a}	2.16^{a}	1.76	0.308
	Uterine	31.0^{b}	31.3^{b}	31.5^{b}	27.0^{b}	2.61	0.217
	Oviduct	42.8^{c}	42.6^{c}	41.9^{c}	40.8^{c}	3.11	0.914
	SEM[4]	5.87	4.81	5.30	4.82		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		

[1]Fatty acids not occurring in proportions of > 0.2 g/100 g total fatty acids in any of the fluids or were recovered in one fluid type only are not displayed.
[2]Interaction Fluid × Dietary treatment: †: P < 0.10.
[3]Plasma (n = 6 for control; n = 5 for grape, acacia, grape + acacia); uterine (n = 2 for control; n = 3 for acacia; n = 4 for grape, grape + acacia); oviduct (n = 2 for control; n = 4 for grape, acacia, grape + acacia).
[4]Maximal SEM.
[a,b,c] Within a column, means without a common superscript differ at P < 0.05.
[y,z] Within a row, means without a common superscript differ at P < 0.05.

Table 8. Effect of diet and fluid on proportions of selected PUFA (g/100 g analysed FA)[1].

		Dietary treatment					
Fatty acid[2]	Fluid[3]	Control	Grape	Acacia	Grape + acacia	SEM[4]	P-value
C18:2*n6*,*cis*	Plasma	1.26[a]	3.13	1.68[a]	1.36[a]	1.289	0.302
	Uterine	6.34[b]	6.98	7.69[ab]	6.59[b]	2.728	0.967
	Oviduct	7.05[b]	7.33	8.01[b]	7.80[b]	2.085	0.945
	SEM[4]	0.959	0.955	1.183	0.920		
	P-value	0.002	0.114	0.013	< 0.001		
C18:2*n6*,*trans*	Uterine	15.5	15.2[b]	15.8[b]	15.1[b]	1.314	0.930
	Oviduct	2.05	1.87[a]	1.50[a]	1.62[a]	1.418	0.909
	SEM[4]	3.949	2.560	2.914	2.560		
	P-value	-	< 0.001	< 0.001	< 0.001		
C20:2*n6*	Plasma	0.16	0.10[a]	0.11[a]	0.12[a]	0.021	0.181
	Oviduct	0.80	0.78[b]	1.02[b]	0.68[b]	0.434	0.162
	SEM[4]	0.177	0.146	0.193	0.109		
	P-value	0.081	0.001	0.004	0.001		
C20:3*n6*	Plasma	0.06[a]	0.06[a]	0.04[a]	0.03[a]	0.021	0.354
**	Oviduct	0.74[b,yz]	1.17[b,z]	1.04[b,z]	0.39[b,y]	0.124	0.012
	SEM[4]	0.114	0.235	0.177	0.060		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
C20:4*n6*	Plasma	0.24[a]	0.58[a]	0.30[a]	0.43[a]	0.182	0.506
	Oviduct	3.97[b]	7.74[b]	5.80[b]	7.82[b]	1.574	0.139
	SEM[4]	0.946	1.302	0.981	1.716		
	P-value	0.023	< 0.001	< 0.001	0.011		
C20:5*n3*	Plasma	0.06[a]	0.07[a]	0.04[a]	0.05[a]	0.025	0.606
	Uterine	6.14[b]	6.81[b]	6.13[b]	7.24[b]	1.927	0.869
	SEM[4]	1.583	1.554	1.245	1.316		
	P-value	0.017	0.013	< 0.001	< 0.001		
C22:6*n3*	Plasma	13.4[b]	10.8[b]	11.5[b]	11.4[b]	1.053	0.190
	Uterine	4.46[a,z]	2.96[a,yz]	1.80[a,y]	3.05[a,yz]	1.174	0.034
	Oviduct	0.93[a]	1.53[a]	1.21[a]	1.78[a]	0.741	0.708
	SEM[4]	1.852	1.275	1.544	1.319		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
Σ polyunsaturated	Plasma	20.4[a]	19.3[a]	18.2[a]	18.3[a]	1.25	0.425
	Uterine	32.4[b]	32.0[b]	31.4[b]	31.9[b]	3.41	0.996
	Oviduct	15.5[a]	19.6[a]	18.6[a]	19.6[a]	1.97	0.393
	SEM[4]	2.03	1.94	1.84	1.93		
	P-value	0.001	0.001	< 0.001	< 0.001		
Σ *n3*	Plasma	13.5[b]	10.9[b]	11.5[c]	11.5[b]	1.056	0.193
	Uterine	10.6[b]	9.76[b]	7.92[b]	10.3[b]	2.872	0.557
	Oviduct	0.93[a]	1.53[a]	1.21[a]	1.78[a]	0.741	0.708
	SEM[4]	1.729	1.375	1.408	1.309		
	P-value	0.001	0.001	< 0.001	< 0.001		
Σ *n6*	Plasma	6.90[a]	8.37[a]	6.74[a]	6.85[a]	1.085	0.399
	Uterine	21.8[c]	22.2[b]	23.5[c]	21.7[b]	2.056	0.812
	Oviduct	14.6[b]	18.1[b]	17.4[b]	17.9[b]	1.471	0.466
	SEM[4]	2.030	1.835	2.183	1.944		
	P-value	< 0.001	< 0.001	< 0.001	< 0.001		
n6/*n3* ratio	Plasma	0.51[a]	0.83[a]	0.61[a]	0.61[a]	0.203	0.254
	Uterine	2.25[b]	2.62[a]	3.01[a]	2.17[a]	0.711	0.095
	Oviduct	15.6[c]	13.9[b]	14.8[b]	18.0[b]	8.308	0.942
	SEM[4]	1.976	1.828	2.001	3.208		
	P-value	< 0.001	< 0.001	< 0.001	0.037		

[1]Fatty acids not occurring in proportions of > 0.2 g/100 g total fatty acids in any of the tissues or were recovered in one fluid type only are not displayed.
[2]Interaction Fluid × Dietary treatment: **: P < 0.01.
[3]Plasma (n = 6 for control; n = 5 for grape, acacia, grape + acacia); uterine (n = 2 for control; n = 3 for acacia; n = 4 for grape, grape + acacia); oviduct (n = 2 for control; n = 4 for grape, acacia, grape + acacia).
[4]Maximal SEM.
[a,b,c] Within a column, means without a common superscript differ at P < 0.05.
[y,z] Within a row, means without a common superscript differ at P < 0.05.

Table 9. Relative expression of selected genes between experimental groups as compared to non-supplemented control group (fold change).

Gene name	Tissue	Dietary treatment			P-value
		Grape n = 6	Acacia n = 5	Grape + acacia n = 5	
ACACA	Endometrium	0.90	0.86	0.75	0.806
	Oviduct	0.91	0.89	1.16	0.346
	Liver	1.12	0.96	1.02	0.927
	Muscle	1.33	0.81	0.93	0.367
CAT	Endometrium	1.08	1.35	1.25	0.954
	Oviduct	1.44	1.97	0.99	0.321
	Liver	1.17	2.36	1.27	0.965
	Muscle	1.12	1.31	1.11	0.498
FABP4	Endometrium	0.54	0.63	1.15	0.297
	Oviduct	0.43*	0.60	0.63	< 0.05
	Liver	1.66	0.80	1.27	0.598
	Muscle	1.31	0.80	2.25	0.141
FAS	Endometrium	0.73	0.75	0.76	0.735
	Oviduct	1.06	1.07	1.03	0.989
	Liver	1.19	1.02	0.85	0.833
	Muscle	1.08	0.92	0.84	0.844
GPX1	Endometrium	1.11	0.77	1.02	0.316
	Oviduct	1.03	0.96	1.09	0.909
	Liver	1.05	0.83	0.95	0.809
	Muscle	1.20	0.87	1.03	0.500
GSR	Endometrium	1.53	1.97	1.02	0.506
	Oviduct	0.93	1.31	0.60	0.424
	Liver	1.07	1.48	0.73	0.246
	Muscle	0.90	2.26	0.86	0.209
HMGCR	Endometrium	0.97	0.91	0.92	0.963
	Oviduct	1.03	0.90	1.20	0.798
	Liver	1.19	1.10	1.04	0.887
	Muscle	1.18	0.92	0.93	0.442
LPL	Endometrium	0.56	0.91	0.66	0.777
	Oviduct	0.78	0.74	0.94	0.290
	Liver	1.28	1.12	0.77	0.485
	Muscle	1.39	0.70	1.00	0.161
PPARγ	Endometrium	1.57	1.17	2.25	0.131
	Oviduct	0.81	0.96	1.27	0.681
	Liver	1.28	1.22	0.88	0.649
	Muscle	1.47	1.05	1.04	0.457
PGC-1α	Endometrium	0.59	1.37	2.52	0.338
	Oviduct	0.50	0.83	1.15	0.328
	Liver	1.32	0.48	0.70	0.283
	Muscle	0.53	0.36	0.41	0.299
PRKAA1	Endometrium	1.01	1.00	1.00	1.000
	Oviduct	1.01	0.97	1.09	0.794
	Liver	1.16	0.83	0.95	0.264
	Muscle	0.95	0.85	0.78	0.639
SCD	Endometrium	0.75	0.58	0.65	0.697
	Oviduct	1.03	0.91	1.13	0.882
	Liver	1.01	0.85	0.81	0.829
	Muscle	0.99	0.74	0.90	0.558
SOD1	Endometrium	1.01	1.04	1.33	0.947
	Oviduct	0.84	1.29	0.96	0.534
	Liver	0.70	1.24	0.84	0.838
	Muscle	0.82	1.73	0.99	0.512
SREBP-1c	Endometrium	0.79	0.87	0.92	0.953
	Oviduct	0.96	1.26	1.16	0.821
	Liver	1.46	1.70	1.32	0.233
	Muscle	0.67	0.66	0.49	0.329

* Within a row, means differ at P < 0.05 from control group.

Discussion

Nutrient management practices present popular means to improve the productivity and health of livestock and subsequently the quality of the foods derived from livestock production systems. The effects of feed supplementation with phenolic compounds on meat and milk characteristics are intensively researched and described in literature [203, 206], but the effects on reproductive tissues and fluids and by that reproduction remain scarcely investigated and therefore need to be elucidated.

Characteristics of the fatty acid profile of reproductive organs and fluids

The endometrial content of FA (g/100g of FA) found in cows [53] is in agreement with our results in sheep for major SFA (i.e. C14:0, C15:0, C16:0 (PA; palmitic acid), C17:0 and C18:0 (SA; stearic acid)). Meier et al. [219] reported comparable SFA concentrations (g/100g of FA) and no differences between endometrial FA profile of gravid and non-gravid uterine horns in cows. In our study, we sampled whole uterus tissue that included peri-, myo- and endometrium including caruncles for analysis of the FA proportions. In cultured human myometrial smooth muscle cells, the addition of docosahexaenoic acid (DHA) and arachidonic acid (AA) to the culture medium resulted in enrichment of long-chain *n*3 PUFA in the membrane lipids [220]. In the caruncular endometrium of mature cows, Burns et al. [160] reported a lower percentage of total FA content for PA, C18:1*n*9 (OA; oleic acid), DHA and total *n*3, likewise a higher percentage for C18:2*n*6 (LA; linoleic acid), C20:5*n*3 (EPA; eicosapentaenoic acid) and C18:3*n*3 (ALA; α-linolenic acid), and finally similar proportions for SA and AA, as compared to our results. Alike, the proportions (mole/100 mole of FA) of AA in feline oviduct and uterus tissue resembled our results, but not those of LA which were two-fold higher in felines [221]. The high proportion of AA in the reproductive tissues as compared to proportions in the liver and muscle tissue, as found in the present experiment, is attributed to the function of the endometrium as a pool for this particular FA, which is necessary for oocyte maturation and development [222]. The AA is a precursor for prostaglandins (PG) of the series 2 (i.e. PGE_2 and $PGF_{2\alpha}$) that are as well involved in embryo-maternal communication and therefore play in important role during the estrous cycle and pregnancy. We also confirmed high proportions of C22:4*n*6 (adrenic acid) and C22:5*n*3 (DPA; docosapentaenoic acid) in the uterus, both precursors of DHA. Adrenic acid was further suggested as a novel anti-inflammatory player in human cartilage [223] and might have similar effects in the uterus. Elevated proportions of DHA were reported to inhibit the synthesis of AA from LA, by that contributing to a reduction in $PGF_{2\alpha}$ production and thereby expressing distinct anti-inflammatory and immunosuppressive effects important in early pregnancy [161]. In addition, our results closely reflected the DHA, *n*3 and *n*6 FA proportions in uterus compared to endometrial biopsies in horses [46]. In the endometrium of sows, the proportions of AA, EPA and DPA were comparable to those found in our study in the

uterus and the oviduct of ewes [224]. Further, LA and DHA were present in higher and lower proportions in the endometrium of sows, respectively, even though the sow diets contained 3.75 % ground flaxseed. This suggests that the rate of biosynthesis of DHA from EPA in the endometrium could be species-specific and elevated in sheep.

The proportions of SFA in plasma were considerably lower in the control group of sheep in the study of Buccioni et al. [225] as compared to our study (40 vs 77 g/100 g FA; reference vs our experiment), caused by extremely high SA proportions (50 g/100 g FA) in our study. Further, MUFA were higher (19 vs 2 g/100 g FA), whereas the proportions of PUFA resembled our results (27 vs 20 g/100 g FA). Similar, Tsiplakou and Zervas [226] reported values of 25 g/100 g FA for SA in sheep plasma. This study showed extremely low plasma proportions of DHA, as compared to our results (0.8 vs 13 g/100 g FA), and extremely high plasma proportions in the case of OA and LA (13 vs 1.6 g/100 g FA; 30 vs 1.2 g/100 g FA, respectively). This is very intriguing and sampling and analytic methods could also have contributed to such contradictory findings.

The proportions of PA and SA were higher in the plasma as compared to the OF and UF, where SA accounted for the second highest proportion of total FA after OA. The third most prevalent FA in OF and UF were PA and C18:2*n*6,*trans*, respectively, followed by LA and PA, respectively, as fourth most prominent FA. The OA was also reported to be the most prevalent FA in reproductive fluids of cow and pig [141]. Overall, the proportions of OA, LA, AA and EPA, were higher in the lipids of the OF and UF, as compared to plasma, reflecting their role as substrates for reproductive processes. Contrarily, SA and LA were reported to be the most prevalent FA in the OF and UF of mares, respectively, both independent of the cycle stage [24]. This suggests species-specific FA requirements of the growing embryo at least during preimplantation development. This might be due to differences in embryo morphology such as that the early horse embryo is encapsulated in contrast to the early embryos of pig, cow and sheep [141]. In our study, the OA proportion was ten-fold higher in OF as compared to plasma. Fayezi et al. [25] suggested that in mammals OA can contribute to normal oocyte and preimplantation embryo development via mechanisms involving metabolic partitioning of FA, change in the membrane structural organization, attenuation of oxidative stress and regulation of intracellular signalling. Oleic acid may prevent detrimental effects of SFA, notably SA and PA, on the embryo developmental competences in bovines [227]. Moreover, OA was shown to improve the ovine oocyte development *in vitro*, and even compensating for the unfavourable effects of PA and SA on the oocyte development [228, 229]. Finally, in bovines, the cumulus cells can desaturate SA into OA, serving as a protective mechanism for the oocyte against the toxicity of SFA present in its environment [230]. The observed high OA proportions in our study correspond well to these findings. Finally, the present results illustrate that the SFA proportions in the OF and UF is not a reflection of the plasma SFA profile. This was also confirmed for the proportions of MUFA and *n*6

FA, which differed between all three matrices. On the other hand, proportions of PUFA in plasma and OF did not differ, whereas the UF contained considerably higher PUFA proportions. The proportions of *n*3 FA in plasma and UF did not differ (except with acacia alone), but were five- to ten-fold higher compared to the proportions observed in the OF. These findings suggested that using plasma for prediction of FA profiles in OF and UF is therefore likely not possible. The only exception based on our results could be made for PUFA and *n*3 FA, where plasma profile resembled the OF and UF FA profile, respectively.

Effect of supplementary dietary phenol sources on FA profile in organs and fluids

The FA profiles found in the liver, muscle and adipose tissue of the present experiment were overall in range of values previously reported in sheep [231-233]. However, we observed differences in proportions of SA and PUFA in muscle tissue, present in lower and higher proportions, respectively, compared to results obtained by Costa et al. [231]. This can be explained by the presence of phenolic compounds in the diets used in our experiment, protecting PUFA from oxidation and thereby saturation. Even though PA proportions in tissues are regulated by the hepatic *de novo* synthesis of PA [234], we observed a reducing effect of grape seed extract supplement on PA proportions in adipose tissue and an interaction between all the tissues and dietary treatments. In an *in vitro* study using tropical plant species, phenols as constituents were suggested to modify the ruminal FA biohydrogenation towards lowering the disappearance of PUFA and thereby the formation of SA [76]. We observed an interaction of tissue and diet on C17:0 and C16:1*n*7 with a significant decrease in proportions of these FA in muscle, adipose (for C17:0) and oviduct (C16:1*n*7) for tissue with acacia alone compared to control, or also in combination with grape seed extract (for C17:0) for muscle and oviduct tissue. Odd chain FA like C17:0 are indicators of rumen microbial activity as these FA are synthesised in the rumen. Jenkins et al. [235] found no correlation between circulating C17:0 and its intake in rats, suggesting its biosynthesis *in vivo*. Interestingly, in our study the plasma proportions of C17:0 proportions are significantly lower in all phenols groups, compared to control. A lowering effect of the polyphenol supplementation was also noted for the plasma proportions of C20:0, C22:0 and C24:0. Generally, MUFA, PUFA and SFA plasma proportions in our study did not confirm the effect reported by Buccioni et al. [225], where tannin extracts were used in diets of grazing ewes.

Despite the already described effects of phenolic supplements on plasma FA proportions, the reproductive fluids remained widely unaffected with the only exception of higher SA proportions in the UF of the grape + acacia group compared to all other groups. Curiously, we found a significant increase with diet in the SA levels in the UF. This finding could reflect a potential adverse effect of feeding phenolic compounds because of the previously described SFA toxicity for the oocyte and

embryo. The higher SA proportion was not observed with grape or acacia only, suggesting that not the type of polyphenol *per se* but the level of polyphenols was responsible for the effect observed. The acacia treatment resulted in a significant decrease of DHA in the UF but not in the OF and plasma. Still, even if tissue levels for DHA did not show a significant diet effect, we observed a decreasing trend for DHA in the oviduct tissue (P = 0.06) with the combined phenolic dietary treatment. This effect of decreased DHA proportions with polyphenol supplementation was also observed in muscle tissue in goats [206]. The plasticity of reproductive tissues towards modifying their FA profiles according to dietary interventions was further confirmed in mares, with incorporation of DHA into endometrial tissue using DHA supplemented diets [46]. In addition, the endometrial levels in heifers were higher for EPA, reduced for AA, and remained constant for DHA, when animals were supplemented with rumen-protected fish oil [47]. Jacobs et al. [46] suggested that increased uterine DHA proportions may improve the uterine environment to support embryonic development. Vedin et al. [236] confirmed that EPA and DHA supplementation in humans reduces $PGF_{2\alpha}$ plasma levels to give rise to anti-inflammatory and neuroprotective lipid mediators. Taking into account the observed partially lower DHA levels in reproductive tissues and fluids, we could suggest a slightly adverse effect on reproductive processes of diet supplementation with high amounts of combined dietary phenolic compounds that were used in our study. However, Zarezadeh et al. [237] described contradictory effects of DHA on oocyte and embryo quality, largely relating this finding to differences in experimental designs and a lack of mechanistic insights regarding the impact of *n*3 PUFA on reproduction.

Examining the effect of diet on reproductive fluids further, the FA profile in the follicular fluid of high-yielding dairy cows fed supplements with high *n*6/*n*3 ratio demonstrated significantly reduced OA and MUFA proportions at cost of PUFA, but increased proportions of LA [44]. However, we did not collect follicular fluid in our study, so the effect of polyphenolic supplementation on follicular fluid FA profiles remains to be elucidated.

Tissue specific gene expression and effects of supplementary dietary phenol sources

We were able to confirm the expression of 24 target genes in the liver, muscle, endometrium and oviduct tissue, with the exception of LEP and GPX4 that were expressed only in the muscle, and EHHADH that was only expressed in both the oviduct and endometrium. For most of the genes that are involved in lipid metabolism and antioxidant defence analysed for their expression, we observed tissue specific expression levels. Interestingly, one of the genes that was not differently expressed between all tissues was SREBP-1c, being described as a key driver for the synthesis of anti-inflammatory FA, such as EPA and DHA [238]. Similarly, no difference between tissues was observed for the expression of GSR, a key antioxidant enzyme involved in the reduction of

glutathione disulphide to the antioxidant glutathione. This finding was unexpected, as an induction of GSR and antioxidant gene expression in general by polyphenol supplementation has repeatedly been shown in tissues of mice and rats [239]. Concerning the effect of polyphenols on antioxidant enzymes, Kerasioti et al. [240] showed no effect of dietary phenolic compounds on SOD activity in liver and spleen of sheep, confirming our results on SOD transcriptional level in all tissues.

In contrast to results described in mice and chicken [241-243], we did not observe any effect of the dietary treatment with phenolic compounds on the expression of genes related to lipid metabolism, although in our study we included the most frequently studied genes in relation to long-chain PUFA metabolism in sheep: LPL, FAS, SCD, ACACA, PRKAA1, PPARγ, HMGCR and SREBP-1c. However, we did not observe any significant differences, not even a trend, between treatments. Daniel [244] and Bhuiyan et al. [245] suggested that in sheep tissues, the OA content depends on SCD mRNA levels. This is in line with our results, since no changes in tissue levels for OA were observed in our study. Similarly, diet (concentrate vs forage) was described to have no effect on ACACA mRNA levels in liver of lambs [246]. A recent study of Odhaib et al. [247] reported no change in expression of LPL, SREBP-1c, nor SCD in muscles of lambs fed with *Nigella sativa* and *Rosmarinus officinalis*, both rich in phytochemicals with antioxidant and anti-inflammatory properties. These observations are in accordance with our results, and can be related to MUFA content being dependent on dietary FA intake [247]. Based on our findings and results from Ponnampalam et al. [248], it is suggested that tissue mRNA expressions may be dependent on the type, duration and level of supplements used in the diet.

Fatty acids regulate gene transcription directly by binding as ligands and controlling the activity of the transcription factor, or indirectly by regulating different signalling pathways controlling the nuclear abundance of transcription factors [249]. Acute beneficial effects at transcription level carried out by phenolic compounds were shown in humans consuming high-phenol virgin olive oil, but if the effects were maintained after prolonged feeding was not investigated [250]. Still, Montero et al. [251] showed that the liver gene expression in obese mice was not significantly modified after one-year diet supplementation with polyphenols. Bearing in mind that the changes in expression of genes occur prior to changes at protein level, we suggest that this could have been the causative reason for the non-observed differences in our study. Thereby, it is suggested that the changes in expression may have happened before the slaughter. Lack of differences in TEP plasma concentration as seen in our study are in accordance with the study of Lepamarai et al. [213] where the phenols in blood were elevated to a certain extent by diet-supplemented grape seed extract.

Conclusion

Our results provide a comprehensive overview of the FA distribution in lipids of reproductive organs and fluids in sheep, and in addition suggest that diet supplementation with phenolic compounds seems to be a strategy with limited effect in modifying their FA profiles. Different from the FA profiles of reproductive organs, FA proportions of liver, muscle and adipose tissue show more plasticity and are affected to a certain degree by polyphenol-rich diets. Unexpectedly, despite uterine FA proportions being completely unaffected by the diet, FA proportions in the UF were even more affected by the diet than those of plasma, whereas the OF FA proportions remained largely unaffected. Such an outcome is partly explained by the use of phenolic compounds that are known to slow down the ruminal biohydrogenation of unsaturated FA and thereby modify the lipid metabolism in the organism. Furthermore, short-term gene expression changes of metabolic and antioxidant enzymes might be involved, even though we could not confirm this in our study, most likely due to the indifferent variations in plasma TEP concentrations at slaughter. The diet effects on FA proportions observed in the reproductive fluids, notably for SA and DHA, emphasize the importance of their presence in the histotroph for the embryo and mother during the periconceptional period. On the other hand, the possibility to partly modify the reproductive fluids by feeding phenolic compounds is encouraging in a way that the reproductive milieu can be influenced via maternal nutrition, which thus has to be optimized for an improved fertility. Still, the effects of maternal dietary polyphenols on reproduction need to be elucidated, especially taking into account the influence of biohydrogenation levels (e.g. EPA and DHA [3]), in small ruminant animals compared with cattle. However, with biohydrogenation not occuring in monogastric animals, such as the horse and the pig, or even mice and rats, a comparison between species for tissue FA profiles and diet effect must be done with caution. In addition to the FA profiles in organs and fluids, this is the first time to our knowledge that such a broad panel of genes was described in sheep, notably in reproductive tissues. Still, immunochemistry was not performed but would be necessary for localization and confirmation of the observed gene expression on the protein level. Apart from tissue differences, under the present experimental conditions we observed a stable gene expression in relation to diet supplementation with phenolic compounds. This was partly supported by the previous findings, leaving a possibility to attribute such results to the presented experimental setup and diets.

Supplementary Table S1. Chemical composition1 of the experimental diets (in g/kg dry matter (DM), except DM that is given as g/kg original substance.

Feeds[2]	DM	OM	CP	EE	NDF	ADF	ADL	TEP[3]	TT[3]	CT[4]
Hay	926	928	92	16.9	595	346	38.8	14.6	4.4	0.4
Concentrate										
Control	891	924	313	46.4	143	59	12.7	4.4	1.6	0.5
Grape	895	923	308	43.1	148	66	16.0	17.7	13.6	7.0
Acacia	892	922	302	43.5	150	65	13.5	14.1	11.3	3.5
Grape + acacia	888	925	301	44.4	157	63	16.6	31.5	26.0	11.1

ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; CT, condensed tannins; DM, dry matter; GE, gross energy; NDF, neutral detergent fiber; OM, organic matter; TP, total phenols; TT, total tannins.

[1]Analysed as described in [252].

[2]Control (high protein concentrate (HPC)), grape (HPC + grape seed extract), acacia (HPC + *Acacia mearnsii* extract), grape + acacia (HPC + *Acacia mearnsii* + grape seed extract).

[3]Given as tannic acid equivalents.

[4]Given as leucocyanidin equivalents.

Supplementary Table S2. Gene symbol, gene name, accession number, forward and reverse sequence, and amplicon size of primers used for gene expression analyses using the Fluidigm Platform (Organism: *Ovis aries*).

Gene symbol	Gene name	Accession number	Primer sequence (5'-3')		bp[2]
			Forward	Reverse	
ACACA	Acetyl-CoA carboxylase alpha	XM_015098292.1	ACCATGCTGGGAGTTGTCTGT[4]	AGAAGTGTATGAGCAGAGAGGACTTG[4]	118
ACO1	Aconitase 1	XM_015093260.1	TTAGCCTCTCCGCCCTTAGT	GGCATTGACACCTAGTGGCT	87
B2M[1]	Beta-2-Microglobulin	NM_001009284.2	CCTTGGTCCTTCTCGGGCTG[3]	TCTGGCGGGTGTCTTGAGTAT[3]	91
CAT	Catalase	XM_012096208.2	CTGGGACCCAACTATCTCCA	AAGTGGGTCCTGTGTTCCAG	179
EHHADH	Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase	XM_004003079.3	GTCCAGGCTGCTGTCCAATA	CCAACAGAGGAGATGGGTCG	194
FABP4	Fatty Acid Binding Protein 4	NM_001114667.1	TGGGATGGGAAATCAACCACC	CGTTCATGACACATTCCAGCA	73
FAS	Fatty acid synthase	XM_012109356.2	CTTAACAGCACGTCCCCCAT	TCCTCGGGCTTGTCTTGTTC	149
GPT	Glutamic--Pyruvic Transaminase	XM_015097626.1	TGCCGAGAGCTCGCAGTTCC	CGCACTCGCCCATGTAGCCC	122
GPX1	Glutathione peroxidase 1	XM_012122111.2	GTGCAACCAGTTTGGGCATC	GTCGGACGTACTTCAGGCAA	71
GPX4	Glutathione peroxidase 4	XM_012116182.2	TCCATTGGTCGGTTCGGC	CTAAAGCTCATCTCGGCGGT	70
GSR	Glutathione-disulfide reductase	XM_015104590.1	TGTCATTGTTGGTGCTGGTT	AGCGTTCTCCAGCTCTTCAG	154
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase	XM_012169928.1	TTCACGCTGGATGCTCTTGT	GTAGTTGGCGAGAACCGACA	114
LEP	Leptin	XM_012131508.2	GGGGCTTGCTAACCATCCAT	AGACCGTTCACACCAAGGAC	178
LIPC	Lipase C, Hepatic Type	XM_012181461.2	AAACTGGGAACACGTCAGGG	GGAGAACCATTGCCTCAGGG	87
LIPE	Lipase E, Hormone Sensitive Type	NM_001128154.1	CTTCTTTGAGGGCGACGAGA	GTTGCGTTTGTAGTGCTCCC	175
LPL	Lipoprotein lipase	NM_001009394.1	CCGCCGACAGGATTACAAGA	CCAGGAATGAGGTGGCAAGT	106
PPARγ	Peroxisome proliferator activated receptor gamma	FJ200441.1	AGAGCAGAGCAAAGAGGTGG	GGTTCACAAAGCCGGGGATA	113
PGC-1α	PPARG Coactivator 1 Alpha	XM_015096413.1	ATGCTGTGTTGGGGGAGATG	TGGCTCGGAGTATCCTGGAA	84
PRKAA1	Protein kinase AMP-activated catalytic subunit alpha 1	XM_012156757.1	ACAGCCGAGAAGCAGAAACA	TTGCCAACCTTCACTTTGCC	101
PTGS2	Prostaglandin-Endoperoxide Synthase 2	NM_001009432.1	GATCCCCAGGGCACAAATCT	GGTGAAAGCTGGTCCTCGTT	96
RPL19[1]	Ribosomal Protein L19	XM_004012836.2	AGCCTGTGACTGTCCATTCC[3]	ACGTTACCTTCTCGGGCATT[3]	126
SCD	Stearoyl-CoA desaturase	FJ513370.1	GATGACATCTATGACCCAACTTACCA[4]	CCCAAGTGTAACAGACCCATGA[4]	100
SLC27A1	Solute Carrier Family 27 Member 1	XM_012128820.2	ACTGTCTGCCCCTGTACCAC	GGCTGGCTGAAAACTTCTTG	102
SOD1	Superoxide dismutase 1	NM_001145185.1	AGAGGCATGTTGGAGACCTG	CAGCGTTGCCAGTCTTTGTA	189
SREBP-1c	Sterol regulatory element binding transcription factor 1	GU206528.1	GGGACAAGGTTTGCTCACATG[4]	GGCAGCTTGTCAGTGTCCACTA[4]	115
STK11	Serine/Threonine Kinase 11	XM_004008774.3	TTGGGAACTATGGGTGGCAG	CGCTGAGAGCAGAGAGATCC	161
UBB[1]	Ubiquitin B	NM_001009202.1	TGGGTTCCTGTGTCGTCTTT	TTGACCTGTGAGTGAATGCGA	75
YWHAZ[1]	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta	NM_001267887.1	GATGAAGCCATTGCTGAACTTGA[3]	CAGCTTCGTCTCCTTGGGTA[3]	124

[1]Reference genes.
[2]Amplicon size in base pairs (bp).
[3]Schultze et al. [253]
[4]Bichi et al. [254]

Supplementary Table S3. Relative expression of selected genes between experimental groups as compared to non-supplemented control group in ewes (fold change).

Gene name	Tissue	Dietary treatment			P-value
		Grape n = 6	Acacia n = 5	Grape + acacia n = 5	
ACO1	Endometrium	1.07	0.85	1.05	0.529
	Oviduct	1.21	1.04	1.34	0.198
	Liver	1.09	0.75	0.99	0.299
	Muscle	1.17	0.78	0.80	0.265
EHHADH	Endometrium	1.47	0.71	0.72	0.809
	Oviduct	0.82	1.38	1.01	0.619
GPT	Endometrium	1.02	0.91	1.17	0.837
	Oviduct	1.13	1.27	1.34	0.606
	Liver	0.90	0.89	0.90	0.949
	Muscle	0.90	0.71	0.94	0.313
GPX4	Muscle	0.54	0.54	0.52	0.095
LEP	Muscle	0.96	0.94	1.08	0.997
LIPC	Endometrium	1.05	0.39	0.74	0.380
	Oviduct	1.01	0.84	1.00	0.886
	Liver	0.72	0.68	0.83	0.632
	Muscle	0.02	0.03	0.03	0.912
LIPE	Endometrium	0.80	1.09	0.92	0.910
	Oviduct	1.15	1.17	0.91	0.970
	Liver	1.25	0.96	0.78	0.497
	Muscle	1.07	1.34	1.18	0.946
PTGS2	Endometrium	0.88	0.92	1.09	0.998
	Oviduct	0.88	1.35	1.80	0.179
	Liver	1.32	1.07	1.07	0.836
	Muscle	2.46	0.86	1.11	0.156
SLC27A1	Endometrium	1.20	1.06	1.26	0.738
	Oviduct	1.10	1.04	1.27	0.412
	Liver	1.08	0.76	0.94	0.498
	Muscle	0.86	0.67	0.68	0.559
STK11	Endometrium	0.55	1.40	0.86	0.299
	Oviduct	1.00	1.59	0.80	0.240
	Liver	0.87	0.88	0.45	0.151
	Muscle	0.74	1.78	0.81	0.240

Chapter 4

The role of leptin in *in vitro* prostanoid synthesis and regulation of gene expression in equine oviduct epithelial cells

Manuscript based on research carried out under the supervision of
B. Drews, K. Giller, V. van der Weijden, S.E. Ulbrich

Abstract

Embryonic loss within 25 days after fertilization is one of the main reasons for low economic performance of horse breeding. These early losses may be caused by malnutrition during the periconceptional period and hamper the embryo development and quality. One of the hormonal factors influenced by malnutrition and potentially affecting fertility is the adipokine leptin. By using short-term (48h) equine oviduct cell (EOEC) culture model, we aimed at exploring the effect of leptin stimulation (concentrations ranging from 2 to 1000 ng/µl) for 6 and 24 h on oviduct cell function in the mare while using stimulation with oxytocin (10 ng/µl) as a positive control. The secretion of the prostanoids prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was measured in the cell culture supernatant using an enzyme-linked immunosorbent assay (ELISA). Total RNA was extracted from cell explants and the expression of genes related to prostanoid metabolism were analysed using the Fluidigm platform. The cell culture was successfully established as confirmed by invariable expression of genes associated with cell functionality (e.g. SOD1, SLC2A1, VEGFA, FOS). The prostanoid synthesis was not significantly affected by leptin stimulation at any concentration. Treatment with oxytocin resulted in lower expression of PTGER4 and ESR1 genes, whereas the expression of PGC-1α, HSPB8 and FGF9 was higher when compared to leptin treatments. In addition, the expression of leptin receptor was confirmed for the first time to our knowledge in the equine oviduct epithelial cells on a transcriptional level. Our results suggest that the stimulation with increasing leptin concentrations has no effect on PGE_2 and $PGF_{2\alpha}$ production by the mare oviduct and exhibits only minor changes in related gene expression. Oxytocin was confirmed to modulate the expression of genes related to prostanoid metabolism. However, because of the restricted number of samples and large variation in cycle stages from the abattoir material used, results of the present experiment need to be interpreted with caution. Future research needs to include more samples from a specific cycle stage, quantification of leptin in the supernatant, and measurements of its possible effect on intracellular calcium levels and ciliary beat frequency. This may help to determine the velocity of the embryo transport through the oviduct and the specific role that leptin may play in the periconceptional period in mares.

Introduction

In the horse, the events of fertilization and embryo transport within the reproductive tract are strongly dependent on a precisely orchestrated interaction between the embryo and the maternal genital tract [188]. After successful fertilization the equine embryo spends first 5 to 6 days in the oviduct. Weber et al. [21] showed that at day 5 embryos are located in the ampullary-isthmic junction section of the oviduct or already entered the uterus. The speed and transport efficiency through the oviduct are supported by two coordinated actions: relaxation of the oviduct and opening of the uterotubal junction [22], but as well by stimulation of ciliary beating of the oviduct epithelial cells. The transport to the uterus is triggered by the isthmic sphincter relaxation, which is driven by PGE_2 secreted by the developing embryo [255]. The stimulation of the ciliary activity of the oviduct epithelium and the relaxation of isthmic oviduct and uterotubal junction by PGE_2 has already been observed in rabbits, hamsters and rats [256, 257]. A recent study showed that administration of PGE_2 on the oviduct surface of mares with known oviduct blockage resulted in clearing of the accumulated debris and successful conceiving within the same breeding season [258]. In addition, the expression of PGE_2 receptors PTGER2 and PTGER4 was confirmed in the luminal epithelium and circular musculature of the oviduct in the mare [259]. These findings point to an inhibitory effect of PGE_2 on contractions of the oviduct musculature in mares.

The prostaglandins (PG) of the 2- and 3-series are derived from the metabolism of cell membrane *n*6 and *n*3 polyunsaturated fatty acids (PUFA), respectively, and are key mediators for a number of biological processes such as cell proliferation, inflammation and immune response [260]. The PG synthesis is controlled by the cyclooxygenase (PTGS) that converts the *n*6 fatty acid arachidonic acid to a common precursor of all prostanoids of the 2-series. These prostanoids are local hormones abundant during ovulation [261]. Several studies have as well demonstrated a stimulation of PGE_2 and $PGF_{2\alpha}$ secretion from equine endometrial explants by the hormone oxytocin [262, 263]. Oxytocin is a nonapeptide with an impressive variety of physiological functions [264], and its actions mediated by specific receptors. It also plays a role in the oviduct contraction or relaxation, in cyclic and/or pregnant females, and in PG synthesis in woman or bitch, for example [265]. However, the stimulation of PGE_2 and $PGF_{2\alpha}$ secretion by oxytocin was not confirmed in the bovine oviduct epithelial cell culture [266], thou it was shown to block the contractile amplitude of the oviducts [267]. A recent study cultured equine oviduct explants from animals in the follicular phase and showed a reduction in PGE_2 production when treated with oxytocin [265].

Leptin is an adipokine, a 16kDa peptide hormone that is secreted mainly from adipose tissue, but its expression was also confirmed in skeletal muscle, placenta, ovary or embryos [268, 269]. It plays a central role in metabolism, by for example regulating energy expenditure and body weight homeostasis [270]. It is also involved in reproduction, by regulating the hypothalamic pituitary gonadal axis [271], and furthermore is involved in embryo development [272]. Zerani et al. [273] demonstrated a stimulation of PGE_2 release by leptin while it inhibited the $PGF_{2\alpha}$ release in the rabbit oviduct. It was further demonstrated that leptin increases PGE_2 production in human endometrial and cartilage cells [274, 275]. Interestingly, low leptin concentrations in the blood of buffalo cows were related to a failure to conceive [276]. The addition of leptin at a concentration of 100 ng/ml during *in vitro* maturation had a beneficial effect on equine oocyte maturation, but on the contrary impaired embryo development [64]. The identification of both leptin and its receptor in equine oocytes as well as embryos suggested a role of leptin as an autocrine/paracrine hormone in oocyte maturation, fertilization and early embryo development. It can be thus suggested that leptin plays an important role in the periconceptional period in the mare, in particular by modulating the sphincter muscle relaxation in the oviduct via stimulation of PGE_2 release.

We hypothesized that circulating plasma leptin can modulate the local prostanoid synthesis in the horse oviduct. Therefore, we incubated equine oviduct explants with human recombinant leptin and measured the secretory activity of the cells. In addition, gene expression changes were evaluated with the focus on prostaglandins, their receptors and synthases. Oviduct explant incubated with oxytocin served as a positive control. Results are reported based on the limited sample size and occurring experimental pitfalls.

Materials & Methods

Animals

Oviduct samples were collected from four healthy adult mares (three Franches-Montagnes and one Haflinger breed) after slaughtering at a commercial abattoir in Switzerland between June and October 2016. The reproductive tract was collected immediately after slaughter and transported on crushed ice to the laboratory. Blood samples were collected from the jugular vein at the moment of slaughter into tubes containing ethylenediaminetetraacetic acid (EDTA), transported on ice and centrifuged (1500 × g for 10 min at 4°C). The collected plasma was frozen at -80°C until further analysis.

For the collection of endometrial tissue, the uterus was flushed with 10 ml in-house made phosphate-buffered saline (PBS). Following, the uterine body and the uterine horns were opened longitudinally. Using surgical scissors, endometrial stripes were carefully cut out and immediately transferred into cryo-tubes. Endometrial samples (n = 3) were snap-frozen in liquid nitrogen and subsequently stored at -80°C for later analysis. The ovaries were cut open and morphological examination included any visible pathologies, the presence and size of *corpus hemmoragicum* (CH), *corpus luteum* (CL), and follicles. These parameters were considered for the evaluation of cycle stage. A cyclic mare was defined by the presence of a CH, CL or medium sized follicles. The progesterone (P4) concentration was measured according to Prakash et al. [158].

The cycle stage was estimated according to the obtained plasma or endometrial tissue P4 concentration, using ELISA and as described in Drews et al. [24].

Isolation and culture of oviduct epithelial cells

The oviducts were cut from the uterus at the uterotubal junction, dissected free from connective tissue and disconnected from the ovary while keeping the infundibulum. After rinsing with PBS, oviducts were rinsed with 70 % ethanol and again with PBS, while taking care of not contaminating the openings. Both oviducts were flushed under a stereo microscope. A rinsing cannula was carefully introduced into the infundibulum, tightened with tweezers, and the oviduct was flushed with 1 ml of PBS. Following, the oviducts were cut open longitudinally starting from the infundibulum until isthmus and fixed on a sterile bench. The equine oviduct epithelial cells (EOEC) from ampulla and ampulla-isthmic junction were scraped using a cell scraper (length 200 mm, width 13 mm; SPL Life Sciences Co Ltd, Korea).

The collected cellular paste was rinsed from cell scraper using 5 ml in-house made hydroxyethyl piperazineethanesulfonic acid (HEPES)-buffered washing medium (Supplementary Table S4) using an 18G needle (1.20 x 80 mm; Henry Schein, Germany) and 1 ml Luer syringe (Henry Schein, Germany). Cells were transferred into a sterile Falcon 15 ml conical centrifuge tube (ThermoFisher Scientific, USA). After 5 min of settling, the washing medium was removed without disturbing the formed cell pellet, and the washing step was repeated with fresh HEPES-buffered washing medium for two more times using the same protocol. After the complete removal of washing medium, 5 ml culture medium (DMEM/F12 with 10 % FCS; ThermoFisher Scientific, USA) were added to the formed cell pellet. Then the cells were immediately seeded for culture in a sterile 24-well plate (TPP Techno Plastic Products AG, Switzerland) with 200 µl of the cell suspension pipetted into each well and

incubated for 24 h at 38.5°C in a humidified incubator with 5 % CO_2 (CO_2CELL 190 – Comfort; MMM Group, Germany). The time span from the slaughter to seeding the cells in culture was between 4 and 6 h.

After the initial culture of the cells for 24 h, only the formed explants were collected and further handled. At the start of EOEC culture, same as at initiation of stimulation (0 h) and at 6 and 24 h of stimulation, explant structure and viability were assessed using an inverted stereo microscope (Zeiss Axiovert 40 CFL, Germany) and recorded using an Olympus XM10 camera (Olympus Corporation, Japan). The cell viability was assessed by visual observation of progressive ciliary activity, which is characterized by the outermost cells of the aggregates being bordered by vigorously beating cilia. At 24 h of incubation, only explants with progressive ciliary activity and a diameter of 100 – 400 µm were transferred into sterile 1.5 ml Eppendorf tubes (Eppendorf AG, Germany). After centrifugation (5 min at 300 x g at room temperature), the supernatant was carefully removed without disturbing the formed cell pellet. Fresh culture medium (200 µl) was added to the cell pellet which was then transferred to a 5 ml Eppendorf tube (Eppendorf AG, Germany) that already contained 3 ml of fresh culture medium. The resulting cell suspension was then equally distributed into the wells of a 24-well dish (250 µl/well). Care was taken to mix the suspension each time before transferring, in order to distribute approximately the same number of explants per well. The explants from ipsi- and contralateral oviducts were treated separately.

Study design

The short-term EOEC culture model design used that included hormonal stimulation is presented in Figure 14. Human recombinant leptin (Sigma-Aldrich, USA) and oxytocin (Sigma-Aldrich, USA) were added to the culture medium that was respectively distributed in the designated wells (250 µl/well) of a sterile 24-well plate (TPP Techno Plastic Products AG, Switzerland). Together with the cell suspension the final volume in the wells was 500 µl including explants and stimulation hormone. The treatments and final concentration of hormones are presented in Table 10.

Table 10. Treatment description used for the short-term EOEC culture.

OXT	CTL	L2	L10	L100	L1000	PBS
Oxytocin (10 g/ml)	Culture medium	Recombinant human leptin (2 ng/ml)	Recombinant human leptin (10 ng/ml)	Recombinant human leptin (100 ng/ml)	Recombinant human leptin (1000 ng/ml)	PBS (100 µl/ml)

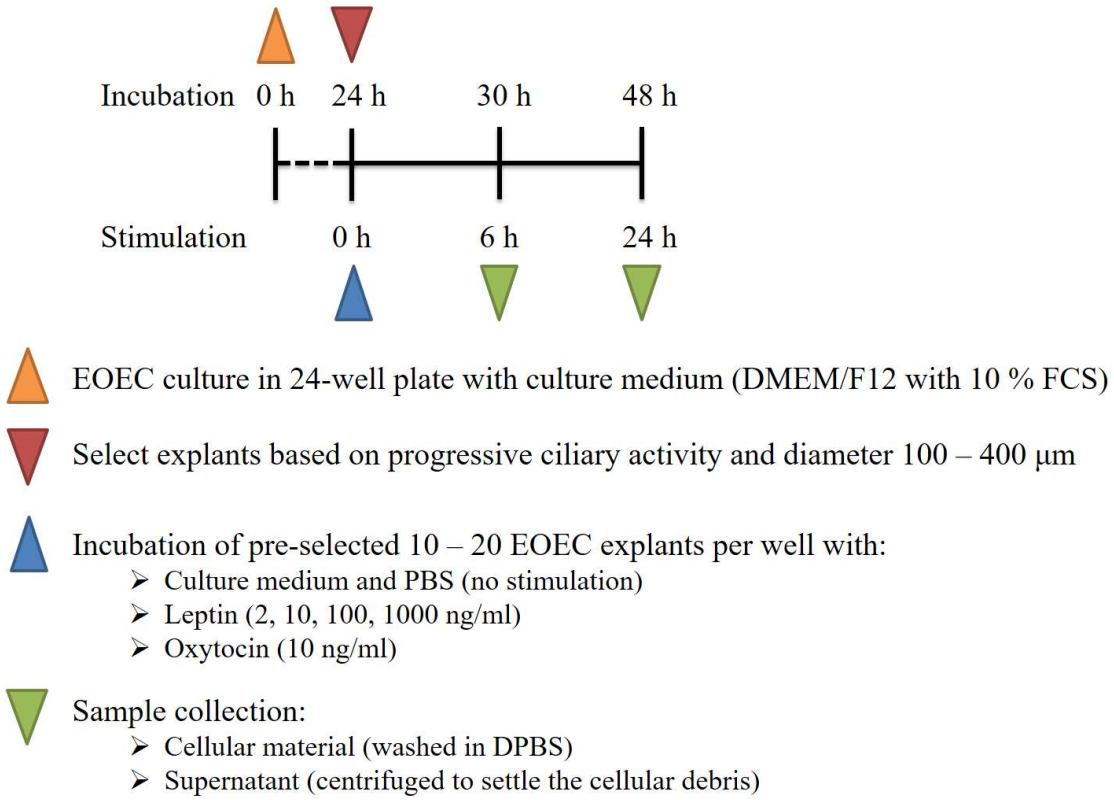

Figure 14. Design of the short-term EOEC culture model.

Explants from one single oviduct were always seeded in two batches, for 6 and 24 h stimulation time, respectively. At these time points, the cellular material and the supernatant were collected for further analysis. The PBS treatment was only seeded and collected in the batch 6 h after stimulation, whereas L1000 only at 24 h after stimulation. This was done in order to observe if high leptin dosage over longer stimulation time has an effect on cell culture.

Collection of cellular material and supernatant

The explants from each well were transferred by pipetting into a sterile 1.5 ml Eppendorf tube. After centrifugation for 5 min at 300 × g at room temperature, the supernatant was removed and the cell pellet was resuspended in 300 µl of Dulbecco's phosphate-buffered saline (DPBS; GIBCO BRL Invitrogen, USA). The centrifugation was repeated and DPBS removed. The washing step was repeated and the cell pellet was immediately frozen at -80°C until further analysis. The supernatant remaining in the wells was transferred into an Eppendorf tube and centrifuged for 5 min at 300 × g at room temperature to remove potential cell debris. The supernatant was transferred into a new tube taking care not to disturb any formed pellet and frozen at -80°C until further analysis.

Prostanoid determination

Culture medium was analysed for PGE_2 and $PGF_{2\alpha}$ using a commercial ELISA (High Sensitivity EIA kit, Enzo Life Sciences AG, Switzerland). The procedure was performed according to the manufacturers' protocol and all the samples were run in duplicates.

Coefficient of Variability (CV) was calculated as inter- and intra-assay CV value. Expression of within-plate consistency (intra-assay % CV) was calculated for all samples, while the expression of plate-to-plate consistency (inter-assay % CV) was computed from three samples, all run on each ELISA plate. For the intra-assay % CV, the standard deviation of the replicates was used, divided by their mean, and converted to the respective % CV value. The intra-assay % CV was set to ≤ 15 %, taking into consideration the limited number of tested samples. Inter-assay % CV from two runs was 17%. In the case of inter-assay % CV, the mean of all three % CV values of the replicates between the plates was used as a measurement of interest. Any sample that had an intra-assay % CV > 15 was excluded, while inter-assay % CV < 18 was a prerequisite for plate-to-plate comparison.

Total RNA extraction and quantitative reverse transcription–polymerase chain reaction

The frozen cell pellets were thawed after addition of RLT lysis buffer (Qiagen, Netherlands) and homogenized using the MagNA Lyser Instrument (Roche, Germany) at 7000 × g for 30 seconds at room temperature. The RNA extraction immediately followed using the RNeasy® Mini kit (Qiagen, Netherlands) according to the manufacturer's protocol. The quantity and quality check was performed for all samples using NanoDrop 8000 (ThermoScientific, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, USA), respectively. Only samples with a RNA integrity number (RIN) of 5.5 or higher were considered for analysis. Reverse transcription was performed using 10 µl of diluted RNA samples containing 60 ng RNA per µl of UltraPure™ DNase/RNase-Free distilled water (Invitrogen, USA) and 10 µl of the GoScript™ Reverse Transcription System master mix (Promega, USA), according to the manufacturer's protocol, using the LabCycler thermocycler (Sensoquest, Germany), to produce cDNA.

Primer pairs for the genes of interest were designed using Primer3 software. A BLAST analysis was performed and primers tested against the NCBI equine sequences (*Equus* (taxid: 9789)) found in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/, accessed 15 June 2017). Corresponding primer sequences were purchased from Microsynth (Balgach, Switzerland). Gene names and symbols, primer sequences, amplicon size, and NCBI accession numbers are summarized in Supplementary Table S5. All the primers were tested by

performing a quantitative reverse transcription–polymerase chain reaction (qRT-PCR) on Bio-Rad CFX Connect Real-Time System (Bio-Rad, USA). The PCR products were analysed on a 10% agarose gel to demonstrate the amplification of a single product of the expected size.

The cDNA was preamplified using the TaqManPreAmp Master Mix (PN 100-5875 C1; Fluidigm, USA). Gene expression was measured using a high throughput gene expression platform – 48.48 Dynamic Array™ IFCs (Integrated Fluidic Circuits) (Fluidigm, USA) based on microfluidic chips. Specific target amplification was detected using EvaGreen® DNA binding dye (PN 100-1208 B, Fluidigm, USA) according to the manufacturer's protocol. The mRNA data was handled in Fluidigm Real-Time PCR Analysis Software 4.5.1. (Fluidigm, USA).

The Normfinder option of the GenEx Pro software (MultiD Analyses AB, Sweden) was consulted to confirm that the geometrical mean of Cycle Threshold (Ct) values from housekeeping genes could be used for normalization of target gene expression data. Accordingly, delta Ct (ΔCt) values per sample were calculated as difference between the Ct value of the target gene and the geometrical mean of Ct values of the reference genes. The ΔCt was used for further statistical analysis. The log2 value of ΔΔCt was used to present the relative quantification of mRNA levels and data was visualized using GraphPad Prism 7 (GraphPad Software Inc., USA).

Statistical analysis

The software package SPSS (SPSS, IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY:IBM Corp.) was used for statistical analysis. Normal distribution of the unstandardized residuals was confirmed by visual inspection and applying the Shapiro-Wilk test. The mixed model procedure and one-way ANOVA were applied to test for differences between the treatments without taking into account the stimulation time, in case of normal distribution. Log values were generated for any non-normally distributed data sets and again tested for normal distribution. If confirmed, they were processed using the mixed model and one-way ANOVA, as previously described. Otherwise, Kruskal-Wallis model was used to report the P-values. The LSD was applied as Post Hoc test. Paired-samples T-test was used for significance between 6 and 24 h exposure within each treatment. All results are presented as mean ± standard error of means (SEM).

Results

Cycle stage of animals

The P4 plasma concentrations of the two mares with blood samples collected were 17.74 and 28.72 ng/ml (23.2 ± 3.9 ng/ml (mean ± SEM)). Endometrial tissue P4 values ranged from 2.88 to 14.21 ng/g with an average value of 8.7 ± 2.8 ng/g (n = 3). A coefficient of correlation between plasma and endometrial P4 levels could not be calculated because of the small number of samples. Based on morphological evaluation of the ovarian functional bodies as well as observed presence of a *corpus luteum* and active follicles (Figure 15), it was suggested that all animals were cyclic (luteal phase) and had a normal ovarian activity.

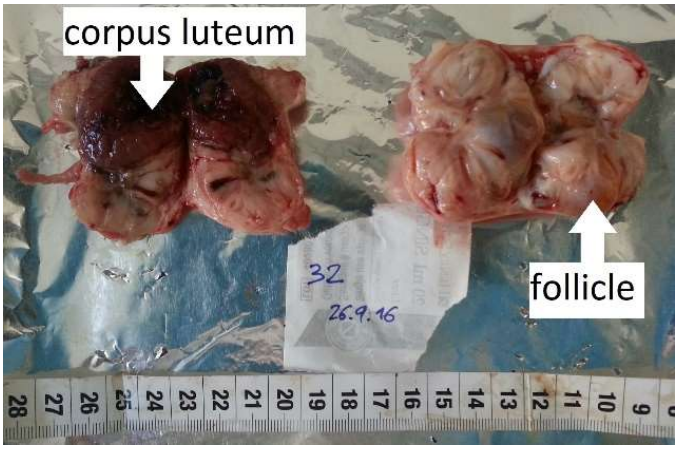

Figure 15. Functional ovarian bodies of a cyclic mare.

Oviduct epithelial cell culture

The collected cellular material was cultured and cells in suspension showed no functional activity. Dead singularized cells were as well identified at this stage (Figure 16a). After 24 h of culture (incubation time), a clear distinction was made between dead single and aggregated cells (Figure 16b). On appearance, the latter were organized in explants bordered by columnar epithelial cells with vigorously beating cilia and a proportionally higher amount of non-ciliated cells. The explants resembled a viable cell culture with intact cells and did not show any major central dark zones (Figure 16c). No explants adhered to the culture dish at any time point. Cell numbers remained relatively constant after initial seeding (Figure 16).

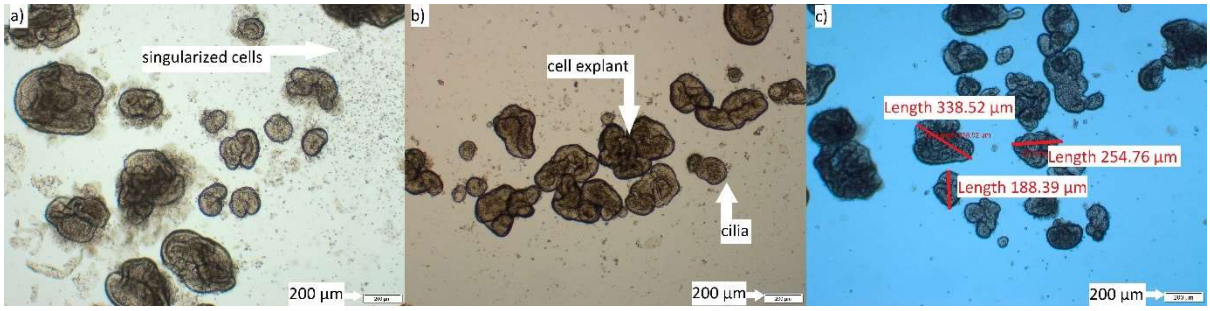

Figure 16. Short-term EOEC culture, showing cells: (a) at 24 h incubation (0 h of stimulation); (b) 6 h and (c) 24 h of stimulation (bar = 200 µm); dead cells, cell explants, ciliary cells, measurement of explants, are respectively shown and indicated with arrows.

Prostanoids in equine oviduct epithelial cell culture

Prostaglandin E_2

The PGE_2 was analysed in the EOEC culture supernatant (n = 22 for 6 h; n = 23 for 24 h; total n = 45). The concentration of PGE_2 ranged from 175 pg/µl to 4.52 ng/µl between all treatments. Figure 17a summarizes the findings. The data is presented as a relation between the measured PGE_2 concentration and total RNA, extracted as per described ($n \geq 4$ for 6 h and $n \geq 2$ for 24 h of stimulation per treatment). The normalization of synthesized PG to total RNA had to be performed in order to allow for the comparison between the stimulation treatments that differed in total DNA quantity. DNA extraction from the samples was not performed and thus total RNA had to be taken as a normalization parameter. The PGE_2 relative change (relation between the PGE_2 concentration and total RNA per sample) showed no significant difference between any of the treatments or time points (additional data to be found in Supplementary Table S6).

Prostaglandin $F_{2\alpha}$

The concentration of $PGF_{2\alpha}$ in the EOEC culture supernatant varied from 1.90 to 30.93 pg/µl between all treatments combined (n = 20 for 6 h; n = 15 for 24 h; total n = 35). The results are presented as a relation between the $PGF_{2\alpha}$ concentration and total RNA per sample ($n \geq 4$ for 6 h and $n \geq 1$ for 24 h of stimulation per treatment), as shown in Figure 17b and Supplementary Table S6. There was no statistical difference between the different treatments. Within treatments, only the control was significantly different with an about 50 % lower $PGF_{2\alpha}$ to total RNA ratio at 24 h compared to 6 h of stimulation. The L10 treatment had only one sample represented, thus no statistical analysis for this treatment could be performed.

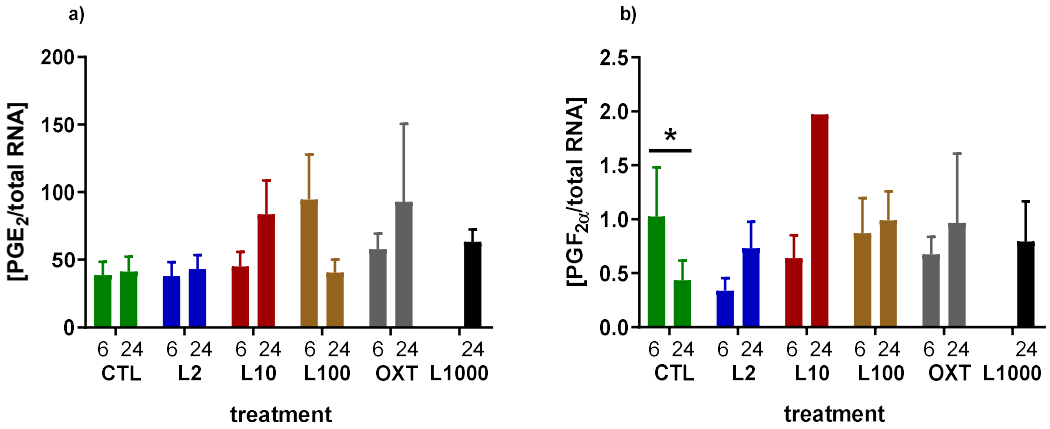

Figure 17. Relative changes of (a) PGE_2 (expressed as PGE_2/total RNA) and (b) $PGF_{2\alpha}$ ($PGF_{2\alpha}$/total RNA) in the EOEC culture supernatant as measured by ELISA (error bars = SEM). Bars of same colour represent the same treatment. The effect of treatments and different time point between treatments was not significant ($P > 0.05$). Treatment with asterisk is significantly different between the time points ($P < 0.05$).

Prostanoid metabolism associated gene expression of the oviduct epithelial cells

Gene expression of PGC-1α, PTGER4 and ESR1 was significantly lower with oxytocin (OXT) stimulation compared to control (CTL) and all leptin treatments (L2, L10, L100) indifferent from the time of stimulation ($P < 0.05$, $P < 0.01$, $P < 0.01$, respectively) (Figure 18). At 24 h of stimulation, a significantly lower expression was also observed for PTGER4 and ESR1 when comparing OXT to all other treatments ($P < 0.01$). The gene expression of OXTR at 24 h of stimulation was significantly lower with L10 and L100 treatment compared to CTL, but increased with OXT ($P < 0.05$). At 24 h of stimulation, INSR showed a strong trend ($P = 0.05$) towards a concentration-dependent decrease in gene expression. When comparing the expression levels between 6 and 24 h of stimulation within each treatment, PTGS2 gene expression was significantly lower for treatment with all tested leptin concentrations at 24 h compared to 6 h, whereas a higher expression was observed with the OXT treatment at 24 h compared to 6 h ($P < 0.05$). A higher expression at 24 h compared to 6 h of stimulation was also observed for the L100 treatment for ESR2 ($P < 0.05$).

Expression patterns of genes related to phospholipid metabolism (PLCG1, SYT5), protein metabolism (HSPA5, CTSL), different cellular processes (S100A6, GJA1, SPINK7), as well as solute carrier family genes (SLC2A5, SLC36A2, SLC03A1) and the progesterone receptor PAQR5, were all not statistically different between the treatments and hours of stimulation. Still, the L100 treatment resulted in a significant decrease in gene expression of SPINK7 and SLC2A5 at 24 h compared to 6 h of stimulation ($P < 0.05$). The gene expression of HSPA5

was higher at 24 h compared to 6 h of stimulation with L100 and OXT ($P < 0.05$). Gene expression results are summarized in Supplementary Table S7.

Among the cell functionality related genes, only FGF9 showed significantly higher expression with OXT as compared to all other treatments independent of time of stimulation and as well at 24 h of stimulation ($P < 0.05$) (Figure 19). The HSPB8 gene expression was significantly higher with OXT compared to all other treatments at 24 h of stimulation ($P < 0.05$). For 6 h of stimulation with L10, significantly lower gene expression was noted for VEGFA and SOD2 compared to 24 h of stimulation ($P < 0.01$). The same was observed for L100 treatment for SLC2A1 and FGF9 ($P < 0.05$ and $P < 0.001$, respectively). The gene expression of FGF9 at 24 h of stimulation with L2 and OXT was significantly higher compared to 6 h of stimulation ($P < 0.05$ and $P = 0.05$, respectively). Treatment OXT at 24 h of stimulation significantly increased IFI6 gene expression compared to 6 h of stimulation ($P < 0.05$).

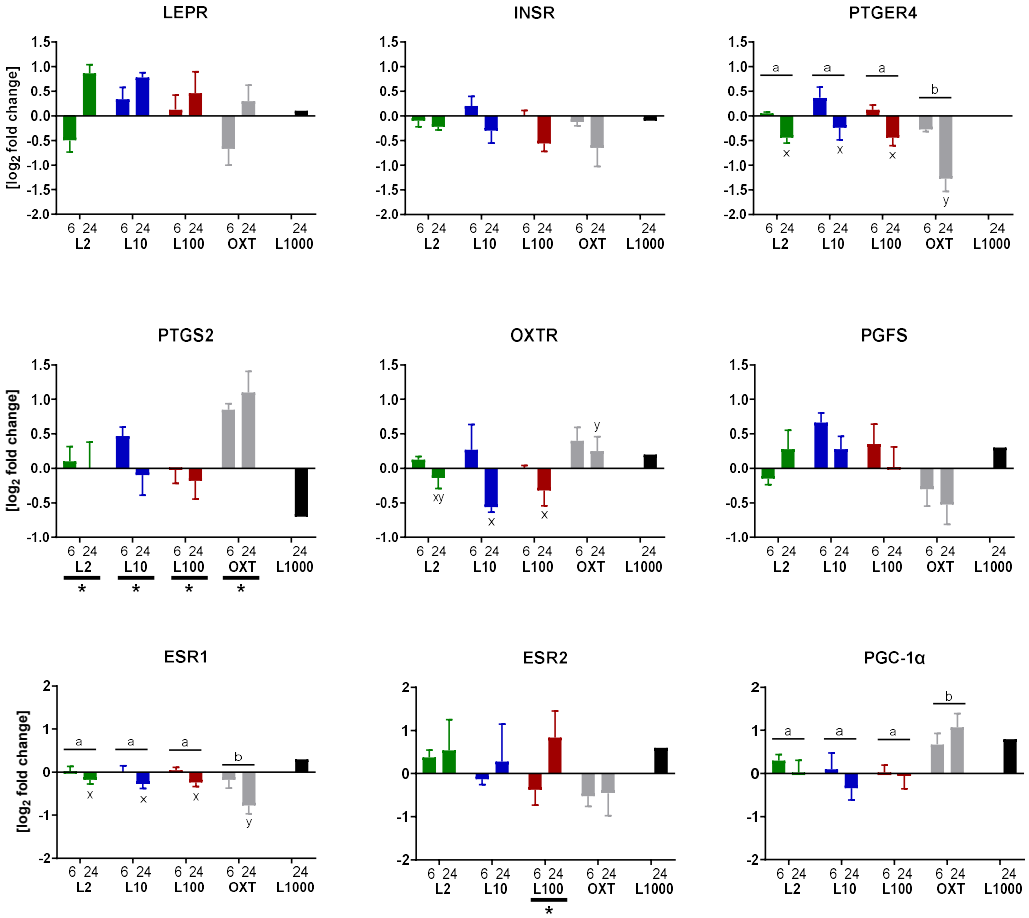

Figure 18. Expression of targeted genes (receptors and synthases) in the EOEC culture analysed using the Fluidigm platform. The results are presented as log2 fold changes in relation to control sample. Bars of the same colour represent the same treatment. The effects of treatment and treatment at specific time point are indicated with superscript letters a, b and x, y, respectively, with asterisk between specific time point within treatment, and were regarded as significant if $P < 0.05$. Bars are represented with SEM values.

There was only one L1000 treatment sample included on the Fluidigm platform, therefore no statistical analysis were performed for this treatment. Still, the respective expression levels for n = 1 are shown in Figure 18 and 19, likewise in Supplementary Table S7.

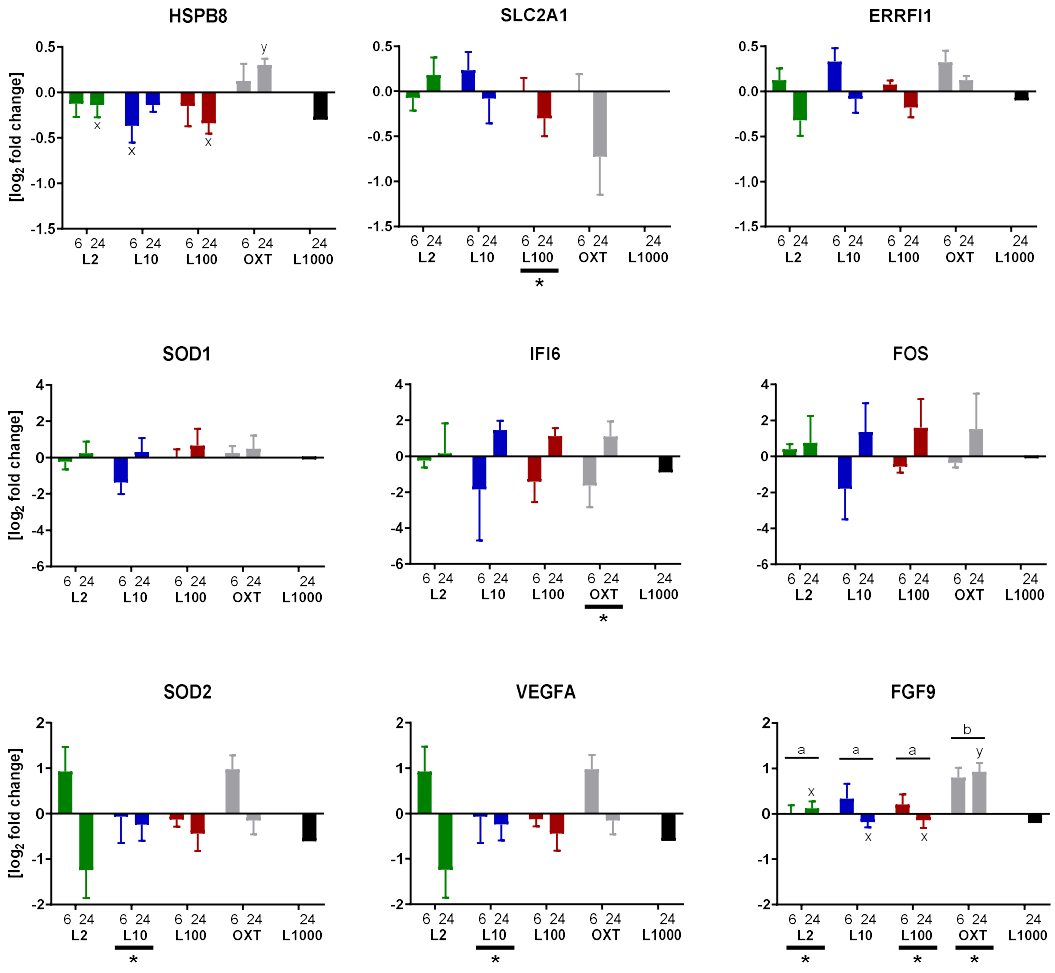

Figure 19. Expression of targeted genes (related to cell functionality) in the EOEC culture analysed using the Fluidigm platform. The results are presented as log2 fold changes in relation to control sample. Bars of same colour represent the same treatment. The effects of treatment and treatment at specific time point are indicated with superscript letters a, b and x, y, respectively, with asterisk between specific time point within treatment, and were regarded as significant if $P < 0.05$. Bars are represented with SEM values.

Discussion

Pregnancy rates in mares tend to be higher on day 4 as compared to day 14 after ovulation, suggesting an early embryonic loss (EEL) that encompasses the period of transport through the oviduct [277]. A failure of oviductal transport of viable embryos is not reported as a clinical complication in mares [278], nevertheless additional processes involved in embryo-maternal signalling may be of importance. Respectively, adequate *in vitro* models are necessary for a

better understanding of EEL in mares. The EOEC culture model used in the present study closely resembles the already described EOEC culture models with a maintained sound morphological and ciliary function, as well as prostanoid production over the course of short-term culture [188, 279]. Moreover, we observed no major changes in the expression of genes related to cell functionality including growth, differentiation, proliferation, transformation and apoptosis (Supplementary Table S7). To the best of the authors' knowledge, this study reports for the first time the expression of 27 genes including ERRFI1, FOS, HSPB8, S100A6, IFI6, GJA and LEPR, in the EOEC model. In a similar EOEC model [188], gene expression of cell functionality markers such as HIF1A, VEGFA, uPA (plasminogen activator, urokinase), GLUT1 (SLC2A1), and PAI1 (plasminogen activator inhibitor-1) remained stable during the first 6 days of culture. In the present study, we observed exceptional downregulation in VEGFA and SLC2A1 expression between 6 and 24 h of leptin stimulation (significant at $P < 0.05$ for VEGFA with L10 and SLC2A1 with L100 treatment). Induced expression of both VEGFA and SLC2A1 is related to apoptosis-like hypoxia-related type of cell degeneration, as reported by Nelis et al. [188]. Thereby we could particularly exclude hypoxia conditions in our EOEC culture model. Based on these described morphological and transcriptional observations we suggest that the EOEC culture was successfully established.

Prostaglandins of conceptus origin are considered to be the trigger for the selective embryo transport along the oviduct and across the utero-tubal junction as well as migration towards the uterus [40]. They mediate their effects by binding to their respective receptors located in the cell membranes of the oviduct epithelium. Ball et al. [259] validated the presence of PGE_2 receptors PTGER2 and PTGER4 in the luminal epithelium in the mare oviduct. Quantitative RT-PCR revealed significantly higher expression of PTGER2 and PTGER4 during the pre-ovulatory phase compared with luteal phase in bovines [280], suggesting the activation of their gene expression in the periconceptional period. The PTGER2 and PTGER4 are involved in smooth muscle relaxation by increasing the amount of cytoplasmic cyclic adenosine mono-phosphate (cAMP). Our results confirm PTGER4 expression in EOEC with significant downregulation by OXT exposure at 6 and 24 h of stimulation. Treating the oviduct explants with OXT was observed to significantly increase the production of PGE_2 by the isthmus during the whole estrous cycle and release of $PGF_{2\alpha}$ by the ampulla during the mid-luteal phase [265]. However, even though we were able to confirm both PGE_2 and $PGF_{2\alpha}$ synthesis by the EOEC, we were not able to confirm an effect of OXT and leptin on PG production since the concentration of both PGE_2 and $PGF_{2\alpha}$ in the cell culture supernatant remained unchanged after the OXT and leptin treatment. We only observed a reduction of $PGF_{2\alpha}$ concentration at 24 h

compared to 6 h of stimulation. A reduction in $PGF_{2\alpha}$ concentrations between 3 and 24 h of incubation in culture medium was also noted using porcine epithelial endometrial cells from mid luteal stage [281].

Treatment with OXT increased the expression of PTGS2 and PGC-1α compared to untreated cells, which is in agreement with the previously reported PTGS2 upregulation in the presence of OXT in human endometrial cells [282] and co-expression of PGC-1α with OXT in zebrafish hypothalamus [283]. Oxytocin treatment was also shown to be responsible for a PTGS-dependent pulsatile PGE_2 release in the rat ileum mucosa [284]. The abundance of OXTR mRNA was increased with OXT treatment compared to unstimulated cells in our model, confirming the gene expression activation of its receptor in the presence of OXT, thereby enhancing its own signalling. However, PTGS2 gene expression is not only enhanced by OXT but also by leptin stimulation. In human endometrial cells, Gao et al. [275] achieved an increase of PGE_2 and PTGS2 production and expression in a dose- and time-dependent manner by leptin stimulation via the JAK2/STAT3, MAPK/ERK and PI3K/AKT pathways. The activation of these pathways by leptin stimulation was further confirmed in ovarian cells, supporting the hypothesis of an involvement of leptin in the activation of PTGS2 gene expression [285]. The presence of leptin protein and receptor has previously been described in canine and porcine endometrium [64, 286], but to our knowledge not yet in the mare oviduct. Therefore, our results show for the first time the presence of leptin receptor transcripts in the epithelial cells of the ampulla section of the equine oviduct. Still, the applied 24 h of stimulation with leptin did not significantly modify its gene expression. We only observed a trend ($P = 0.06$) towards upregulation of 24 h compared to 6 h of stimulation with high leptin (L100) treatment. Likewise, we did not observe any effect concerning the PG synthesis in relation to leptin treatment. A study of Zerani et al. [273] showed that leptin increases $PGF_{2\alpha}$, but inhibits PGE_2 production via a nitric oxide synthase (NOS)-dependent mechanism in the rabbit oviduct. This was confirmed in rats where NOS inhibition increased oviductal mobility and accelerated ovum transport [22]. It may be suggested that there is a species-specific regulation of PG pathways involved in oviduct motility and embryo transport.

In our study, the leptin doses used were based on a range of studies that reported plasma and follicular fluid levels in horses (Supplementary Table S8) [287]. It was advocated that the younger the horse, the higher the ability of adipocytes to release leptin [288]. Finally, we opted for slightly lower doses compared to average literature values taking into account the age factor. The exact increment levels were suggested upon the previously reported studies, with leptin added at 1, 10 and 100 ng/ml to horse oocyte culture medium [268], but as well to ciliated

cells of the rat epithelium [289]. The L1000 treatment in our study reflected a concentration of leptin that was 10-fold higher than the highest experimental dose. This was done in order to demonstrate that the molecular mechanisms and actions are not affected by using these high leptin concentrations. Our results confirmed no influence of leptin on PG concentration, alike gene expression changes.

The endometrial tissue progesterone (P4) concentrations in our study are in range and accordance with the previously reported endometrial P4 concentrations [24]. The P4 concentration in the oviduct tissue and fluid was not measured, but Nelis et al. [290] reported high levels in the oviduct tissue ipsilateral to the ovulation side during diestrous, contrary to other steroid hormones. Therefore the estrous cycle, namely steroid hormones, need to be considered for any *in vitro* model using oviduct cells, since they may additionally alter the transcript abundances [279]. Such models in equines, compared to bovine model, are rather new and optimal conditions are still explored.

Circulating hormones drive the preparation of the reproductive environment for hosting the growing embryo. It was reported that estradiol (E2) downregulates prostanoid production in the EOEC during the follicular phase, but not during the mid-luteal phase [265]. In addition, E2 is essentially involved in the embryo protection via upregulation of the ESR1 receptor that suppresses the inflammatory response and the maternal innate immune system (e.g. secretion of oviduct antimicrobial molecules), both mechanisms that are necessary for embryo survival [42]. In bovine oviduct, E2 originating from follicular fluid was shown to induce isthmic epithelium contraction via a mechanism involving endothelin secretion [291]. Still, the mRNA and protein expression of PTGER2 and PTGER4, involved in relaxation, increase upon E2 exposure in a BOEC model using oviductal smooth muscle [292]. This may suggest subtype tissue specificity in response to E2 stimulation between the oviduct epithelium and smooth muscle, likewise the ampulla and isthmus during different cycle stages. In our study, we showed a significant ESR1 downregulation with leptin and OXT treatment and the effect was even more pronounced at 24 h of stimulation, suggesting alterations in mechanisms that support embryo survival. Oxytocin was described as a negative modulator of estrogen receptors in rat uterus [293], but we could only confirm this for ESR1. On the other hand, we observed a significant upregulation of ESR2 at 24 h as compared to 6 h of stimulation with L100 leptin treatment. ESR2 is cilia-localized and its activation delays the transport of oocyte-cumulus complexes through the oviduct in rats [294]. In the absence of oviductal ESR1 in a knockout model in mice, $PGF_{2\alpha}$ levels in the whole oviduct tissues were lower, whereas PGE_2 levels

increased [295]. Lower concentrations of $PGF_{2\alpha}$ are stimulatory, while higher are inhibitory on contractions of the circular muscle, as seen in rabbit post-ovulatory oviducts *in vitro* [296].

Based on the results of the present study and literature evidence, we suggest that the embryo-produced PG drive the oviduct relaxation in the follicular and early luteal phase in the mare. In the mid- and late luteal phase, when the embryo is already in the uterus, it may be the mother, via prominent systemic $PGF_{2\alpha}$ production under OXT influence, which incites the isthmic muscle contractions. Thereby, the barrier for the oviductal microenvironment can be re-established. In support of the suggested mechanism it was demonstrated that the muscular contractions in the human ampullary-isthmic junction significantly increase after treatment with $PGF_{2\alpha}$ and PGE_2 [297].

As of limited availability of slaughterhouse material, the present study included samples from mid to late luteal phase of aged animals of different breeds, without any previous knowledge on their feeding or reproductive management background. Bearing in mind the limitations of our model, the presented results need to be considered with caution. Further, immunochemistry was not performed but would be necessary to confirm and localize the observed gene expression on the protein level.

Conclusion

We applied a short-term EOEC culture, and a stable expression of genes related to the functionality of cells was encouraging. Thereby the model is proposed as a valuable approach for future studies. A rise in PG levels, notably PGE_2, with time and increasing leptin concentrations in the medium was hypothesized, but not achieved under the tested treatments and protocol. Nevertheless, the synthesis of both PGE_2 and $PGF_{2\alpha}$ by the EOEC was confirmed, same as the expression of a broad panel of 27 genes, including PTGS2, PTGER4, ESR1 and LEPR, with the later for the first time identified in the oviduct of the mare. The time-dependent downregulation of PTGS2, PTGER4, and ESR1, by leptin was contrary to expected, and could only be explained by the cycle-stage and tissue section effect of the used EOEC. As of current standpoint, it is not possible to confirm a major effect of the increasing leptin exposure on the EOEC. Input material selection and improved protocoling would be advocated to further investigate muscular contraction/relaxation in relation to embryo transport across the oviduct within the first days after the fertilization.

Limitations of the experiment

Due to the limited availability of cycle specific material from the local abattoirs, the number of animals used in this project was very low (n = 4). Additionally, we could collect blood samples from only two animals. This greatly reduced the statistical power of our experiment. To clearly answer the research questions, ideally n = 10 animals should be included in such a study. Not only the oviducts, but also the plasma and endometrial tissue, need to be collected from all the animals and in addition, the focus should be on the oviducts ipsilateral to the ovulation side. The cycle stage was previously described to play a major role in a series of events that may lead to different results from the EOEC culture. Thus, a more defined grouping of samples according to the cycle stage would be a great improvement and provide a common basis for further treatments. Therefore, sampling three to five animals of each early and late luteal phase, respectively, would be required. Characterization of early-, mid-, and late-luteal phase, as defined by the presence of a CH, a mature CL (i.e. high P4) and a preovulatory follicle (i.e. low P4), respectively, are important to separate the samples and by this minimize any cyclic effects on the EOEC culture. Further, focus on one specific breed and from a similar age group (ideally mares between 5 and 10 years old), would be an optimal setup for the experiment.

The choice of the ampulla was reasoned by the described epithelial cells collection technique. Still, the isthmus could be of equal importance in relation to the muscular tone changes on a gene expression level. Ideally, a separate culture should be established for the ampulla, isthmus, and ampulla-isthmus junction.

The cellular material needs to be standardized, which would eliminate any experimental design drawbacks. This could be done by determining the cell numbers, percentage of dead/live cells and DNA content, to standardize the amount of live cells per well and treatment. Thus, optimization using StemPro® Accutase® Cell Dissociation (ThermoFisher Scientific, USA) protocol, to dissociate the explants and singularize the cells for the EVE automatic cell counter, could be suggested for this purpose.

Moreover, in the present study, cell explants were not stained, which ruled out a confirmation of cell structure and histology of the EOEC. We could not assess the nature of all cells within explants, other than concluding subjectively based on visual observations using the microscope. Immunochemistry would be another aspect to be considered and included within the experimental design, warranting a confirmation of protein localization in the EOEC.

In our study, we did not consider the E2 or luteinizing hormone (LH) status in plasma or fluids, which could have certainly been of interest. The hormone levels of E2, P4, LH and leptin, therefore need to be measured in the plasma and reproductive tissues of interest, with a new protocol that has to be established. A strong correlation between the plasma and reproductive tissues is expected and has to be validated. If available, the oviduct fluid could also be included in the hormonal analysis if levels are above the detection level using currently available methods. The same is true for cell culture supernatant, which would overall provide valuable additional information and further strengthen the project design and knowledge about the hormonal levels within all the matrices of interest.

Another technical issue is the culture medium composition, since in the present project two batches of EOEC were exposed either to double or only to half of the previsioned stimulation medium, respectively, resulting in unstandardized culturing of a different number of explants, producing ambiguous results. One of the datasets had to be divided by half in order to reflect the applied double concentration factor (i.e. around 40 – 50 explants stimulated instead of 20) and the other had to be multiplied by half as explants were exposed to 1 ml medium instead of 0.5 ml as per standard protocol (i.e. thus being double less exposed to stimulation medium), which most likely biased the outcome due to dilution errors.

Some of the target genes did not perform well on Fluidigm platform for different reasons, among them a noted low expression level. These would ideally be exchanged for other genes that may provide additional information about the cell status, activation of specific PG synthesis pathways, and that may be related to fatty acid metabolism. A suggestion for additional genes to be investigated in this context and their functions is provided in Table 11. Further, antioxidative genes, such as CAT and the ones from GPX family, could be considered.

Table 11. Genes related to fatty acid (FA) and prostaglandin (PG) metabolism [298].

Gene	Function	Description
ACACA	FA synthesis	Catalyzes the rate-limiting reaction in the biogenesis of long-chain FA
PTGS1	PG synthesis	Catalyze the rate-limiting step of PG production and supports muscle contraction
CPT1A	FA metabolism	The mitochondrial oxidation of long-chain FA
DAGLA	FA synthesis	Involved in biosynthesis of the endocannabinoid 2-arachidonoyl-glycerol. Catalyzes the hydrolysis of DAG to 2-arachidonoyl-glycerol
FAS	FA synthesis	Catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated FA
FFAR4	FA receptor	Receptor for medium/long-chain free FA, *n*3 FA. Mediates robust anti-inflammatory effects, in macrophages and fat cells
P19 lipocalin	FA transport	Cytosolic FA binding protein family, important to maintain the pregnancy and transport small hydrophobic ligands from mother to the developing embryo
PLA2G2A	Phospholipid metabolism	Participates in regulation of the phospholipid metabolism in biomembranes, releasing free FA and lysophospholipids
PTGER2	PGE_2 receptor 2	Receptor for PGE_2, with relaxing effect on smooth muscle in the case of subsequent raise in intracellular cAMP
SCD	FA synthesis	Involved in FA biosynthesis, primarily of oleic acid; gives rise to a mixture of 16:1 and 18:1 unsaturated FA, regulating mitochondrial β-fatty acid oxidation
SLC27A1	Lipid metabolism	Regulation of lipid metabolism and long-chain FA-CoA ligase activity
SOCS3	Suppressors of cytokine signaling	Involved in negative regulation of cytokines that signal through the JAK/STAT pathway, inhibits signal transduction by binding to tyrosine kinase receptors including insulin and leptin receptors

Supplementary Table S4. Composition of the washing medium used for the EOEC culture.

Washing medium (HEPES)		
Basic solution		
1 M KCl 50 ml	(0.050 x 74.55 x 1)	3.7275 g
0,1 M NaH_2PO_4 50 ml	(0.050 x 120 x 0.1)	0.6 g
1 M $CaCl_2.2H_2O$ 50 ml	(0.050 x 147.02 x 1)	7.36 g
0,1 M $MgCl_2.6\ h_2O$ 50 ml	(0.050 x 203.3 x 0.1)	1.0165 g
Add water to 50 ml		
Basic stock		
NaCl	(114 mM)	6.666 g
KCl	(3.1 mM)	3.1 ml
NaH_2PO_4	(0.3 mM)	3 ml
$CaCl_2.2H_2O$	(2.1 mM)	2.1 ml
$MgCl_2.6\ h_2O$	(0.4 mM)	4 ml
Phenol red		2 ml
Bicarbonate $NaHCO_3$		0.1680 g
Pyruvate	(1 mM)	0.1100 g
Lactate	(36 mM)	4.0360 g or 3,0576 ml
Gentamycine		5.0 ml (stock 10 mg/ml)
Add water (WAS-TALP) to 1 liter		
HEPES		10 ml
BSA		0.4 g
Adjust osmolarity to 275 – 285		

Supplementary Table S5. Gene symbol, accession number, amplicon size and primer sequences for genes analysed using the Fluidigm platform.

Gene symbol	Accession number	Primer sequence (5'-3')		Amplicon size (bp)
		Forward	reverse	
ACTB	NM_001081838.1	TGATATCGCCGCGCTCGTGG	ATGCCCACCATCACGCCCTG	132
CTSL	XM_023627103.1	TGTCAAGAACAGCTGGGGTG	TTTATCACCCCCGAGTGAAGG	121
ERRFI1	XM_023635858.1	TGCAAGCACCCAAATCCAGCCA	TTACGCTTCACATGGCCGCCT	108
ESR1	XM_023632849.1	CCAGCAGGTGCCCTATTACC	TACCTCCCTGGCGTCGATTA	110
ESR2	XM_023627705.1	TCTGGAAATCTTTGACATGCTC	AGGATACATACTGGAGTTGAGG	118
FGF9	XM_005601094.3	ACGTCAGCTCCACTGTTGCCAAA	AAGCAAGTGGGCACAGGCAGT	129
FOS	XM_001491972.4	GCTCCAGGCGGAGACAGA	AGGGTGAAGGCCTCCTCAGA	231
GAPDH	NM_001163856.1	TTGCCCTCAACGACCACTTT	GGCTCTTACTCCTTGGAGGC	112
GJA1	NM_001309226.1	GTTGCCCAAACTGATGGTG	TAGGTTCTCAGCAAGCCCC	122
H3F3A	XM_005607971.2	CTCCACGTATGCGGCGTGCTAG	CTCCACGTATGCGGCGTGCTAG	146
HHIP	XM_001501985.6	TCCGGCTGGATGTGGACACAGA	AGCACATCTGCCTGGATCGTGGA	131
HSPA5	XM_023628864.1	GTGCCCACCAAGAAGTCTCA	TTGTCTTTCGTCAGGGGTCG	101
HSPB8	XM_001490413.4	AGCCCTGGAAAGTGTGTGTC	TTGCTGCTTCTCCTCGTGTT	110
IFI6	XM_023635180.1	GACCAGTGACAAAGCTTGCG	AGCCATATAGGTCAGCGTGC	173
INSR	XM_023644612.1	CGCAGACCCTTCGAGAAAGT	TGAAGCTCGATGCGATACCC	86
LEPR	XM_023641937.1	ACAATACTCGCGCTGTAGGG	TGCTCTGGTTAAGACTGCGG	116
OXTR	XM_014731360.2	AGCAACGTCAAGCTCATCTC	GAAAGGTGAGGCTTCCTTGG	133
PAQR5	XM_023654740.1	CGCACGTGCAGATGGAAGCCATA	CCGAGGCTGAAGACAAGGCACA	130
PGFS	XM_005606827.3	CCAGCTGCTTTTCAGTTCAAGC	AAGCTATGTGGAGGCTGTCTG	80
PLCG1	XM_005604632.3	CTCCTTTGAAGCTCGCTACC	CGACCGTCAAAATGGTCTG	81
PPARGC1A	XM_023638290.1	GGCTAGTCCTTCCTCCATGC	TTGGCTGGTGCCAGTAAG	93
PTGER4	XM_001499068.5	CGATGAGTATTGAGCGCTACC	AGCCCGCATACATGTAGGAG	134
PTGS2	NM_001081775.2	AAGGAAATGGCTGCGGAGTT	TCCACCAGAAGGGCAGGATA	77
S100A6	NM_001081841.1	CTTCCTTGGGGCTTTGGCTA	AGAGTGTGTCTCCACTTTGCC	79
SLC36A2	XM_005599277.3	ACCATCCCAGTTGAACCCCGTCT	TCAGCTTGACTGGAGCTGGGTCT	101
SLC2A1	NM_001163971.1	CCATGGATCCCAGCAGCAAGAA	CCGGCCAAAGCGGTTAACAA	278
SLC03A1	XM_005602811.3	GGTATCCTGTTCACGATGCT	TGATGTCCAGGTTACTTGTGTC	115
SOD1	NM_001081826.3	AGAGGCATGTTGGAGACCTG	CAGCGTTGCCAGTCTTTGTA	189
SOD2	NM_001082517.2	ATTGCTGGAAGCCATCAAAC	AGCAGGGGGATAAGACCTGT	192
SPINK7	XM_003362870.2	GCCACAGGTGGACTGCAGCATTT	CAGGTATGTGATGGGGCAGGGGAT	70
SYT5	XM_014730820.2	GGCGGGAAAAAGGTGCGGAAGAA	TCACAGGGCACCTCGAAGCTGA	95
UBB	NM_001081862.1	CGTCTCAGGGGTGGCTGTTAGC	TGGGGCTAATGGCTGGAGTGCA	86
VEGFA	NM_001081821.1	AAGAAAATCCCTGTGGGCCT	TTTAACTCAAGCTGCCTCGC	124
YWHAG	XM_023655413.1	TCCTGTCTTTGATCGCCTCT	GAACAGTCCATGTGCCAGTG	112

Supplementary Table S6. Relative change of PGE_2 (PGE_2/total RNA) and $PGF_{2\alpha}$ ($PGF_{2\alpha}$/total RNA) in the EOEC culture, with significance ($P < 0.05$) for treatment, time between and within treatment.

	Treatment											P-value between treatments		
	CTL		L2		L10		L100		L1000	OXT				
	6 h	24 h	6 h	24 h	6 h	24 h	6 h	24 h	24 h	6 h	24 h	at 6 h	at 24 h	at 6 + 24 h
n =	5	4	4	5	5	3	4	6	3	4	2			
PGE_2	38.7	41.3	37.9	43.1	45.1	83.6	94.6	40.5	63.3	57.7	92.9	0.1	0.2	0.4
SEM	9.78	11.1	10.4	10.2	10.9	25.1	33.1	9.5	9.1	11.5	57.7			
P-value	0.3		0.2		0.2		0.1		-	0.6				
n =	4	3	4	3	4	1	4	3	3	4	2			
$PGF_{2\alpha}$	1.03	0.43	0.34	0.73	0.64	1.97	0.87	0.99	0.79	0.67	0.96	0.5	0.3	0.7
SEM	0.46	0.18	0.12	0.25	0.21	-	0.32	0.27	0.37	0.16	0.65			
P-value	< 0.05		0.4		-		0.2		-	0.5				

Supplementary Table S7. Expression of targeted genes in the EOEC culture. The results are shown as fold change in relation to the control sample. Significance ($P < 0.05$) is presented as underline for comparison between 6 and 24 h of stimulation within a specific treatment, and between treatment effect at specific time is marked with a,b or x,y.

Gene name	Treatment [fold change]									P-value between treatments		
	L2		L10		L100		OXT		L1000			
	6 h n ≥ 4	24 h n ≥ 4	6 h n ≥ 2	24 h n ≥ 4	6 h n ≥ 4	24 h n ≥ 4	6 h n ≥ 4	24 h n ≥ 3	24 h n = 1	at 6 h	at 24 h	at 6 + 24 h
CTSL	1.1	1.1	0.8	1.1	1.0	1.0	1.1	1.1	0.9	0.8	1.0	0.8
ERRFI1	1.1	0.8	1.3	1.0	1.1	0.9	1.3	1.1	0.9	0.8	0.3	0.6
ESR1	1.0^{a}	$0.9^{x,a}$	1.0^{a}	$0.8^{x,a}$	1.0^{a}	$0.8^{x,a}$	0.9^{b}	$0.6^{y,b}$	1.2	0.7	< 0.01	< 0.01
ESR2	1.3	2.6	0.9	2.9	0.8	2.9	0.7	0.9	1.5	0.6	0.5	0.4
FGF9	1.0^{a}	$1.1^{x,a}$	1.3^{a}	$0.9^{x,a}$	1.2^{a}	$1.0^{x,a}$	1.8^{b}	$1.9^{y,b}$	0.8	0.2	0.05	< 0.05
FOS	1.4	0.9	0.7	1.0	0.7	1.0	0.8	0.8	0.9	0.2	0.7	0.9
GJA1	1.3	1.2	1.0	1.7	1.0	1.5	1.2	1.6	0.9	1.0	0.4	0.7
HSPA5	1.3	1.5	1.0	2.0	0.9	1.8	1.7	2.4	1.0	0.1	0.5	0.3
HSPB8	0.9	0.9^{x}	0.8	0.9^{x}	0.9	0.8^{x}	1.1	1.2^{y}	0.8	0.6	< 0.05	0.5
IFI6	0.9	3.8	1.0	3.2	0.7	2.6	0.9	2.8	0.5	0.7	0.9	1.0
INSR	1.0	0.9	1.2	0.9	1.0	0.7	0.9	0.7	0.9	0.8	0.1	0.1
LEPR	0.7	1.8	1.3	1.7	1.2	1.6	0.7	1.3	1.0	0.5	0.3	0.5
OXTR	1.1	0.9^{xy}	1.3	0.7^{x}	1.0	0.8^{x}	1.4	1.3^{y}	1.2	0.6	0.05	0.2
PAQR5	0.9	1.3	1.1	1.2	1.1	1.0	0.8	0.9	1.2	0.7	0.5	0.5
PGFS	0.9	1.3	1.6	1.3	1.4	1.1	0.8	0.7	1.2	0.6	0.3	0.5
PLCG1	1.5	1.3	1.3	1.9	0.9	1.6	1.2	2.1	0.9	0.7	0.6	0.4
PGC-1α	1.3^{a}	1.1^{a}	1.2^{a}	0.8^{a}	1.0^{a}	1.0^{a}	1.7^{b}	2.2^{b}	1.8	0.5	0.1	< 0.05
PTGER4	1.0^{a}	$0.7^{x,a}$	1.3^{a}	$0.9^{x,a}$	1.1^{a}	$0.8^{x,a}$	0.8^{b}	$0.4^{y,b}$	1.0	0.2	0.01	0.01
PTGS2	1.1	1.1	1.4	1.0	1.0	0.9	1.8	2.3	0.6	0.4	0.8	0.3
S100A6	1.1	1.2	1.3	0.9	1.0	1.0	1.0	0.9	0.9	0.5	0.7	0.8
SLC03A1	1.2	1.5	1.0	1.6	0.8	1.1	1.3	0.8	0.8	0.6	0.4	0.6
SLC36A2	0.9	1.7	1.5	1.4	1.2	1.2	0.8	0.8	1.8	0.6	0.2	0.3
SLC2A1	1.0	1.2	1.2	1.0	1.0	0.9	1.1	0.7	1.0	1.0	0.1	0.7
SOD1	1.0	1.6	0.5	2.2	1.2	4.5	1.3	2.0	0.9	0.5	0.9	0.8
SOD2	2.4	0.6	1.1	1.0	0.9	0.8	2.1	1.0	0.7	0.2	0.5	1.0
SPINK7	0.9	1.3	1.5	1.2	1.4	1.0	0.8	0.7	1.1	0.4	0.2	0.3
SYT5	0.9	1.1	1.2	1.1	1.1	1.1	0.8	0.9	1.1	0.6	1.0	0.7
VEGFA	2.4	0.6	1.1	1.0	0.9	0.8	2.1	1.0	0.7	0.2	0.5	1.0

[a,b]Effect of treatment at 6 + 24 h of stimulation
[x,y]Effect of treatment at 24 h of stimulation

Supplementary Table S8. Reference values for plasma leptin concentration in horses.

Value [ng/ml]*	Horse characteristics	Reference
0.6	Pregnant mares	[299]
0.8	Non obese	[300]
1.1	Mares with early embryonic death	[299]
1.9	Adult sport	[301]
2.2	Fat vs moderately conditioned mares from postpartum to second ovulation	[302]
2.3	Thin-supplemented	[65]
3.0	10 – 20 weeks postpartum mares	[303]
3.5	300 mixed light breed	[304]
6.9	Fat-restricted	[65]
10.5	16 crossbred naïve (7 geldings, 9 mares) aged 2 – 11 years	[305]
11.0	Obese and insulin-resistant	[300]

*using radioimmunoassay (RIA)

Chapter 5

Conclusive discussion and implications

Difficulties in determining early embryonic nutrient requirements

Fulfilment of the nutritional requirements of the early embryo in order to support pregnancy establishment is the task of foremost priority for the mother. Moreover, the histotroph environment, comprising oviduct (OF) and uterine (UF) fluid, is also indispensable for sperm capacitation as event preceding fertilization. With an increasing understanding of the role of the oviduct at cellular and molecular level, current research signifies the importance of the oviduct for naturally conceived fertilization and preimplantation embryo development [42]. It also promotes the application of these findings to optimize the *in vitro* assisted reproductive technologies. Improved knowledge about the nutrient profiles of the OF and UF in animals is therefore important to establish species-specific range values and references. However, to obtain a high quality of compositional data of the OF and UF collected *in vivo*, there are many challenges. These include the sampling itself, but also differences of the reproductive physiology between species which is reflected in different oocyte and embryo composition and nutrient requirements.

The metabolism of exogenous nutrients such as glucose, lactate, pyruvate, amino acids and fatty acids (FA), by the oocytes and preimplantation mammalian embryos is well described in the literature. The embryo in early stages is dependent on oxidative metabolism and this change to glycolytic metabolism at blastocyst stage. However, the exact requirements of each nutrient at each developmental stage are not completely known. In addition, the role of endogenous stores, particularly lipids, has been largely neglected [306]. Fatty acids are known to provide energy for the oocyte and embryo, but are as well essential components of the membrane lipids the size of which increases rapidly with each cell division, as observed in the increased concentration of linoleic acid in the human embryo in the later stages of preimplantation development [307]. Evidence from animal studies suggest that individual FA may affect the oocyte maturation, fertility and embryo development. Furthermore, a specific FA composition was noted for pig oocytes with intact zona pellucida that was greater than that in cattle and sheep oocytes, confirming a species-specific difference [308]. This finding underlines differences in the sensitivity of oocytes and embryos of different species to handling (i.e. chilling, culture, cryopreservation) and using assisted reproductive technologies (i.e. *in vitro* culture, *in vitro* maturation, intracytoplasmic sperm injection. Similarly, concentrations of

amino acids differ among murine reproductive and follicular fluids *in vivo*, with highest levels found in the OF, and nutrient profiles differing from those of common medium used for *in vitro* murine maturation, fertilization and embryo culture [108]. The observations from our study are in accordance with the assumed dynamic composition of the oviduct histotroph environment with cycle stage. We found that specific FA in the OF of the mare are present in prominent concentrations during early luteal compared to late luteal phase, and no changes were observed for the UF between cycle stages. A changing FA metabolism was also well described in preimplantation human embryos that actively take up individual FA at different rates at different stages of development [307]. Using proteomics as an approach, Maloney et al. [309] showed that also the protein abundancy in the UF of mares significantly differs between estrous and diestrous, notably of ones involved in endometrial defence system (e.g. inflammation, immunity and antimicrobial activity) during the estrous cycle. As well, the presence of a conceptus (and the preovulatory estradiol concentration), was shown to influence the glucose and protein concentrations in the UF of cows [310]. Apart from modifying the composition of the UF, the cyclical changes in the plasma also define the UF volume [290, 311]. In mares, the type and amount of uterine protein fluctuates depending on the reproductive status (i.e. estrous, diestrous and early pregnancy), with certain changes consistent with predicted functions of the proteins identified [312]. Considering that the transport of most FA is rapid and spontaneous across membranes and that the FA enter cells by a viable mechanism of free and passive diffusion, it is suggested that the uptake is controlled only to a certain extent [313]. Changing the ratio of specific FA in the diet *in vivo* and in the culture medium *in vitro* can thereby cause significant dysregulation of cellular processes and embryo development, respectively [314].

Bearing in mind the large differences between species, and thereby different embryo needs, there is no generally ideal environment for the developing embryo across species. Therefore, there is a need to investigate species-specific histotroph composition and how it can be influenced by different endogenous and exogenous environmental factors. By that, knowledge of the histotroph dynamics in relation to its environment remains a task of high priority for understanding and eventually reducing the rate of early embryo loss.

Formation and composition of the oviduct and uterine fluid in the horse and the sheep

The epithelial cells lining the oviduct and endometrium can be regarded as the final barrier that links nutrient supply by the maternal diet at one end and embryonic nutrient supply at the other. Still, as described by Leese et al. [315] little is known about the mechanisms that underlie

the formation of the respective OF and UF. Aviles et al. [316] highlighted that the OF is a complex fluid formed by components secreted from epithelial cells and transudate from blood plasma (BP), containing many metabolic components, whose respective concentrations often differ from those of the UF and BP. For example, Hugentobler et al. [317] observed different concentrations and associations between the energy substrates in the OF, UF and BP in cows and suggested a differential regulation of their secretion by the oviduct and the uterine epithelium. Iritani et al. [318] described that the OF in sows has significantly lower concentrations of dry matter and protein compared to the UF. Moreover, a study of Faulkner et al. [319] using proteomics, identified different proteins including metabolic enzymes, some with antioxidant activity and some involved in modulation of the immune response, to confirm a dynamic nature of the bovine UF composition and its peculiarities in relation to the BP. Overall, the histrotroph contains, apart from the formerly mentioned FA, substrates providing energy (i.e. glucose, lactate, pyruvate), ions, growth factors, amino acids, prostaglandins and steroid hormones, and this with great species-specific differences [320]. Velasquez [321] stated that the analysis of the luminal fluid microenvironments in the reproductive tract is pivotal to elucidate the embryo-maternal signalling mechanisms, as well as preimplantation embryo nutrient supply responsible for a successful reproduction.

The underlying reasons for the insignificant number of studies in this field can partly be related to the complexity and lack of non-invasive techniques of *in vivo* sampling, notably the OF. On the other hand, the BP from animals is easily accessible, but still lacks validated reference ranges. The need for establishment of such ranges is highlighted, but the comparison between the studies is largely limited because of different analytic methods and a great range of values obtained [322]. In our study, the FA profiles in the equine and the sheep OF and UF were for the first time characterized in relation to BP by gas chromatography combined with mass spectrometry. Khandoker et al. [323] described the FA content of the reproductive fluids in rats to be quite similar to that of the BP. In another study using rabbit as a model, it was indicated that in the OF and UF the constituents of FA are reflected, and it was suggested that the FA may be originating from the BP [163]. Moreover, Jordaens et al. [324] did not observe a difference between the non-esterified FA (NEFA) in the BP and OF, indicating a positive correlation and mirrored concentrations between the two matrices. Added, Aguilar [320] suggested that the concentration of nutrients in the OF to be generally below the BP concentrations, advocating overall transport of nutrients across the oviduct via diffusion. In our studies we could confirm a descending order in the absolute FA concentration between the BP, UF and OF, respectively, but in contrast, the composition of FA was different between the BP

and both the OF and UF. This was in line with the findings of Wang et al. [325] in mice, where most FA concentrations in blood serum did not correlate with those in the OF or UF. The FA proportions in the BP and follicular fluid were neither correlated for any FA either in cows [326]. Still, the FA profile and the content of the oocytes and embryos was quite similar to that of the reproductive fluids in rats and rabbits [323]. The FA profile is mostly expressed as percentage of total FA, but in our study, the reported absolute concentrations in the BP and reproductive fluids deserve particular attention. We observed that linoleic (LA), stearic (SA), oleic (OA) and palmitic (PA) acid were the four most prevalent FA in both the BP and reproductive fluids, and the latter contained higher concentrations of arachidonic (AA), eicosapentaenoic and dihomo-γ-linolenic acid (DGLA). For example, in pigs, cows and sheep oocytes, PA, SA and OA, were also the most prominent FA, whereas LA and AA were the most abundant among the polyunsaturated FA, notably for pig oocytes [308]. In our studies, the derivatives of LA were more abundant in the reproductive tissues and fluids as compared to BP and other tissues, as of their role as prostanoid precursors substrates indispensable during the periconceptional period for both the embryo and the mother.

In our study, using the P4 levels as a cycle stage marker, we did not observe any major cycle stage effect on the OF and UF FA concentration in mares, and a significant reduction was observed only for the concentration of OA and PA in the OF between the early and late luteal phase, respectively. As described, both OA and PA are required as substrates in embryo development [141]. Taking into consideration that the embryo is in the oviduct during the early luteal phase, we could assume that increased presence of these FA during this stage ensures a sufficient supply for the growing embryo.

Overall, comparisons between species indicate that the amount and type of lipids present in the oocytes and the embryos of mammals vary considerably [306]. Therefore, we could speculate about an enhanced transport of specific FA from the BP to OF or a higher secretory activity during early luteal phase. Our collective findings suggest distinctive FA profiles of the reproductive tissues and fluids, reflecting species-specific regulation of the uterine and oviduct secretions. As mentioned above, this makes any inter-species comparison rather speculative. Overall, the results provide new knowledge and serve as a basis for further optimisation of culture media in relation to specific FA requirements of the oocyte and the early embryo.

Maternal nutrition and reproductive success

The FA profiles of the lipids in the heart, lung and brain of sheep were shown to be dependent on both dietary and genetic factors in a differentiated way [327]. This effect was further reflected in the endometrium tissue, as shown by Giller et al. [47]. In our sheep study, we did not supplement specific FA, but phenolic compounds, and by that aiming to observe a protective effect of these compounds on the FA to escape ruminal biohydrogenation. Moreover, polyphenols were also described to influence the phospholipid pattern and lipid metabolism, for example in muscles in rats [328]. Our data show that maternal metabolic conditions, associated with supplementation of phenolic compounds, might modify the FA proportions in the reproductive fluids to some extent, but the underlying mechanism is still to be elucidated. Our study showed a distinct FA profile of different reproductive organs and fluids as well as other body tissues, supporting the assumption that specific FA pathways are involved in the periconceptional period. This can be used as a source of information for new research. As reasons for the only weak influence found we suggest either that, the phenol dosages were too low, or that the reproductive organs act as a protective barrier from the environmental influences. This would make these organs more resilient to possibly adverse dietary changes and thus to provide nutrients always adapted to the needs of the embryo. An indication for this is that other body tissues were found to be more variable in the FA profile in response to the diet-supplementation with phenolic compounds.

The dietary phenols did not affect the expression of genes related to lipid metabolism, which was in accordance with some studies in lambs [240, 246, 247], but on contrary to mice and chicken [231-233]. This may suggest a specific gene regulation dependent on the species and level of supplementation. Further evaluation of the effect of different nutritional strategies, and in particular, polyphenol supplementation, on follicular development, oocyte maturation, embryo development, and subsequent offspring, is therefore indicated.

Another type of maternal nutrition could be important for reproductive success. Maternal obesity resulting from overnutrition and defined by increased circulating triglycerides is associated with increased inflammation and endoplasmatic reticulum (ER) stress in mares' endometrium [329]. Sessions-Bresnahan et al. [329] described as well an altered transcript abundance in embryos, especially associated with the trophectoderm, for genes related to lipid homeostasis and mitochondrial, oxidative, and ER stress. In addition, embryos from obese mares were found to have decreased concentrations of lipids important for cell signalling and membrane integrity [85]. Sessions-Bresnahan and Carnevale [330] demonstrated that the intrafollicular environment in the mare is influenced by metabolic diseases and reported this

finding as well in several species including rats, bovine and women. Thus, abnormal regulation of adipokines is implicated in the development of metabolic disorders [331]. In the present doctoral project, the short-term culture of equine oviduct epithelial cells was performed to investigate a possible effect of the obesity-related hormone leptin on the oviductal epithelium and related prostanoid synthesis. Leptin stimulation did not affect the hormone synthesis and that of gene expression only to a limited degree, but our results may encourage and serve as a useful guideline for any similar future research.

Outlook – Future perspectives on the research concerning the importance of fatty acids and polyphenols in animal reproduction

Since intensification of animal husbandry, nutrition and breeding management ever more hold as the key pillars within the field. Improved knowledge of metabolism and physiology of reproduction are decisive drivers for the development of optimized nutritional programs and improved assisted reproductive technologies, respectively.

The present doctoral project was initiated in accordance with the current animal husbandry and research demands. It is well known that malnutrition, and overnutrition in particular, bring not only the human population but also domestic animals into situations where the organisms' energy balance is disturbed. Unfortunately, this trend is evident worldwide and has been increasing during the last decades. Effectively, obesity leads to different metabolic disturbances and diseases likewise in humans and domestic animals. In addition, sportive careers of mares are responsible for late introduction of these animals into breeding, which adds to the problem horse breeding programs are facing. It is speculated that these individuals will experience reproductive disorders more easily and more often than younger mares. Sheep on other hand experience the contrary and the genetic interval pressure imposes early breeding in the ewes, soon after the onset of cyclicity, which can even be artificially provoked. Together, these situations often result in suboptimal fertility performances. Taking into account the constant scientific efforts to describe and understand the early embryo loss and low rates of *in vitro* fertilization success, our results should gain broad acknowledgment and recognition. Moreover, our *in vitro* model may serve as a promising tool to encourage scientists with similar research interests to explore the underlying mechanisms of reproductive issues related to the oviduct environment the early embryo is encountering.

The inability to diagnose pregnancy in mares before day 7 by uterine flushing and more commonly before day 12 after insemination by ultrasound remains a major cause for scarce understanding of the early embryo loss possibly occurring in commercial studs before day 12 after fertilization. Therefore, the complete FA profile in the reproductive fluids for the first time described in the mare and the sheep serves as a valuable basis for any follow up studies, clinical practice, or on a long run, adds as a parameter for defining the FA range for the culture medium commonly used with assisted reproductive technologies.

The experiment that included phenol-supplemented sheep aimed to serve as a scalable model to test for the possible modifications of FA profile in the reproductive fluids and tissues as phenols are known to modify the ruminal and metabolic FA metabolism. The reasoning was to explore the possibilities of altering the histotroph and tissue FA composition, with regards to providing specific FA to support early embryo growth and thereby pregnancy establishment. The supplementation for 10 weeks modified the FA profile in tissue lipids and fluids to some degree, implying as a potential strategy to modify the histotroph composition via dietary means, even though it is still far from being a commercially viable solution. Supplementing specific FA may be another suggested strategy that needs to be tested.

Despite the set of new information on reproductive physiology and nutrition of the horse and the sheep obtained with the present doctoral project, it has to be stated that not all the hypothesized mechanisms were confirmed as expected and still have to be described in other species. Therefore, there is a need for further exploration and conditions to be tested in order to validate the findings presented here and to apply optimized techniques for sample collection and analysis, which should further engage the industry and research interest.

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Scientific contributions

Journal items

B. Drews*, V. Milojevic*, K. Giller, S.E. Ulbrich. Fatty acid profile of blood plasma and oviduct and uterine fluid during early and late luteal phase in the horse. Theriogenology 114 (2018) 258-265. https://doi.org/10.1016/j.theriogenology.2018.04.003
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V. Milojevic, S. Sinz, D. Chiumia, M. Kreuzer, S. Marquardt, K. Giller. Partitioning of fatty acids into tissues and fluids from reproductive organs of ewes as affected by dietary phenolic extracts. In preparation for publication in Theriogenology.

Conference contributions

V. Milojevic, A. Rudolf Vegas, L. Wimel, C. Dubois, M. Robles, P. Chavatte-Palmer, K. Giller, S.E. Ulbrich, B. Drews. The impact of nutritional status on histotroph composition during the periconceptional period in mares. 50th Annual Conference of Physiology and Pathology of Reproduction and 42nd Mutual Conference on Veterinary and Human Reproductive Medicine, Munich, Germany, 15th - 17th February 2017. Reproduction in Domestic Animals 52 (S1) (2017) 37. https://doi.org/10.1111/rda.12909

V. Milojevic, B. Drews, S.E. Ulbrich. Environmental impacts on the maternal periconceptional reproduction tract in ungulates. Symposium for new PhD students. Institute of Agricultural Sciences (S.E. Ulbrich, R. Kühne, E. Ravelhofer, eds) (2015)

Poster presentation

V. Milojevic, S.E. Ulbrich, B. Drews. Fatty acid composition of uterine fluid and blood plasma during the oestrus cycle in the mare. ETH-Schriftenreihe zur Tierernährung (M. Kreuzer, T. Lanzini, A. Liesegang, R. Bruckmaier, H.D. Hess, S.E. Ulbrich), Band 40 (2017) 203-205.

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B. Drews, A. Rudolf Vegas, V.A. van der Weijden, V. Milojevic, A.K. Hankele, G. Schuler, S.E. Ulbrich. Do ovarian steroid hormones control the resumption of embryonic growth following the period of diapause in roe deer (*Capreolus capreolus*)? Submitted in General and Comparative Endocrinology.

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Acknowledgments

Professional

This research would not be possible without the Swiss Government Excellence Scholarship that I was awarded for the period from 2014 to 2017 and thereby I thank everyone involved in supporting my application and during the years when I was a scholarship holder. A special mention goes to Sandra Zweifel who continued supporting me beyond this time. It was indeed great to have such a support when integrating Switzerland.

Enormous efforts were made by different people to collect and analyse the samples. I want to express my gratitude to Pferdemetzgerei Michel AG from Felben-Wellhausen, and Pferdemetzgerei S. Baumgartner from St. Gallen, for their kind assistance and providing the samples for our first and third study. The animal caretaker team at former experimental station of ETH at Chamau is appreciated for all the work in relation to sheep used for the experiments. The abattoir of the University of Zurich and their kind staff are thanked for the assistance provided with the slaughter of sheep. We thank our colleagues from the Functional Genomic Centre Zurich, particularly Dr. Stefan Schauer and Dr. Endre Laczko, for assistance and expertise on FA analysis by the GC-MS, and Carmen Kunz and her team, as well as Nicole Gredig, from the Animal Nutrition group for the same with FA analysis and GC interpretation. Data produced and analysed in relation to gene expression using Fluidigm platform were generated in collaboration with the Genetic Diversity Centre, ETH Zurich. Finally, I need to thank Dr. Pascale Chavatte-Palmer and Dr. Morgane Robles for their input and expertise in horse reproduction and nutrition.

Personal

As one says “congratulation to all connections”, this very much applies to my doctoral project. I started this adventure with sort of “roll the dice” moment, applying last minute with the project, on the way to visit my family and a wedding of a dear friend in Serbia. So, was it a coincidence, destiny, or my intention? Certainly everything a bit.

This is once in a lifetime chance and an adventure for which I am eternally grateful. Still and more than anything, I am thankful to my family and friends, but nonetheless my ancestors, for the strength, courage, energy and wisdom, to accomplish the project – Dr. sc. ETH Zurich.

First and most, I have to thank my supervisors, Prof. Dr. Michael Kreuzer and Dr. Katrin Giller, for recognising my capacities and potential, offering me the chance to work together, and for their generous support, input and knowledge. This project would never be possible

without you. On the other side of this supervision, I have to thank Prof. Dr. Susanne Ulbrich for opening me the door at ETH, and Dr. Barbara Drews for her scientific input, friendship and everlasting encouragements. Prof. Dr. Thomas Peter was the person who was attentive and I am extremely grateful for the things we actually achieved. Within these lines, huge thanks certainly goes to my colleagues that made my every day at work seem as a gathering of good old friends. Vera, here you must be in the first place, same as you are as a researcher to me. An excellent scientist who brought the entire group forward. You are an admirable friend and I will never let the wisdom of yours out of my life. Hisham you are someone with whom I get along extremely easy, we share culture, passion for food and good life, and I trust you for everything. A true friend. Besides, you are a couple with Alba, another important person on this journey. We went hunting together for so many times and if anything, we enjoyed talks and nature at its best. Daniel you will stay as an example for positive vibes and spirit that helped us overcome all the stressors out there. Jochen was always ready to grab a cup of coffee and discuss whatever you can imagine. I learned from him about German culture and I am thankful for that. So many of you others passed by and I thank you as well, and without order – Corina, Anna, Elisa, Susanne S., Sandra, Mara, Sophie, Doro, Madeleine, Michael Broger and his family, and many others. *Summa summarum*: thank you all the members of the Animal Physiology and the Animal Nutrition group. I did not have the time to leave one group, but neither the time to be properly introduced into the other, yet I am lucky to have been sharing my time between the two. Still, some others deserve to be particularly praised. Susanne Meese you become a friend very early and I am happy that we also professionally collaborated at Swissgenetics. I appreciate your support, positive attitude and energy. Professionally, we are indeed very similar. In Madeline Koczura I found the long missing French spirit in Zurich, but she was more than that – she had Slavic origins and attitude. Thus, a great mix for a great friendship. Now the couples: Carmen & Stefan – they were there for me from the beginning and I am happy for them becoming proud parents; Eleni & Nikos – we spent great times together and it is a wonderful friendship I want to keep on; Shiva & Matti – you got my attention from the first moment we met and I enjoy spending time with you; and Nina & Hape – my landlords who trusted me since arrival in Switzerland and made me feel cosy and like home. Nicole Bollier and Kenneth Kronenberg are the people who I must particularly thank for my time in Zurich, as they were supportive and caring to my needs and I hope to have repaid this by doing a good job with their horses. I crossed the path with Malin Olson at some point, and this was an encounter designed by destiny – a great friend and a horse professional. Along comes Max Plank, a person that shares a lot of business knowledge, advices, but as well friendship.

Long time ago I met Prof. Dr. Dejan Škorjanc who told me I have it all to become a researcher – a talent above all, and encouraged me to engage in a scientific career. Well, I did it and thank you for igniting the sparkle. Christophe German is a personality I must thank with all my heart, as he was the one who shaped my attitude and lifestyle in much of what you see today. Merci cher Christophe, je n'oublierai jamais votre générosité. Vous êtes mon père professionnel.

Even though we decided to go separate ways, I must thank you Tanja for all the support and friendship over the long years. The attention and support of your family was particularly appreciated, but most of your brother and my lifelong friend Tićo. I wish you all the best. Hvala.

What to write about Anja, Esma and Jure. This trio, with me a quartet, is the team that wins everything out there. We meet as scholarship fellows, and I will never forget this moment. We spent countless days together and now we become friends for life. Long pages are needed to describe the true friendship of ours. It is out of range. Živeli moji najdraži Anja, Esma i Jure.

Now my most beloved part – the one and only Maša. And Viktor. They are all what one needs to be happy. A life role model for everything you can imagine. I am lucky enough to share my life, time and dreams with you. Bringing projects together is a great privilege and you can always count on me. I love you. Both. Volim te puno, Maša moja. I Viktora volim. Čuvajmo se. Naš susret je bio sve samo ne slučajnost. Hvala ti što si ušla u moj život i dala mi vetar u leđa, a učinio sam sve da ti uzvratim istom merom jer našli smo se baš kad je trebalo. Zahvalan sam ti na svemu što proživljavamo zajedno i jako sam ponosan na nas. Budućnost i neki novi projekti, lepi i srećni, su pred nama. Hvala tebi i tvojoj porodici na svemu.

The family. Never and under no other conditions this would be possible without my parents Klara and Milan who teached me almost everything in my life, but they added a special ingredient to it – parental love. My brother Saša and his wife Ivana, with their kids Aleksa and Andrej, were always out there to support me. My eternal gratitude goes to my grandparents Kata and Gustika, Vida and Janko, who were always curious, full of encouragement and shared unconditional energy. Indeed, I dedicate the thesis to all of them and my love Maša.

Hvala svima koji su bili tu, za mene, na ovom putu. Prvenstveno hvala mojim roditeljima, Klari i Milanu i bratu Saši, na bezuslovnoj podršci i ljubavi. Ne mogu a da ne pomenem i beskrajnu energiju mojih baba Kate i Vide, kao i deda Gustike i Janka, koji nisu više sa nama. Ponosim se svima nama kao i onim što smo kao porodica učinili. Ova moja teza je skromni doprinos tome. Posvećujem ovaj rad svojoj porodici i mojoj Maši.