Deciphering the Embryo-Maternal Dialogue in the Horse Using an Oviduct Explant Model

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Science is not a sacred cow.
Science is like a horse.
Do not worship it, feed it.

Abba Eban, Israeli statesman

CONTENT

CONTENT **1**

LIST OF ABBREVIATIONS **3**

CHAPTER 1 GENERAL INTRODUCTION: **7**

1. EQUINE REPRODUCTION AND PREGNANCY IN A NUTSHELL 8

2.THE MARE'S OVIDUCT 11
- 2.1 Unique features of the equine oviduct 11
- 2.2 The architecture of the oviduct 11
- 2.3 Steroids: key regulators of the oviductal environment 14

3.EQUINE EMBRYO DEVELOPMENT *IN VITRO* 23
- 3.1 Introduction 23
- 3.2 Embryo culture media 23
- 3.3 Oxygen concentration 29
- 3.4 Macromolecules and protein source 29
- 3.5 To renew or not to renew 30
- 3.6 Co-culture 30
  - 3.6.1 Figures and facts about the good, the bad and the ugly 30
  - 3.6.2 The equine embryo and co-culture 35

4.REFERENCES 38

CHAPTER 2 AIMS AND OUTLINES OF THE THESIS **57**

CHAPTER 3 OVIDUCT EXPLANTS: AN EXCELLENT TOOL TO STUDY EARLY REPRODUCTIVE EVENTS IN THE HORSE **59**

PART 3.1: Equine oviduct explant culture: a basic model to decipher embryo-maternal communication 60

PART 3.2: The influence of foetal bovine serum and insulin-transferrin-selenium on oviduct explant viability 93

CHAPTER 4 OF STEROIDS IN THE OVIDUCT *IN VIVO* **107**

CHAPTER 5 OVIDUCTAL FUNCTION BEARS THE STAMP OF STEROIDS AND EMBRYOS *IN VITRO* **159**

PART 5.1: Of steroids and genes in oviductal explants *in vitro* 160

PART 5.2: Of embryos and oviductal genes *in vitro* 207

CHAPTER 6 THE DERBY OF *IN VITRO* EQUINE EMBRYO PRODUCTION: THE RACE TOWARDS THE IMPROVEMENT OF CULTURE CONDITIONS 233

CHAPTER 7 GENERAL DISCUSSION 243

1 EQUINE OVIDUCTAL EXPLANTS 244
1.1 An excellent tool to study early reproductive events 244
1.2 Marginal notes of limitations 247
1.3 Dark cell degeneration 249

2 STEROIDS IN THE EQUINE OVIDUCT 253
2.1 Figures and facts 253
2.2 A weighty role for steroids, conductors of oviductal functionality 254

3 OVIDUCTAL GENE EXPRESSION BEARS THE STAMP OFSTEROIDS AND EMBRYOS 257

4 FINAL CONCLUSIONS 264

5 PROSPECTS FOR THE FUTURE 265
5.1 *In vivo/ex vivo* models 265
5.2 *In vitro* modelling 265

6 REFERENCES 270

SUMMARY 317

SAMENVATTING 321

ACKNOWLEDGEMENTS DANKWOORD 325

CURRICULUM VITAE 333

BIBLIOGRAPHY - BIBLIOGRAFIE 335

LIST OF ABBREVIATIONS

18S	18s Ribosomal RNA
1D-(SDS)-PAGE	1D- (Sodium Dodecyl Sulfate) Polyacrylamide Gel Electrophoresis
3-Beta-HSD	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 1
A2M	Alpha-2-macroglobulin
AC	Adenylyl Cyclase
ACTB	Actin, Beta
AIF	Apoptosis Inducing Factor
AIJ	Ampullary Isthmic Junction
ALB	Albumin
ANOVA	Analysis Of Variance
ANPA	Atrial Natriuretic Peptide
APAF1	Cytoplasmic Adaptor Protein
APES	3-Aminopropyl-Triethoxysilane
BLAST	Basic Local Alignment Search Tool
BOECM	Bovine Oviduct Embryo Culture Medium
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CMRL 1066	Con-Naught Medical Research Laboratories 1066 Medium
CQ	Quantitation Cycle
CR1AA	CR1 Medium With Amino Acids
CSF1	Colony Stimulating Factor
CYP11A1	Cytochrome P450scc
CYP19A1	Aromatase
CYTC	Cytochrome C
CZB	Chatot, Ziomek & Bavister Medium
DABCO	1,4-Diazabicyclo (2.2.2) Octane
DCD	Dark Cell Degeneration
DHEA	Dehydroepiandrosteronsulfaat
DMBT1	Deleted In Malignant Brain Tumor 1
DMEM/F-12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DPBS	Dulbecco's Phosphate-Buffered Saline
DUTP	2'-Deoxyuridine 5'-Triphosphate
ECG	Equine Chorionic Gonadotropin
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ENDOG	Endonuclease G
EOEC	Equine Ovidutal Epithelial Cell Culture
FBS	Foetal Bovine Serum

(continued)

FGF-4	Fibroblast Growth Factor-4
FITC	Fluorescein Isothiocyanate
FSH	Follicle Stimulating Hormone
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GLUT1	Glucose Transporter 1
GM-CSF	Granulocyt-Monocyt Colony Stimulating Factor
HCG	Human Chorionic Gonadotropin
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HGF	Hepatocyte Growth Factor
HIF1A	Hypoxia Inducable Factor, 1a Subunit
HSD3B	Hydroxy-Delta-5-Steroid Dehydrogenase, 3 Beta- And Steroid Delta-Isomerase 1
HTF	Human Tubal Fluid
HTRA2	High Temperature Requirement Protein A2
IAPS	Inhibitor Of Apoptosis Protein
ICAT	Strong Cation Exchange Column
ICSI	Intracytoplasmic Sperm Injection
IGA	Immunoglobulin A
IGF1	Insulin-Like Growth Factor
IL	Interleukin
ISTD	Internal Standard
ITRAQ	Isobaric Tag For Relative And Absolute Quantitation
ITS	Isulin-Transferin-Selenium
IVF	*In Vitro* Fertilization
LH	Luteinizing Hormone
LIF	Leukemia Inhibitory Factor
MAPKAPK	MAP Kinase Associated Protein K
MB2	Menezo’s B2 Medium
MEM	Minimum Essential Medium
MIQE	Minimum Information For Publication Of Quantitative Real-Time Pcr Experiments
MMP2	Matrix Metalloproteinase 2
MMPS	Matrix Metalloproteinases
MRNA	Messenger Ribonucleic Acid
NADH	Nicotinamide Adenine Dinucleotide
Nano-LC/MSMS	Nanoscale Liquid Chromatography Coupled To Tandem Mass Spectrometry
NCBI	National Center For Biotechnology Information
NOS	Nitric Oxide Synthase
OEE	Oviduct Epithelial Explants
OSGS	Oviductal Specific Glycoprotein
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction

(continued)

PDGF	Platelet-Derived Growth Factor
PDI	Protein Disulfide Isomerase
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2alpha
PI	Propidium Iodide
PLA2	Phospholipase A2
PLAU	Urokinase Plasminogen Activator gene
PMSG	Pregnant Mare Serum Gonadotrophin
PR	Progesterone Receptor
PTGER	Prostaglandin E Receptor
PVA	Polyvinylalcohol
PVP	Polyvinyl Pyrrolidone
PZM	Porcine Zygote Medium
RBP	Retinol Binding Protein
RIA	Radioimmunoassay
ROS	Reactive Oxygen Species
RPL32	Ribosomal Protein L32
RPMI-1640	Roswell Park Memorial Institute
RT-PCR	Real-Time Polymerase Chain Reaction
SDHA	Succinate Dehydrogenase Complex, Subunit A
SEM	Standard Error Of The Mean
SERPINE1	Serine (Or Cysteine) Peptidase Inhibitor, Clade E, Member 1 = Pai1
SGLT	Sodium/Glucose Symporter
SLC2A1	Solute Carrier Family 2, Member 1 = Glut1
SOF	Synthetic Oviductal Fluid
SPE	Solid Phase Extraction
STAR	Steroidogenic Acute Regulatory Protein
TALP	Tyrode’s Albumin Lactate Pyruvate
TCM199	Tissue Culture Medium 199
TEM	Transmission Electron Microscopy
TGFA	Transforming Growth Factor Alpha
TGFB	Transforming Growth Factor Beta
TIMP1	Tissue Inhibitor Of Metalloproteinase 1
TIMPS	Tissue Inhibitors Of Metalloproteinases
TK	Tyrosin Kinase
TNF	Tumor Necrosis Factor
TUBA4A	Tubulin, Alpha 4a
TUNEL	Terminal Deoxynucleotidyl Transferase-dUTP Nick End Labeling
UBC	Poly-Ubiquitin C
UHPLC/MS-MS	Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometry
uPAR	Urokinase Plasminogen Activator Receptor
UTJ	Uterotubal Junction

(continued)

VEGFA	Vascular Endothelial Growth Factor A
VEGFR	Vascular Endothelial Growth Factor Receptor
Z-VAD-FMK	Carbobenzoxy-Valyl-Alanyl-Aspartyl-[O-Methyl]-Fluoromethylketone

CHAPTER 1: GENERAL INTRODUCTION

A part of this chapter has been adapted from :

The derby of *in vitro* equine embryo production: an overview of the race towards the improvement of culture conditions

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Ready to submit.

Aut numquam tentes, aut perfice.

Begin niet of zet door.

Ovidius, Roman poet

1 EQUINE REPRODUCTION AND PREGNANCY IN A NUTSHELL

The mare is a poly-oestrous mono-ovulatory seasonal breeder. After fertilization in the ampulla, the zygote embarks on a series of cell divisions that results after 5-6 days in the formation of a compact morula, consisting of blastomeres surrounded by trophoblast cells. These trophoblast cells initiate cavitation or blastocoele formation **(Fig. 1),** resulting in a blastocyst which arrives in the uterus at day 6 post fertilization. In the uterus, a number of important differentiating events take place in the horse conceptus, such as capsule formation, hatching from the zona pellucid, followed by rapid expansion and further differentiation events including gastrulation and subsequent organogenesis (Betteridge 2000). Between day 6, i.e. upon arrival in the uterus, and day 22 after fertilization, the embryo is enveloped in a closely fitting glycocalyx capsule (Betteridge, et al. 1982). As a result, the equine embryo is unable to rearrange and elongate its trophectoderm like its ruminant and porcine counterparts. The spherical embryo migrates completely unattached in the uterine lumen from one horn to the other, propelled by peristaltic myometrial contractions. In the absence of an embryo, the equine uterus produces a potent luteolysin (prostaglandin F2alpha) (Douglas & Ginther 1976) that reaches the ovary through the systemic circulation in order to destroy the corpus luteum at about day 14 of the cycle (Neely, et al. 1979). When an embryo is present, luteolysis must be blocked because the corpus luteum, through its hormone, progesterone, is vital to embryo development, (Sharp 2000) since it stimulates the endometrium to produce appropriate histotrophe which nourishes the developing embryo. The phenomenon of embryo mobility in the mare allows the relatively small embryo to contact the complete uterine mucosa, which is essential for successful pregnancy recognition and maintenance in the mare. The mobile embryo blocks luteolysis (Mcdowell, et al. 1988, Sharp 2000), resulting in elevated progesterone levels and the provision of uterine secretions essential as a nutrient before the formation of a definitive placenta (Spencer, et al. 2004). The gradually increasing uterine tone and decreasing uterine diameter, together with increasing conceptus diameter causes the conceptus to be lodged at the base of one uterine horn around day 16, a phenomenon which is called fixation (Ginther 1995).

At about days 36-38, foetal tissues along the chorionic girdle begin to invade the endometrium and form the endometrial cups which secrete equine chorionic gonadotrophin

(eCG), formerly called pregnant mare serum gonadotrophin (PMSG), peaking around days 60-80 (**Fig. 2**). From then, concentrations decline and the endometrial cups are sloughed around days 120-150 (Allen and Moor 1972, Allen and Wilsher 2009). Equine chorionic gonadotrophin acts in many species as FSH, however, in the mare, it has a predominant LH activity. It stimulates additional ovarian follicular development. These follicles produce high amounts of oestrogen (equilin) from days 38-40 on (Allen and Wilsher 2009, Nett, et al. 1973). Subsequently, the follicles luteinize and contribute to a further increase of serum progesterone concentrations to maintain pregnancy. Progesterone rises to a peak at day 80 and gradually declines to 1-2 ng/ml during mid-late gestation (day 150). The 5α-pregnanes, produced from maternal cholesterol, rise from mid gestation to term.

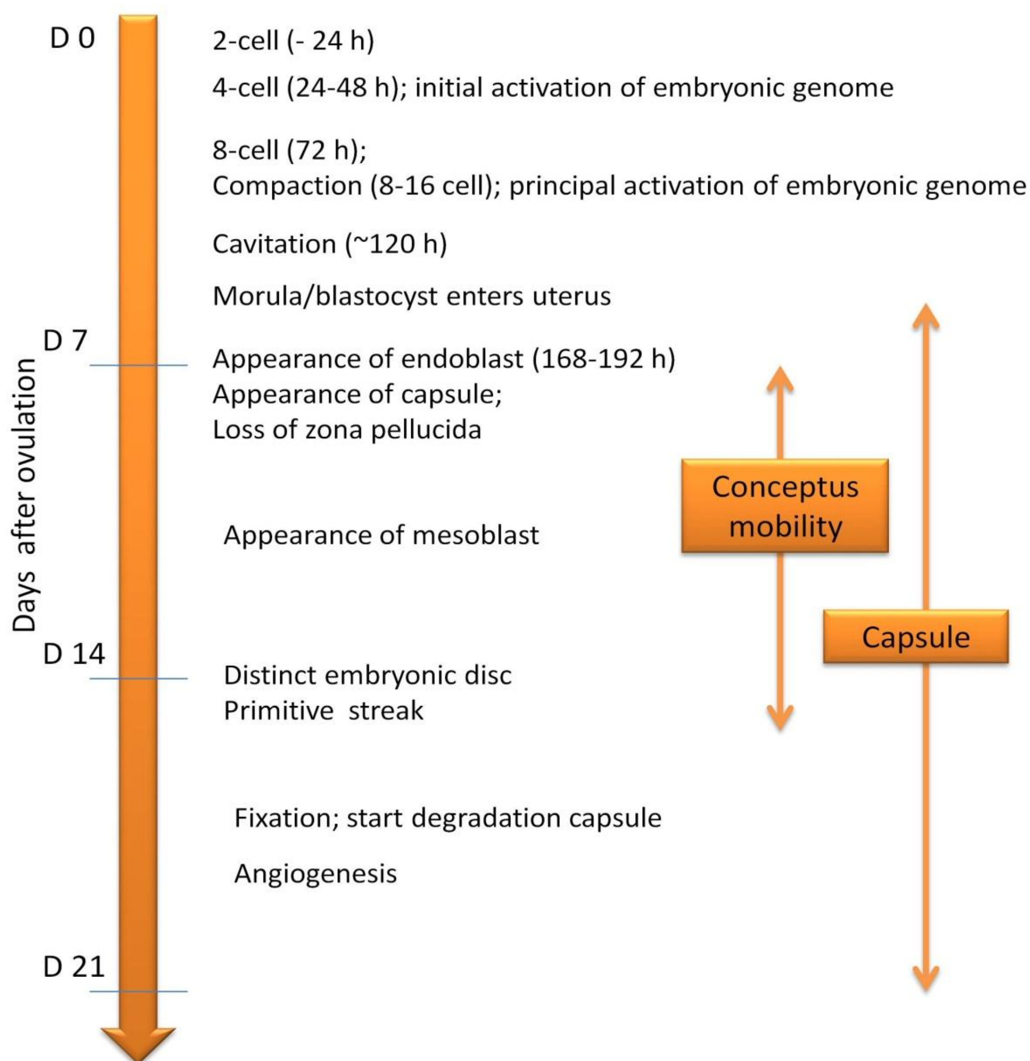

Fig. 1. Approximate timing of some key developmental events during the first 3 weeks of gestation of the equine embryo. Adapted from Betteridge (2000).

Since the endometrial cups are formed from cells from the trophoblastic girdle of the embryo, these cells are foreign for the dam and hence provoke a reaction, which results in dehiscence of endometrial cups at about days 120-150 .

From day 120, placentation starts and it is completed at day 150 (Allen and Wilsher 2009). The mare has a diffuse type of microcotyledonary placenta, which takes over progestagen production to maintain pregnancy from day 120 until birth of the foal around 340-345 days of gestation. From the beginning of the last month of pregnancy, progestagen rises by the production of foetal adrenal 5α-reduced pregnanes, while foetal oestrogens decline after peaking at day 210 and are basal at term (**Fig. 2**) (Holtan, et al. 1991).

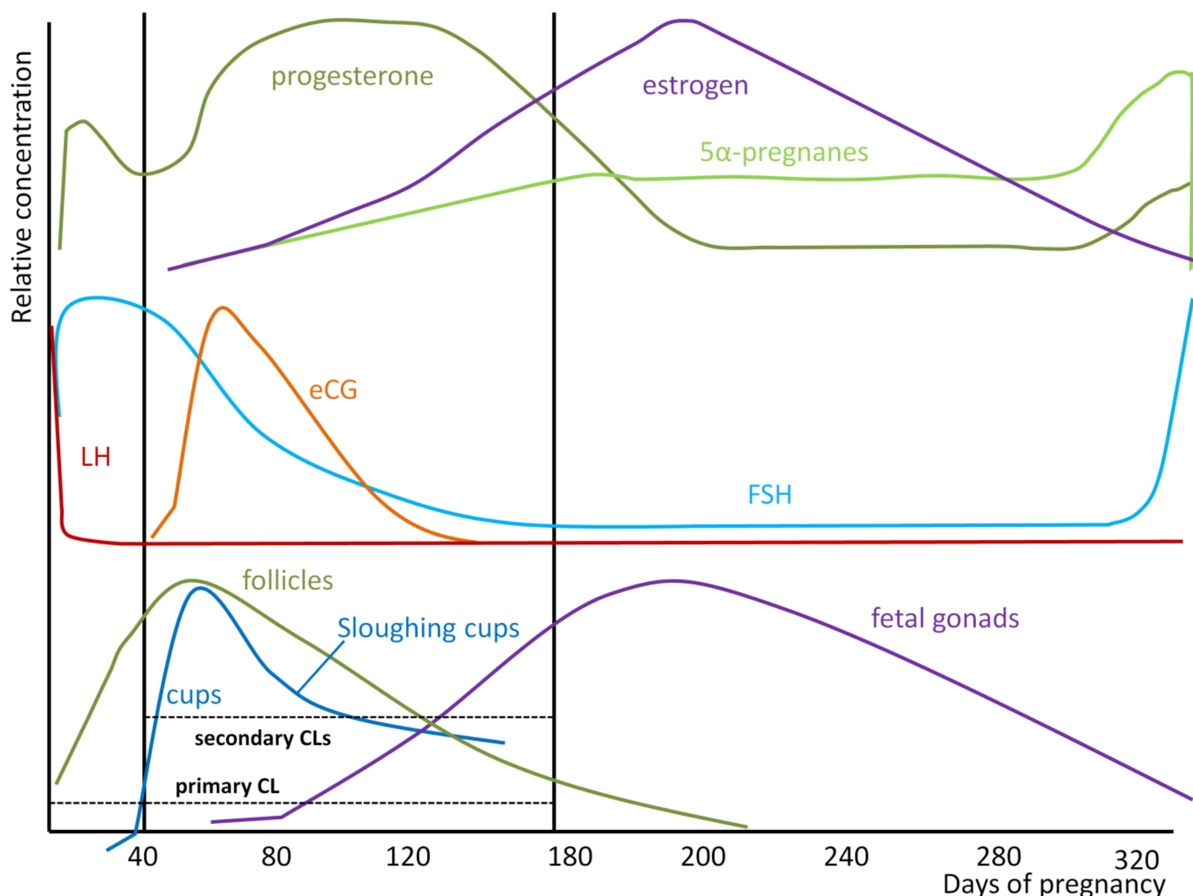

Fig. 2. Relative concentrations, sources and tendencies of fluctuations of progesterone, oestrogens, 5α-pregnanes, eCG, LH and FSH during pregnancy in the mare. (Adapted from: Allen and Moor, 1972; Douglas and Ginther, 1976; McDowell, et al. 1988; Holtan, et al. 1991; Sharp 2000; Spencer, et al. 2004; Allen and Wilsher 2009)

2 THE MARE'S OVIDUCT

1 UNIQUE FEATURES OF THE EQUINE OVIDUCT

Many features in early equine embryo development appear to be unique for this species. In particular, the differential transport of the oocyte and the embryo in the equine oviduct is quite peculiar. If the recently ovulated oocyte remains unfertilized, it discontinues its journey and degenerates in the highly convoluted folds of the ampullary-isthmic junction (Flood, et al. 1979). When the oocyte is fertilized in the ampullary-isthmic region (Hunter and Nichol 1988), the resulting embryo produces prostaglandin E2 (PGE_2) and passes through the utero-tubal junction in order to reach the uterus (Allen 2000, Oguri and Tsutsumi 1972, Wagh and Lippes 1989, Weber, et al. 1991a, b). This finding was in fact an important step forward in revealing what kind of cross-talk is taking place between the equine embryo and the mare's oviduct (Betteridge and Mitchell 1972). Application of PGE_2 on the uterotubal papilla has even been used in clinical conditions, to improve oviductal transport and uterine entry of embryos in subfertile mares with blocked oviducts (Allen, et al. 2006). Despite the fact that the embryo resides for 6 days in the oviduct, it spends nearly all of this period close to the ampullary-isthmic junction, whereas passage through the isthmus takes only a few hours (Weber, et al. 1996). This prolonged period, during which the equine embryo develops in the oviduct, is markedly longer than the 48 and 72 h during which the respective porcine and bovine embryo remain there.

2 THE ARCHITECTURE OF THE OVIDUCT

The oviduct is a highly specialized part of the genital tract with a fimbriated, funnel-shaped infundibulum enveloping the ovulation fossa, a thin-walled ampulla, leading via the ampullary-isthmic junction into the strongly muscular isthmus, and ending into the uterus at the utero-tubal junction **(Fig. 3)**. The infundibulum is adhered to the ovarium at the level of the ovulation fossa and even covers it around the time of ovulation, in order to take up the oocyte after ovulation. The ampulla is the longest and wider part of the oviduct, where oocyte maturation and fertilization take place. The next part is the narrower ampullary-isthmic junction connected to the highly convoluted isthmus, ending up in the distal end of the

oviduct, the utero-tubal junction, which consists of a smooth muscular sphincter in the mare (Nickel, et al. 1987, Yaniz, et al. 2000).

In cross-section, the mucosa of the oviduct is arranged into longitudinal folds: finger-like in the infundibulum, numerous and elaborated in the ampulla and non-branched in the isthmus (Desantis, et al. 2010) **(Fig. 3 h)**. As in other mammals, the epithelium is pseudo-stratified consisting of columnar ciliated and secretory cells (**Fig. 4 a** and **Fig. 4 b**) as two major cell types (Abe 1996). As substantiated by TEM in freshly isolated oviduct explants **(Fig. 4 a)**, all cells contain numerous mitochondria, rough endoplasmic reticulum and microvilli. There is a region-dependent proportion of ciliated cells: the isthmus contains less ciliated cells compared to the ampulla (Abe and Hoshi 2007, Abe, et al. 1999). Cilia are considered to be responsible for oocyte transport (Odor and Blandau 1973). The cilia of the isthmus are enclosed in an amorphous matrix, released from non-ciliated cells (Desantis, et al. 2010). The inner tissue layer of the tunica mucosa, the lamina propria mucosae, consists of fibrous and cellular connective tissue, building the framework for mucosal folds (Ellington 1991) **(Fig. 3 h** and **Fig. 4 b)**. The lamina muscularis or the myosalpinx of the mare is differentially developed dependent on the oviductal segment (**Fig. 3 e, Fig. 3 g**). It is more developed in the ampulla compared with the infundibulum. It consists of a longitudinal, circular and spiral smooth muscle layers, organized as a plexiform structure (Germana, et al. 2002), which could generate a stirring movement of the lumen content (Hodgson, et al. 1977) towards the uterus, especially regarding ovum transport and oviductal clearance.

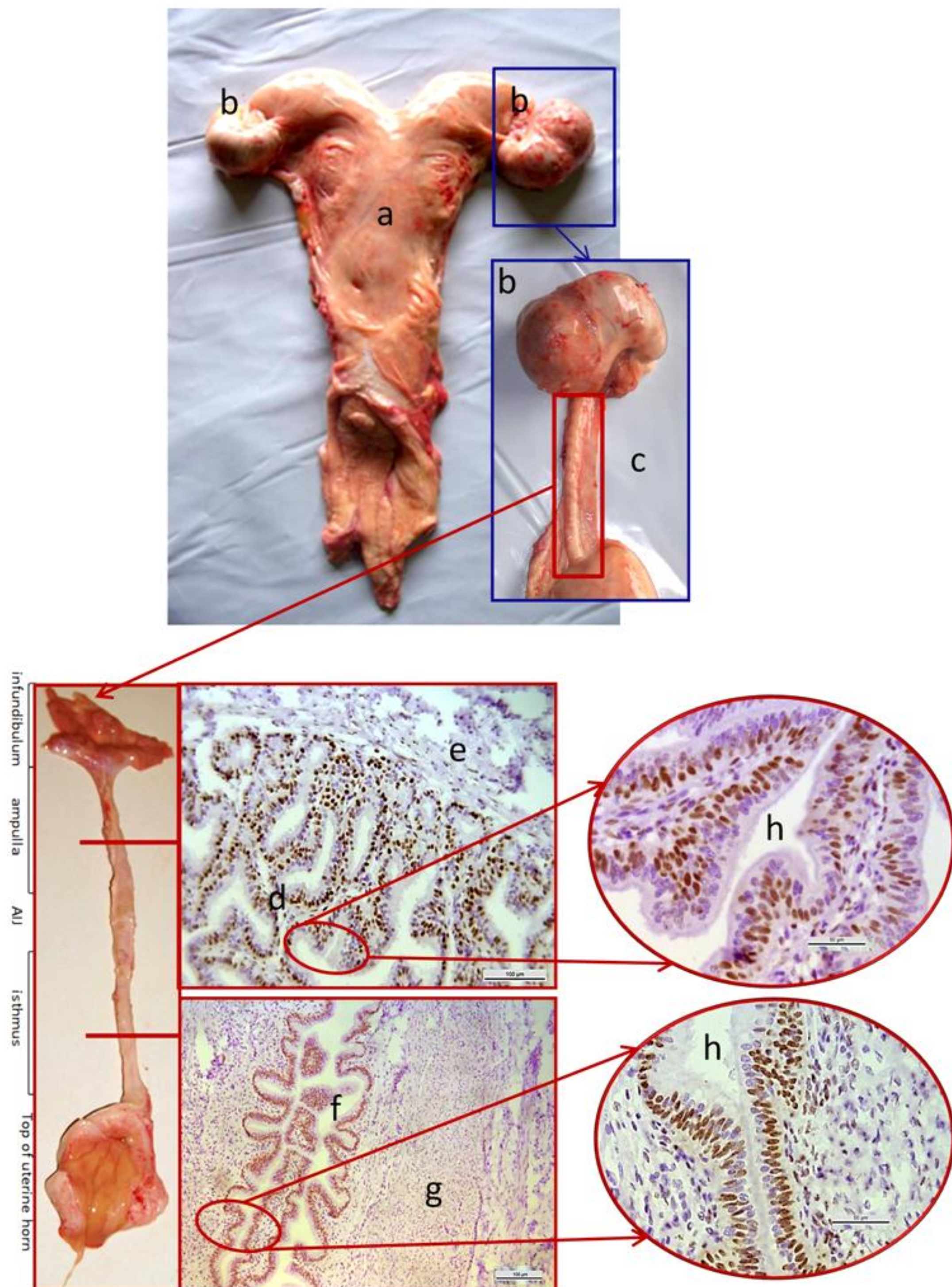

Fig. 3. Uterus (a) of a mare with the two adjacent ovaries (b) and right oviduct (c). The oviduct consists of a funnel-shaped infundibulum, the ampulla with the numerous and very elaborated, finger-like and branched mucosal folds (d; bar = 100 µm) and the rather thin muscular layer (e), the ampullary-isthmic junction (AIJ) and the highly convoluted isthmus with the non-branch mucosal folds (f; bar = 50 µm) and the pronounced muscular wall (g). Both ampullary and isthmic mucosa are lined by a pseudostratified small columnar ciliated epithelium (h; bar = 50 µm).

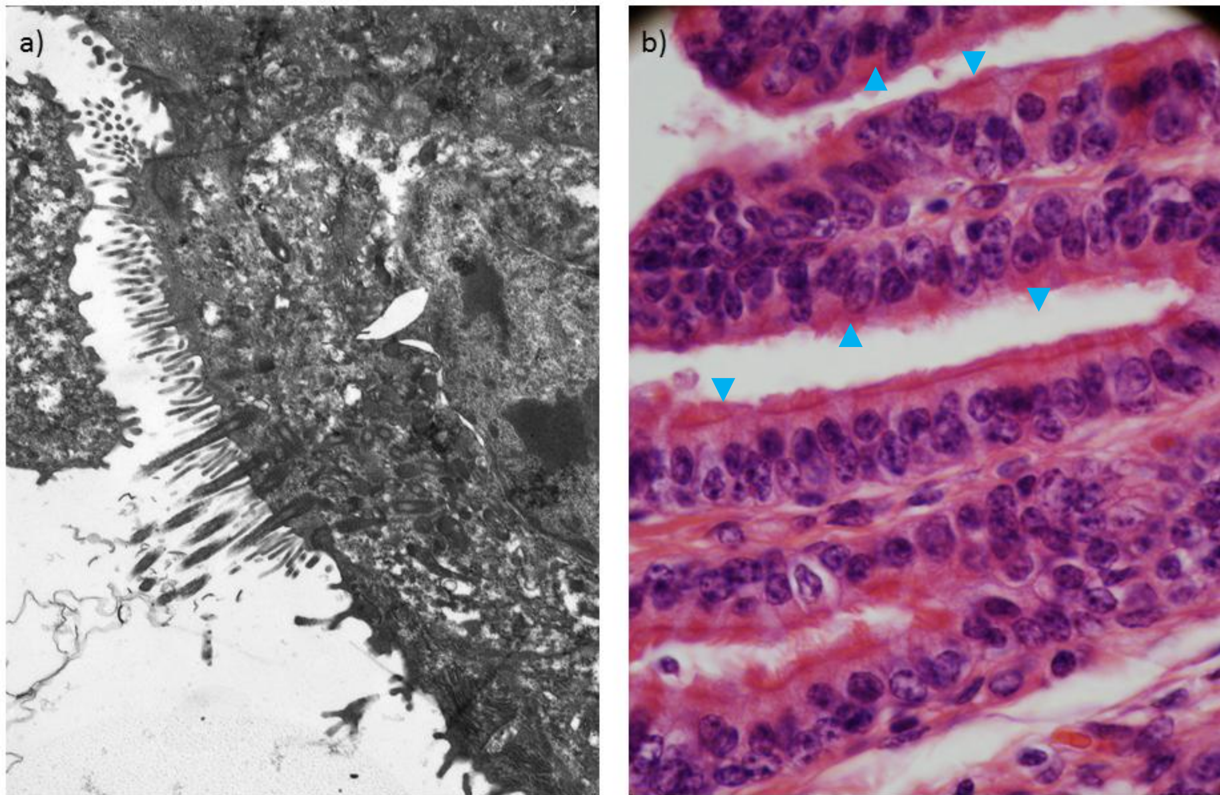

Fig. 4. a) Transmission electron microscopy of freshly isolated equine oviduct explants, consisting of highly differentiated cells with numerous mitochondria and rough endoplasmatic reticulum. (magnification = x 6000) (courtesy K. D'Herde) b) Haematoxylin-eosin staining of *ex vivo* oviductal epithelium bordered by highly differentiated tall columnar epithelial cells with basal nuclei, numerous nucleoli, apical cilia and secretory granules (blue arrowheads) (x 400) (courtesy P. Cornillie).

3 STEROIDS: KEY REGULATORS OF THE OVIDUCTAL ENVIRONMENT

Progesterone and oestradiol influence mammalian embryonic development indirectly by modulating oviductal and endometrial function and secretions by endocrine, paracrine and also autocrine pathways (Wu, et al. 1971). Steroid hormones, being hydrophobic molecules, diffuse freely into all cells. Only their target cells contain specific membraneous, cytoplasmic and/or nuclear proteins that serve as receptors of the hormone. The unbound receptors are associated with an inhibitory complex, containing heat shock proteins (HSP) **(Fig. 5).** Steroid-receptor complexes are phosphorylated to varying levels depending on the presence of regulators and the type of the receptor. Steroid-receptor complexes dimerize and bind to the hormone responsive elements – DNA sequences within promotor regions of genes responsive to the steroid and may also regulate energy metabolism through the mitochondria or modulate

or activate signalling molecules (Maller 2003). The hormone/receptor complex modulates together with transcription factors target gene expression (Beato 1989, Tuohimaa, et al. 1996). In this way, steroids can modulate gene transcription and translation relatively slowly. However, steroids can also induce their effects very rapidly, for instance, by altering membrane fluidity (or by binding to membrane-bound receptors) and subsequently inducing biological responses through amongst others cyclinB and MAP-kinase messenger systems (Maller 2003). An example of the quick mReceptor mediated effects of progesterone is the acrosome reaction in mammalian sperm which is induced within seconds (Cheng, et al. 1998, Levin 2002, Toran-Allerand, et al. 2002).

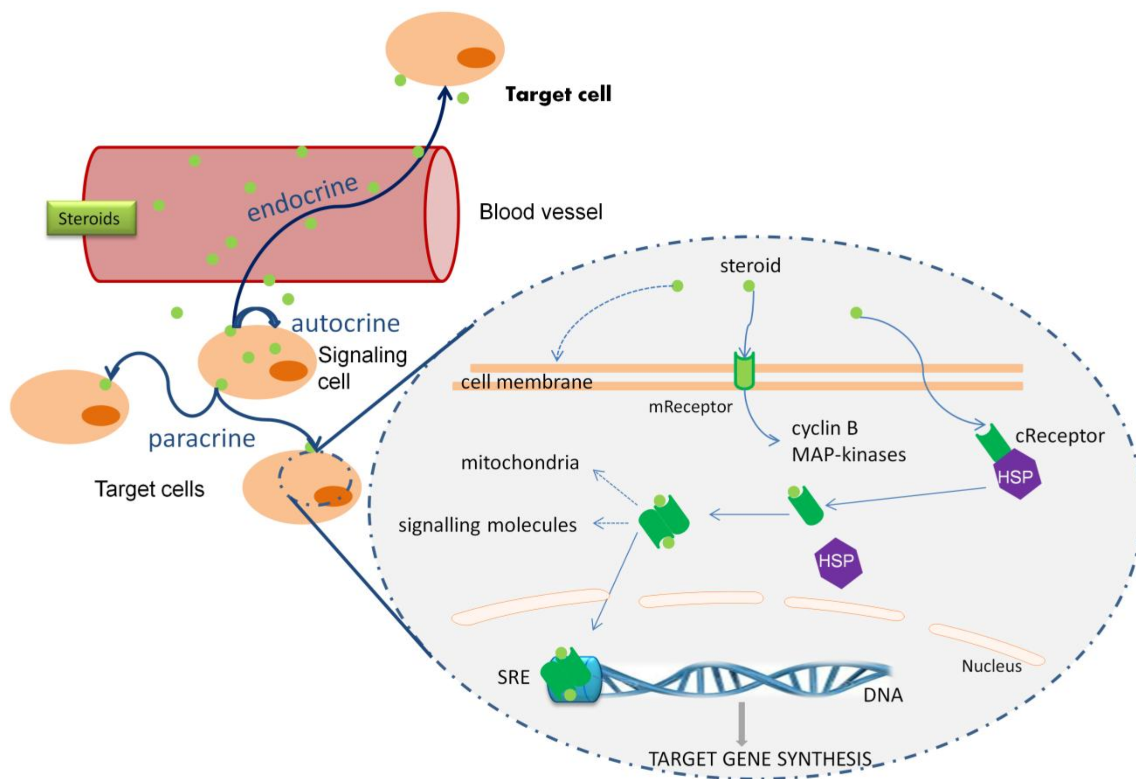

Fig. 5. Schematic overview of endocrine, paracrine and autocrine steroid signaling and the mechanism of steroid triggered hormone responsive genes. cReceptor = cytoplasmic receptor, mReceptor = membranous receptor, HSP = heat shock protein, SRE = steroid responsive element. (Based on: Beato 1989; Tuomihaa,et al. 1996; Maller 2003).

Even small local fluctuations in the steroid concentration affect the oviductal cell gene expression (Bauersachs et al., 2003; 2004) thus changing the oviductal fluid composition. According to Hugentobler *et al.* 2010, progesterone can increase the glycine concentration in the oviduct of cattle by twofold. In mares, Engle et al. (1984) reported cyclic trends in the

oviductal fluid concentrations of histidine, methionine, half-cystine, serine, proline, glycine, alanine, isoleucine and leucine. During oestradiol dominance, oviduct-specific proteins are produced that prepare the oviductal milieu for fertilisation and early embryo development (Buhi 2002, Leese, et al. 2001).

In the follicular phase, cell height and secretory activity and the ratio ciliated/non-ciliated cells increase (Abe 1996, Aguilar, et al. 2012, Buhi, et al. 2000, Donnez and Casanasroux 1985). In the luteal stage, the oviduct epithelium exhibits a regressed status, characterized by a reduction in cellular height and marked deciliation. All of these changes prepare and provide the prerequisites for successful transport of the ovulated oocyte, fertilization and the nourishment, transport and growth of the early embryo.

Key sites for steroid hormone biosynthesis include the ovary, placenta, testis and the adrenal cortex. However, steroids may also be generated *de novo* at lower levels in other tissues (Simpson 1979; Santen 1990; Hanukoglu 1992; Stocco and Clark 1996; Simard *et al.* 2005; LaVoie and King 2009). All steroid hormones are derived from cholesterol **(Fig.6)**. A series of enzymatic steps in the mitochondria and the endoplasmic reticulum of steroidogenic tissues convert cholesterol into all of the other steroid hormones and intermediates (LaVoie and King 2009). The rate limiting step in this process is the transport of free cholesterol from the cytoplasm into mitochondria, mediated by the steroidogenic acute regulatory protein (StAR) (Stocco and Clark 1996), which is in turn rapidly induced by steroidogenic stimuli (LaVoie and King 2009).
Cholesterol is converted to pregnenolone by cytochrome P450 cholesterol side-chain cleavage (P450scc) enzyme (Simpson 1979). Pregnenolone is subsequently metabolized to progesterone by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerases (3-beta-HSDs; **Fig. 6**) (Simard, et al. 2005), or in certain tissues hydroxylated by cytochrome P450 17α-hydroxylase/17,20-lase (P450c17α) (LaVoie and King 2009), resulting in cortisol and androgen synthesis (Hanukoglu 1992). P450 aromatase catalyses the conversion of testosterone into β-oestradiol. It is mainly expressed in granulosa cells but also, besides the gonads, in the mammary gland, the adipocytes, the central nervous system, the skin and the placenta (Hanukoglu 1992, Santen 1990). 17β-hydroxysteroid dehydrogenase catalyses the conversion of the 17-keto and 17β-hydroxy groups in androgens and oestrogens, including androstenedione, DHEA and 17β-oestradiol (Hanukoglu 1992).

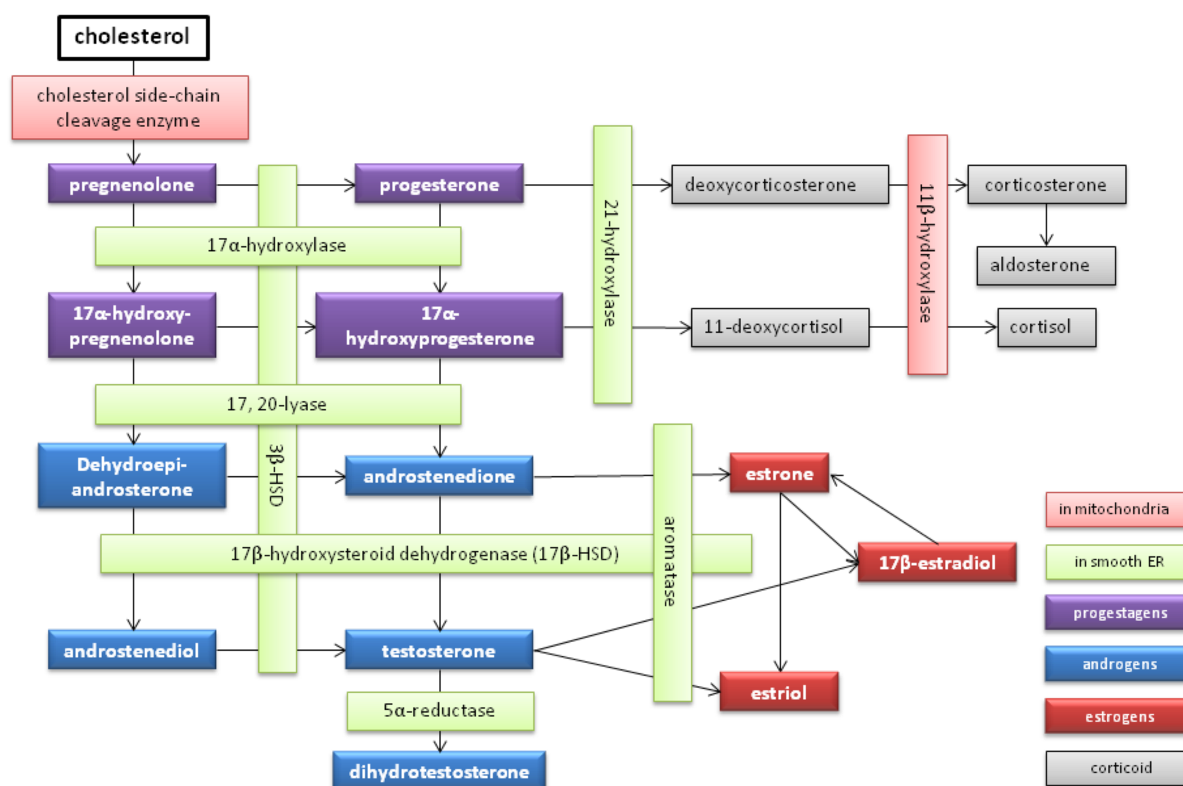

Fig. 6. Overview of steroidogenesis. Based on Hanukoglu (Hanukoglu 1992).

Ovarian steroids induce morphological, biochemical and physiological changes to the oviduct cells and subsequently affect the volume and composition of the oviductal fluid (Georgiou, et al. 2005, Hunter 2012b, Seytanoglu, et al. 2008) and ciliary activity (Bylander, et al. 2010, Wessel, et al. 2004). Consequently, these changes play a key role in the optimization of the microenvironment for final maturation and transport of the gametes, for fertilization and for nourishment, transport and growth of the early embryo (Fazeli 2008, Hunter 2005). In the horse embryo, progesterone and oestrogen receptors have been detected in conceptuses from day 7 to day 14 (Rambags, et al. 2008), indicating that steroids may also act directly to uterine stage embryos. These equine embryos are also able to produce large quantities of oestrogens themselves (Heap, et al. 1982). Next to oestrogens, other steroids which are secreted by horse uterine stage conceptuses are 17-hydroxyprogesterone and androgens (1.0 +/- 0.2 pg of 17-hydroxyprogesterone/embryo, 4.8 +/- 0.6 pg of androstenedione/embryo) (Weber, et al. 1991b). It has been shown that 17α-hydroxyprogesterone synthesized by the equine blastocyst is metabolized to an unidentified steroid by the mare’s endometrium (Goff, et al. 1993). From

this, it is strongly suggestive that embryonic steroids could have an autocrine and paracrine signaling function (**Fig. 5**) (Walters, et al. 2001; Rambags, et al. 2008) and can exert their effects directly.

However, nothing is known about the role of steroids and very little about the influence on the side of ovulation in the development and embryo-maternal communication in the equine oviduct. Only the influence of the oviductal side on prostaglandin receptors and mu-opioid receptors were investigated. Therefore, in CHAPTER 4 and 5 of this thesis, the local concentrations of steroids and their effects are investigated.

4 OVIDUCTAL SECRETIONS IN THE MARE

Since fertilization and early embryo development occur in the oviduct, it could be expected that the formation and the composition of oviductal fluid is well known (**Fig. 7**). Unfortunately, very little is known concerning the formation of oviductal fluid (Leese, et al. 2001), in particular in the mare. Oviductal fluid is the product of serum transsudate and the compounds synthesized by epithelial cells (Leese 1988). In the mare the oviductal fluid secretion rates during estrus and diestrus are respectively 5.08 and 2.82 ml/24h (Campbell, et al. 1979). Concentrations of ions in the oviductal fluid of mares tend to be similar of those in serum (Aguilar and Reyley 2005), except for magnesium. Magnesium concentrations in the oviduct of the mare are 2-5 times higher than plasma concentrations (Campbell, et al. 1979) and much higher than those in other species (Aguilar and Reyley 2005).

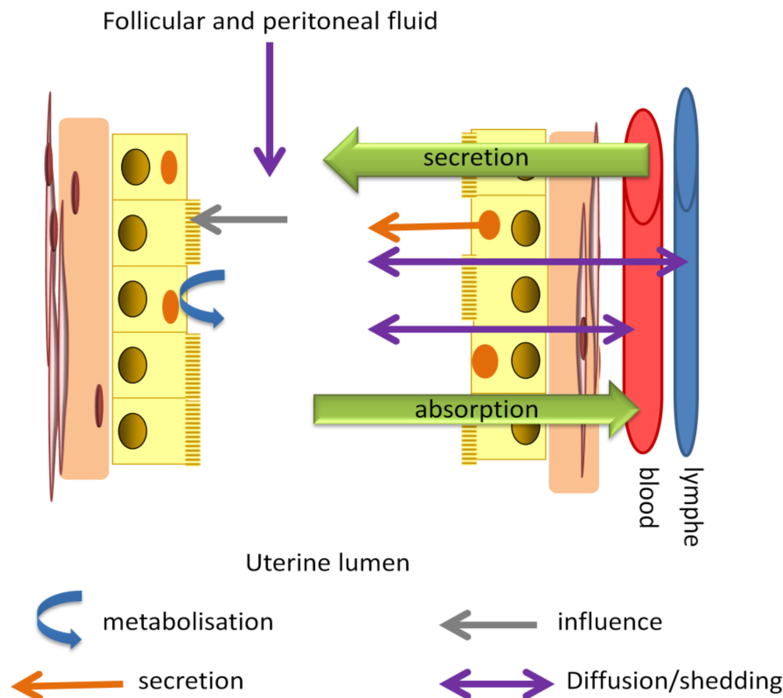

Fig. 7. Formation of oviductal fluid: a mix of secretion, absorption, diffusion and newly synthesized molecules. Based on : Leese 1988.

The energy substrates, glucose, pyruvate and lactate, are important for the nourishment of the oocyte, the spermatozoa and the early embryo. Glucose concentrations in the mare's oviduct range from 2.84 to 5.92 mM (Campbell, et al. 1979). Pyruvate and lactate concentrations in the mare are not known so far. In humans, the main energy substrates are glucose and

pyruvate, both serum transsudates (Brewis, et al. 1992, Leese and Gray 1985). Furthermore, pyruvate can be synthesized by the tubal epithelium from glucose and lactate. Pyruvate does not only act as an energy source, it can also detoxify ammonia with formation of alanine as a result. It is also important in protection against oxidative stress by preventing peroxide-induced injury (Guérin and Ménézo 2011, Morales, et al. 1999). Lactate can also be used as an energy substrate by the embryo. Twenty five % of the lactate in the oviductal fluid is filtered from the blood and 75% is produced by the tubal epithelial cells from vascular glucose (Nichol, et al. 1992). Its function may also be to maintain the proper redox balance and pH. Lactate is converted to pyruvate with generation of reduced nicotinamide adenine dinucleotide (NADH). Consequently, one of the benefits of co-culture may be the production of lactate by the interconversion with pyruvate which is generated during glycolysis (Ouhibi, et al. 1989).

Since supplementation of synthetic oviductal fluid (SOF) with amino acids at *in vivo* concentrations resulted in a higher percentage of ovine blastocysts compared with SOF supplemented with 2% human serum and BSA, amino acids seem to have a beneficial impact on gamete function and embryo survival in sheep (Walker, et al. 1996b). A total of 17 free amino acids were measured in the mare's oviduct **(Table 1),** whereas in other species 23 free amino acids could be identified in oviductal fluid (Engle, et al. 1984). Glycine and alanine levels were found to be the highest, but levels of free amino acids varied during the oestrous cycle. Only traces of histidine, methionine, phenylalanine, threonine and tyrosine were detected in equine oviductal fluid. Histidine and methionine concentrations displayed cyclic variation, whereas phenylalanine, threonine and tyrosine remained relatively constant during the estrous cycle. Glutamic acid had the third highest concentration in equine oviductal fluid at day 13 of the estrous cycle, and decreased until day 21. All detected amino acids were measured in concentrations higher than in plasma and in follicular fluid (Engle, et al. 1984).

Table 1. Concentrations of amino acids in equine oviductal fluid according to Engle et al. (1984).

Amino acid	Concentration (µM/ml)	Amino acid	Concentration (µM/ml)
Alanine	0.14	Lysine	0.053
Arginine	0.031	Methionine	0.014
Aspartic acid	0.022	Phenylalanine	0.026
Cysteine	0.003	Proline	0.048
Glutamic acid	0.057	Serine	0.051
Glycine	0.263	Threonine	0.038
Histidine	0.02	Tyrosine	0.041
Isoleucine	0.025	Valine	0.041
Leucine	0.053		

Very little is known about the identity of proteins, growth factors and macromolecules prevailing in the mare's oviduct. An earlier report (Perkins and Goode 1965) shows a mean protein concentration in ovine oviductal fluid of 29.3 mg/ml. In general, protein concentration in oviductal fluid is approximately 10-15% of that in serum (Leese 1988). Moreover, albumin and immunoglobulin G, derived from serum, are the most abundant proteins and represent about 95% of the total protein content in the oviductal lumen. In pigs (Buhi and Alvarez 1998) and in women (Lippes, et al. 1981), total protein concentration changes during the estrous cycle and in response to fertilization, indicating a critical cyclic variation in total protein content. Nevertheless, the contribution of passive transudate compared to the active oviductal biosynthesis remains to be elucidated (Buhi, et al. 2000). Few attempts have been undertaken to identify proteins in equine oviductal fluid (Willis, et al. 1994) or to detect pregnancy-specific proteins by means of polyacrylamide gel electrophoresis (PAGE), and only retinol binding protein (Mcdowell, et al. 1993), platelet derived growth factor (Eriksen, et al. 1994a, Eriksen, et al. 1994b), osteopontin, atrial natriuretic peptide (Mugnier, et al. 2009) and Deleted in Malignant Brain Tumor 1 (DMBT1) (Ambruosi, et al. 2013) have been identified in oviductal fluid (Ménézo and Guérin 1997).

We determined by means of the Coomassie blue technique the average total protein content of the ipsilateral and contralateral oviduct of non-pregnant and pregnant mares at 3-4 days after

ovulation. Protein content of ipsilateral and contralateral oviducts of non-pregnant mares was 136 and 126 µg and 250 and 278 µg in pregnant mares (Smits, Nelis et al. submitted). Proteins were separated by 1D-PAGE (**Fig. 8**). It might be deduced that certain proteins with a molecular weight between~48 kDa and~62 kDa are more abundantly expressed in oviductal fluid of pregnant compared to non-pregant mares.

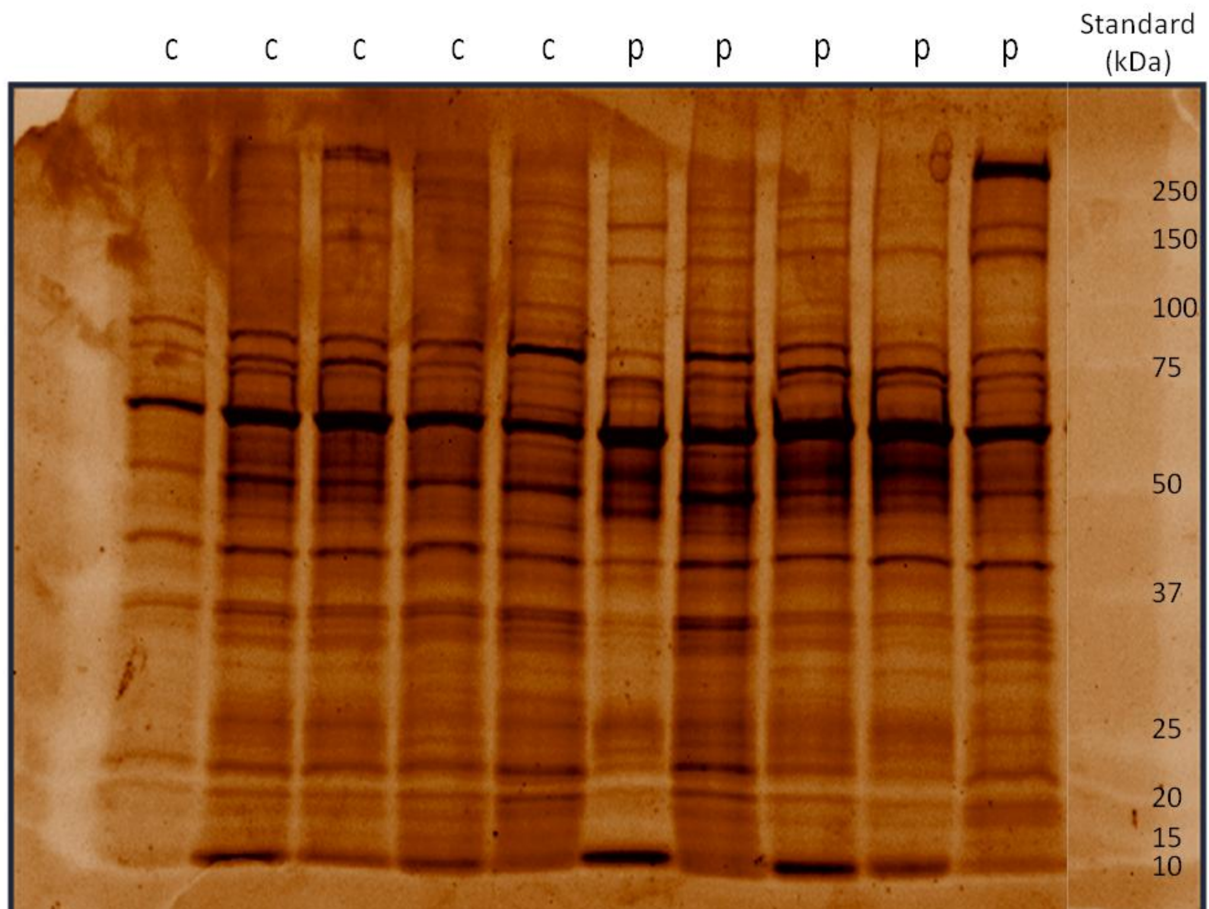

Fig. 8. 1D-PAGE of oviductal fluid from the ipsilateral oviduct of non-pregnant mares (c) and (p) pregnant (3-4 days) mares (4 µg oviductal protein loaded per column). Standard = Precision Plus Protein™ All Blue Standards (Biorad). Preliminary data of Smits, et al submitted.

3 EQUINE EMBRYO DEVELOPMENT *IN VITRO*

1 INTRODUCTION

At present, no reports are available on the production of a viable equine pregnancy or live foal after a complete *in vitro* procedure, including *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and further *in vitro* culture (IVC) of thus produced embryos. Hitherto, only two foals have been documented after conventional IVF of sperm incubation with an *in vivo* matured oocyte (Bezard, et al. 1992, Palmer, et al. 1991). To produce equine embryos *in vitro*, oocytes are fertilized *in vitro* by means of intracytoplasmic sperm injection (ICSI), a technique using micromanipulation to inject an immobilized sperm cell directly into the cytoplasm of a mature oocyte. Compared to other species, progress in the field of *in vitro* production of horse embryos is relatively slow for several reasons. There is a scarcity of equine ovaries, oocytes are much more difficult to retrieve from the follicle (Galli, et al. 2007, Hinrichs 2010), and the procedure of ICSI, which replaced classical IVF in equine embryo production, makes the whole procedure very costly, time-consuming and labour intensive (Hinrichs and Choi 2005). So, research on horse embryo production *in vitro* remained for many years confined to only a few groups which had access to the ovaries and the right equipment (Galli, et al. 2007, Hinrichs and Choi 2005).

2 EMBRYO CULTURE MEDIA

Many embryo culture media have been formulated, based upon the composition of the oviductal fluid, like in ruminants (sheep: synthetic oviductal fluid-SOF (Tervit, et al. 1972), in cattle: bovine oviduct embryo culture medium - BOECM (Leese, et al. 2008)), in pigs (porcine zygote medium – PZM (Yoshioka, et al. 2002) and in humans (human tubal fluid - HTF) (Quinn, et al. 1985)). These media are typically simple salt solutions, supplemented with amino acids and energy substrates, of which the concentrations as they prevail in bovine fluid are listed in **Table 2**. However, oviductal fluid is far more complex than any of these culture media, since many key metabolites and/or growth factors have not been identified yet and are therefore lacking from or are added in unphysiological concentrations to the culture media. In addition, oviductal fluid is continuously changing under the influence of cyclic endocrine changes (Leese *et al.* 2008; Market-Velker *et al.* 2010).

Table 2. Composition (mM) of embryo culture media based on biochemical analysis of genital tract fluids in cattle (BOECM) and sheep (SOF) and on the composition of commercial available media. DMEM/F12 is a somatic cell culture medium. Both SOF and DMEM /F12 give good results in horse embryo culture (data based on Leese, et al. 2008).

Constituent	**Bovine Oviduct Medium for Embryo Culture (BOECM)**	**Synthetic Oviductal fluid (SOF)**	**DMEM/ F12**
Amino acids			
Aspartate	0.143	9.709×10^{-5}	0.050
Glutamate	0.346	9.709×10^{-5}	0.050
Asparagine	0.043	9.709×10^{-5}	0.050
Serine	0.178	9.709×10^{-5}	0.250
Histidine	0.071	1.942×10^{-4}	0.150
Glutamine	0.193	0	0
Glycine	1.496	9.806×10^{-3}	0.250
Threonine	0.164	3.884×10^{-4}	0.449
Arginine	0.136	5.816×10^{-4}	0.699
Taurine	0.050	0	0
Alanine	0.612	9.709×10^{-5}	0.050
Tyrosine	0.059	0	0.214
Methionine	0.042	9.839×10^{-5}	0.116
Tryptophan	0.040	4.854×10^{-5}	0.044
Valine	0.190	3.884×10^{-5}	0.221
Phenylalanine	0.074	1.941×10^{-4}	0.215
Isoleucine	0.092	3.891×10^{-4}	0.416
Leucine	0.200	3.884×10^{-4}	0.451
Lysine	0.229	3.846×10^{-4}	0.499
CystineHCl.H_2O	0	0	0.100
Cystine.2HCl	0	9.703×10^{-5}	0.100
Proline	0	9.455×10^{-5}	0.150

Constituent *(Continued)*	**Bovine Oviduct Medium for Embryo Culture (BOECM)**	**Synthetic Oviductal fluid (SOF)**	**DMEM/ F12**
Alanyl glutamine		9.709×10^{-5}	0
Total amino acid concentration	4.358	0.0150	4.524
Energy substrates			
Glucose	2.3	0.0010	17.51
Lactate	12.4	2.40×10^{-6}	0
Pyruvate	0.10	0.3180	0.5000
Ions			
Chloride	90.0	0.1070	128.8
Phosphate	1.4	0.0010	0.9530
Sodium	1.1	0.4390	151.5
Magnesium	124.1	0.4780	0.7070
Potassium	0.9	6.913	4.157
Calcium	1.7	0.0020	1.050
Sulphate	0	0	0.9530
Nitrate	0	0	3.713×10^{-4}
Hydrogen carbonate	0	24.27	29.02
Iron	0	0	0.0020
Copper	0	0	5.200×10^{-6}
Zinc	0	0	0.0020
Other components			
Sodium hypoxanthine	0	0	0.0150
Linoleic acid	0	0	2.000×10^{-4}
DL-68-thiotic acid	0	0	5.000×10^{-4}
Phenol red	0	0	0.023
Sodium putrescine	0	0	5.000×10^{-4}
Gentamicin	0	1.015×10^{-4}	0
HEPES	0	0.0100	0

Constituent (Continued)	Bovine Oviduct Medium for Embryo Culture (BOECM)	Synthetic Oviductal fluid (SOF)	DMEM/ F12
Foetal calf serum	0	0	0
Serum replacer	0	0	0
Vitamins			
Biotin	0	0	1.434×10^{-5}
Pantothenate	0	0	0.0050
Choline	0	0	0.0640
Folic acid	0	0	0.0060
Inositol	0	0	0.0700
Nicotinamide	0	0	0.0170
Pyridoxine	0	0	0.0100
Riboflavine	0	0	5.82×10^{-4}
Thiamine	0	0	0.0060
Thymidine	0	0	0.0020
Pyridoxal	0	0	5.018×10^{-4}

Although substantial progress has been made in the optimization of embryo culture media in other species, little is known about the effect of a specific medium on the development of equine embryos *in vitro*. A number of media have successfully been used to obtain blastocysts (**Table 3**), although media which are efficiently applied for embryo culture in other species, such as G1/G2 or CZB-medium, were not able to support acceptable blastocyst percentages in the horse (Choi, et al. 2003, Choi, et al. 2004a, Choi, et al. 2004b). Up to now, the best blastocyst rates have been achieved with DMEM/F12 supplemented with 10 % foetal bovine serum at a ratio of 1 µl medium per embryo at 38.2 °C in 5 % O_2, 5 % CO_2 and 90 % N_2 (Choi, et al. 2006, Hinrichs and Choi 2005).

No specific factors which may explain successful blastocyst development in the horse, have been identified. Especially the high glucose content of the DMEM/F-12 medium (17 mM) may be important for the good development achieved, as pointed out by Hinrichs (Hinrichs 2010). Originally developed for somatic cell culture, DMEM/F-12 medium contains much

more glucose than regular embryo culture media. Increasing the glucose concentration in SOF culture media (to 5 mM or even 19 mM) has promoted embryo development to more advanced stages (morulae or blastocysts) especially when they were exposed to high glucose after day 4 (Azuma, et al. 1995). Contrary to other species, horse embryos appear to benefit from high glucose concentrations during early embryo development, which is not surprising since equine oviductal fluid contains 100 to > 300 times more glucose than bovine oviductal fluid (Hugentobler, et al. 2008) **(Table 1)**. Equine blastocyst development appears not to be dependent upon high glucose concentrations but cell allocation may be affected by glucose (Choi, et al. 2015). Most laboratories are now either culturing the horse embryos in DMEM/F-12 and/or in SOF medium until day 5-6 **(Table 3)** both of which are not based on the composition of equine oviductal fluid. Since these media also differ very much as far as the concentration of basic amino acids and energy substrates is concerned **(Table 2)**, it is quite remarkable that satisfactory blastocyst rates can be obtained by embryo culture in both media. This illustrates the flexibility of the equine embryo, to adapt itself to non-physiological circumstances, and may have slowed down further investigations towards improved embryo culture media for horses.

Equine embryos derived by ICSI and cultured *in vitro* yield blastocyst rates similar to those in cattle, although the blastocyst percentages are varying between the labs (10-47%) **(Table 3)** (Lazzari, et al. 2002, Choi, et al. 2006, Galli, et al. 2007; Smits, et al. 2011; Choi, et al. 2015). Foaling rates of over 50% have been reported (Colleoni, et al. 2009, Hinrichs 2010). Nevertheless, there is still room for improvement of equine embryo culture media since *in vitro* produced equine embryos display several morphological and developmental aberrations (Smits, et al. 2011, Tremoleda, et al. 2003). For instance, most equine *in vivo* embryos recovered 7 days after ovulation have reached the blastocyst stage and are larger and contain more cells than their *in vitro* counterparts 7 days after ICSI, with most of them still being at the morula stage (Pomar, et al. 2005). These findings emphasize the importance of embryo-maternal interaction, which is missing during *in vitro* culture of the equine embryo.

Table 3. Blastocyst production (from cleaved oocytes) in different somatic cell and embryo culture media of *in vivo* derived and *in vitro* produced horse embryos after ICSI, in relation to oxygen tension, culture medium and protein source. High oxygen = 20 %, low oxygen = 5 %; ITS = insulin, transferrin, selenium; FBS = foetal bovine serum; BSA = bovine serum albumin; NM = not mentioned; CNM = concentration not mentioned.

Embryo origin	Culture medium	Protein Soure	Oxygen tension	% blastocyst	Reference
	DMEM/F-12	ITS	High	0	Weber *et al.* 1993
	Ham's F12	10 % serum	High	14	White *et al.* 1988
In vitro	DMEM	-	High	0	Li *et al.* 2001
	DMEM/F-12	10 % FBS	Low	NM	Mortensen *et al.* 2010
	DMEM/F-12	10 % FBS	Low	12.5	Smits *et al.* 2012
	DMEM/F-12	10 % FBS	Low	36	Choi *et al.* 2006b
		0.5 % BSA	Low	28	Choi *et al.* 2004a
	DMEM/F-12	10 % FCS	Low	18-19	Choi *et al.* 2015
	G1.2/G1.3	0.8 % BSA	NM	3.4	Choi *et al.* 2004a
		0.8% BSA and 10 % FCS	NM	28	Choi *et al.* 2004a
				NM	Rosati *et al.* 2002
	CR1aa medium	5 % FCS	Low	26	Matsukawa *et al.* 2007
	SOF + MEM	1.6 % BSA	Low	15	Tremoleda *et al.* 2003
	SOF + MEM	0.8 % BSA	Low	0	Rosati *et al.* 2002
	SOF	BSA (CNM)	Low	43-47	Lazzari *et al.* 2002
				20	Galli *et al.* 2002
	GB + 0 mM for 5 days then	10 % FCS	Low	31	Choi *et al.* 2015
	GB + 0 mM for 5 days,	10 % FCS	Low	41	Choi *et al.* 2015
	GB + 5 mM for 5 days then	10 % FCS	Low	46	Choi *et al.* 2015
	GB + 5 mM for 5 days then	10 % FCS	Low	35	Choi *et al.* 2015
	CZB- medium	0.8% BSA		0	Choi *et al.* 2004a

3 OXYGEN CONCENTRATION

The oxygen tension in the oviduct is about 5-6 % (Fischer and Bavister 1993), and as a logical result, bovine, porcine, ovine and caprine embryos are routinely cultured in 5 % oxygen. Further decrease of the oxygen tension, may increase blastocyst rates but may also cause developmental abnormalities in ruminants (Thompson and Peterson 2000). There is only one study in which low (5 % O_2, 5 % CO_2 and 90 % N_2) and high oxygen (5% CO_2 in air or about 20 % O_2) were evaluated for equine *in vitro* embryo development (Choi, et al. 2003). The authors found that the cleavage rate was not different between the 2 gas systems. However, after 96 h of culture in low oxygen, the average nucleus number of horse embryos was almost twice of that found in embryos cultured in 5% CO_2 in air (Choi, et al. 2003). The beneficial effect of reduced oxygen tension on embryo development *in vitro* has been reported in several species, including mice (Gardner and Lane 1996), rabbits (Lindenau and Fischer 1994), sheep and cattle (Tervit, et al. 1972, Thompson, et al. 1990), and humans (Dumoulin, et al. 1999). Reduced oxygen tension may work through suppressing of the production of reactive oxygen species, which can cause developmental aberrations (Goto, et al. 1993). Nevertheless, there are studies reporting no beneficial effects of lower oxygen concentration on embryos (mouse (Nasresfahani, et al. 1992); sheep (Betterbed and Wright 1985) cattle (Khurana and Niemann 2000). This difference might be related to variations of media or culture systems (Bavister 1995) or due to observational methods with a rather low sensitivity.

4 MACROMOLECULES AND PROTEIN SOURCE

Since albumin is the most abundant protein in the female genital tract, supplementation of albumin to the culture medium definitely has a beneficial effect on embryo development by modifying oxidation of pyruvate (Eckert, et al. 1998), by acting as a carrier for hormones, vitamins, bioactive lipids and autocrine ligands (O'Neill 2008) and by neutralizing toxins (Gardner 2008). It provides also surfactant activity and maintains the colloid osmotic pressure, similar to the chemically defined polyvinyl-alcohol (PVA) (Thompson and Peterson 2000).

Although serum contains a range of beneficial compounds like growth factors, nutrients and heavy metal chelators (Vajta, et al. 2010), it may expose the embryos to undefined molecules which are not present in oviductal fluid. In this regard, serum even may have adverse effects on early embryonic development (Vajta, et al. 2010). These induced abnormalities are thought

to cause the "large offspring syndrome" which occurs in cultured and cloned ruminant embryos (Thompson, et al. 1995). Nevertheless, it was not definitely proven that serum addition was responsible for the syndrome and moreover, it also occurred in sheep after *in vitro* culture without serum but with PVA or bovine serum albumin (Rooke, et al. 2007, Sinclair 2008). Since the late 1990s, serum has become an "unwanted component" and was mostly abandoned in ruminant (Walker, et al. 1996a) and human (McEvoy, et al. 2000), but not in equine (Hinrichs 2010) embryo culture medium **(Table 3)**. No comparative studies have been undertaken to compare blastocyst percentages and long-term effects of serum or BSA supplementation in the horse embryo culture media. Up to now, the best results, reported by the lab of K. Hinrichs, Texas A&M University (Choi, et al. 2006, Hinrichs and Choi 2005) and which are routinely achieved, are obtained with DMEM/F-12 with 10% of foetal calf serum.

5 TO RENEW OR NOT TO RENEW

Choi et al (Choi, et al. 2003) revealed that the changing of culture medium at 72 or 96 h had no effect on embryonic development in the horse. Advantages of not renewing the culture medium can be: 1) the embryos are left undisturbed and therefore environmental stress (changes in pH, temperature, humidity…) is reduced, 2) accumulated endogenous and paracrine growth factors are left in place, allowing the embryos to create their own microenvironment and 3) labour intensity, quality control and costs are lower. Negative aspects of not renewing the culture medium could be that toxins may accumulate. These toxins may originate from dead embryos, from healthy embryos, released metabolites, from ageing medium, oil, from the atmosphere and from the used plastic recipients (Vajta, et al. 2010).

6 CO-CULTURE

6.1 Figures and facts about the good, the bad and the ugly

Applied cell types and mode of action

Co-culture of embryos with somatic cells has been used extensively in the early nineties, since at that time, it was the only way to overcome the 8- to 16-cell block (Eyestone and First 1989;

Eyestone *et al.* 1991) in a broad range of species, except for the cat (Swanson *et al.* 1996). This indicates that the then used culture media were suboptimal for embryo development and had to be "conditioned" by the somatic cells.

Although the way in which co-cultured cells exert their beneficial effects is far from fully understood, oviductal cells probably support early embryos by secreting soluble factors which enhance embryonic development (Gandolfi, et al. 1989, Nancarrow and Hill 1994). Various oviduct-derived factors are known to enhance fertilization and embryo development *in vitro* in other species. Two such growth factors which have been elaborately studied in mice and cattle are insulin-like growth factor 1 (IGF1, (Block, et al. 2008, Bonilla, et al. 2010, Byrne, et al. 2002a, b, Jousan and Hansen 2004)) and granulocyte-macrophage colony-stimulating factor (GM-CSF, (Chin, et al. 2009, Sjoblom, et al. 2005)), both of which have important positive effects on embryonic gene expression, leading to increased cell proliferation, decreased apoptosis and normalization of foetal growth. In the horse, only retinol binding protein (Mcdowell, et al. 1993) and PDGF (Eriksen, et al. 1994a, Eriksen, et al. 1994b) have been identified in the oviduct. These growth factors may be added to *in vitro* embryo culture medium in order to investigate their effects on the embryo's transcriptome and/or proteome.

A second possibility in addition to the secretion of embryotrophic substances is that the oviduct cells are capable of removing or reducing metabolites or toxic components (Flood and Shirley 1991, Vanroose, et al. 2001), such as heavy metal ions, hypoxanthine (Bastias, et al. 1993), nicotinamide (Tsai and Gardner 1994) and ammonia (Nancarrow and Hill 1994) in the culture medium. In addition, somatic cells can also modulate antioxidant gene expression in order to protect against oxidative stress by deactivating oxygen free radicals and their products (Harvey, et al. 1995, Minotti and Aust 1989). Furthermore, somatic cells may create a localized low oxygen tension around the embryo as a result of their own oxidative metabolism (Catt 1994) and thereby prevent formation of deleterious radicals (Vanroose, et al. 2001). Also pH as well as glucose, lactate and ion levels can be modulated (Bavister 1992; Rieger, et al. 1995, Vanroose, et al. 2001). These effects do not seem to be limited to oviduct cells from the same species, but co-culture with heterologous embryos and cells also has beneficial effects.

Isolation methods

In earlier studies, to obtain cells for oviduct cell cultures in different species, cells were singularized by enzymatic treatment, using trypsin and pancreatin (Cox and Leese 1997), trypsin (Reischl, et al. 1999, Thibodeaux, et al. 1992) or collagenase (Sun, et al. 1997). Singularized cells in suspension **(Fig. 9 a)** were seeded to attach to the culture dish and grow to a confluent monolayer **(Fig. 9 b).** However, ciliated cells are in constant motion and thus attach slower. As a consequence, the two different cell types, ciliated and secretory cells, with a different adhesion behavior may cause a higher proportion of secretory to ciliated cells **(Fig. 9 a, Fig. 9 b)** (Rottmayer, et al. 2006). Furthermore, enzymatic treatment may damage the ciliated cells due to their bigger cell surface. Therefore, in more recent publications, oviductal cells were obtained by mechanical procedures. Cells are remaining in cell aggregates **(Fig. 9 c)** or explants **(Fig. 9 d)** and cultured in suspension (Boquest and Summers 1999, De Pauw, et al. 2002, Kamishita, et al. 1999, Lim, et al. 1999, Ulbrich, et al. 2003, Yadav, et al. 1998).

Monolayer or primary explant culture?

Monolayers are obtained by seeding cells until a confluent layer of cells is obtained **(Fig. 9 b).** These cells have previously been enzymatically singularized. On the contrary, explants are produced by scraping the oviductal mucosal folds. Due to the constantly beating cilia, the explants remain in suspension **(Fig. d).** Oviduct explants **(Fig. 9 c, Fig. 9 d)** have several advantages when compared to monolayers **(Fig. 9 b)**. First of all, it has been proven that in cattle (Thibodeaux, et al. 1992, Walter 1995) and horses (Dobrinski, et al. 1999, Thomas, et al. 1995) proliferating oviductal cells grown in monolayers dedifferentiate with a concomitant reduction in cell height, loss of beating cilia and loss of secretory granules and bulbous protrusions.

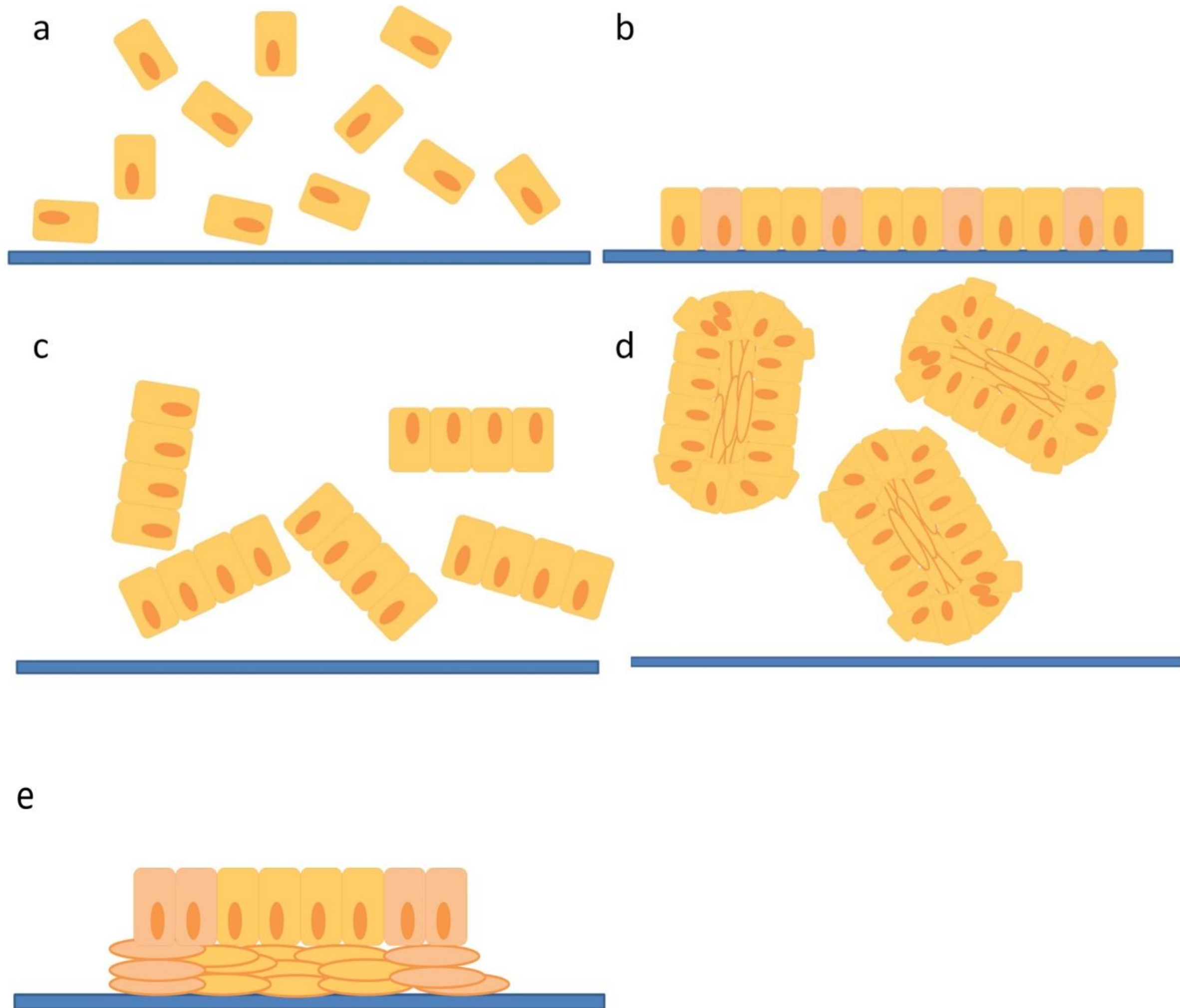

Fig. 9. a) Seeded singularized cells remain in suspension and do not adhere due to their own movement or to stirring of the culture medium. b) Singularized cells attach to the dish and start dividing. c) Explants of one cell type remain in suspension and do not divide. d) explants of two cell types form spherical structures, do not divide and do not adhere due to their own movement. e) Explants of two cell types (ciliated and secretory cells) adhere to the dish (orange cells) and start dividing (pink cells = proliferated cells). Adapted from Rottmayer 2006 and Rottmayer, et al. 2006.

In contrast, the cells bordering the explants maintain their ultrastructural highly differentiated morphology, including numerous mitochondria and rough endoplasmic reticulum, highly similar to the *in vivo* oviduct epithelium. The fact that the ciliation process is the endpoint of differentiation that cannot be induced in an *in vitro* system (Thibodeaux, Myers *et al.* 1992a), this means an important benefit of the explant suspension.

In conclusion, despite the fact that the use of monolayers and resulting cell lines minimizes the risk of disease transmission and batch-dependent variations (Menck, et al. 1997), they are a less solid reflection of the *in vivo* situation (Reischl, et al. 1999, Thibodeaux, et al. 1992, Walter 1995). In our hands, beating cilia in equine oviduct cell aggregates were still detected after 20 days in culture (data not shown). Transmission electron microscopy confirmed the presence of healthy cilia and numerous microvilli (**Fig. 4 a**).

Conditioned media

The use of conditioned media exposed to somatic cells for embryo culture is a technique closely related to co-culture (Orsi and Reischl 2007). Media have been conditioned with oviduct cells in cows (Eyestone and First 1989, Rieger, et al. 1995, Satoh, et al. 1994), in pigs (Vatzias and Hagen 1999) and in mice (Xu, et al. 2001); with granulosa cells in mice (Kobayashi, et al. 1996) and cows (Maeda, et al. 1996, Satoh, et al. 1994); with Vero cells in cows (Maeda, et al. 1996); and with trophoblast cells in horses (Choi, et al. 2001). In conditioned media, there is no interaction between the embryo and the feeder cells (Xu, et al. 2001). Whereas in co-culture systems oviduct cells may alter their secretory profile in response to certain ligands produced by the embryos, a considerable part of the embryo-maternal dialogue cannot take place in conditioned media, making them less appropriate to elaborate on the interaction between the embryo and the oviduct. Moreover, although conditioned media have been reported to have beneficial effects on embryo culture (Maeda, et al. 1996, Satoh, et al. 1994, Vatzias and Hagen 1999), they appear to be less favorable compared to oviduct cell co-culture in cattle, because conditioned media increase the incidence of chromosomal anomalies in cattle embryos (Li, et al. 2004). Furthermore, the only report available about conditioned media tested in the horse, did not show any beneficial effect (Choi, et al. 2001).

6.2 The equine embryo and co-culture

Early studies using *in vivo* derived horse embryos showed that the horse oviduct was a suitable environment for early embryo development, whereas the horse uterus was not suitable to allow embryo survival before day 4 after ovulation **(Table 4).**

Table 4. Survival of *in vivo* derived equine embryos of different ages after transfer to respectively the equine oviduct or uterus.

Day after ovulation in donor when embryo recovered	**Presumed embryonic stage at the time of transfer***	**Further development per cleaved embryo after transfer in synchronous recipients into ****		**Reference**
		Oviduct	Uterus	
1	2-4 cell	---	0/2	Allen & Rowson 1975
2	5-8 cell	5/7	0/7	Weber et al., 1993,
3	16-cell	---	1/5	Allen & Rowson 1975
4	Morula	7/10	5/10	Peyrot et al., 1987
5	32-64 -cell	---	4/4	Allen & Rowson 1975
6	Early blastocyst	---	1/1	Allen & Rowson 1975

*Stages are based on Betteridge 1995
** further development to day 10-21 or as a confirmed pregnancy after day 34 or by birth of a foal

Many embryotrophic factors do not seem to be tissue specific since, e.g. Vero cells, granulosa cells and fibroblast cells next to oviductal cells, were able to sustain equine embryo development and to enhance blastocyst percentage (Ball and Altschul 1990; Ball, et al. 1991; Freeman, et al. 1991; Weber, et al. 1993; Rosati, et al. 2002) (**Table 5** and **Table 6).**

Table 5. Co-culture of *in vivo* derived horse embryos with different cell types and in different media. ML = monolayer; FUB = foetal uterine fibroblast.

Cell type	Medium	% blastocysts	Reference
Trophoblastic vesicles	DMEM/F-12	0	Ball, et al. 1991
Equine FUB ML	Ham's F12	12.5	Ball and Altschul 1990
Bovine FUB ML	Ham's F12	57	Ball and Altschul 1990
Equine oviductal explants	DMEM/F-12	43	Ball, et al. 1991
Equine oviductal tissue	DMEM/F-12	43	Weber, et al. 1993

Table 6. Co-culture of horse embryos produced *in vitro* after ICSI with different cell types and media. Bold: beneficial effect on blastocyst percentage.

Celtype	medium	% blastocysts	Reference
Equine oviductal explants	DMEM/F-12	24 %	Choi, et al. 2004a
Verocell monolayer	Ménézo B2	0 %	Dell'Aquila, et al. 1997
Verocell monolayer	TCM199	8.6 %	Guignot, et al. 1998
Granulosacell monolayer	TCM199	14 %	Rosati, et al. 2002

Although not yet soundly investigated and compared in the horse, there is a trend in many species that fibroblasts perform more poorly as co-culture cells compared to oviductal cells (Gandolfi and Moor 1987, Menezo, et al. 1998, Rexroad and Powell 1988). Therefore, it is generally accepted that oviduct epithelial cells are the most appropriate cells to mimic the post-fertilization's and early embryonic development's environment.

Consequently, this elucidates their superior performance as feeder cells in co-culture in other species than the horse, including human (Gandolfi and Moor 1987, Liu, et al. 1998, Menezo, et al. 1998, Rexroad and Powell 1993).

However, blastocyst rate as the sole parameter for equine embryo development is maybe not sensitive enough. Cell number, apoptotic index, gene expression profile and cryotolerance are

reliable markers for embryo quality and viability (Desai, et al. 2000, Kane, et al. 1997, Kobayashi, et al. 1996, Rizos, et al. 2002, Xu, et al. 2004).

When equine ICSI started to take off, horse zygotes were at first immediately transferred to oviducts of recipient mares in order to create foals (Squires, et al. 2003, Squires, et al. 1996). This was yielding blastocyst rates of about 36% (Choi, et al. 2004a, Hinrichs and Choi 2005). A logical next step would then be to try to culture horse embryos in host oviducts, like those of sheep. By applying this procedure, horse blastocyst development could be more than doubled when compared to total *in vitro* culture (56% in sheep oviduct *vs* 20% after *in vitro* culture out of cleaved oocytes (Lazzari, et al. 2010, Lazzari, et al. 2002). Culture of horse embryos in sheep oviducts, was, however not able to speed up the development of the *in vitro* produced embryos: they were still lagging behind as far as size and cell number was concerned, compared to *in vivo* derived horse embryos (Lazzari, et al. 2010, Tremoleda, et al. 2003). Although the ligated sheep oviduct is probably up to now the best culture system for equine as well as bovine and porcine embryos (Orsi and Reischl 2007), the technique was buried in oblivion due to animal welfare, practical and biosecurity reasons.

In the horse, further research on the topic of somatic cell co-culture was abandoned after the discovery that the basic cell culture medium DMEM/F-12 could support more than 35% blastocyst development of ICSI embryos in low oxygen conditions (Choi, et al. 2006, Hinrichs and Choi 2005). In view of the fact that the oviductal environment represents the optimal environment for early embryo development, progress may still be made. Taking the genital tract as an example: *in vitro* produced equine embryos show qualitatively marked aberrations compared to their *in vivo* counterparts (Smits, et al. 2011, Tremoleda, et al. 2003). Therefore, oviduct explants culture is definitely the way forward towards the deciphering of the embryo-maternal dialogue which could lead to the improvement of the embryo culture protocol.

4 REFERENCES

Abe, H 1996 The mammalian oviductal epithelium: Regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology and Histopathology* **11** 743-768.

Abe, H, and H Hoshi 2007 Regional and cyclic variations in the ultrastructural features of secretory cells in the oviductal epithelium of the Chinese Meishan pig. *Reproduction in Domestic Animals* **42** 292-298.

Abe, H, M Onodera, S Sugawara, T Satoh, and H Hoshi 1999 Ultrastructural features of goat oviductal secretory cells at follicular and luteal phases of the oestrous cycle. *Journal of Anatomy* **195** 515-521.

Aguilar, J, and M Reyley 2005 The uterine tubal fluid: secretion, composition and biological effects. *Animal Reproduction* **2** 91-105.

Aguilar, JJ, J Cuervo-Arango, H Mouguelar, and L Losinno 2012 Histological Characteristics of the Equine Oviductal Mucosa at Different Reproductive Stages. *Journal of Equine Veterinary Science* **32** 99-105.

Allen, WR 2000. The physiology of early pregnancy in the mare., pp. 338-354: IVIS.

Allen, WR, and RM Moor 1972 The origin of the equine endometrial cups. *Journal of Reproduction and Fertility* **29** 313-316.

Allen, WR, and S Wilsher 2009 A Review of Implantation and Early Placentation in the Mare. *Placenta* **30** 1005-1015.

Allen, WR, S Wilsher, L Morris, JS Crowhurst, MH Hillyer, and HN Neal 2006 Laparoscopic application of PGE(2) to re-establish oviducal patency and fertility in infertile mares: a preliminary study. *Equine Veterinary Journal* **38** 454-459.

Ambruosi, B, G Accogli, C Douet, S Canepa, G Pascal, P Monget, C Moros, U Holmskov, J Mollenhauer, C Robbe-Masselot, O Vidal, S Desantis, and G Goudet 2013 Deleted in malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism of fertilization in equine and porcine species. *Reproduction* **146** 119-133.

Araki, Y, M Nohara, H Yoshida-Komiya, T Kuramochi, M Ito, H Hoshi, Y Shinkai, and Y Sendai 2003 Effect of a null mutation of the oviduct-specific glycoprotein gene on mouse fertilization. *Biochemical Journal* **374** 551-557.

Azuma, T, YH Choi, S Hochi, and N Oguri 1995 Effect of glucose in the culture medium on development of horse oocytes matured and microfertilized in vitro. *Reproduction, Fertility and Development* **7** 1067-1071.

Ball, BA, and M Altschul 1990 In vitro development of 4- to 8- cell equine embryos cocultured with trophoblastic vesicles or oviductal explants. . *Theriogenology* **33** 189.

Ball, BA, M Altschul, and JE Ellington 1991 Invitro Development of Day-2 Equine Embryos Cocultured with Oviductal Explants or Trophoblastic Vesicles. *Theriogenology* **35** 669-682.

Ball, BA, SP Brinsko, PGA Thomas, PG Miller, and JE Ellington 1993 Development to Blastocysts of One-Cell to 2-Cell Equine Embryos after Coculture with Uterine Tubal Epithelial-Cells. *American Journal of Veterinary Research* **54** 1139-1144.

Bastias, MC, ST Mcgeebelser, SH Bryan, and JM Vasquez 1993 In-Vitro Deleterious Effect of Hypoxanthine in Ham Nutrient Mixture F-10-Asterisk Culture-Medium on Human Oocyte Fertilization and Early Embryonic-Development. *Fertility and Sterility* **60** 876-880.

Bavister, BD 1992 Coculture for Embryo Development - Is It Really Necessary. *Human Reproduction* **7** 1339-1341.

Bavister, BD 1995 Culture of preimplantation embryos: Facts and artifacts. *Human Reproduction Update* **1** 91-148.

Beato, M 1989 Gene-Regulation by Steroid-Hormones. *Cell* **56** 335-344.

Betterbed, B, and RW Wright 1985 Development of One-Cell Ovine Embryos in 2 Culture Media under 2 Gas Atmospheres. *Theriogenology* **23** 547-553.

Betteridge KJ, and D Mitchell 1972 Retention of Ova by Fallopian Tube in Mares. *Journal of Reproduction and Fertility* **31** 515-515.

Betteridge, KJ 2000 Comparative aspects of equine embryonic development. *Animal Reproduction Science* **60–61** 691-702.

Betteridge, KJ, MD Eaglesome, D Mitchell, PF Flood, and R Beriault 1982 Development of Horse Embryos up to 22 Days after Ovulation - Observations on Fresh Specimens. *Journal of Anatomy* **135** 191-209.

Bezard, J, M Magistrini, I Battut, G Duchamp, and E Palmer 1992 Invitro Fertilization in the Mare. *Recueil De Medecine Veterinaire* **168** 993-1003.

Block, J, C Wrenzycki, H Niemann, D Herrmann, and PJ Hansen 2008 Effects of insulin-like growth factor-1 on cellular and molecular characteristics of bovine blastocysts produced in vitro. *Molecular Reproduction and Development* **75** 895-903.

Bonilla, AQS, M Ozawa, and PJ Hansen 2010 Actions of Insulin-Like Growth Factor I to Increase Development of Bovine Embryos to the Blastocyst Stage. *Biology of Reproduction* 93-93.

Boquest, AC, and PM Summers 1999 Effects of 17 beta-oestradiol or oestrous stage-specific cow serum on the ability of bovine oviductal epithelial cell monolayers to prolong the viability of bull spermatozoa. *Animal Reproduction Science* **57** 1-14.

Brewis, IA, RML Winston, and HJ Leese 1992 Energy-Metabolism of the Human Fallopian-Tube. *Journal of Reproduction and Fertility* **95** 257-262.

Brinsko, SP, GG Ignotz, BA Ball, PGA Thomas, WB Currie, and JE Ellington 1996 Characterization of polypeptides synthesized and secreted by oviductal epithelial cell explants obtained from young, fertile and aged, subfertile mares. *American Journal of Veterinary Research* **57** 1346-1353.

Buhi, WC 2002 Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* **123** 355-362.

Buhi, WC, and IM Alvarez 1998 Purification and identification of three proteins synthesized de novo by porcine oviductal tissue. *Biology of Reproduction* **58** 198-198.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Byrne, AT, J Southgate, DR Brison, and HJ Leese 2002a Effects of insulin-like growth factors I and II on tumour-necrosis-factor-alpha-induced apoptosis in early murine embryos. *Reproduction Fertility and Development* **14** 79-83.

Byrne, AT, J Southgate, DR Brison, and HJ Leese 2002b Regulation of apoptosis in the bovine blastocyst by insulin and the insulin-like growth factor (IGF) superfamily. *Molecular Reproduction and Development* **62** 489-495.

Campbell, DL, LW Douglas, and JC Ramge 1979 Cannulation of the Equine Oviduct and Chemical-Analysis of Oviduct Fluid. *Theriogenology* **12** 47-59.

Carney, EW, C Tobback, JE Ellington, and RH Foote 1990 Coculture of Rabbit 2-Cell Embryos with Rabbit Oviduct Epithelial-Cells and Other Somatic-Cells. *Molecular Reproduction and Development* **27** 209-215.

Catt, JW 1994 Bovine Embryo Coculture. *Cell Biology International* **18** 1155-1162.

Cheng, FP, AR Fazeli, WF Voorhout, JL Tremoleda, MM Bevers, and B Colenbrander 1998 Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances in vitro binding to the zona pellucida. *International Journal of Andrology* **21** 57-66.

Chin, PY, AM Macpherson, JG Thompson, M Lane, and SA Robertson 2009 Stress response genes are suppressed in mouse preimplantation embryos by granulocyte-macrophage colony-stimulating factor (GM-CSF). *Human Reproduction* **24** 2997-3009.

Choi, YH, YG Chung, GE Seidel, and EL Squires 2001 Developmental capacity of equine oocytes matured and cultured in equine trophoblast-conditioned media. *Theriogenology* **56** 329-339.

Choi, YH, CC Love, DD Varner, and K Hinrichs 2006 Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology* **65** 808-819.

Choi, YH, CC Love, DD Varner, LB Love, and K Hinrichs 2003 Effects of gas conditions, time of medium change, and ratio of medium to embryo on in vitro development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219-1229.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004a Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Choi, YH, LM Roasa, CC Love, DD Varner, S Brinsko, and K Hinrichs 2004b Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. *Biology of Reproduction* **70** 1231-1238.

Choi, YH, PJ Ross, IC Velez, BM Garcia, FL Riera, and K Hinrichs 2015 Cell lineage allocation in equine blastocysts produced in vitro under varying glucose concentrations. *Reproduction* doi: 10.1530/REP-14-0662.

Colleoni, S, R Duchi, S Barbacini, D Necchi, G Mari, M Spinaci, G Lazzari, and C Galli 2009 Practical Applications of OPU, ICSI and IVC in Equine Reproduction. *Reproduction in Domestic Animals* **44** 62-62.

Cox, CI, and HJ Leese 1997 Retention of functional characteristics by bovine oviduct and uterine epithelia in vitro. *Animal Reproduction Science* **46** 169-178.

De Pauw, IM, A Van Soom, H Laevens, S Verberckmoes, and A de Kruif 2002 Sperm binding to epithelial oviduct explants in bulls with different nonreturn rates investigated with a new in vitro model. *Biol Reprod* **67** 1073-1079.

Dell'Aquila, ME, YS Cho, P Minoia, V Traina, GM Lacalandra, and F Maritato 1997 Effects of follicular fluid supplementation of in-vitro maturation medium on the fertilization and development of equine oocytes after in-vitro fertilization or intracytoplasmic sperm injection. *Human Reproduction* **12** 2766-2772.

Desai, N, J Lawson, and J Goldfarb 2000 Assessment of growth factor effects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage. *Human Reproduction* **15** 410-418.

Desantis, S, G Ventriglia, S Zizza, AC Guaricci, M Losurdo, A Zarrilli, and M Albrizio 2010 Changes in the expression of the mu-opioid receptor in the mare oviduct during oestrus and anoestrus. *Animal Reproduction Science* **119** 40-49.

Dobrinski, I, JR Jacob, BC Tennant, and BA Ball 1999 Generation of an equine oviductal epithelial cell line for the study of sperm-oviduct interactions. *Theriogenology* **52** 875-885.

Donnez, J, and F Casanasroux 1985 Histophysiology of the Fallopian-Tube. *Contraception Fertilite Sexualite* **13** 1173-1179.

Dumoulin, JCM, CJJ Meijers, M Bras, E Coonen, JPM Geraedts, and JLH Evers 1999 Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Human Reproduction* **14** 465-469.

Eckert, J, PA Pugh, JG Thompson, H Niemann, and HR Tervit 1998 Exogenous protein affects developmental competence and metabolic activity of bovine pre-implantation embryos in vitro. *Reproduction Fertility and Development* **10** 327-332.

Ellington, JE 1991 The Bovine Oviduct and Its Role in Reproduction - a Review of the Literature. *Cornell Veterinarian* **81** 313-328.

Ellington, JE, EW Carney, PB Farrell, ME Simkin, and RH Foote 1990 Bovine 1-2-Cell Embryo Development Using a Simple Medium in 3 Oviduct Epithelial-Cell Coculture Systems. *Biology of Reproduction* **43** 97-104.

Ellington, JE, DD Varner, RC Burghardt, VN Meyers-Wallen, R Barhoumi, SP Brinsko, and BA Ball 1993 Cell-to-cell communication of equine uterine tube (oviduct) cells as determined by anchored cell analysis in culture. *Animal Reproduction Science* **30** 313-324.

Engle, CC, CW Foley, ED Plotka, and DM Witherspoon 1984 Free Amino-Acids and Protein Concentrations in Reproductive-Tract Fluids of the Mare. *Theriogenology* **21** 919-930.

Eriksen, T, O Terkelsen, C Grondahl, and I Brück 1994a The equine oviduct - histology and PGDF localization. *Theriogenology* **41** 191.

Eriksen, T, O Terkelsen, P Hyttel, and T Greve 1994b Ultrastructural Features of Secretory-Cells in the Bovine Oviduct Epithelium. *Anatomy and Embryology* **190** 583-590.

Eyestone, WH, and NL First 1989 Co-Culture of Early Cattle Embryos to the Blastocyst Stage with Oviducal Tissue or in Conditioned Medium. *Journal of Reproduction and Fertility* **85** 715-720.

Feng, HL, XH Wen, T Amet, and SC Presser 1996 Effect of different co-culture systems in early human embryo development. *Human Reproduction* **11** 1525-1528.

Fischer, B, and BD Bavister 1993 Oxygen-Tension in the Oviduct and Uterus of Rhesus-Monkeys, Hamsters and Rabbits. *Journal of Reproduction and Fertility* **99** 673-679.

Flood, LP, and B Shirley 1991 Reduction of Embryotoxicity by Protein in Embryo Culture Media. *Molecular Reproduction and Development* **30** 226-231.

Flood, PF, A Jong, and KJ Betteridge 1979 Location of Eggs Retained in the Oviducts of Mares. *Journal of Reproduction and Fertility* **57** 291-&.

Freeman, DA, JE Butler, JA Weber, RT Geary, and GL Woods 1991 Co-culture of day-5 to day-7 equine embryos in medium with oviductal tissue. *Theriogenology* **36** 815-822.

Freeman, DA, GL Woods, DK Vanderwall, and JA Weber 1992 Embryo-Initiated Oviductal Transport in Mares. *Journal of Reproduction and Fertility* **95** 535-538.

Galli, C, S Colleoni, R Duchi, I Lagutina, and G Lazzari 2007 Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Animal Reproduction Science* **98** 39-55.

Galli, C, G Crotti, P Turini, R Duchi, G Mari, G Zavaglia, G Duchamp, P Daels, and G Lazzari 2002 Frozen-thawed embryos produced by Ovum Pick Up of immature oocytes and ICSI are capable to establish pregnancies in the horse. *Theriogenology* **58** 705-708.

Gandolfi, F, TAL Brevini, L Richardson, CR Brown, and RM Moor 1989 Characterization of Proteins Secreted by Sheep Oviduct Epithelial-Cells and Their Function in Embryonic-Development. *Development* **106** 303-&.

Gandolfi, F, and RM Moor 1987 Stimulation of Early Embryonic-Development in the Sheep by Coculture with Oviduct Epithelial-Cells. *Journal of Reproduction and Fertility* **81** 23-28.

Gardner, DK 2008 Dissection of culture media for embryos: the most important and less important components and characteristics. *Reproduction Fertility and Development* **20** 9-18.

Gardner, DK, and M Lane 1996 Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: Role of amino acids, EDTA and physical parameters. *Human Reproduction* **11** 2703-2712.

Germana, A, R Cassata, S Cristarella, A Scirpo, and U Muglia 2002 Three-dimensional architecture of the myosalpinx in the mare as revealed by scanning electron microscopy. *Anatomical Record* **267** 235-241.

Ginther, OJ 1995 Ultrasonic imaging and animal reproduction. Horses. Book 2. Cross Plains, WI: Equiservices Publishing.

Goff, AK, S Leduc, P Poitras, and D Vaillancourt 1993 Steroid-Synthesis by Equine Conceptuses between Days 7 and 14 and Endometrial Steroid-Metabolism. *Domestic Animal Endocrinology* **10** 229-236.

Goto, Y, Y Noda, T Mori, and M Nakano 1993 Increased Generation of Reactive Oxygen Species in Embryos Cultured Invitro. *Free Radical Biology and Medicine* **15** 69-75.

Guerin, P, and Y Menezo 2011 Review: Role of tubal environment in preimplantation embryogenesis: application to co-culture assays. *Zygote* **19** 47-54.

Guignot, F, M Ottogalli, JM Yvon, and M Magistrini 1998 Preliminary observations in in vitro development of equine embryo after ICSI. *Reproduction Nutrition Development* **38** 653-663.

Hanukoglu, I 1992 Steroidogenic Enzymes - Structure, Function, and Role in Regulation of Steroid-Hormone Biosynthesis. *Journal of Steroid Biochemistry and Molecular Biology* **43** 779-804.

Harvey, MB, MY Arcellanapanlilio, X Zhang, GA Schultz, and AJ Watson 1995 Expression of Genes Encoding Antioxidant Enzymes in Preimplantation Mouse and

Cow Embryos and Primary Bovine Oviduct Cultures Employed for Embryo Coculture. *Biology of Reproduction* **53** 532-540.

Heap, RB, M Hamon, and WR Allen 1982 Studies on estrogen sythesis by the preimplantation equine conceptus. *Journal of Reproduction and Fertility (Supplement)* **32** 343-352.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hinrichs, K, and Y-H Choi 2005 Assisted reproductive techniques in the horse. *Clinical techniques in equine practice* **4** 210-218.

Hodgson, BJ, A Talo, and CJ Pauerstein 1977 Oviductal Ovum Surrogate Movement - Interrelation with Muscular-Activity. *Biology of Reproduction* **16** 394-396.

Holtan, DW, E Houghton, M Silver, AL Fowden, J Ousey, and PD Rossdale 1991 Plasma progestagens in the mare, fetus and newborn foal. *Journal of reproduction and fertility. Supplement* **44** 517-528.

Hugentobler, SA, PG Humpherson, HJ Leese, JM Sreenan, and DG Morris 2008 Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. *Molecular Reproduction and Development* **75** 496-503.

Hugentobler, SA, JM Sreenan, PG Humpherson, HJ Leese, MG Diskin, and DG Morris 2010 Effects of changes in the concentration of systemic progesterone on ions, amino acids and energy substrates in cattle oviduct and uterine fluid and blood. *Reproduction Fertility and Development* **22** 684-694.

Hunter, RHF 1994 Modulation of Gamete and Embryonic Microenvironments by Oviduct Glycoproteins. *Molecular Reproduction and Development* **39** 176-181.

Hunter, RHF 2005 The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction Nutrition Development* **45** 281-290.

Hunter, RHF, and R Nichol 1988 Capacitation Potential of the Fallopian-Tube - a Study Involving Surgical Insemination and the Subsequent Incidence of Polyspermy. *Gamete Research* **21** 255-266.

Jousan, FD, and PJ Hansen 2004 Insulin-like growth factor-I as a survival factor for the bovine preimplantation embryo exposed to heat shock. *Biology of Reproduction* **71** 1665-1670.

Kamishita, H, M Takagi, YH Choi, MP Wijayagunawardane, K Miyazawa, and K Sato 1999 Development of in vitro matured and fertilized bovine embryos cocultured with

bovine oviductal epithelial cells obtained from oviducts ipsilateral to cystic follicles. *Animal Reproduction Science* **56** 201-209.

Kan, FWK, E Roux, and G Bleau 1993 Immunolocalization of Oviductin in Endocytic Compartments in the Blastomeres of Developing Embryos in the Golden-Hamster. *Biology of Reproduction* **48** 77-88.

Kane, MT, PM Morgan, and C Coonan 1997 Peptide growth factors and preimplantation development. *Human Reproduction Update* **3** 137-157.

Kapur, RP, and LV Johnson 1986 Selective Sequestration of an Oviductal Fluid Glycoprotein in the Perivitelline Space of Mouse Oocytes and Embryos. *Journal of Experimental Zoology* **238** 249-260.

Khurana, NK, and H Niemann 2000 Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* **54** 741-756.

Killian, GJ 2004 Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Animal Reproduction Science* **82-3** 141-153.

Kobayashi, M, M Hirako, Y Minato, K Sasaki, R Horiuchi, and I Domeki 1996 Rat hepatoma reuber H-35 cells produce a 2-cell stage-specific inhibitor of the cleavage of mouse embryos. *Biology of Reproduction* **54** 364-370.

LaVoie, HA, and SR King 2009 Transcriptional Regulation of Steroidogenic Genes: STARD1, CYP11A1 and HSD3B. *Experimental Biology and Medicine* **234** 880-907.

Lazzari, G, S Colleoni, I Lagutina, G Crotti, P Turini, I Tessaro, D Brunetti, R Duchi, and C Galli 2010 Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus in vitro culture for different domestic species. *Theriogenology* **73** 748-757.

Lazzari, G, G Crotti, P Turini, R Duchi, G Mari, G Zavaglia, S Barbacini, and C Galli 2002 Equine embryos at the compacted morula and blastocyst stage can be obtained by intracytoplasmic sperm injection (ICSI) of in vitro matured oocytes with frozen-thawed spermatozoa from semen of different fertilities. *Theriogenology* **58** 709-712.

Leese, HJ 1988 The Formation and Function of Oviduct Fluid. *Journal of Reproduction and Fertility* **82** 843-856.

Leese, HJ, and SM Gray 1985 Vascular Perfusion - a Novel Means of Studying Oviduct Function. *American Journal of Physiology* **248** E624-E632.

Leese, HJ, SA Hugentobler, SM Gray, DG Morris, RG Sturmey, SL Whitear, and JM Sreenan 2008 Female reproductive tract fluids: Composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction Fertility and Development* **20** 1-8.

Leese, HJ, JI Tay, J Reischl, and SJ Downing 2001 Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction* **121** 339-346.

Levin, ER 2002 Cellular functions of plasma membrane estrogen receptors. *Steroids* **67** 471-475.

Li, GP, KL White, KI Aston, LN Meerdo, and TD Bunch 2004 Conditioned medium increases the polyploid cell composition of bovine somatic cell nuclear-transferred blastocysts. *Reproduction* **127** 221-228.

Li, XH, LHA Morris, and WR Allen 2001 Influence of co-culture during maturation on the developmental potential of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI). *Reproduction* **121** 925-932.

Lim, JM, Y Mei, B Chen, RA Godke, and W Hansel 1999 Development of bovine IVF oocytes cultured in medium supplemented with a nitric oxide scavenger or inhibitor in a co-culture system. *Theriogenology* **51** 941-949.

Lindenau, A, and B Fischer 1994 Effect of Oxygen Concentration in the Incubators Gas-Phase on the Development of Cultured Preimplantation Rabbit Embryos. *Theriogenology* **41** 889-898.

Lippes, J, J Krasner, LA Alfonso, ED Dacalos, and R Lucero 1981 Human Oviductal Fluid Proteins. *Fertility and Sterility* **36** 623-629.

Liu, LPS, STH Chan, PC Ho, and WSB Yeung 1998 Partial purification of embryotrophic factors from human oviductal cells. *Human Reproduction* **13** 1613-1619.

Maeda, J, F Kotsuji, A Negami, N Kamitani, and T Tominaga 1996 In vitro development of bovine embryos in conditioned media from bovine granulosa cells and Vero cells cultured in exogenous protein- and amino acid-free chemically defined human tubal fluid medium. *Biology of Reproduction* **54** 930-936.

Maller, JL 2003 Signal transduction. Fishing at the cell surface. *Science* **300** 594-595.

Matsukawa, K, S Akagi, N Adachi, F Sato, T Hasegawa, and S Takahashi 2007 In vitro development of equine Oocytes from preserved ovaries after intracytoplasmic sperm injection. *Journal of Reproduction and Development* **53** 877-885.

Mcdowell, KJ, MH Adams, and NM Williams 1993 Characterization of Equine Oviductal Proteins Synthesized and Released at Estrus and at Day 4 after Ovulation in Bred and Nonbred Mares. *Journal of Experimental Zoology* **267** 217-224.

Mcdowell, KJ, DC Sharp, W Grubaugh, WW Thatcher, and CJ Wilcox 1988 Restricted Conceptus Mobility Results in Failure of Pregnancy Maintenance in Mares. *Biology of Reproduction* **39** 340-348.

McEvoy, TG, KD Sinclair, LE Young, I Wilmut, and JJ Robinson 2000 Large offspring syndrome and other consequences of ruminant embryo culture in vitro: Relevance to blastocyst culture in human ART *Human Fertility* **3** 238-246.

Menck, MC, C GuyaderJoly, N Peynot, D LeBourhis, RB Lobo, JP Renard, and Y Heyman 1997 Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems. *Reproduction Nutrition Development* **37** 141-150.

Menezo, Y, and P Guerin 1997 The mammalian oviduct: biochemistry and physiology. *European Journal of Obstetrics & Gynecology and Reproductive Biology* **73** 99-104.

Menezo, Y, A Veiga, and M Benkhalifa 1998 Improved methods for blastocyst formation and culture. *Human Reproduction* **13** 256-265.

Minotti, G, and SD Aust 1989 The Role of Iron in Oxygen Radical Mediated Lipid-Peroxidation. *Chem Biol Interact* **71** 1-19.

Morales, H, P Tilquin, JF Rees, A Massip, F Dessy, and A Van Langendonckt 1999 Pyruvate prevents peroxide-induced injury of in vitro preimplantation bovine embryos. *Molecular Reproduction and Development* **52** 149-157.

Mortensen, CJ, YH Choi, NH Ing, DC Kraemer, MM Vogelsang, and K Hinrichs 2010 Heat shock protein 70 gene expression in equine blastocysts after exposure of oocytes to high temperatures in vitro or in vivo after exercise of donor mares. *Theriogenology* **74** 374-383.

Mugnier, S, M Kervella, C Douet, S Canepa, G Pascal, S Deleuze, G Duchamp, P Monget, and G Goudet 2009 The secretions of oviduct epithelial cells increase the equine in vitro fertilization rate: are osteopontin, atrial natriuretic peptide A and oviductin involved? *Reproductive Biology and Endocrinology* **7**.

Nancarrow, CD, and JL Hill 1994 Coculture, Oviduct Secretion and the Function of Oviduct-Specific Glycoproteins. *Cell Biology International* **18** 1105-1114.

Nasresfahani, MH, NJ Winston, and MH Johnson 1992 Effects of Glucose, Glutamine, Ethylenediaminetetraacetic Acid and Oxygen-Tension on the Concentration of Reactive Oxygen Species and on Development of the Mouse Preimplantation Embryo Invitro. *Journal of Reproduction and Fertility* **96** 219-231.

Neely, DP, GH Stabenfeldt, and CL Sauter 1979 Effect of Exogenous Oxytocin on Luteal Function in Mares. *Journal of Reproduction and Fertility* **55** 303-308.

Nett, TM, DW Holtan, and VL Estergreen 1973 Plasma Estrogens in Pregnant and Postpartum Mares. *Journal of Animal Science* **37** 962-970.

Nichol, R, RHF Hunter, DK Gardner, HJ Leese, and GM Cooke 1992 Concentrations of Energy Substrates in Oviductal Fluid and Blood-Plasma of Pigs during the Periovulatory Period. *Journal of Reproduction and Fertility* **96** 699-707.

Nickel, R, A Schummer, and E Seiferle 1987 Anatomie der Haustiere Band II. *In* V (ed.), Verlag Paul Parey. Berlin.

O'Neill, C 2008 The potential roles for embryotrophic ligands in preimplantation embryo development. *Human Reproduction Update* **14** 275-288.

Odor, DL, and RJ Blandau 1973 Egg Transport over Fimbrial Surface of Rabbit Oviduct under Experimental Conditions. *Fertility and Sterility* **24** 292-300.

Oguri, N, and Y Tsutsumi 1972 Non-Surgical Recovery of Equine Eggs, and an Attempt at Non-Surgical Egg Transfer in Horses. *Journal of Reproduction and Fertility* **31** 187-&.

Orsi, NM, and JB Reischl 2007 Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* **67** 441-458.

Ouhibi, N, Y Menezo, G Benet, and B Nicollet 1989 Culture of epithelial cells derived from the oviduct of different species. *Human Reproduction* **4** 229-235.

Palmer, E, J Bezard, M Magistrini, and G Duchamp 1991 In vitro fertilization in the horse. A retrospective study. *J Reprod Fertil Suppl* **44** 375-384.

Perkins, JL, and L Goode 1965 Protein Lactate and Phosphatase Activity in Oviduct Fluid of Ewes. *Journal of Animal Science* **24** 927-&.

Pomar, FJR, KJ Teerds, A Kidson, B Colenbrander, T Tharasanit, B Aguilar, and BAJ Roelen 2005 Differences in the incidence of apoptosis between in vivo and in vitro produced blastocysts of farm animal species: a comparative study. *Theriogenology* **63** 2254-2268.

Quinn, P, JF Kerin, and GM Warnes 1985 Improved Pregnancy Rate in Human Invitro Fertilization with the Use of a Medium Based on the Composition of Human Tubal Fluid. *Fertility and Sterility* **44** 493-498.

Rambags, BPB, HTA van Tol, MM van den Eng, B Colenbrander, and TAE Stout 2008 Expression of progesterone and oestrogen receptors by early intrauterine equine conceptuses. *Theriogenology* **69** 366-375.

Reischl, J, K Prelle, H Schol, C Neumuller, R Einspanier, F Sinowatz, and E Wolf 1999 Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro. *Cell and Tissue Research* **296** 371-383.

Rexroad, CE, and AM Powell 1988 Co-Culture of Ovine Eggs with Oviductal Cells and Trophoblastic Vesicles. *Theriogenology* **29** 387-397.

Rexroad, CE, and AM Powell 1993 Development of Ovine Embryos Cocultured on Oviductal Cells, Embryonic Fibroblasts, or Sto Cell Monolayers. *Biology of Reproduction* **49** 789-793.

Rieger, D, B Grisart, E Semple, A Vanlangendonckt, KJ Betteridge, and F Dessy 1995 Comparison of the Effects of Oviductal Cell Coculture and Oviductal Cell-Conditioned Medium on the Development and Metabolic-Activity of Cattle Embryos. *Journal of Reproduction and Fertility* **105** 91-98.

Rizos, D, P Lonergan, MP Boland, R Arroyo-Garcia, B Pintado, J de la Fuente, and A Gutierrez-Adan 2002 Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. *Biology of Reproduction* **66** 589-595.

Rooke, JA, TG McEvoy, CJ Ashworth, JJ Robinson, I Wilmut, LE Young, and KD Sinclair 2007 Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* **67** 639-647.

Rosati, I, F Berlinguer, L Bogliolo, G Leoni, S Ledda, and S Naitana 2002 The effect of co-culture on the development of in vitro matured equine oocytes after intracytoplastic sperm injection. *Equine Veterinary Journal* **34** 673-678.

Rottmayer, R 2006 A short-term suspension culture system for bovine oviduct epithelial cells suitable for the study of embryo-maternal communication processes., Faculty of Veterinary Medicine. Munchen: Ludwig-Maximilians-Universität.

Rottmayer, R, SE Ulbrich, S Kolle, K Prelle, C Neumueller, F Sinowatz, HHD Meyer, E Wolf, and S Hiendleder 2006 A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* **132** 637-648.

Santen, RJ 1990 Recent Progress in Development of Aromatase Inhibitors. *European Journal of Cancer* **26** 156-156.

Satoh, T, K Kobayashi, S Yamashita, M Kikuchi, Y Sendai, and H Hoshi 1994 Tissue Inhibitor of Metalloproteinases (Timp-1) Produced by Granulosa and Oviduct Cells Enhances in-Vitro Development of Bovine Embryo. *Biology of Reproduction* **50** 835-844.

Sendai, Y, H Abe, M Kikuchi, T Satoh, and H Hoshi 1994 Purification and Molecular-Cloning of Bovine Oviduct-Specific Glycoprotein. *Biology of Reproduction* **50** 927-934.

Sendai, Y, H Komiya, K Suzuki, T Onuma, M Kikuchi, H Hoshi, and Y Araki 1995 Molecular-Cloning and Characterization of a Mouse Oviduct-Specific Glycoprotein. *Biology of Reproduction* **53** 285-294.

Sharp, DC 2000 The early fetal life of the equine conceptus. *Animal Reproduction Science* **60** 679-689.

Simard, J, ML Ricketts, B Gingras, P Soucy, FA Feltus, and MH Melner 2005 Molecular biology of the 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase gene family. *Endocrine Reviews* **26** 525-582.

Simpson, ER 1979 Cholesterol Side-Chain Cleavage, Cytochrome-P450, and the Control of Steroidogenesis. *Molecular and Cellular Endocrinology* **13** 213-227.

Sinclair, KD 2008 Assisted reproductive technologies and pregnancy outcomes: Mechanistic insights from animal studies. *Seminars in Reproductive Medicine* **26** 153-161.

Sjoblom, C, CT Roberts, M Wikland, and SA Robertson 2005 Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* **146** 2142-2153.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, K, J Govaere, M Hoogewijs, S Piepers, and A Van Soom 2012 A Pilot Comparison of Laser-Assisted vs Piezo Drill ICSI for the In Vitro Production of Horse Embryos. *Reproduction in Domestic Animals* **47** e1-e3.

Spencer, TE, RC Burghardt, GA Johnson, and FW Bazer 2004 Conceptus signals for establishment and maintenance of pregnancy. *Animal Reproduction Science* **82-3** 537-550.

Squires, EL, EM Carnevale, PM McCue, and JE Bruemmer 2003 Embryo technologies in the horse. *Theriogenology* **59** 151-170.

Squires, EL, JM Wilson, H Kato, and A Blaszczyk 1996 A pregnancy after intracytoplasmic sperm injection into equine oocytes matured in vitro. *Theriogenology* **45** 306.

Stocco, DM, and BJ Clark 1996 Regulation of the acute production of steroids in steroidogenic cells. *Endocrine Reviews* **17** 221-244.

Sun, T, ZM Lei, and CV Rao 1997 A novel regulation of the oviductal glycoprotein gene expression by luteinizing hormone in bovine tubal epithelial cells. *Molecular and Cellular Endocrinology* **131** 97-108.

Suzuki, K, Y Sendai, T Onuma, H Hoshi, M Hiroi, and Y Araki 1995 Molecular Characterization of a Hamster Oviduct-Specific Glycoprotein. *Biology of Reproduction* **53** 345-354.

Swanson, WF, TL Roth, and RA Godke 1996 Persistence of the developmental block of in vitro fertilized domestic cat embryos to temporal variations in culture conditions. *Molecular Reproduction and Development* **43** 298-305.

Takeuchi, K, Y Nagata, BA Sandow, and GD Hodgen 1992 Primary Culture of Human Fallopian-Tube Epithelial-Cells and Coculture of Early Mouse Preembryos. *Molecular Reproduction and Development* **32** 236-242.

Tervit, HR, LEA Rowson, and Whitting.Dg 1972 Successful Culture in-Vitro of Sheep and Cattle Ova. *Journal of Reproduction and Fertility* **30** 493.

Thibodeaux, JK, MW Myers, LL Goodeaux, Y Menezo, JD Roussel, JR Broussard, and RA Godke 1992 Evaluating an in vitro culture system of bovine uterine and oviduct epithelial cells for subsequent embryo co-culture. *Reproduction, Fertility and Development* **4** 573-583.

Thomas, PGA, GG Ignotz, BA Ball, PG Miller, SP Brinsko, and B Currie 1995 Isolation, culture, and characterization of equine oviduct epithelial cells in vitro. *Molecular Reproduction and Development* **41** 468-478.

Thompson, JG, DK Gardner, PA Pugh, WH Mcmillan, and HR Tervit 1995 Lamb Birth-Weight Is Affected by Culture System Utilized during in-Vitro Pre-Elongation Development of Ovine Embryos. *Biology of Reproduction* **53** 1385-1391.

Thompson, JG, and AJ Peterson 2000 Bovine embryo culture in vitro: new developments and post-transfer consequences. *Human Reproduction* **15** 59-67.

Thompson, JGE, AC Simpson, PA Pugh, PE Donnelly, and HR Tervit 1990 Effect of Oxygen Concentration on Invitro Development of Preimplantation Sheep and Cattle Embryos. *Journal of Reproduction and Fertility* **89** 573-578.

Toran-Allerand, CD, XP Guan, NJ MacLusky, TL Horvath, S Diano, M Singh, ES Connolly, IS Nethrapalli, and AA Tinnikov 2002 ER-X: A novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *Journal of Neuroscience* **22** 8391-8401.

Tremoleda, JL, TAE Stout, I Lagutina, G Lazzari, MM Bevers, B Colenbrander, and C Galli 2003 Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biology of Reproduction* **69** 1895-1906.

Tsai, FCH, and DK Gardner 1994 Nicotinamide, a Component of Complex Culture Media, Inhibits Mouse Embryo Development in-Vitro and Reduces Subsequent Developmental Potential after Transfer. *Fertility and Sterility* **61** 376-382.

Tuohimaa, P, M Blauer, S Pasanen, S Passinen, A Pekki, R Punnonen, H Syvala, J Valkila, M Wallen, J Valiaho, YH Zhuang, and T Ylikomi 1996 Mechanisms of action of sex steroid hormones: Basic concepts and clinical correlations. *Maturitas* **23** S3-S12.

Ulbrich, SE, A Kettler, and R Einspanier 2003 Expression and localization of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in the bovine oviduct in vivo and in vitro. *Journal of Steroid Biochemistry and Molecular Biology* **84** 279-289.

Vajta, G, L Rienzi, A Cobo, and J Yovich 2010 Embryo culture: can we perform better than nature? *Reproductive Biomedicine Online* **20** 453-469.

Vanroose, G, A Van Soom, and A de Kruif 2001 From co-culture to defined medium: State of the art and practical considerations. *Reproduction in Domestic Animals* **36** 25-28.

Vatzias, G, and DR Hagen 1999 Effects of porcine follicular fluid and oviduct-conditioned media on maturation and fertilization of porcine oocytes in vitro. *Biology of Reproduction* **60** 42-48.

Wagh, PV, and J Lippes 1989 Human Oviductal Fluid Proteins .3. Identification and Partial-Purification. *Fertility and Sterility* **51** 81-88.

Walker, SK, KM Hartwich, and RF Seamark 1996a The production of unusually large offspring following embryo manipulation: Concepts and challenges. *Theriogenology* **45** 111-120.

Walker, SK, JL Hill, DO Kleemann, and CD Nancarrow 1996b Development of ovine embryos in synthetic oviductal fluid containing amino acids at oviductal fluid concentrations. *Biology of Reproduction* **55** 703-708.

Walter, I 1995 Culture of Bovine Oviduct Epithelial-Cells (Boec). *Anatomical Record* **243** 347-356.

Walters, KW, JF Roser, and GB Anderson 2001 Maternal-conceptus signalling during early pregnancy in mares: oestrogen and insulin-like growth factor 1. *Reproduction* **121** 331-338.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991a Prostaglandin E2 hastens oviductal transport of equine embryos. *Biol Reprod* **45** 544-546.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991b Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45** 540-543.

Weber, JA, GL Woods, and JJ Aguilar 1996 Location of equine oviductal embryos on day 5 post ovulation and oviductal transport time of day 5 embryos autotransferred to the contralateral oviduct. *Theriogenology* **46** 1477-1483.

Weber, JA, GL Woods, DA Freeman, and DK Vanderwall 1993 Oviductal and uterine influence on the development of Day-2 equine embryos in vivo and in vitro. *Theriogenology* **40** 689-698.

Wegner, CC, and GJ Killian 1991 Invitro and Invivo Association of an Oviduct Estrus-Associated Protein with Bovine Zona-Pellucida. *Molecular Reproduction and Development* **29** 77-84.

Wiemer, KE, PL Casey, DC Devore, and RA Godke 1988 The Culture of Equine Embryos Using New Monolayer-Cultures Derived from Fetal Uterine Fibroblast Cells. *Theriogenology* **29** 327-327.

Wiemer, KE, DI Hoffman, WS Maxson, S Eager, B Muhlberger, I Fiore, and M Cuervo 1993 Embryonic Morphology and Rate of Implantation of Human Embryos Following Coculture on Bovine Oviductal Epithelial-Cells. *Human Reproduction* **8** 97-101.

Willis, P, KNC Sekhar, P Brooks, and RA Fayrerhosken 1994 Electrophoretic Characterization of Equine Oviductal Fluid. *Journal of Experimental Zoology* **268** 477-485.

Wu, JT, Z Dickmann, and DC Johnson 1971 Effects of Oestrogen and Progesterone on Development, Oviductal Transport and Uterine Retention of Eggs in Hypophysectomized Pregnant Rats. *Journal of Endocrinology* **51** 569.

Xu, J, TM Cheung, ST-h Chan, PC Ho, and WSB Yeung 2000 Human oviductal cells reduce the incidence of apoptosis in cocultured mouse embryos. *Fertility and Sterility* **74** 1215-1219.

Xu, JS, STH Chan, WWM Lee, KF Lee, and WSB Yeung 2004 Differential growth, cell proliferation, and apoptosis of mouse embryo in various culture media and in coculture. *Molecular Reproduction and Development* **68** 72-80.

Xu, JS, TM Cheung, STH Chan, PC Ho, and WSB Yeung 2001 Temporal effect of human oviductal cell and its derived embryotrophic factors on mouse embryo development. *Biology of Reproduction* **65** 1481-1488.

Yadav, PS, A Saini, A Kumar, and GC Jain 1998 Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Animal Reproduction Science* **51** 301-306.

Yaniz, JL, F Lopez-Gatius, P Santolaria, and KJ Mullins 2000 Study of the functional anatomy of bovine oviductal mucosa. *Anatomical Record* **260** 268-278.

Yoshioka, K, C Suzuki, A Tanaka, IMK Anas, and S Iwamura 2002 Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of Reproduction* **66** 112-119.

CHAPTER 2 AIMS AND OUTLINES OF THE THESIS

Aim for the moon.
If you miss,
you may hit a star.
W. Clement Stone, businessman and writer

The equine embryo has an intense relationship with the Fallopian tube or oviduct, in which it resides during the first 6 days of its development. The oviduct is under constant cyclic influences of changing hormone levels of oestradiol and progesterone, which then also affect the developing embryo. Because it is difficult to get access to oviductal stage embryos and/or oviductal contents in living mares, so far, details concerning embryo-maternal communication are less well-known at this early stage of life in the horse.

The general goal of this thesis was to gain more insight in the embryo-maternal communication in the horse, using both the mare's oviduct and an *in vitro* oviduct explant model.

In order to achieve this goal, the following specific aims were pursued :

1. To optimize and validate an equine oviduct explant culture system, in order to be able to study steroid-induced changes in the oviduct and the interaction between the oviduct and the developing horse embryo *in vitro* (CHAPTER 3).
2. To determine local steroid concentrations as they prevail at the follicular and the luteal stage in the equine oviduct *in vivo* (CHAPTER 4).
3. To mimic the *in vivo* oviduct environment by exposing oviduct explants *in vitro* to typical follicular and luteal concentrations of steroids, and monitoring the explant's response by measuring different parameters (CHAPTER 5.1).
4. To investigate whether the addition of steroids (CHAPTER 5.1) and of equine embryos to oviduct explants *in vitro* (CHAPTER 5.2) affect the gene expression of the oviduct cells.
5. To improve equine embryo culture *in vitro*, by changing the temperature at which equine embryos are cultured *in vitro* to the body temperature of the mare (CHAPTER 6)

CHAPTER 3 OVIDUCT EXPLANTS: A TOOL TO STUDY EARLY REPRODUCTIVE EVENTS IN THE HORSE

The most exciting phrase to hear in science,

the one that heralds new discoveries,

is not "Eureka!"

but: "That's funny…".

Isaak Asimov, author and biochemist

PART 3.1: EQUINE OVIDUCT EXPLANT CULTURE: A BASIC MODEL TO DECIPHER EMBRYO-MATERNAL COMMUNICATION

Adapted from:

Equine oviduct explant culture: a basic model to decipher embryo-maternal communication.

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EQUINE OVIDUCT EXPLANT CULTURE: A BASIC MODEL TO DECIPHER EMBRYO-MATERNAL COMMUNICATION

ABSTRACT

Equine embryos remain for 6 days in the oviduct and thus there is a need for an *in vitro* model to study embryo– oviductal interactions in the horse, since this subtle way of communication is very difficult to analyse *in vivo*. Until now, no equine oviduct explant culture model has been characterised both morphologically and functionally. Therefore, we established a culture system for equine oviduct explants that maintained epithelial morphology during 6 days of culture, as revealed by light microscopy and transmission electron microscopy. We demonstrated the presence of highly differentiated, tall columnar, pseudostratified epithelium with basal nuclei, numerous nucleoli, secretory granules and apical cilia, which is very similar to the *in vivo* situation. Both epithelium and stromal cells originating from the lamina propria are represented in the explants. Moreover, at least 98% of the cells remained membrane intact and fewer than 2% of the cells were apoptotic after 6 days of culture. Although dark-cell degeneration, which is a hypoxia-related type of cell death, was observed in the center of the explants, quantitative real-time PCR failed to detect upregulation of the hypoxia-related marker genes *HIF1A*, *VEGFA*, *PLAU*, *GLUT1* and *PAI1*. Since the explants remained morphologically and functionally intact and since the system is easy to set up, it appears to be an excellent tool for proteome, transcriptome and miRNome analysis in order to unravel embryo–maternal interactions in the horse.

INTRODUCTION

Co-culture of bovine embryos with somatic cells has been used extensively in the early 1990s; since at that time, it has been the only way to overcome the 8- to 16-cell block (Eyestone and First 1989, Eyestone, et al. 1991). This indicates that the culture media used then were suboptimal for bovine embryo development and had to be ‘conditioned’ first by somatic cells. For a few years, co-culture became a routine technique to produce embryos *in vitro* and in many cases, oviduct epithelial cells were used e.g. in cattle (Vansoom, et al. 1992) and in pigs

(Hardy and Spanos 2002). Also in the horse, the benefits of oviduct co-culture were investigated using *in vivo*-collected equine embryos (Ball, et al. 1991, Ball, et al. 1993, Ball and Miller 1992, Brinsko, et al. 1994). At the end of the 1990s, semi-defined media were developed that were equally suited to culture embryos from the zygote to the blastocyst stage *in vitro*, with blastocyst rates of 35% reported for cattle embryos (in modified SOF with BSA; (Holm, et al. 1996, Lonergan, et al. 1991). Horse embryos produced by intracytoplasmic sperm injection (ICSI) can be successfully cultured to the blastocyst stage in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) plus serum (Carnevale, et al. 2000, Choi, et al. 2006, Choi, et al. 2004a, Hinrichs and Choi 2005, Smits, et al. 2012b, Yuan, et al. 2003) with percentages ranging between 10 and 35%. Since then, embryo–somatic cell co-culture has been abandoned and is considered to be a less desirable technique because it is labour intensive, may introduce pathogens and is less defined than cell-free medium (Bavister 1995, De Pauw, et al. 2003). However, in view of the fact that the oviductal environment represents the optimal environment for early embryo development, we want to reconsider the use of oviduct epithelial explants, especially for co-culture in *in vitro*-produced horse embryos to enable the study of embryo– maternal communication in the horse at the level of gene expression in an *in vitro* model that approximates the *in vivo* situation as closely as possible.

At present, molecular biology has progressed enormously compared with twenty years ago, making it possible to analyse subtle changes in gene expression at the level of single embryos or using very low amounts of tissue (Merkl, et al. 2010). Unfortunately, this does not allow us to study embryo–maternal interactions *in vivo*, ''because some interactions are very short or very local, making it very challenging to localise an embryo of only a few hundred µm in diameter in a large-size reproductive tract'' (Ulbrich, et al. 2013b). For the reasons mentioned above, we think it is timely now to reintroduce oviduct explants in horse-embryo culture, not for the purpose of increasing embryo production, but to study signaling events during the first embryonic cleavage divisions *in vitro*. To this end, the most important prerequisite is to preserve the 3D architecture of the oviduct by preventing epithelial cell dedifferentiation. Since monolayers dedifferentiate with a concomitant loss of important morphological characteristics, an explant culture system more closely resembles the *in vivo* situation (Walter 1995), since it is produced by stripping epithelial folds from the oviduct, which consist of both epithelial and stromal cells. In order to be used as an *in vitro* model to study embryo–oviduct interactions in the horse, the oviduct explants have to remain viable and exert normal gene expression for, 6 days, which is the time spent in the oviduct by the horse embryo. One

salient feature of the equine oviduct explants was that many of them showed central darkening after a few days of culture, while still displaying vigorous ciliary activity, a feature that we had never observed with bovine oviduct explants (De Pauw, et al. 2002). Therefore the aims of this study were: (1) to characterise equine oviduct explants morphologically by means of light, immunofluorescence and transmission electron microscopy over time and (2) to assess whether the central dark zones in the explants were induced by hypoxia by quantifying the expression of hypoxia-related genes with an embryotrophic function in explants at Day 0 and Day 6 of culture. In this paper, we show that epithelial morphology of equine oviduct explants is preserved during 6 days of culture, that the dark zones are caused by dark-cell degeneration and that hypoxia-related gene expression is not changed during culture, indicating that culture conditions are not hypoxic. The equine oviduct explant model is at present being used to study the importance of endocrine and paracrine signaling during early embryo development in the horse.

MATERIALS AND METHODS

Animals

Oviducts (three replicates of 7 to 10 oviducts) were obtained at a slaughterhouse from healthy warmblood mares aged 5–22 years and without any visible reproductive tract pathologies. Only ipsilateral oviducts of mares showing a recent corpus luteum or a corpus hemorrhagicum on the ovaries, indicating ovulation had occurred not later than 5 days earlier (Pierson and Ginther 1985), were used. Determination of progesterone concentration by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC/MS-MS) in another study (Nelis, et al. 2015a) revealed a positive predicting value of 94% between the presence of a corpus hemorrhagicum and a progesterone concentration lower than 2 ng/mL.

Preparation and morphological evaluation of oviduct explants

Oviducts of mares were trimmed of excess connective tissue, closed at both ends and transported in sterile 0.9% saline and gentamycin (50 mg/mL; Invitrogen, Merelbeke, Belgium) on ice. In the laboratory oviducts were washed in phosphate-buffered saline (PBS) and the epithelial cells were obtained by scraping the ampullary–isthmic region of the

longitudinally opened oviduct. The harvested cellular material was put in a tube containing home-made 10x 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Tyrode's albumin–lactate–pyruvate (TALP) and was left to settle for 10 min, after which the cell pellet was resuspended in 3 mL of fresh HEPES-buffered TALP washing medium. The process of sedimentation was repeated twice. Afterwards, the harvested cellular material was washed and cultured in DMEM/F12 (Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), 50 mg/mL gentamycin and 2.5 mg/mL amphotericin-B (Fungizone; Invitrogen) at 38.5°C in a humidified atmosphere with 5% CO_2 in air. The time-span from slaughter of mares to seeding of cells was 3 to 4 h. The DMEM/F12-FBS culture medium was refreshed the first and the fourth day after cell isolation. Only cultures with more than 99% of membrane-intact cells, determined by Trypan blue staining (Sigma-Aldrich, Diegem, Belgium), 2 to 4 h after start of culture (= Day 0) were used. In the first experiment we compared two culture systems: the first were 50 µL droplets under oil containing 5 explants with a diameter less than 200 µm per droplet and the second was identical to the first but contained only one vesicle with a diameter larger than 600 µm. Frozen damaged explants were used as a membrane-damaged control. These explants were subjected to three subsequent freeze (-20°C) –thaw (38°C) cycles in PBS. Every 24 h, 20 to 30 explants were fixed in 4% formaldehyde for 24 h and processed for haematoxylin–eosin staining. Next, ciliary activity and central dark-zone formation, as shown in **Fig. 1*a–c***, was evaluated every 24 h in the whole number of explants present in at least 20 droplets, containing 20–30 explants each. Explants were considered to show ciliary activity when bordered by vigorously beating cilia, clearly seen on the inverted microscope (400x magnification). To lower interpretative bias, all explants were counted by two researchers and the mean value was used for statistical analysis.

Fluorescence microscopy

To determine the presence of membrane-damaged cells, 10 explants were stained after every 24 h of culture with the nucleic stain SYBR14 and propidium iodide (PI; LIVE/DEAD Sperm Viability Kit; Molecular Probes, Leiden, The Netherlands; (Garner, et al. 1994). The explants were washed in HEPES-buffered washing medium, incubated in 5 mL of a 1:50 SYBR14 dilution in HEPES-buffered washing medium, for 15 min at 37°C, followed by a 5 min incubation with 5 mL PI. The stained explants were mounted in 1,4-diazabicyclo[2.2.2]octane

(DABCO) on siliconised glass slides. Explants that were frozen in PBS at -20°C for 24 h, subsequently thawed at 38°C and stained were used as a membrane-damaged control. To detect apoptotic and necrotic cells, epithelial explants showing dark zones after 6 days of culture were stained with a combination of Hoechst, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and anti-caspase based on previous publications (Gjorret, et al. 2007, Vandaele, et al. 2007, Wydooghe, et al. 2011). Explants were fixed in 4% paraformaldehyde for 20 min at room temperature and stored in PBS containing 0.5% bovine serum albumin (PBS–BSA; Sigma–Aldrich, Bornem, Belgium) at 4°C until the staining was performed. Positive-control explants were incubated overnight with 0.5 mM staurosporine. Fixed explants were permeabilised with 0.5% Triton X-100 (Sigma–Aldrich, Bornem) in PBS for 1 h and washed again in polyvinylpyrrolidon (PVP) solution. The explants were held for 1 h in 0.5% Triton X-100 in PBS at room temperature. Subsequently, the explants were washed three times for 2 min in PBS–BSA. After washing, the explants were blocked overnight in 10% goat serum (Invitrogen) and 0.05% Tween 20 in PBS at 4°C. Explants serving as negative controls remained in blocking solution. The test explants were washed two times for 15 min at room temperature and incubated overnight at 4°C in rabbit active caspase-3 antibody (0.768 mg/mL in blocking solution; Cell Signaling Technology, Leiden, The Netherlands). After another wash step (two times for 15 min), test explants and negative controls were transferred to goat anti-rabbit Texas Red antibody (20 mg/mL in blocking solution; Molecular Probes, Merelbeke, Belgium) for 1 h at room temperature.

For TUNEL staining, positive and negative controls were treated with DNase (50 U/ml in PBS) for 1 h at 37°C to ensure detection of strand breaks by TUNEL (*In Situ* Cell Detection kit; Boehringer, Mannheim, Germany). After washing, positive controls and samples were incubated in fluorescein (FITC)-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37°C in the dark. Meanwhile, the negative control was incubated in nucleotide mixture only in the absence of transferase. After a second washing, controls and samples were incubated in RNase A (50 mg/mL in PBS) for 1 h at room temperature. The explants were washed twice and transferred to Hoechst 33342 (50 mg/mL in PBS–BSA; Molecular Probes, Life Technologies Europe B.V., Ghent, Belgium) for 10 min at room temperature. Evaluation of the explants was performed the next day by fluorescence microscopy, with a U-M3DAFITR (Olympus, Aartselaar, Belgium) filter cub, a triple bandpass filter for 4^0,6-diamidino-2-phenylindole (DAPI) (440/40 filter, 405 nm laser), FITC (525/50 filter, 488 nm laser) and

Texas Red (595/50 filter, 561 nm laser). The number of cells of at least 10 explants from each of the three replicates was determined by counting the Hoechst-positive cells. Next, the percentage of anti-caspase-3 and TUNEL-positive cells was determined by counting all the Hoechst-, TUNEL- and anti-caspase-3-positive cells in at least 10 explants. After counting, per explant, the ratio of TUNEL- and/or anti-caspase-3-positive cells of the Hoechst-positive cells was determined. Images were acquired with a C1si confocal laser-scanning microscope (Nikon BeLux, Brussels, Belgium) using a Plan Apo 40x objective with a numerical aperture of 0.95 (Nikon). All filters and lasers were purchased from CVI Melles Griot (Albuquerque, NM, USA).

Transmission electron microscopy

At Days 0, 3 and 6 of culture, oviduct explants were fixed in 0.2 M sodium cacodylate-buffered formaldehyde and post-fixed with osmium tetroxide. After rinsing, cells were pelleted by centrifugation (for 5 min at 200*g* at room temperature) in 10% BSA supplemented with 1% glutaraldehyde. The pellet was then dehydrated and embedded in epoxy resin (LX-112; Ladd Industries, Williston, ND, USA). Sections were made with a Reichert Jung (Depew, NY, USA) Ultracut E ultra-microtome.

Semi-thin sections (2 µm) were stained with toluidine blue to select the most appropriate regions for ultrathin sectioning. Next, ultra-thin sections (90 nm) were made and stained with uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope (Jeol Europe, Zaventem, Belgium) at 80 kV.

RNA extraction and quantitative RT-PCR

Primer design and validation, RNA extraction and quantitative reverse transcription–polymerase chain reaction (RT-qPCR) were performed according to the MIQE-guidelines (Bustin, et al. 2009). Oviduct explants were cultured in 50 µL drops in DMEM/F12–10% FBS. At Days 0 and 6 of culture, between 40 to 70 explants per sample (n = 12 per group) were washed in Dulbecco’s Phosphate-Buffered Saline (DPBS; GIBCO BRL Invitrogen, Life Technologies Europe B.V., Ghent, Belgium) and conserved at -80°C in RNase-free water containing 10% RNasin Plus RNase inhibitor (Promega, Leiden, The Netherlands), 5%

dithiothreitol (Promega) and 0.8% Igepal CA-630 (Sigma-Aldrich, Bornem) until analysis.

The primers (**Table 1**; Integrated DNA Technologies, Leuven, Belgium and Sigma-Aldrich, Bornem, Belgium) for the genes of interest (*VEGFA*, *HIF1A*, *GLUT1*, *PAI1*, *PLAU*) were designed by means of Primer3 software (Misener, et al. 1999) (http://frodo.wi.mit.edu/primer3/, accessed 10 May 2011), based on horse sequences found in the NCBI GenBank (http://www. ncbi.nlm.nih.gov/, accessed 10 May 2011). To distinguish genomic DNA amplification, to provide specificity and to avoid secondary structures in the primer region, primers were selected over intron–exon boundaries, tested using a BLAST analysis against the NCBI database and characterised with MFold (Zuker 2003) (http://mfold.rna.albany.edu/?q=mfold/DNA- Folding-Form, accessed 10 May 2011). The amplicons were run on a 2% agarose gel and confirmed by nucleotide sequenc-ing. All primers are listed in **Tables 1 and 2.**

All RT-qPCR reactions were performed in duplicate with 2.5 µL of sample, 7.5 µL of the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA), 0.6 µL of 5 or 10 mM forward and reverse primer and 3.8 µL water. A blank, a melting curve and a 5- or 10-fold serial dilution series of pooled oviductal cDNA were included for each gene to check for contamination and specificity and to acquire PCR efficiencies **(Tables 1, 2)** based on a relative standard curve. Calculation of the Cq values, PCR efficiencies, correlation coefficients and analysis of the melting curves was performed by means of iCycler iQ Optical System Software Version 3.0a (Biorad, Nazareth, Belgium). All quantification cycle (C_q) values were converted into raw data using these PCR efficiencies and normalised by dividing them by their respective normalisation factors. This normalisation factor was determined per sample by calculating the geometric mean of the validated reference genes as determined for each type of explant. Therefore, a set of stable reference genes was identified in 10 samples of post-ovulatory oviduct explants at Day 0 and in 10 samples after 6 days of culture. Eight reference genes were selected based on previous studies (Bogaert, et al. 2006, Cappelli, et al. 2008, Goossens, et al. 2005, Smits 2009). The selected genes (*ACTB*, *GAPDH*, *HPRT*, *RPL32*, *SDHA*, *TUBA4A*, *18S* and *UBC*; **Table 2**) belonged to different functional classes, which reduced the chance of co-regulation. Primers for *ACTB*, *HPRT1*, *RPL32*, *TUBA4A* and *UBC* were provided by Bogaert, et al. (2006). Primers for *GAPDH*, *SDHA and 18S* were available from previous research in equine blastocysts (Smits, et al. 2009).

Table 1 For each gene the NCBI GenBank accession number, the sequence of both forward

and reverse primers, the size of the amplicon and the optimal primer annealing temperature are listed. F = forward primer; R = reverse primer.

Gene	GenBank accession number	Primer sequence (5'–3')	Amplicon size (bp)	Ta °C	Efficiency
PLAU	XM_001502951.4	F: AAAGTCCCTCCTCTCCTC R: CGAAGAAGGAGGACTACATT	249	61	92
VEGFA	NM_001081821	F: ACTGCCGTCCAATCGAGA R: ATCAAACCTCACCAAAGCCA	193	61	97
HIF1A	XM_001493206	F: TGCTGGAGACACAATCATA R: GAGTTTCAGAGGCAGGTAAT	167	59	87
GLUT1	NM_001163971.1	F: CCAGAAGGTGATCGAGGAAT R: CAGTTTTGAGAAGCCCATGA	238	60	118
PAI1	XM_001492517	F: ACTCGGAAGCAGATCCAAGA R: CAGGTGGACTTTTCAGAGGTG	223	61	87

The mRNA expression of five genes – glucose transporter 1 (*GLUT1*), vascular endothelial growth factor (*VEGFA*), hypoxia-inducible factor 1a subunit (*HIF1A*), plasminogen activator inhibitor 1 (*PAI1*) and urokinase plasminogen activator inhibitor (*PLAU*) was evaluated. These genes are upregulated under hypoxic culture conditions and can be used as marker genes for hypoxia. Moreover, these factors play an important role in early embryonic development (Buhi 2002, Carmeliet, et al. 1996, Liao, et al. 2007, Wrenzycki, et al. 2001b), so down-regulation of these genes could indicate a loss of functionality of the cells.

Table 2 For each gene the NCBI GenBank accession number, the sequence of both forward and reverse primers, the size of the amplicon and the optimal primer annealing temperature are listed. The RTPrimerDB ID http://rtprimerdb.org (accessed 10 April 2011) F = forward primer; R = reverse primer.

Gene	GenBank accession number	Primer sequence (5'-3')	Amplicon size (bp)	Ta (°C)	Efficiency (%)
ACTB	AF035774	CCAGCACGATGAAGATCAAG GTGGACAATGAGGCCAGAAT	88	60	101
GAPDH	AF157626	CAGAACATCATCCCTGCTTC ATGCCTGCTTCACCACCTTC	187	59	97
HPRT1	AY372182	GGCAAAACAATGCAAACCTT CAAGGGCATATCCTACGACAA	163	57	93
RPL32	XM_008531004	AGCCATCTACTCGGCGTCA TCCAATGCCTCTGGGTTTC	149	60	89
SDHA	XM_001490889	TCCATCGCATAAGAGCAAAG GGTGGAACTGAACGAACTCC	159	59	99
TUBA4A	XM_001491910	GCCCTACAACTCCATCCTGA ATGGCTTCATTGTCCACCA	78	60	104
UBC	AF506969	GCAAGACCATCACCCTGGA CTAACAGCCACCCCTGAGAC	206	60	100
18 S	AJ311673	GACCATAAACGATGCCGACT TCTGTCAATCCTGTCCGTGT	219	60	92

Measurement of glucose and lactic acid consumption by oviduct explants

Oviduct explants were cultured in 5% CO_2 in air or in 5% CO_2, 5% O_2 or in 90% N_2 at 38°C as described earlier. At Days 0, 1, 3 and 6 of culture, samples of culture medium were taken and frozen at -80°C until analysis using an UV enzymatic method using the Roche Cobas

8000 according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). A calibration line to determine the efficiency was drawn up along with 10 samples per day of sampling. The efficiency of the UV enzymatic analyses was 98% for glucose and 99% for lactic acid. The limit of detection was 0.1 mM for glucose and 0.2 mM for lactic acid.

Statistical analysis

For analysis of the Gaussian-distributed data of the morphological evaluation of the explants and glucose and lactic acid concentration, repeated-measures with Greenhouse–Geisser correction with the general linear model procedure was implemented. Post hoc test for multiple comparisons with Bonferroni correction was performed. For gene-expression studies, analysis of variance (ANOVA) and calculation of standard errors of means (s.e.m.) were performed with the general linear model procedure as implemented in SPSS 19 for Windows (SPSS IBM, Brussels, Belgium). Paired-samples *t*-test and Wilcoxon-signed rank test was used in comparisons of target gene expression differences throughout the culture period, depending on whether or not a Gaussian distribution was obtained after logarithmic transformation of the data. Differences were considered to be significant at $P < 0.05$. Statistical analysis and graph plotting were performed with SPSS 19. Power analysis and sample-size calculation (a = 0.05; power = 0.90–0.95) for gene-expression studies and other experiments were performed using Piface version 1.7 (Lenth 2007) (University of Iowa; http://homepage. stat.uiowa.edu/,rlenth/Power/; accessed 28 March 2011), and G*Power 3.1.3 (Faul, et al. 2007) (Heinrich Heine Universität Düsseldorf; http://www.psycho.uni-duesseldorf.de/abteilungen/ aap/gpower3/download-and-register; accessed 1 August 2012), respectively.

RESULTS

Morphological features of equine oviductal explants during culture

From 6 h of culture the formation of spherical structures was observed. More than 95% of the explants smaller than 200 µm or larger than 600 µm were still showing ciliary activity after 6

days of culture. Over time, we saw that progressively more explants were showing central darkening (**Fig. 1*a*, *b***). At Days 5 and 6, a significantly lower percentage of smaller explants was showing central dark zones compared with the larger-sized vesicles (P = 0.01; **Fig. 2**).

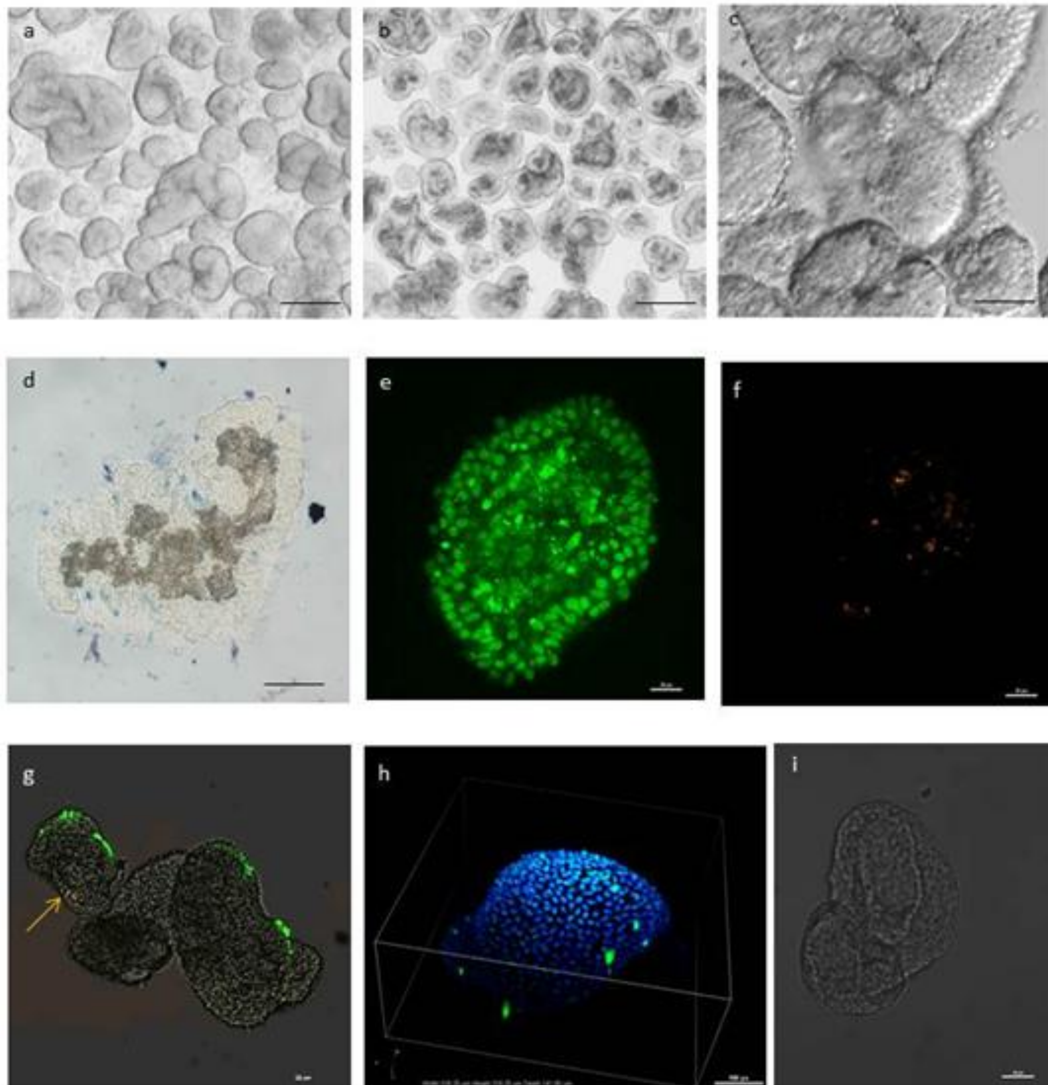

Fig. 1. (*a*, *c*) Inverted microscopic image of equine oviduct explants after 1 day of culture and (*b*) after 6 days of culture, showing central dark zones. (*d*) Trypan blue and (*e*) SYBR14 staining revealed that more than 98% of the cells were membrane intact after 6 days of culture (green in SYBR14, translucent in Trypan blue staining). (*f*) Very few membrane-damaged cells were stained orange by propidium iodide. (*g*) Confocal transmission view of the combined staining of TUNEL (green) and anti-caspase-3 (orange; arrow). (*h*) Confocal 3D view of oviduct explants stained with Hoechst, TUNEL and anti-caspase-3. Fewer than 2% of the cells in the explants were TUNEL and/or anti-caspase-3 positive after 6 days of culture. (*i*) Staining negative confocal transmission view for background autofluorescence subtraction. (*a*, *b*, *h*) Bar = 100 µm; (*c*) Bar = 50 µm; (*d*–*g*, *i*) Bar = 20 µm.

There was no significant difference (P = 0.9) in ciliary activity and the percentage of explants showing central black zones related to culture in low-oxygen atmosphere (90% N_2, 5% CO_2, 5% O_2) and in 5% CO_2 in air (data not shown).

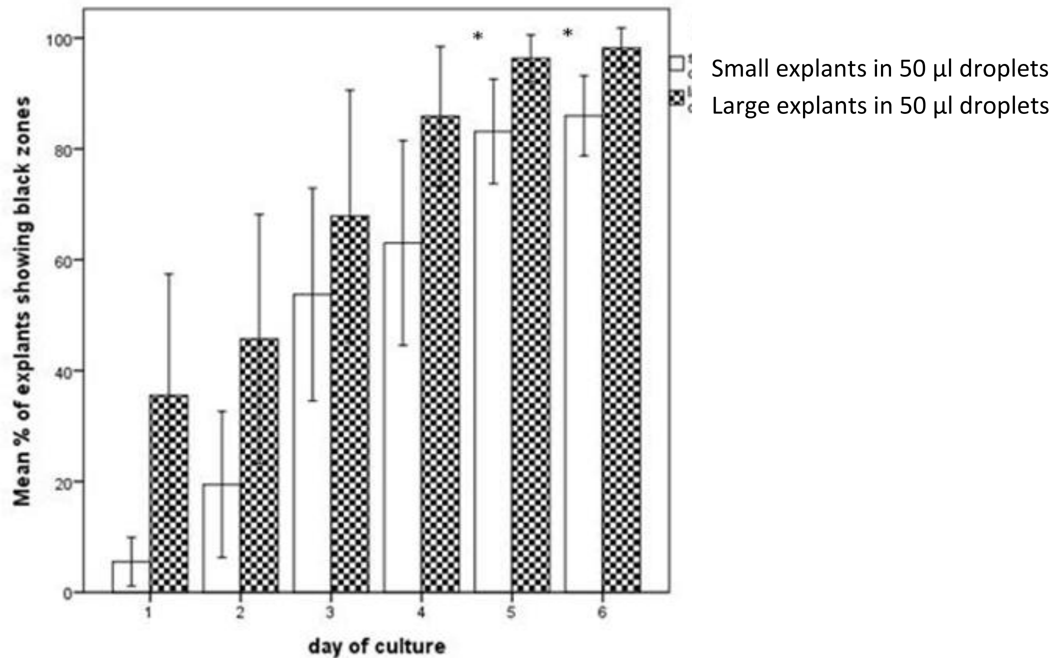

Fig. 2. Progressively increasing percentage of explants showing central dark zones counted every 24 h during a culture period of 6 days in small (<200 µm) compared with large (>600 µm) explants. * P = 0.05. Repeated-measures ANOVA with Greenhouse–Heisser correction; post hoc tests with Bonferroni correction.

Haematoxylin–eosin staining demonstrated that between Day 0 and Day 6 of culture the explants **(Fig. 3*a*)** were bordered by highly differentiated tall columnar epithelial cells with basal nuclei, numerous nucleoli, apical cilia and secretory granules, very similar to *ex vivo* samples **(Fig. 3*b*)**. In the spherical explant structures, stromal cells originating from the lamina propria were also observed. Trypan blue **(Fig. 1*d*)** and SYBR14/PI **(Fig. 1*e*, *f*)** staining demonstrated that more than 95% of the cells remained membrane intact after 6 days of culture.

Hoechst, TUNEL and anti-caspase-3 staining revealed that only 2% of the cells, scattered over the whole explants, contained fragmented DNA **(Fig. 1*g*, *h***; green TUNEL-positive cells**)** and fewer than 1% showed caspase-3-positive cells **(Fig. 1*g***; orange arrow**)** after a culture period of 6 days.

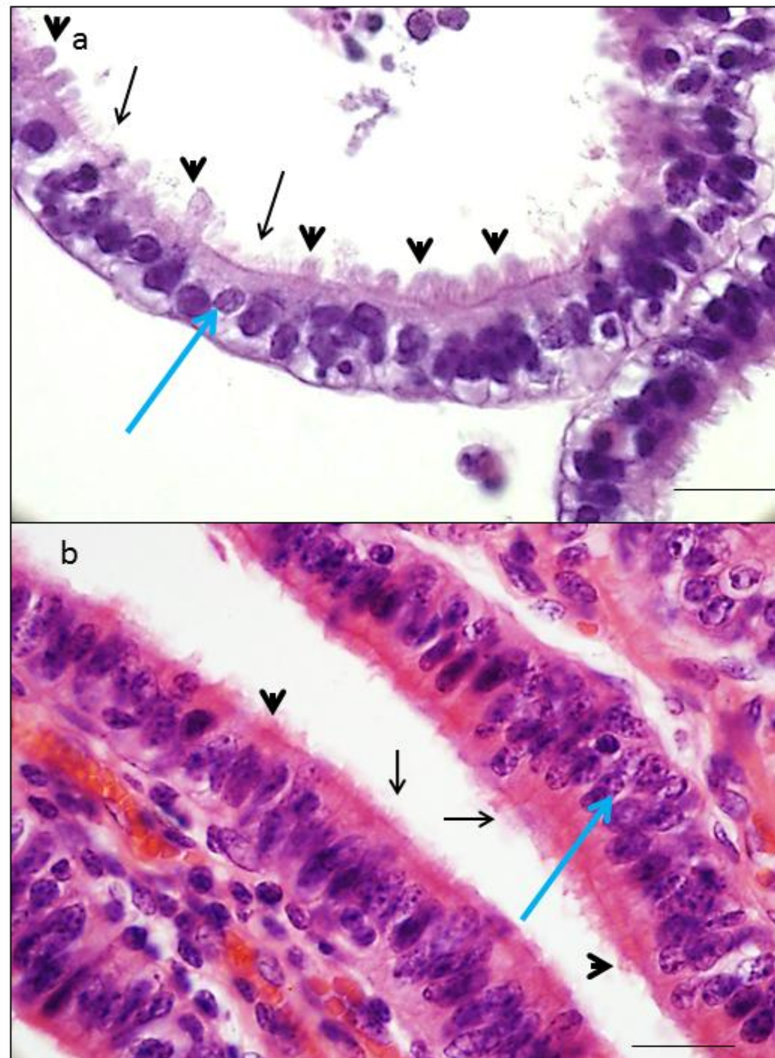

Fig. 3. (*a*) Close-up of the epithelium, bordering an oviduct explant; the cells are highly differentiated with tall columnar, pseudostratified epithelium, with basal nuclei, numerous nucleoli (blue arrow), secretory granules (arrowheads) and apical cilia (black arrows), very similar to (*b*) the *in vivo* situation. Haematoxylin–eosin staining, bar = 25 µm.

Transmission electron microscopy confirmed the presence of cell polarity during the 6 days of culture as evidenced by the presence of cilia and microvilli on the apical cell surface and of tight junctions on the lateral cell surfaces **(Fig. 4)**. In explants showing central darkening, originating from mares in the postovulatory cycle stage, conspicuous dark nuclear and cytoplasmic regions were visible without the hallmark ultrastructural features of either apoptotic or necrotic cell death as described previously (Krysko, et al. 2008). In some cases these dark cells were engulfed by neighbouring healthy cells.

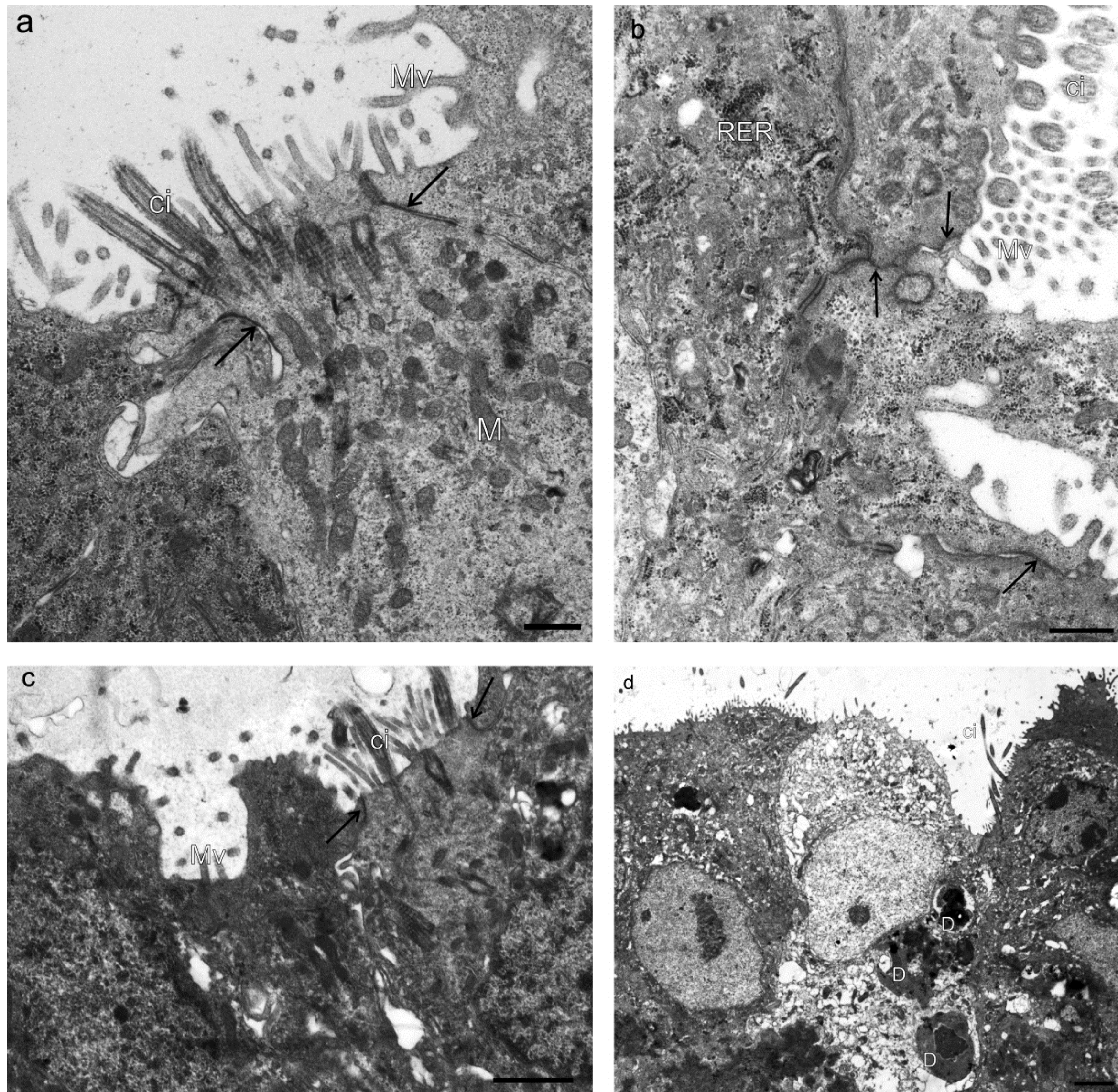

Fig. 4. Transmission electron microscopic images of oviduct explants. (*a*) *Ex vivo* **e**xplant, bar = 500 nm; (*b*) explant cultured for 1 day, bar = 500 nm; (*c*) explant cultured for 5 days, bar = 1 µm; (*d*) explant cultured for 6 days with dark zone, bar = 2 µm. During the whole culture period, the explants were bordered by highly differentiated epithelial cells with apical cilia (ci) and microvilli (Mv), intact junctional complexes (arrowheads) and numerous mitochondria (M) and rough endoplasmic reticulum (RER). Explants showing central dark zones contain cells undergoing dark-cell degeneration (D), hallmarked by their shrunken and very electron-dense appearance without recognizable organelles in the cytoplasm, their nuclei containing condensed chromatin and areas of vacuolization in the cytoplasm.

Gene expression of equine oviduct explants during culture

Quantitative real-time PCR results of post-ovulatory oviduct explants on Day 0 and Day 6 of culture were normalised against the geometric mean of an optimal number of reference genes.

Results of the reference gene stability, as determined by geNorm, are shown in **Fig. 5**. The optimal number of genes was determined with geNorm by means of the pairwise variations ($V_{n/nþ1}$) between the sequential normalisation fac-tors (NF_n and $NF_{nþ1}$) after successive inclusion of less-stable reference genes **(Fig. 6)**. The value of the pairwise variations reduces until 0.137 for $V_{4/5}$. This suggests that the inclusion of a fifth reference gene contributes to the stability. Therefore it is recommended to use the five most-stable genes for RT-qPCR in post-ovulatory oviduct explants. When segregating Day 0 and Day 6, on Day 0 the most stable reference genes were *ACTB*, *UBB*, *18S*, *TUBA4A*, *SDHA*, *HPRT* and on Day 6, *ACTB*, *SDHA*, *UBB*, *RPL32*, *HPRT* were the most stable (data not shown). When both groups of explants (Day 0 and Day 6) were included, the most stable genes were *UBB*, *ACTB*, *18S*, *RPL32* and *SDHA*. The geometric mean of these latter genes was used for normalisation of the test genes. The M values **(Fig. 5)** ranged between 0.4 and 0.8, which indicates relatively good stability.

A highly significant downregulation of *GLUT1* (P = 0.0005) and a slightly significant downregulation of *PAI1* (paired-samples *t*-test) and *PLAU* (Wilcoxon-signed rank test) was observed after 6 days of culture (P = 0.03 and P = 0.01). Normalised expression values of *GLUT1* and *PAI1* were loga-rithmically transformed to obtain a Gaussian distribution. There were no differences in expression levels of *HIF1A* and *VEGFA* between Day 0 and Day 6 of culture **(Fig. 7)**.

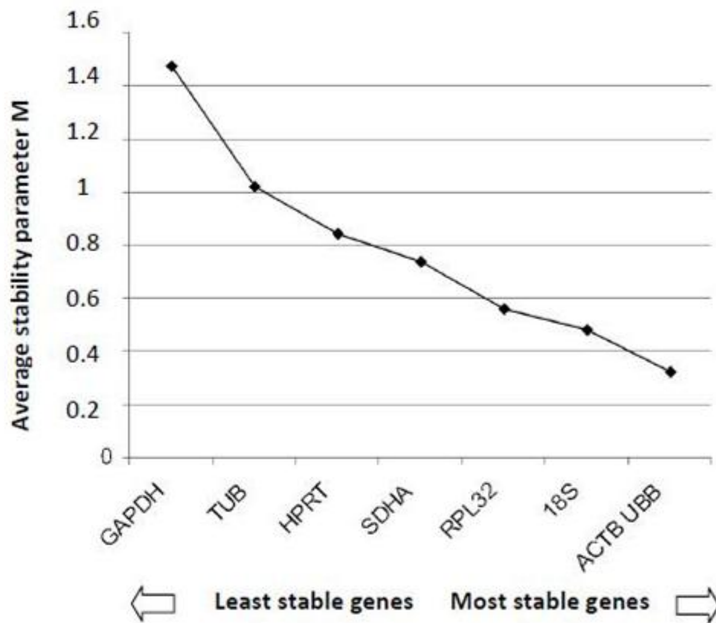

Fig. 5. Average expression-stability values of post-ovulatory oviduct explants. The average stability values of the control genes were calculated with geNorm. In this population *UBB*, *ACTB*, *18S* and *RPL32* were found to be the most stable.

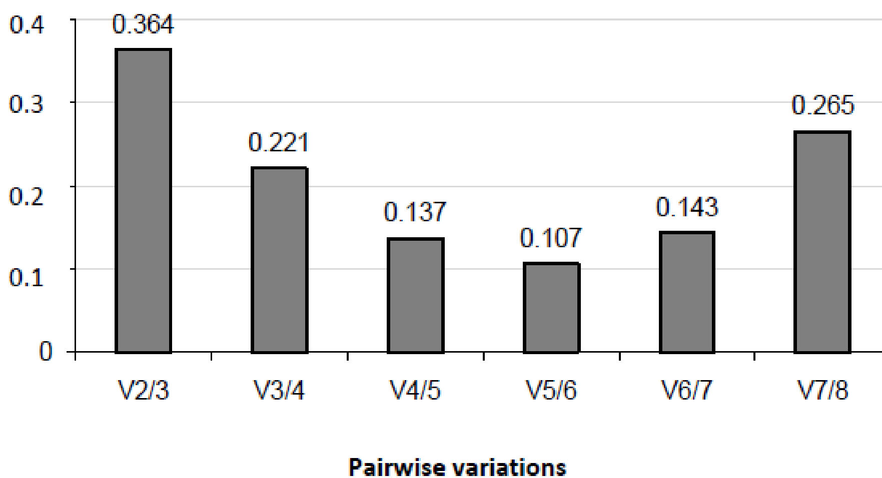

Fig. 6. Determination of the optimal number of control genes for normal-isation in post-ovulatory explants. The optimal number of control genes for normalisation was calculated by geNorm. The value of the pairwise variations reduces until 0.137 for $V_{4/5}$, which indicates that the inclusion a fifth reference gene contributes to the stability. Therefore the average of the five most stable genes is recommended to determine a reliable normalisation factor.

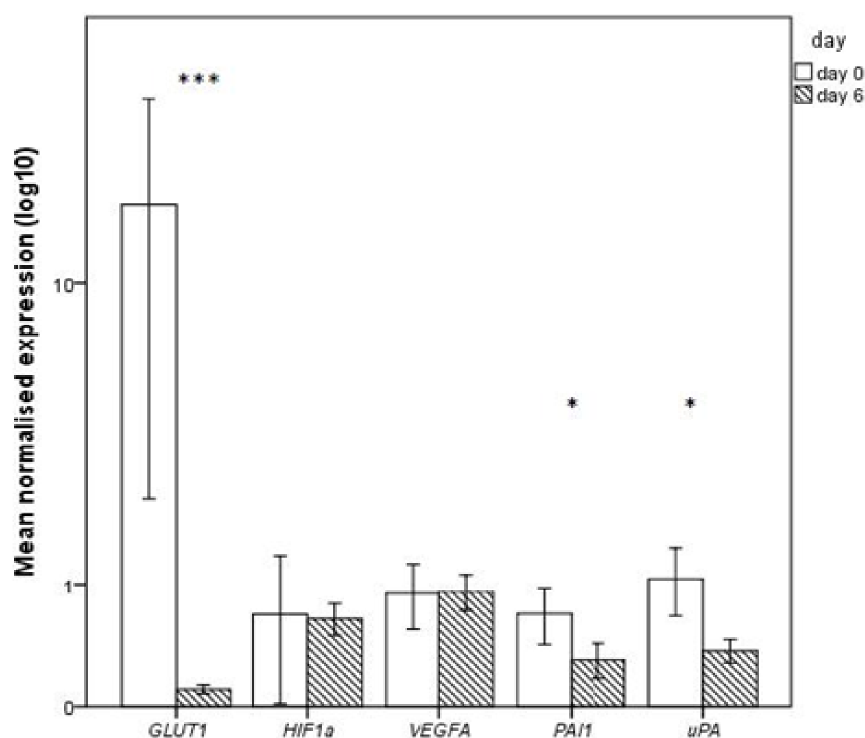

Fig. 7. Differential normalised gene expression in oviduct explants between Day 0 and Day 6 of culture in 5% CO_2 in air as determined by RT-qPCR. Mean (+/- s.e.m.) expression of five genes determined by RT-qPCR in oviduct explants. After 6 days of culture, mean *GLUT1*, *PAI* and *PLAU* expression were significantly lower. *P = 0.05, ***P = 0.0005; paired-samples *t*-test or Wilcoxon-signed rank test.

Glucose and lactic acid changes during culture

After 3 days of culture glucose consumption was 5.4 +/- 0.8 mM, so the glucose concentration in the culture medium had significantly dropped from 17 mM to 11.6 +/- 0.8 mM. Lactic acid concentrations increased from 0 to 4.4 mM in 5% CO_2 in air (P = 0.005). Glucose and lactic acid concentration did not change during the subsequent 3 days of culture (P = 0.9). In 5% O_2, 5% CO_2 and 90% N_2, glucose consumption significantly increased to 8.3 mM on Day 3 and to 11.3 mM on Day 6 (P = 0.005 and 0.0005, respectively) whereas lactic acid concentration increased to 18.8 mM (P = 0.0005) on Day 3 and did not change during the following 3 days of culture **(Fig. 8)**.

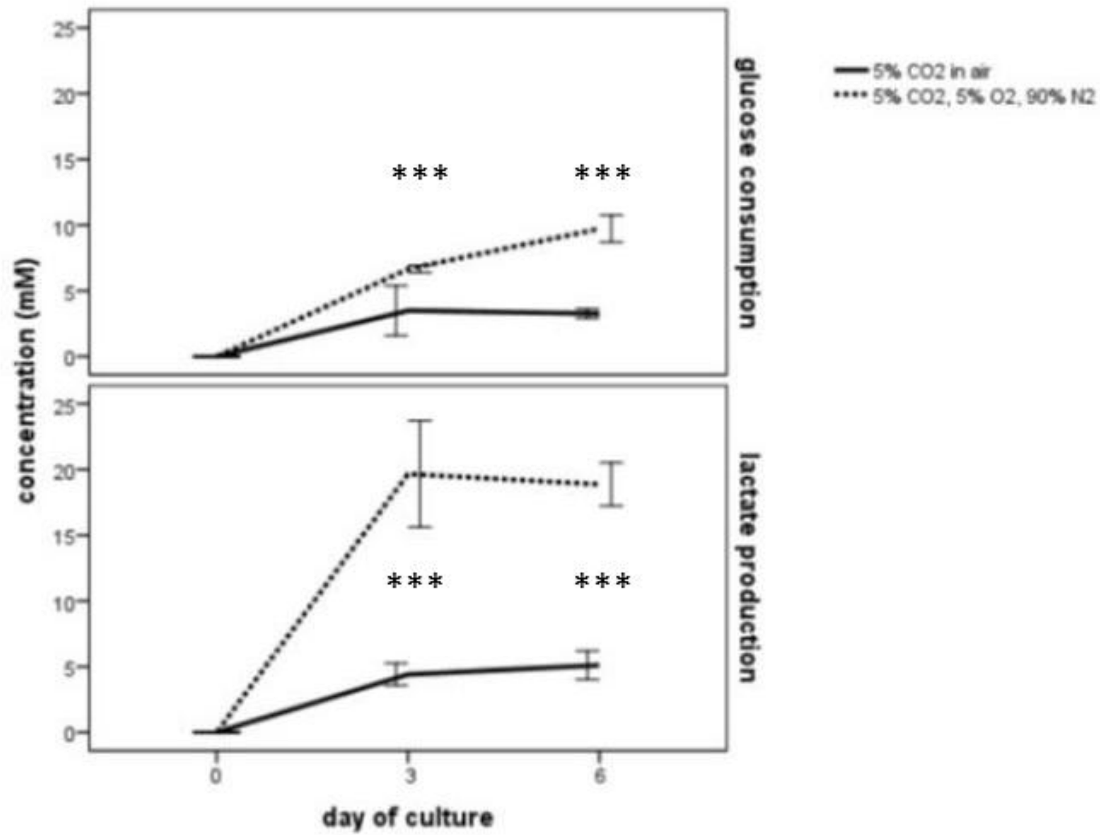

Fig. 8. Mean glucose consumption and lactic acid production (mM) measured in medium containing equine oviduct explants over 6 days of culture under high- or low-oxygen atmosphere. The glucose consumption and the lactic acid production of the explants in the low-oxygen environment were significantly higher than in higher-oxygen conditions. ***P = 0.001; repeated-measures ANOVA with Greenhouse–Heisser correction; post hoc tests with Bonferroni correction.

DISCUSSION

This study is the first to describe the morphology, ultrastructure, glucose consumption, lactic acid production and related gene expression of an equine oviduct explant culture system showing preservation of epithelial differentiation for 6 days of culture, which is equal to the time spent in the oviduct by the equine embryo. This is a significant finding since this model will be used for the further study of differentiation markers and signaling molecules to unravel embryo–maternal interaction at the level of gene expression in the horse. The first objective of this study was to obtain equine oviduct explants with preservation of morphological and ultrastructural features. Oviduct explants have several advantages when compared with monolayers. First of all, it has been proven that in cattle (Thibodeaux, et al. 1992a, Walter 1995) and horses (Dobrinski, et al. 1999, Thomas, et al. 1995a) proliferating oviductal cells grown in monolayers dedifferentiate with a concomitant reduction in cell height, loss of beating cilia and loss of secretory granules and bulbous protrusions. Despite the fact that the use of monolayers and resulting cell lines minimises the risk of disease transmission and batch-dependent variations (Menck, et al. 1997), they are a less solid reflection of the *in vivo* situation (Reischl, et al. 1999, Thibodeaux, et al. 1992a, Walter 1995). In our hands, beating cilia in equine oviduct cell aggregates were still detected after 20 days in culture (data not shown). Transmission electron microscopy confirmed the presence of healthy cilia and numerous microvilli **(Fig. 4)**. Furthermore, the cells bordering the explants maintained their ultrastructural highly differentiated morphology, including numerous mitochondria and rough endoplasmic reticulum, highly similar to the oviduct epithelium *ex vivo* **(Fig. 4)**. Since the ciliation process is stated to be the endpoint of differentiation that cannot be induced in an *in vitro* system (Thibodeaux and Godke 1992), this means an important benefit of the explant suspension. Next, the oviduct is a biosynthetic active and secretory organ that releases macromolecules throughout the oestrous cycle (Buhi, et al. 2000). The explants in our culture system were showing numerous secretory granules after 6 days of culture, indicating that the functional integrity was preserved. A limitation in studying embryo–oviduct inter-actions *in vitro* is the impossibility to study regional and tem-poral differences. Furthermore, in the horse, it is rather difficult to determine the exact postovulatory cycle stage. To lower this bias, 7–10 oviducts ipsilateral to ovulation were pooled. A major advantage of explants compared with monolayers is of practical importance: explants can be used within 6 to 12 h after harvest whereas monolayers can be used only after

several days (Rottmayer, et al. 2006).

Different media have been used to culture oviduct cells *in vitro* in different species, such as DMEM/F12, Menezo's B2 medium (MB2), Roswell Park Memorial Institute (RPMI-1640 medium), Tissue Culture Medium-199 (TCM-199), Con-naught Medical Research Laboratories 1066 (CMRL 1066) (Abe and Hoshi 1997, De Pauw, et al. 2002, Ulbrich, et al. 2003). Since equine embryos apparently benefit from high glucose concentrations during early development (Choi, et al. 2004b), DMEM/F12 medium was selected for explant culture since it contains high levels of glucose (17.5 mM) and is a suitable medium for equine embryo culture (Hinrichs 2010, Smits, et al. 2011). Interestingly, glucose consumption by the oviduct explants was almost 2-fold higher under low oxygen tension (5% CO_2, 5% O_2, 90% N_2) compared with 5% CO_2 in air **(Fig. 8)**, indicating that cellular activity is much higher (Leclerc, et al. 2003). Concomitantly, we observed more than 3-fold higher lactic acid production in the low oxygen tension condition. This can be explained by the enhanced generation of lactic acid by anaerobic glycolysis when oxygen is limited (Heiden, et al. 2009).

One salient finding of this study was that, over time, the number of explants showing central dark zones increased gradually, reaching 85% in explants smaller than 200 μm and 98% in explants larger than 600 μm at Day 6 of culture **(Figs 1*b*, 3)**. This finding has never been reported before in oviduct explant culture. These dark zones could not be attributed to membrane-damaged cells, since Trypan blue and SYBR14/PI staining showed that the percentage of membrane-intact cells was over 95% during the whole culture period **(Fig. 1*d*–*f*)**. Acceptable values of 90% membrane-intact cells are reported as an adequate criterion for cell isolation and culture (Cox and Leese 1997, Mishra, et al. 2003, Reischl, et al. 1999, Ulbrich, et al. 2003). Since the dark zones did not seem to consist of membrane-damaged cells, the presence of apoptotic cells was evaluated. Over the whole culture period, fewer than 2% of the cells in the explants were TUNEL positive and fewer than 1% were anti-caspase-3 positive. The difference in the percentage of cells containing fragmented DNA (TUNEL positive) and anti-caspase-3 positive cells can be explained by the fact that TUNEL is prone to artefacts (Hardy and Spanos 2002) and that only a portion of the TUNEL-positive cells stain for the caspase-3 cleavage product, suggesting caspase-3 activation may not be required for apoptosis in all cells (Namura, et al. 1998). These results indicate that the central dark zones did not consist of a cluster of apoptotic nor necrotic cells since TUNEL is a marker for

DNA fragmentation (Stadelmann and Lassmann 2000). Transmission electron microscopy (TEM) was performed to elucidate the ultrastructural features of the oviduct explants. This technique revealed only a few cells with ultrastructural changes typical of apoptosis such as plasma membrane blebbing but also showed epithelial cells undergoing dark-cell degeneration, characterised by strong cytoplasmic condensation, chromatin clumping and ruffling of the cell membrane but no blebbing of the nucleus or plasma membrane (Leist and Jaattela 2001).

Dark-cell degeneration is stated to be an apoptosis-like hypoxia-related type of cell degeneration described hitherto in Huntington’s disease, in neuronal cell degeneration (Leist and Jaattela 2001) and chondrocytes (Roach and Clarke 2000). In neuronal cells, dark-cell degeneration is stated to be caused by hypoxia (Barenberg, et al. 2001). Destructive levels of glutamate have been reported to mediate hypoxic-induced neuronal death, caused by the positive feedback of glutamate on its own release (Hardy, et al. 2002).

We hypothesized that the dark-cell degeneration in the explants was caused by hypoxic culture conditions as described in neuronal cells. Although a concentration of 5% oxygen, as applied in our experiments, is the physiological oviductal concentration of mammals (Fischer and Bavister 1993), due to the lack of microvasculature, the explants might (partly) suffer from hypoxia. Therefore, we evaluated the mRNA expression of five hypoxia-related genes. Hypoxia-inducible factor (*HIF1A*) is a transcription factor with a central role in the hypoxia response. Its activity is regulated by the oxygen-dependent degradation of the HIF1A protein (Chi, et al. 2006). Hypoxia induces not only the expression of HIF1A in mammalian cells but also regulates the expression of growth factors such as *VEGFA*, a potent angiogenic factor with an essential role in embryonic vasculogenesis and angiogenesis in mice (Carmeliet, et al. 1996, Ferrara, et al. 1996). In cattle, *VEGFA* secretion is increased before ovulation and therefore creates an optimal environment for gamete maturation, fertilisation and early embryonic development (Wijayagunawardane, et al. 2005). Furthermore, *VEGFA* enhances *in vitro* maturation of bovine oocytes and accelerates early embryonic development (Gabler, et al. 1999, Luo, et al. 2002). Wijayagunawardane, et al. (2005) hypothesize that *VEGFA* regulates oviductal embryo transport in cattle. The promoter region of the *VEGFA* gene has hypoxia-responsive elements that respond to *HIF1A* (Raleigh, et al. 1998). *VEGFA* mRNA levels are dramatically increased within a few hours of exposing different cell cultures to

hypoxia (0% O_2; (Shweiki, et al. 1992)), which stimulates neovascularization (Fan, et al. 2009). In this study, there was no change in expression of *HIF1A* nor *VEGFA* after 6 days of culture under different oxygen tensions.

The expression of glucose transporter 1 (*GLUT1*) is also upregulated under hypoxic conditions and mediated by *HIF1A*. Expression of the *GLUT1* gene is frequently used to describe differences between different culture systems (Wathes, et al. 1998, Wrenzycki, et al. 1998a, Wrenzycki, et al. 2001b). Furthermore, *GLUT1* plays an important role in the transfer of glucose from the oviduct epithelium into the lumen and in maintaining adequate glucose concentration in the oviductal fluid (Tadokoro, et al. 1995). It also mediates cellular glucose incorporation into embryonic cells and is necessary for transition from the morula to blastocyst stage (Leese, et al. 1995). Our results revealed a 5-fold downregulation of *GLUT1* after a culture period of 6 days. This can be explained by the autoregulatory mechanism of cells by downregulating or induction of degradation of the principal glucose transporter *GLUT1*-mRNA to protect against deleterious effects of hyperglycaemic culture conditions (Hahn, et al. 1998). Despite the high glucose consumption by the explants and the concomitant drop in glucose concentration **(Fig. 8)**, there was a downregulation of *GLUT1*. This can be explained as a negative feedback caused by the non-physiologically high glucose concentration in the medium, even after 6 days of culture (7–12 mM), since the *in vivo* glucose concentration in oviductal fluid of the mare is only 2.84–5.92 mM (Campbell, et al. 1979). However, modified DMEM/F12 medium with a high glucose concentration of 17 mM is superior to media with no glucose or low glucose concentration to support equine embryonic development *in vitro* (Choi, et al. 2004a). Since the explants, especially in the low-oxygen embryo-culture environment, seem to consume high amounts of glucose **(Fig. 8)**, it may be advisable to add glucose daily in embryo–oviduct co-culture experiments.

Plasminogen activator inhibitor 1 (*PAI1*) is also regulated by hypoxia and *HIF1A*. It may have a function in protecting the zona pellucida, the preimplantation embryo and oviductal tissue from proteolytic degradation by active proteases such as urokinase plasminogen activator (PLAU) and matrix metalloproteinases present in the oviductal environment (Buhi 2002, Kouba, et al. 2000a). Further, it may have a function in the regulation of extracellular matrix turnover and remodeling and may be involved in early cleavage-stage embryonic development (Kouba, et al. 2000a). In this experiment, both *PAI1* and *PLAU* were slightly

downregulated after 6 days of culture under atmospheric oxygen (0.01, P = 0.05). This is another indication of the fact that the explants did not suffer hypoxia, since under hypoxic conditions these factors are supposed to be upregulated (Liao, et al. 2007). In bovine oviducts *ex vivo*, *PLAU* expression was significantly higher during the preovulatory phase compared with Days 1 to 5 after ovulation (Gabler, et al. 2001), indicating hormonally regulated expression. Therefore, the downregulation observed in our experiment might be caused either by the presence of PLAU inhibitors such as PAI1, PAI2 and *nexin* (Blasi 1997) in the serum or produced by the explants, or by the lack of hormonal stimulation in our culture system.

In conclusion, our culture system sustains equine oviduct explants bordered by highly differentiated, functional and intact epithelial cells showing vigorous ciliary activity during 6 days of culture. Furthermore, only a negligible percentage (1–2%) of the cells in the explants shows features of apoptosis or necrosis and therefore the explants mimic the *in vivo* situation very closely. Although dark-cell degeneration, which is a hypoxia related type of cell death, was observed, no proof of hypoxia could be observed at the level of mRNA expression.
Since our culture system is lacking hormonal stimulation and since *in vivo* oviductal hormone concentrations in the horse are high experiments with steroid hormone supplementation are ongoing and promising (Nelis, et al. 2015b, Nelis, et al. 2013, Nelis, et al. 2011). Furthermore, in order to finally validate our model, co-culture experiments with ICSI-produced embryos are planned. So, although the cause of the dark-cell degeneration needs to be clarified, the oviduct explant system is definitely the basis of a robust model suitable to study embryo–oviduct interactions in the horse.

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REFERENCES

Abe, H, and H Hoshi 1997 Bovine oviductal epithelial cells: Their cell culture and applications in studies for reproductive biology. *Cytotechnology* **23** 171-183.

Ball, BA, M Altschul, and JE Ellington 1991 Invitro Development of Day-2 Equine Embryos Cocultured with Oviductal Explants or Trophoblastic Vesicles. *Theriogenology* **35** 669-682.

Ball, BA, SP Brinsko, PGA Thomas, PG Miller, and JE Ellington 1993 Development to Blastocysts of One-Cell to 2-Cell Equine Embryos after Coculture with Uterine Tubal Epithelial-Cells. *American Journal of Veterinary Research* **54** 1139-1144.

Ball, BA, and PG Miller 1992 Survival of Equine Embryos Cocultured with Equine Oviductal Epithelium from the 4-Cell to 8-Cell to the Blastocyst Stage after Transfer to Synchronous Recipient Mares. *Theriogenology* **37** 979-991.

Barenberg, P, H Strahlendorf, and J Strahlendorf 2001 Hypoxia induces an excitotoxic-type of dark cell degeneration in cerebellar Purkinje neurons. *Neuroscience Research* **40** 245-254.

Bavister, BD 1995 Culture of preimplantation embryos: Facts and artifacts. *Human Reproduction Update* **1** 91-148.

Blasi, F 1997 uPA, uPAR, PAI-I: key intersection of proteolytic, adhesive and chemotactic highways? *Immunology Today* **18** 415-417.

Bogaert, L, M Van Poucke, C De Baere, L Peelman, F Gasthuys, and A Martens 2006 Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *Bmc Biotechnology* **6**.

Brinsko, SP, BA Ball, PG Miller, PGA Thomas, and JE Ellington 1994 In-Vitro Development of Day-2 Embryos Obtained from Young, Fertile Mares and Aged, Subfertile Mares. *Journal of Reproduction and Fertility* **102** 371-378.

Buhi, WC 2002 Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* **123** 355-362.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Bustin, SA, V Benes, JA Garson, J Hellemans, J Huggett, M Kubista, R Mueller, T Nolan, MW Pfaffl, GL Shipley, J Vandesompele, and CT Wittwer 2009 The

MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55** 611-622.

Campbell, DL, LW Douglas, and JC Ramge 1979 Cannulation of the Equine Oviduct and Chemical-Analysis of Oviduct Fluid. *Theriogenology* **12** 47-59.

Cappelli, K, M Felicetti, S Capomaccio, G Spinsanti, M Silvestrelli, and AV Supplizi 2008 Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. *Bmc Molecular Biology* **9**.

Carmeliet, P, N Mackman, L Moons, T Luther, P Gressens, I VanVlaenderen, H Demunck, M Kasper, G Breier, P Evrard, M Muller, W Risau, T Edgington, and D Collen 1996 Role of tissue factor in embryonic blood vessel development. *Nature* **383** 73-75.

Carnevale, EM, LJ Maclellan, TJ Scott, MA Coutinho da Silva, CF Scoggin, and EL Squires 2000. Effects of oocyte maturity and methods of collection, culture and insemination on equine oocyte transfer., p. 34. Ed. KBaJW WR Allen: R&W Publications, Newmarket.

Chi, JT, Z Wang, DSA Nuyten, EH Rodriguez, ME Schaner, A Salim, Y Wang, GB Kristensen, A Helland, AL Borresen-Dale, A Giaccia, MT Longaker, T Hastie, GP Yang, MJ van de Vijver, and PO Brown 2006 Gene expression programs in response to hypoxia: Cell type specificity and prognostic significance in human cancers. *Plos Medicine* **3** 395-409.

Choi, YH, CC Love, DD Varner, and K Hinrichs 2006 Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology* **65** 808-819.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004a Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Choi, YH, LM Roasa, CC Love, DD Varner, S Brinsko, and K Hinrichs 2004b Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. *Biology of Reproduction* **70** 1231-1238.

Cox, CI, and HJ Leese 1997 Retention of functional characteristics by bovine oviduct and uterine epithelia in vitro. *Animal Reproduction Science* **46** 169-178.

De Pauw, IM, A Van Soom, H Laevens, S Verberckmoes, and A de Kruif 2002 Sperm binding to epithelial oviduct explants in bulls with different nonreturn rates investigated with a new in vitro model. *Biol Reprod* **67** 1073-1079.

De Pauw, IMC, A Van Soom, K Mintiens, S Verberckmoes, and A de Kruif 2003 In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. *Theriogenology* **59** 1093-1107.

Dobrinski, I, JR Jacob, BC Tennant, and BA Ball 1999 Generation of an equine oviductal epithelial cell line for the study of sperm-oviduct interactions. *Theriogenology* **52** 875-885.

Eyestone, WH, and NL First 1989 Co-Culture of Early Cattle Embryos to the Blastocyst Stage with Oviducal Tissue or in Conditioned Medium. *Journal of Reproduction and Fertility* **85** 715-720.

Eyestone, WH, JM Jones, and NL First 1991 Some Factors Affecting the Efficacy of Oviduct Tissue-Conditioned Medium for the Culture of Early Bovine Embryos. *Journal of Reproduction and Fertility* **92** 59-64.

Fan, X, CJ Heijnen, MA van der Kooij, F Groenendaal, and F van Bel 2009 The role and regulation of hypoxia-inducible factor-1 alpha expression in brain development and neonatal hypoxic-ischemic brain injury. *Brain Research Reviews* **62** 99-108.

Faul, F, E Erdfelder, AG Lang, and A Buchner 2007 G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39** 175-191.

Ferrara, N, K CarverMoore, H Chen, M Dowd, L Lu, KS OShea, L PowellBraxton, KJ Hillan, and MW Moore 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380** 439-442.

Fischer, B, and BD Bavister 1993 Oxygen-Tension in the Oviduct and Uterus of Rhesus-Monkeys, Hamsters and Rabbits. *Journal of Reproduction and Fertility* **99** 673-679.

Gabler, C, A Einspanier, D Schams, and R Einspanier 1999 Expression of vascular endothelial growth factor (VEGF) and its corresponding receptors (flt-1 and flk-1) in the bovine oviduct. *Molecular Reproduction and Development* **53** 376-383.

Gabler, C, GJ Killian, and R Einspanier 2001 Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction* **122** 121-130.

Garner, DL, LA Johnson, ST Yue, BL Roth, and RP Haugland 1994 Dual DNA Staining Assessment of Bovine Sperm Viability Using Sybr-14 and Propidium Iodide. *Journal of Andrology* **15** 620-629.

Gjorret, JO, D Fabian, B Avery, and P Maddox-Hyttel 2007 Active caspase-3 and ultrastructural evidence of apoptosis in spontaneous and induced cell death in bovine in vitro produced pre-implantation embryos. *Molecular Reproduction and Development* **74** 961-971.

Goossens, JF, C Foulon, AL Villard, JY Puy, I Lefebvre, C Perigaud, C Vaccher, and JP Bonte 2005 Column selection and method development for the separation of nucleoside phosphotriester diastereoisomers, new potential anti-viral drugs. Application to cellular extract analysis. *Biomed Chromatogr* **19** 415-425.

Hahn, T, S Barth, U Weiss, W Mosgoeller, and G Desoye 1998 Sustained hyperglycemia in vitro down-regulates the GLUT1 glucose transport system of cultured human term placental trophoblast: a mechanism to protect fetal development? *Faseb Journal* **12** 1221-1231.

Hardy, K, and S Spanos 2002 Growth factor expression and function in the human and mouse preimplantation embryo. *Journal of Endocrinology* **172** 221-236.

Hardy, K, C Wright, S Rice, M Tachataki, R Roberts, D Morgan, S Spanos, and D Taylor 2002 Future developments in assisted reproduction in humans. *Reproduction* **123** 171-183.

Heiden, MGV, LC Cantley, and CB Thompson 2009 Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* **324** 1029-1033.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hinrichs, K, and Y-H Choi 2005 Assisted reproductive techniques in the horse. *Clinical techniques in equine practice* **4** 210-218.

Holm, P, SK Walker, and RF Seamark 1996 Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *Journal of Reproduction and Fertility* **107** 175-181.

Kouba, AJ, IM Alvarez, and WC Buhi 2000 Identification and localization of plasminogen activator inhibitor-1 within the porcine oviduct. *Biology of Reproduction* **62** 501-510.

Krysko, DV, T Vanden Berghe, K D'Herde, and P Vandenabeele 2008 Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods* **44** 205-221.

Leclerc, E, Y Sakai, and T Fujii 2003 Cell Culture in 3-Dimensional Microfluidic Structure of PDMS (polydimethylsiloxane). *Biomedical Microdevices* **5** 109-114.

Leese, HJ, M Alexiou, MT Comer, VK Lamb, and JG Thompson 1995 Assessment of embryo nutritional requirements and role of co-culture techniques. *Assisted Reproduction* **7** 11-23.

Leist, M, and M Jaattela 2001 Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2** 589-598.

Lenth, RV 2007 Statistical power calculations. *Journal of Animal Science* **85** E24-E29.

Liao, H, MC Hyman, DA Lawrence, and DJ Pinsky 2007 Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1 alpha, and C/EBP alpha. *Faseb Journal* **21** 935-949.

Lonergan, P, H Sharif, P Monaghan, H Wahid, M Gallagher, and I Gordon 1991 Factors affecting embryo yield following maturation, fertilization and culture in vitro of bovine oocytes. . *Isr. J. Agric. Res.* **30** 78.

Luo, HL, K Kimura, M Aoki, and M Hirako 2002 Vascular endothelial growth factor (VEGF) promotes the early development of bovine embryo in the presence of cumulus cells. *Journal of Veterinary Medical Science* **64** 967-971.

Menck, MC, C GuyaderJoly, N Peynot, D LeBourhis, RB Lobo, JP Renard, and Y Heyman 1997 Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems. *Reproduction Nutrition Development* **37** 141-150.

Merkl, M, SE Ulbrich, C Otzdorff, N Herbach, R Wanke, E Wolf, J Handler, and S Bauersachs 2010 Microarray Analysis of Equine Endometrium at Days 8 and 12 of Pregnancy. *Biology of Reproduction* **83** 874-886.

Misener, S, S Krawetz, S Rozen, and H Skaletsky 1999 Primer3 on the WWW for general users and for biologist programmers., Bioinformatics Methods and Protocols., pp. 365-386. Merelbeke, Belgium: S. Misener and S. Krawetz.

Mishra, S, ZM Lei, and CV Rao 2003 A novel role of luteinizing hormone in the embryo development in cocultures. *Biology of Reproduction* **68** 1455-1462.

Namura, S, JM Zhu, K Fink, M Endres, A Srinivasan, KJ Tomaselli, JY Yuan, and MA Moskowitz 1998 Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *Journal of Neuroscience* **18** 3659-3668.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015a Steroids in the equine

oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*, http://dx.doi.org/10.1071/RD15044.

Nelis, H, B Wojciechowicz, A Franczak, B Leemans, K D'Herde, K Goossens, P Cornillie, L Peelman, A Van Soom, and K Smits 2015b Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and immunoreactive steroidogenic protein expression in equine oviduct explants in vitro. *Reproduction Fertility Development* Epub June 18th. *Reproduction, Fertility and Development*, Epub March 10th http://dx.doi.org/10.1071/RD14483

Nelis, HM, K Goossens, B Leemans, L Peelman, and A Van Soom 2013 Steroid-Regulated Mrna Expression in Oviduct Epithelial Cells in the Mare. *Reproduction Fertility and Development* **25** 258-258.

Nelis, HM, L Vanhaecke, K Roels, and A Van Soom 2011 Serum steroid fluctuations during early pregnancy in mares, measured by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. *Reproduction in Domestic Animals* **46 (s3)** 134.

Pierson, RA, and OJ Ginther 1985 Ultrasonic evaluation of the corpus luteum of the mare. *Theriogenology* **23** 795-806.

Raleigh, JA, DP Calkins-Adams, LH Rinker, CA Ballenger, MC Weissler, WC Fowler, DB Novotny, and MA Varia 1998 Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Research* **58** 3765-3768.

Reischl, J, K Prelle, H Schol, C Neumuller, R Einspanier, F Sinowatz, and E Wolf 1999 Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro. *Cell and Tissue Research* **296** 371-383.

Roach, HI, and NMP Clarke 2000 Physiological cell death of chondrocytes in vivo is not confined to apoptosis - New observations on the mammalian growth plate. *Journal of Bone and Joint Surgery-British Volume* **82B** 601-613.

Rottmayer, R, SE Ulbrich, S Kolle, K Prelle, C Neumueller, F Sinowatz, HHD Meyer, E Wolf, and S Hiendleder 2006 A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* **132** 637-648.

Shweiki, D, A Itin, D Soffer, and E Keshet 1992 Vascular Endothelial Growth-Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis. *Nature* **359** 843-845.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, K, J Govaere, LJ Peelman, K Goossens, DC de Graaf, D Vercauteren, L Vandaele, M Hoogewijs, E Wydooghe, T Stout, and A Van Soom 2012 Influence of the uterine environment on the development of in vitro-produced equine embryos. *Reproduction* **143** 173-181.

Smits, KG, K.; Van Soom, A.; Govaere, J.; Hoogewijs, M.; Vanhaesebrouck, E.; Galli, C.; Colleoni, S.; Vandesompele, J.; Peelman, L. 2009 Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246.

Stadelmann, C, and H Lassmann 2000 Detection of apoptosis in tissue sections. *Cell and Tissue Research* **301** 19-31.

Tadokoro, C, Y Yoshimoto, M Sakata, T Imai, M Yamaguchi, H Kurachi, Y Oka, T Maeda, and A Miyake 1995 Expression and Localization of Glucose-Transporter-1 (Glut1) in the Rat Oviduct - a Possible Supplier of Glucose to Embryo during Early Embryonic-Development. *Biochem Biophys Res Commun* **214** 1211-1218.

Thibodeaux, JK, and RA Godke 1992 Invitro Enhancement of Early-Stage Embryos with Coculture. *Archives of Pathology & Laboratory Medicine* **116** 364-372.

Thibodeaux, JK, Y Menezo, JD Roussel, W Hansel, LL Goodeaux, DL Thompson, and RA Godke 1992 Coculture of Invitro Fertilized Bovine Embryos with Oviductal Epithelial-Cells Originating from Different Stages of the Estrous-Cycle. *Journal of Dairy Science* **75** 1448-1455.

Thomas, PGA, GG Ignotz, BA Ball, PG Miller, SP Brinsko, and B Currie 1995 Isolation, Culture, and Characterization of Equine Oviduct Epithelial-Cells in-Vitro. *Molecular Reproduction and Development* **41** 468-478.

Ulbrich, SE, A Kettler, and R Einspanier 2003 Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. *The Journal of Steroid Biochemistry and Molecular Biology* **84** 279-289.

Ulbrich, SE, E Wolf, and S Bauersachs 2013 Hosting the preimplantation embryo: potentials and limitations of different approaches for analysing embryo-endometrium interactions in cattle. *Reproduction Fertility and Development* **25** 62-70.

Vandaele, L, B Mateusen, DGD Maes, A de Kruif, and A van Soom 2007 Temporal detection of caspase-3 and-7 in bovine in vitro produced embryos of different developmental capacity. *Reproduction* **133** 709-718.

Vansoom, A, I Vanvlaenderen, AR Mahmoudzadeh, H Deluyker, and A Dekruif 1992 Compaction Rate of Invitro Fertilized Bovine Embryos Related to the Interval from Insemination to 1st Cleavage. *Theriogenology* **38** 905-919.

Walter, I 1995 Culture of Bovine Oviduct Epithelial-Cells (Boec). *Anatomical Record* **243** 347-356.

Wathes, DC, TS Reynolds, RS Robinson, and KR Stevenson 1998 Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of Dairy Science* **81** 1778-1789.

Wijayagunawardane, MPB, SP Kodithuwakku, D Yamamoto, and A Miyamoto 2005 Vascular endothelial growth factor system in the cow oviduct: A possible involvement in the regulation of oviductal motility and embryo transport. *Molecular Reproduction and Development* **72** 511-520.

Wrenzycki, C, D Herrmann, JW Carnwath, and H Niemann 1998 Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA. *Journal of Reproduction and Fertility* **112** 387-398.

Wrenzycki, C, D Wells, D Herrmann, A Miller, J Oliver, R Tervit, and H Niemann 2001 Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biology of Reproduction* **65** 309-317.

Wydooghe, E, L Vandaele, J Beek, H Favoreel, B Heindryckx, P De Sutter, and A Van Soom 2011 Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Analytical Biochemistry* **416** 228-230.

Yuan, YQ, A Van Soom, FOJ Coopman, K Mintiens, ML Boerjan, A Van Zeveren, A de Kruif, and LJ Peelman 2003 Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology* **59** 1585-1596.

Zuker, M 2003 Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31** 3406-3415.

PART 3.2: THE INFLUENCE OF FOETAL BOVINE SERUM AND INSULIN-TRANSFERRIN-SELENIUM ON OVIDUCT EXPLANT VIABILITY

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AETE Conference 2015, Ghent, Belgium.

ABSTRACT

It has been previously demonstrated that equine oviduct explants are an excellent tool to unravel embryo-maternal interactions. The system is easy to set-up and the equine oviduct explants remain *in vitro* functionally intact and highly differentiated. Although the outer surface of the explants does not undergo ultrastructural or functional changes, dark cell degeneration (DCD), as exteriorized by dark central zones in the explants, is observed inside the explants. Since serum has been reported to negatively affect cell and embryo culture the effect of serum and the serum replacer insulin-transferrin-selenium on the prevalence of DCD, the percentage of explants showing ciliary activity, membrane integrity and ultrastructure was assessed. Therefore, ipsilateral oviducts from mares in the early postovulatory cycle stage were gathered. Oviduct explants were harvested by scraping and cultured for 6 days in 50 µl drops under oil in 5 % CO2 in air in DMEM/F12 (unsupplemented medium) or DMEM/F12 with 10 % foetal bovine serum (FBS) or in DMEM/F12 supplemented with 5 µg/ml insulin and transferrin and 5 ng/ml selenium selenite (ITS). One droplet contained one µl (20-30 explants) of explant suspension. Three replicates of 60 droplets per treatment group were cultured. Using an inverted microscope, every 24 h, the percentage of explants with dark zones and the percentage of explants showing ciliary activity were determined and compared between the groups. In addition, membrane integrity, as evidenced by Trypan blue staining, was compared by recording the percentage of membrane-damaged cells. At day 0, 3 and 6, ultrastructure was assessed by TEM.

Compared to ITS (68 %, $P < 0.0005$) and the unsupplemented medium (67 %, $P < 0.0005$), FBS seems to protect (36 %) against the development of DCD during the first 2 days of

culture while it fails to do so from day 3 on. From then on, the prevalence of DCD was the lowest in the unsupplemented group (81 %) compared to ITS and FBS (87-92 %, $P < 0.0005$). FBS and in lesser extent ITS seem to sustain ciliary activity (respectively 97 and 94 %, $P < 0.0005$) compared with the unsupplemented medium (87 %, $P < 0.0005$). In all groups, as shown by Trypan blue staining, the explants consisted of more than 98 % membrane intact cells ($P = 0.9$). TEM revealed that there was no qualitative difference in the development of DCD. The outer surface of all explants in all groups was, similar to the *in vivo* situation, highly differentiated and intact.

In conclusion, FBS and ITS supplementation sustains ciliary activity. while ITS seems to enhance the development of DCD, components of FBS, which may be depleted after 2 days of culture, turn out to protect partly against DCD. Since the toxic margin of insulin and transferrin is known to be far above the applied levels in our culture system, next to other factors present in the culture system, selenium may play a role in the development of DCD. Further research is needed to unravel the exact cause and/or trigger in the development in DCD in oviduct explants.

INTRODUCTION

To maintain the integrity and differentiation status of cultured cells, the culture medium must provide all essential components for metabolism, growth and proliferation, ensuring that the microenvironment imitates as much as possible the *in vivo* status. These components include precursors and substrates for cell anabolism and energy metabolism, vitamins and trace elements to primarily fulfill catalytic functions and electrolytes to maintain among others osmolarity and pH (Bottenstein, et al. 1979). Fetal bovine serum (FBS) is almost universally added to semi-defined cell culture media such as DMEM/F12. In particular for *in vitro* culture of equine embryos, addition of serum to the culture medium is compulsory (Choi, et al. 2003, Choi, et al. 2004a, Hinrichs 2010, Smits, et al. 2011). It provides essential factors for somatic as well as embryonic cell growth and differentiation. Serum contains growth factors and hormones, binding and transport proteins, amino acids and trace elements, fatty acids and lipids, protease inhibitors, direct or indirect detoxifying agents and molecules such as albumin to maintain (colloid) osmotic pressure (Bottenstein, et al. 1979). As a drawback, serum is a rather ill-defined parameter (Bjare 1992) in cell culture which also hampers proteome

research since abundant proteins like albumin overshadow and hide less abundant proteins in HPLC/MS-MS output. In addition, compositional between-batch differences are likely to occur whereas the endotoxin level may also vary and influence culture results. It has also been reported that FBS alters the morphology and biochemistry of cultured cells (Gardner, et al. 1994, Shamsuddin and Rodriguezmartinez 1994, Thompson, et al. 1995) and lowers embryo survival and offspring vitality (Thompson, et al. 1998). Therefore, serum free media have been developed during the last decades. It turned out that most cell lines benefit from DMEM/F12 supplemented with the serum replacers insulin, transferrin and selenium (ITS) (Bottenstein, et al. 1979, Gstraunthaler 2003). Insulin is vital for glucose transport into cells and might also initiate IGF activity. Transferrin is a cellular iron transporting protein. Selenium (added as sodium selenite) is a trace element which serves as a cofactor of selenium-dependent enzymes such as gluthatione peroxidase and reductase (Bottenstein, et al. 1979, Gstraunthaler 2003).

Since in our equine oviduct cell explant culture system (Nelis et al 2013; CHAPTER 3.1) dark cell degeneration, a caspase-independent way of cell death, was observed *in vitro* but not *in vivo*, we hypothesized that the supplemented FBS may be involved in the cause of dark cell degeneration. Therefore, oviduct explants were cultured in unsupplemented DMEM/F12, in DMEM/F12 with FBS or in DMEM/F12 with ITS. The prevalence of dark zones, the percentage of explants showing ciliary activity, membrane integrity and ultrastructure were compared between the culture conditions.

MATERIALS AND METHODS

Animals

Oviducts (three replicates of 7 to 10 oviducts) were obtained at a slaughterhouse (Euromeat Group, Moeskroen, Belgium) from healthy warmblood mares aged 3–20 years and without any visible reproductive tract pathologies. Only ipsilateral oviducts of mares showing a recent corpus luteum or a corpus hemorrhagicum on the ovaries were used, indicating that ovulation had occurred not later than 5 days earlier (Nelis, et al. 2015a, Pierson and Ginther 1985).

Preparation and morphological evaluation of oviduct explants

Explants were prepared as extensively described earlier (Nelis 2014). The harvested cellular material was washed and cultured in DMEM/F12 (Invitrogen) (unsupplemented medium), or in DMEM/F12 supplemented with either 10% fetal bovine serum (FBS; Invitrogen) or with 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml sodium selenite (ITS) **(Figure 1)**. All media were supplemented with 50 mg/ml gentamycin and 2.5 mg/ml amphotericin-B (Fungizone; Invitrogen). Three replicates were performed. Per treatment group, each replicate contained 4 plates of 15 droplets (50 µl) containing 20-30 explants (1 µl explants suspension) per droplet. Culture was conducted at 38.5°C in a humidified atmosphere with 5% CO_2 in air. The time-span from slaughter of mares to seeding of cells was 3 to 4 h. Only cultures with more than 99% of membrane-intact cells, determined by Trypan blue staining (Sigma-Aldrich, Diegem, Belgium), 2 to 4 h after start of culture (= Day 0) were used. Frozen damaged explants were used as a membrane-damaged control. These explants were subjected to three subsequent freeze (-20°C) –thaw (38°C) cycles in PBS. The percentage of explants showing central dark-zones and the percentage of explants showing ciliary activity were evaluated every 24 h in the whole number of explants. Explants were considered to show ciliary activity when bordered by vigorously beating cilia, clearly seen on the inverted microscope (400 x magnification). To lower interpretative bias, all explants were counted by two researchers and the mean value was used for statistical analysis.

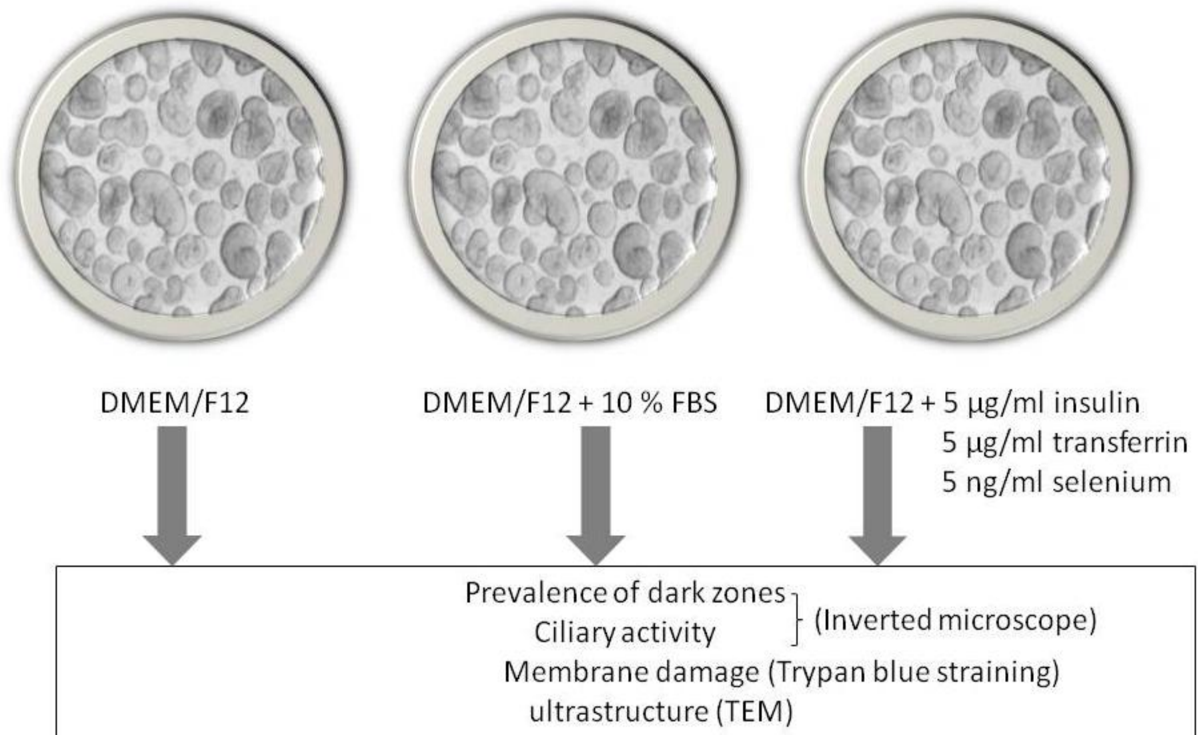

Fig. 1. Experimental set-up : explants were cultured in DMEM/F12, DMEM/F12 with foetal bovine serum (FBS) or insulin (5µg/ml), transferrin (5µg/ml), selenium (5 ng /ml) (ITS). For six days, every 24 hours, the prevalence of explants showing central dark zones and ciliary activity were determined using an inverted microscope as well as membrane damage by Trypan blue staining. At day 0, 3 and 6, the ultrastructure was evaluated by TEM.

Transmission electron microscopy

At Days 0, 3 and 6 of culture, oviduct explants **(Fig. 1)** were fixed in 0.2 M sodium cacodylate-buffered formaldehyde and post-fixed with osmium tetroxide. After rinsing, cells were pelleted by centrifugation (for 5 min at 200*g* at room temperature) in 10% BSA supplemented with 1% glutaraldehyde. The pellet was then dehydrated and embedded in epoxy resin (LX-112; Ladd Industries, Williston, ND, USA). Sections were made with a Reichert Jung (Depew, NY, USA) Ultracut E ultra-microtome.

Semi-thin sections (2 mm) were stained with toluidine blue to select the most appropriate regions for ultrathin sectioning. Next, ultra-thin sections (90 nm) were made and stained with

uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope (Jeol Europe, Zaventem, Belgium) at 80 kV.

RESULTS

Prevalence of dark zones is influenced by FBS and ITS without qualitatively affecting ultrastructure

During the first two days of culture, significantly less explants showed dark zones in the medium with FBS (36%, $P < 0.0005$), compared to ITS (68 %) or the unsupplemented medium (67 %). From day 4 on, explants in DMEM/F12 without FBS or ITS supplementation exhibited far less dark zones (81 %, $P < 0.0005$) compared to FBS (87 %) or ITS (92 %) supplemented medium (**Fig. 2**).

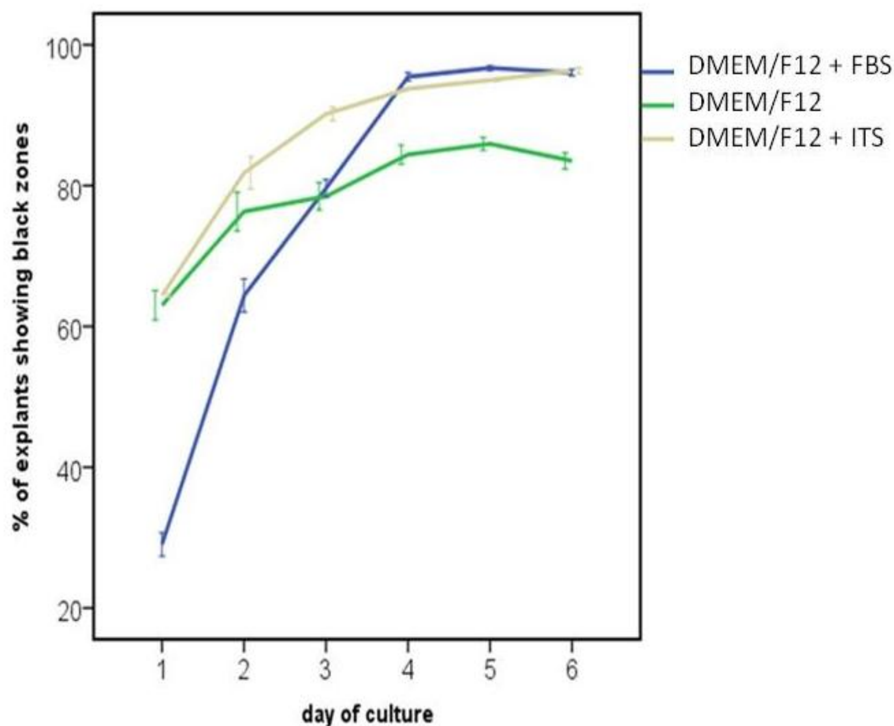

Fig. 2. The percentage of oviduct explants showing dark zones during a culture period of 6 days in 3 different media.

From days 3 on, as confirmed by transmission electron microscopy, explants in all groups showed central darkening with hallmarks of dark cell degeneration (DCD), as described previously. However, no qualitative differences in DCD development were observed between

the 3 groups during the culture period **(Fig. 3)**. Cell polarity and differentiation were maintained during the 6 days of culture as evidenced by the presence of cilia and microvilli on the apical surface and of tight junctions on the lateral surfaces.

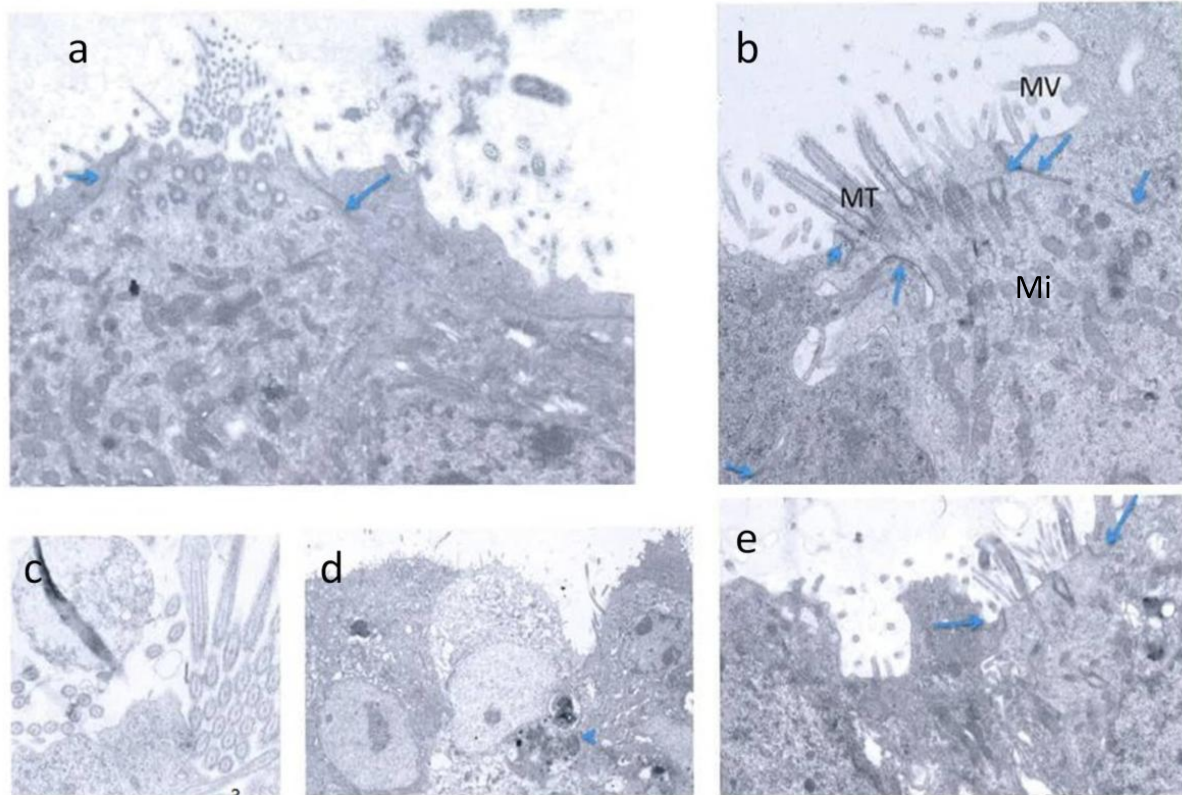

Fig. 3. Transmission electron microscopy of oviduct explants. During the whole culture period, the explants were bordered by highly differentiated epithelial cells with apical cilia (MT) and microvilli (Mv), intact junctional complexes (arrows) and numerous mitochondria (Mit) and rough endoplasmic reticulum. Explants showing central dark zones contain cells undergoing dark-cell degeneration (arrowhead, d), hallmarked by their shrunken and very electron-dense appearance without recognisable organelles in the cytoplasm, their nuclei containing condensed chromatin and areas of vacuolisation in the cytoplasm. No differences were observed in explants cultured in different media. Magnification x 5000

Ciliary activity is supported by FBS and ITS

During the culture period of 6 days, the number of explants showing ciliary activity, , was significantly the highest (97 %; $P < 0.0005$) in the medium with FBS followed by the medium with ITS (94 %; $P < 0.0005$) compared with unsupplemented DMEM/F12 (91 %) **(Fig. 4)**. Whereas the percentage of explants with ciliary activity only dropped slowly during the

whole culture period in the FBS and ITS supplemented media, in the unsupplemented DMEM/F12, a steep decrease in ciliary activity was observed from day 4 on.

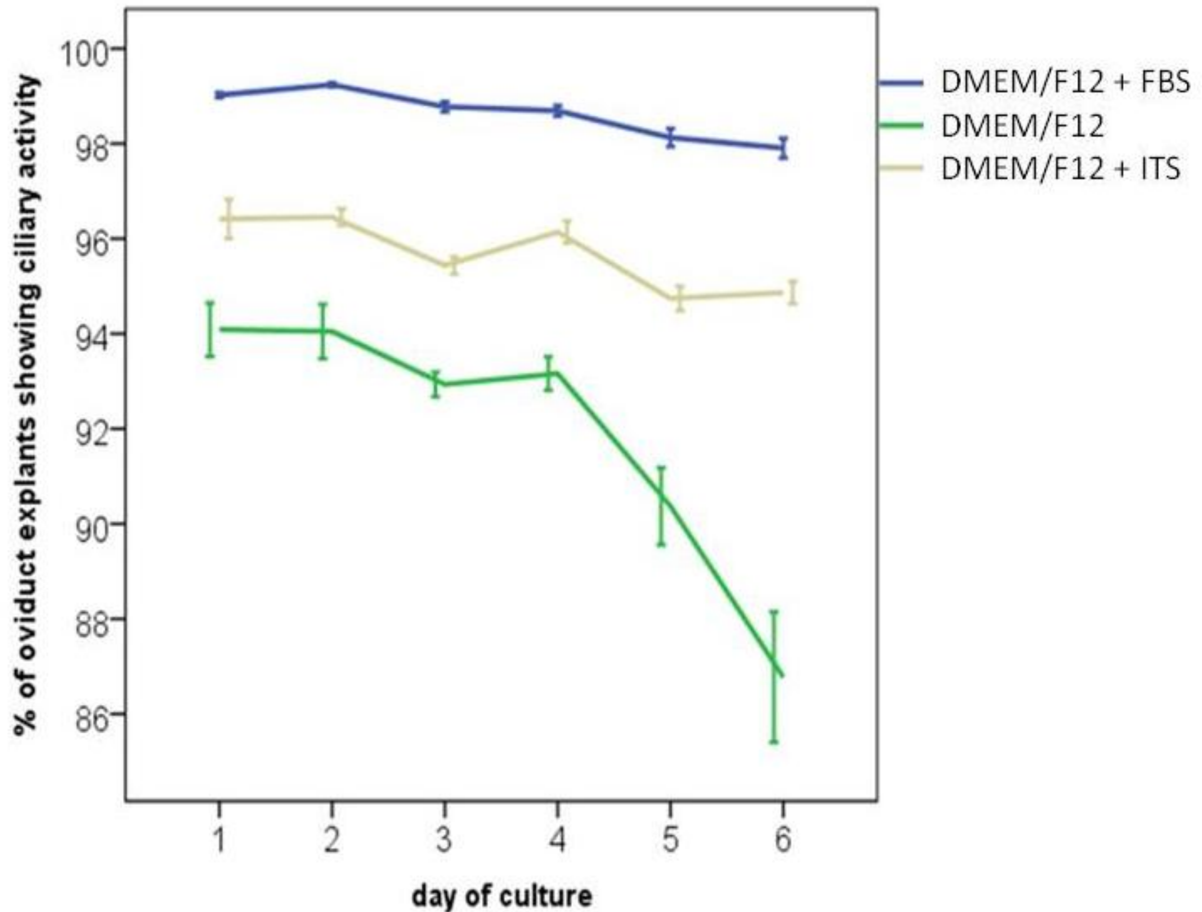

Fig. 4. The percentage of oviduct explants showing vigorous ciliary activity during a culture period of 6 days in 3 different media.

Membrane integrity is not affected by FBS or ITS

In all groups **(Fig. 5**), more than 98 % of the cells were membrane intact during the culture period of six days. No differences between the media were observed ($P > 0.05$). Zones with DCD were not showing more membrane damaged cells compared to regions without DCD. Frozen damaged negative controls consisted of 100 % membrane damaged cells whereas explants stained 2 to 20 hours after harvesting showed more than 99% of membrane intact cells and served as positive controls.

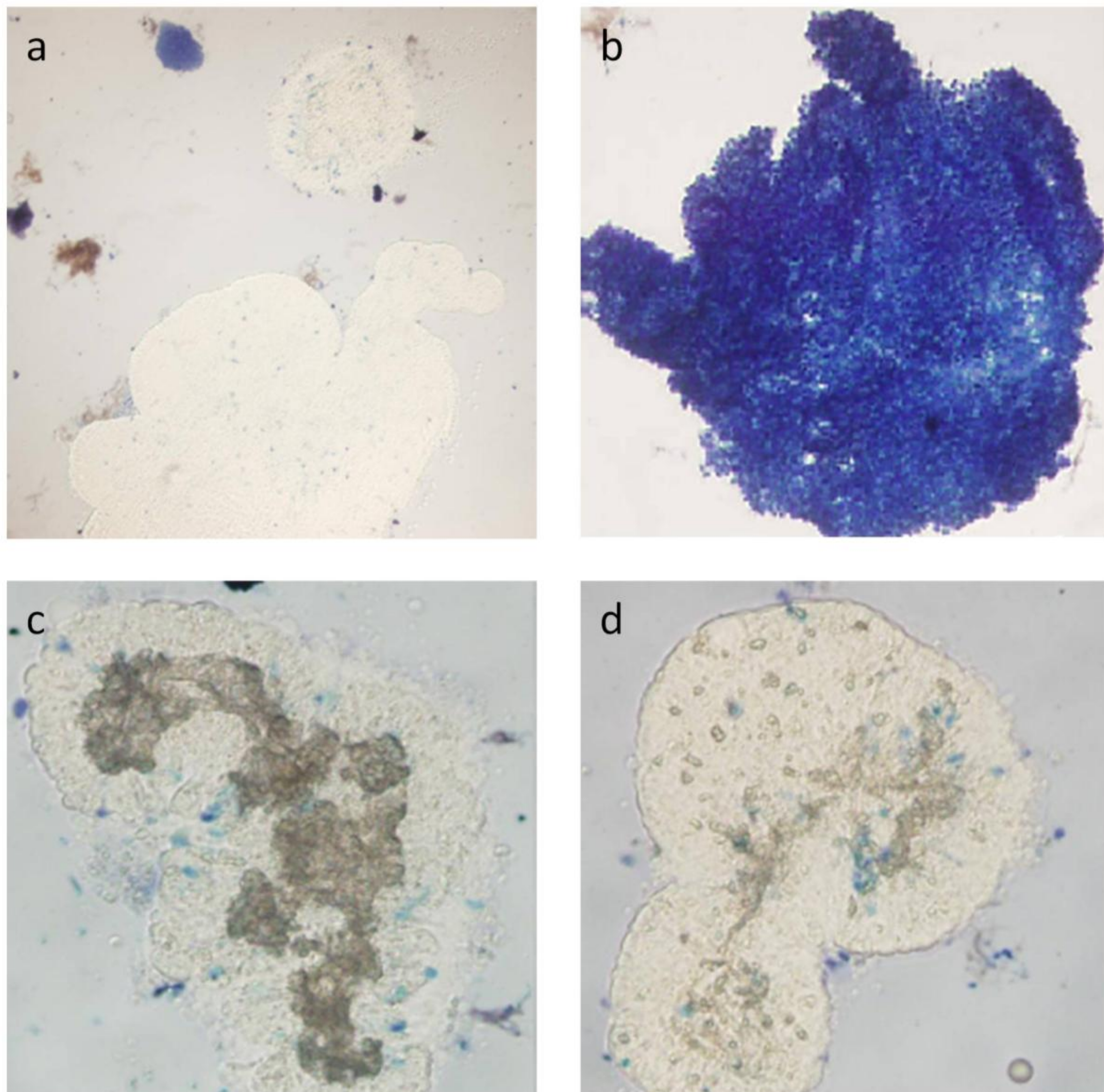

Fig. 5. Trypan Blue staining of oviduct explants after 2 hours (a), 6 days (c) and 20 h (d) after isolation. The white cells are membrane intact while the blue cells are membrane-damaged. The regions showing dark zones (c, d) do not contain membrane damaged cells. (b) Oviduct explants after several freeze-thaw cycles (-20 °C) served as control (b). Magnification = x 200.

DISCUSSION

Whereas the addition of FBS is beneficial to maintain ciliary activity in equine oviduct explants *in vitro* (**Fig. 4**), it seems also beneficial until at least two days of culture in preventing the development of dark cell degeneration (DCD) **(Fig. 2, Fig. 3)**, exteriorized by the central dark zones. From day three on, it fails to do so at the level of DCD **(Fig. 2)**. The addition of insulin, transferrin and selenium, compared with unsupplemented DMEM/F12 and DMEM/F12 with FBS, enhanced the development of dark zones but sustained superiorly ciliary activity compared with unsupplemented DMEM/F12 without affecting membrane integrity **(Fig. 5)**. From these results, it may be suggested that components of ITS may play a role in the development of dark cell degeneration whereas components in FBS, which may be depleted after two days of culture, may protect against the phenomenon of DCD **(Fig. 2)**.

The positive effect of serum on ciliary activity (Figure 4) may be explained by the considerable amounts of steroids or other components in the supplemented serum. It has indeed been reported earlier that DMEM/F12 with 10 % of FBS contains around and about 100 pg/ml progesterone and oestrogens and 90 pg/ml testosterone (CHAPTER 4) (Nelis, et al. 2015a). Moreover, oviductal cilia are steroid responsive (CHAPTER 5.1) (Aguilar, et al. 2012, Mahmood, et al. 1998). On its turn, ciliary activity was superior in the group supplemented with ITS compared to the unsupplemented group (DMEM/F12). Insulin has been reported to be relatively non-ciliotoxic as it does not change ciliary beat frequency or pattern (Jian and Po 1993). Therefore, it is assumed selenium sustains ciliary activity. Even though the toxic margin of selenium/selenite for ciliotoxicity is rather small (Lag, et al. 1984), it may contribute to the integrity of ciliary function by protecting the cells from oxidative damage. It is indeed a key molecule for selenium-dependent anti-oxidative enzymes such as glutathione peroxidase (Bhabak and Mugesh 2010).

It has been reported that insulin and IGFs increase caspase independent cell death and also the proportion of TUNEL positive murine embryonal cells (Chi, et al. 2000). TUNEL is a staining method which detects fragmented DNA (Stadelmann and Lassmann 2000). However, it has been shown earlier (Nelis, et al. 2014) that the proportion of TUNEL positive cells after 6 days culture in medium supplemented with FBS, which contains considerable levels of insulin (6-14 µU/ml) (Gstraunthaler 2003), was not increased. Although it should be born in

mind that cell media are highly cell specific (Gstraunthaler 2003), given the current findings, it might be suggested that insulin is not the main cause of the observed dark cell degeneration.

Transferrin is a 80 kDa protein, that can sequester free traces of iron to prevent highly toxic radical formation, and it sustains cell division in certain cell lines (Guilbert and Iscove 1976, Messmer 1973). It has been reported to inhibit cell division and growth *in vitro* at more than 100 µg/ml, which is 20-fold higher than supplied in our system (Bottenstein, et al. 1979). Thus, it may be assumed that transferrin is also not a catalysator of the observed dark cell degeneration in oviduct explants.
In contrast to transferrin, the toxic margin of sodium selenite is reported to be much narrower. While selenium (from sodium selenite) enhances cell division at concentrations of lower than or equal to 30 nM selenium (which approaches the applied concentration of 5 ng/ml in the current experiment) (Bottenstein, et al. 1979, Wydooghe, et al. 2014), it is reported to be cytotoxic at higher levels as demonstrated by a dramatically decreased cell division rate (Bottenstein, et al. 1979). Moreover, selenite has been reported to induce caspase-independent cell death in several types of cells via the mitochondrial pathway by a combination of indirect and direct effects (Chung, et al. 2006, Hu, et al. 2006). For instance, it has been reported that selenite induces time- and dose-dependent oxidative stress resulting in suppressed DNA synthesis and DNA damage, which in turn activates the p53-dependent and the p38 pathway in cervical carcinoma cells without affecting caspase-3, 8 and 9 (Rudolf, et al. 2008). Even though bovine embryos seem to benefit from 5 ng/ml selenium in the culture medium (Wydooghe, et al. 2014), it may activate cell death pathways in oviductal cells *in vitro* which results in dark cell degeneration, exteriorized by the central dark zones. Since the proportion of explants showing dark zones is highly significantly lower in DMEM/F12 medium compared with the same medium supplemented with ITS or 10 % of FBS, it is concluded that, next to other factors present in the culture system, selenium/selenite may play a role in the development of DCD. Further research is needed to unravel the exact cause and/or trigger in the development of DCD in equine oviductal explants.

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REFERENCES

Aguilar, JJ, J Cuervo-Arango, H Mouguelar, and L Losinno 2012 Histological Characteristics of the Equine Oviductal Mucosa at Different Reproductive Stages. *Journal of Equine Veterinary Science* **32** 99-105.

Bhabak, KP, and G Mugesh 2010 Functional Mimics of Glutathione Peroxidase: Bioinspired Synthetic Antioxidants. *Accounts of Chemical Research* **43** 1408-1419.

Bjare, U 1992 Serum-Free Cell-Culture. *Pharmacology & Therapeutics* **53** 355-374.

Bottenstein, J, I Hayashi, S Hutchings, H Masui, J Mather, DB McClure, R Ohasa, A Rizzino, G Sato, G Serrero, R Wolfe, and R Wu 1979 The growth of cells in serum-free hormone-supplemented media. *Methods Enzymology* **58** 94-109.

Chi, MMY, AL Schlein, and KH Moley 2000 High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141** 4784-4792.

Choi, YH, CC Love, DD Varner, LB Love, and K Hinrichs 2003 Effects of gas conditions, time of medium change, and ratio of medium to embryo on in vitro development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219-1229.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004 Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Chung, YW, TS Kim, SY Lee, SH Lee, Y Choi, N Kim, BM Min, DW Jeong, and IY Kim 2006 Selenite-induced apoptosis of osteoclasts mediated by the mitochondrial pathway. *Toxicol Lett* **160** 143-150.

Fernandez-Gonzalez, R, P Moreira, A Bilbao, A Jimenez, M Perez-Crespo, MA Ramirez, FR De Fonseca, B Pintado, and A Gutierrez-Adan 2004 Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proceedings of the National Academy of Sciences of the United States of America* **101** 5880-5885.

Gardner, DK, M Lane, A Spitzer, and PA Batt 1994 Enhanced Rates of Cleavage and Development for Sheep Zygotes Cultured to the Blastocyst Stage in-Vitro in the Absence of Serum and Somatic-Cells - Amino-Acids, Vitamins, and Culturing Embryos in Groups Stimulate Development. *Biology of Reproduction* **50** 390-400.

Gstraunthaler, G 2003 Alternatives to the use of fetal bovine serum: Serum-free cell culture. *Altex-Alternativen Zu Tierexperimenten* **20** 275-281.

Guilbert, LJ, and NN Iscove 1976 Partial Replacement of Serum by Selenite, Transferrin, Albumin and Lecithin in Hematopoietic Cell-Cultures. *Nature* **263** 594-595.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hu, H, C Jiang, T Schuster, GX Li, PT Daniel, and J Lu 2006 Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway. *Mol Cancer Ther* **5** 1873-1882.

Jian, L, and ALW Po 1993 Effects of Insulin and Nasal Absorption Enhancers on Ciliary Activity. *International Journal of Pharmaceutics* **95** 101-104.

Lag, M, G Paulsen, and J Jonsen 1984 Effect of Sodium Selenite on the Ciliary Activity, Adenosine-Triphosphate, and Protein-Synthesis in Mouse Trachea Organ-Cultures. *Journal of Toxicology and Environmental Health* **13** 857-864.

Mahmood, T, E Saridogan, S Smutna, AM Habib, and O Djahanbakhch 1998 The effect of ovarian steroids on epithelial ciliary beat frequency in the human Fallopian tube. *Human Reproduction* **13** 2991-2994.

Messmer, TO 1973 Nature of Iron Requirement for Chinese-Hamster V79 Cells in Tissue-Culture Medium. *Experimental Cell Research* **77** 404-408.

Nelis, H, K D'Herde, K Goossens, L Vandenberghe, B Leemans, K Forier, K Smits, K Braeckmans, L Peelman, and A Van Soom 2014 Equine oviduct explant culture: a basic model to decipher embryo–maternal communication. *Reproduction, Fertility and Development* **26** 954-966.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015 Steroids in the equine oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*.

Pierson, RA, and OJ Ginther 1985 Ultrasonic evaluation of the corpus luteum of the mare. *Theriogenology* **23** 795-806.

Rudolf, E, K Rudolf, and M Cervinka 2008 Selenium activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells. *Cell Biol Toxicol* **24** 123-141.

Shamsuddin, M, and H Rodriguezmartinez 1994 Fine-Structure of Bovine Blastocysts Developed Either in Serum-Free Medium or in Conventional Coculture with Oviduct Epithelial-Cells. *Journal of Veterinary Medicine Series a-Zentralblatt Fur Veterinarmedizin Reihe a-Physiology Pathology Clinical Medicine* **41** 307-316.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Stadelmann, C, and H Lassmann 2000 Detection of apoptosis in tissue sections. *Cell and Tissue Research* **301** 19-31.

Thompson, JG, NW Allen, LT McGowan, ACS Bell, MG Lambert, and HR Tervit 1998 Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. *Theriogenology* **49** 1239-1249.

Thompson, JG, DK Gardner, PA Pugh, WH Mcmillan, and HR Tervit 1995 Lamb Birth-Weight Is Affected by Culture System Utilized during in-Vitro Pre-Elongation Development of Ovine Embryos. *Biology of Reproduction* **53** 1385-1391.

Wydooghe, E, S Heras, J Dewulf, S Piepers, E Van den Abbeel, P De Sutter, L Vandaele, and A Van Soom 2014 Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reprod Fertil Dev* **26** 717-724.

CHAPTER 4 OF STEROIDS IN THE OVIDUCT *IN VIVO*

Men love to wonder,

and that is the seed of science

Ralph Waldo Emerson, essayist

CHAPTER 4 OF STEROIDS IN THE OVIDUCT *IN VIVO*

Adapted from:

Steroids in the equine oviduct: synthesis, local concentrations and receptor expression.

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ABSTRACT

Steroids play an important role in mammalian reproduction and early pregnancy. Whereas systemic changes in steroid concentrations have been well documented, it is not clear how these correlate with local steroid concentrations in the genital tract. We hypothesized that, in the horse, the pre-implantation embryo may be subjected to high local steroid concentrations for several days. Therefore, we measured progesterone, 17-hydroxyprogesterone, 17β-oestradiol, testosterone and 17α-testosterone concentrations in equine oviductal tissue by means of ultra-high performance liquid chromatography coupled to tandem mass spectrometry and progesterone, 17β-oestradiol, oestrone and testosterone in oviduct fluid using radioimmunoassay, with reference to cycle stage and side of ovulation. Progesterone concentrations were high in oviductal tissue and fluid ipsilateral to the ovulation side during diestrus, whereas other steroid hormone concentrations were not influenced by the side of ovulation. These results suggest that the high ipsilateral progesterone concentration is induced by 1) the contribution from follicular fluid in the oviduct and the diffusion of follicular fluid steroids after ovulation; 2) a local transfer of steroids via blood or lymph, 3) local synthesis of progesterone in the oviduct, as evidenced by the expression of steroidogenic enzymes and, 4) the paracrine contribution from follicular cells. These data provide a basis to study the importance of endocrine and paracrine signalling during early embryonic development in the horse.

INTRODUCTION

Oestradiol and progesterone are ovarian steroids which are secreted during the follicular and the luteal phase of the mare's oestrous cycle respectively and which exert both systemic and local effects. Systemic effects are very obvious and include changes in the mare's behaviour and gross changes in her reproductive tract (Daels and Hughes 1993, Hayes, et al. 1985). During oestrus, plasma progesterone concentrations are < 2 ng/ml (Daels and Hughes 1993, Hayes, et al. 1985), while oestrogen concentrations are high (around 140 pg/ml; (Pattison, et al. 1974). Under the influence of oestrogen, the mare displays oestrous behaviour and the endometrial folds in the uterus become oedematous and can be visualized easily by transrectal ultrasound. During dioestrus, circulating progesterone concentrations are high which prepares

the uterus for possible pregnancy. The mare rejects the advances of the stallion and endometrial oedema disappears (Daels and Hughes 1993).

Local effects of ovarian steroids on the genital tract are more subtle and can only be detected by histological and ultrastructural examinations. Furthermore, changes in local steroid concentrations affect gene expression (Chen, et al. 2013b, Desantis, et al. 2010, Nelis, et al. 2013, Rottmayer, et al. 2006) and, accordingly, the morphology and secretory activity of oviductal cells. Oestradiol stimulates epithelial cell hypertrophy, secretion and ciliogenesis, while progesterone is associated with atrophy and deciliation of epithelial cells in the mammalian oviduct (Georgiou, et al. 2005, Hunter 2012b, Seytanoglu, et al. 2008). Progesterone has been reported as being a chemoattractant for mammalian spermatozoa (Eisenbach and Giojalas 2006, Guidobaldi, et al. 2008, Teves, et al. 2006). Also in humans, sperm migrates preferably to the ipsilateral oviduct (Wildt, et al. 1998). In cattle sheep and pigs, concentrations of progesterone, 17β-oestradiol, prostaglandin E2, prostaglandin F2α and endothelin, as measured by an immunoassay, have been shown to be higher in the ipsilateral than in the contralateral oviduct (Ballester, et al. 2014, Pope, et al. 1982, Staples, et al. 1982, Wijayagunawardane, et al. 1999). The highest oviductal activity was observed at oestrus in the ipsilateral oviduct (Bennett, et al. 1988, Ruckebusch and Bayard 1975). All these changes prepare for successful transport of gametes and fertilization (Suarez 2008, Suarez and Ho 2003). In the horse, no information is available concerning local steroid concentrations in the oviduct and their fluctuations during the oestrous cycle.

In the present study, we adapted an ultra-high performance liquid chromatography coupled to tandem mass spectrometry (U-HPLC-MS/MS) protocol, that was developed previously to detect steroids in bovine muscle tissue, to measure progesterone, 17-hydroxyprogesterone, 17β-oestradiol, testosterone and 17α-testosterone concentrations in equine oviductal tissue, as well as in serum and follicular fluid. This technique of U-HPLC-MS/MS is a precise, and highly reproducible method for steroid quantification. It allows measurement of actual concentrations of steroids in the oviduct and, subsequently, to compare these with systemic concentrations of steroids in pre-and postovulatory serum. To evaluate local steroid concentrations in equine oviductal fluid, radioimmunoassay (RIA) was used based upon an earlier optimized and validated protocol (Franczak and Bogacki 2009, Szafranska, et al. 2002). Additionally, immunoreactive protein staining intensity of a set of catalytic enzymes involved in the steroidogenic pathway was determined in order to investigate the origin of the steroids

found. In addition, since progesterone receptors can potentially sequester large amounts of the steroid, their involvement was investigated by localizing and quantifying them in oviductal tissue.

MATERIALS AND METHODS

Steroid concentrations and production in the oviduct

Sampling

Oviductal tissue, follicular fluid, and serum. Oviducts and serum samples were recovered and frozen within 2h after slaughter from healthy mares with either a preovulatory follicle in their ovaries and distinct uterine oedema (preovulatory phase; n = 15), or a corpus hemorrhagicum or recent corpus luteum without uterine oedema (early postovulatory phase; n = 15). Follicular fluid was also collected from the preovulatory follicles. Determination of progesterone concentrations by UHPLC-MS/MS in a preliminary experiment revealed a positive predictive value of 94% between the presence of a corpus hemorrhagicum and a serum progesterone concentration of < 2 ng/ml. Ginther, et al. 2007 also measured serum progesterone concentrations of < 2 ng/ml during the early postovulatory period.

Oviductal fluid. Ipsi- and contralateral oviducts were collected from slaughtered mares in the pre- (n = 18) and postovulatory (n = 18) stages of the oestrous cycle and ligated in the slaughterhouse before being transported to the laboratory on ice. Here, each oviduct was flushed twice with 1 ml PBS supplemented with 5% BSA. To concentrate the steroids, the samples were then evaporated using a Centrivap Cold Trap (Labconco, Kansas City, MO, USA). After reduction to dryness, the samples were pooled according to cycle stage and side of ovulation. This resulted in 4 pools of samples, each containing the dry matter of the oviductal fluid of 18 oviducts.

U-HPLC-MS/MS

Progesterone, 17-hydroxyprogesterone, 17β-oestradiol, testosterone, and 17α-testosterone concentrations were measured in individual samples of pre- and postovulatory oviduct tissue, pre-and postovulatory serum and in the follicular fluid of the preovulatory mares.

Reagents and chemicals

Standards for progesterone, 17-hydroxyprogesterone, 17β-oestradiol, 17α-testosterone, and testosterone were purchased from Sigma-Aldrich (Diegem, Belgium). The internal standards 17β-oestradiol-d3, medroxyprogesteron acetate-d3, and 17β-testosterone-d2 were obtained from RIKILT (Wageningen, The Netherlands). Progesterone-d9 was provided by Toronto Research Chemicals (New York, USA). Solvents were of analytical grade when used for extraction and purification steps, and of LC–MS Optima grade when applied to UHPLC–MS/MS. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively. Primary stock solutions were prepared in methanol at a concentration of 1000 ng/L and stored in dark glass bottles at -20 °C. Ultrapure water was produced using an Arium 611 UV system (Sartorius Stedim Biotech, Aubagne, France). Oasis strong anion exchange MAX 6 cc 150 mg cartridges were obtained from Waters EDC (Etten-Leur, The Netherlands); Isolute Si (500 mg in 10 ml) and NH2 (100 mg in 1 ml) cartridges were purchased from Biotage (Uppsala, Sweden).

Steroid extraction from serum and follicular fluid

Serum and follicular fluid samples were thawed and centrifuged for 10 min at 4000 g. Aliquots of 0.5 ml for progesterone analysis and 5 ml for 17-hydroxyprogesterone, 17β-oestradiol, testosterone, and 17α-testosterone analyses were pipetted into 15 ml polypropylene centrifuge tubes. Samples were then processed as described by McDonald *et al.* (McDonald, et al. 2010). Briefly, 0.5 or 5 ml phosphorus acid (4%) and 50 ng/ml internal standard was added to each sample. The mixture was vortexed for 1 min and mixed on a mechanical shaker for 10 min prior to solid phase extraction (SPE). Oasis strong anion exchange MAX 6 cc 150 mg cartridges were conditioned with 6 ml ethanol and 6 ml water. The samples were decanted into the SPE cartridges, the centrifuge tubes were washed with a further 6 ml water and this too was decanted into the SPE. The cartridges were then washed with 6 ml of 5% ammonia and 3 ml of n-hexane and subsequently dried for 2 min under vacuum. Analytes were eluted with 2 times 2 ml ethyl acetate into new centrifuge tubes and dried under nitrogen at 40 °C. The analytes were then dissolved in 65 µl methanol plus 65 µl water and vortexed for 30 sec before being centrifuged for 10 minutes at 9000 g (Sorvall RC5PLUS centrifuge) before being stored at -20 °C until analysed. Together with the serum samples calibration series ranging from 0–25 ng/ml for progesterone (0, 0.1, 0.5, 1.0, 2.5, 10, 25 ng/ml) and 0–100

pg/ml (0, 5, 10, 25, 50, 75, 100 pg/ml) for 17β-oestradiol, testosterone and 17α-testosterone and 17-hydroxyprogesterone were created.

The limits of detection and quantification in serum were respectively 0.4 and 1.3 ng/ml for progesterone, 63.3 pg/ml for 17β- oestradiol, 12 and 40 pg/ml for 17α-testosterone and testosterone and 10 and 33.3 pg/ml for 17-hydroxyprogesterone. The limits of detection and quantification in follicular fluid were 0.44 and 1.5 ng/ml for progesterone, 2.3 and 7.7 ng/ml for 17β-oestradiol, 5.4 and 18 ng/ml for 17α-testosterone , 7.3 and 24.3 ng/ml for testosterone and 2.76 and 9.2 ng/ml 17-hydroxyprogesterone.

Steroid extraction and purification of steroids from oviducts

Extraction and clean-up of the samples was based on a previously validated protocol (Vanhaecke, et al. 2011) using the following procedure. Aliquots of oviductal tissue (2.5 ± 0.1 g wet weight of complete oviduct) were placed in 100 ml Sovirel glass flasks and mixed with 50 µl of an internal standard (500 ng/ml) at a level corresponding to 2.5 µg/kg. The flasks were kept at room temperature for 15 min before 2 ml of sodium acetate buffer and 8 ml of ultrapure water were added to each. Next, the flasks were subjected to microwave (Zanker, The Netherlands) treatment for 72 sec at 100 Watts before the contents were transferred to 50 ml polypropylene tubes and homogenized for 1 min using an Ultra-turrax instrument. Methanol (10 ml) was added to the tubes which were then vortexed for 1 min before being centrifuged at 9000 g at 4 °C for 10 min. Any tissue residues were then removed from the supernatant by filtration over a cotton plug. Addition of 5 ml n-hexane, followed by rigorous shaking for 2 min and centrifugation at 9000 g at 4 °C for 10 min allowed elimination of the more lipophilic matrix fraction by discarding the hexane fraction. The analytes were then extracted by adding 20 ml diethyl ether to the water–methanol mixture and vigorously shaking for 2 min. The samples were allowed to settle before the ether layer was transferred to a 15 ml polypropylene flask and evaporated to dryness at 60 °C under nitrogen. Next, the residue was reconstituted in 0.5 ml chloroform, vortexed for 1 min and diluted with 5 ml hexane just prior to SPE. The solution was applied onto Si cartridges (500 mg), which had been preconditioned twice with 2.5 ml of hexane, the tubes which had contained the extract were rinsed with 5 ml of hexane and this too was applied onto the Si cartridges. NH_2 cartridges (100 mg) were placed underneath the Si cartridges which were rinsed with 5 ml hexane and allowed to run dry. Finally, each cartridge was eluted with 5 ml

chloroform:acetone (4:1, v/v) and the eluates were reduced to dryness under nitrogen at 45 °C before being reconstituted with 125 µl of methanol:water (50:50, v/v). A 10 µl aliquot was then injected onto the UHPLC column. Together with the oviductal samples, a calibration series which ranged from 0–75 ng/g for progesterone (0, 0.5, 2.5, 5.0, 10, 25, 50 and 75 ng/ml) and 0–5 ng/ml (0, 0.01, 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 ng/g) for 17β-oestradiol, testosterone, 17α-testosterone, and 17-hydroxyprogesterone were injected onto the UHPLC column.

The limits of detection were 24 ng/g for progesterone, 3 ng/g for 17β-oestradiol, 0.9 ng/g for 17α-testosterone and testosterone and 0.4 ng/g for 17-hydroxyprogesterone. The limits of quantification were 80 ng/g for progesterone, 10 ng/g for 17β-oestradiol, 3 ng/g for testosterone and 17α-testosterone and 1.3 ng/g for 17-hydroxyprogesterone.

Instrumentation and steroid quantification

The LC system consisted of a Thermo Fisher Scientific (San José, CA, USA) Accela UHPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic separation was achieved by reversed phase chromatography and gradient elution. Separation of the steroids was carried out on a Hypersil Gold C18 column (1.9 µm, 100 mm x 2.1 mm, Thermo Fisher Scientific), kept at 30 °C. The mobile phase consisted of water and methanol and was pumped at a flow rate of 0.3 ml min^{-1}. Optimized separation of all analytes was obtained using a linear gradient starting with a mixture of 50% water and methanol. After 0.5 min the amount of methanol was increased to 65% and kept at this concentration for 2.75 min. Next, the amount of methanol was increased to 100% in 0.5 min and kept at this concentration for 2 min. Finally, the column was allowed to re-equilibrate for 2.25 min at initial conditions before each run. All the analytes could be separated in a total run time of 8 min.
MS/MS analysis was performed on a triple quadrupole mass analyzer (TSQ Vantage, Thermo Fisher Scientific, San José, CA, USA), fitted with an atmospheric pressure chemical ionization source (APCI) operating in positive ion mode. The following working conditions were applied: spray voltage at 4 (+) kV; vaporizer and capillary temperature at 320 and 300 °C, respectively; sheath and auxiliary gas at 35 and 10 arbitrary units (a.u.), respectively; cycle time of 0.8 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da FWHM. Precursor ion, S-lens RF amplitude, and collision energy (CE) in Q2 were optimized individually per compound or transition, as was reported previously. Instrument control and

data processing were carried out by Xcalibur 2.1 software (Thermo Fisher Scientific, San José, CA, USA).

Prior to analysis of the samples, a standard mixture of the targeted compounds was injected to check the operational conditions of the devices. To every sample, a mixture of procedure ISTD was added at a concentration of 2.5 µg kg^{-1} prior to the extraction. Identification of the steroids was based on their retention time relative to the retention time of the ISTD of choice and on the ion ratios of the product ions. After identification, the analyte concentration was quantified by fitting its area ratio in a seven (serum and follicular fluid) or eight (tissue)-point calibration curve, established by blank meat samples fortified with the ISTDs at 2.5 µg kg^{-1} and the steroids in the range of 0–25 ng/ml for progesterone and 0–100 pg/ml for 17β-oestradiol, testosterone, 17α-testosterone, and 17-hydroxyprogesterone. Area ratios were determined by integration of the area of an analyte under the specific extracted chromatograms with reference to the integrated area of the ISTD. Regression coefficients of the calibration lines were all > 0.99. Limits of detection were calculated as 3*SD/slope. Limits of quantification were calculated as 10*SD/slope.

Statistical analysis

For oviductal tissue, follicular fluid and serum, independent samples t-test, paired-samples-t-test, Mann-Whitney-U-test, or Wilcoxon-signed rank test were applied, based upon data distribution. Correlations between oviductal tissue, serum and follicular fluid concentrations were calculated by means of Kendall's-τ correlation coefficients. Linear and curvilinear/polynomial regression was applied to identify linear and non-linear associations. Differences were considered significant at $P < 0.05$. Statistical analysis and graph plotting was performed with SPSS 21. Power analysis and sample size calculation ($\alpha = 0.05$; power = 0.85–0.99) were performed using G*Power 3.1.3 (Faul, et al. 2007).

Radioimmunoassay

Concentrations of 17β-oestradiol, progesterone, oestrone, and testosterone in preovulatory and postovulatory oviductal fluid were determined using the radioimmunoassay (RIA) method described by Ciereszko (Ciereszko 1999) and validated before (Franczak and Bogacki 2009).

The cross reactivities of the antisera against steroids have been previously reported (Szafranska, et al. 2002).
The efficiency of extraction and the coefficient of correlation between the added and recovered amounts of different hormone concentrations for 17β-oestradiol, progesterone, oestrone, and testosterone were 85.10% and 0.988, 85.60% and 0.989, 86.40% and 0.978, and 85.10% and 0.992, respectively. The limits of sensitivity of the assays were 0.5 pg/ml for 17β-oestradiol and testosterone, and 1 pg/ml for progesterone and oestrone. For each hormone all the samples were included in the same assay. The intra-assay coefficient of variation for 17β-oestradiol was 1.17%, for progesterone 1.08%, for oestrone 0.45%, and for testosterone 0.69%.

Localization of steroidogenic enzymes and progesterone receptors

Epifluorescence and confocal microscopy to detect aromatase, StAR and cytochrome P450scc protein

Oviductal samples were snap-frozen and stored in liquid nitrogen. Expression of aromatase, steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (cytochrome P450scc) protein in oviductal cross-sections was determined using indirect immunofluorescent staining as described earlier (Franczak, et al. 2013). In short, oviductal samples were sectioned at 7-µm. Approximately 30 min before staining the slides were brought up to room temperature, washed in PBS, dried, and then incubated with goat serum for 1 h to prevent nonspecific binding. After further rinsing with PBS and drying, the sections were incubated overnight (20 h) at 4 °C with the primary antibodies, each at a concentration of 1 µg/ml (rabbit anti-human aromatase, cytochrome P450scc and StAR polyclonal antibodies; Sigma Aldrich, Poznarí, Poland). The specificity of antibodies was evaluated initially *in silico* using Protein BLAST. The similarities of detectable immunogens with equine immunogens for anti-aromatase, anti-cytochrome P450scc and anti-StAR antibodies were 75%, 80% and 95%, respectively. For each staining a negative control, with dilution buffer used instead of primary antibody, was performed. Positive control tissues were follicular wall tissue and corpus luteum. Negative control tissue was connective tissue of oviductal tunica serosa. Next day, after washing with PBS and drying, the sections were incubated for 1 h at room temperature with the second antibody (goat anti-rabbit IgG

conjugated with biotin; Chemicon, Warszawa, Poland) at a concentration of 1.25 µg/ml. The sections were then rinsed thoroughly in PBS, dried, and incubated with Cy3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) to visualize the antigen–antibody complex. The sections were then mounted using Fluoroshield with 40,6-diamidino-2-phenylindole (Sigma Aldrich) to counterstain DNA and examined under an epifluorescent microscope (Olympus BX51). They were photographed at 500× magnification using Cell ˇF software version 3.4 (Olympus, Warszawa, Poland).

The intensity of fluorescent emission was evaluated after the conversion of the photographs to grayscale by measuring the average grey intensity with Cell ˇF software (Franczak, et al. 2006). The grey intensity was measured in 12 regions of interests (epithelium) for each of 4 biological replicates.

Immunohistochemistry to detect progesterone receptors and 3β-hydroxysteroid dehydrogenase/Δ5→4-isomerase

Staining for progesterone receptors (PR) and 3β-hydroxysteroid dehydrogenase/Δ5→4-isomerase (3-beta-HSD) was performed as described previously (De Bosschere, et al. 2002, Van den Broeck, et al. 2002). Briefly, for PR, the oviductal tissue samples were fixed for 24 h in 10% phosphate-buffered formaldehyde (pH 6.7) at room temperature and dehydrated with increasing concentrations of ethanol. Subsequently, the tissue samples were embedded in paraffin wax and sectioned at 5 µm (Microm HM360, Prosan, Merelbeke, Belgium). The sections were mounted on 3-aminopropyl-triethoxysilane-coated slides (APES, Sigma, St. Louis, MO, USA) and dried overnight at 37 °C. Next, they were dewaxed with xylene and dehydrated using decreasing concentrations of ethanol before being submitted to heat-mediated antigen retrieval in 1:10 citrate-based solution (pH 6; Antigen Retrieval Citra solution, Biogenex, Fremont, USA). After quenching of endogenous peroxidases with 3% H_2O_2, blocking was performed using 20% bovine serum. Next, the sections were incubated in a wet box for 1 h at room temperature while covered with 75 µl of a 1/100 dilution of the primary mouse anti-human PR antibody (MA5-12642, clone hPRa2, Thermo scientific inc., Perbio Science BVBA, Aalst, Belgium) in 2% BSA (Sigma-Aldrich, Diegem, Belgium); this antibody was reported by the manufacturer to cross-react with the equine progesterone receptor. Rabbit anti-mouse gamma globulin conjugated to biotin (DAKO, Prosan, Merelbeke,

Belgium) was used at a dilution of 1/500 as the secondary antibody and peroxidase-conjugated streptavidine (1/3000) was employed as the enzyme label. A 6 min incubation with 3,3'-diaminobenzidine hydrochloride (DAB) chromogen substrate (Liquid DAB+, DAKO, Prosan, Merelbeke, Belgium) resulted in a brown staining of the nucleus. The sections were counterstained with hematoxylin and subsequently dehydrated with increasing concentrations of ethanol and, finally, with xylene. Positive and negative controls were included in every staining procedure (Burry 2011). Positive controls were equine endometrium known to be positive for PR (Silva, et al. 2014, Wilsher, et al. 2011), while similar tissue sections were incubated with dilution buffer instead of the primary antibody, then biotinylated secondary antibody, or the peroxidase-conjugated streptavidine served as negative controls. Equine connective tissue of the oviductal tunica serosa served as negative control tissue. Other sections were also incubated with DAB alone to exclude the possibility of residual endogenous peroxidase activity. For each oviductal region, at least four technical replicates per each of five biological replicates were included.

3-Beta-HSD staining was performed similarly. The cross reactivity of rabbit anti-human 3-beta-HSD (Ab 154385, Abcam, Cambridge, UK) with equine tissue was initially *in silico* confirmed by subjecting the equine HSD3B sequence (gi|126352310) and the human HSD3B2 sequence (gi|112770) to NCBI protein BLAST analysis (last accessed: July 2013; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq&QUERY=&SUBJECTS=). This revealed a cover query of 99% and 75% identities. Goat anti-rabbit gamma globulin conjugated to biotin (DAKO, Prosan, Merelbeke, Belgium) was used as the secondary antibody. The staining protocol, including negative controls, antibody and reagent concentrations, was identical to the PR staining protocol as mentioned above. In a preliminary trial, primary antibody concentrations of 1/50, 1/100 and 1/500 were evaluated. To confirm reactivity and specificity of the PR antibody, equine corpus luteum was used as the positive control tissue and serosal connective tissue of the oviduct was included as a negative control.

Quantification and statistical analysis

To quantify progesterone receptor (PR-) positive cells, the ratio of epithelial PR-negative cells (purple)/PR-positive nuclei (brown) was determined in each sample by means of Image J software (Maryland, USA; (Schneider, et al. 2012). Per oviductal region, all the cells were evaluated at a magnification of 400x in 3 different fields (n = 961–1735) of the various sections for each biological replicate (Bologna-Molina, et al. 2011). To determine significant differences in the percentages of PR- positive cells, binary logistic regression was implemented with the Nagelkerke pseudo R^2 and χ^2 fit tests. To quantify 3-beta-HSD immunoreactive protein, chromogen intensity was analysed via a reciprocal intensity approach (Nguyen, et al. 2013). At least 12 similar regions of interest were analysed for each tissue slice of the four technical replicates per each of five biological replicates within one group.

To compare staining intensities, one-way ANOVA with post-hoc LSD test was applied. To elucidate relationships between staining intensities and steroid concentrations, Spearman's rho or Pearson correlation coefficients were calculated and linear and polynomial/curvilinear regression model fit was applied. Statistical analysis was performed with SPSS 21 and graph plotting by means of Excel 2010 and sample size calculation ($\alpha = 0.05$; power = 0.85–0.99) were performed using G*Power 3.1.3 (Faul, et al. 2007).

RESULTS

High progesterone concentrations in the ipsilateral postovulatory oviductal tissue measured using U-HPLC-MS/MS

Our main finding was that progesterone values in the postovulatory stage were much higher ($P < 0.0005$) in the ipsilateral (median value 700 ng/g) compared with the contralateral oviduct (median value 18.7 ng/g) (Table 1); this overt difference was not apparent in the preovulatory oviducts. Progesterone concentrations were lower in the preovulatory than in the postovulatory stage, both in the ipsilateral and contralateral oviducts ($P < 0.0005$). 17β-oestradiol concentrations were higher in the preovulatory than the postovulatory stage both in the ipsilateral and contralateral oviducts ($P < 0.0005$) and similarly 17α-testosterone concentrations were slightly higher ($P = 0.011$) in the preovulatory stage than the

postovulatory stage in the ipsi- and contralateral oviducts. Concentrations of 17-hydroxyprogestrone or testosterone were not significantly influenced by the cycle stage or the side relative to ovulation -).

Table 1. Median values and interquartile range of local concentrations of progesterone, 17β-oestradiol, 17-hydroxyprogesterone, 17α-testosterone and testosterone measured by U-HPLC-MS/MS in equine oviducts with reference to side of ovulation and cycle stage. P-values are provided for the comparison between preovulatory (pre) and postovulatory (post) and between ipsilateral (ipsi) and contralateral (contra) oviducts. *** $P < 0.0005$, * $0.005 < P < 0.05$; NS = not significant.

	Stage	Side	Median value (ng/g)	Interquar-tile range (ng/g)	P – value (pre vs post)	P – value (ipsi vs contra)
Progesterone	Pre	Ipsi	18.7	75.0	< 0.0005***	NS
		Contra	20.6	28.7	< 0.0005***	
	Post	Ipsi	700	825		< 0.0005***
		Contr	69.0	127		
17β-Oestradiol	Pre	Ipsl	63.3	115	< 0.0005***	NS
		Contra	80.8	123	< 0.0005***	
	Post	Ipsi	2.40	9.40		NS
		Contra	4.60	26.0		
17-Hydroxyprogesterone	Pre	Ipsi	25.0	123	NS	NS
		Contra	26.0	169	NS	
	Post	Ipsi	35.5	35.2		NS
		Contra	14.0	11.0		
17α-Testosterone	Pre	Ipsi	7.0	13.3	0.011*	NS
		Contra	5.60	29.0	0.011*	
	Post	Ipsi	4.20	3.50		NS
		Contra	2.30	3.00		
Testosterone	Pre	Ipsi	1.40	4.50	NS	NS
		Contra	0.900	9.00	NS	
	Post	Ipsi	1.70	2.90		NS
		Contra	1.20	2.10		

Correlation coefficients (Kendall's τ) between ipsi- and contralateral oviducts are represented in Table 2. Ipsi- and contralateral steroid concentrations are strongly correlated, indicating a systemic influence. However, this is much less so for progesterone, suggesting an additional local control mechanism.

Table 2. Correlation coefficients (Kendall's τ or Pearson's) with reference to the cycle stage between ipsilateral and contralateral oviductal tissue progesterone, 17β-oestradiol, 17-hydroxyprogesterone, 17α-testosterone and testosterone concentrations. * 0.01 < P < 0.05; ** 0.005 < P < 0.05; *** P < 0.005.

	Preovulatory	**Postovulatory**
Progesterone	0.228	0.535*
17β-oestradiol	0.600***	0.657***
17-Hydroxyprogesterone	0.390*	0.905***
17α-Testosterone	0.562***	0.524***
Testosterone	0.517**	0.941***

Steroid concentrations in oviductal fluid differ with reference to side of ovulation and cycle stage as measured with RIA

Preliminary, the average volume of fluid in equine oviducts other than those included for steroid measurement was measured, and based on an earlier report on equine oviductal fluid volume (Campbell, et al. 1979), the volume was set to be 50 µl in the postovulatory stage and 100 µl at the preovulatory stage. Based on these findings and the absolute quantities of steroids that had been measured in the oviduct samples, progesterone, 17β-oestradiol, oestrone and testosterone concentrations were calculated in oviductal fluid **(Table 3)**. At the postovulatory stage, again a high progesterone concentration (103.087 ng/ml) was detected in the ipsilateral oviduct fluid; it was 6.5 times higher than that of the contralateral side (15.760 ng/ml) and 31.2 times higher than in the preovulatory stage (3.300 ng/ml). The preovulatory contralateral oestrone concentration (9.868 ng/ml) was 54.5 times higher than the postovulatory concentration (0.181 ng/ml), but this difference was not observed on the ipsilateral side (respectively 0.131 and 0.544 ng/ml). No marked differences in the order of

magnitude of 17β-oestradiol and testosterone levels were detected when comparing ipsilateral and contralateral or preovulatory and postovulatory oviductal fluid.

Table 3. Progesterone, 17β-oestradiol, oestrone and testosterone concentrations (ng/ml) in equine oviductal fluid, calculated from pooled oviductal flushes and assuming that the oviduct contains on average 100 µl of oviductal fluid in the preovulatory and 50 µl in the postovulatory cycle stage (Own unpublished observations; Campbell et al. 1979).

	Preovulatory		**Postovulatory**	
	Ipsilateral	**Contralateral**	**Ipsilateral**	**Contralateral**
Progesterone	3.30	11.6	103	15.8
17β-Oestradiol	0.168	0.147	0.512	0.385
Oestrone	0.131	9.87	0.544	0.181
Testosterone	0.547	0.376	1.09	0.874

High concentrations of steroids in follicular fluid versus serum, measured using U-HPLC-MS/MS

Mean progesterone, 17β-oestradiol, 17-hydroxyprogesterone, testosterone and 17α-testosterone concentrations (ng/ml) were compared between follicular fluid and preovulatory and postovulatory serum (**Table 4**). The concentrations of all these steroids were significantly higher in follicular fluid compared to preovulatory ($P < 0.005$) and postovulatory ($0.0005 < P < 0.05$) serum.

Immunoreactive protein expression of steroidogenic enzymes aromatase, cytochrome P450scc, StAR and 3-beta-HSD in the oviduct is dependent on cycle stage and side of ovulation

The epithelial cells lining both the ampulla and the isthmus were found to be positive for StAR, cytochrome P450scc, aromatase and 3-beta-HSD, except for the postovulatory contralateral ampulla and the ipsilateral ampulla which were negative for StAR and cytochrome P450scc respectively (**Fig. 1; Fig. 2. Fig. 3**).

Table 4. Mean concentrations of progesterone, 17β-oestradiol, 17-hydroxyprogesterone, 17α-testosterone and testosterone (ng/ml) measured by U-HPLC-MS/MS in equine serum and follicular fluid from the same mare and in postovulatory serum. SEM = standard error of the mean.

	Serum preovulatory		Serum postovulatory		Follicular fluid	
	Mean	**SEM**	**Mean**	**SEM**	**Mean**	**SEM**
Progesterone	0.768	0.355	2.57	0.244	14.3	4.43
17β-Oestradiol	0.882	0.382	< 0.010	< 0.010	59.2	13.9
17-Hydroxyprogesterone	0.726	0.363	0.442	0.089	5.82	1.3
17α-Testosterone	0.164	0.046	0.001	$2.04 . 10^{-4}$	2.77	0.393
Testosterone	0.001	$1.00 . 10^{-}$	0.001	$5.67 . 10^{-6}$	1.76	0.437

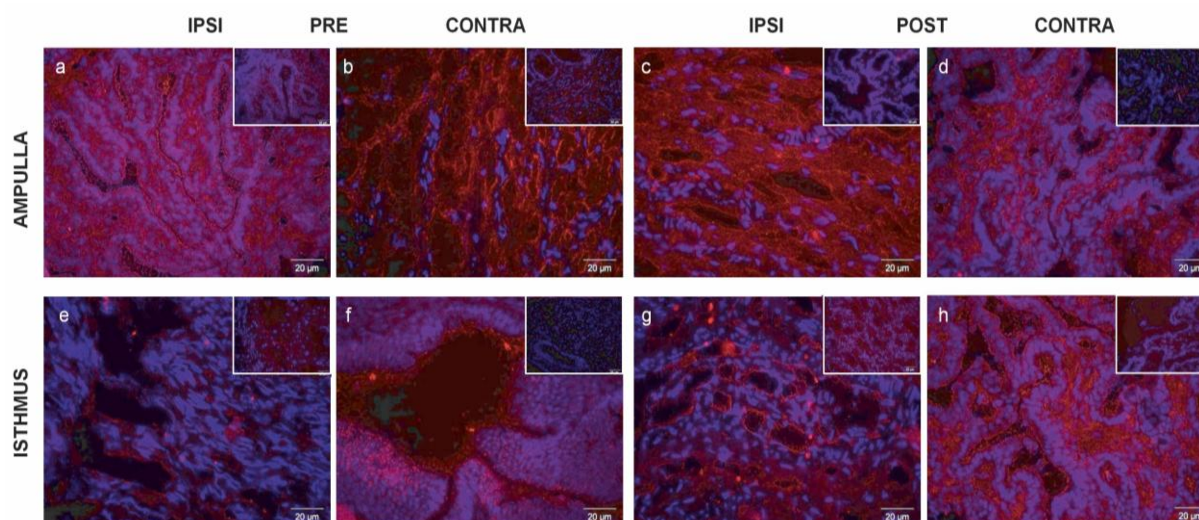


Figure 1. Epifluorescent pictures for aromatase in the equine oviduct with reference to cycle stage, location (ampulla [a–d] or isthmus [e–h]) and side of ovulation. ISPI = ipsilateral to ovulation (a, e, c, g), CONTRA = contralateral to ovulation (b, f, d, h), PRE = preovulatory (a, b, e, f), POST = postovulatory (c, d, g, h). Blue = DAPI nuclear counterstaining; red = aromatase positive tissue. All oviductal fragments were found to be aromatase positive compared to the negative control slide without primary antibody (upper right corner). Bar = 20 µm.

No specific cell type expressing steroidogenic immunoreactive protein could be identified as the signal was present in all types of oviductal cells. No specific pattern which may explain

the ipsilateral postovulatory oviductal high progesterone concentrations could be detected **(Fig. 2)**. Both preovulatory and postovulatory oviductal epithelium stained positively for 3-beta-HSD compared to the negative controls **(Fig. 3).** The chromogen intensity was the strongest in the contralateral isthmus epithelium **(Fig. 2 d)**. In each part, chromogen intensity was significantly higher in the isthmus compared to the ampulla ($P < 0.0001$; **Fig. 2d**). Smooth muscle cells lining the small oviductal veins and arteries, the lamina (propria) muscularis and in the surrounding connective tissue were also strongly positively stained **(Fig. 3)**. Compared to the negative controls, the cytoplasm and the nuclei of cumulus cells both stained strongly ($P < 0.0001$) for 3-beta-HSD **(Suppl. 1 f)**. Positive tissue control samples (follicular wall or corpus luteum) were markedly positive **(Suppl. 1)**.

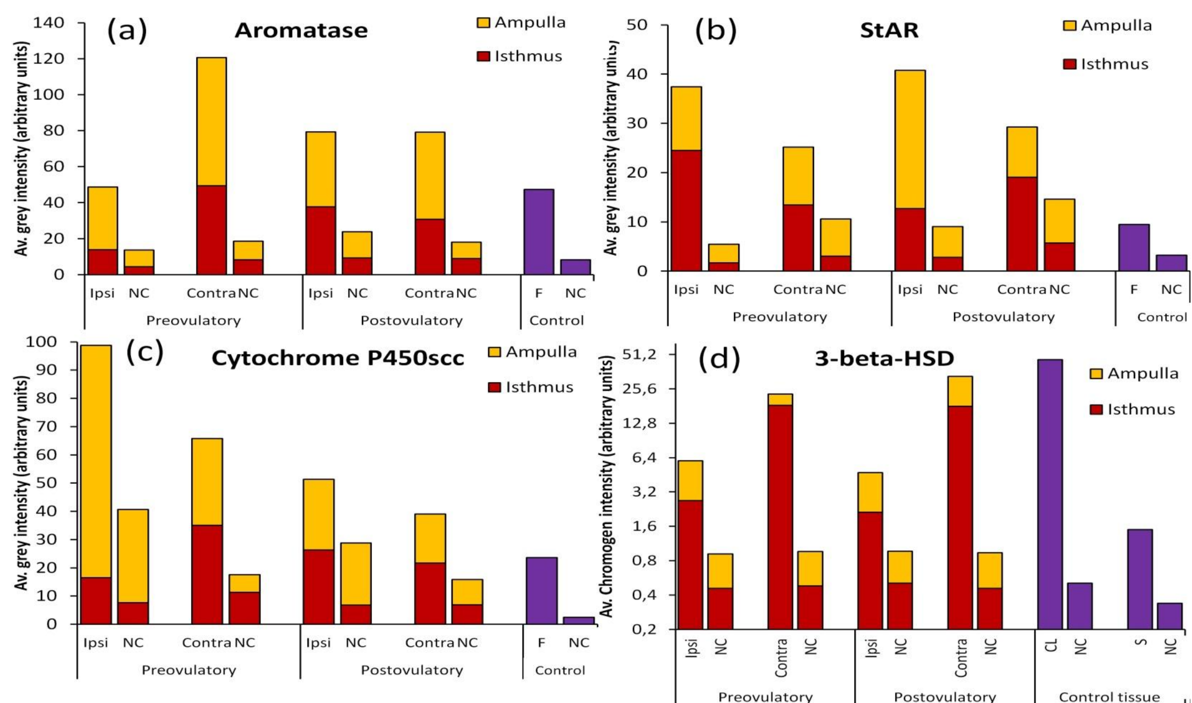


Fig. 2. Average gray intensity (a-c) and chromogen intensity (d) in arbitrary units of slides of equine oviductal tissue stained by immunofluorescensce (a-c) and immunohistochemistry (d) to detect immunoreactive protein of aromatase, cholesterol side-chain cleavage enzyme (cytochrome P450scc), steroidogenic acute regulatory protein (StAR) and 3β-hydroxysteroid dehydrogenase/Δ5→4-isomerase (3- beta-HSD). NC = Negative antibody controls. Positive controls were follicular wall (F) and corpus luteum (CL). Negative tissue control is stroma of equine oviductal tunica serosa (S). One-way ANOVA. All intensities (ampulla versus isthmus and pre- versus postovulatory) were significantly different, except the contralateral ampulla for StAR and the ipsilateral ampulla for cytochrome P450scc in the postovulatory stage.

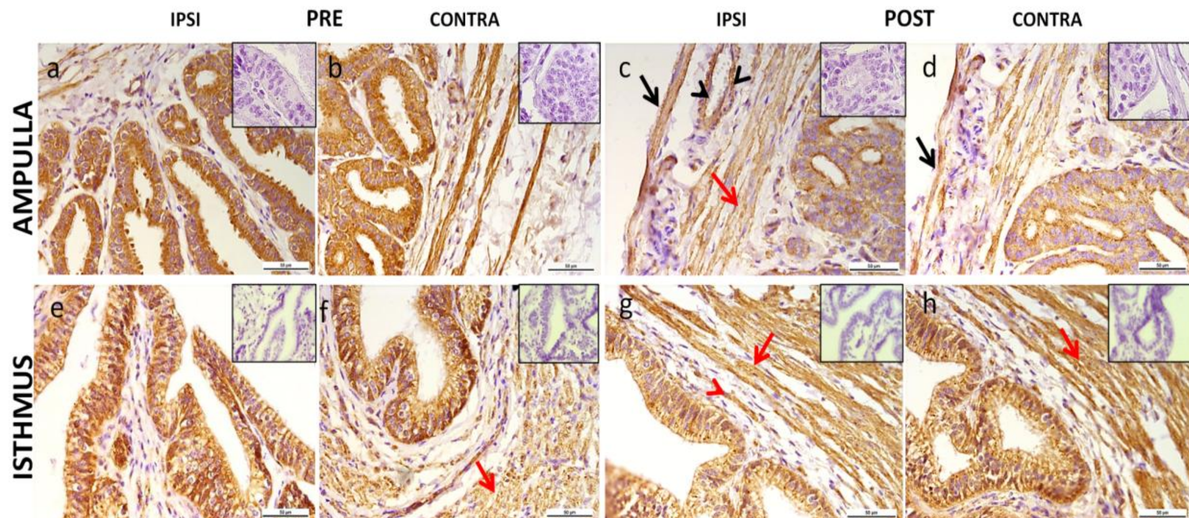
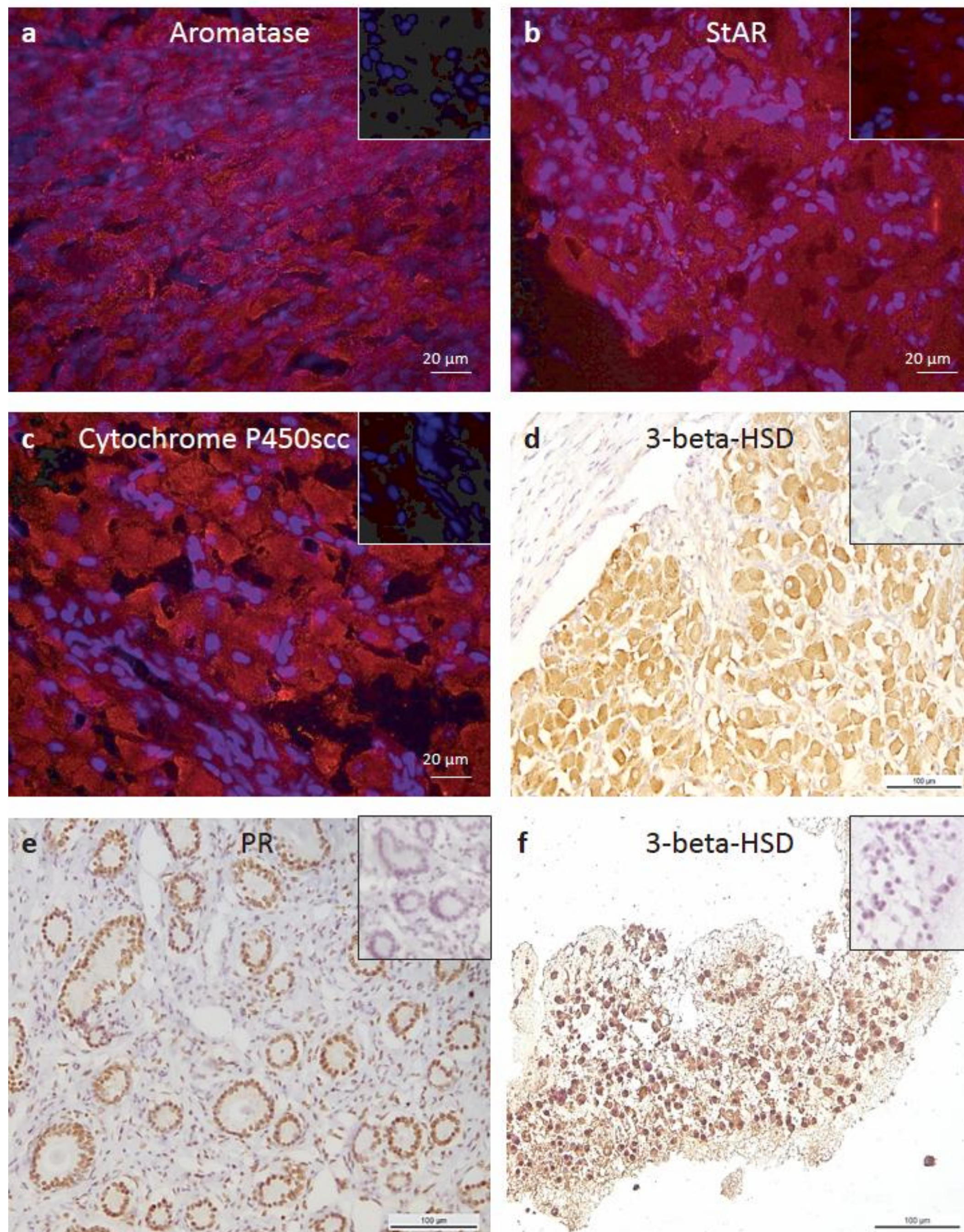


Fig. 3. 3β-hydroxysteroid dehydrogenase/Δ5 → 4-isomerase (3-beta-HSD) immunohistochemical staining (brown) with hematoxylin counterstaining (purple) of oviductal tissue, related to cycle stage, location (ampulla [a – d] or isthmus [e – h]) and side of ovulation. IPSI = ipsilateral to ovulation (a, c, e, g), CONTRA = contralateral to ovulation (b, d, f, h), PRE = preovulatory (a, b, e, f), POST = postovulatory (c, d, g, h). 3-beta-HSD is highly abundant in the cytoplasm of epithelial cells and smooth muscle cells of the lamina muscularis propria (red arrow head), the lamina muscularis (red arrows) and also in the wall of arteries and veins in the oviductal wall (black arrow heads) and surrounding connective tissue (black arrow). A negative control without primary antibody is included in the upper right corner of each photograph. Bar = 50 μm.

Correlations

In the preovulatory stage of the cycle, no correlations were observed between serum and follicular fluid. However, follicular fluid progesterone, 17-hydroxyprogesterone and 17α-testosterone concentrations were positively correlated with postovulatory serum concentrations (respectively $P = 0.008$, $P = 0.026$, $P = 0.026$ and $r = 0.846$, $r = 0.6$, $r = 0.643$; **Table 5**). Follicular fluid progesterone concentrations are positively associated with preovulatory oviductal tissue concentrations ($R^2 = 0.453$, $F(1,13) = 9.10$, $P = 0.015$) whereas no association with postovulatory concentrations could be detected suggesting a local transfer and a minimal shedding of follicular fluid in the oviduct after ovulation. Follicular fluid 17β-oestradiol/progesterone ratio is significantly correlated to preovulatory ipsilateral cytochrome P450scc staining intensity ($r = 0.770$, $P = 0.003$) but not to contralateral or postovulatory intensities ($P > 0.05$). Similarly, follicular fluid progesterone is negatively correlated to

preovulatory ipsilateral (r = 0.650, P = 0.016) but not to contralateral or postovulatory 3-beta-HSD expression (P > 0.05).



Suppl. 1. Epifluorescent (a-c) and immunohistochemically (d-f) positive tissue control pictures and negative control slides without primary antibody (right upper corners) in the equine oviduct for aromatase (a), StAR (b), cytochrome P450scc (c), 3-beta-HSD (d, f) and PR (e). Positive control tissue was equine follicular wall (a), corpus luteum (b-d) and endometrium (e). 3-Beta-HSD staining (brown) with hematoxylin counterstaining (purple) of equine cumulus cells (f). Both the cytoplasm and the nuclei of cumulus cells stain strongly. Bar a-c = 20 µm, bar d-f = 100 µm.

Table 5. Correlation coefficients (Kendall's τ or Pearson's) between preovulatory/postovulatory serum and follicular fluid progesterone, 17β-estradiol, 17-hydroxyprogesterone, 17α-testosterone and testosterone concentrations. * $0.01 < P < 0.05$; ** $0.005 < P < 0.01$.

	Preovulatory	Postovulatory
Progesterone	0.139	0.846**
17β-Estradiol	0.428	-0.445
17-Hydroxyprogesterone	0.555	0.600*
17α-Testosterone	-0.530	0.643*
Testosterone	-0.217	0.333

The percentage of progesterone receptor positive nuclei is decreased in the epithelium of the preovulatory contralateral ampulla and increased in the postovulatory contralateral isthmus

Ratios of PR positive to PR negative cells **(Fig. 4** and **Fig. 5)** were higher in the epithelium of the ipsilateral preovulatory ampulla (66 %) than in the epithelium of the contralateral preovulatory ampulla (44.8%; $P < 0.0001$; odds ratio = 2.38), whereas in the postovulatory oviduct, the contralateral epithelium of the isthmus contained more progesterone positive (93.5%) nuclei than the ipsilateral isthmus epithelium (66.6%; $P < 0.0001$; odds ratio = 7.22). Overall, significantly more PR positive nuclei were detected in the postovulatory contralateral oviduct compared with the ipsilateral side ($P < 0.0001$). Also smooth muscle cells in the lamina propria muscularis **(Fig. 4e**, red arrow**)** and the lamina muscularis and smooth muscle cells in the surrounding connective tissue of the tunica serosa all stained positively. Smooth muscle cell nuclei lining the small oviductal veins and arteries were also positive.

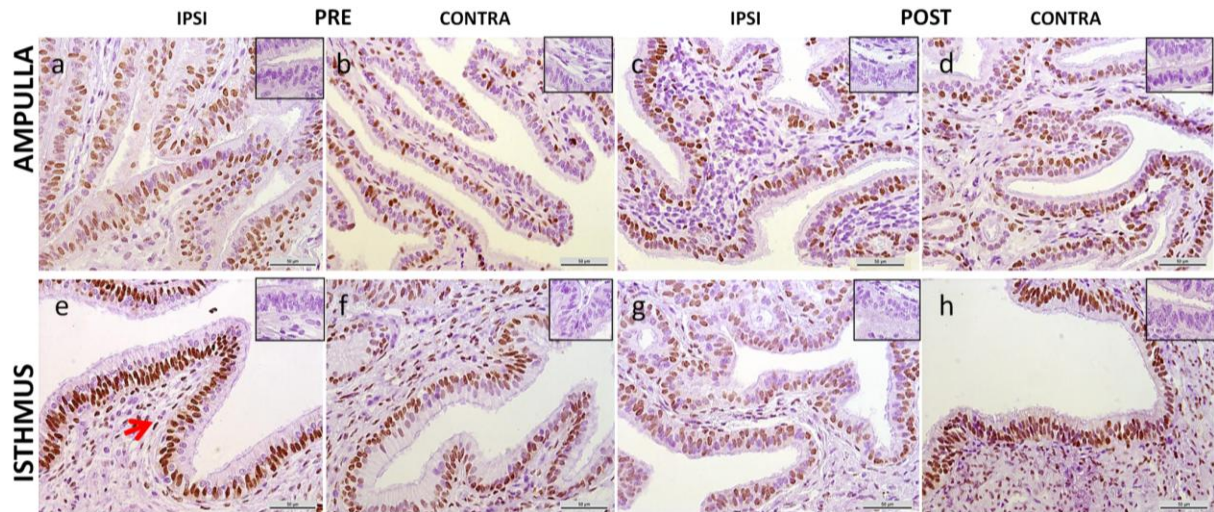


Fig. 4. Progesterone receptors in equine oviductal tissue stained with immunohistochemistry (brown) and counterstained with hematoxylin (purple) in oviductal tissue, related to cycle stage, location (ampulla [a–d] or isthmus [e–h] and side of ovulation. IPSI = ipsilateral to ovulation (a, c, e, g), CONTRA = contralateral to ovulation (b, d, f, h), PRE = preovulatory (a, b, e, f), POST = postovulatory (c, d, g, h). Progesterone receptors (brown) were present in epithelial nuclei as well as in the smooth muscle cell nuclei of the lamina muscularis propria (red arrow) and the lamina muscularis. A negative control without primary antibody is included in the upper right corner of each photograph. Bar = 50 µm.

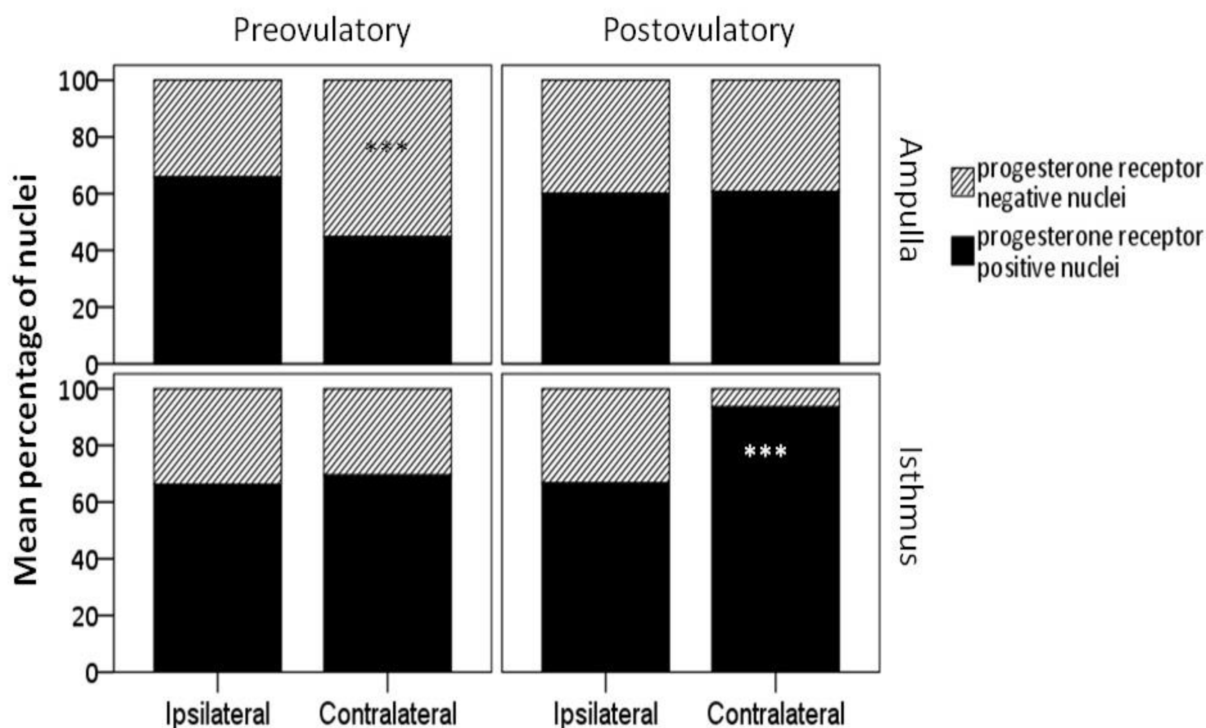


Fig. 5. Percentage of progesterone receptor positive and negative epithelial nuclei on immunohistochemical slides in the preovulatory and postovulatory cycle stage. The percentage of progesterone receptor positive nuclei was highly significantly lower in the contralateral ampulla compared to the isthmus and the ipsilateral ampulla and isthmus in the preovulatory cycle stage. In the postovulatory cycle stage, the percentage of progesterone receptor positive nuclei in the contralateral isthmus was highly elevated. Binary logistic regression. *** = $P < 0.0001$

DISCUSSION

Using U-HPLC-MS/MS in this study, we determined for the first time local concentrations of 17β-oestradiol, progesterone, 17-hydroxyprogesterone, testosterone and 17α-testosterone in the ipsi- and contralateral oviduct of the mare, at two biologically different stages of the oestrous cycle. Furthermore, using RIA, 17β-oestradiol, progesterone, testosterone and oestrone concentrations were quantified in oviductal fluid. We found that oviductal steroid concentrations, progesterone receptor expression, staining intensity of steroidogenic proteins and tissue steroidogenic activity are all dependent on both the cycle stage of the mare and the side of ovulation.

The most significant finding was that ipsilateral postovulatory oviductal tissue and oviductal fluid progesterone concentrations are dramatically higher than those on the contralateral side. In oviductal tissue, this phenomenon has only been described once in the cow (Wijayagunawardane, et al. 1996), a species in which a countercurrent transfer mechanism between the uterine vein and ovarian artery is present (Kotwica, et al. 1982). In the cow, a substantial number of genes which are involved in important reproductive biological processes have been shown to be differentially expressed in the postovulatory ipsilateral oviduct, when compared to the contralateral oviduct, (Bauersachs, et al. 2003a). These genes are hypothesized to play a role in providing the optimal conditions for the early reproductive processes and to pave the way for the early embryo. These unilateral effects may result from local progesterone-induced influences of the ipsilateral ovary or the cumulus-oocyte complex and/or from local steroidogenic processes (Bauersachs, et al. 2003a) Thus, next to the cycle stage-specific endocrine changes, local effects of steroids, in particular progesterone, may play a role as well in the oviduct (Reischl, et al. 1999). Also in humans, the biological relevance of different local progesterone concentrations has been illustrated by the fact that sperm moves preferably to the ipsilateral oviduct (Wildt, et al. 1998), confirming the chemotactic properties of progesterone (Eisenbach and Giojalas 2006).

Now, using the new and highly sensitive U-HPLC-MS/MS assay method we have confirmed the same marked difference between ipsilateral and contralateral oviduct steroid levels in the horse, in which no countercurrent exchange mechanism exists (Ginther, et al. 1972). Although there may be some transport of progesterone via the local circulation towards the oviduct, we nonetheless hypothesize that the high local concentration of progesterone found in the oviduct may be caused by 5 different mechanisms (Hunter 2012b, Wijayagunawardane, et al. 1996); 1) a peritoneal route, involving progesterone uptake from follicular fluid shed during ovulation, 2) the presence of a local transfer system from the ovary to the oviduct via blood or lymph; 3) the expression of high affinity receptors for progesterone in the oviduct; 4) local synthesis of progesterone in the oviduct; and 5) paracrine contribution of progesterone by follicular cells shed into the oviduct at ovulation.

Follicular fluid shed into the oviduct or the peritoneal cavity

A preovulatory follicle has a mean diameter of about 45 mm, which is equivalent to a volume of 47.7 ml. In line with the observations of Watson et al. (Watson, et al. 2002), the average

progesterone concentration in fluid from follicles close to ovulation was 14.32 ± 4.40 ng/ml (Table 4), which means that such a follicle contains about 683.06 ± 207.70 ng of progesterone in total. Likewise, total concentrations for other follicular steroids are about 2825.4 ± 666.1 ng for 17β-oestradiol, 277 ± 66.1 ng for 17-hydroxyprogesterone, 132.1 ± 18.7 ng for 17α-testosterone and 83.8 ± 20.8 ng for testosterone. These follicular steroids, in particular progesterone, may reach the ipsilateral oviduct in two putative ways that may explain the higher oviductal progesterone concentrations. A first possibility is the abovarian direction in which the cilia beat within the infundibulum (Yaniz, et al. 2002). A second possible route is by diffusion from the peritoneal fluid through pores within the visceral peritoneum that cover the oviduct (Abuhijleh, et al. 1995, Ginther, et al. 2008, Leak and Rahil 1978). The latter route is much more likely than the former since in the mare, the majority of the large amount of follicular fluid shed at ovulation passes from the fimbriae into the peritoneal cavity (Ginther, et al. 2008). Moreover, curvilinear regression analysis showed that follicular fluid 17β-oestradiol and its ratios with progesterone could significantly predict postovulatory ipsilateral but not contralateral oviductal progesterone concentrations and progesterone/17β-oestradiol ratio (data not shown). Also follicular fluid progesterone concentration is positively associated with oviductal 17β-oestradiol/progesterone ratio. The causal relationship between these parameters might suggest an ipsilateral influence of follicular fluid shed in the oviduct or peritoneal cavity on the oviductal concentrations. Thus, these results may suggest that oviductal fluid steroids shed in the peritoneal cavity diffuse into the oviduct after ovulation. However, since follicular fluid contains besides progesterone also high amounts of other steroids, another mechanism is required to explain the selective increase of progesterone in the ipsilateral oviduct.

Local transfer of steroids from the ovary to the oviduct

Local transfer of steroids from the ovary to the oviduct via a counter-current system including ovarian veins or lymph vessels which anastomose in the mesovarium has been described in sows, cows and women (Bendz 1977, Bendz, et al. 1979, Cicinelli, et al. 2004, Einer-Jensen and Hunter 2000, Einerjensen and Mccracken 1981, Gabler, et al. 1999, Ginther 1974, 1976, Mccracken, et al. 1984, Stefanczyk-Krzymowska, et al. 2002). In contrast to pigs and cows, in which a countercurrent arterio-venous system exists, the equine uterine vein and its tributaries perfuse only a small area of the local tissues. However, anatomically, the idea of a counter-current exchange between the ovarian venous drainage and the uterine tubal arterial supply

may not be rejected due to the intense contact between the accessory ovarian vein and the ovarian branch of the ovarian vein, which supplies a major part of the uterine tube **(Fig. 6;** (Barone 2001). In sheep it has been shown that the oviductal vein which drains the oviduct and the cranial part of the uterus, adjacent to the ovary with the corpus luteum, contributes to the ipsilateral elevated progesterone concentrations in uterine tissue. After resection of the oviductal vein indeed, the ipsilateral uterine progesterone concentration was not higher anymore compared to the contralateral side (Weems, et al. 1989).

Local elevated progesterone concentrations could also originate from transport or diffusion of hormones by the extensive network of lymphatic vessels in the mesovarium. In sheep and goats, the utero-ovarian lymph progesterone concentrations were between 10 to 1000-fold higher than those in peripheral plasma. Moreover, lymph progesterone concentrations equalled plasma concentrations after ipsilateral ovariectomy (Staples, et al. 1982). Local diffusion with resulting high concentrations of hormones in tissue surrounding the ovary was also evidenced in the cow: a progesterone gradient in tissue ipsilateral but not contralateral to the corpus luteum was demonstrated (Pope, et al. 1982). Also in the mare, a similar phenomenon has been described during early pregnancy (d24-80): a 220-fold increased progesterone concentration in the venous effluent from the corpus luteum bearing ovary compared with the contralateral side was reported while contralateral concentrations approached those measured in the jugular vein (Squires and Ginther 1975). These findings most likely also explain the 100-fold increase of the ipsilateral postovulatory oviductal progesterone concentration in mares **(Table 1)**. Indeed, follicular fluid progesterone and 17β-oestradiol concentrations and their ratios are associated with oviductal progesterone and 17β-oestradiol concentrations. The one-sided (unilateral) influence of a dominant follicle or corpus luteum via a regional vascular/lymphatic transfer probably may also account for the unilateral elevated steroidogenic enzyme staining intensity of aromatase, StAR, cytochrome P450scc and 3-beta-HSD as depicted in **Fig. 2**. The associations between follicular fluid steroid concentrations and unilateral enzyme expression may suggest indeed a unilateral influence of follicular fluid on preovulatory enzyme expression probably accomplished by a local vascular and/or lymphatic transfer system. Nonetheless, one should bear in mind that the biological relevance and significance of associations between variables should be interpreted with caution. Steroid determination in ovarian arteries, venes and lymph vessels would substantiate this hypothesis definitively.

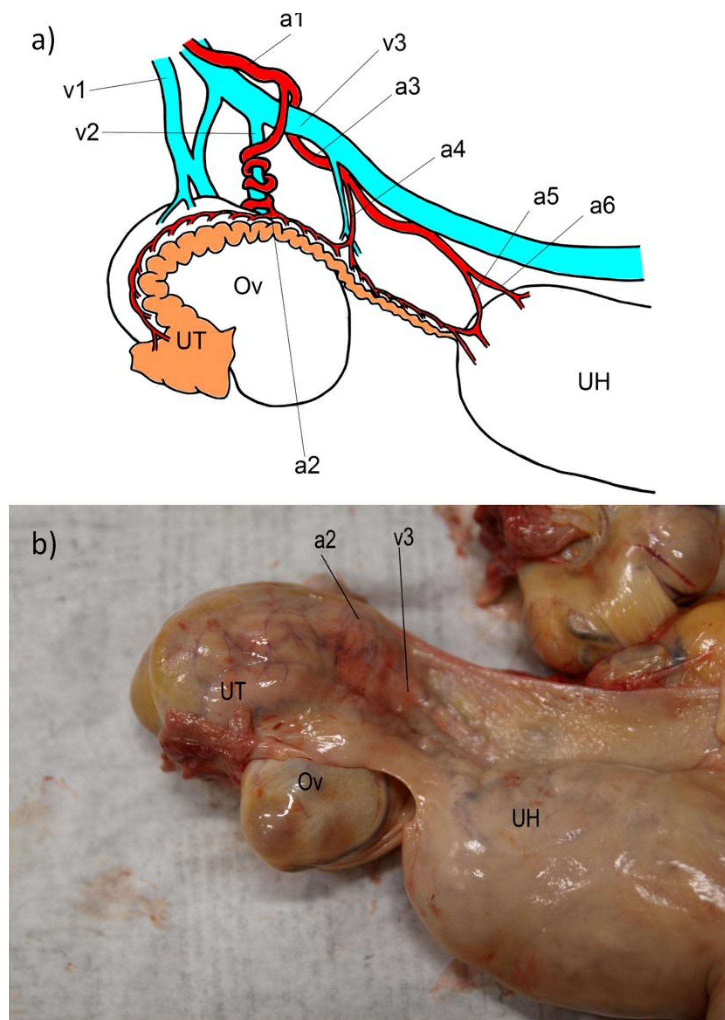


Fig. 6. a) Schematic drawing of the vasculature at the level of the left uterine tube in the mare (left lateral view, redrawn after Barone (2001). Courtesy: Pieter Cornillie. The ovarian vein (v1) lies topographically isolated from the ovarian artery (a1) and drains directly into the caudal vena cava. However, an accessory ovarian vein (v2), which is a tributary of the uterine branch (v3) of the ovarian vein (ramus uterinus), is heavily encircled by a coiled ovarian branch of the ovarian artery, which not only supplies the ovary (OV), but also a large part of the uterine tube (UT) through its cranial tubal branch (a2). The remainder of the uterine tube is supplied by derivatives of the uterine branch of the ovarian artery (a3), i.e. the middle (a4) and caudal tubal branch (a5), before the uterine branch disperses in the uterine horn (UH) as cranial artery of the uterine horn (a6). b) *Ex vivo* equine ovary, oviduct and top of the uterine horn with adjacent tissues and blood vessels. Courtesy: Pieter Cornillie (6 a) and Jan Govaere (6 b).

A difference between steroid concentrations in arterial and systemic blood endorses the assumption that local counter-current transfer of modulating factors, even though very species specific (Einer-Jensen and Hunter 2005), modulates the function of the female genital tract

(Hunter, et al. 1983, Stefanczyk-Krzymowska, et al. 2002). For instance, the transfer of progesterone from the corpus luteum may inhibit follicle growth more in the ipsilateral compared to the contralateral ovary (Fukuda, et al. 1997). This may explain the tendency of alternation of ovulation between the ovaries (Fukuda, et al. 1997). Similarly, high levels of oestradiol produced in the large follicle reach the ovarian artery where it induces an increased blood flow (Cicinelli, et al. 2004). Also other substances, which have been demonstrated to be transferred between the ovarian vein and artery such as prostaglandins, inert gases, peptides and other steroids as described in mice, pigs, sheep, cows and women (Einer-Jensen and Hunter 2000, Einerjensen 1988), may unilaterally optimize the oviductal milieu for gamete transport and maturation, fertilization and early embryonic development (Einer-Jensen and Hunter 2005). Indeed, spermatozoa tend to move to the ipsilateral oviduct and human embryos prefer to nidate on the uterine wall ipsilateral to the side of ovulation (Kunz, et al. 1998).

Expression of high-capacity progesterone receptors

Another explanation for the high progesterone concentrations in the ipsilateral oviduct could be the presence of high-capacity progesterone receptors (PRs) on the ipsilateral side. Hypothetically, an upregulation of PRs along the oviduct could indicate increased amounts of functionally active hormone-receptor complexes (Ulbrich, et al. 2003) which could subsequently sequestrate large amounts of progesterone. This hypothesis could also explain the dramatically increased level of progesterone in the ipsilateral oviduct shortly after ovulation (Hartt, et al. 2005, Watson, et al. 1992) (Table 1). If so, it should be possible to demonstrate high expression of PRs in the ipsilateral postovulatory oviduct. Immunohistochemistry did indeed reveal that PRs are abundantly expressed in the luminal epithelium of the oviduct **(Fig. 4)**. However, many more progesterone receptor positive nuclei were present in the isthmic portion of the contralateral postovulatory oviduct, than in the ipsilateral oviduct **(Fig. 4 g, h** and **Fig. 5).** This finding might be explained by the fact that an elevated progesterone concentration in the ipsilateral oviduct may decrease its own receptor expression by means of a negative feedback mechanism, as has been described previously in guinea pigs (Hai, et al. 1977). Therefore, the high ipsilateral progesterone concentration observed in the present study seems to be associated with a downregulation of PRs, and as such the hypothesis that the elevated ipsilateral progesterone is due to an increase in PRs can be rejected. The significant differences in PR expression in relation to the target regions

(ampulla and isthmus), the side (ipsilateral or contralateral) and the stage of the cycle (pre and postovulatory) do reflect their functional importance and are indicative of the fact that progesterone, the PR ligand, exerts its effect locally in the oviduct. Since PR expression is very species-specific, further elaboration on the regulation of the finely tuned differences in PR expression, and the interaction of PR with its ligands, including progesterone and 17-hydroxyprogesterone, is needed.

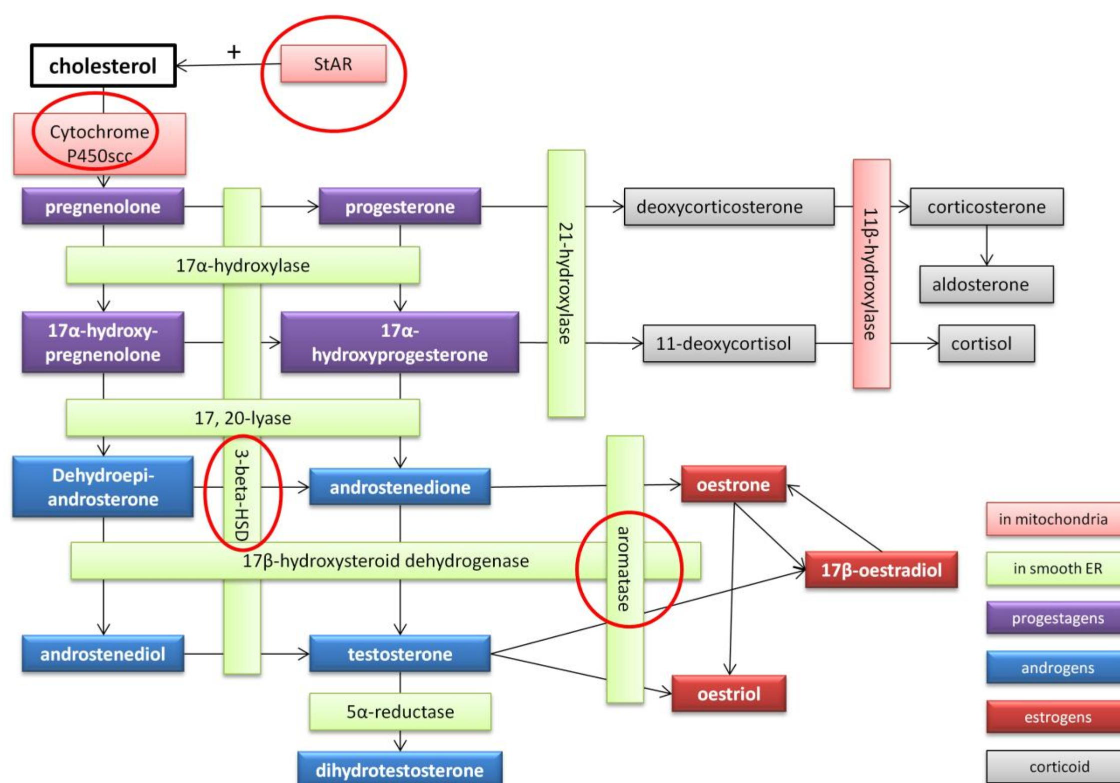
Local synthesis of progesterone in the oviduct

A further mechanism to possibly explain the elevated progesterone concentrations in ipsilateral oviductal tissue and oviductal fluid is its local production. **Fig. 2** shows that StAR, cytochrome P450scc, aromatase and 3-beta-HSD immunoreactive proteins are present in the equine oviduct which is suggestive of local steroidogenesis **(Suppl. 2).** In the rabbit, progesterone synthesis by the oviduct epithelium and associated muscle cells has been put forward in previous studies (Richardson and Oliphant 1981, Spilman and Wilks 1976, Takeda, et al. 1978). The murine oocyte also stimulates increased production of progesterone by the oviduct during oocyte transport (Kendle and Lee 1980). Even though no apparent differences in the steroidogenic enzymes could explain the selective increase of progesterone in the ipsilateral postovulatory oviduct, the overall expression of these immunoreactive proteins clearly demonstrates the steroidogenic capacity of the oviduct.

In the ipsilateral postovulatory equine oviduct, no clear upregulation of StAR or cytochrome P450scc was detected which could explain the high progesterone production.

Aromatase activity has been demonstrated before in the oviduct of the frog (Kobayashi, et al. 1996), the woman (Li, et al. 2003) and the rat (Tetsuka, et al. 1998). Little is known about the regulation of this enzyme. It may influence the actions of either androgen and oestrogen *in vivo* (Hillier, et al. 1980) and may be involved in the regulation of androgen receptors *in vivo* (Shao, et al. 2007b). The presence of immunoreactive aromatase protein demonstrates that the equine oviduct is able to convert androgens to oestrogens **(Suppl. 2)**. Since oestrone synthesis is also catalyzed by aromatase and since high concentrations of oestrone, measured in oviductal fluid (Table 3) in the contralateral preovulatory oviduct, are consistent with the relatively higher expression of aromatase by the contralateral oviduct, the elevated contralateral oviductal fluid concentrations of oestrone could be explained by local synthesis, catalyzed by aromatase.

3β-Hydroxysteroiddehydrogenase (3-beta-HSD) catalyses **(Suppl. 2)** the conversion of pregnenolone to progesterone (LaVoie and King 2009). Its expression is enhanced by, amongst other factors, oestradiol and suppressed by testosterone (Heggland, et al. 1997) and progesterone (Munabi, et al. 1983). However, it should be noted that regulation of the HSD3B gene is species and cell type specific (Simard, et al. 2005). The lack of association between immunoreactive protein expression of 3-beta-HSD and progesterone production levels indicates that other variants, haplotypes or enzymes may be involved in equine oviductal progesterone biosynthesis and metabolism pathways (Olson, et al. 2007)



Suppl. 2. Overview of steroidogenesis (based on Lavoie and King 2009). The enzymes discussed in the paper are encircled in red.

Paracrine contribution of follicular cells in the oviduct

The follicle cells shed together with the oocyte and the follicular fluid in the oviduct at the time of ovulation, could be another source of steroids that might affect local oviductal steroid concentrations. This could explain the selectively elevated progesterone concentration without a concomitant increase in concentrations of 17β-oestradiol, 17-hydroxyprogesterone and

testosterone in the ipsilateral oviduct in the postovulatory phase. Many viable follicle cells in suspension have been shown to be present in the neighbourhood of an oocyte or a developing embryo (Gardner, et al. 1996). They are able to act in a paracrine manner to produce large amounts of progesterone and oestradiol (Reverchon, et al. 2012), androgens, and other signalling molecules. Equine granulosa cells show immunoreactive 3-beta-HSD protein (data not shown) which may confirm their progesterone producing capabilities, as described in other species (Chabrolle, et al. 2009, Schuetz and Dubin 1981, Smith, et al. 2011, Stoklosowa, et al. 1982). Porcine granulosa cells are reported to produce in 48 h 114 ng progesterone per 10^5 granulosa cells (Stoklosowa, et al. 1982). This could explain the ipsilateral elevated progesterone concentrations without increased levels of other steroids.

In conclusion, it can be deduced that the most plausible explanation for establishment of the elevated progesterone concentration in the ipsilateral oviduct of the mare is a combination of 1) the contribution from follicular fluid in the oviduct and the diffusion of follicular fluid steroids after ovulation; 2) a local transfer of steroids via blood or lymph, 3) local synthesis of progesterone in the oviduct, as evidenced by the expression of steroidogenic enzymes and, 4) the paracrine contribution from follicular cells

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REFERENCES

Abuhijleh, MF, OA Habbal, and ST Moqattash 1995 The Role of the Diaphragm in Lymphatic Absorption from the Peritoneal-Cavity. *Journal of Anatomy* **186** 453-467.

Aguilar, JJ, J Cuervo-Arango, H Mouguelar, and L Losinno 2012 Histological Characteristics of the Equine Oviductal Mucosa at Different Reproductive Stages. *Journal of Equine Veterinary Science* **32** 99-105.

Alkhalaf, M, AY Propper, G Chaminadas, and GL Adessi 1992 Ultrastructural-Changes in Guinea-Pig Endometrial Cells during the Estrous-Cycle. *Journal of Morphology* **214** 83-96.

Armstron.Dt, and DL Black 1966 Influence of Luteinizing Hormone on Corpus Luteum Metabolism and Progesterone Biosynthesis Throughout Bovine Estrous Cycle. *Endocrinology* **78** 937-&.

Ball, BA, and M Altschul 1990 In vitro development of 4- to 8- cell equine embryos cocultured with trophoblastic vesicles or oviductal explants. . *Theriogenology* **33** 189.

Ball, BA, KE Scoggin, MHT Troedsson, and EL Squires 2013 Characterization of prostaglandin E-2 receptors (EP2, EP4) in the horse oviduct. *Animal Reproduction Science* **142** 35-41.

Ballester, L, J Romero-Aguirregomezcorta, C Soriano-Ubeda, C Matas, R Romar, and P Coy 2014 Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency. *Fertility and Sterility* **102** 1762-U1598.

Barone, R 2001 Anatomie comparée des mammifères domestiques. *In* Vigot (ed.), Anatomie comparée des mammifères domestiques., pp. 322. Paruis.

Bauersachs, S, H Blum, S Mallok, H Wenigerkind, S Rief, K Prelle, and E Wolf 2003 Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: A transcriptomics approach. *Biology of Reproduction* **68** 1170-1177.

Bendz, A 1977 Anatomical Basis for a Possible Counter Current Exchange Mechanism in Human Adnex. *Prostaglandins* **13** 355-362.

Bendz, A, N Einerjensen, O Lundgren, and PO Janson 1979 Exchange of Kr-85 between the Blood-Vessels of the Human Uterine Adnexa. *Journal of Reproduction and Fertility* **57** 137-142.

Bennett, WA, TL Watts, WD Blair, SJ Waldhalm, and JW Fuquay 1988 Patterns of Oviducal Motility in the Cow during the Estrous-Cycle. *Journal of Reproduction and Fertility* **83** 537-543.

Bhabak, KP, and G Mugesh 2010 Functional Mimics of Glutathione Peroxidase: Bioinspired Synthetic Antioxidants. *Accounts of Chemical Research* **43** 1408-1419.

Bhatnagar, P, VE Papaioannou, and JD Biggers 1995 Csf-1 and Mouse Preimplantation Development in-Vitro. *Development* **121** 1333-1339.

Bjare, U 1992 Serum-Free Cell-Culture. *Pharmacology & Therapeutics* **53** 355-374.

Bogaert, L, M Van Poucke, C De Baere, L Peelman, F Gasthuys, and A Martens 2006 Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *Bmc Biotechnology* **6**.

Bologna-Molina, R, P Damian-Matsumura, and N Molina-Frechero 2011 An easy cell counting method for immunohistochemistry that does not use an image analysis program. *Histopathology* **59** 801-803.

Bonnans, C, J Chou, and Z Werb 2014 Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology* **15** 786-801.

Bottenstein, J, I Hayashi, S Hutchings, H Masui, J Mather, DB McClure, R Ohasa, A Rizzino, G Sato, G Serrero, R Wolfe, and R Wu 1979 The growth of cells in serum-free hormone-supplemented media. *Methods Enzymology* **58** 94-109.

Buhi, WC, and IM Alvarez 2003 Identification, characterization and localization of three proteins expressed by the porcine oviduct. *Theriogenology* **60** 225-238.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Buhi, WC, JL Vallet, and FW Bazer 1989 Denovo Synthesis and Release of Polypeptides from Cyclic and Early Pregnant Porcine Oviductal Tissue in Explant Culture. *Journal of Experimental Zoology* **252** 79-88.

Burry, RW 2011 Controls for Immunocytochemistry: An Update. *Journal of Histochemistry & Cytochemistry* **59** 6-12.

Bustin, SA, V Benes, JA Garson, J Hellemans, J Huggett, M Kubista, R Mueller, T Nolan, MW Pfaffl, GL Shipley, J Vandesompele, and CT Wittwer 2009 The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55** 611-622.

Bylander, A, M Nutu, R Wellander, M Goksor, H Billig, and DGJ Larsson 2010 Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. *Reproductive Biology and Endocrinology* **8**.

Campbell, DL, LW Douglas, and JC Ramge 1979 Cannulation of the Equine Oviduct and Chemical-Analysis of Oviduct Fluid. *Theriogenology* **12** 47-59.

Cappelli, K, M Felicetti, S Capomaccio, G Spinsanti, M Silvestrelli, and AV Supplizi 2008 Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. *Bmc Molecular Biology* **9**.

Chabrolle, C, L Tosca, C Rame, P Lecomte, D Royere, and J Dupont 2009 Adiponectin increases insulin-like growth factor I-induced progesterone and estradiol secretion in human granulosa cells. *Fertility and Sterility* **92** 1988-1996.

Chase, CC, RP Delvecchio, SB Smith, and RD Randel 1992 Invitro Metabolism of Glucose by Bovine Reproductive Tissues Obtained during the Estrous-Cycle and after Calving. *Journal of Animal Science* **70** 1496-1508.

Chegini, N, Y Zhao, and FW Mclean 1994 Expression of Messenger-Ribonucleic-Acid and Presence of Immunoreactive Proteins for Epidermal Growth-Factor (Egf), Transforming Growth-Factor-Alpha (Tgf-Alpha) and Egf/Tgf-Alpha Receptors and I-125 Egf Binding-Sites in Human Fallopian-Tube. *Biology of Reproduction* **50** 1049-1058.

Chen, S, R Einspanier, and J Schoen 2013a In Vitro Mimicking of Estrous Cycle Stages in Porcine Oviduct Epithelium Cells: Estradiol and Progesterone Regulate Differentiation, Gene Expression, and Cellular Function. *Biology of Reproduction* **89**.

Chen, S, R Einspanier, and J Schoen 2013b Long-term culture of primary porcine oviduct epithelial cells: Validation of a comprehensive in vitro model for reproductive science. *Theriogenology* **80** 862-869.

Chi, MMY, AL Schlein, and KH Moley 2000 High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141** 4784-4792.

Choi, YH, CC Love, DD Varner, LB Love, and K Hinrichs 2003 Effects of gas conditions, time of medium change, and ratio of medium to embryo on in vitro development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219-1229.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004 Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Christensen, AK, and SW Gillim 1969 The correlation of fine structure and function in steroid-secreting cells with emphasis on those of the gonads. . *In* K McKerns (ed.), The Gonads, pp. 415-490. New York: Meredith Corporation.

Chung, YW, TS Kim, SY Lee, SH Lee, Y Choi, N Kim, BM Min, DW Jeong, and IY Kim 2006 Selenite-induced apoptosis of osteoclasts mediated by the mitochondrial pathway. *Toxicol Lett* **160** 143-150.

Cicinelli, E, N Einer-Jensen, M Cignarelli, L Mangiacotti, D Luisi, and S Schonauer 2004 Preferential transfer of endogenous ovarian steroid hormones to the uterus during both the follicular and luteal phases. *Human Reproduction* **19** 2001-2004.

Ciereszko, R 1999 Radioimmunologiczne oznaczanie stężenia hormonów steroidowych w płynach biologicznych [Radioimmunoassay of steroid hormones in biological fluids. *In* J Przala (ed.), Animal Physiology., pp. 157-163. Olsztyn: UWM Press.

Cohen, PE, LY Zhu, and JW Pollard 1997 Absence of colony stimulating factor-1 in osteopetrotic (csfm(op)/csfm(op)) mice disrupts estrous cycles and ovulation. *Biology of Reproduction* **56** 110-118.

Daels, P, and JP Hughes 1993 The normal oestrous cycle. *In* A McKinnon and PLAM Vos (ed.), Equine Reproduction, pp. 121-133. Lea & Febiger.

De Bosschere, H, R Ducatelle, H Vermeirsch, P Simoens, and M Coryn 2002 Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. *Animal Reproduction Science* **70** 251-259.

Desantis, S, G Ventriglia, S Zizza, AC Guaricci, M Losurdo, A Zarrilli, and M Albrizio 2010 Changes in the expression of the mu-opioid receptor in the mare oviduct during oestrus and anoestrus. *Animal Reproduction Science* **119** 40-49.

Desantis, S, S Zizza, G Accogli, F Acone, R Rossi, and L Resta 2011 Morphometric and ultrastructural features of the mare oviduct epithelium during oestrus. *Theriogenology* **75** 671-678.

Diaz, MJ, S Giunta, J Valz-Gianinet, S Pereyra-Alfonso, V Flores, and D Miceli 2000 Proteases with plasminogen activator activity in hamster oviduct. *Molecular Reproduction and Development* **55** 47-54.

Duc-Goiran, P, TM Mignot, C Bourgeois, and F Ferre 1999 Embryo-maternal interactions at the implantation site: a delicate equilibrium. *European Journal of Obstetrics Gynecology and Reproductive Biology* **83** 85-100.

Duong, TD, and CA Erickson 2004 MMP-2 plays an essential role in producing epithelial-mesenchymal transformations in the avian embryo. *Developmental Dynamics* **229** 42-53.

Einer-Jensen, N, and RHF Hunter 2000 Physiological and pharmacological aspects of local transfer of substances in the ovarian adnexa in women. *Human Reproduction Update* **6** 132-138.

Einer-Jensen, N, and RHF Hunter 2005 Counter-current transfer in reproductive biology. *Reproduction* **129** 9-18.

Einerjensen, N 1988 Countercurrent Transfer in the Ovarian Pedicle and Its Physiological Implications. *Oxford Reviews of Reproductive Biology* **10** 348-381.

Einerjensen, N, and JA Mccracken 1981 Physiological-Aspects of Corpus-Luteum Blood-Flow and of the Counter System in the Ovarian Pedicle of the Sheep. *Acta Veterinaria Scandinavica* 89-101.

Eisenbach, M, and LC Giojalas 2006 Sperm guidance in mammals - an unpaved road to the egg. *Nature Reviews Molecular Cell Biology* **7** 276-285.

Faul, F, E Erdfelder, AG Lang, and A Buchner 2007 G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39** 175-191.

Fazeli, A 2008 Maternal communication with gametes and embryos. *Theriogenology* **70** 1182-1187.

Fernandez-Gonzalez, R, P Moreira, A Bilbao, A Jimenez, M Perez-Crespo, MA Ramirez, FR De Fonseca, B Pintado, and A Gutierrez-Adan 2004 Long-term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes, development, and behavior. *Proceedings of the National Academy of Sciences of the United States of America* **101** 5880-5885.

Franczak, A, and M Bogacki 2009 Local and Systemic Effects of Embryos on Uterine Tissues During Early Pregnancy in Pigs. *Journal of Reproduction and Development* **55** 262-272.

Franczak, A, G Kotwica, B Kurowicka, A Oponowicz, I Woclawek-Potocka, and BK Petroff 2006 Expression of enzymes of cyclooxygenase pathway and secretion of

prostaglandin E-2 and F-2 alpha by porcine myometrium during luteolysis and early pregnancy. *Theriogenology* **66** 1049-1056.

Franczak, A, B Wojciechowicz, A Zmijewska, J Kolakowska, and G Kotwica 2013 The effect of interleukin 1 beta and interleukin 6 on estradiol-17 beta secretion in the endometrium of pig during early pregnancy and the estrous cycle. *Theriogenology* **80** 90-98.

Frolova, A, L Flessner, M Chi, ST Kim, N Foyouzi-Yousefi, KH Moley, and H Moley 2009 Facilitative Glucose Transporter Type 1 Is Differentially Regulated by Progesterone and Estrogen in Murine and Human Endometrial Stromal Cells. *Endocrinology* **150** 1512-1520.

Fukuda, M, K Fukuda, CY Andersen, and AG Byskov 1997 Does corpus luteum locally affect follicular growth negatively? *Human Reproduction* **12** 1024-1027.

Gabler, C, A Einspanier, D Schams, and R Einspanier 1999 Expression of vascular endothelial growth factor (VEGF) and its corresponding receptors (flt-1 and flk-1) in the bovine oviduct. *Molecular Reproduction and Development* **53** 376-383.

Gabler, C, GJ Killian, and R Einspanier 2001 Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction* **122** 121-130.

Gardner, DK, M Lane, I Calderon, and J Leeton 1996 Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertility and Sterility* **65** 349-353.

Gardner, DK, M Lane, A Spitzer, and PA Batt 1994 Enhanced Rates of Cleavage and Development for Sheep Zygotes Cultured to the Blastocyst Stage in-Vitro in the Absence of Serum and Somatic-Cells - Amino-Acids, Vitamins, and Culturing Embryos in Groups Stimulate Development. *Biology of Reproduction* **50** 390-400.

Georgiou, AS, E Sostaric, CH Wong, APL Snijders, PC Wright, HD Moore, and A Fazeli 2005 Gametes alter the oviductal secretory proteome. *Molecular & Cellular Proteomics* **4** 1785-1796.

Ginther, OJ 1974 Internal Regulation of Physiological Processes through Local Venoarterial Pathways - Review. *Journal of Animal Science* **39** 550-564.

Ginther, OJ 1976 Comparative anatomy of the uteroovarian vasculature., Veterinary Scope, pp. 1-17. Kalamazoo, Michigan: Upjohn Co.

Ginther, OJ 1992 Reproductive biology of the mare. Basic and applied aspects. Cross Plains, Wisconsin: Cross Plains, Equiservices.

Ginther, OJ, MC Garcia, EL Squires, and Steffenh.Wp 1972 Anatomy of Vasculature of Uterus and Ovaries in Mare. *American Journal of Veterinary Research* **33** 1561-1586.

Ginther, OJ, EL Gastal, MO Gastal, and MA Beg 2008 Passage of postovulatory follicular fluid into the peritoneal cavity and the effect on concentrations of circulating hormones in mares. *Animal Reproduction Science* **107** 1-8.

Ginther, OJ, EL Gastal, MO Gastal, MD Utt, and MA Beg 2007 Luteal blood flow and progesterone production in mares. *Animal Reproduction Science* **99** 213-220.

Goossens, K, A Van Soom, M Van Poucke, L Vandaele, J Vandesompele, A Van Zeveren, and LJ Peelman 2007 Identification and expression analysis of genes associated with bovine blastocyst formation. *Bmc Developmental Biology* **7**.

Gstraunthaler, G 2003 Alternatives to the use of fetal bovine serum: Serum-free cell culture. *Altex-Alternativen Zu Tierexperimenten* **20** 275-281.

Guidobaldi, HA, ME Teves, DR Unates, A Anastasia, and LC Giojalas 2008 Progesterone from the Cumulus Cells Is the Sperm Chemoattractant Secreted by the Rabbit Oocyte Cumulus Complex. *Plos One* **3**.

Guilbert, LJ, and NN Iscove 1976 Partial Replacement of Serum by Selenite, Transferrin, Albumin and Lecithin in Hematopoietic Cell-Cultures. *Nature* **263** 594-595.

Hai, MTV, F Logeat, M Warembourg, and E Milgrom 1977 Hormonal-Control of Progesterone Receptors. *Annals of the New York Academy of Sciences* **286** 199-209.

Hanukoglu, I 1992 Steroidogenic Enzymes - Structure, Function, and Role in Regulation of Steroid-Hormone Biosynthesis. *Journal of Steroid Biochemistry and Molecular Biology* **43** 779-804.

Hartt, LS, SJ Carling, MM Joyce, GA Johnson, DK Vanderwall, and TL Ott 2005 Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. *Reproduction* **130** 241-250.

Hashizume, K, T Takahashi, M Shimizu, J Todoroki, A Shimada, M Hirata, T Sato, and A Ito 2003 Matrix-metalloproteinases-2 and-9 production in bovine endometrial cell culture. *Journal of Reproduction and Development* **49** 45-53.

Hayes, KEN, RA Pierson, ST Scraba, and OJ Ginther 1985 Effects of Estrous-Cycle and Season on Ultrasonic Uterine Anatomy in Mares. *Theriogenology* **24** 465-477.

Heggland, SJ, SA Signs, and JRD Stalvey 1997 Testosterone decreases 3 beta-hydroxysteroid dehydrogenase-isomerase messenger ribonucleic acid in cultured mouse Leydig cells by a strain-specific mechanism. *Journal of Andrology* **18** 646-655.

Hillier, SG, AMJ Vandenboogaard, LE Reichert, and EV Vanhall 1980 Alterations in Granulosa-Cell Aromatase-Activity Accompanying Preovulatory Follicular Development in the Rat Ovary with Evidence That 5-Alpha-Reduced C-19 Steroids Inhibit the Aromatase Reaction Invitro. *Journal of Endocrinology* **84** 409-419.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hu, H, C Jiang, T Schuster, GX Li, PT Daniel, and J Lu 2006 Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway. *Mol Cancer Ther* **5** 1873-1882.

Hunter, RHF 2005 The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction Nutrition Development* **45** 281-290.

Hunter, RHF 2012 Components of oviduct physiology in eutherian mammals. *Biological Reviews* **87** 244-255.

Hunter, RHF, B Cook, and NL Poyser 1983 Regulation of Oviduct Function in Pigs by Local Transfer of Ovarian-Steroids and Prostaglandins - a Mechanism to Influence Sperm Transport. *European Journal of Obstetrics Gynecology and Reproductive Biology* **14** 225-232.

Jian, L, and ALW Po 1993 Effects of Insulin and Nasal Absorption Enhancers on Ciliary Activity. *International Journal of Pharmaceutics* **95** 101-104.

Jones, JI, and DR Clemmons 1995 Insulin-Like Growth-Factors and Their Binding-Proteins - Biological Actions. *Endocrine Reviews* **16** 3-34.

Kendle, KE, and B Lee 1980 Investigation of the Influence of Progesterone on Mouse Embryo Transport by Using Anti-Progestational Steroids. *Journal of Reproduction and Fertility* **58** 253-258.

Kennedy, TG, KD Brown, and TJ Vaughan 1994 Expression of the Genes for the Epidermal Growth-Factor Receptor and Its Ligands in Porcine Oviduct and Endometrium. *Biology of Reproduction* **50** 751-756.

Kervancioglu, ME, E Saridogan, T Atasu, T Camlibel, A Demircan, B Sarikamis, and O Djahanbakhch 1997 Human Fallopian tube epithelial cell co-culture increases

fertilization rates in male factor infertility but not in tubal or unexplained infertility. *Hum Reprod* **12** 1253-1258.

Kim, HY, J Sohn, GT Wijewickrama, P Edirisinghe, T Gherezghiher, M Hemachandra, PY Lu, RE Chandrasena, ME Molloy, DA Tonetti, and GR Thatcher 2010 Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens. *Bioorg Med Chem* **18** 809-821.

Kim, NH, and AR Menino 1995 Effects of Stimulators of Protein Kinase-a and Kinase-C and Modulators of Phosphorylation on Plasminogen-Activator Activity in Porcine Oocyte-Cumulus Cell Complexes during in-Vitro Maturation. *Molecular Reproduction and Development* **40** 364-370.

King, SR, and HA LaVoie 2012 Gonadal transactivation of STARD1, CYP11A1 and HSD3B. *Frontiers in Bioscience-Landmark* **17** 824-846.

Kobayashi, F, SJ Zimniski, and KN Smalley 1996 Characterization of oviductal aromatase in the northern leopard frog, Rana pipens. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **113** 653-657.

Kotwica, J, GL Williams, and MJ Marchello 1982 Countercurrent Transfer of Testosterone by the Ovarian Vascular Pedicle of the Cow - Evidence for a Relationship to Follicular Steroidogenesis. *Biology of Reproduction* **27** 778-789.

Kouba, AJ, BR Burkhardt, IM Alvarez, MM Goodenow, and WC Buhi 2000 Oviductal plasminogen activator inhibitor-1 (PAI-1): mRNA, protein, and hormonal regulation during the estrous cycle and early pregnancy in the pig. *Molecular Reproduction and Development* **56** 378-386.

Krysko, DV, T Vanden Berghe, K D'Herde, and P Vandenabeele 2008 Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods* **44** 205-221.

Kunz, G, M Herbertz, M Noe, and G Leyendecker 1998 Sonographic evidence for the involvement of the utero-ovarian counter-current system in the ovarian control of directed uterine sperm transport. *Human Reproduction Update* **4** 667-672.

Lag, M, G Paulsen, and J Jonsen 1984 Effect of Sodium Selenite on the Ciliary Activity, Adenosine-Triphosphate, and Protein-Synthesis in Mouse Trachea Organ-Cultures. *Journal of Toxicology and Environmental Health* **13** 857-864.

LaVoie, HA, and SR King 2009 Transcriptional Regulation of Steroidogenic Genes: STARD1, CYP11A1 and HSD3B. *Experimental Biology and Medicine* **234** 880-907.

Leak, LV, and K Rahil 1978 Permeability of Diaphragmatic Mesothelium - Ultrastructural Basis for Stomata. *American Journal of Anatomy* **151** 557-593.

Leclerc, E, Y Sakai, and T Fujii 2003 Cell Culture in 3-Dimensional Microfluidic Structure of PDMS (polydimethylsiloxane). *Biomedical Microdevices* **5** 109-114.

Lei, ZM, and CV Rao 1992 Expression of Epidermal Growth-Factor (Egf) Receptor and Its Ligands, Egf and Transforming Growth Factor-Alpha, in Human Fallopian-Tubes. *Endocrinology* **131** 947-957.

Leist, M, and M Jaattela 2001 Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2** 589-598.

Lenth, RV 2007 Statistical power calculations. *Journal of Animal Science* **85** E24-E29.

Leroith, D, H Werner, D Beitnerjohnson, and CT Roberts 1995a Molecular and Cellular Aspects of the Insulin-Like Growth-Factor-I Receptor. *Endocrine Reviews* **16** 143-163.

LeRoith, D, H Werner, S Neuenschwander, T Kalebic, and LJ Helman 1995b The role of the insulin-like growth factor-I receptor in cancer. *Receptor Activation by Antigens, Cytokines, Hormones, and Growth Factors* **766** 402-408.

Levanon, K, V Ng, HY Piao, Y Zhang, MC Chang, MH Roh, DW Kindelberger, MS Hirsch, CP Crum, JA Marto, and R Drapkin 2010 Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* **29** 1103-1113.

Li, Y, L Qin, ZH Xiao, YL Wang, R Herva, JH Leng, JH Lang, V Isomaa, and YS Piao 2003 Expression of P450 aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 at fetal-maternal interface during tubal pregnancy. *Journal of Steroid Biochemistry and Molecular Biology* **87** 241-246.

Mahmood, T, E Saridogan, S Smutna, AM Habib, and O Djahanbakhch 1998 The effect of ovarian steroids on epithelial ciliary beat frequency in the human Fallopian tube. *Human Reproduction* **13** 2991-2994.

Mccracken, JA, W Schramm, and N Einerjensen 1984 The Structure of Steroids and Their Diffusion through Blood-Vessel Walls in a Countercurrent System. *Steroids* **43** 293-303.

McCue, PM, and EL Squires 2002 Persistent anovulatory follicles in the mare. *Theriogenology* **58** 541-543.

McDonald, M, E Malone, and J McBride 2010 Confirmation of Hormones in Animal Serum by Liquid Chromatography/Tandem Mass Spectrometry According to European Commission Decision 2002/657. *Journal of Aoac International* **93** 343-349.

McKinnon, AO 1997 Ovarian abnormalities. *In* MAO Ranaten N.W. (ed.), Equine diagnostic ultrasonography, pp. 233-251. Baltimore: Williams and Wilkins.

Messmer, TO 1973 Nature of Iron Requirement for Chinese-Hamster V79 Cells in Tissue-Culture Medium. *Experimental Cell Research* **77** 404-408.

Miessen, K, S Sharbati, R Einspanier, and J Schoen 2011 Modelling the porcine oviduct epithelium: A polarized in vitro system suitable for long-term cultivation. *Theriogenology* **76** 900-910.

Morishige, KI, H Kurachi, K Amemiya, H Adachi, K Adachi, Y Sakoyama, A Miyake, and O Tanizawa 1993 Menstrual Stage-Specific Expression of Epidermal Growth-Factor and Transforming Growth-Factor-Alpha in Human Oviduct Epithelium and Their Role in Early Embryogenesis. *Endocrinology* **133** 199-207.

Munabi, AK, FG Cassorla, DG Pfeiffer, BD Albertson, and DL Loriaux 1983 The effects of estradiol and progesterone on rat ovarian 17-hydroxylase and 3ß-hydroxysteroid dehydrogenase activities. *Steroids* **41** 95-98.

Nelis, H, K D'Herde, K Goossens, L Vandenberghe, B Leemans, K Forier, K Smits, K Braeckmans, L Peelman, and A Van Soom 2014 Equine oviduct explant culture: a basic model to decipher embryo–maternal communication. *Reproduction, Fertility and Development* **26** 954-966.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015 Steroids in the equine oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*.

Nelis, HM, K Goossens, B Leemans, L Peelman, and A Van Soom 2013 Steroid-Regulated Mrna Expression in Oviduct Epithelial Cells in the Mare. *Reproduction Fertility and Development* **25** 258-258.

Nguyen, DH, T Zhou, J Shu, and J-H Mao 2013 Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity., Cancer InCytes, edn Summer 2013. Santa Monica.

Nielsen, OB, F de Paoli, and K Overgaard 2001 Protective effects of lactic acid on force production in rat skeletal muscle. *Journal of Physiology-London* **536** 161-166.

Ohe, T, M Hirobe, and T Mashino 2000 Novel metabolic pathway of estrone and 17 beta-estradiol catalyzed by cytochrome P-450. *Drug Metabolism and Disposition* **28** 110-112.

Olson, SH, EV Bandera, and I Orlow 2007 Variants in estrogen biosynthesis genes, sex steroid hormone levels, and endometrial cancer: A HuGE review. *American Journal of Epidemiology* **165** 235-245.

Page-McCaw, A, AJ Ewald, and Z Werb 2007 Matrix metalloproteinases and the regulation of tissue remodelling. *Nature Reviews Molecular Cell Biology* **8** 221-233.

Pampfer, S, S Tabibzadeh, FC Chuan, and JW Pollard 1991 Expression of Colony-Stimulating Factor-I (Csf-1) Messenger-Rna in Human Endometrial Glands during the Menstrual-Cycle - Molecular-Cloning of a Novel Transcript That Predicts a Cell-Surface Form of Csf-1. *Molecular Endocrinology* **5** 1931-1938.

Pattison, ML, CL Chen, ST Kelley, and GW Brandt 1974 Luteinizing-Hormone and Estradiol in Peripheral-Blood of Mares during Estrous-Cycle. *Biology of Reproduction* **11** 245-250.

Pepper, MS, D Belin, R Montesano, L Orci, and JD Vassalli 1990 Transforming Growth Factor-Beta-1 Modulates Basic Fibroblast Growth-Factor Induced Proteolytic and Angiogenic Properties of Endothelial-Cells Invitro. *Journal of Cell Biology* **111** 743-755.

Pfeifer, TL, and N Chegini 1994 Immunohistochemical Localization of Insulin-Like Growth-Factor (Igf-I), Igf-I Receptor, and Igf Binding-Proteins-1-4 in Human Fallopian-Tube at Various Reproductive Stages. *Biology of Reproduction* **50** 281-289.

Pierson, RA 1993 Folliculogenesis and ovulation. . *In* VJL McKinnon A.O. (ed.), Equine reproduction. , pp. 151-161. Philadelphia: Lea and Febiger.

Pierson, RA, and OJ Ginther 1985 Ultrasonic evaluation of the corpus luteum of the mare. *Theriogenology* **23** 795-806.

Pope, WF, RR Maurer, and F Stormshak 1982 Distribution of progesterone in the uterus, broad ligament, and uterine arteries of beef cows. *The Anatomical Record* **203** 245-249.

Rajagopal, M, TL Tollner, WE Finkbeiner, GN Cherr, and JH Widdicombe 2006 Differentiated structure and function of primary cultures of monkey oviductal epithelium. *In Vitro Cellular & Developmental Biology-Animal* **42** 248-254.

Reischl, J, K Prelle, H Schol, C Neumuller, R Einspanier, F Sinowatz, and E Wolf 1999 Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro. *Cell and Tissue Research* **296** 371-383.

Reverchon, M, M Cornuau, C Rame, F Guerif, D Royere, and J Dupont 2012 Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells. *Human Reproduction* **27** 1790-1800.

Richardson, LL, and G Oliphant 1981 Steroid Concentrations in Rabbit Oviducal Fluid during Estrus and Pseudopregnancy. *Journal of Reproduction and Fertility* **62** 427-431.

Rodgers, WH, LM Matrisian, LC Giudice, B Dsupin, P Cannon, C Svitek, F Gorstein, and KG Osteen 1994 Patterns of Matrix Metalloproteinase Expression in Cycling Endometrium Imply Differential Functions and Regulation by Steroid-Hormones. *Journal of Clinical Investigation* **94** 946-953.

Roldan-Olarte, M, M Jimenez-Diaz, and DC Miceli 2005 Plasminogen detection in oocytes and plasminogen activator activities in the porcine oviduct during the estrous cycle. *Zygote* **13** 115-123.

Rose, IA, and JVB Warms 1966 Control of Glycolysis in Human Red Blood Cell. *Journal of Biological Chemistry* **241** 4848-&.

Rottmayer, R, SE Ulbrich, S Kolle, K Prelle, C Neumueller, F Sinowatz, HHD Meyer, E Wolf, and S Hiendleder 2006 A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* **132** 637-648.

Ruckebusch, Y, and F Bayard 1975 Motility of Oviduct and Uterus of Cow during Estrous-Cycle. *Journal of Reproduction and Fertility* **43** 23-32.

Schell, DL, PA Mavrogianis, AT Fazleabas, and HG Verhage 1994 Epidermal Growth-Factor, Transforming Growth-Factor-Alpha, and Epidermal Growth-Factor Receptor Localization in the Baboon (Papio-Anubis) Oviduct during Steroid Treatment and the Menstrual-Cycle. *Journal of the Society for Gynecologic Investigation* **1** 269-276.

Schmidt, A, R Einspanier, W Amselgruber, F Sinowatz, and D Schams 1994 Expression of Insulin-Like Growth-Factor-1 (Igf-1) in the Bovine Oviduct during the Estrous-Cycle. *Experimental and Clinical Endocrinology* **102** 364-369.

Schneider, CA, WS Rasband, and KW Eliceiri 2012 NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9** 671-675.

Schuetz, AW, and NH Dubin 1981 Progesterone and Prostaglandin Secretion by Ovulated Rat Cumulus Cell-Oocyte Complexes. *Endocrinology* **108** 457-463.

Seytanoglu, A, AS Georgiou, E Sostaric, PF Watson, WV Holt, and A Fazeli 2008 Oviductal cell proteome alterations during the reproductive cycle in pigs. *Journal of Proteome Research* **7** 2825-2833.

Shamsuddin, M, and H Rodriguezmartinez 1994 Fine-Structure of Bovine Blastocysts Developed Either in Serum-Free Medium or in Conventional Coculture with Oviduct Epithelial-Cells. *Journal of Veterinary Medicine Series a-Zentralblatt Fur Veterinarmedizin Reihe a-Physiology Pathology Clinical Medicine* **41** 307-316.

Shao, R, E Egecioglu, B Weijdegard, JJ Kopchick, J Fernandez-Rodriguez, N Andersson, and H Billig 2007a Dynamic regulation of estrogen receptor-alpha isoform expression in the mouse fallopian tube: mechanistic insight into estrogen-dependent production and secretion of insulin-like growth factors. *American Journal of Physiology-Endocrinology and Metabolism* **293** E1430-E1442.

Shao, RJ, K Ljungstrom, B Weijdegard, E Egecioglu, J Fernandez-Rodriguez, FP Zhang, A Thurin-Kjellberg, C Bergh, and H Billig 2007b Estrogen-induced upregulation of AR expression and enhancement of AR nuclear translocation in mouse fallopian tubes in vivo. *American Journal of Physiology-Endocrinology and Metabolism* **292** E604-E614.

Silva, ES, KE Scoggin, IF Canisso, MHT Troedsson, EL Squires, and BA Ball 2014 Expression of receptors for ovarian steroids and prostaglandin E2 in the endometrium and myometrium of mares during estrus, diestrus and early pregnancy. *Animal Reproduction Science* **151** 169-181.

Simard, J, ML Ricketts, B Gingras, P Soucy, FA Feltus, and MH Melner 2005 Molecular biology of the 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase gene family. *Endocrine Reviews* **26** 525-582.

Smith, OF, AA Ogunsola, AO Ladokun, and A Ajadi 2011 Synergistic effects of insulin-like growth factor ii (IGF-II) and leutinizing hormone (LH) on steroid hormone secretion by cultured bovine granulosa cells. *Indian Journal of Animal Sciences* **81** 1116-1120.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally

important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, KG, K.; Van Soom, A.; Govaere, J.; Hoogewijs, M.; Vanhaesebrouck, E.; Galli, C.; Colleoni, S.; Vandesompele, J.; Peelman, L. 2009 Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246.

Spilman, CH, and JW Wilks 1976 Peripheral Plasma Progesterone during Egg Transport in Rabbit. *Proceedings of the Society for Experimental Biology and Medicine* **151** 726-729.

Squires, EL, and OJ Ginther 1975 Collection Technique and Progesterone Concentration of Ovarian and Uterine Venous-Blood in Mares. *Journal of Animal Science* **40** 275-281.

Stadelmann, C, and H Lassmann 2000 Detection of apoptosis in tissue sections. *Cell and Tissue Research* **301** 19-31.

Staples, LD, IR Fleet, and RB Heap 1982 Anatomy of the Utero-Ovarian Lymphatic Network and the Composition of Afferent Lymph in Relation to the Establishment of Pregnancy in the Sheep and Goat. *Journal of Reproduction and Fertility* **64** 409-420.

Stefanczyk-Krzymowska, S, T Krzymowski, B Wasowska, and J Chlopek 2002 Retrograde transfer of ovarian steroid hormones to the ovary in the porcine periovarian vascular complex. *Experimental Physiology* **87** 361-371.

Stevenson, KR, and DC Wathes 1996 Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *Journal of Reproduction and Fertility* **108** 31-40.

Stocco, DM 2001 StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* **63** 193-213.

Stocco, DM, XJ Wang, Y Jo, and PR Manna 2005 Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: More complicated than we thought. *Molecular Endocrinology* **19** 2647-2659.

Stoklosowa, S, E Gregoraszczuk, and CP Channing 1982 Estrogen and Progesterone Secretion by Isolated Cultured Porcine Thecal and Granulosa-Cells. *Biology of Reproduction* **26** 943-952.

Suarez, S, K Redfern, P Raynor, F Martin, and DM Phillips 1991 Attachment of Boar Sperm to Mucosal Explants of Oviduct Invitro - Possible Role in Formation of a Sperm Reservoir. *Biology of Reproduction* **44** 998-1004.

Suarez, SS 2008 Regulation of sperm storage and movement in the mammalian oviduct. *International Journal of Developmental Biology* **52** 455-462.

Suarez, SS, and HC Ho 2003 Hyperactivated motility in sperm. *Reproduction in Domestic Animals* **38** 119-124.

Sukerkar, PA, KW MacRenaris, TR Townsend, RA Ahmed, JE Burdette, and TJ Meade 2011 Synthesis and Biological Evaluation of Water-Soluble Progesterone-Conjugated Probes for Magnetic Resonance Imaging of Hormone Related Cancers. *Bioconjugate Chemistry* **22** 2304-2316.

Swan, CL, MC Agostini, PM Bartlewski, V Feyles, RJ Urban, and PJ Chedrese 2002 Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: Control of transcriptional activity of the cytochrome P450 side-chain cleavage gene. *Biology of Reproduction* **66** 959-965.

Szafranska, B, A Ziecik, and S Okrasa 2002 Primary antisera against selected steroids or proteins and secondary antisera against gamma-globulins--an available tool for studies of reproductive processes. *Reproductive Biology* **2** 187-204.

Tadokoro, C, Y Yoshimoto, M Sakata, T Imai, M Yamaguchi, H Kurachi, Y Oka, T Maeda, and A Miyake 1995 Expression and Localization of Glucose-Transporter-1 (Glut1) in the Rat Oviduct - a Possible Supplier of Glucose to Embryo during Early Embryonic-Development. *Biochem Biophys Res Commun* **214** 1211-1218.

Takeda, T, Y Tsutsumi, S Hara, and M Ida 1978 Effects of Prostaglandin-F2-Alpha on Egg Transport and Invivo Egg Recovery from Vaginas of Rabbits. *Fertility and Sterility* **30** 79-85.

Tetsuka, M, M Milne, and SG Hillier 1998 Expression of oestrogen receptor isoforms in relation to enzymes of oestrogen synthesis in rat ovary. *Molecular and Cellular Endocrinology* **141** 29-35.

Teves, ME, F Barbano, HA Guidobaldi, R Sanchez, W Miska, and LC Giojalas 2006 Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertility and Sterility* **86** 745-749.

Thomas, PGA, BA Ball, and SP Brinsko 1994 Interaction of Equine Spermatozoa with Oviduct Epithelial-Cell Explants Is Affected by Estrous-Cycle and Anatomic Origin of Explant. *Biology of Reproduction* **51** 222-228.

Thompson, JG, NW Allen, LT McGowan, ACS Bell, MG Lambert, and HR Tervit 1998 Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. *Theriogenology* **49** 1239-1249.

Thompson, JG, DK Gardner, PA Pugh, WH Mcmillan, and HR Tervit 1995 Lamb Birth-Weight Is Affected by Culture System Utilized during in-Vitro Pre-Elongation Development of Ovine Embryos. *Biology of Reproduction* **53** 1385-1391.

Tibbetts, TA, M Mendoza-Meneses, BW O'Malley, and OM Conneely 1998 Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biology of Reproduction* **59** 1143-1152.

Ulbrich, SE, A Kettler, and R Einspanier 2003 Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. *The Journal of Steroid Biochemistry and Molecular Biology* **84** 279-289.

Ullrich, A, A Gray, AW Tam, T Yangfeng, M Tsubokawa, C Collins, W Henzel, T Lebon, S Kathuria, E Chen, S Jacobs, U Francke, J Ramachandran, and Y Fujitayamaguchi 1986 Insulin-Like Growth Factor-I Receptor Primary Structure - Comparison with Insulin-Receptor Suggests Structural Determinants That Define Functional Specificity. *Embo Journal* **5** 2503-2512.

Urban, RJ, JC Garmey, MA Shupnik, and JD Veldhuis 1991 Follicle-Stimulating-Hormone Increases Concentrations of Messenger-Ribonucleic-Acid Encoding Cytochrome-P450 Cholesterol Side-Chain Cleavage Enzyme in Primary Cultures of Porcine Granulosa-Cells. *Endocrinology* **128** 2000-2007.

Van den Broeck, W, M D'haeseleer, M Coryn, and P Simoens 2002 Cell-specific distribution of progesterone receptors in the bovine ovary. *Reproduction in Domestic Animals* **37** 164-170.

Vandesompele, J, K De Preter, F Pattyn, B Poppe, N Van Roy, A De Paepe, and F Speleman 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**.

Vanhaecke, L, JV Bussche, K Wille, K Bekaert, and HF De Brabander 2011 Ultra-high performance liquid chromatography-tandem mass spectrometry in high-throughput confirmation and quantification of 34 anabolic steroids in bovine muscle. *Analytica Chimica Acta* **700** 70-77.

Walter, I 1995 Culture of Bovine Oviduct Epithelial-Cells (Boec). *Anatomical Record* **243** 347-356.

Watson, AJ, LC Barcroft, DH Bets, PA DeSousa, E Gilfoyle, J Looye, J PierreLouis, and QA Winger 1996 Maternal and embryonic control of bovine pre-attachment development: Expression of oviductal and embryonic genes. *Archiv Fur Tierzucht-Archives of Animal Breeding* **39** 55-69.

Watson, ED, SB Skolnik, and HG Zanecosky 1992 Progesterone and Estrogen-Receptor Distribution in the Endometrium of the Mare. *Theriogenology* **38** 575-580.

Watson, ED, R Thomassen, M Steele, M Heald, R Leask, NP Groome, and SC Riley 2002 Concentrations of inhibin, progesterone and oestradiol in fluid from dominant and subordinate follicles from mares during spring transition and the breeding season. *Animal Reproduction Science* **74** 55-67.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991a Prostaglandin E2 hastens oviductal transport of equine embryos. *Biol Reprod* **45** 544-546.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991b Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45** 540-543.

Weems, CW, YS Weems, CN Lee, and DL Vincent 1989 Progesterone in uterine and arterial tissue and in jugular and uterine venous plasma of sheep. *Biology of Reproduction* **41** 1-6.

Wessel, T, U Schuchter, and H Walt 2004 Ciliary motility in bovine oviducts for sensing rapid non-genomic reactions upon exposure to progesterone. *Hormone and Metabolic Research* **36** 136-141.

West, NB, and RM Brenner 1985 Progesterone-Mediated Suppression of Estradiol Receptors in Cynomolgus Macaque Cervix, Endometrium and Oviduct during Sequential Estradiol-Progesterone Treatment. *Journal of Steroid Biochemistry and Molecular Biology* **22** 29-37.

West, NB, HG Verhage, and RM Brenner 1977 Changes in Nuclear Estradiol Receptor and Cell Structure during Estrous Cycles and Pregnancy in Oviduct and Uterus of Cats. *Biology of Reproduction* **17** 138-143.

Wijayagunawardane, MPB, WA Cerbito, A Miyamoto, TJ Acosta, M Takagi, K Miyazawa, and K Sato 1996 Oviductal progesterone concentration and its spatial distribution in cyclic and early pregnant cows. *Theriogenology* **46** 1149-1158.

Wijayagunawardane, MPB, YH Choi, A Miyamoto, H Kamishita, S Fujimoto, M Takagi, and K Sato 1999 Effect of ovarian steroids and oxytocin on the production of

prostaglandin E-2, prostaglandin F-2 alpha and endothelin-1 from cow oviductal epithelial cell monolayers in vitro. *Animal Reproduction Science* **56** 11-17.

Wijayagunawardane, MPB, A Miyamoto, WA Cerbito, TJ Acosta, M Takagi, and K Sato 1998 Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* **49** 607-618.

Wildt, L, S Kissler, P Licht, and W Becker 1998 Sperm transport in the human female genital tract and its modulation by oxytocin as assessed by hysterosalpingoscintigraphy, hysterotonography, electrohysterography and Doppler sonography. *Human Reproduction Update* **4** 655-666.

Wilsher, S, S Gower, and WR Allen 2011 Immunohistochemical localisation of progesterone and oestrogen receptors at the placental interface in mares during early pregnancy. *Animal Reproduction Science* **129** 200-208.

Wollenhaupt, K, A Kettler, Br, K-P ssow, F Schneider, W Kanitz, and R Einspanier 2001 Regulation of the expression and bioactivation of the epidermal growth factor receptor system by estradiol in pig oviduct and endometrium. *Reproduction, Fertility and Development* **13** 167-176.

Wollenhaupt, K, U Tiemann, R Einspanier, F Schneider, W Kanitz, and KP Brussow 1997 Characterization of the epidermal growth factor receptor in pig oviduct and endometrium. *Journal of Reproduction and Fertility* **111** 173-181.

Wydooghe, E, S Heras, J Dewulf, S Piepers, E Van den Abbeel, P De Sutter, L Vandaele, and A Van Soom 2014 Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reprod Fertil Dev* **26** 717-724.

Yamada, M, T Horiuchi, T Oribe, S Yamamoto, H Matsushita, and PA Gentry 1996 Plasminogen activator activity in the bovine oocyte-cumulus complex and early embryo. *Journal of Veterinary Medical Science* **58** 317-322.

Yaniz, JL, M Lopez-Bejar, P Santolaria, J Rutllant, and F Lopez-Gatius 2002 Intraperitoneal insemination in mammals: A review. *Reproduction in Domestic Animals* **37** 75-80.

Zhao, Y, N Chegini, and KC Flanders 1994 Human Fallopian-Tube Expresses Transforming Growth-Factor (Tgf-Beta) Isoforms, Tgf-Beta Type I-Iii Receptor Messenger-Ribonucleic-Acid and Protein, and Contains [I-125] Tgf-Beta-Binding Sites. *Journal of Clinical Endocrinology & Metabolism* **79** 1177-1184.

Zhao, YG, AZ Xiao, XM Cao, and C Zhu 2002 Expression of matrix metalloproteinase-2,-9 and tissue inhibitors of metalloproteinase-1,-2,-3 mRNAs in rat uterus during early pregnancy. *Molecular Reproduction and Development* **62** 149-158.

CHAPTER 5 OVIDUCTAL FUNCTION BEARS THE STAMP OF STEROIDS AND EMBRYOS *IN VITRO*

The science of today
is the technology of tomorrow.

Edward Teller, physicist

PART 5.1: OF STEROIDS AND GENES IN OVIDUCTAL EXPLANTS *IN VITRO*

Adapted from:

Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and immunoreactive steroidogenic protein expression in equine oviduct explants *in vitro*.

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STEROIDS AFFECT GENE EXPRESSION, CILIARY ACTIVITY, GLUCOSE UPTAKE, PROGESTERONE RECEPTOR EXPRESSION AND IMMUNOREACTIVE STEROIDOGENIC PROTEIN EXPRESSION IN EQUINE OVIDUCT EXPLANTS *IN VITRO*.

ABSTRACT

The oviduct undergoes dramatic functional and morphological changes throughout the estrous cycle of the mare. To unravel the effects of steroids on the morphology, functionality and gene expression of the equine oviduct, an *in vitro* oviduct explant culture system was stimulated with physiological concentrations of progesterone and 17β-oestradiol. Four conditions were compared: unsupplemented preovulatory explants, preovulatory explants which were stimulated with postovulatory hormone concentrations, unsupplemented postovulatory explants and postovulatory explants which were stimulated with preovulatory hormone concentrations. The modulating effects of both steroids on oviduct explants at different levels were investigated: 1) ciliary activity, 2) glucose consumption and lactate production pattern, 3) ultrastructure, 4) mRNA expression of embryotrophic genes, 5) steroidogenic capacities of oviductal explants, and 6) progesterone receptor expression.

The present paper shows that the equine oviduct is an organ with potential steroidogenic capacities which is highly responsive to local changes in progesterone and 17β-oestradiol concentrations at the level of morphology, functionality and gene expression of the oviduct. These data provide a basis to study the importance of endocrine and paracrine signalling during early embryonic development in the horse.

INTRODUCTION

The reproductive cycle in mammals consists of two stages: the preovulatory follicular and the postovulatory luteal stage. These stages are induced by fluctuations of steroid hormones, mainly progesterone and 17β-oestradiol, before and after ovulation (Seytanoglu, et al. 2008). Ovarian steroids induce morphological, biochemical and physiological changes to the oviduct cells and subsequently affect the volume and composition of the oviductal fluid (Georgiou, et al. 2005, Hunter 2012b, Seytanoglu, et al. 2008) and ciliary activity (Bylander, et al. 2010, Wessel, et al. 2004). These changes play a key role in the optimization of the

microenvironment for final maturation and transport of the gametes, for fertilization and for nourishment, transport and growth of the early embryo (Fazeli 2008, Hunter 2005).

Important local paracrine embryo-maternal interactions are difficult to capture in *in vivo* studies because the equine oviduct can only be reached surgically or *post mortem*; moreover, the exact location of the embryo is hard to pinpoint. Therefore, an *in vitro* model which approximates the *in vivo* situation as closely as possible provides the ideal basis to study the oviductal response to steroids, gametes or embryos. In this study an oviduct epithelial explant culture model, on which has been reported earlier (Nelis, et al. 2014), was further optimized by unravelling its response to physiological concentrations of progesterone and oestradiol. It has been demonstrated that *in vivo* concentrations of progesterone can be very high in the equine oviductal tissue and fluid ipsilateral to the ovulation side (Nelis et al., 2015). This phenomenon has also been described in the bovine oviduct (Wijayagunawardane, et al. 1998). Apart from the endocrine hormones influencing the oviduct, the oviduct itself appears to have potential steroidogenic properties. Immunoreactive steroidogenic enzymes, all of which have catalytic properties in steroidogenesis, are abundantly present in the mare's oviduct (Nelis, et al. 2015a), hallmarking the steroidogenic capacity of the oviduct and the importance of local steroids for reproductive events taking place during the oviductal stage of equine embryo development.

In vitro culture of oviduct epithelial explants has been developed in several species such as horses (Ball and Altschul 1990, Nelis, et al. 2014, Thomas, et al. 1994), cattle (Rottmayer, et al. 2006, Walter 1995), pigs (Buhi, et al. 1989, Miessen, et al. 2011, Suarez, et al. 1991), humans (Kervancioglu, et al. 1997, Levanon, et al. 2010) and monkeys (Rajagopal, et al. 2006). However, little information exists on how steroid hormone concentrations influence morphology, function and gene expression in the oviduct. Only in the cow (Rottmayer, et al. 2006) and the sow (Chen, et al. 2013a) the effect of hormonal changes on oviduct epithelial cells has been studied during *in vitro* culture. In the pig, 17β-oestradiol and progesterone affect cellular polarity, transformation of ciliated and secretory cells, as well as electrical conductivity of the oviduct epithelium and expression of hormone receptors and oviductal glycoprotein (Chen, et al. 2013a). In bovine oviductal cells, 17β-oestradiol increases progesterone receptor mRNA expression (Rottmayer, et al. 2006). In the horse, only two studies compared the mRNA expression in oviductal tissue from mares in oestrus and post-ovulation. No influence of the oestrous cycle on prostaglandin E2 receptors was found (Ball,

et al. 2013). Another study reported that mRNA of μ-opioid receptors were mainly upregulated during oestrus (Desantis, et al. 2010). In addition, equine embryos apparently benefit from high glucose concentrations (17 mM) during early development (Choi, et al. 2004a, Hinrichs 2010, Smits, et al. 2011). However, no data is available concerning the glucose consumption in oviduct explants in response to steroids. Moreover, since in this article postovulatory oviduct cells turned out to produce considerable higher amounts of progesterone compared to preovulatory explants, the expression of enzymes involved in the progesterone synthesis pathway may be steroid regulated. It has been suggested earlier that immunoreactive protein expression of steroidogenic enzymes in the equine oviduct is cycle-stage dependent (Nelis, et al. 2015a). However, no literature data is available about the direct regulation of steroidogenic immunoreactive proteins in the equine oviduct by progesterone and 17β-oestradiol.

The aim of the present study was to induce cycle stage-related alterations *in vitro* to verify whether the *in vivo* situation could be mimicked in oviduct explants in response to steroids. For that purpose, preovulatory explants were stimulated with hormone concentrations as they prevail in the postovulatory stage and vice versa. The influence of these steroid hormones on ciliary activity, and the effect on glucose consumption and lactate production as well as the ultrastructure, the mRNA expression of a set of embryotrophic genes, the steroidogenic capacities and the progesterone receptor expression in the equine oviduct explants were assessed **(Fig. 1)**.

MATERIALS AND METHODS

Animals

Oviducts were collected during the breeding season (March - August) in a local slaughterhouse (Euro Meat Group, Moeskroen, Belgium) from healthy mares with a preovulatory follicle and distinct uterine oedema, indicating oestradiol dominance (late follicular phase) or a recent corpus luteum/hemorrhagicum and without uterine oedema, indicating recent ovulation (early luteal phase) (Pierson and Ginther 1985). Determination of progesterone concentration in oviductal tissue by U-HPLC-MS/MS revealed a positive predicting value of 94% between the presence of a recent corpus luteum/hemorrhagicum and a serum progesterone concentration lower than 2 ng/ml (Nelis, et al. 2015a). This association

was confirmed earlier (Ginther, et al. 2007, Pierson and Ginther 1985). Mares with ovaries showing signs of atretic or anovulatory follicles (Ginther 1992, McCue and Squires 2002, McKinnon 1997, Pierson 1993) were not included.

Culture and hormone supplementation of oviduct explants

Preparation and selection of oviduct explants

Oviduct epithelial explants were obtained as described earlier (Nelis, et al. 2014). Briefly, the harvested cellular material, obtained by scraping the ampulla and the ampullary-isthmic junction of ipsilateral oviducts, was washed and cultured in DMEM/F12 (Invitrogen, Merelbeke, Belgium) supplemented with 10% FCS (Fetal calf serum, Invitrogen, Merelbeke, Belgium), 50 µg/ml gentamycin (Invitrogen, Merelbeke, Belgium) and 2.5 µg/ml amphotericin-B (Fungizone, Invitrogen, Merelbeke, Belgium). One µl of explants suspension, coinciding with 30-35 oviduct explants with a diameter of less than 200 µm, was cultured in 50 µl under mineral oil or 10 µl in 500 µl without oil overlay at 38.5° C in a humidified atmosphere with 5% CO_2 in air (Nelis et al. 2014). Viability was judged at the start and after culture by Trypan blue staining (Sigma-Aldrich, Diegem, Belgium) and by evaluating ciliary activity at the start and at the end of culture **(Fig. 1)**. Only oviduct explants with more than 98% of membrane intact cells, determined by Trypan blue staining, 2 to 4 hours after start (= day 0) of culture were used.

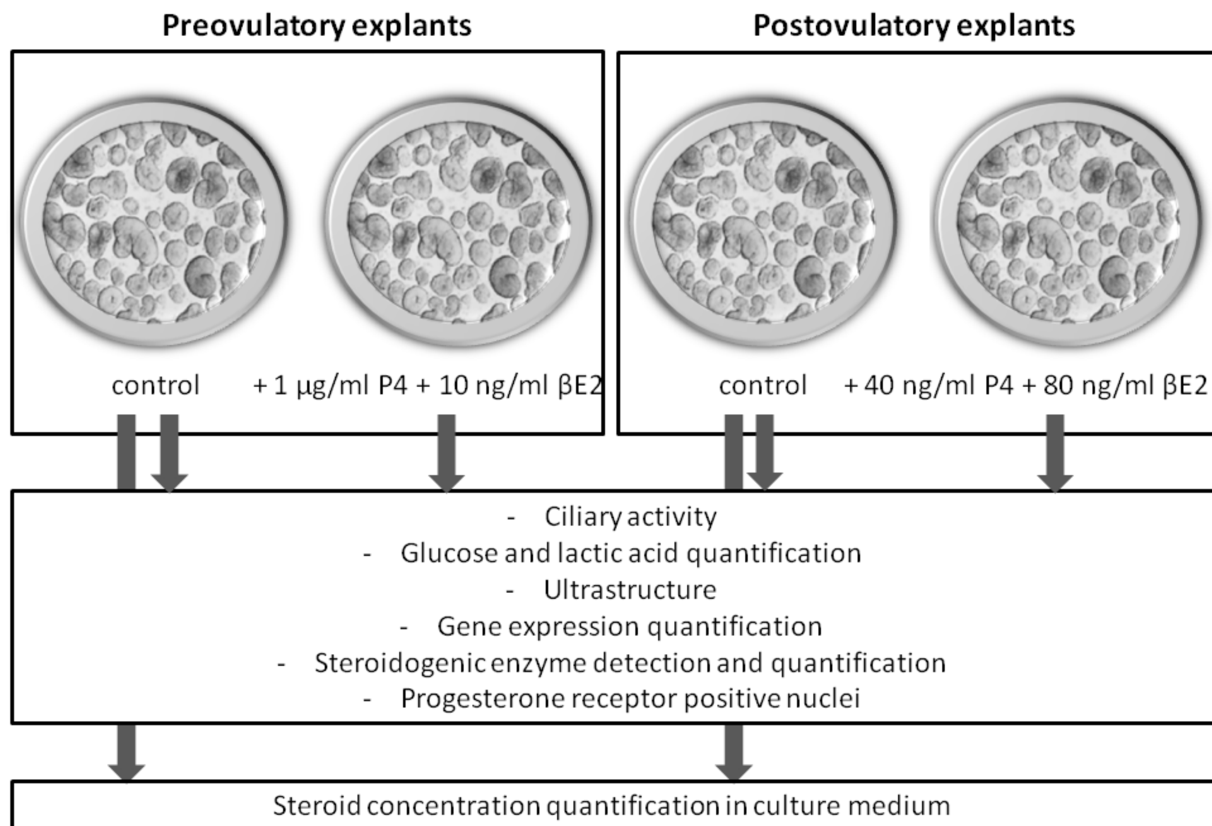

Fig. 1. Experimental set-up. Oviductal explants originating from mares in the preovulatory or the postovulatory cycle stage were cultured in DMEM/F12 supplemented with 10% fetal calf serum. The pre- and postovulatory explants were cultured supplemented with respectively 1 µg/ml or 40 ng/ml cell-culture tested water soluble progesterone and 10 ng/ml or 80 ng/ml cell culture tested water soluble 17β-estradiol. Control = no hormone supplementation, hormone = hormone supplementation. After 3 days of culture, ciliary activity, glucose consumption and lactic acid production, the ultrastructure, gene expression, steroidogenic enzyme expression and the number of progesterone receptor positive nuclei were assessed.

Hormone supplementation

To gain insights in the hormone responsiveness of equine oviduct epithelial cells, the *in vivo* situation was mimicked as closely as possible *in vitro* by adding 17β-oestradiol and progesterone concentrations as those measured in equine ipsilateral oviductal tissue with reference to cycle stage. Mean concentrations of progesterone in oviductal tissue *in vivo* were 48 ± 18.6 ng/g in the preovulatory stage and 1098 ± 296.5 ng/g in the postovulatory stage. Mean preovulatory concentrations of 17β-oestradiol were 81.3 ± 21.2 ng/g and postovulatory concentrations were 12.7 ± 5.9 ng/g (Nelis, et al. 2015a). Based upon these *in vivo* concentrations and assuming a density of 1000 kg/m^3 for oviductal tissue, the *in vitro* culture

medium was supplemented with physiological concentrations of steroids and the explants were cultured for 3 days. The explants taken at the preovulatory cycle stage were cultured with 1 µg/m1 cell-culture tested water soluble progesterone (progesterone: 2-hydroxypropyl-β-cyclodextrin complex, P7556, Sigma-Aldrich BVBA, Diegem, Belgium), and 10 ng/ml cell culture tested water soluble 17β-oestradiol (cyclodextrin-encapsulated 17β-oestradiol, E4389, Sigma-Aldrich BVBA, Diegem, Belgium), while the explants taken at the postovulatory stage were cultured with 40 ng/ml soluble progesterone and 80 ng/ml soluble 17β-oestradiol **(Fig. 1)**. These water soluble molecules were validated and proven to cross cell membranes and activate oestrogen (Kim, et al. 2010) and progesterone (Sukerkar, et al. 2011) receptors *in vitro*. In each group, a control group without hormone supplementation was included.

Ultrastructural and functional effects of steroid supplementation on equine oviduct explants *in vitro*

Evaluation of ciliary activity

Ciliary activity was evaluated in the whole number of explants present in 3 replicates of at least 20 droplets, containing 30-35 explants each. Explants were considered showing ciliary activity when bordered by vigorously beating cilia, clearly seen on the inverted microscope (400× magnification). To lower interpretative bias in the assessment of ciliary activity, culture plate labels were blinded and all explants were counted twice and the mean value was used for statistical analysis. To compare ciliary activity, binary logistic regression was implemented. Differences were considered significant at $P < 0.05$. Statistical analysis and graph plotting was performed with SPSS 21 for windows (SPSS IBM, Brussels, Belgium).

Quantification of glucose consumption and lactic acid production

At day 0 and day 3 of culture of preovulatory and postovulatory oviduct explants with or without hormone supplementation, 10 samples (from 3 replicates) of 250 µl medium were taken and frozen at -80 °C until analysis. The samples were analysed (Medic Lab/Zoolyx, Aalst, Belgium) with a UV enzymatic method using the Roche Cobas 8000 according to the manufacturers recommendations (Roche Diagnostics, Mannheim, Germany). A calibration line to determine the efficiency was drawn up along with 10 samples per day of sampling. The

efficiency of the UV enzymatic analyses was 98% for glucose and 99% for lactic acid. The limit of detection was 2 mg/dl for glucose and 1.8 mg/dl for lactic acid. To detect statistically significant differences, repeated measures analysis of variance (ANOVA) with Greenhouse-Geisser correction with the general linear model procedure as implemented in SPSS 19 was performed.

Transmission electron microscopy

After 3 days of culture, the oviduct explants in the pre- and postovulatory cycle stage (from 7 mares in each group) with and without steroid supplementation as described above, were pooled per group and fixed in 0.2 M sodium cacodylate buffered formaldehyde and post-fixed with osmium tetroxide. After rinsing, cells were pelleted by centrifugation in 10% BSA supplemented with 1% glutaraldehyde. The pellet was then dehydrated and embedded in epoxy resin (LX-112 Ladd Industries, Williston, ND, USA). Sections were made with a Reichert Jung Ultracut E ultra-microtome (Depew, NY, USA). Semi-thin sections (2 µm) were stained with toluidine blue to select the most appropriate regions for ultrathin sectioning. Next, ultra-thin sections (90 nm) were made and stained with uranyl acetate and lead citrate solutions before examining under a Jeol EX II transmission electron microscope (Jeol Europe, Zaventem, Belgium) at 80 kV.

Gene expression

Experimental set-up

In a first experiment, the mRNA expression of 11 genes, plasminogen activator inhibitor 1 (*PAI1*), urokinase plasminogen activator inhibitor (*PLAU*), transforming growth factor α (*TGFA*), tissue inhibitor of matrix metalloproteinase-1 (*TIMP1*), matrix metalloproteinase (*MMP*2), colony stimulating factor (*CSF1*), prostaglandin receptor E2 (*PTGER2*) and E4 (*PTGER4*), insulin-like growth factor-1 (*IGF1*), vascular endothelial growth factor (*VEGFA*) and glucose transporter 1 (*GLUT1*) was evaluated in pre- and postovulatory oviduct explants without hormone supplementation to elucidate hormone dependency. These genes were selected based on their embryotrophic function and their putative steroid responsiveness (Buhi, et al. 2000).

In a second experiment, the influence of hormone supplementation on the expression of the genes that were found to be significantly up- or downregulated in the first experiment (*TGFA, MMP2, CSF1, PAI1, GLUT1, PLAU*) was further examined by comparing the expression in preovulatory and postovulatory oviduct explants cultured with and without hormone supplementation.

RNA extraction and quantitative RT-PCR

After 3 days of culture, ciliary activity was evaluated, the explants were washed 2 times in DPBS (Gibco Invitrogen, Ghent, Belgium), subsequently snap-frozen in lysis buffer and stored in liquid nitrogen in cryovials. Lysis buffer constituted of 10% RNasin Plus RNase inhibitor (Promega, The Netherlands), 5% dithiothreitol (Promega, The Netherlands), 0.8% Igepal CA-630 (Sigma, Belgium) in RNase free water (Qiagen, Venlo, The Netherlands). Total RNA was extracted with the RNeasy kit (Qiagen) according to the manufacturers' instructions, including DNAse digestion, as described earlier (Nelis, et al. 2014, Smits, et al. 2011). Primer design and validation, RNA extraction and quantitative RT-PCR were performed according to the MIQE-guidelines (Bustin, et al. 2009) and as also reported earlier (Nelis, et al. 2014). The primers (Integrated DNA Technologies, Leuven, Belgium and Sigma-Aldrich, Bornem, Belgium) for the genes of interest (*PAI1, PLAU, TGFA, TIMP1, MMP2, CSF1, PTGER2, PTGER4, VEGFA, IGF1, GLUT1*) are summarized in **Table 2.** For normalization, eight candidate reference genes **(Table 1)** were evaluated based on previous studies (Bogaert, et al. 2006, Cappelli, et al. 2008, Goossens, et al. 2007, Smits 2009). The most stable reference genes were selected with GeNorm (Vandesompele, et al. 2002).

An optimal set of reference genes: UBB, ACTB and RPL32

Quantitative real-time PCR results of pre- and postovulatory oviduct explants were normalized against the geometric mean of the expression of an optimal number of reference genes as determined with geNorm (Vandesompele, et al. 2002). When taking only postovulatory explants into account, the most stable reference genes were *ACTB, UBB, 18S, RPL32, SDHA* and *HPRT*. For preovulatory explants, *ACTB, RPL32, UBB, SDHA* and *HPRT* were the most stable reference genes. Taking into account both preovulatory and postovulatory explants, *ACTB, UBB* and *RPL32* turned out to be the most reliable reference

genes. Therefore, the geometric mean of *ACTB, UBB* and *RPL32* was used for normalization of the test genes.

Table 1. Primers for RT-qPCR of the reference genes. For each reference gene, the NCBI GenBank accession number, the sequence of both forward and reverse primer, the size of the amplicon, the optimal primer annealing temperature and amplification efficiency are listed.

Gene	GenBank accession number	Primer sequence (5'-3')	Amplicon size (bp)	Ta (°C)	Efficiency (%)
ACTB	AF035774	CCAGCACGATGAAGATCAAG GTGGACAATGAGGCCAGAAT	88	60	101
GAPDH	AF157626	CAGAACATCATCCCTGCTTC ATGCCTGCTTCACCACCTTC	187	59	97
HPRT1	AY372182	GGCAAAACAATGCAAACCTT CAAGGGCATATCCTACGACAA	163	57	93
RPL32	XM_008531004	AGCCATCTACTCGGCGTCA TCCAATGCCTCTGGGTTTC	149	60	89
SDHA	XM_001490889	TCCATCGCATAAGAGCAAAG GGTGGAACTGAACGAACTCC	159	59	99
TUBA4A	XM_001491910	GCCCTACAACTCCATCCTGA ATGGCTTCATTGTCCACCA	78	60	104
UBC	AF506969	GCAAGACCATCACCCTGGA CTAACAGCCACCCCTGAGAC	206	60	100
18 S	AJ311673	GACCATAAACGATGCCGACT TCTGTCAATCCTGTCCGTGT	219	60	92

Table 2. Primers for RT-qPCR of the target genes. For each gene, the NCBI GenBank accession number, the sequence of both forward and reverse primer, the size of the amplicon and the optimal primer annealing temperature and amplification efficiency are listed.

Gene	GenBank accession number	Primer sequence (5'-3')	Amplicon size (bp)	Ta (°C)	Efficiency (%)
PLAU	XM_001502951.4	AAAGTCCCTCCTCTCCTC CGAAGAAGGAGGACTACATT	249	61	92
VEGFA	NM_001081821	ACTGCCGTCCAATCGAGA ATCAAACCTCACCAAAGCCA	193	61	97
GLUT1	NM_001163971.1	CCAGAAGGTGATCGAGGAAT CAGTTTTGAGAAGCCCATGA	238	57	118
PAI1	XM_001492517	ACTCGGAAGCAGATCCAAGA CAGGTGGACTTTTCAGAGGTG	223	61	87
CSF1	XM_005610548	TTCGGTTTGTAGACCCAGAC TGCTCTTCATAGTCCTTGGTG	193	56	108
IGF1	NM_001082498	TTCTACCTGGCCCTGTGCT CTGTCTCCACACACGAACTGA	108	59.5	103
TGFA	XM_005599909	GATTCCCACAGTCAGTTCTGC ACATGCGATGATGAGGACAG	210	60	105
MMP2	XM_001493281	AGGGCACATCCTACGACAG AGATGTGGTGCGCGACTA	240	61	90.9
PTGER2	NM_001127352.1	GGACCACCTCATCCTCCTG CGGCCTAAAGATGGCAAAG	181	60	94
PTGER4	XM_001499068.3	CATCTTACTCATCCCACCT GAGGCATTTGATCTTCTCTATCG	229	60	103
TIMP1	NM_001082515	AGAAGTCAACCAGACCACCTTAC ATACTTCCACAGGTCGGAGA	152	61	96

Statistical analysis

An independent samples t-tests with bootstrapping or an independent samples Mann-Whitney U-test were used to compare target gene expression differences, depending on whether or not a Gaussian distribution was obtained after logarithmic transformation of the data. Differences

were considered significant at $P < 0.05$. To elucidate relationships between the expression of genes, Spearman's rho or Pearson correlation coefficients were calculated and linear and polynomial/curvilinear regression model fit was applied. Statistical analysis and graph plotting was performed with SPSS 21. Power analysis and sample size calculation ($\alpha = 0.05$; power = 0.85 - 0.99) for gene expression studies were performed using Piface version 1.7 (Lenth 2007); (University of Iowa; http://homepage.stat.uiowa.edu/_rlenth/Power/; accessed 28 March 2013).

Steroid quantification by means of RIA

After 3 days of culture without hormone supplementation, culture medium from the preovulatory group, culture medium from the postovulatory group and control medium, cultured without explants, was pooled per group and concentrated using a Centrivap Cold Trap (Labconco, Kansas City, MO, USA). The three pools each contained culture medium from 7 replicates. After reduction to dryness, the pools were dissolved in 2 ml PBS. Due to the limitations in availability of oviducts of healthy mares in the optimal cycle stage and in order to exceed the limits of detection, only one pool per group was created and measured.
Concentrations of 17β-oestradiol, progesterone, oestrone and testosterone were determined with the radioimmunoassay method described (Ciereszko 1999) and validated earlier (Franczak and Bogacki 2009). The cross reactivities of the antisera against steroids have been previously reported (Szafranska, et al. 2002). The efficiency of extraction and the coefficient of correlation between the added and recovered amount of different hormone concentrations for oestradiol, progesterone, oestrone and testosterone were 85.10% and 0.988, 85.60% and 0.989, 86.40% and 0.978, and 85.10% and 0.992, respectively. The assay sensitivity for oestradiol and testosterone was 0.5 pg/ml, for progesterone and oestrone 1 pg/ml. All samples were measured within one assay per one hormone. The intra-assay coefficient of variation for 17β-oestradiol was 1.17%, for progesterone 1.08%, for oestrone 0.45%, and for testosterone 0.69%.

Epifluorescence of aromatase, cytochrome P450scc and StAR

Oviductal epithelium explants were snap-frozen and stored in liquid nitrogen. Expression of aromatase (aromatase), steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (cytochrome P450scc) protein in oviductal epithelium explants was

determined using indirect immunofluorescent staining as described earlier (Franczak, et al. 2013). In short, explants were immobilized on Menzel-Gläser SuperFrost® Plus microscope slides (Thermo Scientific, Braunshweig, Germany). Approximately 30 minutes before staining, slides were transferred to room temperature, washed with PBS, dried, and then incubated with goat serum for 1 hour to prevent nonspecific binding of antibodies. After washing with PBS and drying, samples were incubated overnight (20 hours) at 4 °C with primary antibodies at the concentration of 1 µg/mL - rabbit anti-human cytochrome aromatase (A7981), StAR (HPA027318) and cytochrome P450scc (HPA016436) polyclonal antibodies (Sigma Aldrich, St. Louis, MO, USA). The specificity of antibodies was initially evaluated *in silico* using Protein BLAST and confirmed in optimization experiments. The similarities of detectable immunogens with equine immunogens for anti-aromatase, anti- StAR and anti-cytochrome P450scc antibodies were 75%, 95% and 80%, respectively. For each staining, a negative control with dilution buffer used instead of primary antibody, was performed to ensure that visible signal is antigen-specific. Positive tissue control samples were both equine corpus luteum and follicular wall. The next day, after washing with PBS and drying, samples were incubated for 1 hour at room temperature (approximately 21 °C) with a secondary antibody at the concentration of 1.25 µg/mL (goat anti-rabbit IgG conjugated with biotin; Merck-Millipore, Darmstadt, Germany). Subsequently, samples were washed with PBS, dried, and incubated with Cy3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) to visualize the antigen–antibody complex. Preparations were mounted with use of Fluoroshield with 40,6-diamidino-2-phenylindole (Sigma Aldrich) to counterstain DNA and subsequently examined using an epifluorescent microscope (Olympus BX51). Pictures were taken at 500× magnification with Cell ˇF software version 3.4 (Olympus). The intensity of fluorescent emission was evaluated after the conversion of the photographs to grayscale by measuring the average grey intensity using Cell ˇF software. The grey intensity was measured in 12 regions of interests for each biological replicate, averaged and subjected to One-way ANOVA statistical analysis. To compare staining intensities, one-way ANOVA with post-hoc LSD test was applied. Statistical analysis was performed with SPSS 21 and graph plotting by means of Excel 2010. Sample size calculation ($\alpha = 0.05$; power = 0.85–0.99) was performed using G*Power 3.1.3 (Faul, et al. 2007).

Immunohistochemistry of progesterone receptors and 3-beta-HSD

Staining for progesterone receptors (PR) and 3β-hydroxysteroid dehydrogenase/$\Delta5 \rightarrow 4$-isomerase (3-beta-HSD) was performed as described previously (De Bosschere, et al. 2002, Van den Broeck, et al. 2002). Briefly, for PR, the oviductal tissue samples were fixed for 24 h in 10% phosphate-buffered formaldehyde (pH 6.7) at room temperature, embedded in methylcellulose (Methocel, Sigma Aldrich) and dehydrated with increasing concentrations of ethanol. Subsequently, the tissue samples were embedded in paraffin wax and sectioned at 5 µm (Microm HM360, Prosan, Merelbeke, Belgium). The sections were mounted on 3-aminopropyl-triethoxysilane-coated slides (APES, Sigma, St. Louis, MO, USA) and dried overnight at 37 °C. Next, they were dewaxed with xylene and dehydrated using decreasing concentrations of ethanol before being submitted to heat-mediated antigen retrieval in 1:10 citrate-based solution (pH 6; Antigen Retrieval Citra solution, Biogenex, Fremont, USA). After quenching of endogenous peroxidases with 3% H_2O_2, blocking was performed using 20% bovine serum. Next, the sections were incubated in a wet box for 1 h at room temperature while covered with 75 µl of a 1/100 dilution of the primary mouse anti-human PR antibody (MA5-12642, clone hPRa2, Thermo scientific inc., Perbio Science BVBA, Aalst, Belgium) in 2% BSA (Sigma-Aldrich, Diegem, Belgium); this antibody was reported by the manufacturer to cross-react with the equine progesterone receptor. Rabbit anti-mouse gamma globulin conjugated to biotin (DAKO, Prosan, Merelbeke, Belgium) was used at a dilution of 1/500 as the secondary antibody and peroxidase-conjugated streptavidin (1/3000) was employed as the enzyme label. A 6 min incubation with 3,3'-diaminobenzidine hydrochloride (DAB) chromogen substrate (Liquid DAB+, DAKO, Prosan, Merelbeke, Belgium) resulted in a brown staining of the nucleus. The sections were counterstained with hematoxylin and subsequently dehydrated with increasing concentrations of ethanol and, finally, with xylene. Positive and negative controls were included in every staining procedure (Burry 2011). Positive controls were equine endometrium known to be positive for PR, while similar tissue sections were incubated with dilution buffer instead of the primary antibody, then biotinylated secondary antibody, or the peroxidase-conjugated streptavidin served as negative controls. Equine connective tissue of the oviductal tunica serosa served as negative control tissue. Other sections were also incubated with DAB alone to exclude the possibility of residual endogenous peroxidase activity. At least 20 sections of each group in each of 3 replicates were stained.

3-Beta-HSD staining was performed similarly. Rabbit anti-human 3-beta-HSD (Ab 154385, Abcam, Cambridge, UK) was known to cross-react with equine tissue and was used as the primary antibody. This cross reactivity was confirmed by subjecting the equine HSD3B sequence (gi|126352310) and the human HSD3B2 sequence (gi|112770) to NCBI protein BLAST analysis (last accessed: July 2013; http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&BLAST_SPEC=blast2seq&QUERY=&SUBJECTS=). This revealed a cover query of 99% and 75% identities. Goat anti-rabbit gamma globulin conjugated to biotin (DAKO, Prosan, Merelbeke, Belgium) was used as the secondary antibody. The staining protocol, including negative controls, number of sections, antibody and reagent concentrations, was identical to the PR staining protocol as mentioned above. Preliminary, primary antibody concentrations of 1/50, 1/100 and 1/500 were evaluated. Equine corpus luteum was used as the positive control tissue. Ovarian stroma was included as negative control tissue. To quantify progesterone receptor (PR)- positive cells, the ratio of epithelial PR-negative cells (purple) / PR-positive nuclei (brown) was determined in each sample by means of Image J software (Maryland, USA; (Schneider, et al. 2012). All the cells at 200x magnification in at least 20 sections in each replicate of each group were evaluated (Bologna-Molina, et al. 2011). To determine significant differences in the percentages of PR- positive cells, binary logistic regression with the Nagelkerke pseudo R^2 and χ^2 fit tests was implemented. To quantify 3β-hydroxysteroid dehydrogenase/Δ5→4-isomerase immunoreactive protein, chromogen intensity via a reciprocal intensity approach was implemented (Nguyen, et al. 2013). At least 10 similar regions of interest for each section were analyzed.

RESULTS

Ultrastructure and functional responses of oviduct explants to steroids *in vitro*

Ciliary activity

Ciliary activity was lower in the postovulatory compared with the preovulatory unsupplemented explants (P = 0.002) **(Fig. 2)**. Supplementation of oviduct explants originating from mares in the preovulatory cycle stage with 1 µg/ml of progesterone and 10 ng/ml of 17β-oestradiol decreased ciliary activity after three days of culture from 91% in the control group to 56% in the hormone treated group (P < 0.001). Supplementation with 40 ng/ml of progesterone and 80 ng/ml of 17β-oestradiol also reduced ciliary activity in postovulatory explants (P = 0.017) **(Fig. 2)**.

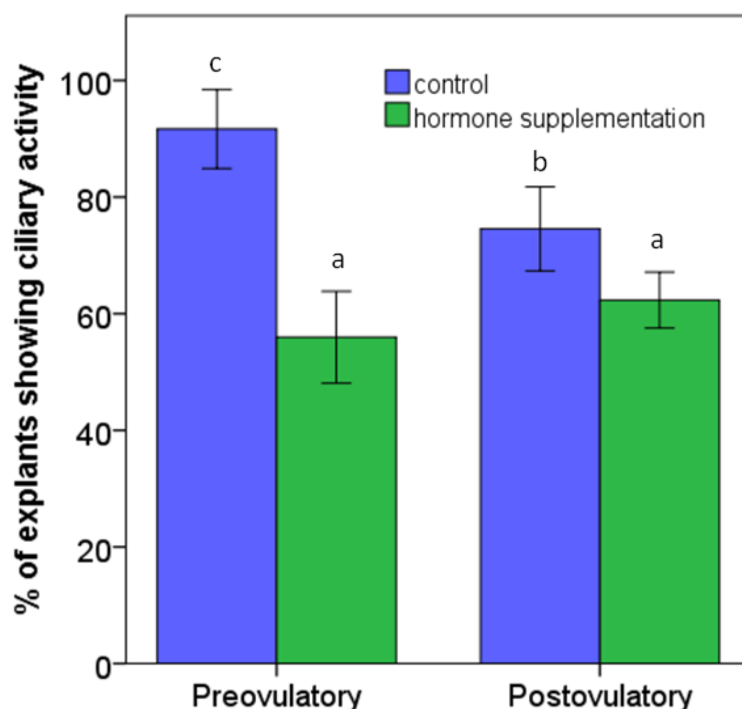

Fig. 2. Percentage of equine oviduct explants showing vigorous ciliary activity *in vitro* with or without hormone supplementation. Preovulatory explants were cultured with or without supplementation of 1 µg/ml of progesterone and 10 ng/ml of 17β-estradiol whereas postovulatory oviduct explants were exposed to 40 ng/ml of progesterone and 80 ng/ml of 17β-estradiol. One-way analysis of variance (ANOVA); error bars (2 x standard error of means) with different label are significantly different (P < 0.05).

Glucose consumption and lactic acid production in response to steroids in vitro

The average glucose consumption was higher in the postovulatory explants than in the preovulatory explants. Hormone supplementation during three days of culture did not affect glucose consumption nor lactate production in the preovulatory explants. In the postovulatory explants cultured in medium supplemented with 40 ng/ml progesterone and 80 ng/ml oestradiol, glucose consumption was significantly increased (P = 0.002) compared to the control group **(Fig. 3)**. Lactate production was not altered in response to hormone supplementation.

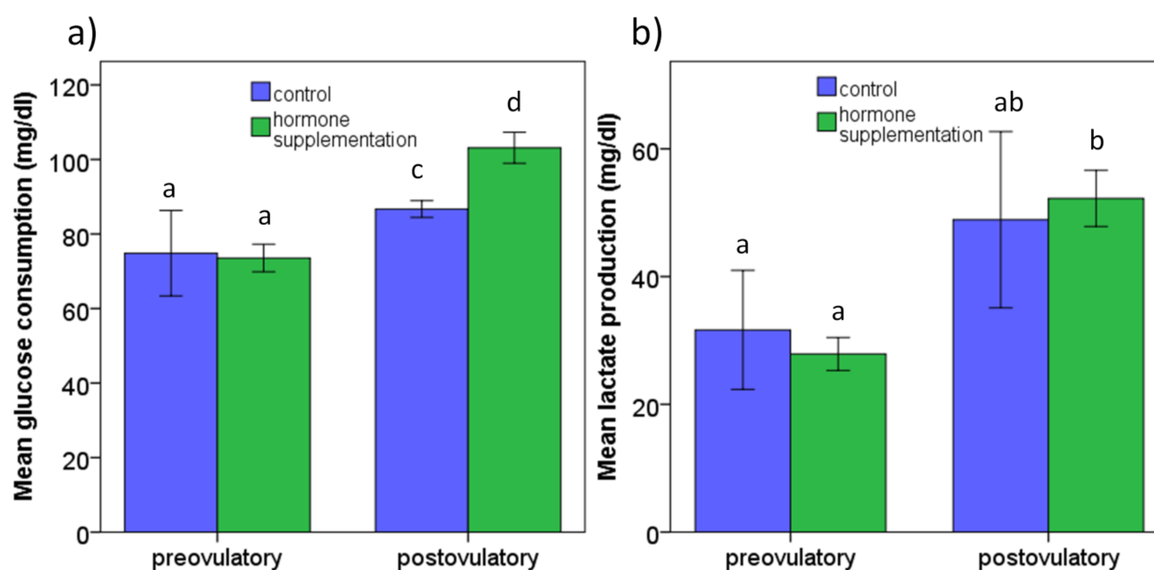

Fig. 3. Average glucose consumption (a) and lactate production (b) of preovulatory and postovulatory oviduct explants after 3 days of culture quantified by means of an UV enzymatic method. The pre- and postovulatory explants were cultured supplemented with respectively 1 µg/ml or 40 ng/ml cell-culture tested water soluble progesterone and 10 ng/ml or 80 ng/ml cell culture tested water soluble 17β-oestradiol. Control = no hormone supplementation, hormone = hormone supplementation. Error bars (± 2 x standard error of means) with different label are significantly different (P < 0.05). One-way analysis of variance (ANOVA).

Transmission electron microscopy

Transmission electron microscopy confirmed the presence of cell polarity during the three days of culture as evidenced by the presence of cilia and microvilli on the apical cell surface and of tight junctions on the lateral cell surfaces **(Fig. 4)**. No qualitative differences were

observed between the four groups with regard to the differentiation status and the polarity of the cells and to the presence of dark cells. In none of the four conditions an ultrastructural signature of active steroidogenesis, i. e. an elaborate smooth endoplasmic reticulum and the presence of mitochondria with tubulovesicular cristae was observed. The explants in both control groups (B, D) as well as in both hormone treated groups (A, C) showed conspicuous dark nuclear and cytoplasmic regions without classical ultrastructural features of apoptotic cell death as described previously (Krysko, et al. 2008) and reported earlier in oviduct explants *in vitro* (Nelis, et al. 2014). The cytoplasm surrounding the condensed chromatin did not contain recognizable organelles. In some cases these dark cells were engulfed by neighbouring healthy cells.

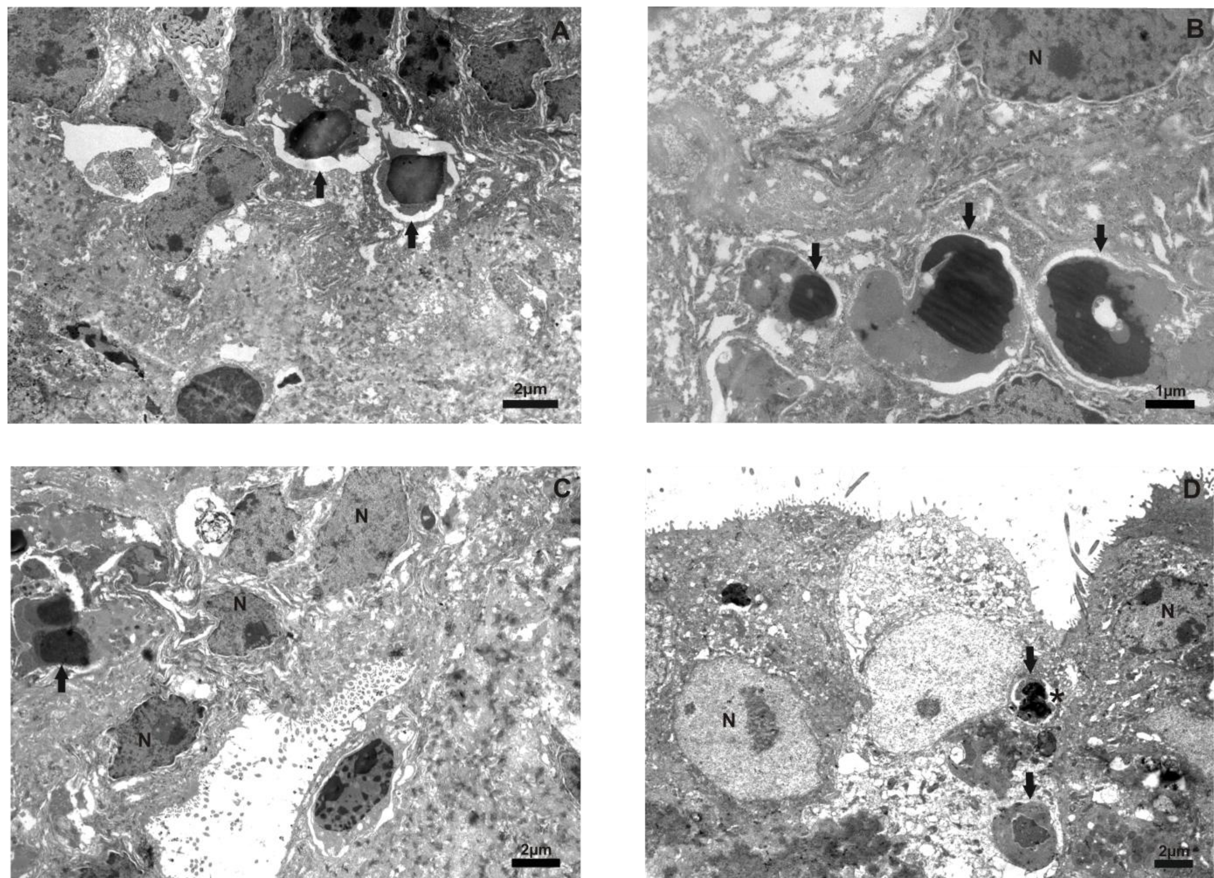

Fig. 4 Transmission electron micrographs. A. Postovulator oviduct explants after hormone supplementation. B. Postovulatory explants without hormone supplementation. C. Preovulatory explants after hormone supplementation. D Preovulatory explants without hormone supplementation. In the four conditions dark cells are apparent (arrows) within regions with healthy cells displaying normal nuclei (N). The dark cells are engulfed by neighbouring healthy cells (asterisk). No morphological differences were observed between the four conditions. Bar = 2 µm (a-c); bar = 1 µm (d).

Gene expression

Gene expression according to the cycle stage

A highly significant upregulation of *PAI1* and *PLAU* (P < 0.005), and a significant upregulation of *GLUT1, CSF1, TGFA* and *MMP2* were observed (0.01 < P < 0.05) in the postovulatory explants compared to the preovulatory explants. There were no differences in the expression levels of *VEGFA*, *TIMP1*,_PTGER2 and *PTGER4* in postovulatory oviduct cells **(Fig. 5)**. *VEGFA* expression showed a strong correlation (r > 0.80, P = 0.002) in the postovulatory stage and a moderate correlation in the preovulatory stage (r > 0.60, P = 0.04) with expression of *PTGER2* and *PTGER4.* In the postovulatory but not in the preovulatory stage, there is a highly positive correlation between the expression of *TIMP1* and *MMP2* (r = 0.82, P = 0.001), between *PAI1* and *PLAU* (r = 0.96, P = 0.001), and between *PLAU* with *MMP2* (r = 0.84, P = 0.018) and *TIMP1* (r = 0.90, P < 0.0005).

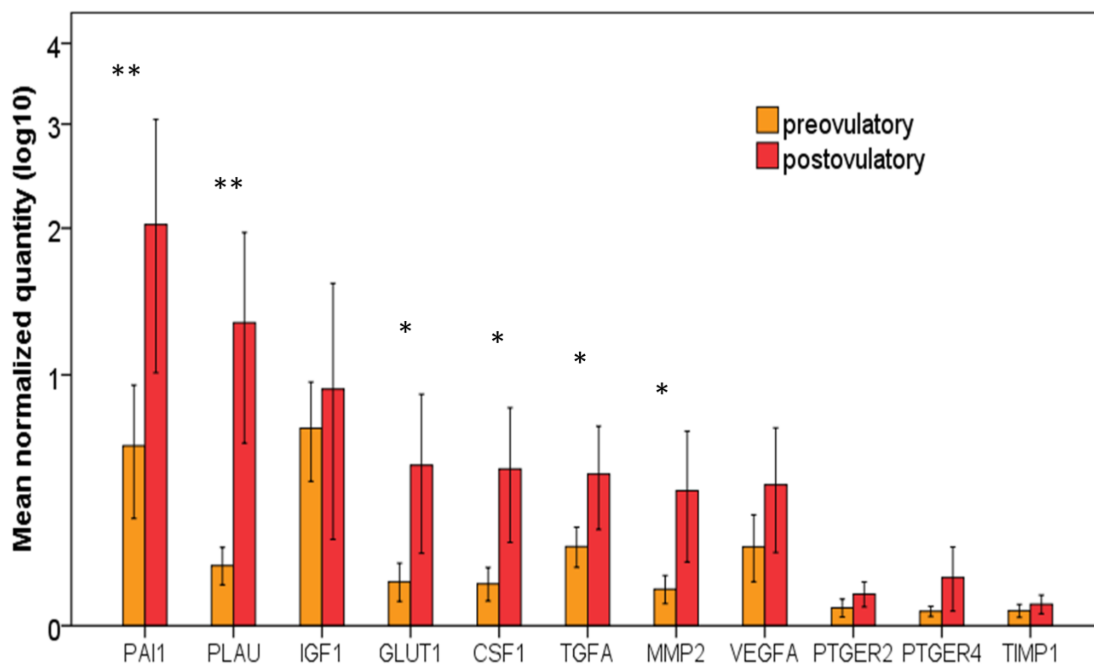

Fig. 5. Differential normalised gene expression (mean ± 2 x S.E.M.) in oviduct explants in the pre- and the postovulatory cycle stage as determined by RT-qPCR. In the postovulatory phase, expression levels of *PAI1, uPA, GLUT1, CSF1, TGFA* and *MMP2* were significantly higher compared with the preovulatory phase. * 0.01 < *P* < 0.05, ** 0.005 < *P* < 0.01; Independent samples t-test or Mann-Whitney U-test.

Gene expression changes in response to hormone supplementation

Supplementation of oviduct explants originating from mares in the preovulatory cycle stage with 1 µg/ml of progesterone and 10 ng/ml of 17β-oestradiol induced after three days of culture an upregulation of *PAI1* (P = 0.012), *GLUT1* (P = 0.01), *CSF1* (P = 0.04) and *MMP2* (P = 0.03), but not of *PLAU* and *TGFA* (P = 0.96 and 0.9) **(Fig. 6)**. Supplementation of oviduct explants originating from mares in the postovulatory cycle stage with 40 ng/ml of progesterone and 80 ng/ml of 17β-oestradiol induced after three days of culture a downregulation of *CFS1* and an upregulation of *GLUT1* **(Fig. 7)**.

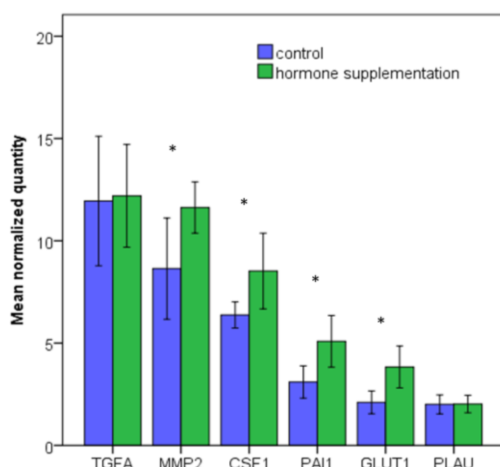

Fig. 6. Differential normalised gene expression (mean ± 2 x S.E.M.) as determined by RT-qPCR in oviduct explants derived from mares in the preovulatory cycle stage and cultured for 3 days with or without supplementation of 1 µg/ml progesterone and 10 ng/ml 17β-estradiol. Mean *PAI1, GLUT1, CSF1* and *MMP2* expression are significantly upregulated in the hormone treated explants. * 0.01 < *P* < 0.05. Paired samples t-test or Wilcoxon-signed rank test.

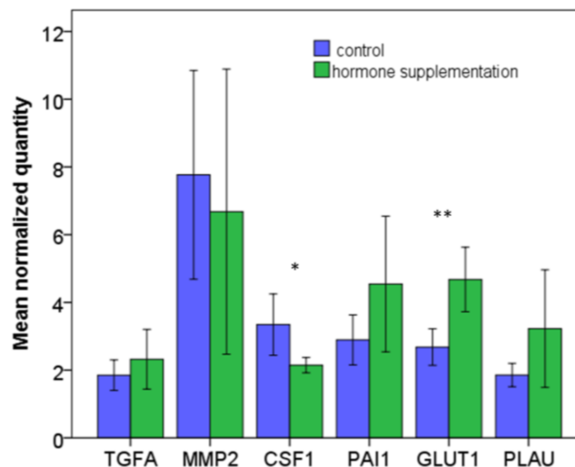

Fig. 7. Differential normalised gene expression (mean ± 2 x S.E.M.) as determined by RT-qPCR in oviduct explants derived from mares in the postovulatory cycle stage cultured for 2.5 days with or without supplementation of 80 ng/ml 17β-estradiol and 40 ng/ml progesterone. Mean *CSF1* expression is significantly lower, whereas *GLUT1* is strongly upregulated. * 0.01 < *P* < 0.05, ** 0.005 < P < 0.01. Paired samples t-test

Steroid determination in oviduct explant culture medium by means of RIA

The progesterone concentration in the culture medium of postovulatory explants was 84 times higher (6659.0 pg/ml) compared to the control medium without oviduct explants (79.1 pg/ml). In the preovulatory culture medium, the progesterone level was in the same range (55.4 pg/ml) as in the control group. 17β-Oestradiol, testosterone and oestrone concentrations were lower in the pre- and postovulatory group compared with the control group **(Table 3)**.

Table 3. 17β-oestradiol, progesterone, testosterone concentrations (pg/ml) in pooled cultured medium after 3 days of cultures without (control) or with (pre- or postovulatory) explants.

	control	**preovulatory**	**postovulatory**
17β-Estradiol	23.1	5.6	11.4
Progesterone	79.1	55.4	6.66×10^3
Testosterone	87.4	7.70	49.7
Estrone	60.1	14.5	21.2

Immunoreactive steroidogenic protein detection in oviduct explants by means of epifluorescence and immunohistochemistry

Immunoreactive protein was clearly detected for aromatase, cytochrome P450scc and StAR as determined by epifluorescence, as well as for 3-beta-HSD as determined by immunohistochemistry **(Fig. 8; Fig. 9)** in both the unsupplemented control and the hormone-treated groups. Hormone supplementation to the preovulatory explants induced a downregulation of aromatase ($P < 0.0005$) and an upregulation of cytochrome P450scc ($P < 0.005$) whereas StAR and 3-beta-HSD intensity remained unchanged. Steroid supplementation to postovulatory explants increased the expression of aromatase, cytochrome P450scc, StAR and 3-beta-HSD immunoreactive proteins ($P < 0.005$, 0.0005, 0.0005 and respectively 0.0005). Staining intensity of positive tissue control samples were compared to negative tissue and negative antibody controls, which stained positive for all enzymes. Negative antibody and tissue controls were approximately zero **(Fig. 9 a-c)** for aromatase, cytochrome P450scc and StAR and very low for 3-beta-HSD **(Fig. 9 d)**. Positive control

slides (follicular wall for aromatase and StAR and corpus luteum for cytochrome P450scc and corpus luteum for 3-beta-HSD) stained strongly positive **(Fig. 9)**.

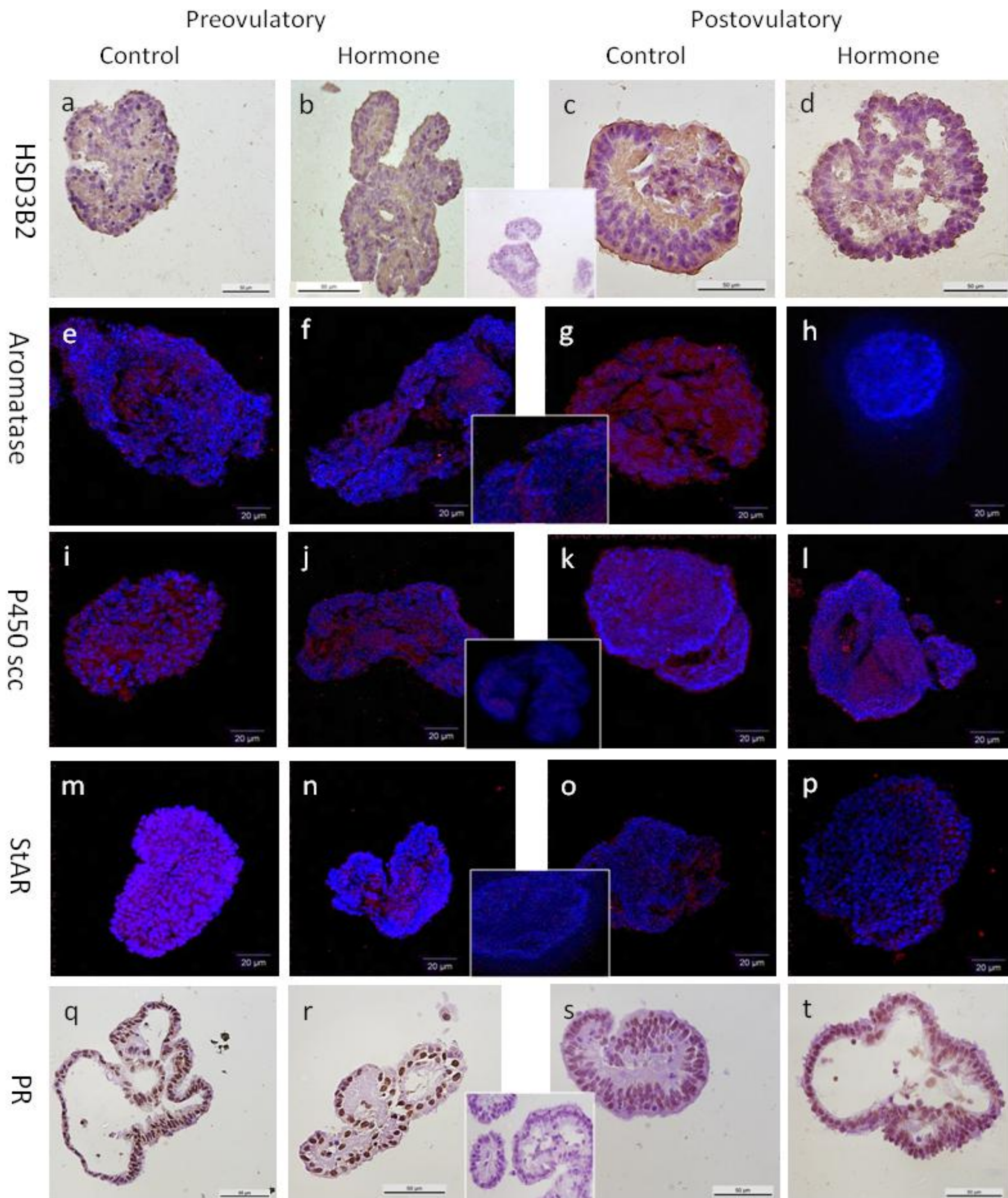

Fig. 8. Epifluorescent pictures for aromatase (e-h), P450 scc (i-l) and StAR (m-p). 3β-hydroxysteroid dehydrogenase/Δ5→4-isomerase (brown) stained with immunohistochemistry and hematoxylin counterstaining (purple) (a-d). Immunohistochemistry staining for progesterone receptor (PR, brown) with hematoxylin counterstaining (purple) (q-t). A negative control for each staining is added in the middle of the row. Bar a-d and q-t = 50 µm; Bar e-p = 20 µm

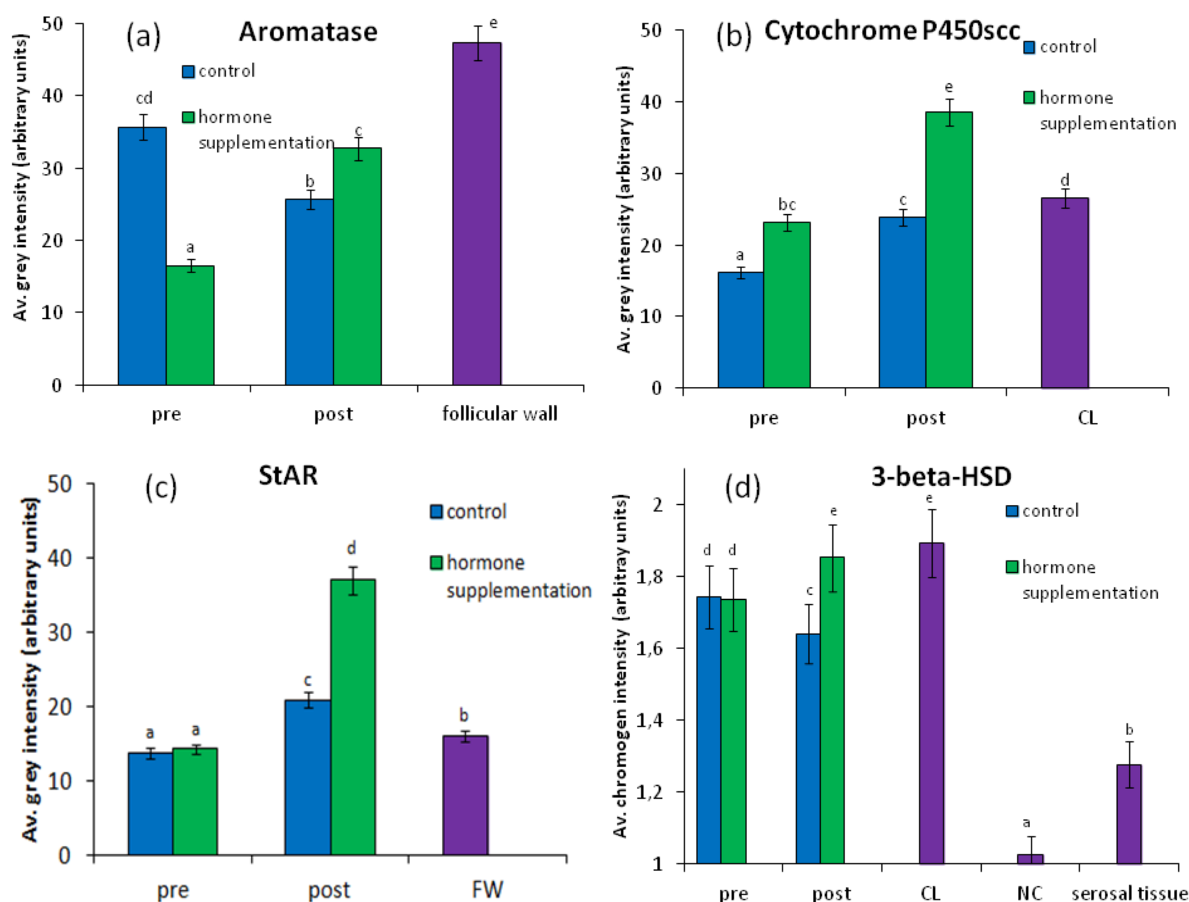

Fig. 9. Average gray intensity (a-c) and chromogen intensity (d) in arbitrary units of slides equine oviduct explants stained by immunofluorescence (a-c) and immunohistochemistry (d) to detect immunoreactive protein of aromatase (P450AROM), P450SCC, STAR and HSD3B. Negative controls (NC) were negative for P450AROM, P450SCC and STAR. Follicular wall and corpus luteum (CL) served as positive control tissue.. Serosal (oviductal) tissue was negative control tissue. Error bars (confidence interval) with different label are significantly different. One-way ANOVA.

Progesterone receptors

Ratios of progesterone positive/negative cells **(Fig. 8 q-t)** were significantly lower in the postovulatory explants when compared to the preovulatory explants ($P < 0.0005$) **(Fig. 10)**. Supplementation of preovulatory explants with 1 µg/ml progesterone and 10 ng/ml 17β-oestradiol induced a highly significant decrease ($P < 0.0005$) in the ratio of progesterone receptor positive/negative nuclei. Ratios were comparable in the postovulatory explants

cultured with and without supplementation of 40 ng/ml of progesterone and 80 ng/ml of 17β-oestradiol.

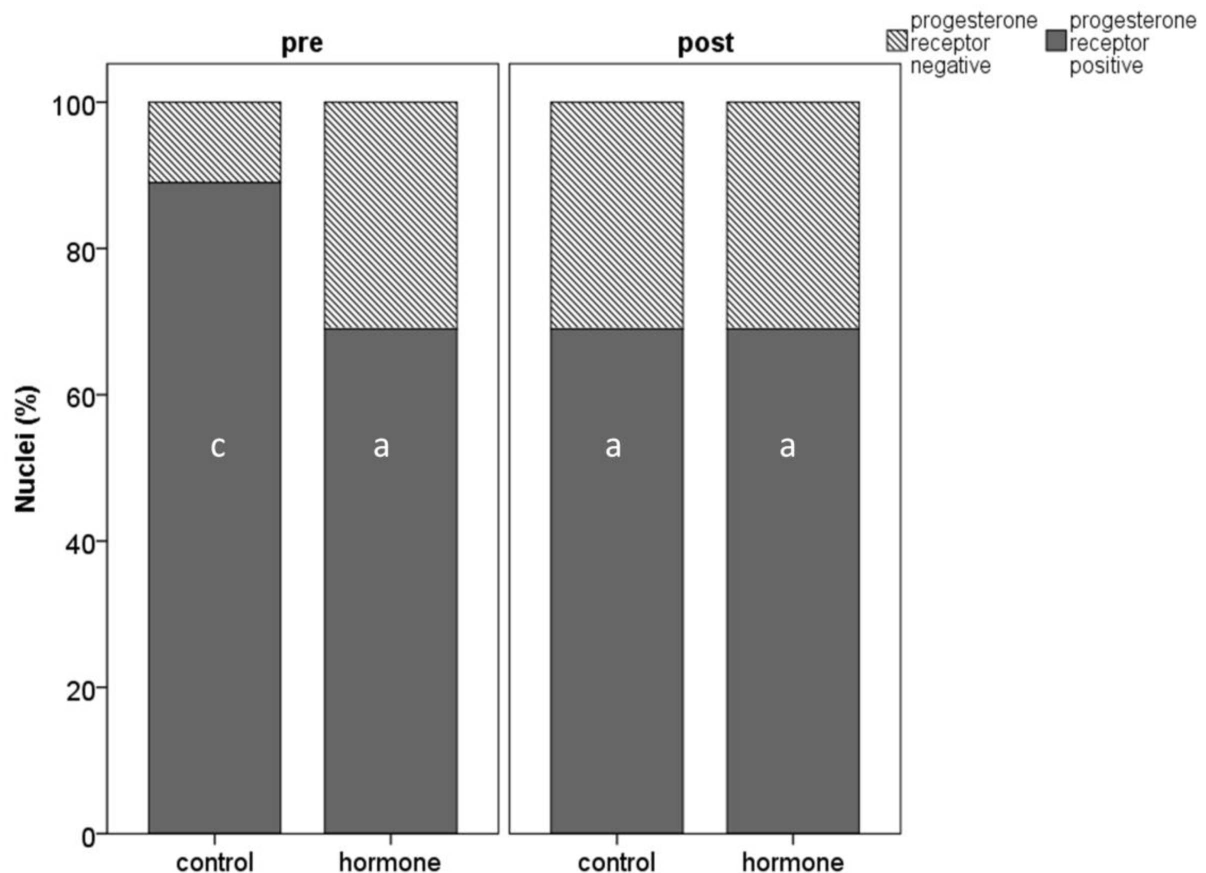

Fig. 10. Percentage of progesterone receptor positive and negative nuclei in oviduct epithelial cells as determined by immunohistochemistry. Preovulatory explants (pre) and postovulatory explants (post) were cultured with or without respectively 1 µg/ml or 40 ng/ml cell-culture tested water soluble progesterone and 10 ng/ml or 80 ng/ml cell culture tested water soluble 17β-oestradiol supplementation. The percentage of progesterone receptor positive nuclei was significantly higher in the unsupplemented preovulatory oviduct explants, when compared to the three other groups. Binary logistic regression; different labels in the bars correspond to significant differences.

DISCUSSION

This report shows that steroid hormones influence the equine oviduct at the level of ciliary activity, glucose consumption, mRNA expression, steroidogenesis and progesterone receptor expression. This effect is present in the oviduct *in vivo*, since differences were observed between oviduct epithelial explants originating from mares in oestrus when compared to those from mares in the postovulatory stage. Moreover, this influence was also observed in oviduct explants *in vitro* after the addition of hormones to the culture medium. Interestingly, supplementation of the culture medium of preovulatory oviduct explants with steroid hormones in concentrations as they prevail in the postovulatory mare induced a change in the oviduct explants, approaching the *in vivo* postovulatory situation, at the level of ciliary activity, gene expression and progesterone receptor expression. The effect of preovulatory hormone levels on postovulatory explants was less pronounced, as explained below.

The phenotype of the epithelial explants is very similar to that of oviduct epithelium collected *in vivo* (Nelis, et al. 2014). It consists of polarized, highly differentiated epithelial cells with basal nuclei and apical cilia and microvilli, as shown by histology **(Fig. 8)** and TEM **(Fig. 4)**. No apparent influence of steroid hormones on the oviductal ultrastructure was observed. However the 4 experimental groups were not compared morphometrically with regard to quantitative features of different subcellular compartments such as number, form and dimension of organelles (golgi apparatus, rough and smooth endoplasmic reticulum, mitochondria and nucleus). It is to be expected but not an absolute rule that increased steroidogenic activity *in vitro* as measured in the culture medium of postovulatory explants is accompanied by alterations in 2 subcellular compartments i.e the smooth endoplasmic reticulum and the mitochondrial compartment. The smooth endoplasmic reticulum would become more abundant, the mitochondria adapt a configuration with tubulovesicular cristae (Christensen and Gillim 1969). The dark cell degeneration (Krysko, et al. 2008, Leist and Jaattela 2001), as described earlier in oviductal explants *in vitro* (Nelis *et al.* 2013) but which was not detected *in vivo* (Desantis, et al. 2011), was observed in both preovulatory and postovulatory, as well as in both hormone treated and untreated explants **(Fig. 4)**. Although this hypothesis needs to be confirmed, the dark cell degeneration is speculated to be induced by a caspase-non-dependent apoptotic process, established amongst others by hypoxia, nutrient depletion, waste accumulation, mechanical agitation, reactive oxygen species (Nelis, et al. 2014).

The ciliary beating was the highest in the unsupplemented preovulatory explants. This coincides with the physiological situation, as ciliary activity is stimulated by oestrogen and decreased by progesterone (Mahmood, et al. 1998). The treatment of preovulatory explants with progesterone and 17β-oestradiol concentrations as they prevail physiologically in the postovulatory oviduct induced a strong decrease in ciliary activity without jeopardizing the vitality of the explants **(Fig. 2)**. This could be explained by the concentration-dependent reduction in ciliary activity caused by progesterone (Mahmood, et al. 1998), without decrease in the proportion of ciliated/non ciliated cells (Aguilar, et al. 2012). Despite the fact that high levels of 17β-oestradiol may counteract the progesterone mediated reduction in ciliary beating (Mahmood, et al. 1998), the addition of 17β-oestradiol to the postovulatory explants apparently did not reach a sufficient level to obtain this effect **(Fig. 2)**. It should also be noted that more obvious effects could have been obtained when using oviducts from mares in the late luteal stage. However, in the light of embryo co-culture, since the scope of the present paper is to study oviduct explants that provide the most physiological environment for embryos, explants from early postovulatory oviducts were studied.

Next to the effect on ciliary activity, 17β-oestradiol and progesterone can also influence the glucose consumption in the oviduct **(Fig. 3)**. Horse embryos are cultured *in vitro* at relatively high levels (17 mM) of glucose (Hinrichs 2010) and that is why this parameter is especially interesting. Glucose consumption by the postovulatory oviduct explants was higher **(Fig. 3)** compared to the preovulatory explants, indicating a higher cellular activity (Leclerc, et al. 2003) in order to optimize the microenvironment for gamete and embryo development and transport. Similarly, in bovine endometrium, maximum metabolic activity and glucose consumption was observed during progesterone dominance (Chase, et al. 1992). Nevertheless, the addition of postovulatory concentrations of progesterone (1 μg/ml) and 17β-oestradiol (10 ng/ml) to preovulatory explants could not simulate glucose consumption as measured in the postovulatory explants. Higher glucose consumption is not only observed in tissues that are influenced by high progesterone concentrations, but also in tissues that produce progesterone themselves. An enhanced oxygen and glucose consumption was observed in progesterone producing luteal tissue compared with luteal tissues which lost their progesterone producing ability (Armstron and Black 1966). This finding is in line with the fact that postovulatory explants, which produce 100 times more progesterone compared with preovulatory explants **(Table 3)**, consume significantly more glucose than their preovulatory counterparts **(Fig. 3)**.

GLUT1, which plays an important role in the glucose transfer from the oviduct epithelium into the lumen along a gradient, maintains the adequate glucose concentration in the oviductal fluid to support the developing embryo (Tadokoro, et al. 1995). Progesterone upregulates while estrogen downregulates *GLUT1* expression and glucose uptake (Frolova, et al. 2009). This was confirmed in our study, as *GLUT1* expression was significantly higher in the postovulatory explants than in the preovulatory explants and as addition of postovulatory hormone concentrations to preovulatory explants induced upregulation of *GLUT1* expression. Surprisingly, *GLUT1* was also upregulated in postovulatory explants stimulated with preovulatory high 17β-oestradiol and low progesterone concentrations. This may be explained by a more explicit effect of progesterone, when compared to 17β-oestradiol and to the considerable amount of accumulating autologously produced progesterone **(Table 3)** which may induce *GLUT1* expression in the postovulatory explants or by other factors which directly or indirectly upregulate *GLUT*.

Lactic acid production was not concomitantly enhanced in response to steroid stimulation. In mammalian cells, except in muscle cells and red blood cells, lactic acid fermentation of pyruvate is most likely occurring as a consequence of pyruvate accumulation following enhanced glycolysis (Nielsen, et al. 2001, Rose and Warms 1966).

The mRNA expression of 11 genes, *PAI1*, *PLAU*, *TGFA*, *TIMP1*, *MMP*2, *CSF*, *PTGER2*, *PTGER4*, *IGF1*, *VEGFA* and *GLUT1* was evaluated in pre- and postovulatory oviduct explants to elucidate hormone dependency. *PAI1, TGFA, MMP2, CSF1, GLUT1* and *PLAU* expressions were upregulated in the postovulatory stage **(Fig. 5)** which strongly suggests their steroid-responsiveness and it is indicating that these proteins may play a relevant role in fertilization, the gamete- and embryo-maternal dialogue and/or early embryonic development (Buhi and Alvarez 2003).

IGF1, VEGFA, TIMP1, PTGER2 and PTGER4 do not seem to be predominantly steroid regulated. Similar to the postovulatory *in vivo* situation, upregulation of *MMP2, CSF1, PAI1* and *GLUT1* could be induced in preovulatory explants after three days of exposure to postovulatory hormone concentrations **(Fig. 6)**. *TGFA* and *PLAU* expression was not altered after hormone supplementation, indicating that other (intermediate) factors may be involved in their regulation which are absent *in vitro.* In turn, addition of preovulatory concentrations of steroids to postovulatory explants could induce a downregulation only in *CSF1* **(Fig. 7)**. Overall, this may suggest a predominant stimulatory effect of progesterone on the modulation

of *MMP2, CSF1, PAI1* and *GLUT1*. In the equine oviduct indeed, *PAI1* with *PLAU* and also *TIMP1* with *MMP2* are positively associated in the postovulatory but not the preovulatory stage, confirming their predominant role in maintaining and stabilizing the oviductal milieu in the anticipation of fertilization and the arrival of the embryo. This also suggests that these proteins may play a relevant role in fertilization, the gamete- and embryo-maternal dialogue and/or early embryonic development (Buhi and Alvarez 2003).

MMP2 is involved in tissue remodelling (Bonnans, et al. 2014, Duc-Goiran, et al. 1999) and cell motility during embryogenesis (Duong and Erickson 2004, Page-McCaw, et al. 2007). Moreover, together with its inhibitor TIMP1, its protein and gene expression is strictly controlled and balanced in order to regulate degrading, reconstruction of the extracellular matrix and consequently cellular adhesion, cell migration and proliferation (Duong and Erickson 2004, Zhao, et al. 2002). Indeed, mRNA expression of *MMP2* is strongly positively associated with *TIMP1, TGFA, PAI1, PLAU, CSF* and *GLUT1* in the postovulatory stage and with *PAI1* and *TIMP1* in the preovulatory phase. In bovine endometrial cells *in vitro*, MMP2 expression is enhanced by high doses of progesterone (Hashizume, et al. 2003). Also in the horse oviduct, *MMP2* was increased in the early luteal phase **(Fig. 5)**. Moreover, supplementation of postovulatory hormones characterized by high progesterone concentrations to preovulatory explants upregulated mRNA expression of *MMP2* **(Fig. 6)**. In contrast, in the bovine oviduct, *MMP2* mRNA is downregulated after ovulation (Gabler, et al. 2001). This finding could be explained by the species, cell-type and tissue specific nature of the expression of *MMPs* (Rodgers, et al. 1994). As in the cow (Gabler, et al. 2001), *PLAU* expression was higher after ovulation **(Fig. 5)**. PLAU may rebuild or degrade cumulus cells during fertilization or early development (Roldan-Olarte, et al. 2005) and/or could be involved in sperm capacitation or sperm-egg interaction (Diaz, et al. 2000). An important inhibitor of *PLAU, PAI1,* was also upregulated in the postovulatory phase. However, supplementation of postovulatory or preovulatory concentrations of progesterone and 17β-oestradiol could not mimic this phenomenon **(Fig. 6; Fig. 7)**. *PAI1*, upregulated in the same order of magnitude as *PLAU* **(Fig. 5)** may act as a stabilizer of extracellular matrix integrity of the oviduct epithelium and the zona pellucida by neutralizing the inherent plasminogen activator activity of cumulus-oocyte complexes (Kim and Menino 1995, Yamada, et al. 1996), the embryo and/or the oviduct itself (Gabler, et al. 2001, Kouba, et al. 2000b).

CSF1 (or M-CSF), a hematopoietic growth factor, plays a role in the regulation of macrophages in the reproductive tract as well as its influence on other cells like trophoblast cells and oocytes (Cohen, et al. 1997). CSF1, which was significantly upregulated in the postovulatory oviduct explants, is proven to stimulate blastocyst development (Pampfer, et al. 1991b), to accelerate blastocoel formation and to increase the number of trophectodermal cells (Bhatnagar, et al. 1995).

Also expression of *TGFA,* together with TIMP1 fulfilling an antiproteolytic function (Pepper, et al. 1990), is dependent on cell type and hormonal status (Chegini, et al. 1994, Pfeifer and Chegini 1994, Schell, et al. 1994, Schmidt, et al. 1994, Stevenson and Wathes 1996, Zhao, et al. 1994). *TGFA* is also expressed in the human (Morishige, et al. 1993) and porcine (Wollenhaupt, et al. 2001) oviduct. TGFα exerts its action through epidermal growth factor receptors (EGF-R) (Wollenhaupt, et al. 2001). In porcine oviductal tissue, oestrogen stimulates EGF-R protein concentration (Wollenhaupt, et al. 1997). Progesterone and oestradiol together caused a more intense increase than did each steroid alone (Watson, et al. 1996). Reports concerning the steroid responsiveness of oviductal *TGFA* are conflicting: oestrogen dependency (Lei and Rao 1992) as well as unresponsiveness (Kennedy, et al. 1994) to steroids have been described. In our experiments, *TGFA* was upregulated in the early postovulatory phase compared to the preovulatory phase, indicating a stimulatory experiment of progesterone in the horse **(Fig. 5)**. The comparable levels of IGF1 expression shortly before and after ovulation might be due to its spatial and regional distribution. This is confirmed by findings in sows (Buhi and Alvarez 1998) and cows (Schmidt, et al. 1994). IGF1 concentrations were higher on day 0 and day 2 after ovulation. In contrast, in oviducts of mice, IGF1 is regulated by oestradiol through (putative co-localized) estrogen receptors α (Shao, et al. 2007a). In the oviductal lumen, locally produced IGF1 may regulate embryo development directly or indirectly via IGF1Rs (LeRoith, et al. 1995b), present on the oviduct epithelium, resulting in differentiation, mitogenesis and inhibition of apoptosis (Jones and Clemmons 1995, Leroith, et al. 1995a, Ullrich, et al. 1986).

Especially in the horse, the fertilization and early embryo development are strongly dependent on a precisely orchestrated interaction between the embryo and the maternal genital tract. The equine embryo indeed produces prostaglandin E_2 (PGE_2). PGE2 binds to PTGER2 or PTGER4, resulting in a relaxation of the oviduct which hastens the oviductal transport of equine embryos and opens up the uterotubal papilla in order to allow uterine entry (Weber, et

al. 1991a, b). However, mRNA expression of *PTGER2* and *PTGER4* was not significantly altered (P = 0.2) in the equine oviduct in the postovulatory stage, coinciding with the physiological stage at which interaction with the embryo occurs *in vivo*.

These results show that preovulatory oviduct explants, primed by steroids *in vivo*, are responsive to *in vitro* stimulation with postovulatory oviductal progesterone and 17β-oestradiol concentrations and approach the *in vivo* condition at the level of functionality and gene expression. This confirms again that our explant model remains functional and responsive for at least three days (Nelis, et al. 2014). The novel finding that equine epithelial explants are capable of producing large amounts of progesterone and are able to remove considerable amounts of oestrone, 17β-oestradiol and testosterone from the culture medium further substantiates the functional intactness of the culture system **(Table 3)**. This substantial progesterone production by the postovulatory oviduct explants coincides with findings in other species (Kendle and Lee 1980, Richardson and Oliphant 1981, Spilman and Wilks 1976, Takeda, et al. 1978). While progesterone concentrations were significantly increased in medium conditioned by the postovulatory explants, concentrations of 17β-oestradiol, oestrone and testosterone in the medium were similar in the presence of preovulatory and postovulatory explants. Lower concentrations of these hormones were detected in the presence of oviduct explants, when compared to the explant-free control medium, indicating that the oviduct explants might bind or metabolize 17β-oestradiol, oestrone and testosterone from the culture medium. The steroids measured in explant free medium originates from the foetal calf serum **(Table 3)** (Gstraunthaler 2003).

To gain insight in the mechanisms underlying the production and the metabolism of steroid hormones by the oviduct cells, the presence of the immunoreactive proteins of four enzymes involved in the steroidogenesis was localized and quantified in the explants. Steroidogenic acute regulatory protein (StAR), which initiates and catalyses steroidogenesis by delivering cholesterol from the outer mitochondrial membrane to the inner membrane (LaVoie and King 2009), was significantly higher in the postovulatory explants **(Fig. 9)**. Therefore, increased StAR in the postovulatory stage could be at the basis of the higher progesterone production, as observed in the postovulatory stage. StAR was further increased after supplementation with preovulatory hormones, which could be due to a reported stimulatory effect of 17β-oestradiol (Townson *et al.* 1996). The regulation of StAR is very complex and not fully understood (LaVoie and King 2009, Stocco 2001, Stocco, et al. 2005). Our results suggest higher StAR

immunoreactive protein expression in the postovulatory explants, under progesterone dominance, and a further increase by supplemental addition of preovulatory hormones, characterized by a high oestradiol concentration. Therefore, both hormones could have a stimulatory effect on StAR.

Immunoreactive protein expression of cytochrome P450 cholesterol side chain cleavage (cytochrome P450scc; CYP11A1 gene), which converts cholesterol to pregnenolone (King and LaVoie 2012), is also significantly higher in the postovulatory compared with the preovulatory explants **(Fig. 9)**. Therefore cytochrome P450scc is the second possible candidate enzyme involved in the high progesterone production by the postovulatory explants. Cytochrome P450scc is reported to be stimulated by amongst others oestradiol (Urban, et al. 1991) and progesterone (Swan, et al. 2002). This coincides with our observations *in vitro*, where cytochrome P450scc in both pre- and postovulatory equine oviduct explants was upregulated after supplementation of the culture medium with progesterone and 17β-oestradiol **(Fig. 9)**.

3β-Hydroxysteroiddehydrogenase (3-beta-HSD) catalyses the conversion of pregnenolone to progesterone (LaVoie and King 2009). In the rat, its expression is enhanced by 17β-oestradiol (Munabi, et al. 1983) and suppressed by progesterone (Munabi, et al. 1983) but the regulation of the HSD3B gene is species and cell type specific (Simard, et al. 2005). This was confirmed in our model as 3-beta-HSD immunoreactive protein expression was higher in preovulatory oviduct explants than in unstimulated postovulatory explants. Moreover, expression of 3-beta-HSD could be induced in the postovulatory explants by culture in the presence of hormone concentrations as they prevail preovulatory *in vivo.* A similar event was observed in rat ovarian cells (Munabi, et al. 1983): progesterone reduces whereas 17β-oestradiol increases 3-beta-HSD activity. Overall stimulation of 3-beta-HSD by preovulatory hormones in our study coincided with literature findings, but 3-beta-HSD could not explain the high progesterone production in the postovulatory stage.

Besides the support for a steroid induced capacity to produce progesterone, our findings also provide evidence that the equine oviduct may convert testosterone to 17β-oestradiol, catalyzed by aromatase **(Fig. 8; Fig. 9;** (Nelis, et al. 2015a). Oviduct aromatase activity has been demonstrated before in the frog (Kobayashi, et al. 1996), in human (Li, et al. 2003) and in the rat oviduct (Tetsuka, et al. 1998). Literature data concerning the endocrine regulation of

aromatase expression by 17β-oestradiol and progesterone is contradictory. In equine oviduct explants, immunoreactive protein of aromatase was stimulated by preovulatory hormones and reduced by postovulatory hormones, suggesting a possible positive feedback by 17β-oestradiol and a stimulatory effect of progesterone. Apart from the potential aromatase activity, metabolisation of testosterone by the equine oviduct was also suggested by a reduced concentration of testosterone in the culture medium in the presence of equine oviduct explants. Testosterone concentrations in the control medium, originating from the supplementation of FCS to the culture medium, were higher than the concentrations in the medium with explants. Moreover, testosterone levels in the medium with the postovulatory explants were higher compared with its preovulatory counterpart **(Table 3)**, again suggesting increased metabolisation of testosterone in the preovulatory stage, which coincides with the increased presence of aromatase immunoreactive protein. Also 17β-oestradiol and oestrone concentrations seem to be much lower in the culture medium of the explants compared with the control medium **(Table 3)**. Oestrone and 17β-oestradiol may be converted to oestriol, as described in the liver and placenta (Hanukoglu 1992) or might be converted by a variety of isoenzymes of P450 into quinols (Ohe, et al. 2000).

The observed high concentrations of progesterone associated with the postovulatory condition *in vivo* and *in vitro* have further repercussions on the progesterone receptor expression. Significantly lower ratios of progesterone receptor positive nuclei were observed in the oviductal explants from mares in the postovulatory stage, when compared with the preovulatory explants **(Fig. 10)**, confirming previous findings *in vivo* (Nelis, et al. 2015a). Again, this effect could be elegantly mimicked *in vitro*, as addition of postovulatory hormones to preovulatory explants resulted in a significant downregulation of the progesterone receptors **(Fig. 10)**. Our hypothesis is that an elevated progesterone concentration in the ipsilateral oviduct may decrease its own receptor expression by means of a negative feedback mechanism, as has been described previously in guinea pigs (Alkhalaf, et al. 1992, Hai, et al. 1977), macaques (West and Brenner 1985), cats (West, et al. 1977) and mice (Tibbetts, et al. 1998). Results obtained in bovine oviduct cells stimulated with progesterone and oestradiol *in vitro* (Ulbrich, et al. 2003) confirm the downregulating capacity of progesterone on its own receptor.

In conclusion, for the first time, we used an equine simulation of the pre- and the postovulatory changes in progesterone and oestradiol concentrations and showed that they

were able to modify ciliary activity, energy metabolism, gene expression, immunoreactive steroidogenic enzyme expression and progesterone receptor expression in oviductal explants. Furthermore, a set of embryotrophic genes was shown to be upregulated in the oviductal epithelium originating from mares in the postovulatory cycle stage. In addition, these cycle-related changes indicate the importance of steroids, especially progesterone, in fertilization, embryo growth and viability, as well as of the oviduct, both as a target of steroids and as a site of steroid biosynthesis and metabolism. An overview of the findings is presented in **Fig. 11**. These findings are of significant value and can be implemented in *in vitro* oviduct-embryo co-culture models (Nelis, et al. 2014) in order to elaborate on the deciphering of endocrine, autocrine and paracrine signalling during early embryo development in the horse.

	Preovularory explants + postovulatory steroids		**Postovulatory explants + preovulatory steroids**	
	Outcome	***In vivo*-like effect?**	**Outcome**	***In vivo*-like effect?**
Ciliary activity	Decreased	✓	Decreased	✗
Glucose consumption	No effect	✗	Increased	✗
Lactate production	No effect	✗	No effect	✗
Ultrastructure	Intact	✓	Intact	✓
Gene expression PAI1	Upregulated	✓	No effect	✗
PLAU	No effect	✗	No effect	✗
GLUT1	Upregulated	✓	Upregulated	✗
CSF1	Upregulated	✓	Upregulated	✓
TGFA	No effect	✗	No effect	✗
MMP2	Upregulated	✓	No effect	✗
Aromatase	Downregulated	✓	Upregulated	✓
Cytochrome P450 scc	Upregulated	✓	Upregulated	✗
StAR	No effect	✗	Upregulated	✗
3-Beta-HSD	No effect	✗	Upregulated	✓
Progesterone receptor	Downregulated	✓	No effect	✗

Fig. 11 A synthesis of the outcomes after the stimulation of preovulatory explants with postovulatory concentrations of progesterone and 17β-oestradiol and vice versa.

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REFERENCES

Aguilar, JJ, J Cuervo-Arango, H Mouguelar, and L Losinno 2012 Histological Characteristics of the Equine Oviductal Mucosa at Different Reproductive Stages. *Journal of Equine Veterinary Science* **32** 99-105.

Alkhalaf, M, AY Propper, G Chaminadas, and GL Adessi 1992 Ultrastructural-Changes in Guinea-Pig Endometrial Cells during the Estrous-Cycle. *Journal of Morphology* **214** 83-96.

Armstron.Dt, and DL Black 1966 Influence of Luteinizing Hormone on Corpus Luteum Metabolism and Progesterone Biosynthesis Throughout Bovine Estrous Cycle. *Endocrinology* **78** 937-&.

Ball, BA, and M Altschul 1990 In vitro development of 4- to 8- cell equine embryos cocultured with trophoblastic vesicles or oviductal explants. . *Theriogenology* **33** 189.

Ball, BA, KE Scoggin, MHT Troedsson, and EL Squires 2013 Characterization of prostaglandin E-2 receptors (EP2, EP4) in the horse oviduct. *Animal Reproduction Science* **142** 35-41.

Bhatnagar, P, VE Papaioannou, and JD Biggers 1995 Csf-1 and Mouse Preimplantation Development in-Vitro. *Development* **121** 1333-1339.

Bogaert, L, M Van Poucke, C De Baere, L Peelman, F Gasthuys, and A Martens 2006 Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *Bmc Biotechnology* **6**.

Bologna-Molina, R, P Damian-Matsumura, and N Molina-Frechero 2011 An easy cell counting method for immunohistochemistry that does not use an image analysis program. *Histopathology* **59** 801-803.

Bonnans, C, J Chou, and Z Werb 2014 Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology* **15** 786-801.

Buhi, WC, and IM Alvarez 2003 Identification, characterization and localization of three proteins expressed by the porcine oviduct. *Theriogenology* **60** 225-238.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Buhi, WC, JL Vallet, and FW Bazer 1989 Denovo Synthesis and Release of Polypeptides from Cyclic and Early Pregnant Porcine Oviductal Tissue in Explant Culture. *Journal of Experimental Zoology* **252** 79-88.

Burry, RW 2011 Controls for Immunocytochemistry: An Update. *Journal of Histochemistry & Cytochemistry* **59** 6-12.

Bustin, SA, V Benes, JA Garson, J Hellemans, J Huggett, M Kubista, R Mueller, T Nolan, MW Pfaffl, GL Shipley, J Vandesompele, and CT Wittwer 2009 The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55** 611-622.

Bylander, A, M Nutu, R Wellander, M Goksor, H Billig, and DGJ Larsson 2010 Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. *Reproductive Biology and Endocrinology* **8**.

Cappelli, K, M Felicetti, S Capomaccio, G Spinsanti, M Silvestrelli, and AV Supplizi 2008 Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. *Bmc Molecular Biology* **9**.

Chase, CC, RP Delvecchio, SB Smith, and RD Randel 1992 Invitro Metabolism of Glucose by Bovine Reproductive Tissues Obtained during the Estrous-Cycle and after Calving. *Journal of Animal Science* **70** 1496-1508.

Chegini, N, Y Zhao, and FW Mclean 1994 Expression of Messenger-Ribonucleic-Acid and Presence of Immunoreactive Proteins for Epidermal Growth-Factor (Egf), Transforming Growth-Factor-Alpha (Tgf-Alpha) and Egf/Tgf-Alpha Receptors and I-125 Egf Binding-Sites in Human Fallopian-Tube. *Biology of Reproduction* **50** 1049-1058.

Chen, S, R Einspanier, and J Schoen 2013 In Vitro Mimicking of Estrous Cycle Stages in Porcine Oviduct Epithelium Cells: Estradiol and Progesterone Regulate Differentiation, Gene Expression, and Cellular Function. *Biology of Reproduction* **89**.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004 Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Christensen, AK, and SW Gillim 1969 The correlation of fine structure and function in steroid-secreting cells with emphasis on those of the gonads. . *In* K McKerns (ed.), The Gonads, pp. 415-490. New York: Meredith Corporation.

Ciereszko, R 1999 Radioimmunologiczne oznaczanie stężenia hormonów steroidowych w płynach biologicznych [Radioimmunoassay of steroid hormones in biological fluids. *In* J Przala (ed.), Animal Physiology., pp. 157-163. Olsztyn: UWM Press.

Cohen, PE, LY Zhu, and JW Pollard 1997 Absence of colony stimulating factor-1 in osteopetrotic (csfm(op)/csfm(op)) mice disrupts estrous cycles and ovulation. *Biology of Reproduction* **56** 110-118.

De Bosschere, H, R Ducatelle, H Vermeirsch, P Simoens, and M Coryn 2002 Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. *Animal Reproduction Science* **70** 251-259.

Desantis, S, G Ventriglia, S Zizza, AC Guaricci, M Losurdo, A Zarrilli, and M Albrizio 2010 Changes in the expression of the mu-opioid receptor in the mare oviduct during oestrus and anoestrus. *Animal Reproduction Science* **119** 40-49.

Desantis, S, S Zizza, G Accogli, F Acone, R Rossi, and L Resta 2011 Morphometric and ultrastructural features of the mare oviduct epithelium during oestrus. *Theriogenology* **75** 671-678.

Diaz, MJ, S Giunta, J Valz-Gianinet, S Pereyra-Alfonso, V Flores, and D Miceli 2000 Proteases with plasminogen activator activity in hamster oviduct. *Molecular Reproduction and Development* **55** 47-54.

Duc-Goiran, P, TM Mignot, C Bourgeois, and F Ferre 1999 Embryo-maternal interactions at the implantation site: a delicate equilibrium. *European Journal of Obstetrics Gynecology and Reproductive Biology* **83** 85-100.

Duong, TD, and CA Erickson 2004 MMP-2 plays an essential role in producing epithelial-mesenchymal transformations in the avian embryo. *Developmental Dynamics* **229** 42-53.

Faul, F, E Erdfelder, AG Lang, and A Buchner 2007 G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39** 175-191.

Fazeli, A 2008 Maternal communication with gametes and embryos. *Theriogenology* **70** 1182-1187.

Franczak, A, and M Bogacki 2009 Local and Systemic Effects of Embryos on Uterine Tissues During Early Pregnancy in Pigs. *Journal of Reproduction and Development* **55** 262-272.

Franczak, A, B Wojciechowicz, A Zmijewska, J Kolakowska, and G Kotwica 2013 The effect of interleukin 1 beta and interleukin 6 on estradiol-17 beta secretion in the endometrium of pig during early pregnancy and the estrous cycle. *Theriogenology* **80** 90-98.

Frolova, A, L Flessner, M Chi, ST Kim, N Foyouzi-Yousefi, KH Moley, and H Moley 2009 Facilitative Glucose Transporter Type 1 Is Differentially Regulated by Progesterone and Estrogen in Murine and Human Endometrial Stromal Cells. *Endocrinology* **150** 1512-1520.

Gabler, C, GJ Killian, and R Einspanier 2001 Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction* **122** 121-130.

Georgiou, AS, E Sostaric, CH Wong, APL Snijders, PC Wright, HD Moore, and A Fazeli 2005 Gametes alter the oviductal secretory proteome. *Molecular & Cellular Proteomics* **4** 1785-1796.

Ginther, OJ 1992 Reproductive biology of the mare. Basic and applied aspects. Cross Plains, Wisconsin: Cross Plains, Equiservices.

Ginther, OJ, EL Gastal, MO Gastal, MD Utt, and MA Beg 2007 Luteal blood flow and progesterone production in mares. *Animal Reproduction Science* **99** 213-220.

Goossens, K, A Van Soom, M Van Poucke, L Vandaele, J Vandesompele, A Van Zeveren, and LJ Peelman 2007 Identification and expression analysis of genes associated with bovine blastocyst formation. *Bmc Developmental Biology* **7**.

Gstraunthaler, G 2003 Alternatives to the use of fetal bovine serum: Serum-free cell culture. *Altex-Alternativen Zu Tierexperimenten* **20** 275-281.

Hai, MTV, F Logeat, M Warembourg, and E Milgrom 1977 Hormonal-Control of Progesterone Receptors. *Annals of the New York Academy of Sciences* **286** 199-209.

Hanukoglu, I 1992 Steroidogenic Enzymes - Structure, Function, and Role in Regulation of Steroid-Hormone Biosynthesis. *Journal of Steroid Biochemistry and Molecular Biology* **43** 779-804.

Hashizume, K, T Takahashi, M Shimizu, J Todoroki, A Shimada, M Hirata, T Sato, and A Ito 2003 Matrix-metalloproteinases-2 and-9 production in bovine endometrial cell culture. *Journal of Reproduction and Development* **49** 45-53.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hunter, RHF 2005 The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction Nutrition Development* **45** 281-290.

Hunter, RHF 2012 Components of oviduct physiology in eutherian mammals. *Biological Reviews* **87** 244-255.

Jones, JI, and DR Clemmons 1995 Insulin-Like Growth-Factors and Their Binding-Proteins - Biological Actions. *Endocrine Reviews* **16** 3-34.

Kendle, KE, and B Lee 1980 Investigation of the Influence of Progesterone on Mouse Embryo Transport by Using Anti-Progestational Steroids. *Journal of Reproduction and Fertility* **58** 253-258.

Kennedy, TG, KD Brown, and TJ Vaughan 1994 Expression of the Genes for the Epidermal Growth-Factor Receptor and Its Ligands in Porcine Oviduct and Endometrium. *Biology of Reproduction* **50** 751-756.

Kervancioglu, ME, E Saridogan, T Atasu, T Camlibel, A Demircan, B Sarikamis, and O Djahanbakhch 1997 Human Fallopian tube epithelial cell co-culture increases fertilization rates in male factor infertility but not in tubal or unexplained infertility. *Hum Reprod* **12** 1253-1258.

Kim, HY, J Sohn, GT Wijewickrama, P Edirisinghe, T Gherezghiher, M Hemachandra, PY Lu, RE Chandrasena, ME Molloy, DA Tonetti, and GR Thatcher 2010 Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens. *Bioorg Med Chem* **18** 809-821.

Kim, NH, and AR Menino 1995 Effects of Stimulators of Protein Kinase-a and Kinase-C and Modulators of Phosphorylation on Plasminogen-Activator Activity in Porcine Oocyte-Cumulus Cell Complexes during in-Vitro Maturation. *Molecular Reproduction and Development* **40** 364-370.

King, SR, and HA LaVoie 2012 Gonadal transactivation of STARD1, CYP11A1 and HSD3B. *Frontiers in Bioscience-Landmark* **17** 824-846.

Kobayashi, F, SJ Zimniski, and KN Smalley 1996 Characterization of oviductal aromatase in the northern leopard frog, Rana pipens. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **113** 653-657.

Kouba, AJ, BR Burkhardt, IM Alvarez, MM Goodenow, and WC Buhi 2000 Oviductal plasminogen activator inhibitor-1 (PAI-1): mRNA, protein, and hormonal regulation during the estrous cycle and early pregnancy in the pig. *Molecular Reproduction and Development* **56** 378-386.

Krysko, DV, T Vanden Berghe, K D'Herde, and P Vandenabeele 2008 Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods* **44** 205-221.

LaVoie, HA, and SR King 2009 Transcriptional Regulation of Steroidogenic Genes: STARD1, CYP11A1 and HSD3B. *Experimental Biology and Medicine* **234** 880-907.

Leclerc, E, Y Sakai, and T Fujii 2003 Cell Culture in 3-Dimensional Microfluidic Structure of PDMS (polydimethylsiloxane). *Biomedical Microdevices* **5** 109-114.

Lei, ZM, and CV Rao 1992 Expression of Epidermal Growth-Factor (Egf) Receptor and Its Ligands, Egf and Transforming Growth Factor-Alpha, in Human Fallopian-Tubes. *Endocrinology* **131** 947-957.

Leist, M, and M Jaattela 2001 Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2** 589-598.

Lenth, RV 2007 Statistical power calculations. *Journal of Animal Science* **85** E24-E29.

Leroith, D, H Werner, D Beitnerjohnson, and CT Roberts 1995a Molecular and Cellular Aspects of the Insulin-Like Growth-Factor-I Receptor. *Endocrine Reviews* **16** 143-163.

LeRoith, D, H Werner, S Neuenschwander, T Kalebic, and LJ Helman 1995b The role of the insulin-like growth factor-I receptor in cancer. *Receptor Activation by Antigens, Cytokines, Hormones, and Growth Factors* **766** 402-408.

Levanon, K, V Ng, HY Piao, Y Zhang, MC Chang, MH Roh, DW Kindelberger, MS Hirsch, CP Crum, JA Marto, and R Drapkin 2010 Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* **29** 1103-1113.

Li, Y, L Qin, ZH Xiao, YL Wang, R Herva, JH Leng, JH Lang, V Isomaa, and YS Piao 2003 Expression of P450 aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 at fetal-maternal interface during tubal pregnancy. *Journal of Steroid Biochemistry and Molecular Biology* **87** 241-246.

Mahmood, T, E Saridogan, S Smutna, AM Habib, and O Djahanbakhch 1998 The effect of ovarian steroids on epithelial ciliary beat frequency in the human Fallopian tube. *Human Reproduction* **13** 2991-2994.

McCue, PM, and EL Squires 2002 Persistent anovulatory follicles in the mare. *Theriogenology* **58** 541-543.

McKinnon, AO 1997 Ovarian abnormalities. *In* MAO Ranaten N.W. (ed.), Equine diagnostic ultrasonography, pp. 233-251. Baltimore: Williams and Wilkins.

Miessen, K, S Sharbati, R Einspanier, and J Schoen 2011 Modelling the porcine oviduct epithelium: A polarized in vitro system suitable for long-term cultivation. *Theriogenology* **76** 900-910.

Morishige, KI, H Kurachi, K Amemiya, H Adachi, K Adachi, Y Sakoyama, A Miyake, and O Tanizawa 1993 Menstrual Stage-Specific Expression of Epidermal Growth-

Factor and Transforming Growth-Factor-Alpha in Human Oviduct Epithelium and Their Role in Early Embryogenesis. *Endocrinology* **133** 199-207.

Munabi, AK, FG Cassorla, DG Pfeiffer, BD Albertson, and DL Loriaux 1983 The effects of estradiol and progesterone on rat ovarian 17-hydroxylase and 3ß-hydroxysteroid dehydrogenase activities. *Steroids* **41** 95-98.

Nelis, H, K D'Herde, K Goossens, L Vandenberghe, B Leemans, K Forier, K Smits, K Braeckmans, L Peelman, and A Van Soom 2014 Equine oviduct explant culture: a basic model to decipher embryo–maternal communication. *Reproduction, Fertility and Development* **26** 954-966.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015 Steroids in the equine oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*.

Nguyen, DH, T Zhou, J Shu, and J-H Mao 2013 Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity., Cancer InCytes, edn Summer 2013. Santa Monica.

Nielsen, OB, F de Paoli, and K Overgaard 2001 Protective effects of lactic acid on force production in rat skeletal muscle. *Journal of Physiology-London* **536** 161-166.

Ohe, T, M Hirobe, and T Mashino 2000 Novel metabolic pathway of estrone and 17 beta-estradiol catalyzed by cytochrome P-450. *Drug Metabolism and Disposition* **28** 110-112.

Page-McCaw, A, AJ Ewald, and Z Werb 2007 Matrix metalloproteinases and the regulation of tissue remodelling. *Nature Reviews Molecular Cell Biology* **8** 221-233.

Pampfer, S, S Tabibzadeh, FC Chuan, and JW Pollard 1991 Expression of Colony-Stimulating Factor-I (Csf-1) Messenger-Rna in Human Endometrial Glands during the Menstrual-Cycle - Molecular-Cloning of a Novel Transcript That Predicts a Cell-Surface Form of Csf-1. *Molecular Endocrinology* **5** 1931-1938.

Pepper, MS, D Belin, R Montesano, L Orci, and JD Vassalli 1990 Transforming Growth Factor-Beta-1 Modulates Basic Fibroblast Growth-Factor Induced Proteolytic and Angiogenic Properties of Endothelial-Cells Invitro. *Journal of Cell Biology* **111** 743-755.

Pfeifer, TL, and N Chegini 1994 Immunohistochemical Localization of Insulin-Like Growth-Factor (Igf-I), Igf-I Receptor, and Igf Binding-Proteins-1-4 in Human Fallopian-Tube at Various Reproductive Stages. *Biology of Reproduction* **50** 281-289.

Pierson, RA 1993 Folliculogenesis and ovulation. . *In* VJL McKinnon A.O. (ed.), Equine reproduction. , pp. 151-161. Philadelphia: Lea and Febiger.

Pierson, RA, and OJ Ginther 1985 Ultrasonic evaluation of the corpus luteum of the mare. *Theriogenology* **23** 795-806.

Rajagopal, M, TL Tollner, WE Finkbeiner, GN Cherr, and JH Widdicombe 2006 Differentiated structure and function of primary cultures of monkey oviductal epithelium. *In Vitro Cellular & Developmental Biology-Animal* **42** 248-254.

Richardson, LL, and G Oliphant 1981 Steroid Concentrations in Rabbit Oviducal Fluid during Estrus and Pseudopregnancy. *Journal of Reproduction and Fertility* **62** 427-431.

Rodgers, WH, LM Matrisian, LC Giudice, B Dsupin, P Cannon, C Svitek, F Gorstein, and KG Osteen 1994 Patterns of Matrix Metalloproteinase Expression in Cycling Endometrium Imply Differential Functions and Regulation by Steroid-Hormones. *Journal of Clinical Investigation* **94** 946-953.

Roldan-Olarte, M, M Jimenez-Diaz, and DC Miceli 2005 Plasminogen detection in oocytes and plasminogen activator activities in the porcine oviduct during the estrous cycle. *Zygote* **13** 115-123.

Rose, IA, and JVB Warms 1966 Control of Glycolysis in Human Red Blood Cell. *Journal of Biological Chemistry* **241** 4848-&.

Rottmayer, R, SE Ulbrich, S Kolle, K Prelle, C Neumueller, F Sinowatz, HHD Meyer, E Wolf, and S Hiendleder 2006 A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* **132** 637-648.

Schell, DL, PA Mavrogianis, AT Fazleabas, and HG Verhage 1994 Epidermal Growth-Factor, Transforming Growth-Factor-Alpha, and Epidermal Growth-Factor Receptor Localization in the Baboon (Papio-Anubis) Oviduct during Steroid Treatment and the Menstrual-Cycle. *Journal of the Society for Gynecologic Investigation* **1** 269-276.

Schmidt, A, R Einspanier, W Amselgruber, F Sinowatz, and D Schams 1994 Expression of Insulin-Like Growth-Factor-1 (Igf-1) in the Bovine Oviduct during the Estrous-Cycle. *Experimental and Clinical Endocrinology* **102** 364-369.

Schneider, CA, WS Rasband, and KW Eliceiri 2012 NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9** 671-675.

Seytanoglu, A, AS Georgiou, E Sostaric, PF Watson, WV Holt, and A Fazeli 2008 Oviductal cell proteome alterations during the reproductive cycle in pigs. *Journal of Proteome Research* **7** 2825-2833.

Shao, R, E Egecioglu, B Weijdegard, JJ Kopchick, J Fernandez-Rodriguez, N Andersson, and H Billig 2007 Dynamic regulation of estrogen receptor-alpha isoform expression in the mouse fallopian tube: mechanistic insight into estrogen-dependent production and secretion of insulin-like growth factors. *American Journal of Physiology-Endocrinology and Metabolism* **293** E1430-E1442.

Simard, J, ML Ricketts, B Gingras, P Soucy, FA Feltus, and MH Melner 2005 Molecular biology of the 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase gene family. *Endocrine Reviews* **26** 525-582.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, KG, K.; Van Soom, A.; Govaere, J.; Hoogewijs, M.; Vanhaesebrouck, E.; Galli, C.; Colleoni, S.; Vandesompele, J.; Peelman, L. 2009 Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246.

Spilman, CH, and JW Wilks 1976 Peripheral Plasma Progesterone during Egg Transport in Rabbit. *Proceedings of the Society for Experimental Biology and Medicine* **151** 726-729.

Stevenson, KR, and DC Wathes 1996 Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *Journal of Reproduction and Fertility* **108** 31-40.

Stocco, DM 2001 StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* **63** 193-213.

Stocco, DM, XJ Wang, Y Jo, and PR Manna 2005 Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: More complicated than we thought. *Molecular Endocrinology* **19** 2647-2659.

Suarez, S, K Redfern, P Raynor, F Martin, and DM Phillips 1991 Attachment of Boar Sperm to Mucosal Explants of Oviduct Invitro - Possible Role in Formation of a Sperm Reservoir. *Biology of Reproduction* **44** 998-1004.

Sukerkar, PA, KW MacRenaris, TR Townsend, RA Ahmed, JE Burdette, and TJ Meade 2011 Synthesis and Biological Evaluation of Water-Soluble Progesterone-Conjugated Probes for Magnetic Resonance Imaging of Hormone Related Cancers. *Bioconjugate Chemistry* **22** 2304-2316.

Swan, CL, MC Agostini, PM Bartlewski, V Feyles, RJ Urban, and PJ Chedrese 2002 Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: Control of transcriptional activity of the cytochrome P450 side-chain cleavage gene. *Biology of Reproduction* **66** 959-965.

Szafranska, B, A Ziecik, and S Okrasa 2002 Primary antisera against selected steroids or proteins and secondary antisera against gamma-globulins--an available tool for studies of reproductive processes. *Reproductive Biology* **2** 187-204.

Tadokoro, C, Y Yoshimoto, M Sakata, T Imai, M Yamaguchi, H Kurachi, Y Oka, T Maeda, and A Miyake 1995 Expression and Localization of Glucose-Transporter-1 (Glut1) in the Rat Oviduct - a Possible Supplier of Glucose to Embryo during Early Embryonic-Development. *Biochem Biophys Res Commun* **214** 1211-1218.

Takeda, T, Y Tsutsumi, S Hara, and M Ida 1978 Effects of Prostaglandin-F2-Alpha on Egg Transport and Invivo Egg Recovery from Vaginas of Rabbits. *Fertility and Sterility* **30** 79-85.

Tetsuka, M, M Milne, and SG Hillier 1998 Expression of oestrogen receptor isoforms in relation to enzymes of oestrogen synthesis in rat ovary. *Molecular and Cellular Endocrinology* **141** 29-35.

Thomas, PGA, BA Ball, and SP Brinsko 1994 Interaction of Equine Spermatozoa with Oviduct Epithelial-Cell Explants Is Affected by Estrous-Cycle and Anatomic Origin of Explant. *Biology of Reproduction* **51** 222-228.

Tibbetts, TA, M Mendoza-Meneses, BW O'Malley, and OM Conneely 1998 Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biology of Reproduction* **59** 1143-1152.

Ulbrich, SE, A Kettler, and R Einspanier 2003 Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. *The Journal of Steroid Biochemistry and Molecular Biology* **84** 279-289.

Ullrich, A, A Gray, AW Tam, T Yangfeng, M Tsubokawa, C Collins, W Henzel, T Lebon, S Kathuria, E Chen, S Jacobs, U Francke, J Ramachandran, and Y Fujitayamaguchi 1986 Insulin-Like Growth Factor-I Receptor Primary Structure - Comparison with Insulin-Receptor Suggests Structural Determinants That Define Functional Specificity. *Embo Journal* **5** 2503-2512.

Urban, RJ, JC Garmey, MA Shupnik, and JD Veldhuis 1991 Follicle-Stimulating-Hormone Increases Concentrations of Messenger-Ribonucleic-Acid Encoding Cytochrome-P450 Cholesterol Side-Chain Cleavage Enzyme in Primary Cultures of Porcine Granulosa-Cells. *Endocrinology* **128** 2000-2007.

Van den Broeck, W, M D'haeseleer, M Coryn, and P Simoens 2002 Cell-specific distribution of progesterone receptors in the bovine ovary. *Reproduction in Domestic Animals* **37** 164-170.

Vandesompele, J, K De Preter, F Pattyn, B Poppe, N Van Roy, A De Paepe, and F Speleman 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**.

Walter, I 1995 Culture of Bovine Oviduct Epithelial-Cells (Boec). *Anatomical Record* **243** 347-356.

Watson, AJ, LC Barcroft, DH Bets, PA DeSousa, E Gilfoyle, J Looye, J PierreLouis, and QA Winger 1996 Maternal and embryonic control of bovine pre-attachment development: Expression of oviductal and embryonic genes. *Archiv Fur Tierzucht-Archives of Animal Breeding* **39** 55-69.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991a Prostaglandin E2 hastens oviductal transport of equine embryos. *Biol Reprod* **45** 544-546.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991b Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45** 540-543.

Wessel, T, U Schuchter, and H Walt 2004 Ciliary motility in bovine oviducts for sensing rapid non-genomic reactions upon exposure to progesterone. *Hormone and Metabolic Research* **36** 136-141.

West, NB, and RM Brenner 1985 Progesterone-Mediated Suppression of Estradiol Receptors in Cynomolgus Macaque Cervix, Endometrium and Oviduct during Sequential Estradiol-Progesterone Treatment. *Journal of Steroid Biochemistry and Molecular Biology* **22** 29-37.

West, NB, HG Verhage, and RM Brenner 1977 Changes in Nuclear Estradiol Receptor and Cell Structure during Estrous Cycles and Pregnancy in Oviduct and Uterus of Cats. *Biology of Reproduction* **17** 138-143.

Wijayagunawardane, MPB, A Miyamoto, WA Cerbito, TJ Acosta, M Takagi, and K Sato 1998 Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* **49** 607-618.

Wollenhaupt, K, A Kettler, Br, K-P ssow, F Schneider, W Kanitz, and R Einspanier 2001 Regulation of the expression and bioactivation of the epidermal growth factor receptor system by estradiol in pig oviduct and endometrium. *Reproduction, Fertility and Development* **13** 167-176.

Wollenhaupt, K, U Tiemann, R Einspanier, F Schneider, W Kanitz, and KP Brussow 1997 Characterization of the epidermal growth factor receptor in pig oviduct and endometrium. *Journal of Reproduction and Fertility* **111** 173-181.

Yamada, M, T Horiuchi, T Oribe, S Yamamoto, H Matsushita, and PA Gentry 1996 Plasminogen activator activity in the bovine oocyte-cumulus complex and early embryo. *Journal of Veterinary Medical Science* **58** 317-322.

Zhao, Y, N Chegini, and KC Flanders 1994 Human Fallopian-Tube Expresses Transforming Growth-Factor (Tgf-Beta) Isoforms, Tgf-Beta Type I-Iii Receptor Messenger-Ribonucleic-Acid and Protein, and Contains [I-125] Tgf-Beta-Binding Sites. *Journal of Clinical Endocrinology & Metabolism* **79** 1177-1184.

Zhao, YG, AZ Xiao, XM Cao, and C Zhu 2002 Expression of matrix metalloproteinase-2,-9 and tissue inhibitors of metalloproteinase-1,-2,-3 mRNAs in rat uterus during early pregnancy. *Molecular Reproduction and Development* **62** 149-158.

PART 5.2: OF EMBRYOS AND OVIDUCTAL GENES *IN VITRO*

Adapted from:

Co-culture with equine embryos affects gene expression in equine oviductal epithelial explants.

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CO-CULTURE WITH EQUINE EMBRYOS AFFECTS GENE EXPRESSION IN EQUINE OVIDUCTAL EPITHELIAL EXPLANTS

ABSTRACT

The equine embryo spends an exceptionally long time in the oviduct. While studies in other species demonstrated an effect of the presence of embryos on oviductal gene expression and protein production, little is known about this interaction in the horse. The aim of this study was to determine the effect of co-culture of horse embryos and oviduct epithelial explants on function and gene expression of those explants. Equine oviduct explants and embryos were produced *in vitro* and cultured in 50 µl-droplets of DMEM-F12 with 10% FCS. After 9.5 days of co-culture, explants were evaluated for ciliary activity and subsequently frozen for gene expression analysis of 11 embryotrophic genes by RT-qPCR. The ciliary beating of the explants was not affected, but co-culture affected gene expression. The explants cultured in the presence of embryos showed a significant upregulation of *PTGER2*, *TIMP1* ($0.0005 < P < 0.005$) and *TGFA* and *MMP2* ($0.01 < P < 0.05$). A tendency ($0.05 < P < 0.09$) of upregulation was observed for *CSF1* and *PAI1*. Expression of *HIF1A, TGFA, PTGER4, VEGFA* and *PLAU* was unaffected. The upregulated genes have been described to stimulate embryonic development and embryo transport to the uterus, and to modulate oviductal matrix turnover. In conclusion, co-culture did not affect ciliary activity of oviduct explants, but expression of 4 embryotrophic genes was significantly upregulated indicating that the equine embryo can convey its presence to the oviduct explant *in vitro*.

INTRODUCTION

The dynamic, fine-tuned environment of the oviduct provides the optimal environment for early embryonic development. The oviduct epithelium alters its secretory profile in response to cycle-stage related and steroid-induced changes (Bauersachs, et al. 2003b, Bauersachs, et al. 2004). The oviduct does not only respond to endocrine signals and to signals from gametes (Bauersachs, et al. 2003b, Fazeli, et al. 2004, Georgiou, et al. 2005, Kodithuwakku, et al. 2007) but also to embryo-secreted factors. Indeed, porcine, murine and bovine embryos *in vivo* influence the oviduct in order to create their own micro-environment by modulating the oviductal transcriptome and proteome (Chang, et al. 2000, Lee, et al. 2002, Maillo, et al. 2015). A myriad of proteins are involved in the dialogue between the oviduct and the embryo.

The molecules performing this dialogue include growth factors, cytokines, angiogenic factors, apoptotic factors and adhesion molecules (Hill 2006). These ligands and receptors exert their effects in a precisely orchestrated interaction between the embryo and the maternal genital tract, resulting in the activation of signal transduction pathways, inducing changes in the embryonic (Cordova, et al. 2014, Lee, et al. 2001, Lloyd, et al. 2009) or the oviductal transcriptomic profile (Bauersachs, et al. 2003b, Fazeli, et al. 2004, Georgiou, et al. 2005, Kodithuwakku, et al. 2007, Lee, et al. 2002). In the horse, a striking and unique example of an early maternal response to the embryo's signaling is the stage-specific production of PGE2 by the equine conceptus in order to relax the oviductal smooth muscle cells of the uterotubal junction to allow uterine entry (Freeman, et al. 1992, Weber, et al. 1991a, b). The intimate dialogue between the mature oocyte, the fertilizing sperm and the oviduct seems to be crucial since conventional *in vitro* fertilization in the horse yields discouraging and non-reproducible results (Hinrichs 2010). Moreover, equine embryos produced *in vitro* by ICSI are genetically, functionally, morphologically and developmentally aberrant compared with their *in vivo* counterparts (Smits, et al. 2011, Smits, et al. 2012b, Tremoleda, et al. 2003). This emphasizes that especially in the horse, early embryo development is critically dependent on the efficient interplay with the oviduct.

It can be assumed that the oviduct represents the most optimal environment for the early-stage equine embryo. Hence, we suppose that oviductal cells derived from the mare provide specific mitogenic factors that would normally be present in the oviduct or non-specific factors that improve the culture environment by reduction of oxygen tension, by removing waste products or by providing substrates and co-factors (Orsi and Reischl 2007). There are some data confirming this hypothesis. Successful attempts have been undertaken to improve *in vitro* fertilization and blastocyst rates using an oviductal co-culture system in humans, cows and pigs (Bongso and Fong 1993, Ellington, et al. 1990, Eyestone and First 1989, Rexroad 1989, Sakkas, et al. 1989, Smith, et al. 1992, Xu, et al. 2000). However, in the horse, a positive effect of oviduct co-culture has up to now been described with *in vivo* (Ball, et al. 1993, Freeman, et al. 1991, Weber, et al. 1993) but not with *in vitro*-derived (Choi, et al. 2004b) embryos. Moreover, very few signals involved in the embryo-maternal interplay have been identified in the horse so far (Ambruosi, et al. 2013, Mugnier, et al. 2009, Weber, et al. 1991b) and data on alterations in the gene expression of the equine oviduct in response to the presence of developing embryos are lacking. In the light of the improvement of *in vitro*

equine embryo production and the optimization of a (semi-)defined medium, a thorough understanding of the underlying mechanisms of the embryo-oviductal interplay is required. The aim of the study was to elucidate the role of developing embryos on the gene expression in the oviduct. Therefore, the mRNA expression of a set of genes is evaluated in oviductal cells cultured with or without equine zygotes produced by ICSI. These genes, plasminogen activator inhibitor 1 (*PAI1*), urokinase plasminogen activator inhibitor (*PLAU*), transforming growth factor α (*TGFA*), tissue inhibitor of matrix metalloproteinase-1 (*TIMP1*), matrix metalloproteinase (*MMP*2), colony stimulating factor (*CSF1*), prostaglandin receptor E2 (*PTGER2*) and E4 (*PTGER4*), vascular endothelial growth factor (*VEGFA*), hypoxia inducible factor 1α (*HIF1A*) and glucose transporter 1 (*GLUT1*) were selected based on their embryotrophic, embryo protective function (Ball, et al. 2013, Buhi, et al. 2000, Duc-Goiran, et al. 1999) or hypoxia-marking capacity (Chi, et al. 2006). In addition, the effects on ciliary activity and the occurrence of dark cell degeneration, as described earlier in our oviduct explant model (Nelis, et al. 2014), were assessed. The influence of co-culture on blastocyst percentage and blastocyst diameter was also evaluated.

MATERIALS AND METHODS

Equine embryo production

Oocytes were collected, matured and fertilized and zygotes were cultured as described earlier (Smits, et al. 2010, Smits, et al. 2012a). Briefly, ovaries were collected from slaughtered mares and all follicles larger than 5 mm were aspirated. The oocytes were matured during 26 hours in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12; Invitrogen) based medium in an atmosphere containing 5% CO_2 in air (Galli *et al.*, 2007). After the removal of the surrounding cumulus cells by means of gentle pipetting, mature oocytes with a polar body were fertilized by Piezo-driven ICSI. Frozen sperm from a stallion of proven fertility was thawed, centrifuged over a 90%/45% Percoll® gradient, washed with calcium free Tyrode's Albumin-Lactate-Pyruvate (TALP) solution and centrifuged again at 400 x g for 10 minutes. During ICSI the oocytes were kept in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered SOF medium and the sperm in 9% polyvinylpyrrolidone in PBS. All manipulations were performed on a heated plate (38.5 °C) of an inverted microscope. ICSI was performed using a Piezo Drill (*Prime Tech* Ltd., Ibaraki,

Japan) as described before (Smits, et al. 2012a). The injected oocytes were cultured in groups of 14-21 embryos in 50 µl droplets of DMEM-F12 with 10% fetal calf serum at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2. On day 2.5 after fertilization, the embryos which were not cleaved, were removed and half of the medium was changed. On day 6, again half of the medium was changed and on day 9.5 the embryonic development was evaluated. The size of the blastocysts was measured using a stereomicroscope fitted with an eyepiece micrometer.

Equine oviduct explant culture and evaluation

Oviduct epithelial explants were obtained as described in Nelis et al., (2012).

Vitality was judged by evaluating ciliary activity. Only cultures with more than 99% of membrane intact cells, determined by Trypan blue staining (Sigma-Aldrich, Diegem, Belgium), 18 h after start of culture were used. The time span from slaughter of mares to seeding of cells was approximately 3 to 4 h. Explants were considered showing ciliary activity when bordered by vigorously beating cilia, clearly seen on the inverted microscope (400x magnification). The number of explants showing dark zones (Nelis, et al. 2014) was also determined in each group.

Experimental designs

Equine oviduct explants (n = 30-35) were cultured without **(Fig. 1 a)** and with **(Fig. 1 b)** equine ICSI-derived zygotes (n = 14-21). In a third group, equine zygotes were cultured without explants **(Fig. 1 c)**. Ciliary activity, black zones and gene expression in oviduct explants were evaluated after 9.5 days of culture. Embryo cleavage rate and, blastocyst percentage and diameter were evaluated after respectively 2.5 and 9.5 days.

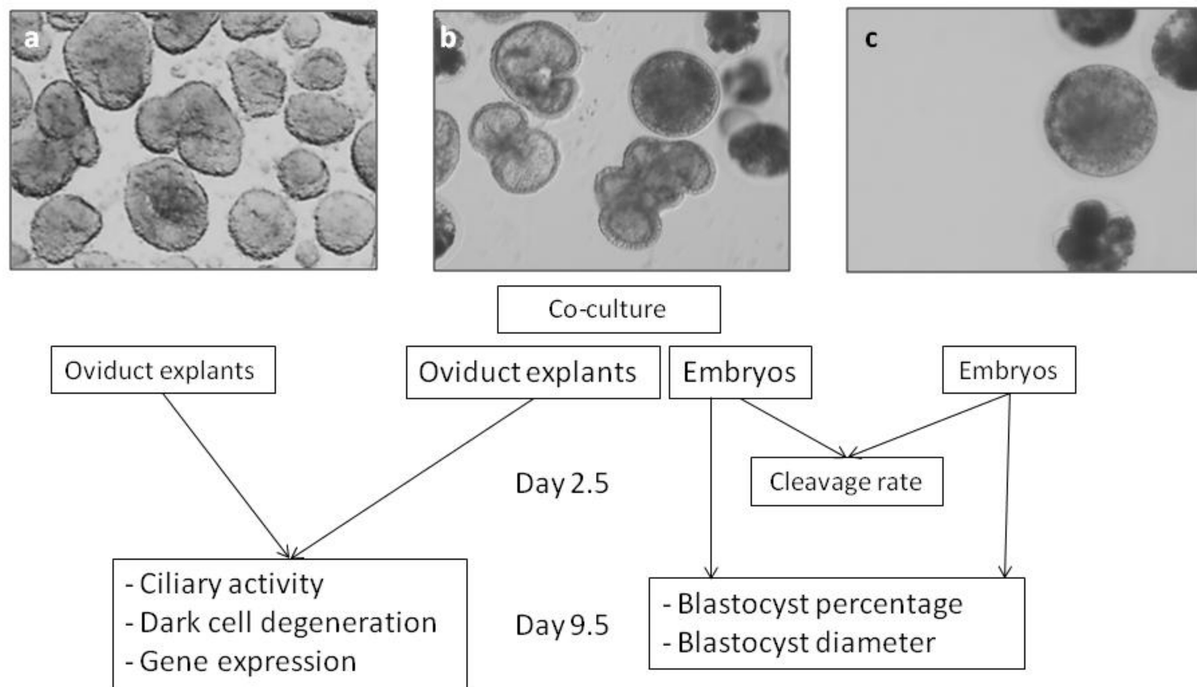

Fig. 1. Experimental set-up. Oviduct explants were cultured without (a) and with equine developing ICSI-derived embryos (b) for 9.5 days. The effect of the presence of the embryos on ciliary activity, the prevalence of dark cell degeneration and gene expression was assessed. On the other hand, the effect of co-culture on cleavage rate and blastocyst percentage and diameter was evaluated. Embryos cultured without oviduct cells served as control (c).

RNA extraction and quantitative RT-PCR

After 9.5 days of culture, ciliary activity and the prevalence of dark cell degeneration was evaluated as aforementioned. The explants were washed 2 times in PBS (14190, Gibco, Invitrogen, Ghent, Belgium) and subsequently snap-frozen in lysis buffer and stored at -80°C in cryovials. Lysis buffer constituted of 10% RNasin Plus RNase inhibitor (Promega, Leiden, The Netherlands), 5% dithiothreitol (Promega, Leiden, The Netherlands), 0.8% Igepal CA-630 (Sigma Aldrich, Diegem Belgium) in RNase free water (Qiagen, Venlo, The Netherlands). Total RNA was extracted with the RNeasy mini kit (Qiagen) according to the manufacturers' instructions, including DNAse digestion and transcribed into cDNA (iScript cDNA synthesis kit, Bio-Rad, Nazareth, Belgium) as described earlier (Nelis, et al. 2014). Primer design and validation, RNA extraction and quantitative RT-PCR were performed according to the MIQE-guidelines (Bustin, et al. 2009) and as also reported earlier (Nelis, et al. 2014). The sequences of forward and reverse primers, optimal primer annealing temperature, and amplification

efficiency of all primers (Integrated DNA Technologies, Leuven, Belgium and Sigma-Aldrich, Bornem, Belgium) of the genes of interest (*CSF1, GLUT1, HIF1A, MMP2, PAI1, PTGER2, PTGER4, TGFA, TIMP1, PLAU* and *VGFA)* and of the reference genes (*ACTB, UBB* and *RPL32*) have been reported earlier (Nelis, et al. 2014, Nelis, et al. 2015b).

All 252 RT-qPCR reactions (9 explant samples per group (n = 2); 3 reference genes and 11 target genes) were performed in duplicate with 2.5 µl of sample, 7.5 µl of the KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Woburn, MA, USA), 0.6 µl of 10 µM forward and reverse primer and 3.8 µl of water. A blank, a melting curve and a 5- or 10-fold serial dilution series of pooled oviductal cDNA were included for each gene to check for contamination and specificity and to acquire PCR efficiencies (Nelis, et al. 2014, Nelis, et al. 2015b) based on a relative standard curve. Calculation of the Cq values, PCR efficiencies, correlation coefficients and analysis of the melting curves was performed by means of iCycler iQ Optical System Software Version 3.0a (Biorad, Nazareth, Belgium). All quantification cycle (C_q) values were converted into raw data using these PCR efficiencies and normalised by dividing them by their respective normalisation factor. This normalisation factor was determined per sample by calculating the geometric mean of the validated reference genes *UBB, ACTB* and *RPL32* (Bogaert, et al. 2006, Nelis, et al. 2014, Nelis, et al. 2015b, Smits 2009).

Statistical analysis

To determine significant differences in the blastocyst rate, binary logistic regression with the Nagelkerke pseudo R^2 and χ^2 fit tests was implemented. Independent samples t-tests or independent samples Mann-Whitney U-test as implemented in SPSS 19 for Windows (SPSS IBM, Brussels, Belgium) were used to compare target gene expression differences and blastocyst diameters, depending on whether or not a Gaussian distribution was obtained after logarithmic transformation of the data. Differences were considered significant at $P < 0.05$. Statistical analysis and graph plotting was performed with SPSS 21. Power analysis and sample size calculation ($\alpha = 0.05$; power = 0.85 - 0.99) for gene expression studies were performed using Piface version 1.7 (Lenth 2007); University of Iowa; http://homepage.stat.uiowa.edu/,rlenth/Power/; accessed July 2013).

RESULTS

The influence of co-culture on blastocyst rate and diameter

Of the total number of 82 and 84 injected oocytes (3 replicates), 53 and 52 zygotes reached the cleavage stage in the control respectively the co-cultured group. Six (11%), respectively 12 (23%) of these 2-cell stage embryos reached the blastocyst stage. A tendency for increased blastocyst rate in the co-culture group could be observed, albeit not significant (P = 0.1; **Table 1**). No significant difference in blastocyst diameter was detected (P > 0.05; **Table 1**).

Table 1. Percentage of cleaved oocytes that develop to the blastocyst stage and blastocyst diameter when cultured with and without oviduct explants for 9.5 days (co-culture) . SD = standard deviation.

	Injected oocytes	Cleaved zygotes (% of injected oocytes)	Blastocyst stage (% of cleaved oocytes)	Blastocyst diameter +/- SD (μm)
no co-culture	82	53 (65 %)	6 (11 %)	153.4 +/- 14.9
co-culture	84	52 (62 %)	12 (23 %)	154.6 +/- 13.3

The influence on the functionality and morphology of oviduct explants

No significant differences were observed in the percentage of explants showing dark cell degeneration. 49 (64/313) of the control and 42 % (55/131) of the co-cultured explants showed dark cell degeneration (P = 0.264) whereas 59 (77/131) and 60 % (78/131) showed ciliary activity (P = 0.269).

The influence of co-culture on the gene expression of oviduct explants

A highly significant upregulation of the RNA expression of *PTGER2* (2-fold) and *TIMP1* (4-fold) (0.0005 < P <0.005), and a significant upregulation of the RNA expression of *MMP2* (3.5-fold) and *TGFA* (2-fold) were observed (0.01< P < 0.05) in the co-cultured explants compared to the control preovulatory explants. A tendency to upregulation (0.05 < P < 0.09;

1.5-fold) of both *CSF1* and *PAI1* expression in oviduct explants with embryo co-culture was shown. There were no differences in expression levels of *GLUT1, HIF1A*, *PLAU, PTGER4* and *VEGFA* between both groups **(Fig. 2**).

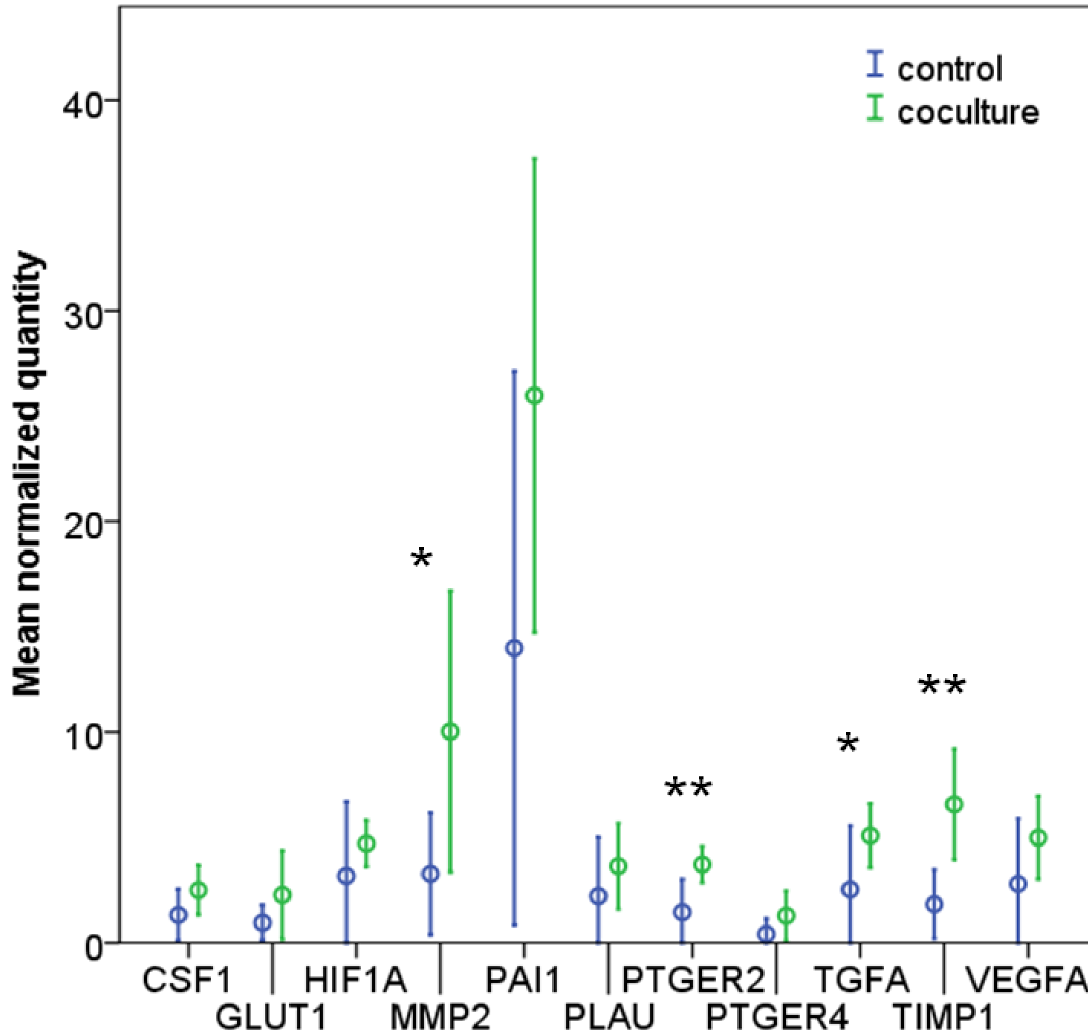

Fig. 2. Mean normalized gene mRNA expression of oviduct explants cultured with and without equine developing embryos. *MMP2, PTGER2*, *TGFA* and *TIMP1* were compared to the control group upregulated in response to the presence of embryos. Independent samples t-tests or independent samples Mann-Whitney U-test. Error bars = 95 % confidence interval with mean value. * 0.005 < P < 0.05; ** 0.0005 < P < 0.005.

DISCUSSION

In this study, we demonstrated that the equine embryo is able to signal its presence to the oviduct cells *in vitro* by modulating the oviduct's gene expression profile in order to establish an environment in favour of the optimal requirements of the early developing embryo.

The finding that the oviduct is able to respond to embryonic stimuli *in vitro* by altering its transcriptome has also recently been solidly established in the cow by means of microarray screening of the oviductal transcriptome (Schmaltz-Panneau, et al. 2014). In total, 33 genes, mostly involved in the delicate balance between pregnancy recognition and protection against the oviductal immune response, were upregulated in cultured oviductal cells as a reply to embryonic stimuli (Schmaltz-Panneau, et al. 2014). A similar effect on the oviductal transcriptome in response to early-stage embryos has also been reported in the *in vivo* embryo-bearing bovine (Maillo, et al. 2015), porcine (Alminana, et al. 2012) and human (Hess, et al. 2013) oviduct.

In our study, the mRNA expression of *PAI1*, *PLAU, TGFA*, *TIMP1, MMP*2, *CSF1*, *PTGER2*, *PTGER4*, *VEGFA*, *HIF1A* and *GLUT1* was evaluated in oviduct explants using RT-qPCR after 9.5 days of co-culture with ICSI-derived equine embryos. It was demonstrated earlier that these genes play a role in early reproductive processes (Ball, et al. 2013, Bhatnagar, et al. 1995, Buhi and Alvarez 2003, Buhi, et al. 1997, 2000, Chi, et al. 2006, Duc-Goiran, et al. 1999, Merkl, et al. 2010, Nelis, et al. 2015b, Nelis, et al. 2013, Roldan-Olarte, et al. 2012, Ulbrich, et al. 2011).

TIMP1, MMP2 and *PTGER2* were markedly upregulated (2 – 4-fold; $P < 0.05$) while *CSF1* and *PAI1* showed a tendency to upregulation (1.5-fold; $0.05 < P < 0.09$) in response to embryonic stimuli **(Fig. 2)**. It could consequently been deduced that these factors participate in the optimization of the oviductal milieu during the embryo's passage through the oviduct.

Indeed, MMPs and TIMPs are crucial factors in the remodeling of extracellular matrices (ECM) (Ulbrich, et al. 2011, Visse and Nagase 2003). The ECM consists of mainly collagen and proteoglycans through which metabolites, nutrients, ions and growth factors may diffuse (Ulbrich, et al. 2011). Thus, the ECM functions as a "mailbox" between the oviduct and the embryo (Wolf, et al. 2003a). MMPs degrade the ECM but contribute at the same time to the activation and liberation of growth factors, such as TGFA (Whiteside, et al. 2001) which was also upregulated in our study **(Fig. 2)**. These activated growth factors support embryo

development by modulating differentiation, cell migration and vascularisation (Nagase, et al. 2006). The inhibitors of MMPs, TIMPs, counterbalance the proteolytic effects of MMPs on both the oviduct and the embryo, in particular the zona pellucida (Buhi, et al. 1997, 2000) in order to create a well-balanced ECM turn-over (Nagase, et al. 2006, Ulbrich, et al. 2011). The biological importance of TIMPs has been substantiated *in vitro* in cows (Hwang, et al. 2000) and *in vivo* in mice (Nothnick 2001). Indeed, adding TIMP1 to bovine embryo culture medium increased the percentage of morulae or blastocysts (Hwang, et al. 2000) while TIMP-deficient mice exhibit lower pregnancy rates (Nothnick 2001). Interestingly, *TIMPs* were also upregulated in endometrial cells in mares (Klein, et al. 2010, Merkl, et al. 2010), cows (Ulbrich, et al. 2011), sheep (Gray, et al. 2006) and women (Giudice 1999) in response to an embryo during the first 2 weeks of pregnancy. This corroborates that TIMPs also play a considerable role in the uterus during the period of embryo mobility and attachment/implantation.

TGFA is also upregulated in co-cultured oviduct explants **(Fig. 2)**. The importance of TGFA in early embryo development is further substantiated by the fact that 4-cell porcine embryos *in vivo* are already able to strongly upregulate *TGFA* oviductal mRNA (Chang, et al. 2000). Moreover, TGFA reduces the number of apoptotic cells in mouse embryos (Brison and Schultz 1998) and has an effect on proliferation and differentiation of mammalian embryonic cells and extra embryonic tissues (Lysiak, et al. 1993, Reneker, et al. 1995).

As an obvious example that the equine embryo is able to communicate with its environment from day 5 on, it has been demonstrated that the equine embryo regulates its own transport through the oviduct by producing PGE2 in order to relax the smooth muscle cells to allow uterine entry (Freeman, et al. 1992, Weber, et al. 1991a, b). The fact that in the co-cultured explants, *PTGER2* but not *PTGER4* was upregulated **(Fig. 2)** may substantiate further the interaction of embryo-derived PGE2 with the oviductal PTGER2 but not with PTGER4, in order to open up the uterotubal papilla (Freeman, et al. 1992, Weber, et al. 1991a, b). Given the widespread distribution of PTGER2 in epithelial, endothelial and in muscular cells of the oviduct (Ball, et al. 2013) it could be deduced that, next to modulating muscular activity, PGE2 exerts diverse other effects on the oviduct (Ball, et al. 2013). Since *PTGER2* is expressed in both secretory and ciliated cells (Ball, et al. 2013), a regulatory role of PGE2 on oviductal secretion (Desantis, et al. 2011) as well as on oviductal ciliary activity may be suggested. Indeed, embryo-derived PGE2 has been described to increase ciliary beat in

laboratory species (Hermoso, et al. 2001, Verdugo, et al. 1980), indicating that next to steroids (Nelis, et al. 2015b), also the equine embryo may modulate oviductal ciliary activity. However, in the co-cultured group, the percentage of explants showing ciliary activity was not different compared to the control group. A similar observation has been reported in the cow and in women *in vivo* (Kolle, et al. 2009, Patek, et al. 1972, Verhage, et al. 1979). The presence of an embryo may keep the ciliary beating relatively constant to ensure mucociliar clearance (Kolle, et al. 2009, Patek, et al. 1972, Verhage, et al. 1979). Live cell imaging may be an interesting approach to visualize tiny changes in ciliary beat frequency and pattern of the oviductal cells in the presence or absence of an embryo (Kalab, et al. 1993, Kolle, et al. 2009, Kolle, et al. 2010, Lyons, et al. 2006). The importance of PGE2 and PTGERs in the embryo-maternal dialogue is further emphasized by the fact that *PTGER2* and *PTGER4* are also upregulated in the equine endometrium as a response to the presence of a day 8-12 embryo while potential target genes of conceptus derived PGE2 are concomitantly enhanced (Klein, et al. 2010, Merkl, et al. 2010).

CSF1 and *PAI1* showed a tendency for upregulation (1.5 fold, $0.05 < P < 0.09$) after co-culture, suggesting that both factors might also participate in the optimization of the oviductal milieu of the pregnant mare. Indeed, the receptor for CSF1 has been detected in human embryos (Duc-Goiran, et al. 1999, Sharkey 1998) while CSF1 protein has also been reported in murine oviducts (Arceci, et al. 1992, Pampfer, et al. 1991a). In the murine uterus, CSF1 expression was 100-fold during pregancy while depletion of CSF1 resulted in failure of initiation of embryonic DNA synthesis (Matsushime, et al. 1991), hallmarking its importance in early embryonic development.
PAI1 inhibits the proteolytic activity of uPA (gene = PLAU) and may thus contribute further to a predominantly antiproteolytic environment to protect the embryo, in particular the zona pellucida, from oviductal or embryonic uPA and MMP activity (Kouba, et al. 1998).

It should nevertheless be borne in mind that no protein content or activity of the proteases and their inhibitors was determined. Due to the presence of inhibitors at the posttranscriptional level, the mRNA expression may remain high while enzyme activity is decreased (Gabler, et al. 2001). Basal PAI1 activity may inhibit uPA activity while *PLAU* mRNA levels remain high. Moreover, next to PAI1, PAI2 and nexin, 2 other inhibitors of *PLAU* (Blasi 1997) may for instance bias the interpretation of the relevance of mRNA fluctuations.

Next to *PTGER4* and *PLAU,* embryo co-culture does not seem to influence the expression of *GLUT1, HIF1A* and *VEGFA*. GLUT1 plays an important role in the transport of glucose down a concentration gradient (Tadokoro, et al. 1995). It also mediates cellular glucose incorporation into embryonic cells and is necessary for transition from the morula to blastocyst stage (Leese 1995). The medium DMEM/F12 which is very suitable for equine embryo culture (Choi, et al. 2003, Hinrichs 2010, Smits, et al. 2011), contains unphysiologically high glucose levels (17 mM versus 2.8-5.2 mM (Campbell, et al. 1979). Apparently, since no embryo-induced *GLUT1* up- or downregulation could be observed, it might be concluded that the medium glucose concentrations meet the embryo's requirements. *GLUT1*, similar to *VEGFA*, is also upregulated and mediated by HIF1A under hypoxic conditions and thus are markers of hypoxia (Petroski 2008, van den Driesche, et al. 2008, Wagh and Lippes 1989, Wang, et al. 1995). Hypoxia-inducible factor (*HIF1A*) is a transcription factor with a central role in the hypoxia response. Its activity is regulated by the oxygen-dependent degradation of the HIF1A protein (Chi, et al. 2006). In response to hypoxia, HIF1A stimulates angiogenesis via *VEGFA* (Petroski 2008, van den Driesche, et al. 2008, Wang, et al. 1995, Wrenzycki, et al. 2001a, Wrenzycki, et al. 1998b) and glucose uptake via *GLUT1* (Wrenzycki, et al. 2001a, Wrenzycki, et al. 1998b). VEGFA is a potent angiogenic factor with an essential role in embryonic vasculogenesis and angiogenesis in mice (Carmeliet, et al. 1996, Ferrara, et al. 1996, Risau 1997). In cattle, it is supposed to be involved in oviductal embryo transport (Gabler, et al. 1999). Taken together, since neither *HIF1A* and *VEGFA* neither *GLUT1* were upregulated in response to embryonic stimuli, it could be concluded that, at least at the level of the oviduct explants, no hypoxia prevails in our culture system.

While the developing embryos were able to alter the oviductal gene expression profile, the percentage of explants showing ciliary activity (vide supra) and the prevalence of explants showing dark cell degeneration (DCD) were not modified as a consequence of embryo-oviduct interaction. As previously demonstrated (Nelis, et al. 2014, Nelis, et al. 2015b), the oviductal explants showed phagocytosis of cells suffering from dark cell-degeneration (DCD) without any observed features of autophage cell death. This phenomenon was not detected *in vivo* (Desantis, et al. 2011). The percentage of oviduct cells showing DCD was not lower in the group co-cultured with embryos. Despite the observation of DCD, in both groups, the cells bordering the oviductal explants maintained their highly differentiated, ciliated status and

intact cell membrane and did not seem to be functionally hampered during the culture period as was previously described (Nelis, et al. 2014).

This present and the aforementioned studies provide unequivocal evidence that the preattachment embryo communicates its presence to the oviduct in the horse and other domestic species. On the other hand, there is also very convincing evidence that oviduct-derived factors modify the embryonic transcriptome in order to support proliferation, differentiation, cell migration and vascularization (Carter, et al. 2010, Cordova, et al. 2014, Gad, et al. 2012, Goovaerts, et al. 2011, Lee, et al. 2001, Lloyd, et al. 2009, Maillo, et al. 2015, Rief, et al. 2002), which may *in vitro* be translated to an improvement of embryo quality and blastocyst percentage in ruminants, rodents and pigs (Donnay, et al. 1997, Lee, et al. 2001, Schmaltz-Panneau, et al. 2014, Yadav, et al. 1998). Despite the fact that earlier reports have demonstrated a beneficial effect on blastocyst percentage of oviduct co-culture using *in vivo* collected equine embryos (Ball, et al. 1991, Ball and Miller 1992, Brinsko, et al. 1994, Freeman, et al. 1991), in line with our results, co-culture of oviduct explants with *in vitro* produced equine embryos does not seem to enhance nor to reduce blastocyst percentages (Choi, et al. 2004b) or blastocyst diameter (Table 1). This could be explained by the fact that blastocyst rate and diameter (Mckinnon, et al. 1988) as sole parameters for the assessment of equine embryo development are maybe not sensitive enough.

In conclusion, in order to create an environment in the oviduct ideal for early embryo development, we have shown that equine oviductal explants *in vitro* are able to crosstalk in a paracrine and/or an autocrine way with developing *in vitro* derived equine embryos. However, little is known about underlying regulatory mechanisms. In order to decipher the embryo-maternal dialogue, the co-culture model will be used in future for proteome, transcriptome and/or microRNome studies.

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REFERENCES

Ambruosi, B, G Accogli, C Douet, S Canepa, G Pascal, P Monget, C Moros, U Holmskov, J Mollenhauer, C Robbe-Masselot, O Vidal, S Desantis, and G Goudet 2013 Deleted in malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism of fertilization in equine and porcine species. *Reproduction* **146** 119-133.

Arceci, RJ, S Pampfer, and JW Pollard 1992 Role and Expression of Colony-Stimulating Factor-I and Steel Factor Receptors and Their Ligands during Pregnancy in the Mouse. *Reproduction Fertility and Development* **4** 619-632.

Ball, BA 1996 Scanning electron microscopy of the equine oviduct and observations on ciliary currents in vitro at day 2 after ovulation. *Theriogenology* **46** 1305-1311.

Ball, BA, and M Altschul 1990 In vitro development of 4- to 8- cell equine embryos cocultured with trophoblastic vesicles or oviductal explants. . *Theriogenology* **33** 189.

Ball, BA, M Altschul, and JE Ellington 1991 Invitro Development of Day-2 Equine Embryos Cocultured with Oviductal Explants or Trophoblastic Vesicles. *Theriogenology* **35** 669-682.

Ball, BA, SP Brinsko, PGA Thomas, PG Miller, and JE Ellington 1993 Development to Blastocysts of One-Cell to 2-Cell Equine Embryos after Coculture with Uterine Tubal Epithelial-Cells. *American Journal of Veterinary Research* **54** 1139-1144.

Ball, BA, and PG Miller 1992 Survival of Equine Embryos Cocultured with Equine Oviductal Epithelium from the 4-Cell to 8-Cell to the Blastocyst Stage after Transfer to Synchronous Recipient Mares. *Theriogenology* **37** 979-991.

Bauersachs, S, H Blum, S Mallok, H Wenigerkind, S Rief, K Prelle, and E Wolf 2003 Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: a transcriptomics approach. *Biol Reprod* **68** 1170-1177.

Bauersachs, S, S Rehfeld, SE Ulbrich, S Mallok, K Prelle, H Wenigerkind, R Einspanier, H Blum, and E Wolf 2004 Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *Journal of Molecular Endocrinology* **32** 449-466.

Bavister, BD 1995 Culture of preimplantation embryos: Facts and artifacts. *Human Reproduction Update* **1** 91-148.

Blasi, F 1997 uPA, uPAR, PAI-I: key intersection of proteolytic, adhesive and chemotactic highways? *Immunology Today* **18** 415-417.

Bogaert, L, M Van Poucke, C De Baere, L Peelman, F Gasthuys, and A Martens 2006 Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *Bmc Biotechnology* **6**.

Bongso, A, and CY Fong 1993 The Effect of Coculture on Human Zygote Development. *Current Opinion in Obstetrics & Gynecology* **5** 585-593.

Brinsko, SP, BA Ball, PG Miller, PGA Thomas, and JE Ellington 1994 In-Vitro Development of Day-2 Embryos Obtained from Young, Fertile Mares and Aged, Subfertile Mares. *Journal of Reproduction and Fertility* **102** 371-378.

Brison, DR, and RM Schultz 1997 Apoptosis during mouse blastocyst formation: Evidence for a role for survival factors including transforming growth factor alpha. *Biology of Reproduction* **56** 1088-1096.

Brison, DR, and RM Schultz 1998 Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. *Biology of Reproduction* **59** 136-144.

Buhi, WC 2002 Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* **123** 355-362.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Bustin, SA, V Benes, JA Garson, J Hellemans, J Huggett, M Kubista, R Mueller, T Nolan, MW Pfaffl, GL Shipley, J Vandesompele, and CT Wittwer 2009 The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55** 611-622.

Campbell, DL, LW Douglas, and JC Ramge 1979 Cannulation of the Equine Oviduct and Chemical-Analysis of Oviduct Fluid. *Theriogenology* **12** 47-59.

Carmeliet, P, N Mackman, L Moons, T Luther, P Gressens, I VanVlaenderen, H Demunck, M Kasper, G Breier, P Evrard, M Muller, W Risau, T Edgington, and D Collen 1996 Role of tissue factor in embryonic blood vessel development. *Nature* **383** 73-75.

Chang, HS, WTK Cheng, HK Wu, and KB Choo 2000 Identification of genes expressed in the epithelium of porcine oviduct containing early embryos at various stages of development. *Molecular Reproduction and Development* **56** 331-335.

Chi, JT, Z Wang, DSA Nuyten, EH Rodriguez, ME Schaner, A Salim, Y Wang, GB Kristensen, A Helland, AL Borresen-Dale, A Giaccia, MT Longaker, T Hastie, GP Yang, MJ van de Vijver, and PO Brown 2006 Gene expression programs in

response to hypoxia: Cell type specificity and prognostic significance in human cancers. *Plos Medicine* **3** 395-409.

Choi, YH, CC Love, DD Varner, LB Love, and K Hinrichs 2003 Effects of gas conditions, time of medium change, and ratio of medium to embryo on in vitro development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219-1229.

Choi, Y.H., Velez, I.C., Macias-Garcia, B. and K Hinrichs 2015. Timing Factors Affecting Blastocyst Development in Equine Somatic Cell Nuclear Transfer. *Cell Reprogram* **17** 124-130.

Cross, JC, Z Werb, and SJ Fisher 1994 Implantation and the Placenta - Key Pieces of the Development Puzzle. *Science* **266** 1508-1518.

Dell'Aquila, ME, YS Cho, P Minoia, V Traina, S Fusco, GM Lacalandra, and F Maritato 1997 Intracytoplasmic sperm injection (ICSI) versus conventional IVF on abottoir-derived and in vitro-matured equine oocytes. *Theriogenology* **47** 1139-1156.

Desai, N, and J Goldfarb 1998 Co-cultured human embryos may be subjected to widely different microenvironments: pattern of growth factor cytokine release by Vero cells during the co-culture interval. *Human Reproduction* **13** 1600-1605.

Desantis, S, G Ventriglia, S Zizza, AC Guaricci, M Losurdo, A Zarrilli, and M Albrizio 2010 Changes in the expression of the mu-opioid receptor in the mare oviduct during oestrus and anoestrus. *Animal Reproduction Science* **119** 40-49.

Desantis, S, S Zizza, G Accogli, F Acone, R Rossi, and L Resta 2011 Morphometric and ultrastructural features of the mare oviduct epithelium during oestrus. *Theriogenology* **75** 671-678.

Duc-Goiran, P, TM Mignot, C Bourgeois, and F Ferre 1999 Embryo-maternal interactions at the implantation site: a delicate equilibrium. *European Journal of Obstetrics Gynecology and Reproductive Biology* **83** 85-100.

Ellington, JE, EW Carney, PB Farrell, ME Simkin, and RH Foote 1990 Bovine 1-2-Cell Embryo Development Using a Simple Medium in 3 Oviduct Epithelial-Cell Coculture Systems. *Biology of Reproduction* **43** 97-104.

Ellington, JE, GG Ignotz, BA Ball, VN Meyerswallen, and WB Currie 1993 Denovo Protein-Synthesis by Bovine Uterine Tube (Oviduct) Epithelial-Cells Changes during Coculture with Bull Spermatozoa. *Biology of Reproduction* **48** 851-856.

Eyestone, WH, and NL First 1989 Co-Culture of Early Cattle Embryos to the Blastocyst Stage with Oviducal Tissue or in Conditioned Medium. *Journal of Reproduction and Fertility* **85** 715-720.

Fazeli, A, NA Affara, M Hubank, and WV Holt 2004 Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biology of Reproduction* **71** 60-65.

Ferrara, N, K CarverMoore, H Chen, M Dowd, L Lu, KS OShea, L PowellBraxton, KJ Hillan, and MW Moore 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380** 439-442.

Freeman, DA, JE Butler, JA Weber, RT Geary, and GL Woods 1991 Co-culture of day-5 to day-7 equine embryos in medium with oviductal tissue. *Theriogenology* **36** 815-822.

Freeman, DA, GL Woods, DK Vanderwall, and JA Weber 1992 Embryo-Initiated Oviductal Transport in Mares. *Journal of Reproduction and Fertility* **95** 535-538.

Gabler, C, A Einspanier, D Schams, and R Einspanier 1999 Expression of vascular endothelial growth factor (VEGF) and its corresponding receptors (flt-1 and flk-1) in the bovine oviduct. *Molecular Reproduction and Development* **53** 376-383.

Gabler, C, GJ Killian, and R Einspanier 2001 Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction* **122** 121-130.

Galluzzi, L, I Vitale, JM Abrams, ES Alnemri, EH Baehrecke, MV Blagosklonny, TM Dawson, VL Dawson, WS El-Deiry, S Fulda, E Gottlieb, DR Green, MO Hengartner, O Kepp, RA Knight, S Kumar, SA Lipton, X Lu, F Madeo, W Malorni, P Mehlen, G Nunez, ME Peter, M Piacentini, DC Rubinsztein, Y Shi, HU Simon, P Vandenabeele, E White, J Yuan, B Zhivotovsky, G Melino, and G Kroemer 2012 Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death and Differentiation* **19** 107-120.

Garcia, DC, DC Miceli, PA Valdecantos, EV Garcia, and M Roldan-Olarte 2014 Expression of urokinase type plasminogen activator receptor (uPAR) in the bovine oviduct: Relationship with uPA effect on oviductal epithelial cells. *Research in Veterinary Science* **97** 118-123.

Georgiou, AS, E Sostaric, CH Wong, APL Snijders, PC Wright, HD Moore, and A Fazeli 2005 Gametes alter the oviductal secretory proteome. *Molecular & Cellular Proteomics* **4** 1785-1796.

Grobner, MA, and AR Menino 1994 Plasminogen-Activator Production and Enhanced Development in Medium Containing Plasminogen or Plasmin by Rabbit Embryos in-Vitro. *Journal of Reproduction and Fertility* **101** 467-475.

Harvey, MB, KJ Leco, MY Arcellanapanlilio, X Zhang, DR Edwards, and GA Schultz 1995 Proteinase Expression in Early Mouse Embryos Is Regulated by Leukemia Inhibitory Factor and Epidermal Growth-Factor. *Development* **121** 1005-1014.

Hermoso, M, N Barrera, B Morales, S Perez, and M Villalon 2001 Platelet activating factor increases ciliary activity in the hamster oviduct through epithelial production of prostaglandin E-2. *Pflugers Archiv-European Journal of Physiology* **442** 336-345.

Hill, AJ 2006 Maternal–Embryonic Cross-Talk. *Annals of the New York Academy of Sciences* **943** 17-25.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hinrichs, K, CC Love, SP Brinsko, YH Choi, and DD Varner 2002 In vitro fertilization of in vitro-matured equine oocytes: Effect of maturation medium, duration of maturation, and sperm calcium ionophore treatment, and comparison with rates of fertilization in vivo after oviductal transfer. *Biology of Reproduction* **67** 256-262.

Hwang, W, H Kim, E Lee, J Lim, S Roh, T Shin, K Hwang, and B Lee 2000 Purification and embryotropic roles of tissue inhibitor of metalloproteinase-1 in development of "HanWoo" (Bos taurus coreanae) oocytes co-cultured with bovine oviduct epithelial cells. *Journal of Veterinary Medical Science* **62** 1-5.

Kalab, P, RM Schultz, and GS Kopf 1993 Modifications of the Mouse Zona-Pellucida during Oocyte Maturation - Inhibitory Effects of Follicular-Fluid, Fetuin, and Alpha-2hs-Glycoprotein. *Biology of Reproduction* **49** 561-567.

Kodithuwakku, SP, A Miyamoto, and MPB Wijayagunawardane 2007 Spermatozoa stimulate prostaglandin synthesis and secretion in bovine oviductial epithelial cells. *Reproduction* **133** 1087-1094.

Kolle, S, S Dubielzig, S Reese, A Wehrend, P Konig, and W Kummer 2009 Ciliary Transport, Gamete Interaction, and Effects of the Early Embryo in the Oviduct: Ex

Vivo Analyses Using a New Digital Videomicroscopic System in the Cow. *Biology of Reproduction* **81** 267-274.

Kolle, S, S Reese, and W Kummer 2010 New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. *Theriogenology* **73** 786-795.

Kouba, AJ, IM Alvarez, and WC Buhi 1998 Characterization of pig oviductal plasminogen activator inhibitor (PAI)-1 during early pregnancy. *Biology of Reproduction* **58** 172-172.

Lalou, C, C Silve, R Rosato, B Segovia, and M Binoux 1994 Interactions between Insulin-Like Growth-Factor-I (Igf-I) and the System of Plasminogen Activators and Their Inhibitors in the Control of Igf-Binding Protein-3 Production and Proteolysis in Human Osteosarcoma Cells. *Endocrinology* **135** 2318-2326.

Lee, KF, YQ Yao, KL Kwok, JS Xu, and WS Yeung 2002 Early developing embryos affect the gene expression patterns in the mouse oviduct. *Biochem Biophys Res Commun* **292** 564-570.

Leese, HJ 1988 The Formation and Function of Oviduct Fluid. *Journal of Reproduction and Fertility* **82** 843-856.

Leese, HJ 1995 Metabolic control during preimplantation mammalian development. *Human Reproduction Update* **1** 63-72.

Lenth, RV 2007 Statistical power calculations. *Journal of Animal Science* **85** E24-E29.

Li, XH, LHA Morris, and WR Allen 2001 Influence of co-culture during maturation on the developmental potential of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI). *Reproduction* **121** 925-932.

Lyons, RA, E Saridogan, and O Djahanbakhch 2006 The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update* **12** 363-372.

Lyons, RM, J Keskioja, and HL Moses 1988 Proteolytic Activation of Latent Transforming Growth Factor-Beta from Fibroblast-Conditioned Medium. *Journal of Cell Biology* **106** 1659-1665.

Lysiak, JJ, VKM Han, and PK Lala 1993 Localization of Transforming Growth-Factor-Alpha in the Human Placenta and Decidua - Role in Trophoblast Growth. *Biology of Reproduction* **49** 885-894.

Makarevich, AV, and M Markkula 2002 Apoptosis and cell proliferation potential of bovine embryos stimulated with insulin-like growth factor I during in vitro maturation and culture. *Biology of Reproduction* **66** 386-392.

Matsushime, H, MF Roussel, RA Ashmun, and CJ Sherr 1991 Colony-Stimulating Factor-I Regulates Novel Cyclins during the G1 Phase of the Cell-Cycle. *Cell* **65** 701-713.

Mccomb, P, L Langley, M Villalon, and P Verdugo 1986 The Oviductal Cilia and Kartageners-Syndrome. *Fertility and Sterility* **46** 412-416.

Mckinnon, AO, EL Squires, EM Carnevale, JL Voss, and GE Seidel 1988 Heterogenous and Xenogenous Fertilization of Equine Oocytes. *Theriogenology* **29** 278-278.

McPartlin, LA, SS Suarez, CA Czaya, K Hinrichs, and SJ Bedford-Guaus 2009 Hyperactivation of Stallion Sperm Is Required for Successful In Vitro Fertilization of Equine Oocytes. *Biology of Reproduction* **81** 199-206.

Menino, AR, AR Dyk, CS Gardiner, MA Grobner, MA Kaaekuahiwi, and JS Williams 1989 The Effects of Plasminogen on Invitro Ovine Embryo Development. *Biology of Reproduction* **41** 899-905.

Menino, AR, and JS Williams 1987 Activation of Plasminogen by the Early Bovine Embryo. *Biology of Reproduction* **36** 1289-1295.

Mugnier, S, M Kervella, C Douet, S Canepa, G Pascal, S Deleuze, G Duchamp, P Monget, and G Goudet 2009 The secretions of oviduct epithelial cells increase the equine in vitro fertilization rate: are osteopontin, atrial natriuretic peptide A and oviductin involved? *Reproductive Biology and Endocrinology* **7**.

Nelis, H, K D'Herde, K Goossens, L Vandenberghe, B Leemans, K Forier, K Smits, K Braeckmans, L Peelman, and A Van Soom 2014 Equine oviduct explant culture: a basic model to decipher embryo–maternal communication. *Reproduction, Fertility and Development* **26** 954-966.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015a Steroids in the equine oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*.

Nelis, H, B Wojciechowicz, A Franczak, B Leemans, K D'Herde, K Goossens, P Cornillie, L Peelman, A Van Soom, and K Smits 2015b Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and

immunoreactive steroidogenic protein expression in equine oviduct explants in vitro. *Reprod Fertil Dev*.

Orsi, NM, and JB Reischl 2007 Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* **67** 441-458.

Ortiz, ME, P Bedregal, MI Carvajal, and HB Croxatto 1986 Fertilized and Unfertilized Ova Are Transported at Different Rates by the Hamster Oviduct. *Biology of Reproduction* **34** 777-781.

Ortiz, ME, C Llados, and HB Croxatto 1989 Embryos of Different Ages Transferred to the Rat Oviduct Enter the Uterus at Different Times. *Biology of Reproduction* **41** 381-384.

Palmer, E, J Bezard, M Magistrini, and G Duchamp 1991 In vitro fertilization in the horse. A retrospective study. *J Reprod Fertil Suppl* **44** 375-384.

Pampfer, S, RJ Arceci, and JW Pollard 1991 Role of Colony Stimulating Factor-I (Csf-1) and Other Lympho-Hematopoietic Growth-Factors in Mouse Preimplantation Development. *Bioessays* **13** 535-540.

Papanikolaou, T, GS Amiridis, I Dimitriadis, E Vainas, and CA Rekkas 2008 Effect of plasmin, plasminogen activators and a plasmin inhibitor on bovine in vitro embryo production. *Reproduction Fertility and Development* **20** 320-327.

Patek, E, Johannis.E, and L Nilsson 1972 Scanning Electron-Microscopic Study of Human Fallopian Tube .1. Proliferative and Secretory Stages. *Fertility and Sterility* **23** 459-&.

Pepper, MS, D Belin, R Montesano, L Orci, and JD Vassalli 1990 Transforming Growth Factor-Beta-1 Modulates Basic Fibroblast Growth-Factor Induced Proteolytic and Angiogenic Properties of Endothelial-Cells Invitro. *Journal of Cell Biology* **111** 743-755.

Petroski, MD 2008 The ubiquitin system, disease, and drug discovery. *Bmc Biochemistry* **9**.

Reneker, LXW, DW Silversides, K Patel, and PA Overbeek 1995 Tgf-Alpha Can Act as a Chemoattractant to Perioptic Mesenchymal Cells in Developing Mouse Eyes. *Development* **121** 1669-1680.

Rexroad, CE 1989 Co-Culture of Domestic-Animal Embryos. *Theriogenology* **31** 105-114.

Risau, W 1997 Mechanisms of angiogenesis. *Nature* **386** 671-674.

Rizos, D, P Lonergan, MP Boland, R Arroyo-Garcia, B Pintado, J de la Fuente, and A Gutierrez-Adan 2002 Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. *Biology of Reproduction* **66** 589-595.

Roldan-Olarte, M, DC Garcia, M Jimenez-Diaz, PA Valdecantos, and DC Miceli 2012 In vivo and in vitro expression of the plasminogen activators and urokinase type plasminogen activator receptor (u-PAR) in the pig oviduct. *Animal Reproduction Science* **136** 90-99.

Sakkas, D, AO Trounson, and I Kola 1989 Invivo Cleavage Rates and Viability Obtained for Early Cleavage Mouse Embryos in Co-Culture with Oviduct Cells. *Reproduction Fertility and Development* **1** 127-136.

Sato, Y, and DB Rifkin 1989 Inhibition of Endothelial-Cell Movement by Pericytes and Smooth-Muscle Cells - Activation of a Latent Transforming Growth Factor-Beta-1-Like Molecule by Plasmin during Co-Culture. *Journal of Cell Biology* **109** 309-315.

Schmaltz-Panneau, B, A Cordova, S Dhorne-Pollet, C Hennequet-Antier, S Uzbekova, E Martinot, S Doret, P Martin, P Mermillod, and Y Locatelli 2014 Early bovine embryos regulate oviduct epithelial cell gene expression during in vitro co-culture. *Animal Reproduction Science* **149** 103-116.

Sharkey, A 1998 Cytokines and implantation. *Reviews of Reproduction* **3** 52-61.

Smith, S, M Schmidt, B Purwantara, and T Greve 1992 Oviduct Epithelial-Cell Coculture of Early Porcine Embryos. *Acta Veterinaria Scandinavica* **33** 349-355.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, K, J Govaere, M Hoogewijs, C De Schauwer, E Van Haesebrouck, M Van Poucke, L Peelman, M van den Berg, T Vullers, and A Van Soom 2010 Birth of the first ICSI foal in the Benelux. *Vlaams Diergeneeskundig Tijdschrift* **79** 134-138.

Smits, K, J Govaere, M Hoogewijs, S Piepers, and A Van Soom 2012a A Pilot Comparison of Laser-Assisted vs Piezo Drill ICSI for the In Vitro Production of Horse Embryos. *Reproduction in Domestic Animals* **47** e1-e3.

Smits, K, J Govaere, LJ Peelman, K Goossens, DC de Graaf, D Vercauteren, L Vandaele, M Hoogewijs, E Wydooghe, T Stout, and A Van Soom 2012b Influence of the uterine environment on the development of in vitro-produced equine embryos. *Reproduction* **143** 173-181.

Smits, KG, K.; Van Soom, A.; Govaere, J.; Hoogewijs, M.; Vanhaesebrouck, E.; Galli, C.; Colleoni, S.; Vandesompele, J.; Peelman, L. 2009 Selection of reference genes

for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246.

Tadokoro, C, Y Yoshimoto, M Sakata, T Imai, M Yamaguchi, H Kurachi, Y Oka, T Maeda, and A Miyake 1995 Expression and Localization of Glucose-Transporter-1 (Glut1) in the Rat Oviduct - a Possible Supplier of Glucose to Embryo during Early Embryonic-Development. *Biochem Biophys Res Commun* **214** 1211-1218.

Thomas, PGA, GG Ignotz, BA Ball, SP Brinsko, and WB Currie 1995 Effect of Coculture with Stallion Spermatozoa on De-Novo Protein-Synthesis and Secretion by Equine Oviduct Epithelial-Cells. *American Journal of Veterinary Research* **56** 1657-1662.

Tremoleda, JL, TAE Stout, I Lagutina, G Lazzari, MM Bevers, B Colenbrander, and C Galli 2003 Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biology of Reproduction* **69** 1895-1906.

van den Driesche, S, M Myers, E Gay, KJ Thong, and WC Duncan 2008 HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *Molecular Human Reproduction* **14** 455-464.

VanSoom, A, M Boerjan, MT Ysebaert, and A DeKruif 1996 Cell allocation to the inner cell mass and the trophectoderm in bovine embryos cultured in two different media. *Molecular Reproduction and Development* **45** 171-182.

Verdugo, P, RE Rumery, and PY Tam 1980 Hormonal-Control of Oviductal Ciliary Activity - Effect of Prostaglandins. *Fertility and Sterility* **33** 193-196.

Verhage, HG, ML Bareither, RC Jaffe, and M Akbar 1979 Cyclic Changes in Ciliation, Secretion and Cell Height of the Oviductal Epithelium in Women. *American Journal of Anatomy* **156** 505-521.

Wagh, PV, and J Lippes 1989 Human Oviductal Fluid Proteins .3. Identification and Partial-Purification. *Fertility and Sterility* **51** 81-88.

Wang, GL, BH Jiang, EA Rue, and GL Semenza 1995 Hypoxia-Inducible Factor-1 Is a Basic-Helix-Loop-Helix-Pas Heterodimer Regulated by Cellular O-2 Tension. *Proceedings of the National Academy of Sciences of the United States of America* **92** 5510-5514.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991a Prostaglandin E2 hastens oviductal transport of equine embryos. *Biol Reprod* **45** 544-546.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991b Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45** 540-543.

Weber, JA, GL Woods, DA Freeman, and DK Vanderwall 1993 Oviductal and uterine influence on the development of Day-2 equine embryos in vivo and in vitro. *Theriogenology* **40** 689-698.

Whiteside, EJ, M Kan, MM Jackson, JG Thompson, C McNaughton, AC Herington, and MB Harvey 2001 Urokinase-type plasminogen activator (uPA) and matrix metalloproteinase-9 (MMP-9) expression and activity during early embryo development in the cow. *Anatomy and Embryology* **204** 477-483.

Wolf, E, GJ Arnold, S Bauersachs, HM Beier, H Blum, R Einspanier, T Fröhlich, A Herrler, S Hiendleder, S Kölle, K Prelle, HD Reichenbach, M Stojkovic, H Wenigerkind, and F Sinowatz 2003 Embryo-Maternal Communication in Bovine – Strategies for Deciphering a Complex Cross-Talk. *Reproduction in Domestic Animals* **38** 276-289.

Wrenzycki, C, D Herrmann, L Keskintepe, A Martins, S Sirisathien, B Brackett, and H Niemann 2001 Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Human Reproduction* **16** 893-901.

Wrenzycki, C, D Herrmann, E Lemme, K Korsawe, JW Carnwath, and H Niemann 1998 Transcriptional level of developmentally important genes in bovine preimplantation embryos generated in vitro. *Theriogenology* **49** 191-191.

Xu, J, TM Cheung, ST-h Chan, PC Ho, and WSB Yeung 2000 Human oviductal cells reduce the incidence of apoptosis in cocultured mouse embryos. *Fertility and Sterility* **74** 1215-1219.

Xu, JS, STH Chan, WWM Lee, KF Lee, and WSB Yeung 2004 Differential growth, cell proliferation, and apoptosis of mouse embryo in various culture media and in coculture. *Molecular Reproduction and Development* **68** 72-80.

Zhang, X, GM Kidder, C Zhang, F Khamsi, and DT Armstrong 1994 Expression of Plasminogen-Activator Genes and Enzymatic-Activities in Rat Preimplantation Embryos. *Journal of Reproduction and Fertility* **101** 235-240.

CHAPTER 6 THE DERBY OF *IN VITRO* EQUINE EMBRYO PRODUCTION: THE RACE TOWARDS THE IMPROVEMENT OF CULTURE CONDITIONS

The characteristic of scientific progress is
our knowing
that we do not know.

Gaston Bachelard, philosopher

OF EQUINE EMBRYO *IN VITRO* CULTURE TEMPERATURE

Based on:

Culturing equine oocytes and embryos at the physiological temperature of the mare.

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OF EQUINE EMBRYO *IN VITRO* CULTURE TEMPERATURE

ABSTRACT

It is generally accepted that the incubation temperature is an important parameter for successful embryo culture, but in the horse, this variable has not been investigated so far. The objective of this experiment was to investigate the effect of a decrease in maturation and culture temperature from 38.5°C to 37.3°C on maturation and blastocyst rate and on blastocyst size.

Oocytes from slaughtered mares were matured in DMEM-F12-based medium (Galli *et al.* 2007) in 5% CO_2 in air at either 37.3 °C (group 1) or 38.5 °C (group 2) for 30-32 h. Oocytes with a polar body were fertilized by piezo drill intracytoplasmic sperm injection (ICSI) and cultured in DMEM-F12 with 10% fetal calf serum in 5% CO_2, 5% O_2, and 90% N_2 for 9.5 days. Group 1 was cultured at 37.3 °C and group 2 at 38.5 °C. At day 9.5, the size of the blastocysts were measured using a stereo microscope fitted with an eyepiece micrometer.

After maturation, about one third of the oocytes in each group were degenerated. Of the non-degenerated oocytes, 90.1% (155/172) and 89.7% (130/145) were mature in group 1 vs 2 respectively. Cleavage rate at day 2.5 after ICSI was 44% in group 1 and 47% in group 2. Six out of 68 (8.8%) in group 1 and three out of the 61 cleaved embryos in group 2 (5%) reached the blastocyst stage. Percentages of degenerated and matured oocytes, cleavage and blastocyst rates did not differ significantly between groups ($P > 0.5$, Pearson Chi-square test). Interestingly blastocyst size at day 9 was significantly larger ($P=0.02$; Mann-Whitney U test) in group 2 (166 +/- 4.8 µm in diameter) than in group 1 (134 +/- 6.7 µm in diameter).

Maturation of oocytes and culture of embryos at the physiological temperature of 37.3°C did not influence nuclear maturation or blastocyst rates significantly, although blastocyst rates were numerically higher at 37.3°C ($P > 0.05$). Blastocyst size was significantly larger after culture at 38.5°C. These preliminary data show that there is apparently no clear benefit in using the physiological temperature of 37.3°C for horse embryo production *in vitro*.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) is the method of choice for production of equine embryos *in vitro*. Despite the quick progress in the optimization of embryo culture conditions in other species, little is known about the effect of specific environmental factors such as temperature on equine embryonic development. Moreover, blastocyst rates are varying markedly amongst research groups between 5 (Jarazo, et al. 2011), 10 (Smits, et al. 2010), 17.8 (Galli, et al. 2001) and 38% (Hinrichs and Choi 2005). Moreover, the applied culture temperatures are varying respectively between 39, 38.5, 38 and 38.2°C. Since blastocyst rates in mice decrease significantly at mild hyperthermic conditions (38.5°C-39°C) by impairing cytoplasmic maturation (Wang, et al. 2009), we hypothesized that the lowest physiological temperature of 37.3°C in healthy mares (Robertshaw, 2005) approaches more the optimal embryo culture temperature compared to 38.5°C. Therefore, to test this hypothesis, we compared oocyte maturation, cleavage and blastocyst rate as well as blastocyst diameter between both temperatures.

MATERIALS AND METHODS

Oocytes were collected, matured and fertilized and zygotes were cultured as described earlier (Smits, et al. 2010, Smits, et al. 2012a). Half (n = 264) of the oocytes were matured at 37.3°C and the other half (n = 242) at 38.5°C. After fertilization, zygotes from oocytes matured at 37.3°C respectively 38.5°C, were cultured at respectively 37.3°C and 38.5°C.

On day 9.5 the embryonic development was evaluated. The size of the blastocysts was measured using a stereomicroscope fitted with an eyepiece micrometer.

To determine significant differences in the blastocyst rate, binary logistic regression with the Nagelkerke pseudo R^2 and χ^2 fit tests was implemented. Differences were considered significant at $P < 0.05$. Statistical analysis and graph plotting was performed with SPSS 21.

RESULTS

The outcome of maturation, fertilization and blastocyst rate are depicted in **Fig. 1.**, After maturation, in group 1 (n = 264) 35% and in group 2 (n = 242) 40% of the oocytes were degenerated. Of the non-degenerated oocytes, maturation was respectively 90.1% (155/172)

and 89.7% (130/145). Cleavage rate was 47% in group 1 and 49% in group 2. Six out of 68 (9.6%) in group 1 and three out of the 61 cleaved embryos in group 2 (5.2 %) reached the blastocyst stage. Although the percentage of degenerated oocytes (P = 0.8), the maturation (P = 0.9), cleavage (P = 0.7) neither blastocyst rates differed significantly (P = 0.2), blastocyst size was significantly larger (P = 0.02; Mann-Whitney U test) in group 2 (166 +/- 4.8 µm) than in group 1 (134 +/- 6.7 µm) (**Fig. 2**).

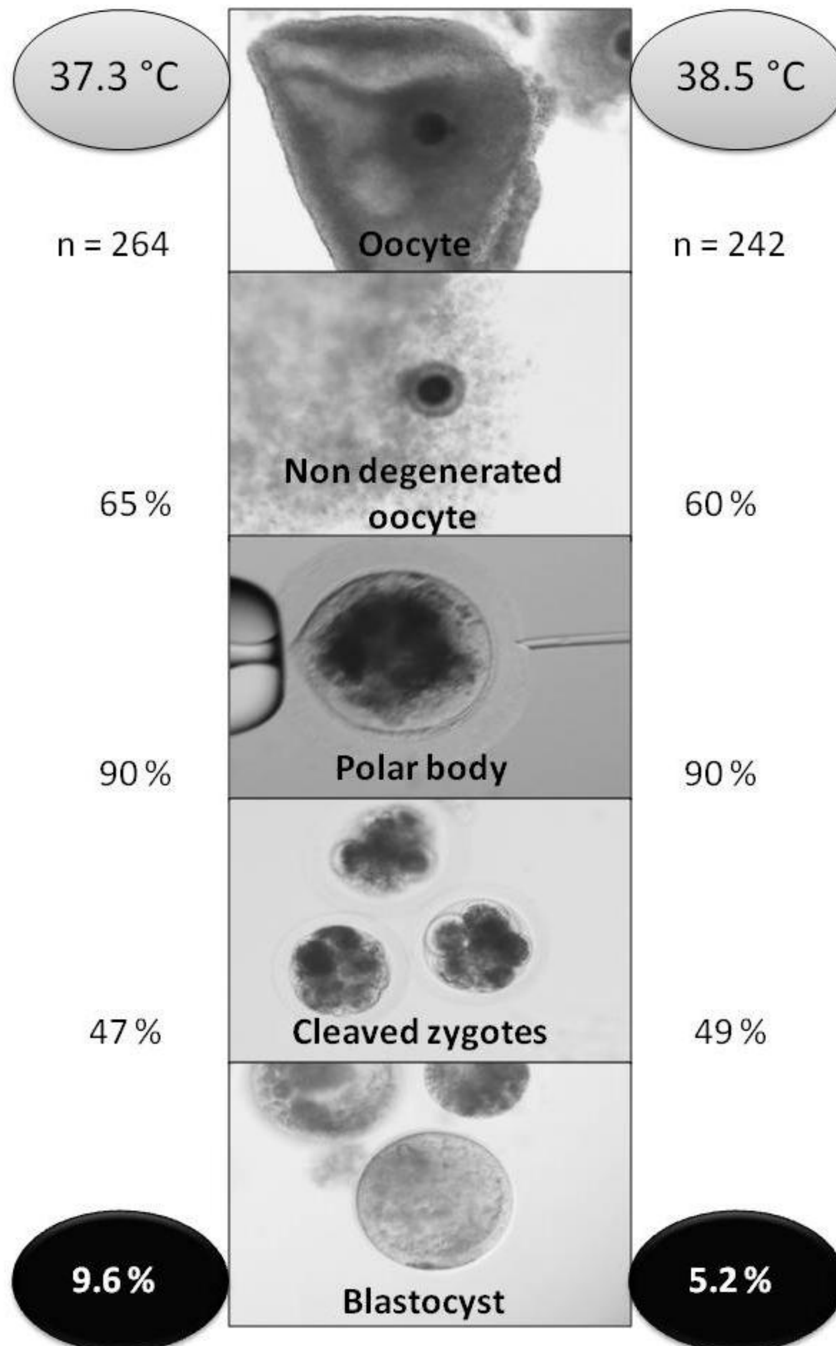


Fig. 1 Low temperature (37.3°C) versus high temperature (38.5°C) outcome of *in vitro* maturation and fertilization of oocytes and blastocyst development.IVM = *in vitro* maturation; ICSI = intracytoplasmic sperm injection; IVC = *in vitro* culture.

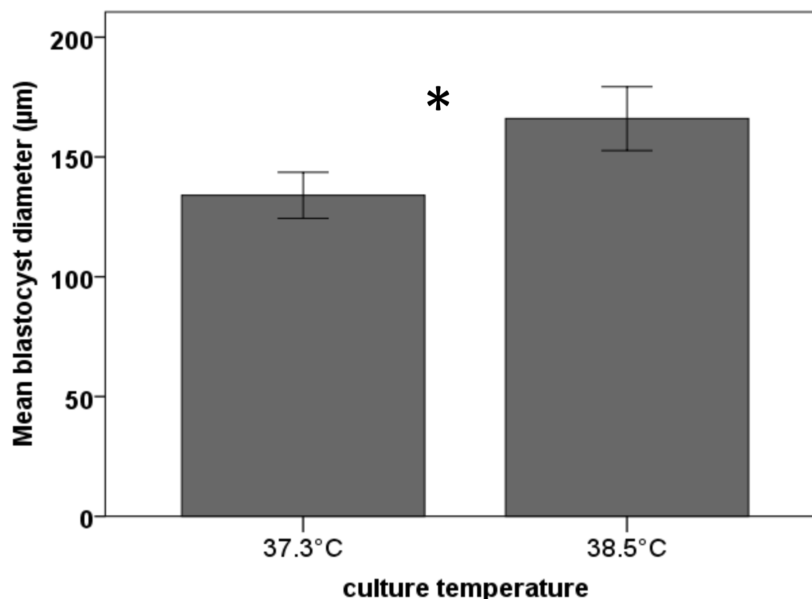


Fig. 2. Mean blastocyst diameter after oocyte maturation and embryo culture. ** P = 0.02. Independent samples Mann-Whitney U-test. Error bar = 2 standard error of mean.

DISCUSSION

These results show that maturation of oocytes and culture of zygotes at 37.3°C does not change nuclear maturation neither cleavage rate (P > 0.05). Blastocyst rate showed a slight tendency to be higher in the group cultured at 37.3°C (P = 0.2). Since cytoplasmic rather than nuclear components determine preimplantation developmental capacity (Wang, et al. 2009) and the developmental parameters did not change significantly, we may conclude that cytoplasmic maturation does not differ at 38.5°C compared to 37.3°C. Nevertheless, it should be borne in mind that blastocysts percentages as a sole factor to assess *in vitro* embryo development does not always reflects the aberrations which may occur at the molecular, functional or ultrastructural level. Therefore, gene expression profile, apoptotic index, cell number, and cryotolerance are markers which represent more quality and viability (Desai, et al. 2000, Orsi and Reischl 2007, Rizos, et al. 2002, Xu, et al. 2004).

The size of blastocysts was significantly smaller (P = 0.02) in the group of oocytes matured and embryos cultured at 37.3°C compared with the group matured and cultured at 38.5°C. Since blastocyst size is a parameter of embryo viability (Mckinnon, et al. 1988), maturation and culture at 38.5°C may be recommended rather than culture at 37.3°C.

In conclusion, out of the many factors that influence early embryo development in the oviduct, we provided evidence that an equine embryo culture temperature of 38.5°C is recommended rather than 37.3°C.

REFERENCES

Desai, N, J Lawson, and J Goldfarb 2000 Assessment of growth factor effects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage. *Human Reproduction* **15** 410-418.

Galli, C, G Crotti, C Notari, P Turini, R Duchi, and G Lazzari 2001 Embryo production by ovum pick up from live donors. *Theriogenology* **55** 1341-1357.

Hinrichs, K, and Y-H Choi 2005 Assisted reproductive techniques in the horse. *Clinical techniques in equine practice* **4** 210-218.

Jarazo, J, A Gambini, A De Stefano, L Muredas, JG Oriol, and D Salamone 2011 203 equine embryo in vitro development after intracytoplasmic sperm injection followed by chemical activation. *Reproduction, Fertility and Development* **24** 214-214.

Mckinnon, AO, EL Squires, EM Carnevale, JL Voss, and GE Seidel 1988 Heterogenous and Xenogenous Fertilization of Equine Oocytes. *Theriogenology* **29** 278-278.

Orsi, NM, and JB Reischl 2007 Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* **67** 441-458.

Rizos, D, P Lonergan, MP Boland, R Arroyo-Garcia, B Pintado, J de la Fuente, and A Gutierrez-Adan 2002 Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. *Biology of Reproduction* **66** 589-595.

Robertshaw, D 2004 Temperature Regulation and Thermal Environment, in *Dukes' Physiology of Domestic Animals,* 12th ed., Reece W.O., Ed., Cornell University.

Smits, K, J Govaere, M Hoogewijs, C De Schauwer, E Van Haesebrouck, M Van Poucke, L Peelman, M van den Berg, T Vullers, and A Van Soom 2010 Birth of the first ICSI foal in the Benelux. *Vlaams Diergeneeskundig Tijdschrift* **79** 134-138.

Smits, K, J Govaere, M Hoogewijs, S Piepers, and A Van Soom 2012 A Pilot Comparison of Laser-Assisted vs Piezo Drill ICSI for the In Vitro Production of Horse Embryos. *Reproduction in Domestic Animals* **47** e1-e3.

Wang, JZ, HS Sui, DQ Miao, N Liu, P Zhou, L Ge, and JH Tan 2009 Effects of heat stress during in vitro maturation on cytoplasmic versus nuclear components of mouse oocytes. *Reproduction* **137** 181-189.

Xu, JS, STH Chan, WWM Lee, KF Lee, and WSB Yeung 2004 Differential growth, cell proliferation, and apoptosis of mouse embryo in various culture media and in coculture. *Molecular Reproduction and Development* **68** 72-80.

CHAPTER 7: GENERAL DISCUSSION

The aim of argument, or of discussion,

should not be victory, but progress.

Joseph Joubert, moralist

1 EQUINE OVIDUCTAL EXPLANTS

1.1 A MODEL TO STUDY EARLY REPRODUCTIVE EVENTS

The complex and dynamic oviductal environment is pivotal during the early reproductive events such as oocyte maturation, sperm capacitation, fertilization, embryonic development and embryo-maternal communication **(Fig. 1)**. To gain a better insight into the complexity of endocrine, paracrine and autocrine signalling and biochemical pathways, appropriate models are essential.

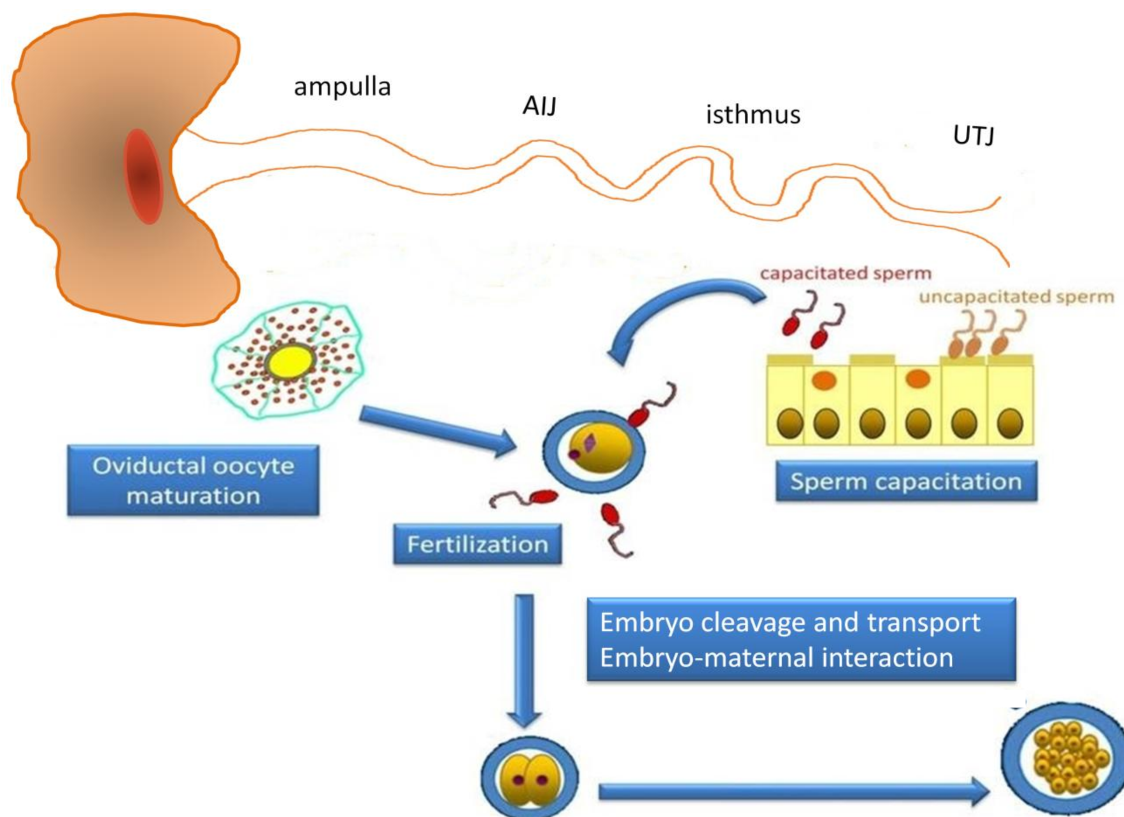


Fig. 1. Oviductal events such oocyte maturation, sperm capacitation, fertilization, embryonic development and embryo-maternal communication are up to now poorly understood in the horse. All these processes can be studied *in vitro* using our oviductal explant model. AIJ = ampullary-isthmic junction; UTJ = uterotubal junction. Adapted from Avilés, et al. 2010 and Smits 2010.

Although *in vivo* models are the gold standard, it is difficult to investigate molecular processes within a large space like the equine oviduct. It is for instance difficult to locate gametes and the embryo in the oviduct and to unravel local paracrine and autocrine events,

which are essential in elucidating intra- and extracellular molecular pathways and processes. Moreover, the equine oviduct can only be reached surgically or post mortem. In addition, *in vivo* experiments with horses conflict with animal welfare and are time consuming and expensive.

The ligated sheep oviduct could be an excellent preimplantation culture system for equine as well as bovine and porcine embryos (Lazzari, et al. 2010, Orsi and Reischl 2007, Rizos, et al. 2010). However, there are some differences between the development of equine and ruminant embryos. Moreover, the surgical procedure may introduce a bias. Therefore, the technique of temporary culture in the sheep oviduct was quickly abandoned. This evolution was enhanced for reasons of animal welfare, and for practical and biosecurity issues.

Since the oviductal environment is the most appropriate for early embryonic development, co-culture of embryos with equine oviduct explants is a suitable model to study embryo-maternal interaction (Wolf, et al. 2003b). Indeed, co-culture with explants allows paracrine interaction between epithelial and in a lesser extent stromal cells (**Fig. 2 c),** whereas in conventional embryo culture, only autocrine interactions can occur **(Fig. 2 b)**. Whereas in co-culture systems oviduct cells may alter their secretory profile in response to certain ligands produced by the embryos (**Fig. 2 c**), a considerable part of the embryo-maternal dialogue cannot take place in (semi)defined or conditioned media, making them less appropriate (**Fig. 2 b**) to elaborate on the interaction between the embryo and the oviduct (**Fig. 2 a**). During *in vitro* culture without co-culture, embryos are deprived of endocrine as well as paracrine signals from epithelial, stromal and muscle cells, whereas *in vivo* paracrine interactions between embryos are rather unlikely to occur **(Fig. 2 b)**.

During the period of co-culture, the observed effects on embryos and somatic cells are resulting on the one hand from the reciprocal messages between them, but on the other hand also from the combined effect of excreted waste products and the consumption and depletion of nutrients and growth factors (Lee, et al. 2002). Whereas in co-culture the oviductal cells may be able to remove deleterious factors produced by the embryos, in conventional embryo culture, all waste products are accumulating in the culture medium. In addition, the superior performance of oviductal cells in sustaining embryonic development in other species than the horse, including human, compared to conventional culture media and other somatic cells, has

been frequently confirmed (Gandolfi and Moor 1987, Liu, et al. 1998, Menezo, et al. 1998, Rexroad and Powell 1993).

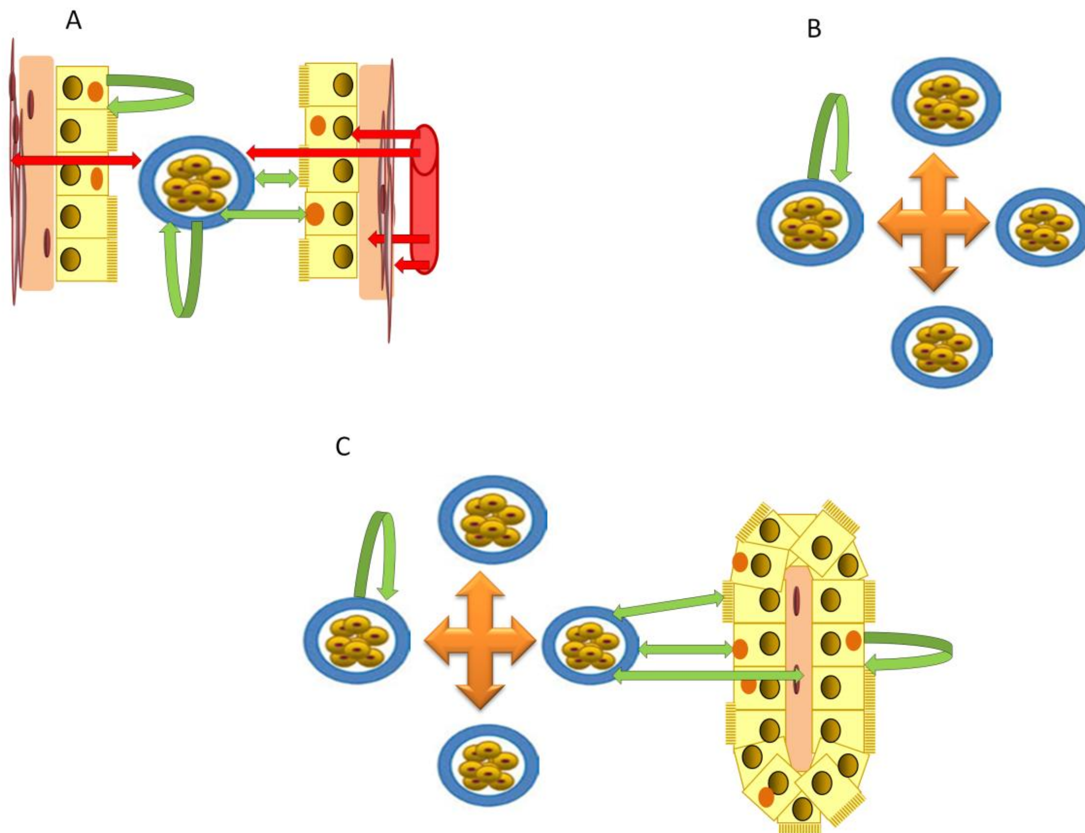


Fig. 2 Schematic representation of possible interactions *in vivo* (A), *in vitro* without (B) and with (C) co-culture. *In vivo*, endocrine, autocrine and paracrine interactions are possible between embryos and the oviduct. The embryo is continuously nourished in response to its signals to the oviduct in function of its changing needs. Waste products are efficiently removed and/or neutralized (A). In conventional embryo culture (B), only paracrine and autocrine interactions between the embryos are possible. The supply of embryotrophic products and the removal of toxic products are dependent on the medium replacement protocol and the embryo's capacity to neutralize waste products. In an oviductal explant co-culture system, autocrine and paracrine interactions are possible between embryos and the oviduct. Nutrients are delivered and waste products removed by the oviductal cells, the latter which respond to the embryonic signals.

To guarantee optimal environmental conditions, the culture system must meet the requirements for embryos as well as oviductal cells. Moreover, to mimic the *in vivo* situation, the cells must keep their differentiation status. In our culture system (CHAPTER 3) the cells

bordering the explants are maintaining their ultrastructural highly differentiated morphology during at least six days of culture, including cilia, numerous mitochondria and rough endoplasmic reticulum, highly similar to the oviduct epithelium *ex vivo*. Since the ciliation process is stated to be the endpoint of differentiation that cannot be induced in an *in vitro* system (Thibodeaux, et al. 1992b), this means an important benefit of the explant suspension. Furthermore, only a negligible percentage (1–2%) of the cells in the explants shows features of apoptosis or necrosis and therefore the explants mimic the *in vivo* situation very well). Although dark-cell degeneration, which is probably a hypoxia related type of cell death, was observed (CHAPTER 3), no proof of hypoxia could be observed at the level of mRNA expression.

Moreover, oviduct explants respond at the level of protein, progesterone receptor and gene expression as well as glucose metabolism to stimuli such as steroid supplementation (CHAPTER 5.1). This confirms the functionality and vitality of our culture system and the superiority of oviductal cells as compared to epithelial monolayers. Although monolayers and resulting cell lines provide more standardized culture conditions, it has been proven in cattle (Thibodeaux, et al. 1992b, Walter 1995) and horses (Dobrinski, et al. 1999, Thomas, et al. 1995b) that monolayers of oviductal cells dedifferentiate and consist of cells with reduced height, less beating cilia and loss of secretory granules. Therefore, they are far less mimicking the *in vivo* situation (Reischl, et al. 1999, Thibodeaux, et al. 1992b, Walter 1995), despite the fact that the use of monolayers and resulting cell lines minimizes the risk of disease transmission (Menck, et al. 1997).

1.2 MARGINAL NOTES OF LIMITATIONS

Although co-cultured somatic cells may neutralize toxic components, nutrient depletion and the accumulation of particular toxic components can still occur in the oviduct explant co-culture. To overcome this, the introduction of dynamic perfusion culture systems **(Fig. 3)**, using permeable cell supports (Reischl, et al. 1997) or sequential media (different culture media for culturing at different stages of development), as already applied in human assisted reproduction, could open up new perspectives. However, it is difficult to imitate the dynamic changes of the composition of the oviductal fluid: embryos have different requirements depending on their stage of development and some culture conditions may induce

dedifferentiation in somatic cells (Lee, et al. 2002, Wolf, et al. 2003b), since the composition of the medium and serum supplementation have obvious effects on cell function, integrity and morphology (CHAPTER 3.2; (Rief, et al. 2002).

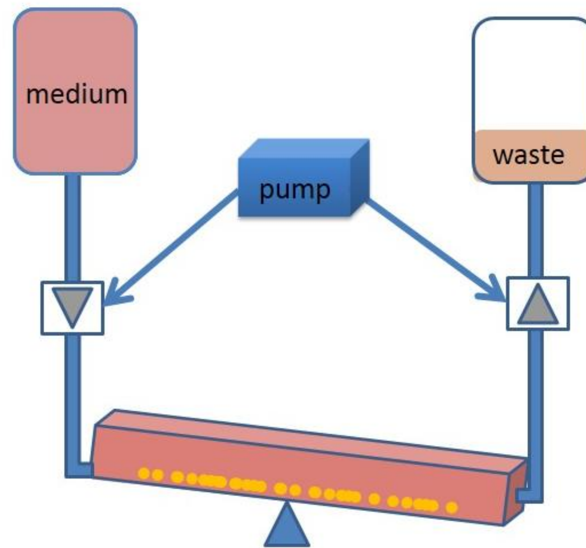


Fig. 3. A dynamic culture system with a continuing supply of fresh culture medium and removal of waste products.

Also the environmental temperature may play a considerable role during cell culture. A temperature gradient exists in the oviduct. In rabbits, sheep and pigs (Hunter 2012a), the caudal isthmus is reported to be 1-2 °C cooler than the ampulla at oestrus. These gradients may be involved in sperm storage and transport (thermotaxis) (Hunter 2012a). Even small fluctuations in temperature may have biochemical and/or molecular consequences by altering the folding and final conformation of certain proteins in the oviduct and the embryo. Unfortunately, these temperature gradients cannot be imitated *in vitro* unless high-tech equipment is being applied. In CHAPTER 6, it was shown that lowering the *in vitro* culture temperature from 38.5° C to 37.3° C decreased blastocyst diameter significantly (P = 0.02) with a tendency of increased blastocyst rate. Even though 37.3° C approaches more the physiologic body temperature of the mare, a temperature of 38.5° C seems to be more beneficial to sustain intact *in vitro* equine embryo development. Indeed, blastocyst diameter seems to be a superior parameter of embryo viability compared with blastocyst percentage (Mckinnon, et al. 1988).

Equine embryos are generally cultured in small amounts of medium (1 - 2 µl/embryo; (Choi, et al. 2004a, Galli, et al. 2007, Hinrichs 2010, Smits 2010, Smits, et al. 2012a). As a consequence, taken into account the scarce availability of equine oviducts/ovaries/oocytes/embryos and thus the rather low sample sizes in experiments with equine embryos compared with bovine and porcine experimental set-ups, sample pooling is sometimes the only option to meet the detection limits of certain techniques. For instance, to obtain raw data concerning steroid concentrations using RIA in oviduct explant culture medium, all culture medium of all replicates was pooled per test group and concentrated in order to ensure that the outcome comes within the reach of the limits of detection (CHAPTER 5.1). Since the basic assumption of sample pooling is biological averaging, the measure taken on the pool of samples is equal to the average of the same measure taken on each of the individual samples which contributed and will therefore not reduce biological variation (Zhang and Gant 2005). Consequently, the average values of the measurements, either individual or pooled, will always be the same (Leger and Didrichsons 1994).

1.3 DARK CELL DEGENERATION

In our cell culture from day 2 on (CHAPTER 3), the oviductal explants showed phagocytosis of cells suffering from dark cell-degeneration without any observed features of autophage cell death. Nevertheless, the cells bordering the explants maintained their highly differentiated, ciliated status and intact cell membrane and did not seem to be functionally hampered (CHAPTER 3, CHAPTER 5). Moreover, only very few cells were observed to be Trypan blue, propidium iodide (SYBR14/PI), TUNEL and/or caspase-positive (CHAPTER 3) indicating that only very few cells show respectively membrane damage (Trypan blue, PI), DNA fragmentation (TUNEL) and/or apoptosis (caspase). It may be suggested that an intrinsic caspase-independent mechanism **(Fig. 4)** with atypical morphology may be detected. Hypoxia, mechanical or environmental agitation, nutrient depletion and waste product accumulation may be other likely causes of caspase-independent apoptosis. To definitively exclude caspase involvement, Z-VAD-fmk, the classical caspase inhibitor could be added to oviduct explant culture medium (Galluzzi, et al. 2012b).

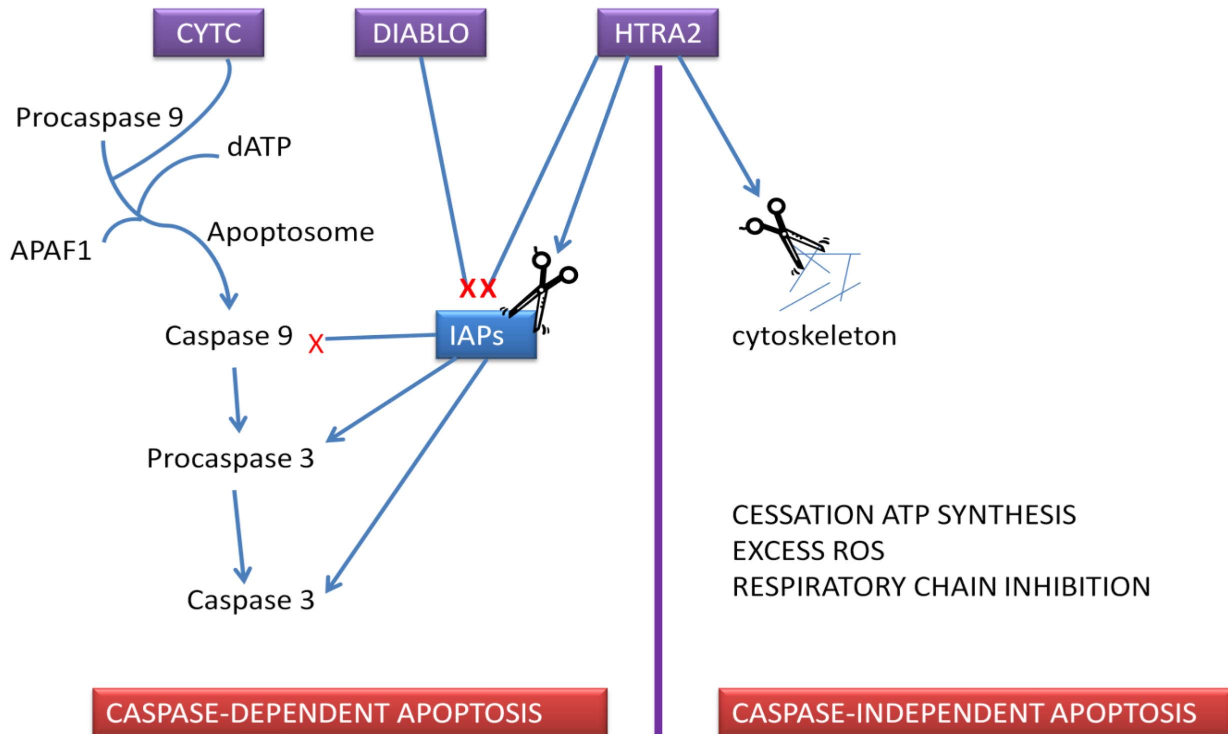


Fig. 4. Caspase-dependent and independent apoptosis. IAPs: inhibitor of apoptosis protein; CYTC: cytochrome c; DIABLO: direct IAP-binding protein; HTRA2: high temperature requirement protein A2; APAF1: cytoplasmic adaptor protein; ROS: reactive oxygen species. Adapted from Galluzzi, et al. 2012a.

It was hypothesized that the dark cell degeneration was induced by hypoxia (CHAPTER 3.1) as a putative consequence of hampered oxygen diffusion in the spherical oviductal explants. However, the oviductal expression of several markers for hypoxia, namely *HIFA* (Lin, et al. 2011), *VEGFA* (Fan, et al. 2009, Shweiki, et al. 1992) and *GLUT1* (Wrenzycki, et al. 2001a, Wrenzycki, et al. 1998b), was not increased after 6 days of culture (CHAPTER 3.1). Co-culture with equine embryos during 9 days did not increase the expression of these genes in the oviductal explants either. (CHAPTER 5.2). Therefore hypoxia is not likely to occur in our culture system. Moreover, culture at high (5 % CO_2 in air) and low (5 % O_2, 5 % CO_2, 90 % N_2) oxygen tension yielded a comparable incidence of explants showing DCD (CHAPTER 3.1).

A second putative cause for the DCD may be the occurrence of hypoglycemia in the culture medium (Galluzzi, et al. 2012a). Following the very high glucose concentration in equine embryo culture medium (17 mM) compared with the physiological oviductal concentrations

(3-5 mM; (Campbell, et al. 1979), hypoglycemia is very unlikely in the culture system, despite the measured high glucose consumption by the explants and the concomitant drop in glucose concentration (CHAPTER 3.1). In addition, long-term culture (6 days) of post- and preovulatory explants cultured without steroid supplementation, a spectacular decrease of *GLUT1* was noted (CHAPTER 3.1), rather indicating hyperglycemic conditions. The compensatory direct downregulating effect of high glucose concentrations on glucose transporters 1 (and 2) has also been observed in mouse embryos (Coutinho da Silva 2008, Moley, et al. 1998a, Moley, et al. 1998b). As a consequence of the strong downregulation of GLUTs, cellular hypoglycemia may arise which may activate apoptosis inducing factors (AIFs) which on its turn results in caspase-independent cell death (Santiago, et al. 2007) (**Fig. 4**). It should be noted that other (insulin sensitive) GLUTs, insulin or other factors involved in cellular energy metabolism may be involved.

Since it is reported that the addition of anti-apoptotic factors which are activated by insulin or IGF1 receptors, may decrease the number of explants showing the hallmarks of caspase-non dependent cell death (Purcell and Moley 2009), in a third hypothesis, the effect on the dark cell degeneration of 10% serum supplementation or 5 µg/ml insulin + 5 µg/ml transferrin + 5 ng/ml selenium (ITS) to the regular medium DMEM/F12 was investigated (CHAPTER 3.2). The addition of FBS seems to protect the oviductal explants to some extent against dark cell degeneration during the first 2 days of culture, whereas it seems to enhance the development of dark cell degeneration from day 3 on. It may be suggested that serum contains factors which partly protect against DCD, but which may be consumed after 2 days of culture. The addition of insulin, transferrin and selenium, enhanced the development of dark zones compared to unsupplemented DMEM/F12 or DMEM/F12 with 10% FBS. Thus, it could be concluded that components of ITS play a role in the development of dark cell degeneration. It has indeed been reported that insulin and IGFs increase caspase independent cell death and also the proportion of TUNEL positive cells (Chi, et al. 2000). However, it has been shown earlier (CHAPTER 3.1)(Nelis, et al. 2014) that the proportion of TUNEL positive cells after 6 days culture in medium supplemented with FBS, which contains considerable levels of insulin (6-14 µU/ml) (Gstraunthaler 2003), was not increased. Consequently, medium supplemented with 10 % FBS contains comparable levels of insulin as measured in (human) oviductal fluid (< 2 µU/ml) (Chi, et al. 2000) or in our medium supplemented with 5 µg/ml insulin. Thus, the DCD is not likely to be induced by insulin. Toxicity levels of transferrin are reported to be 20-

fold higher than 5 ng/ml as applied in our culture system (Bottenstein, et al. 1979). Therefore, also transferrin is not considered as being the principal cause of the DCD. Contrastingly, the toxic margin for selenium (selenite) in certain cell lines is 5 ng/ml whereas it promotes bovine embryo development at this concentration (Bottenstein, et al. 1979, Wydooghe, et al. 2014), which is equal to the concentration in our experiment. As a consequence, it is likely that selenium or its by-products enhances DCD in the medium supplemented with ITS compared to unsupplemented DMEM/F12 and during the first 2 days with the DMEM/F12 supplemented with 10 % of FBS.

2 STEROIDS IN THE EQUINE OVIDUCT

2.1 FIGURES AND FACTS

Tissue progesterone and 17β-oestradiol concentrations in the equine oviduct are obviously cycle stage dependent whereas testosterone and 17β-hydroxyprogesterone concentrations are not significantly altered. Median progesterone concentrations in the postovulatory ipsilateral oviduct tissue are 37 times increased when comparing with the preovulatory oviduct, whereas this increase is only three times at the contralateral side. The same tendency was observed in oviductal fluid of ipsilateral oviducts.

The most plausible explanation for the ipsilateral elevated progesterone concentration is a combination of 1) a local transfer between the ovary/corpus luteum and the oviduct, 2) the ipsilateral contribution of progesterone produced by the granulosa cells, and 3) local oviductal progesterone production. Since follicular fluid progesterone and 17β-oestradiol concentrations and their ratios were associated with oviductal progesterone and 17β-oestradiol concentrations, a local transfer mechanism of steroids between the ipsilateral corpus luteum bearing ovary could indeed contribute to the ipsilateral dramatically increased local oviductal concentrations (Barone 2001). Equine granulosa cells as well as granulosa cells from other species have been reported earlier to produce high amounts of progesterone in mammalian species (Chabrolle, et al. 2009, Schuetz and Dubin 1981, Smith, et al. 2011, Stoklosowa, et al. 1982). However, this cannot clarify the high levels of *in vitro* produced progesterone, since no granulosa cells were present in the culture system. Local production of progesterone by oviduct explants was obviously confirmed *in vitro* (CHAPTER 5.1). However, the expression in the oviduct of none of the key enzymes, 3-beta-HSD, StAR and cytochrome P450scc which are involved in the progesterone synthesis pathway, was markedly stronger in the ipsilateral oviduct. Nevertheless, their expression is region- and cycle-stage dependent in *in vivo* oviducts. The progesterone producing capacity of oviduct explants was confirmed *in vitro*. Indeed, progesterone production by oviduct explants originating from ipsilateral postovulatory oviducts produced 100 times more progesterone than explants from preovulatory oviducts (CHAPTER 5.1). Again, also *in vitro*, none of the investigated steroidogenic enzymes were upregulated. Nevertheless, the steroid responsiveness of the steroidogenic enzymes was confirmed *in vitro* in oviduct explants (CHAPTER 5.1).

From the *in vitro* and *in vivo* findings, it may be concluded that the drastically increased ipsilateral postovulatory progesterone concentrations are highly likely to be induced by oviductal synthesis, but (iso)enzymes other than 3-beta-HSD seem to be involved.

In CHAPTER 4, the concentrations of progesterone, 17β-oestradiol, testosterone and hydroxyprogesterone in equine oviductal tissue were determined by the highly powerful technique U-HPLC-MS/MS (Vanhaecke, et al. 2011), whereas RIA was applied to measure steroids in oviductal fluid (Szafranska, et al. 2002). Due to the tiny amount of oviductal fluid in each oviduct, which is about 50-100 µl on average, the limits of detection of U-HPLC-MS/MS for steroids in serum (10 pg/ml for 17α- 17β-hydroxytestosterone and 17-hydroxyprogesterone, 0.4 ng/ml for progesterone and 19 pg/ml for β-oestradiol) were approximated. Moreover, due to optimization issues, it was inevitable to switch to RIA. Even though the RIA results could be consistently higher compared with (U-HP)LC-MS/MS due to lower specificity, interference and matrix effects as reported before (Fernandes, et al. 2011), Nonetheless, the high correlation between RIA and MS, as described in Hsing et al. (2007) and in Dorgan et al. (2002) suggest that these two methods are very likely to yield similar proportions between pre- and postovulatory stage and ipsilateral and contralateral side. RIA may have caused a slight overestimation of the real values of steroid concentrations in the oviductal fluid, but the conclusion, on which is elaborated on in the aforementioned paragraphs, remains the same.

2.2 A WEIGHTY ROLE FOR STEROIDS, CONDUCTORS OF OVIDUCTAL FUNCTIONALITY

The cumulus-oocyte complex and spermatozoa enter the oviduct from opposite ends. The oocyte travels around 12 h to reach the ampullary-isthmic junction where fertilization occurs. After 6 days the embryo moves on to the uterus. In synchrony with the contraction of tubal musculature and the flow of oviductal secretions, gamete and embryo transport is established by ciliary activity (Buhi, et al. 1997, Jansen 1984, Killian 2004, Lyons, et al. 2006). Meanwhile, the oviductal epithelium prepares and establishes the optimal environment for gamete transport and maturation, fertilization and embryonic development and transport (Aguilar and Reyley 2005, Besenfelder, et al. 2012, Wolf, et al. 2003b). In order to accomplish this task, the oviduct epithelium undergoes cycle dependent changes, which precisely and timely prepare oviductal function in correspondence to the stage of embryonic

development and the site where the early embryo contacts the epithelium. These epithelial cycle dependent changes are conducted by steroids, with progesterone and 17β-oestradiol playing a leading role (Abe and Hoshi 2008, Nakahari, et al. 2011).

Data on the effect of progesterone and oestradiol on ciliary beat are contradictory. Most reports describe an accelerating effect of oestradiol and a decelerating effect induced by progesterone (Lyons, et al. 2002, Lyons, et al. 2006, Nakahari, et al. 2011, Orihuela, et al. 2001). The latter is confirmed by the prevention of progesterone induced ciliary beat frequency inhibition by mifepristone, which is a progesterone receptor blocker (Mahmood, et al. 1998). Moreover, in mammals, LH is inducing expression of progesterone receptors in ciliated cells, suggesting a regulatory role for progesterone on ciliary beat frequency and subsequent gamete and embryo transport (Akison and Robker 2012, Nishimura, et al. 2010). In CHAPTER 5.1, it has been shown that *in vitro*, the addition of postovulatory concentrations of 17β-oestradiol and progesterone decreased the number of explants showing ciliary activity, which may suggest that progesterone overshadows the effects of 17β-oestradiol and that progesterone is depressing indeed ciliary activity or is decreasing the number of ciliated cells. However, it should also be noted that progesterone and 17β-oestradiol may exert their actions through intermediate molecules and pathways which may be absent *in vitro*. Consequently, these other factors may alter or overshadow the effect of steroids on ciliary activity *in vivo*. Moreover, since it is only possible to count explants showing vigorous ciliary activity, the use of digital cameras would be interesting to visualize changes in ciliary beat frequency and pattern, and to determine the ratio of cells which are showing increased or decreased ciliary activity (Lyons, et al. 2006).

The steroids produced by the oviduct may not only exert their actions by modulating the oviductal environment, they may also act directly on the developing embryo. Indeed, the presence of membrane-associated progesterone (and oestrogen) receptors on early equine embryos (Rambags, et al. 2008) could confirm this hypothesis. If progesterone does have direct effects on embryonic development, this is most likely mediated by membrane-associated and not by nuclear progesterone receptors (Falkenstein, et al. 1996). This results in activation of cyclin B and MAP-kinases (Maller 2003), respectively regulators of mitosis (Pines 2006) and embryonic processes such as differentiation and angiogenesis (Kuida and Boucher 2004). Oestrogens can directly influence developmental processes such as embryonic brain gene expression (Beyer, et al. 2003), embryonic neural stem cell differentiation and

proliferation (Brannvall, et al. 2002). Up to now, no attempts have been undertaken to assess the effects of steroids on the development of *in vitro* produced equine embryos.

3 OVIDUCTAL GENE EXPRESSION BEARS THE STAMP OF STEROIDS AND EMBRYOS

In synchrony with ciliary and muscular activity, the epithelial cells prepare and provide the optimal environment for transport of gametes, fertilization and early embryonic development (Aguilar and Reyley 2005) and concurrent embryo-maternal crosstalk. This crosstalk is facilitated by 1) cytokines such as *CSF1*, 2) growth factors such as IGFs, EGFs and TGFA, 3) angiogenic factors such as VEGF, 4) adhesion molecules such as galectins and 5) apoptotic factors such as galectin-3-bp (Aguilar and Reyley 2005). To fulfill this task, the epithelium that lines the oviductal lumen, serves as an interface to determine the composition of the oviductal fluid, which is crucial for embryonic survival (Besenfelder, et al. 2012, Wolf, et al. 2003b). The composition of oviductal fluid is strongly regulated by both steroids and the presence of gametes or an embryo (CHAPTER 5.1 and CHAPTER 5.2). Steroids may alter expression and secretion patterns and may alter diffusion of, for instance, amino acids or steroids by modulating the permeability of membranes in a spatio-temporal pattern (Carlson, et al. 1970, Ehrenwald, et al. 1990, Killian, et al. 1989). On the other hand, the oviductal-stage early equine embryo synthesizes already PGE2 in order to relax the smooth muscle cells of the uterotubal junction (Weber, et al. 1991a, b). This demonstrates that embryo-maternal communication is already established at this stage. It also confirms that also the embryo is able to modulate oviductal function and gene expression. Indeed, the murine transcriptomic profile is reported to be altered in response to the presence of embryos (Lee, et al. 2002). In addition, the oviductal proteome is altered as well in response to developing embryos (Seytanoglu, et al. 2008).

In order to understand the molecular mechanisms of embryo-maternal interaction, an in-depth understanding of the temporal (Bauersachs, et al. 2007, Bauersachs, et al. 2004, Lapointe and Bilodeau 2003, Lapointe, et al. 2006, Swangchan-Uthai, et al. 2011) and regional (Arganaraz, et al. 2012, Jeoung, et al. 2010, Kubota, et al. 2009) transcriptomic changes in the oviduct as well as the embryo-induced gene expression patterns is required. Therefore, in CHAPTER 4 and CHAPTER 5.1 the effects of respectively steroids *in vivo* and *in vitro* and the effects of *in vitro* developing embryos (CHAPTER 5.2) on the mRNA expression of 11 putative steroid-regulated embryotrophic genes such as proteases (*MMP2*, *PLAU*) and their inhibitors (*TIMP1*, *PAI1*), growth factors (*CSF1*, *TGFA*, *VEGFA*) and glucose transporters (*GLUT1*) and

prostaglandin E2 receptors (*PTGER2, PTGER4*) were investigated in oviductal cells. The results show that the equine oviduct is a source of both proteases and their specific inhibitors (Gabler, et al. 2001, Kouba, et al. 1998, 2000a) such as PLAU/PAI and MMP2/TIMP1. Moreover, the expression of mRNA measured by RT-qPCR of the proteases and inhibitors is cycle-stage dependent and responds also to progesterone and oestradiol exposure as well as to the presence of developing equine embryos *in vitro* (**Fig. 5, Fig. 6**).

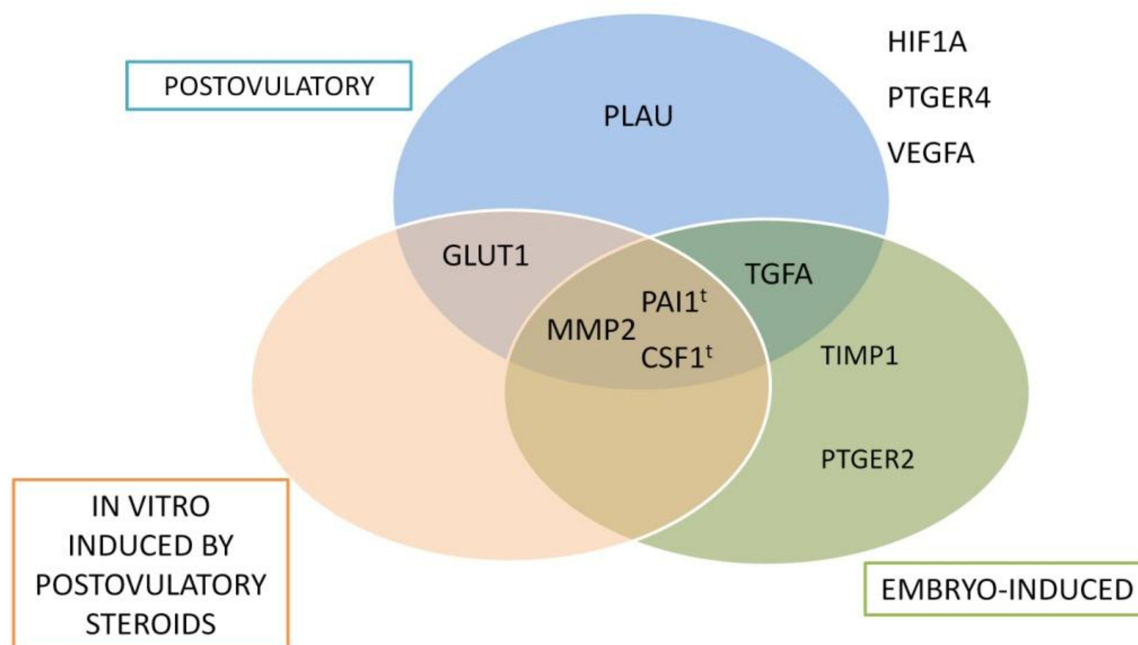


Fig. 5 Venn diagram of all investigated factors, except *IGFI* (due to technical restrictions in co-cultured explants). MRNA of all factors depicted in the figure was expressed in the equine oviduct. *HIF1A*, *PTGER4* and *VEGFA* did not respond to steroids neither to embryonic signalling. *PLAU, MMP2*, *PAI1*, *CSF1, GLUT1* and *TGFA* were upregulated in oviduct explants in the postovulatory phase. *MMP2, PAI1, CSF1* and *GLUT1* could also be induced *in vitro* by adding postovulatory concentrations of progesterone and oestradiol to preovulatory explants. *MMP2* was also upregulated in explants co-cultured with equine embryos. A similar tendency (t) was observed in *PAI1* and *CSF1*. *TIMP1* and *PTGER2* could be induced by embryos but do not seem to be steroid-regulated whereas *GLUT1* seems to be steroid-regulated without responding to embryonic stimuli.

MMP2, TGFA, and to a lesser extent to *PAI1* and *CSF1* mRNA expression responds to both postovulatory steroids as well as to embryonic signalling by ICSI-derived equine embryos (**Fig. 5**). Moreover, the steroid-effect can be reproduced *in vitro* in *MMP2, PAI1* and *CSF1*.

TIMP1 and *PTGER2-2* are not responsive to steroids but do firmly answer to embryonic factors whereas *PLAU* and *GLUT1* solely reply to steroids. The latter effect is inducible *in vitro* in *GLUT1* but not in *PLAU* **(Fig. 5)**. The expression of the hypoxia markers *HIF1A* and *VEGFA* (van den Driesche, et al. 2008) was not altered by steroids neither by embryos, indicating that no hypoxia prevails in our culture system.

Interestingly, the ratio of *PLAU/PAI1* and *MMP2/TIMP1* expression was respectively 4 and 4.5 times lower in postovulatory explants with embryo co-culture compared to postovulatory explants without embryo co-culture (CHAPTER 5.2). This confirms the hypothesis that the oviduct responds to the embryo's signalling by establishing a predominantly antiproteolytic environment.

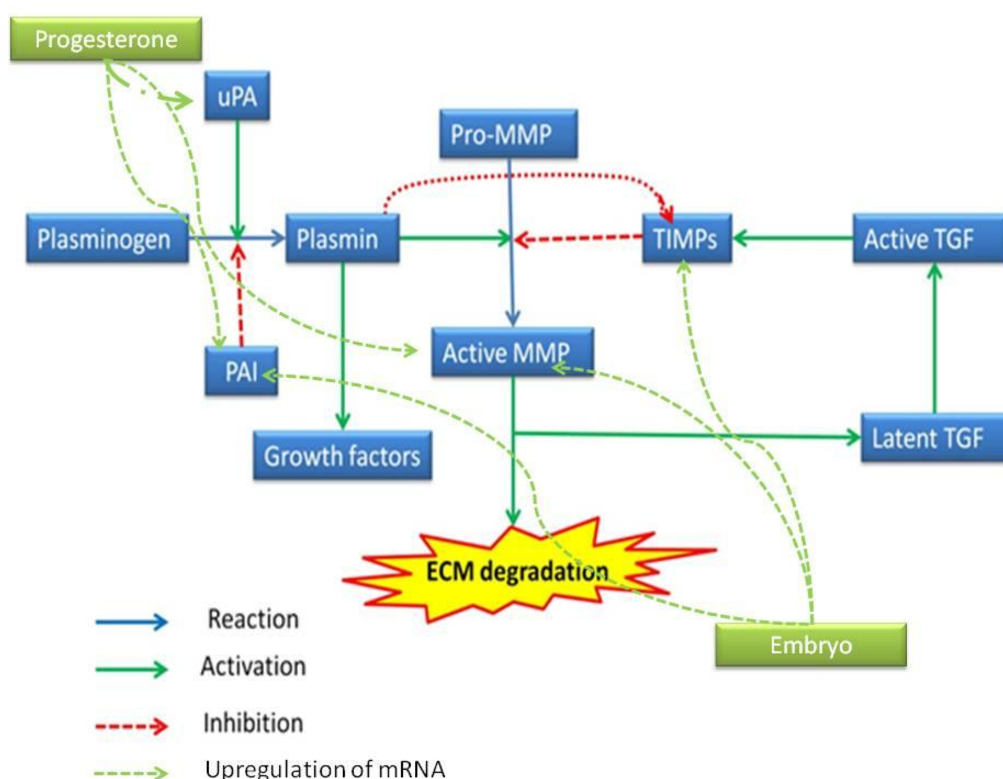


Fig. 6 The plasminogen-plasmin pathway. Plasminogen is activated by urokinase plasminogen activator (uPA), which is inhibited by plasminogen activator inhibitor 1 (PAI1). On its turn, plasmin activates matrix metalloproteinases (MMPs) and growth factors. MMPs are inhibited by tissue inhibitors of MMPs (TIMPs), the latter are proteolysed by plasmin. Active MMPs degrade the extracellular matrix (ECM). Progesterone is able to increase mRNA expression of *PLAU* (uPA), *PAI1* and *MMP2* in equine oviduct explants *in vivo* and *in vitro*. mRNA expression of *PAI1, MMP2* and *TIMP1* is increased in response to embryonic signalling during *in vitro* co-culture.

Extracellular matrix degradation is initiated by the generation of plasmin from its inactive form plasminogen, catalyzed by uPA (*PLAU* = gene), whereas *PAI1* is the potent physiological inhibitor of PAs. The former controls therefore the activation of plasminogen and subsequently maintains and stabilizes the oviductal epithelium and milieu **(Fig. 6**). Consequently, *PAI1* might act to prevent premature nidation of the embryo (Kouba, et al. 1998). However, since the early equine embryo lacks invasive potential (Bazer, et al. 2009), it is possible that the *PAI1,* produced by the oviductal epithelium protects the oviduct from embryo-induced proteolysis (Kouba, et al. 1998). Since PAI1 is slightly upregulated in response to embryonic messages without inducing PLAU, an anti-proteolytic milieu may be created. PAI1 may also counterbalance embryo-derived uPA. Indeed, the equine embryo, like the human (Duc-Goiran, et al. 1999) and mouse and rat embryo (Harvey, et al. 1995) may produce additional uPA to facilitate its transport through the oviduct (Harvey, et al. 1995, Zhang, et al. 1994). The fact that the uPA receptor has also been detected in the bovine and porcine oviduct (Garcia, et al. 2014, Roldan-Olarte, et al. 2012) may support this hypothesis. PAI1 acts in concert with TIMP1 (Kouba, et al. 1998, 2000a), the specific inhibitor of MMP2 **(Fig. 6**). MMP2, also known as gelatinase A, degrades collagen types IV, V, VII and X, elastin and denatured collagens (Lijnen 2002) with subsequent pericellular proteolysis.

Altogether, these observations could sustain again the hypothesis that the embryo produces factors which enhance predominantly *PAI1* production and simultaneously *TIMP1* levels in order to counteract the proteolytic activities to protect the embryo, in particular the zona pellucida, from oviductal or embryonic plasminogen activator activity (Kouba, et al. 2000a). However, it should be borne in mind that no protein content or activity of the proteases and their inhibitors was determined. Due to the presence of inhibitors, the mRNA expression may remain high while enzyme activity is decreased (Gabler, et al. 2001). Basal *PAI1* activity may inhibit *PLAU* activity while *PLAU* mRNA levels remain high. Moreover, next to *PAI1*, PAI2 and nexin, 2 other inhibitors of *PLAU* (Blasi 1997) may, for instance, bias the interpretation of the relevance of mRNA fluctuations.

Next to *MMP2* and *PAI*, *TGFA* and *CSF1* are upregulated in postovulatory explants, whereas it is additionally upregulated as a result of co-culture with embryos. TGFA interacts with and mediates its biological effects through EGF receptors and may be modulated by glucose (Daniels, et al. 1993, Paria, et al. 1990). TGFA may exert autocrine and paracrine effects, inducing cell proliferation (Lyons, et al. 1988) and suppressing progesterone production in

steroidogenic tissue (Roberts and Skinner 1991). Since EGF receptors, like in human (Cross, et al. 1994, Duc-Goiran, et al. 1999) may also be present on equine embryos, the embryo may in a positive feedback further stimulate oviductal TGFA expression. Plasmin may also induce TGFA, like it induces other growth factors such as EGF, FGFs, IGFs and VEGFs (Gabler, et al. 2001, Lee, et al. 2005, Taipale and KeskiOja 1997). Also CSF1 receptors have been reported in human embryos (Duc-Goiran, et al. 1999, Sharkey 1998). These are highly likely to be expressed also on equine concepti, since co-culture slightly concomitantly enhances CSF1 expression in oviduct explants, while postovulatory steroids induced a strong upregulation **(Fig. 5)**. Levels in mice uteri have been reported to increase 1000 fold in response to pregnancy. Its importance in embryonic development has been confirmed in depletion studies in which failure of initiation of DNA synthesis has been shown (Matsushime, et al. 1991). Moreover, it has been detected in mouse oviducts (Arceci, et al. 1989, Pampfer, et al. 1991b).

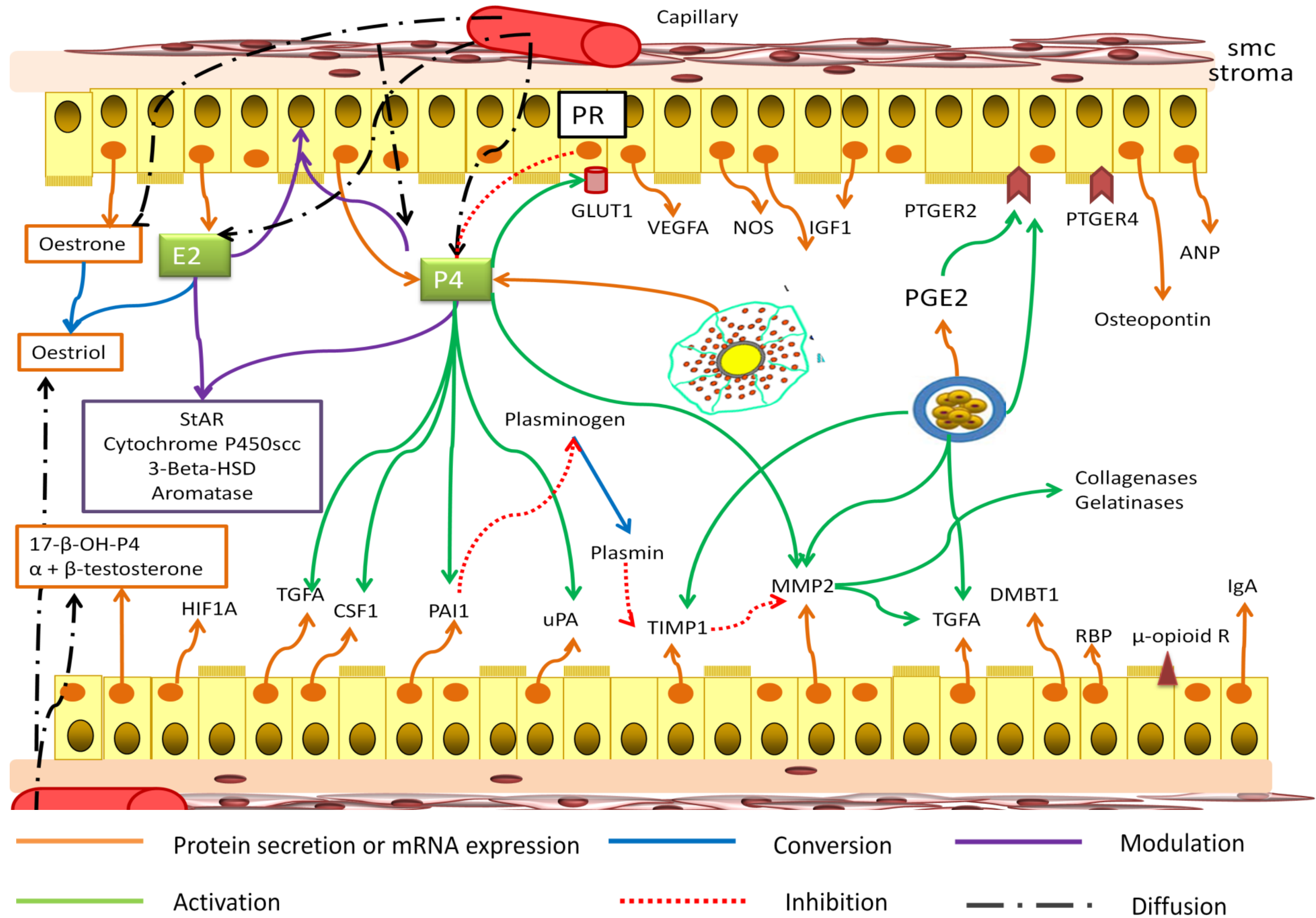
Comparable to the expression pattern of *PLAU, GLUT1* is upregulated in postovulatory explants compared to the preovulatory counterparts (CHAPTER 5.1), but its expression is not altered in response to embryos. In addition, *in vitro* oviductal epithelial glucose consumption is higher in postovulatory derived oviduct epithelium compared with the preovulatory stage (CHAPTER 5.1). In endometrial cancer cells a similar trend has been observed in response to the higher energy demand in response to increased transcriptory and translatory activity (Medina, et al. 2004). Taken into account the elevated glucose consumption in steroid-stimulated cells, it is highly likely that a similar mechanism prevails in oviductal cells. Progesterone and β-oestradiol supplementation to the culture medium induced an upregulation of *GLUT1* in both preovulatory and postovulatory explants. Higher glucose consumption is not only observed in tissues that are influenced by high progesterone concentrations, but also in tissues that produce progesterone themselves. An enhanced oxygen and glucose consumption was observed in progesterone producing luteal tissue compared with luteal tissues which lost their progesterone producing ability (Armstron and Black 1966). This finding is in line with the fact that postovulatory explants, which produce 100 times more progesterone compared with preovulatory explants, consume significantly more glucose than their preovulatory counterparts (CHAPTER 5.1). Co-culture did not further stimulate GLUT1 expression even though the equine embryo is reported to produce considerable amounts of progesterone and oestradiol (Marsan, et al. 1987, Walters, et al. 2001, Zavy, et al. 1979)

which could directly or indirectly enhance GLUT1 expression. Even though, nonetheless the embryos may produce GLUT1-enhancing steroids, the embryos may also produce factors, which decreases GLUT1 expression, such as calreticulin, which was also detected in the equine oviduct (Smits, Nelis, et al. submitted) in response to the high glucose concentrations (Totary-Jain, et al. 2005). Furthermore, other GLUTs which work against the gradient such as SGLT1 and SGLT2 (De Vos, et al. 1995) may be involved and other factors and energy substrates may play a role in the embryo-oviductal energy metabolisms and dialogue.

PTGER2 and PTGER4 expression was not altered in response to steroid stimulation *in vivo* or *in vitro*. In contrast, co-culture induced a strong upregulation of PTGER2 but not PTGER4. This may indicate that the embryo-derived PGE2 interacts with PTGER2 rather than PTGER4 in order to open the uterotubal papilla (Weber et al., 1991a; b; Freeman et al., 1992).

In conclusion, to cope with the changing needs during the early reproductive events, the oviduct modifies its gene expression in response to cycle related local steroid fluctuations and embryonic signalling.

Fig. 7 presents an overview of all current knowledge of genes, enzymes, proteins and receptors demonstrated in the oviductal epithelium or oviductal fluid of the mare, based on the findings in this thesis, own unpublished results and on literature data. Retinol binding protein (RBP) (McDowell, et al. 1993), deleted in malignant brain tumor 1 (DMBT1) (Ambruosi, et al. 2013), μ-opioid receptor (Desantis, et al. 2010), and osteopontin and atrial natriuretic peptide (ANP A) (Mugnier, et al. 2009) were detected in oviduct fluid and/or oviductal epithelium.


Capillary
smc
stroma
PR
GLUT1
VEGFA
NOS
IGF1
PTGER2
PTGER4
ANP
Oestrone
E2
P4
Oestriol
PGE2
Osteopontin
StAR
Cytochrome P450scc
3-Beta-HSD
Aromatase
Plasminogen
Plasmin
Collagenases
Gelatinases
17-β-OH-P4
α + β-testosterone
TGFA
HIF1A
CSF1
PAI1
uPA
TIMP1
MMP2
TGFA
DMBT1
IgA
RBP
μ-opioid R
Protein secretion or mRNA expression
Conversion
Modulation
Activation
Inhibition
Diffusion

4 FINAL CONCLUSIONS

In conclusion, for the first time, we optimized an equine oviduct explant model and used it to simulate the effect of pre- and postovulatory changes in progesterone and oestradiol concentrations upon oviduct epithelial cells. In this way we showed that steroids were able to modify ciliary activity, energy metabolism, gene expression, immunoreactive steroidogenic enzyme expression and progesterone receptor expression in oviductal explants. Furthermore, a set of embryotrophic genes was shown to be upregulated in the oviductal epithelium originating from mares in the postovulatory cycle stage *in vivo* and *in vitro*. These cycle-related changes indicate the importance of steroids, especially progesterone, in fertilization, embryo growth and viability, as well as of the oviduct, both as a target of steroids and as a site of steroid biosynthesis and metabolism. Moreover, it was confirmed that embryos are capable of changing the oviductal gene expression *in vitro* and the proteome *in vivo*, confirming that the embryo does communicate with its environment. These findings are of significant value and will be implemented in an *in vitro* oviduct-embryo co-culture model in order to elaborate further on the deciphering of endocrine, autocrine and paracrine signalling during early embryo development in the horse.

5 PROSPECTS FOR THE FUTURE

5.1 *IN VIVO/EX VIVO* MODELS

In vivo models with proteome (nano-UHPLC-MS/MS), transcriptome (micro-array and next generation sequencing) and/or lipidome analysis (for instance electrospray ionization tandem mass spectrometry (Murphy, et al. 2001)) are without doubt the gold standard to investigate embryo-maternal and gamete-maternal interaction. However, the investigation of the specific interactions between the gametes and embryo and the oviduct *in vivo* is almost impossible due to the very local character of the interactions, the very small amounts of material and the difficult access of the oviduct in large animals *in vivo*. As a consequence, only major alterations in expression patterns can be determined.
An interesting approach to decipher the contribution of a single molecule is to transfer gamete or blastocyst-sized gelatin beads carrying the absorbed target molecule (Glattauer, et al. 2010, Hwang, et al. 2000, Ma, et al. 2013) into the oviduct. These carrier beads may mimic the presence of gametes or an embryo and provoke a maternal response. As already performed in mice (Paria, et al. 2002), the maternal signals can be captured *in vivo* by means of transcriptome and proteome analysis (Paria, et al. 2001). However, it is difficult to distinguish between gamete and embryo-induced effects (Fazeli 2008).

The combination of transcriptome data of oviductal ligands and embryonic receptors and vice versa, will also provide putative interacting factors which offers a very robust base for target confirmation studies (Ulbrich, et al. 2013a, Ulbrich, et al. 2013b).

5.2 *IN VITRO* MODELLING

Evaluation of specific effects

As already mentioned before, embryo-maternal interactions are difficult to capture *in vivo* since the exact location of the embryo is almost impossible to determine. As a consequence, upregulated molecules with a low abundance may be diluted to undetectable levels. This could be circumvented using *in vitro* oviduct explants. Oviduct explants could be attached to a culture plate and co-cultured with embryos. To unravel and compare the response of embryos on for instance ciliated and non-ciliated cells, single cell isolation by laser capture

microdissection **(Fig. 8)** (Espina, et al. 2006) with subsequent transcriptome (next generation sequencing and microarray) proteome (nano-UHPLC-MS/MS) and/or lipidome analysis (using for instance electrospray ionization tandem mass spectrometry (Murphy; et al. 2001)) may identify critical factors in the embryo-maternal interplay.

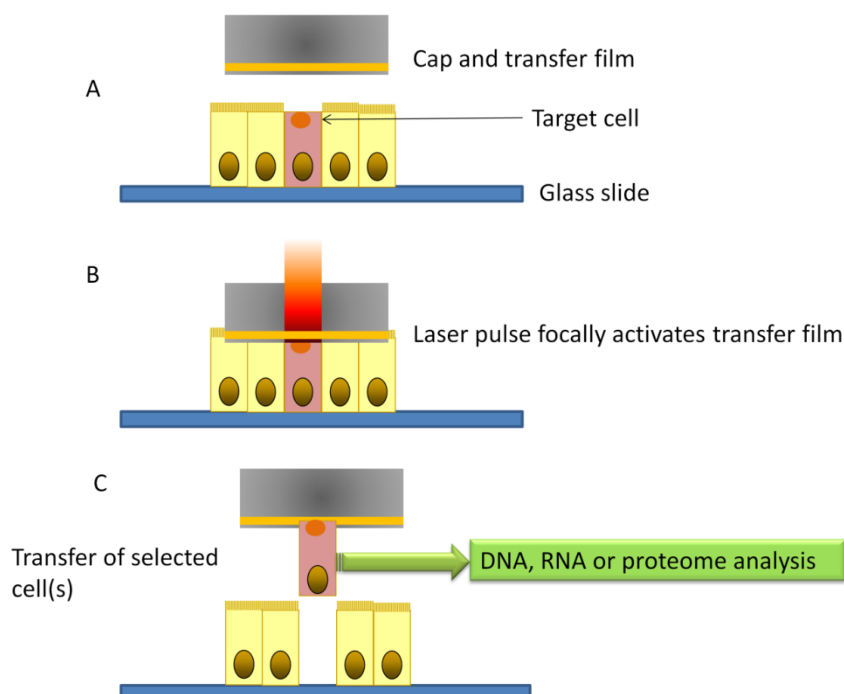


Fig. 8. Laser capture microdissection. Tissue slide with target cell(s) fixed on a glass slide (A). Using a laser pulse which activates a transfer film (B), the target cell(s) are isolated from the tissue sample (C). Adapted from Espina, et al. 2006.

Further confirmation

In order to unravel the pathways which may play a role in oocyte-, sperm- and embryo-maternal interaction an *in vitro* model can be used, such as the oviduct explant. To confirm functional relevance of putative candidate genes, these genes should be overexpressed or knocked down or recombinant protein could be added to the culture medium. The development of a suitable *in vitro* system (CHAPTER 3; CHAPTER 5) combined with the developments in vector design (Davis 2002, Kaufman 2000) and RNA interference (RNAi) (Azorsa, et al. 2006, Fire, et al. 1998, Timmons, et al. 2003) will boost these investigations. RNA interference, a technique for selectively inhibiting specific genes **(Fig. 9)**, may help to

identify processes and signalling pathways by performing large scale screening (Kupferschmidt 2013).

How promising the newest biotechnological techniques may be the establishment of the hierarchy of molecular relationships and the distinction whether signalling pathways operate as a network, in parallel or independently (Paria, et al. 2002), remains a considerable challenge. Another challenge is to unravel critical factors within the same gene family or within the same gene via alternative splicing. For instance, when EGF, TGFA and amphiregulin are deleted, their function is taken over by other members of the same gene family, which obscures the relative significance of individual genes contributing to embryo-maternal interaction (Lim, et al. 2002).

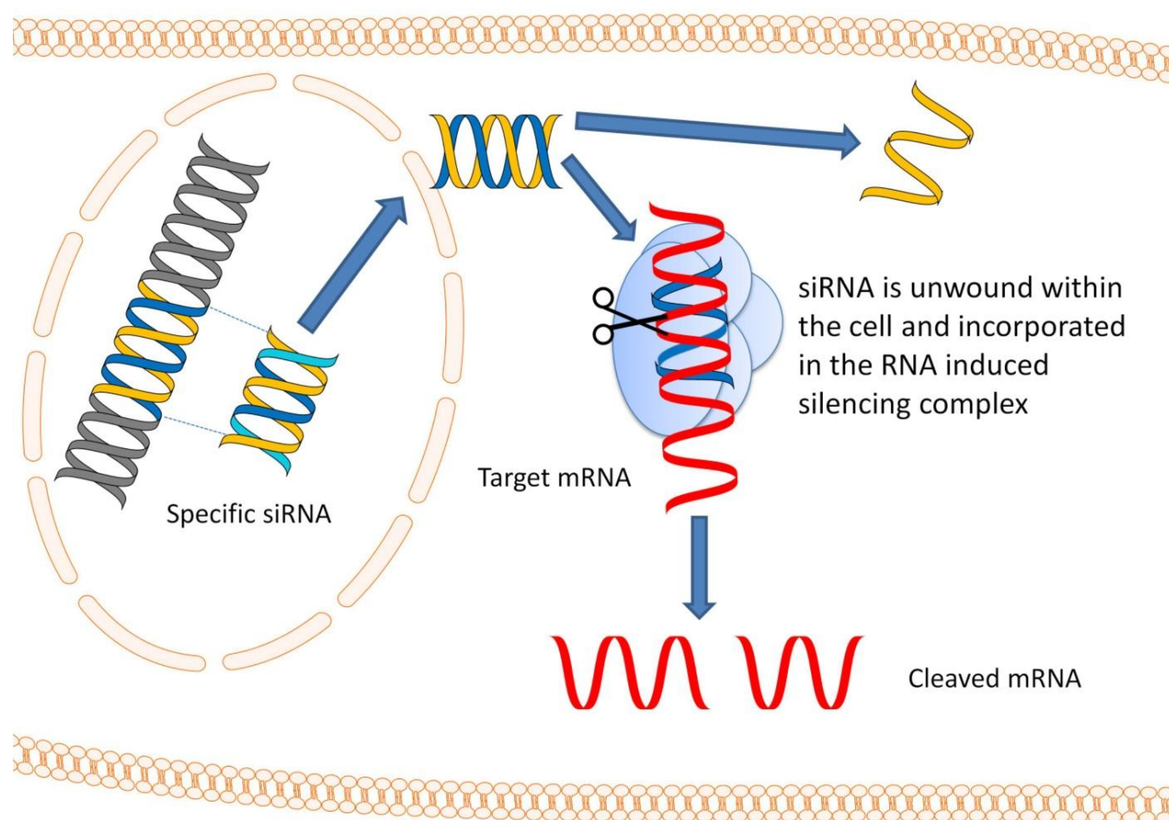


Fig. 9. Gene silencing through RNA interference. RNAi technology takes advantage of the cell’s natural machinery, facilitated by short interfering RNA molecules, to effectively knock down expression of a gene of interest. There are several ways to induce RNAi, synthetic molecules, RNAi vectors, and *in vitro* dicing. In mammalian cells, short pieces of dsRNA, short interfering RNA (siRNA), initiate the specific degradation of a targeted cellular mRNA. Adapted from: Azorsa, et al. 2006. Picture drawn using MOTIFOLIO PPT drawing toolkit®.

In vitro fertilization

Up to now, conventional *in vitro* fertilization results in equine species are disappointing. The lack of hyperactivated motility under standard *in vitro* capacitation conditions has been proposed as the main reason why *in vitro* fertilization fails in the horse (Hinrichs and Loux 2012). To elucidate the conditions that trigger hyperactivated motility of stallion spermatozoa in the mare's reproductive tract and to determine whether release of capacitated sperm from oviduct epithelial cells could be achieved under *in vitro* conditions, an oviductal cell model is indispensable (Lagutina, et al. 2007, Leemans, et al. 2014, Leemans, et al. 2011). For instance, oviduct-derived osteopontin and atrial natriuretic peptide have been reported as potentially involved in equine fertilization (Mugnier, et al. 2009).On the other hand, also the effect of spermatozoa on oviductal cells could be studied *in vitro* using oviduct cells (Abe 1996, Aldarmahi, et al. 2014, Aldarmahi, et al. 2012).

Inadequate maturation of the horse oocyte, which is an obstacle for *in vitro* production of equine embryos, may also benefit from the use of the oviduct explant model. The proportion of equine oocytes that reach the metaphase II stage of development after culture *in vitro* for 20 to 40 h is much lower than for other domestic species (Li, et al. 2001). Interestingly, encouraging pregnancy rates were reported when metaphase I oocytes, obtained by oocyte aspiration 24 h after administration of gonadotrophins, were transferred simultaneously with spermatozoa into the oviducts of recipient mares (Carnevale, et al. 2000). This proves that equine oocytes can complete maturation in the oviduct and be fertilized successfully thereafter *in vivo*.
This illustrates also the important role of the oviduct in equine oocyte maturation and the great potential of our oviduct explants model in gaining knowledge of the conditions required to achieve satisfactory equine maturation and fertilization rates. In addition, it appears to be an excellent tool for proteome, transcriptome and miRNome analysis in order to unravel embryo–maternal interactions in the horse to improve *in vitro* embryo development.

Towards the improvement of equine embryo culture medium

After the functional confirmation of particular target molecules, these molecules may be added to equine embryo culture media in order to increase blactocyst quality.

Nevertheless, up to now, most studies have only investigated the effect of a single factor while *in vivo*, a coordinated crosstalk between the embryo and its environment is enabled by a complex network of several interacting factors. A complex mixture of carefully determined concentrations of embryotrophic factors would reproduce natural conditions more closely. An *in vitro* system allows to capture and unravel pathways and reactions induced by for instance one single factor without the presence of other influences which may obscure its effect. For example, the importance of a growth factor for embryonic development could be first confirmed by detecting its respective receptors on equine embryos and subsequently, its regulation, biological functions and involvement in embryo-maternal communication could be studied *in vitro* whereas *in vivo*, numerous factors may overshadow the single factor's effects. Like this, the equine embryo culture medium could be enriched with maternally derived proteins and other (growth) factors (Richter 2008) which contribute to the improved development of embryos (Roudebush, et al. 2004).

In order to design an optimal appropriate horse-specific (co-)culture medium, gaining knowledge in the energy metabolism of both the embryo and the oviduct is imperative (Leese, et al. 2008). Up to now, very little is known concerning the energy and oxygen requirement of developing embryos. To gain insights in metabolic pathways, both enzyme-linked fluorescence assays and radiolabelled substrates to detect the appearance and disapprearance of a particular substrate from culture media have been reported (Rieger , et al., 1992, Guerif, et al 2013).
To derive knowledge about oxidative metabolism, oxugen consumption of embryos and oviduct cells may be measured using pyrene fluorescence (Houghton , et al. 1996) and nanorespirometry (Lopes, et al. 2010). In addition, metabolic inhibitors such as cyanide, can be added to the culture medium. From this, it can be proven whether or not a certain pathway is essential for development (Brison & Leese 1994).

Taken together, the modifications induced by co-cultured oviduct explants, mimicking oviductal secretions in response to the embryo's needs, could be the basis of the improvement of equine embryo culture conditions.

6 REFERENCES

Abe, H 1996 The mammalian oviductal epithelium: Regional variations in cytological and functional aspects of the oviductal secretory cells. *Histology and Histopathology* **11** 743-768.

Abe, H, and H Hoshi 1997 Bovine oviductal epithelial cells: Their cell culture and applications in studies for reproductive biology. *Cytotechnology* **23** 171-183.

Abe, H, and H Hoshi 2008 Morphometric and ultrastructural changes in ciliated cells of the oviductal epithelium in prolific Chinese Meishan and Large White pigs during the oestrous cycle. *Reproduction in Domestic Animals* **43** 66-73.

Abuhijleh, MF, OA Habbal, and ST Moqattash 1995 The Role of the Diaphragm in Lymphatic Absorption from the Peritoneal-Cavity. *Journal of Anatomy* **186** 453-467.

Aguilar, J, and M Reyley 2005 The uterine tubal fluid: secretion, composition and biological effects. *Animal Reproduction* **2** 91-105.

Aguilar, JJ, J Cuervo-Arango, H Mouguelar, and L Losinno 2012 Histological Characteristics of the Equine Oviductal Mucosa at Different Reproductive Stages. *Journal of Equine Veterinary Science* **32** 99-105.

Akahani, S, P NangiaMakker, H Inohara, HRC Kim, and A Raz 1997 Galectin-3: A novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Research* **57** 5272-5276.

Akison, LK, and RL Robker 2012 The Critical Roles of Progesterone Receptor (PGR) in Ovulation, Oocyte Developmental Competence and Oviductal Transport in Mammalian Reproduction. *Reproduction in Domestic Animals* **47** 288-296.

Aldarmahi, A, S Elliott, J Russell, and A Fazeli 2014 Effects of spermatozoa-oviductal cell coincubation time and oviductal cell age on spermatozoa-oviduct interactions. *Reproduction Fertility and Development* **26** 358-365.

Aldarmahi, A, S Elliott, J Russell, T Klonisch, S Hombach-Klonisch, and A Fazeli 2012 Characterisation of an in vitro system to study maternal communication with spermatozoa. *Reproduction Fertility and Development* **24** 988-998.

Alkhalaf, M, AY Propper, G Chaminadas, and GL Adessi 1992 Ultrastructural-Changes in Guinea-Pig Endometrial Cells during the Estrous-Cycle. *Journal of Morphology* **214** 83-96.

Alminana, C, PR Heath, S Wilkinson, J Sanchez-Osorio, C Cuello, I Parrilla, MA Gil, JL Vazquez, JM Vazquez, J Roca, EA Martinez, and A Fazeli 2012 Early Developing Pig Embryos Mediate Their Own Environment in the Maternal Tract. *Plos One* **7**.

Ambruosi, B, G Accogli, C Douet, S Canepa, G Pascal, P Monget, C Moros, U Holmskov, J Mollenhauer, C Robbe-Masselot, O Vidal, S Desantis, and G Goudet 2013 Deleted in malignant brain tumor 1 is secreted in the oviduct and involved in the mechanism of fertilization in equine and porcine species. *Reproduction* **146** 119-133.

Arceci, RJ, S Pampfer, and JW Pollard 1992 Role and Expression of Colony-Stimulating Factor-I and Steel Factor Receptors and Their Ligands during Pregnancy in the Mouse. *Reproduction Fertility and Development* **4** 619-632.

Arceci, RJ, F Shanahan, ER Stanley, and JW Pollard 1989 Temporal Expression and Location of Colony-Stimulating Factor-I (Csf-1) and Its Receptor in the Female Reproductive-Tract Are Consistent with Csf-1-Regulated Placental Development. *Proceedings of the National Academy of Sciences of the United States of America* **86** 8818-8822.

Arganaraz, ME, SA Apichela, and DC Miceli 2012 LEFTY2 expression and localization in rat oviduct during early pregnancy. *Zygote* **20** 53-60.

Armstron, D, and DL Black 1966 Influence of Luteinizing Hormone on Corpus Luteum Metabolism and Progesterone Biosynthesis Throughout Bovine Estrous Cycle. *Endocrinology* **78** 937-&.

Arrigo, AP 2005 In search of the molecular mechanism by which small stress proteins counteract apoptosis during cellular differentiation. *Journal of Cellular Biochemistry* **94** 241-246.

Avilés, M, A Gutiérrez-Adán, and P Coy 2010 Oviductal secretions: will they be key factors for the future ARTs? *Molecular Human Reproduction* **16** 896-906.

Azorsa, DO, S Mousses, and NJ Caplen 2006 Potential for Therapeutics and Functional Genomics. *In* CGJaMJ During. (ed.), Peptide Nucleic Acids, Morpholinos, and Related Antisense Biomolecules, pp. 13.

Azoury, J, MH Verlhac, and J Dumont 2009 Actin filaments: key players in the control of asymmetric divisions in mouse oocytes. *Biology of the Cell* **101** 69-76.

Ball, BA, and M Altschul 1990 In vitro development of 4- to 8- cell equine embryos cocultured with trophoblastic vesicles or oviductal explants. . *Theriogenology* **33** 189.

Ball, BA, M Altschul, and JE Ellington 1991 Invitro Development of Day-2 Equine Embryos Cocultured with Oviductal Explants or Trophoblastic Vesicles. *Theriogenology* **35** 669-682.

Ball, BA, SP Brinsko, PGA Thomas, PG Miller, and JE Ellington 1993 Development to Blastocysts of One-Cell to 2-Cell Equine Embryos after Coculture with Uterine Tubal Epithelial-Cells. *American Journal of Veterinary Research* **54** 1139-1144.

Ball, BA, and PG Miller 1992 Survival of Equine Embryos Cocultured with Equine Oviductal Epithelium from the 4-Cell to 8-Cell to the Blastocyst Stage after Transfer to Synchronous Recipient Mares. *Theriogenology* **37** 979-991.

Ball, BA, KE Scoggin, MHT Troedsson, and EL Squires 2013 Characterization of prostaglandin E-2 receptors (EP2, EP4) in the horse oviduct. *Animal Reproduction Science* **142** 35-41.

Ballester, L, J Romero-Aguirregomezcorta, C Soriano-Ubeda, C Matas, R Romar, and P Coy 2014 Timing of oviductal fluid collection, steroid concentrations, and sperm preservation method affect porcine in vitro fertilization efficiency. *Fertility and Sterility* **102** 1762-U1598.

Barenberg, P, H Strahlendorf, and J Strahlendorf 2001 Hypoxia induces an excitotoxic-type of dark cell degeneration in cerebellar Purkinje neurons. *Neuroscience Research* **40** 245-254.

Barone, R 2001 Anatomie comparée des mammifères domestiques. *In* Vigot (ed.), Anatomie comparée des mammifères domestiques., pp. 322. Paruis.

Bauersachs, S, H Blum, S Mallok, H Wenigerkind, S Rief, K Prelle, and E Wolf 2003a Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: A transcriptomics approach. *Biology of Reproduction* **68** 1170-1177.

Bauersachs, S, H Blum, S Mallok, H Wenigerkind, S Rief, K Prelle, and E Wolf 2003b Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: a transcriptomics approach. *Biol Reprod* **68** 1170-1177.

Bauersachs, S, K Mitko, H Blum, and E Wolf 2007 Technical note: Bovine oviduct and endometrium array version 1: A tailored tool for studying bovine endometrium biology and pathophysiology. *Journal of Dairy Science* **90** 4420-4423.

Bauersachs, S, S Rehfeld, SE Ulbrich, S Mallok, K Prelle, H Wenigerkind, R Einspanier, H Blum, and E Wolf 2004 Monitoring gene expression changes in bovine oviduct

epithelial cells during the oestrous cycle. *Journal of Molecular Endocrinology* **32** 449-466.

Bavister, BD 1995 Culture of preimplantation embryos: Facts and artifacts. *Human Reproduction Update* **1** 91-148.

Bazer, FW, TE Spencer, GA Johnson, RC Burghardt, and GY Wu 2009 Comparative aspects of implantation. *Reproduction* **138** 195-209.

Bendz, A 1977 Anatomical Basis for a Possible Counter Current Exchange Mechanism in Human Adnex. *Prostaglandins* **13** 355-362.

Bendz, A, N Einerjensen, O Lundgren, and PO Janson 1979 Exchange of Kr-85 between the Blood-Vessels of the Human Uterine Adnexa. *Journal of Reproduction and Fertility* **57** 137-142.

Bennett, WA, TL Watts, WD Blair, SJ Waldhalm, and JW Fuquay 1988 Patterns of Oviducal Motility in the Cow during the Estrous-Cycle. *Journal of Reproduction and Fertility* **83** 537-543.

Besenfelder, U, V Havlicek, and G Brem 2012 Role of the Oviduct in Early Embryo Development. *Reproduction in Domestic Animals* **47** 156-163.

Beyer, C, J Pawlak, V Brito, M Karolczak, T Ivanova, and E Kuppers 2003 Regulation of gene expression in the developing midbrain by estrogen - Implication of classical and nonclassical steroid signaling. *Steroids and the Nervous System* **1007** 17-28.

Bhabak, KP, and G Mugesh 2010 Functional Mimics of Glutathione Peroxidase: Bioinspired Synthetic Antioxidants. *Accounts of Chemical Research* **43** 1408-1419.

Bhatnagar, P, VE Papaioannou, and JD Biggers 1995 Csf-1 and Mouse Preimplantation Development in-Vitro. *Development* **121** 1333-1339.

Bjare, U 1992 Serum-Free Cell-Culture. *Pharmacology & Therapeutics* **53** 355-374.

Black and Douglas L. 2003. Mechanisms of alternative pre-messenger RNA splicing. *Annual Reviews of Biochemistry* **72** 291–336.

Blasi, F 1997 uPA, uPAR, PAI-I: key intersection of proteolytic, adhesive and chemotactic highways? *Immunology Today* **18** 415-417.

Bogaert, L, M Van Poucke, C De Baere, L Peelman, F Gasthuys, and A Martens 2006 Selection of a set of reliable reference genes for quantitative real-time PCR in normal equine skin and in equine sarcoids. *Bmc Biotechnology* **6**.

Bologna-Molina, R, P Damian-Matsumura, and N Molina-Frechero 2011 An easy cell counting method for immunohistochemistry that does not use an image analysis program. *Histopathology* **59** 801-803.

Bongso, A, and CY Fong 1993 The Effect of Coculture on Human Zygote Development. *Current Opinion in Obstetrics & Gynecology* **5** 585-593.

Bonnans, C, J Chou, and Z Werb 2014 Remodelling the extracellular matrix in development and disease. *Nature Reviews Molecular Cell Biology* **15** 786-801.

Bottenstein, J, I Hayashi, S Hutchings, H Masui, J Mather, DB McClure, R Ohasa, A Rizzino, G Sato, G Serrero, R Wolfe, and R Wu 1979 The growth of cells in serum-free hormone-supplemented media. *Methods Enzymology* **58** 94-109.

Bouchie, JL, H Hansen, and EP Feener 1998 Natriuretic factors and nitric oxide suppress plasminogen activator inhibitor-1 expression in vascular smooth muscle cells - Role of cGMP in the regulation of the plasminogen system. *Arteriosclerosis Thrombosis and Vascular Biology* **18** 1771-1779.

Brannvall, K, L Korhonen, and D Lindholm 2002 Estrogen-receptor-dependent regulation of neural stem cell proliferation and differentiation. *Molecular and Cellular Neuroscience* **21** 512-520.

Brinsko, SP, BA Ball, PG Miller, PGA Thomas, and JE Ellington 1994 In-Vitro Development of Day-2 Embryos Obtained from Young, Fertile Mares and Aged, Subfertile Mares. *Journal of Reproduction and Fertility* **102** 371-378.

Brison, DR, and RM Schultz 1998 Increased incidence of apoptosis in transforming growth factor alpha-deficient mouse blastocysts. *Biology of Reproduction* **59** 136-144.

Brison, DR, and HJ Leese 1994 Blastocoele Cavity Formation by Preimplantation Rat Embryos in the Presence of Cyanide and Other Inhibitors of Oxidative-Phosphorylation. *Journal of Reproduction and Fertility* **101** 305-309.

Buhi, WC 2002 Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* **123** 355-362.

Buhi, WC, and IM Alvarez 2003 Identification, characterization and localization of three proteins expressed by the porcine oviduct. *Theriogenology* **60** 225-238.

Buhi, WC, IM Alvarez, and AJ Kouba 1997 Oviductal regulation of fertilization and early embryonic development. *Journal of Reproduction and Fertility* 285-300.

Buhi, WC, IM Alvarez, and AJ Kouba 2000 Secreted proteins of the oviduct. *Cells Tissues Organs* **166** 165-179.

Buhi, WC, JL Vallet, and FW Bazer 1989 Denovo Synthesis and Release of Polypeptides from Cyclic and Early Pregnant Porcine Oviductal Tissue in Explant Culture. *Journal of Experimental Zoology* **252** 79-88.

Burry, RW 2011 Controls for Immunocytochemistry: An Update. *Journal of Histochemistry & Cytochemistry* **59** 6-12.

Bustin, SA, V Benes, JA Garson, J Hellemans, J Huggett, M Kubista, R Mueller, T Nolan, MW Pfaffl, GL Shipley, J Vandesompele, and CT Wittwer 2009 The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry* **55** 611-622.

Bylander, A, M Nutu, R Wellander, M Goksor, H Billig, and DGJ Larsson 2010 Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. *Reproductive Biology and Endocrinology* **8**.

Calder, MD, P Madan, and AJ Watson 2008 Bovine oocytes and early embryos express Staufen and ELAVL RNA-binding proteins. *Zygote* **16** 161-168.

Campbell, DL, LW Douglas, and JC Ramge 1979 Cannulation of the Equine Oviduct and Chemical-Analysis of Oviduct Fluid. *Theriogenology* **12** 47-59.

Cappelli, K, M Felicetti, S Capomaccio, G Spinsanti, M Silvestrelli, and AV Supplizi 2008 Exercise induced stress in horses: Selection of the most stable reference genes for quantitative RT-PCR normalization. *Bmc Molecular Biology* **9**.

Carlson, D, DL Black, and GR Howe 1970 Oviduct Secretion in Cow. *Journal of Reproduction and Fertility* **22** 549-&.

Carmeliet, P, N Mackman, L Moons, T Luther, P Gressens, I VanVlaenderen, H Demunck, M Kasper, G Breier, P Evrard, M Muller, W Risau, T Edgington, and D Collen 1996 Role of tissue factor in embryonic blood vessel development. *Nature* **383** 73-75.

Carnevale, EM, LJ Maclellan, TJ Scott, MA Coutinho da Silva, CF Scoggin, and EL Squires 2000. Effects of oocyte maturity and methods of collection, culture and insemination on equine oocyte transfer., p. 34. Ed. KBaJW WR Allen: R&W Publications, Newmarket.

Carter, F, F Rings, S Mamo, M Holker, A Kuzmany, U Besenfelder, V Havlicek, JP Mehta, D Tesfaye, K Schellander, and P Lonergan 2010 Effect of Elevated Circulating Progesterone Concentration on Bovine Blastocyst Development and

Global Transcriptome Following Endoscopic Transfer of In Vitro Produced Embryos to the Bovine Oviduct. *Biology of Reproduction* **83** 707-719.

Chabrolle, C, L Tosca, C Rame, P Lecomte, D Royere, and J Dupont 2009 Adiponectin increases insulin-like growth factor I-induced progesterone and estradiol secretion in human granulosa cells. *Fertility and Sterility* **92** 1988-1996.

Chang, HS, WTK Cheng, HK Wu, and KB Choo 2000 Identification of genes expressed in the epithelium of porcine oviduct containing early embryos at various stages of development. *Molecular Reproduction and Development* **56** 331-335.

Chase, CC, RP Delvecchio, SB Smith, and RD Randel 1992 Invitro Metabolism of Glucose by Bovine Reproductive Tissues Obtained during the Estrous-Cycle and after Calving. *Journal of Animal Science* **70** 1496-1508.

Chegini, N, Y Zhao, and FW Mclean 1994 Expression of Messenger-Ribonucleic-Acid and Presence of Immunoreactive Proteins for Epidermal Growth-Factor (Egf), Transforming Growth-Factor-Alpha (Tgf-Alpha) and Egf/Tgf-Alpha Receptors and I-125 Egf Binding-Sites in Human Fallopian-Tube. *Biology of Reproduction* **50** 1049-1058.

Chen, S, R Einspanier, and J Schoen 2013a In Vitro Mimicking of Estrous Cycle Stages in Porcine Oviduct Epithelium Cells: Estradiol and Progesterone Regulate Differentiation, Gene Expression, and Cellular Function. *Biology of Reproduction* **89**.

Chen, S, R Einspanier, and J Schoen 2013b Long-term culture of primary porcine oviduct epithelial cells: Validation of a comprehensive in vitro model for reproductive science. *Theriogenology* **80** 862-869.

Chi, JT, Z Wang, DSA Nuyten, EH Rodriguez, ME Schaner, A Salim, Y Wang, GB Kristensen, A Helland, AL Borresen-Dale, A Giaccia, MT Longaker, T Hastie, GP Yang, MJ van de Vijver, and PO Brown 2006 Gene expression programs in response to hypoxia: Cell type specificity and prognostic significance in human cancers. *Plos Medicine* **3** 395-409.

Chi, MMY, AL Schlein, and KH Moley 2000 High insulin-like growth factor 1 (IGF-1) and insulin concentrations trigger apoptosis in the mouse blastocyst via down-regulation of the IGF-1 receptor. *Endocrinology* **141** 4784-4792.

Choi, YH, CC Love, DD Varner, and K Hinrichs 2006 Equine blastocyst development after intracytoplasmic injection of sperm subjected to two freeze-thaw cycles. *Theriogenology* **65** 808-819.

Choi, YH, CC Love, DD Varner, LB Love, and K Hinrichs 2003 Effects of gas conditions, time of medium change, and ratio of medium to embryo on in vitro development of horse oocytes fertilized by intracytoplasmic sperm injection. *Theriogenology* **59** 1219-1229.

Choi, YH, LB Love, DD Varner, and K Hinrichs 2004a Factors affecting developmental competence of equine oocytes after intracytoplasmic sperm injection. *Reproduction* **127** 187-194.

Choi, YH, LM Roasa, CC Love, DD Varner, S Brinsko, and K Hinrichs 2004b Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. *Biology of Reproduction* **70** 1231-1238.

Christensen, AK, and SW Gillim 1969 The correlation of fine structure and function in steroid-secreting cells with emphasis on those of the gonads. . *In* K McKerns (ed.), The Gonads, pp. 415-490. New York: Meredith Corporation.

Chung, YW, TS Kim, SY Lee, SH Lee, Y Choi, N Kim, BM Min, DW Jeong, and IY Kim 2006 Selenite-induced apoptosis of osteoclasts mediated by the mitochondrial pathway. *Toxicol Lett* **160** 143-150.

Cicinelli, E, N Einer-Jensen, M Cignarelli, L Mangiacotti, D Luisi, and S Schonauer 2004 Preferential transfer of endogenous ovarian steroid hormones to the uterus during both the follicular and luteal phases. *Human Reproduction* **19** 2001-2004.

Ciereszko, R 1999 Radioimmunologiczne oznaczanie stężenia hormonów steroidowych w płynach biologicznych [Radioimmunoassay of steroid hormones in biological fluids. *In* J Przala (ed.), Animal Physiology., pp. 157-163. Olsztyn: UWM Press.

Cohen, PE, LY Zhu, and JW Pollard 1997 Absence of colony stimulating factor-1 in osteopetrotic (csfm(op)/csfm(op)) mice disrupts estrous cycles and ovulation. *Biology of Reproduction* **56** 110-118.

Colegrove-Otero, LJ, A Devaux, and N Standart 2005 The Xenopus ELAV protein ElrB represses Vg1 mRNA translation during oogenesis. *Molecular and Cellular Biology* **25** 9028-9039.

Cordova, A, C Perreau, S Uzbekova, C Ponsart, Y Locatelli, and P Mermillod 2014 Development rate and gene expression of IVP bovine embryos cocultured with bovine oviduct epithelial cells at early or late stage of preimplantation development. *Theriogenology* **81** 1163-1173.

Coutinho da Silva, MA 2008 When should a mare go for assisted reproduction? *Theriogenology* **70** 441-444.

Cox, CI, and HJ Leese 1997 Retention of functional characteristics by bovine oviduct and uterine epithelia in vitro. *Animal Reproduction Science* **46** 169-178.

Cross, JC, Z Werb, and SJ Fisher 1994 Implantation and the Placenta - Key Pieces of the Development Puzzle. *Science* **266** 1508-1518.

Daels, P, and JP Hughes 1993 The normal oestrous cycle. *In* A McKinnon and PLAM Vos (ed.), Equine Reproduction, pp. 121-133. Lea & Febiger.

Daniels, MC, P Kansal, TM Smith, AJ Paterson, JE Kudlow, and DA Mcclain 1993 Glucose Regulation of Transforming Growth-Factor-Alpha Expression Is Mediated by Products of the Hexosamine Biosynthesis Pathway. *Molecular Endocrinology* **7** 1041-1048.

Davis, ME 2002 Non-viral gene delivery systems. *Current Opinion in Biotechnology* **13** 128-131.

De Bosschere, H, R Ducatelle, H Vermeirsch, P Simoens, and M Coryn 2002 Estrogen-alpha and progesterone receptor expression in cystic endometrial hyperplasia and pyometra in the bitch. *Animal Reproduction Science* **70** 251-259.

De Pauw, IM, A Van Soom, H Laevens, S Verberckmoes, and A de Kruif 2002 Sperm binding to epithelial oviduct explants in bulls with different nonreturn rates investigated with a new in vitro model. *Biol Reprod* **67** 1073-1079.

De Pauw, IMC, A Van Soom, K Mintiens, S Verberckmoes, and A de Kruif 2003 In vitro survival of bovine spermatozoa stored at room temperature under epididymal conditions. *Theriogenology* **59** 1093-1107.

De Vos, A, H Heimberg, E Quartier, P Huypens, L Bouwens, D Pipeleers, and F Schuit 1995 Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. *The Journal of Clinical Investigation* **96** 2489-2495.

Dell'Aquila, ME, M De Felici, S Massari, F Maritato, and P Minoia 1999 Effects of fetuin on zona pellucida hardening and fertilizability of equine oocytes matured in vitro. *Biology of Reproduction* **61** 533-540.

Denecke, B, S Graber, C Schafer, A Heiss, M Woltje, and W Jahnen-Dechent 2003 Tissue distribution and activity testing suggest a similar but not identical function of fetuin-B and fetuin-A. *Biochemical Journal* **376** 135-145.

Desai, N, J Lawson, and J Goldfarb 2000 Assessment of growth factor effects on post-thaw development of cryopreserved mouse morulae to the blastocyst stage. *Human Reproduction* **15** 410-418.

Desantis, S, G Ventriglia, S Zizza, AC Guaricci, M Losurdo, A Zarrilli, and M Albrizio 2010 Changes in the expression of the mu-opioid receptor in the mare oviduct during oestrus and anoestrus. *Animal Reproduction Science* **119** 40-49.

Desantis, S, S Zizza, G Accogli, F Acone, R Rossi, and L Resta 2011 Morphometric and ultrastructural features of the mare oviduct epithelium during oestrus. *Theriogenology* **75** 671-678.

Diaz, MJ, S Giunta, J Valz-Gianinet, S Pereyra-Alfonso, V Flores, and D Miceli 2000 Proteases with plasminogen activator activity in hamster oviduct. *Molecular Reproduction and Development* **55** 47-54.

Dobrinski, I, JR Jacob, BC Tennant, and BA Ball 1999 Generation of an equine oviductal epithelial cell line for the study of sperm-oviduct interactions. *Theriogenology* **52** 875-885.

Donnay, I, A VanLangendonckt, P Auquier, B Grisart, A Vansteenbrugge, A Massip, and F Dessy 1997 Effects of co-culture and embryo number on the in vitro development of bovine embryos. *Theriogenology* **47** 1549-1561.

Dorgan, JF, TR Fears, RP McMahon, LA Friedman, BH Patterson, and SF Greenhut 2002 Measurement of steroid sex hormones in serum: a comparison of radioimmunoassay and mass spectrometry. *Steroids* **67** 151-158.

Duc-Goiran, P, TM Mignot, C Bourgeois, and F Ferre 1999 Embryo-maternal interactions at the implantation site: a delicate equilibrium. *European Journal of Obstetrics Gynecology and Reproductive Biology* **83** 85-100.

Duong, TD, and CA Erickson 2004 MMP-2 plays an essential role in producing epithelial-mesenchymal transformations in the avian embryo. *Developmental Dynamics* **229** 42-53.

Duverger, O, L Paslaru, and M Morange 2004 HSP25 is involved in two steps of the differentiation of PAM212 keratinocytes. *Journal of Biological Chemistry* **279** 10252-10260.

Ehrenwald, E, RH Foote, and JE Parks 1990 Bovine Oviductal Fluid Components and Their Potential Role in Sperm Cholesterol Efflux. *Molecular Reproduction and Development* **25** 195-204.

Einer-Jensen, N, and RHF Hunter 2000 Physiological and pharmacological aspects of local transfer of substances in the ovarian adnexa in women. *Human Reproduction Update* **6** 132-138.

Einer-Jensen, N, and RHF Hunter 2005 Counter-current transfer in reproductive biology. *Reproduction* **129** 9-18.

Einerjensen, N 1988 Countercurrent Transfer in the Ovarian Pedicle and Its Physiological Implications. *Oxford Reviews of Reproductive Biology* **10** 348-381.

Einerjensen, N, and JA Mccracken 1981 Physiological-Aspects of Corpus-Luteum Blood-Flow and of the Counter System in the Ovarian Pedicle of the Sheep. *Acta Veterinaria Scandinavica* 89-101.

Eisenbach, M, and LC Giojalas 2006 Sperm guidance in mammals - an unpaved road to the egg. *Nature Reviews Molecular Cell Biology* **7** 276-285.

Ellington, JE, EW Carney, PB Farrell, ME Simkin, and RH Foote 1990 Bovine 1-2-Cell Embryo Development Using a Simple Medium in 3 Oviduct Epithelial-Cell Coculture Systems. *Biology of Reproduction* **43** 97-104.

Espina, V, JD Wulfkuhle, VS Calvert, A VanMeter, WD Zhou, G Coukos, DH Geho, EF Petricoin, and LA Liotta 2006 Laser-capture microdissection. *Nature Protocols* **1** 586-603.

Eyestone, WH, and NL First 1989 Co-Culture of Early Cattle Embryos to the Blastocyst Stage with Oviducal Tissue or in Conditioned Medium. *Journal of Reproduction and Fertility* **85** 715-720.

Eyestone, WH, JM Jones, and NL First 1991 Some Factors Affecting the Efficacy of Oviduct Tissue-Conditioned Medium for the Culture of Early Bovine Embryos. *Journal of Reproduction and Fertility* **92** 59-64.

Falkenstein, E, C Meyer, C Eisen, PC Scriba, and M Wehling 1996 Full-length cDNA sequence of a progesterone membrane-binding protein from porcine vascular smooth muscle cells. *Biochem Biophys Res Commun* **229** 86-89.

Fan, X, CJ Heijnen, MA van der Kooij, F Groenendaal, and F van Bel 2009 The role and regulation of hypoxia-inducible factor-1 alpha expression in brain development and neonatal hypoxic-ischemic brain injury. *Brain Research Reviews* **62** 99-108.

Faul, F, E Erdfelder, AG Lang, and A Buchner 2007 G*Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behavior Research Methods* **39** 175-191.

Fazeli, A 2008 Maternal communication with gametes and embryos. *Theriogenology* **70** 1182-1187.

Fazeli, A, NA Affara, M Hubank, and WV Holt 2004 Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biology of Reproduction* **71** 60-65.

Fernandes, RMT, GC Gomes, AM Porcari, JRV Pimentel, PM Porciuncula, HA Martins, PHP Miguez, JL da Costa, PH Amaral, F Perecin, EC Meurer, PV Furtado, RC Simas, MN Eberlin, CR Ferreira, and EH Madureira 2011 LC-MS/MS quantitation of plasma progesterone in cattle. *Theriogenology* **76** 1266-1274.

Ferrara, N, K CarverMoore, H Chen, M Dowd, L Lu, KS OShea, L PowellBraxton, KJ Hillan, and MW Moore 1996 Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* **380** 439-442.

Fire, A, SQ Xu, MK Montgomery, SA Kostas, SE Driver, and CC Mello 1998 Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391** 806-811.

Fischer, B, and BD Bavister 1993 Oxygen-Tension in the Oviduct and Uterus of Rhesus-Monkeys, Hamsters and Rabbits. *Journal of Reproduction and Fertility* **99** 673-679.

Forrester, MT, M Benhar, and JS Stamler 2006 Nitrosative stress in the ER: A new role for S-nitrosylation in Neurodegenerative diseases. *Acs Chemical Biology* **1** 355-358.

Franczak, A, and M Bogacki 2009 Local and Systemic Effects of Embryos on Uterine Tissues During Early Pregnancy in Pigs. *Journal of Reproduction and Development* **55** 262-272.

Franczak, A, G Kotwica, B Kurowicka, A Oponowicz, I Woclawek-Potocka, and BK Petroff 2006 Expression of enzymes of cyclooxygenase pathway and secretion of prostaglandin E-2 and F-2 alpha by porcine myometrium during luteolysis and early pregnancy. *Theriogenology* **66** 1049-1056.

Franczak, A, B Wojciechowicz, A Zmijewska, J Kolakowska, and G Kotwica 2013 The effect of interleukin 1 beta and interleukin 6 on estradiol-17 beta secretion in the endometrium of pig during early pregnancy and the estrous cycle. *Theriogenology* **80** 90-98.

Freeman, DA, JE Butler, JA Weber, RT Geary, and GL Woods 1991 Co-culture of day-5 to day-7 equine embryos in medium with oviductal tissue. *Theriogenology* **36** 815-822.

Freeman, DA, GL Woods, DK Vanderwall, and JA Weber 1992 Embryo-Initiated Oviductal Transport in Mares. *Journal of Reproduction and Fertility* **95** 535-538.

Frolova, A, L Flessner, M Chi, ST Kim, N Foyouzi-Yousefi, KH Moley, and H Moley 2009 Facilitative Glucose Transporter Type 1 Is Differentially Regulated by Progesterone and Estrogen in Murine and Human Endometrial Stromal Cells. *Endocrinology* **150** 1512-1520.

Fukuda, M, K Fukuda, CY Andersen, and AG Byskov 1997 Does corpus luteum locally affect follicular growth negatively? *Human Reproduction* **12** 1024-1027.

Gabler, C, A Einspanier, D Schams, and R Einspanier 1999 Expression of vascular endothelial growth factor (VEGF) and its corresponding receptors (flt-1 and flk-1) in the bovine oviduct. *Molecular Reproduction and Development* **53** 376-383.

Gabler, C, GJ Killian, and R Einspanier 2001 Differential expression of extracellular matrix components in the bovine oviduct during the oestrous cycle. *Reproduction* **122** 121-130.

Gad, A, M Hoelker, U Besenfelder, V Havlicek, U Cinar, F Rings, E Held, I Dufort, MA Sirard, K Schellander, and D Tesfaye 2012 Molecular Mechanisms and Pathways Involved in Bovine Embryonic Genome Activation and Their Regulation by Alternative In Vivo and In Vitro Culture Conditions. *Biology of Reproduction* **87**.

Galli, C, S Colleoni, R Duchi, I Lagutina, and G Lazzari 2007 Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranging from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. *Animal Reproduction Science* **98** 39-55.

Galli, C, G Crotti, C Notari, P Turini, R Duchi, and G Lazzari 2001 Embryo production by ovum pick up from live donors. *Theriogenology* **55** 1341-1357.

Gallicano, GI 2001 Composition, regulation, and function of the cytoskeleton in mammalian eggs and embryos. *Frontiers in Bioscience* **6** D1089-D1108.

Galluzzi, L, I Vitale, JM Abrams, ES Alnemri, EH Baehrecke, MV Blagosklonny, TM Dawson, VL Dawson, WS El-Deiry, S Fulda, E Gottlieb, DR Green, MO Hengartner, O Kepp, RA Knight, S Kumar, SA Lipton, X Lu, F Madeo, W Malorni, P Mehlen, G Nunez, ME Peter, M Piacentini, DC Rubinsztein, Y Shi, HU Simon, P Vandenabeele, E White, J Yuan, B Zhivotovsky, G Melino, and G Kroemer 2012a Molecular definitions of cell death subroutines: recommendations of

the Nomenclature Committee on Cell Death 2012. *Cell Death and Differentiation* **19** 107-120.

Galluzzi, L, I Vitale, JM Abrams, ES Alnemri, EH Baehrecke, MV Blagosklonny, TM Dawson, VL Dawson, WS El-Deiry, S Fulda, E Gottlieb, DR Green, MO Hengartner, O Kepp, RA Knight, S Kumar, SA Lipton, X Lu, F Madeo, W Malorni, P Mehlen, G Nunez, ME Peter, M Piacentini, DC Rubinsztein, Y Shi, HU Simon, P Vandenabeele, E White, J Yuan, B Zhivotovsky, G Melino, and G Kroemer 2012b Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death and Differentiation* **19** 107-120.

Gandolfi, F, and RM Moor 1987 Stimulation of Early Embryonic-Development in the Sheep by Coculture with Oviduct Epithelial-Cells. *Journal of Reproduction and Fertility* **81** 23-28.

Garcia, DC, DC Miceli, PA Valdecantos, EV Garcia, and M Roldan-Olarte 2014 Expression of urokinase type plasminogen activator receptor (uPAR) in the bovine oviduct: Relationship with uPA effect on oviductal epithelial cells. *Research in Veterinary Science* **97** 118-123.

Gardner, DK, M Lane, I Calderon, and J Leeton 1996 Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertility and Sterility* **65** 349-353.

Gardner, DK, M Lane, A Spitzer, and PA Batt 1994 Enhanced Rates of Cleavage and Development for Sheep Zygotes Cultured to the Blastocyst Stage in-Vitro in the Absence of Serum and Somatic-Cells - Amino-Acids, Vitamins, and Culturing Embryos in Groups Stimulate Development. *Biology of Reproduction* **50** 390-400.

Garner, DL, LA Johnson, ST Yue, BL Roth, and RP Haugland 1994 Dual DNA Staining Assessment of Bovine Sperm Viability Using Sybr-14 and Propidium Iodide. *Journal of Andrology* **15** 620-629.

Georgiou, AS, E Sostaric, CH Wong, APL Snijders, PC Wright, HD Moore, and A Fazeli 2005 Gametes alter the oviductal secretory proteome. *Molecular & Cellular Proteomics* **4** 1785-1796.

Ghosh, M, HL Aguila, J Michaud, YX Ai, MT Wu, A Hemmes, A Ristimaki, CY Guo, H Furneaux, and T Hla 2009 Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. *Journal of Clinical Investigation* **119** 3530-3543.

Ginther, OJ 1974 Internal Regulation of Physiological Processes through Local Venoarterial Pathways - Review. *Journal of Animal Science* **39** 550-564.

Ginther, OJ 1976 Comparative anatomy of the uteroovarian vasculature., Veterinary Scope, pp. 1-17. Kalamazoo, Michigan: Upjohn Co.

Ginther, OJ 1992 Reproductive biology of the mare. Basic and applied aspects. Cross Plains, Wisconsin: Cross Plains, Equiservices.

Ginther, OJ, MC Garcia, EL Squires, and Steffenh.Wp 1972 Anatomy of Vasculature of Uterus and Ovaries in Mare. *American Journal of Veterinary Research* **33** 1561-1586.

Ginther, OJ, EL Gastal, MO Gastal, and MA Beg 2008 Passage of postovulatory follicular fluid into the peritoneal cavity and the effect on concentrations of circulating hormones in mares. *Animal Reproduction Science* **107** 1-8.

Ginther, OJ, EL Gastal, MO Gastal, MD Utt, and MA Beg 2007 Luteal blood flow and progesterone production in mares. *Animal Reproduction Science* **99** 213-220.

Giudice, LC 1999 Genes associated with embryonic attachment and implantation and the role of progesterone. *Journal of Reproductive Medicine* **44** 165-171.

Gjorret, JO, D Fabian, B Avery, and P Maddox-Hyttel 2007 Active caspase-3 and ultrastructural evidence of apoptosis in spontaneous and induced cell death in bovine in vitro produced pre-implantation embryos. *Molecular Reproduction and Development* **74** 961-971.

Glattauer, V, JF White, WB Tsai, CC Tsai, TA Tebb, SJ Danon, JA Werkmeister, and JAM Ramshaw 2010 Preparation of resorbable collagen-based beads for direct use in tissue engineering and cell therapy applications. *Journal of Biomedical Materials Research Part A* **92A** 1301-1309.

Good, PJ 1995 A Conserved Family of Elav-Like Genes in Vertebrates. *Proceedings of the National Academy of Sciences of the United States of America* **92** 4557-4561.

Goossens, JF, C Foulon, AL Villard, JY Puy, I Lefebvre, C Perigaud, C Vaccher, and JP Bonte 2005 Column selection and method development for the separation of nucleoside phosphotriester diastereoisomers, new potential anti-viral drugs. Application to cellular extract analysis. *Biomed Chromatogr* **19** 415-425.

Goossens, K, A Van Soom, M Van Poucke, L Vandaele, J Vandesompele, A Van Zeveren, and LJ Peelman 2007 Identification and expression analysis of genes associated with bovine blastocyst formation. *Bmc Developmental Biology* **7**.

Goovaerts, IGF, JLMR Leroy, D Rizos, P Bermejo-Alvarez, A Gutierrez-Adan, EPA Jorssen, and PEJ Bols 2011 Single in vitro bovine embryo production: Coculture with autologous cumulus cells, developmental competence, embryo quality and gene expression profiles. *Theriogenology* **76** 1293-1303.

Gray, CA, CA Abbey, PD Beremand, YS Choi, JL Farmer, DL Adelson, TL Thomas, FW Bazer, and TE Spencer 2006 Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. *Biology of Reproduction* **74** 383-394.

Gruber, CW, M Cemazar, B Heras, JL Martin, and DJ Craik 2006 Protein disulfide isomerase: the structure of oxidative folding. *Trends in Biochemical Sciences* **31** 455-464.

Gstraunthaler, G 2003 Alternatives to the use of fetal bovine serum: Serum-free cell culture. *Altex-Alternativen Zu Tierexperimenten* **20** 275-281.

Guerif, F, P McKeegan, HJ Leese, and RG Sturmey 2013 A Simple Approach for COnsumption and RElease (CORE) Analysis of Metabolic Activity in Single Mammalian Embryos. *Plos One* **8**.

Guidobaldi, HA, ME Teves, DR Unates, A Anastasia, and LC Giojalas 2008 Progesterone from the Cumulus Cells Is the Sperm Chemoattractant Secreted by the Rabbit Oocyte Cumulus Complex. *Plos One* **3**.

Guilbert, LJ, and NN Iscove 1976 Partial Replacement of Serum by Selenite, Transferrin, Albumin and Lecithin in Hematopoietic Cell-Cultures. *Nature* **263** 594-595.

Guilbert, LJ, B Winklerlowen, A Smith, DR Branch, and M Garcialloret 1993 Analysis of the Synergistic Stimulation of Mouse Macrophage Proliferation by Macrophage-Colony-Stimulating Factor (Csf-1) and Tumor-Necrosis-Factor-Alpha (Tnf-Alpha). *Journal of Leukocyte Biology* **54** 65-72.

Guo, B, W Wang, SJ Li, YS Han, L Zhang, XM Zhang, JX Liu, and ZP Yue 2012 Differential Expression and Regulation of Angiopoietin-2 in Mouse Uterus During Preimplantation Period. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology* **295** 338-346.

Hahn, T, S Barth, U Weiss, W Mosgoeller, and G Desoye 1998 Sustained hyperglycemia in vitro down-regulates the GLUT1 glucose transport system of cultured human term placental trophoblast: a mechanism to protect fetal development? *Faseb Journal* **12** 1221-1231.

Hai, MTV, F Logeat, M Warembourg, and E Milgrom 1977 Hormonal-Control of Progesterone Receptors. *Annals of the New York Academy of Sciences* **286** 199-209.

Hanukoglu, I 1992 Steroidogenic Enzymes - Structure, Function, and Role in Regulation of Steroid-Hormone Biosynthesis. *Journal of Steroid Biochemistry and Molecular Biology* **43** 779-804.

Hardy, K, and S Spanos 2002 Growth factor expression and function in the human and mouse preimplantation embryo. *Journal of Endocrinology* **172** 221-236.

Hardy, K, C Wright, S Rice, M Tachataki, R Roberts, D Morgan, S Spanos, and D Taylor 2002 Future developments in assisted reproduction in humans. *Reproduction* **123** 171-183.

Hartt, LS, SJ Carling, MM Joyce, GA Johnson, DK Vanderwall, and TL Ott 2005 Temporal and spatial associations of oestrogen receptor alpha and progesterone receptor in the endometrium of cyclic and early pregnant mares. *Reproduction* **130** 241-250.

Harvey, MB, KJ Leco, MY Arcellanapanlilio, X Zhang, DR Edwards, and GA Schultz 1995 Proteinase Expression in Early Mouse Embryos Is Regulated by Leukemia Inhibitory Factor and Epidermal Growth-Factor. *Development* **121** 1005-1014.

Hashizume, K, T Takahashi, M Shimizu, J Todoroki, A Shimada, M Hirata, T Sato, and A Ito 2003 Matrix-metalloproteinases-2 and-9 production in bovine endometrial cell culture. *Journal of Reproduction and Development* **49** 45-53.

Hayes, KEN, RA Pierson, ST Scraba, and OJ Ginther 1985 Effects of Estrous-Cycle and Season on Ultrasonic Uterine Anatomy in Mares. *Theriogenology* **24** 465-477.

Heggland, SJ, SA Signs, and JRD Stalvey 1997 Testosterone decreases 3 beta-hydroxysteroid dehydrogenase-isomerase messenger ribonucleic acid in cultured mouse Leydig cells by a strain-specific mechanism. *Journal of Andrology* **18** 646-655.

Heiden, MGV, LC Cantley, and CB Thompson 2009 Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science* **324** 1029-1033.

Hermoso, M, N Barrera, B Morales, S Perez, and M Villalon 2001 Platelet activating factor increases ciliary activity in the hamster oviduct through epithelial production of prostaglandin E-2. *Pflugers Archiv-European Journal of Physiology* **442** 336-345.

Hess, AP, S Talbi, AE Hamilton, DM Baston-Buest, M Nyegaard, JC Irwin, F Barragan, JS Kruessel, A Germeyer, and LC Giudice 2013 The human oviduct transcriptome

reveals an anti-inflammatory, anti-angiogenic, secretory and matrix-stable environment during embryo transit. *Reproductive Biomedicine Online* **27** 423-435.

Hill, AJ 2006 Maternal–Embryonic Cross-Talk. *Annals of the New York Academy of Sciences* **943** 17-25.

Hillier, SG, AMJ Vandenboogaard, LE Reichert, and EV Vanhall 1980 Alterations in Granulosa-Cell Aromatase-Activity Accompanying Preovulatory Follicular Development in the Rat Ovary with Evidence That 5-Alpha-Reduced C-19 Steroids Inhibit the Aromatase Reaction Invitro. *Journal of Endocrinology* **84** 409-419.

Hinrichs, K 2010 In Vitro Production of Equine Embryos: State of the Art. *Reproduction in Domestic Animals* **45** 3-8.

Hinrichs, K, and Y-H Choi 2005 Assisted reproductive techniques in the horse. *Clinical techniques in equine practice* **4** 210-218.

Hinrichs, K, and SC Loux 2012 Hyperactivated Sperm Motility: Are Equine Sperm Different? *Journal of Equine Veterinary Science* **32** 441-444.

Holm, P, SK Walker, and RF Seamark 1996 Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *Journal of Reproduction and Fertility* **107** 175-181.

Houghton, FD, JA Hawkhead, PG Humpherson, JE Hogg, AH Balen, AJ Rutherford, and HJ Leese 2002 Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction* **17** 999-1005.

Hsing, AW, FZ Stanczyk, A Belanger, P Schroeder, L Chang, RT Falk, and TR Fears 2007 Reproducibility of serum sex steroid assays in men by RIA and mass spectrometry. *Cancer Epidemiol Biomarkers Prev* **16** 1004-1008.

Hu, H, C Jiang, T Schuster, GX Li, PT Daniel, and J Lu 2006 Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway. *Mol Cancer Ther* **5** 1873-1882.

Hunter, RH 2012a Temperature gradients in female reproductive tissues. *Reproductive Biomedicine Online* **24** 377-380.

Hunter, RHF 2005 The Fallopian tubes in domestic mammals: how vital is their physiological activity? *Reproduction Nutrition Development* **45** 281-290.

Hunter, RHF 2012b Components of oviduct physiology in eutherian mammals. *Biological Reviews* **87** 244-255.

Hunter, RHF, B Cook, and NL Poyser 1983 Regulation of Oviduct Function in Pigs by Local Transfer of Ovarian-Steroids and Prostaglandins - a Mechanism to Influence Sperm Transport. *European Journal of Obstetrics Gynecology and Reproductive Biology* **14** 225-232.

Hwang, W, H Kim, E Lee, J Lim, S Roh, T Shin, K Hwang, and B Lee 2000 Purification and embryotropic roles of tissue inhibitor of metalloproteinase-1 in development of "HanWoo" (Bos taurus coreanae) oocytes co-cultured with bovine oviduct epithelial cells. *Journal of Veterinary Medical Science* **62** 1-5.

Jansen, RP 1984 Endocrine response in the fallopian tube. *Endocrine Reviews* **5** 525-551.

Jarazo, J, A Gambini, A De Stefano, L Muredas, JG Oriol, and D Salamone 2011 203 equine embryo in vitro development after intracytoplasmic sperm injection followed by chemical activation. *Reproduction, Fertility and Development* **24** 214-214.

Jeoung, M, S Lee, HK Hawng, YP Cheon, YK Jeong, MC Gye, M Iglarz, C Ko, and PJ Bridges 2010 Identification of a Novel Role for Endothelins within the Oviduct. *Endocrinology* **151** 2858-2867.

Jian, L, and ALW Po 1993 Effects of Insulin and Nasal Absorption Enhancers on Ciliary Activity. *International Journal of Pharmaceutics* **95** 101-104.

Jones, JI, and DR Clemmons 1995 Insulin-Like Growth-Factors and Their Binding-Proteins - Biological Actions. *Endocrine Reviews* **16** 3-34.

Kase, S, SK He, S Sonoda, M Kitamura, C Spee, E Wawrousek, SJ Ryan, R Kannan, and DR Hinton 2010 alpha B-crystallin regulation of angiogenesis by modulation of VEGF. *Blood* **115** 3398-3406.

Katsanou, V, O Papadaki, S Milatos, PJ Blackshear, P Anderson, G Kollias, and DL Kontoyiannis 2005 HuR as a negative posttranscriptional modulator in inflammation. *Molecular Cell* **19** 777-789.

Kaufman, RJ 2000 Overview of vector design for mammalian gene expression. *Molecular Biotechnology* **16** 151-160.

Kendle, KE, and B Lee 1980 Investigation of the Influence of Progesterone on Mouse Embryo Transport by Using Anti-Progestational Steroids. *Journal of Reproduction and Fertility* **58** 253-258.

Kennedy, TG, KD Brown, and TJ Vaughan 1994 Expression of the Genes for the Epidermal Growth-Factor Receptor and Its Ligands in Porcine Oviduct and Endometrium. *Biology of Reproduction* **50** 751-756.

Kervancioglu, ME, E Saridogan, T Atasu, T Camlibel, A Demircan, B Sarikamis, and O Djahanbakhch 1997 Human Fallopian tube epithelial cell co-culture increases fertilization rates in male factor infertility but not in tubal or unexplained infertility. *Hum Reprod* **12** 1253-1258.

Killian, GJ 2004 Evidence for the role of oviduct secretions in sperm function, fertilization and embryo development. *Animal Reproduction Science* **82-3** 141-153.

Killian, GJ, DA Chapman, JF Kavanaugh, DR Deaver, and HB Wiggin 1989 Changes in Phospholipids, Cholesterol and Protein-Content of Oviduct Fluid of Cows during the Estrous-Cycle. *Journal of Reproduction and Fertility* **86** 419-426.

Kim, HY, J Sohn, GT Wijewickrama, P Edirisinghe, T Gherezghiher, M Hemachandra, PY Lu, RE Chandrasena, ME Molloy, DA Tonetti, and GR Thatcher 2010 Click synthesis of estradiol-cyclodextrin conjugates as cell compartment selective estrogens. *Bioorg Med Chem* **18** 809-821.

Kim, NH, and AR Menino 1995 Effects of Stimulators of Protein Kinase-a and Kinase-C and Modulators of Phosphorylation on Plasminogen-Activator Activity in Porcine Oocyte-Cumulus Cell Complexes during in-Vitro Maturation. *Molecular Reproduction and Development* **40** 364-370.

King, SR, and HA LaVoie 2012 Gonadal transactivation of STARD1, CYP11A1 and HSD3B. *Frontiers in Bioscience-Landmark* **17** 824-846.

Klein, C, KE Scoggin, AD Ealy, and MHT Troedsson 2010 Transcriptional Profiling of Equine Endometrium During the Time of Maternal Recognition of Pregnancy. *Biology of Reproduction* **83** 102-113.

Kobayashi, F, SJ Zimniski, and KN Smalley 1996 Characterization of oviductal aromatase in the northern leopard frog, Rana pipens. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **113** 653-657.

Kodithuwakku, SP, A Miyamoto, and MPB Wijayagunawardane 2007 Spermatozoa stimulate prostaglandin synthesis and secretion in bovine oviductial epithelial cells. *Reproduction* **133** 1087-1094.

Kolle, S, S Dubielzig, S Reese, A Wehrend, P Konig, and W Kummer 2009 Ciliary Transport, Gamete Interaction, and Effects of the Early Embryo in the Oviduct: Ex Vivo Analyses Using a New Digital Videomicroscopic System in the Cow. *Biology of Reproduction* **81** 267-274.

Kolle, S, S Reese, and W Kummer 2010 New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. *Theriogenology* **73** 786-795.

Kotwica, J, GL Williams, and MJ Marchello 1982 Countercurrent Transfer of Testosterone by the Ovarian Vascular Pedicle of the Cow - Evidence for a Relationship to Follicular Steroidogenesis. *Biology of Reproduction* **27** 778-789.

Kouba, AJ, IM Alvarez, and WC Buhi 1998 Characterization of pig oviductal plasminogen activator inhibitor (PAI)-1 during early pregnancy. *Biology of Reproduction* **58** 172-172.

Kouba, AJ, IM Alvarez, and WC Buhi 2000a Identification and localization of plasminogen activator inhibitor-1 within the porcine oviduct. *Biology of Reproduction* **62** 501-510.

Kouba, AJ, BR Burkhardt, IM Alvarez, MM Goodenow, and WC Buhi 2000b Oviductal plasminogen activator inhibitor-1 (PAI-1): mRNA, protein, and hormonal regulation during the estrous cycle and early pregnancy in the pig. *Molecular Reproduction and Development* **56** 378-386.

Krysko, DV, T Vanden Berghe, K D'Herde, and P Vandenabeele 2008 Apoptosis and necrosis: Detection, discrimination and phagocytosis. *Methods* **44** 205-221.

Kubota, K, Y Omori, S Ikeda, and T Minegishi 2009 Expression and Cyclic Change of Betaglycan in the Rat Oviduct. *Journal of Reproduction and Development* **55** 200-205.

Kuida, K, and DM Boucher 2004 Functions of MAP kinases: insights from gene-targeting studies. *J Biochem* **135** 653-656.

Kunz, G, M Herbertz, M Noe, and G Leyendecker 1998 Sonographic evidence for the involvement of the utero-ovarian counter-current system in the ovarian control of directed uterine sperm transport. *Human Reproduction Update* **4** 667-672.

Kupferschmidt, K 2013 A Lethal Dose of RNA. *Science* **341** 732-733.

Lag, M, G Paulsen, and J Jonsen 1984 Effect of Sodium Selenite on the Ciliary Activity, Adenosine-Triphosphate, and Protein-Synthesis in Mouse Trachea Organ-Cultures. *Journal of Toxicology and Environmental Health* **13** 857-864.

Lagutina, I, G Lazzari, R Duchi, P Turini, I Tessaro, D Brunetti, S Colleoni, G Crotti, and C Galli 2007 Comparative aspects of somatic cell nuclear transfer with conventional and zona-free method in cattle, horse, pig and sheep. *Theriogenology* **67** 90-98.

Lapointe, J, and JF Bilodeau 2003 Antioxidant defenses are modulated in the cow oviduct during the estrous cycle. *Biology of Reproduction* **68** 1157-1164.

LaVoie, HA, and SR King 2009 Transcriptional Regulation of Steroidogenic Genes: STARD1, CYP11A1 and HSD3B. *Experimental Biology and Medicine* **234** 880-907.

Lazzari, G, S Colleoni, I Lagutina, G Crotti, P Turini, I Tessaro, D Brunetti, R Duchi, and C Galli 2010 Short-term and long-term effects of embryo culture in the surrogate sheep oviduct versus in vitro culture for different domestic species. *Theriogenology* **73** 748-757.

Leak, LV, and K Rahil 1978 Permeability of Diaphragmatic Mesothelium - Ultrastructural Basis for Stomata. *American Journal of Anatomy* **151** 557-593.

Leclerc, E, Y Sakai, and T Fujii 2003 Cell Culture in 3-Dimensional Microfluidic Structure of PDMS (polydimethylsiloxane). *Biomedical Microdevices* **5** 109-114.

Lee, AS 2001 The glucose-regulated proteins: stress induction and clinical applications. *Trends in Biochemical Sciences* **26** 504-510.

Lee, KF, JFC Chow, JS Xu, STH Chan, SM Ip, and WSB Yeung 2001 A comparative study of gene expression in murine embryos developed in vivo, cultured in vitro, and cocultured with human oviductal cells using messenger ribonucleic acid differential display. *Biology of Reproduction* **64** 910-917.

Lee, KF, YQ Yao, KL Kwok, JS Xu, and WS Yeung 2002 Early developing embryos affect the gene expression patterns in the mouse oviduct. *Biochem Biophys Res Commun* **292** 564-570.

Lee, S, SM Jilani, GV Nikolova, D Carpizo, and ML Iruela-Arispe 2005 Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. *Journal of Cell Biology* **169** 681-691.

Leemans, B, BM Gadella, E Sostaric, H Nelis, TAE Stout, M Hoogewijs, and A Van Soom 2014 Oviduct Binding and Elevated Environmental pH Induce Protein Tyrosine Phosphorylation in Stallion Spermatozoa. *Biology of Reproduction* **91**.

Leemans, B, HM Nelis, M Hoogewijs, L Vandaele, and A Van Soom 2011. Whittens medium maintains oviduct cell viability and facilitates oviduct-sperm binding in the horse.

Leese, HJ 1995 Metabolic control during preimplantation mammalian development. *Human Reproduction Update* **1** 63-72.

Leese, HJ, M Alexiou, MT Comer, VK Lamb, and JG Thompson 1995 Assessment of embryo nutritional requirements and role of co-culture techniques. *Assisted Reproduction* **7** 11-23.

Leese, HJ, SA Hugentobler, SM Gray, DG Morris, RG Sturmey, SL Whitear, and JM Sreenan 2008 Female reproductive tract fluids: Composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction Fertility and Development* **20** 1-8.

Leger, DW, and IA Didrichsons 1994 An Assessment of Data Pooling and Some Alternatives. *Animal Behaviour* **48** 823-832.

Lei, ZM, and CV Rao 1992 Expression of Epidermal Growth-Factor (Egf) Receptor and Its Ligands, Egf and Transforming Growth Factor-Alpha, in Human Fallopian-Tubes. *Endocrinology* **131** 947-957.

Leist, M, and M Jaattela 2001 Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* **2** 589-598.

Lenth, RV 2007 Statistical power calculations. *Journal of Animal Science* **85** E24-E29.

Leroith, D, H Werner, D Beitnerjohnson, and CT Roberts 1995a Molecular and Cellular Aspects of the Insulin-Like Growth-Factor-I Receptor. *Endocrine Reviews* **16** 143-163.

LeRoith, D, H Werner, S Neuenschwander, T Kalebic, and LJ Helman 1995b The role of the insulin-like growth factor-I receptor in cancer. *Receptor Activation by Antigens, Cytokines, Hormones, and Growth Factors* **766** 402-408.

Levanon, K, V Ng, HY Piao, Y Zhang, MC Chang, MH Roh, DW Kindelberger, MS Hirsch, CP Crum, JA Marto, and R Drapkin 2010 Primary ex vivo cultures of human fallopian tube epithelium as a model for serous ovarian carcinogenesis. *Oncogene* **29** 1103-1113.

Li, XH, LHA Morris, and WR Allen 2001 Influence of co-culture during maturation on the developmental potential of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI). *Reproduction* **121** 925-932.

Li, Y, L Qin, ZH Xiao, YL Wang, R Herva, JH Leng, JH Lang, V Isomaa, and YS Piao 2003 Expression of P450 aromatase and 17 beta-hydroxysteroid dehydrogenase type 1 at fetal-maternal interface during tubal pregnancy. *Journal of Steroid Biochemistry and Molecular Biology* **87** 241-246.

Liao, H, MC Hyman, DA Lawrence, and DJ Pinsky 2007 Molecular regulation of the PAI-1 gene by hypoxia: contributions of Egr-1, HIF-1 alpha, and C/EBP alpha. *Faseb Journal* **21** 935-949.

Lijnen, HR 2002 Matrix metalloproteinases and cellular fibrinolytic activity. *Biochemistry-Moscow* **67** 92-98.

Lim, HJ, H Song, BC Paria, J Reese, SK Das, and SK Dey 2002 Molecules in blastocyst implantation: Uterine and embryonic perspectives. *Vitamins and Hormones - Advances in Research and Applications, Vol 64* **64** 43-76.

Lin, Q, X Cong, and Z Yun 2011 Differential hypoxic regulation of hypoxia-inducible factors 1alpha and 2alpha. *Mol Cancer Res* **9** 757-765.

Liu, LPS, STH Chan, PC Ho, and WSB Yeung 1998 Partial purification of embryotrophic factors from human oviductal cells. *Human Reproduction* **13** 1613-1619.

Liu, YY, and BG Szaro 2011 hnRNP K post-transcriptionally co-regulates multiple cytoskeletal genes needed for axonogenesis. *Development* **138** 3079-3090.

Lloyd, RE, R Romar, C Matas, A Gutierrez-Adan, WV Holt, and P Coy 2009 Effects of oviductal fluid on the development, quality, and gene expression of porcine blastocysts produced in vitro. *Reproduction* **137** 679-687.

Lonergan, P, H Sharif, P Monaghan, H Wahid, M Gallagher, and I Gordon 1991 Factors affecting embryo yield following maturation, fertilization and culture in vitro of bovine oocytes. . *Isr. J. Agric. Res.* **30** 78.

Lundstrom, J, and A Holmgren 1990 Protein Disulfide-Isomerase Is a Substrate for Thioredoxin Reductase and Has Thioredoxin-Like Activity. *Journal of Biological Chemistry* **265** 9114-9120.

Luo, HL, K Kimura, M Aoki, and M Hirako 2002 Vascular endothelial growth factor (VEGF) promotes the early development of bovine embryo in the presence of cumulus cells. *Journal of Veterinary Medical Science* **64** 967-971.

Lyons, RA, O Djahanbakhch, T Mahmood, E Saridogan, S Sattar, MT Sheaff, AA Naftalin, and R Chenoy 2002 Fallopian tube ciliary beat frequency in relation to the stage of menstrual cycle and anatomical site. *Hum Reprod* **17** 584-588.

Lyons, RA, E Saridogan, and O Djahanbakhch 2006 The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update* **12** 363-372.

Lyons, RM, J Keskioja, and HL Moses 1988 Proteolytic Activation of Latent Transforming Growth Factor-Beta from Fibroblast-Conditioned Medium. *Journal of Cell Biology* **106** 1659-1665.

Lysiak, JJ, VKM Han, and PK Lala 1993 Localization of Transforming Growth-Factor-Alpha in the Human Placenta and Decidua - Role in Trophoblast Growth. *Biology of Reproduction* **49** 885-894.

Ma, SH, M Natoli, X Liu, MP Neubauer, FM Watt, A Fery, and WTS Huck 2013 Monodisperse collagen-gelatin beads as potential platforms for 3D cell culturing. *Journal of Materials Chemistry B* **1** 5128-5136.

Mahmood, T, E Saridogan, S Smutna, AM Habib, and O Djahanbakhch 1998 The effect of ovarian steroids on epithelial ciliary beat frequency in the human Fallopian tube. *Human Reproduction* **13** 2991-2994.

Maillo, V, P Ó Gaora, N Forde, U Besenfelder, H Vitezslav, GW Burns, TE Spencer, A Gutierrez-Adan, P Lonergan, and D Rizos 2015 Oviduct-embryo interactions in cattle: two-way traffic or a one-way street? *Biology of Reproduction*.

Malcuit, C, MC Trask, L Santiago, E Beaudoin, KD Tremblay, and J Mager 2009 Identification of novel oocyte and granulosa cell markers. *Gene Expression Patterns* **9** 404-410.

Maller, JL 2003 Signal transduction. Fishing at the cell surface. *Science* **300** 594-595.

Mariani, ML, M Souto, MA Fanelli, and DR Ciocca 2000 Constitutive expression of heat shock proteins hsp25 and hsp70 in the rat oviduct during neonatal development, the oestrous cycle and early pregnancy. *Journal of Reproduction and Fertility* **120** 217-223.

Marsan, C, AK Goff, J Sirois, and KJ Betteridge 1987 Steroid secretion by different cell types of the horse conceptus. *J Reprod Fertil Suppl* **35** 363-369.

Matsushime, H, MF Roussel, RA Ashmun, and CJ Sherr 1991 Colony-Stimulating Factor-I Regulates Novel Cyclins during the G1 Phase of the Cell-Cycle. *Cell* **65** 701-713.

McClung, HM, SL Thomas, P Osenkowski, M Toth, P Menon, A Raze, R Fridman, and SA Rempel 2007 SPARC upregulates MT1-MMP expression, MMP-2 activation, and the secretion and cleavage of galectin-3 in U87MG glioma cells. *Neuroscience Letters* **419** 172-177.

Mccracken, JA, W Schramm, and N Einerjensen 1984 The Structure of Steroids and Their Diffusion through Blood-Vessel Walls in a Countercurrent System. *Steroids* **43** 293-303.

McCue, PM, and EL Squires 2002 Persistent anovulatory follicles in the mare. *Theriogenology* **58** 541-543.

McDonald, M, E Malone, and J McBride 2010 Confirmation of Hormones in Animal Serum by Liquid Chromatography/Tandem Mass Spectrometry According to European Commission Decision 2002/657. *Journal of Aoac International* **93** 343-349.

McKinnon, AO 1997 Ovarian abnormalities. *In* MAO Ranaten N.W. (ed.), Equine diagnostic ultrasonography, pp. 233-251. Baltimore: Williams and Wilkins.

Mckinnon, AO, EL Squires, EM Carnevale, JL Voss, and GE Seidel 1988 Heterogenous and Xenogenous Fertilization of Equine Oocytes. *Theriogenology* **29** 278-278.

Medina, RA, AM Meneses, JC Vera, C Guzman, F Nualart, F Rodriguez, M de los Angeles Garcia, S Kato, N Espinoza, C Monso, A Carvajal, M Pinto, and GI Owen 2004 Differential regulation of glucose transporter expression by estrogen and progesterone in Ishikawa endometrial cancer cells. *Journal of Endocrinology* **182** 467-478.

Mehlen, P, A Mehlen, J Godet, and AP Arrigo 1997 hsp27 as a switch between differentiation and apoptosis in murine embryonic stem cells. *Journal of Biological Chemistry* **272** 31657-31665.

Menck, MC, C GuyaderJoly, N Peynot, D LeBourhis, RB Lobo, JP Renard, and Y Heyman 1997 Beneficial effects of Vero cells for developing IVF bovine eggs in two different coculture systems. *Reproduction Nutrition Development* **37** 141-150.

Menezo, Y, A Veiga, and M Benkhalifa 1998 Improved methods for blastocyst formation and culture. *Human Reproduction* **13** 256-265.

Merkl, M, SE Ulbrich, C Otzdorff, N Herbach, R Wanke, E Wolf, J Handler, and S Bauersachs 2010 Microarray Analysis of Equine Endometrium at Days 8 and 12 of Pregnancy. *Biology of Reproduction* **83** 874-886.

Messmer, TO 1973 Nature of Iron Requirement for Chinese-Hamster V79 Cells in Tissue-Culture Medium. *Experimental Cell Research* **77** 404-408.

Miessen, K, S Sharbati, R Einspanier, and J Schoen 2011 Modelling the porcine oviduct epithelium: A polarized in vitro system suitable for long-term cultivation. *Theriogenology* **76** 900-910.

Misener, S, S Krawetz, S Rozen, and H Skaletsky 1999 Primer3 on the WWW for general users and for biologist programmers., Bioinformatics Methods and Protocols., pp. 365-386. Merelbeke, Belgium: S. Misener and S. Krawetz.

Mishra, S, ZM Lei, and CV Rao 2003 A novel role of luteinizing hormone in the embryo development in cocultures. *Biology of Reproduction* **68** 1455-1462.

Moley, KH, MM Chi, CM Knudson, SJ Korsmeyer, and MM Mueckler 1998a Hyperglycemia induces apoptosis in pre-implantation embryos through cell death effector pathways. *Nature Medicine* **4** 1421-1424.

Moley, KH, MM Chi, and MM Mueckler 1998b Maternal hyperglycemia alters glucose transport and utilization in mouse preimplantation embryos. *American Journal of Physiology* **275** E38-47.

Morishige, KI, H Kurachi, K Amemiya, H Adachi, K Adachi, Y Sakoyama, A Miyake, and O Tanizawa 1993 Menstrual Stage-Specific Expression of Epidermal Growth-Factor and Transforming Growth-Factor-Alpha in Human Oviduct Epithelium and Their Role in Early Embryogenesis. *Endocrinology* **133** 199-207.

Mugnier, S, M Kervella, C Douet, S Canepa, G Pascal, S Deleuze, G Duchamp, P Monget, and G Goudet 2009 The secretions of oviduct epithelial cells increase the equine in vitro fertilization rate: are osteopontin, atrial natriuretic peptide A and oviductin involved? *Reproductive Biology and Endocrinology* **7**.

Munabi, AK, FG Cassorla, DG Pfeiffer, BD Albertson, and DL Loriaux 1983 The effects of estradiol and progesterone on rat ovarian 17-hydroxylase and 3ß-hydroxysteroid dehydrogenase activities. *Steroids* **41** 95-98.

Murphy RC, Fiedler J, J Hevko 2001 "Analysis of nonvolatile lipids by mass spectrometry". *Chem. Rev.* **101** (2): 479–526.

Muthuchamy, M, L Pajak, P Howles, T Doetschman, and DF Wieczorek 1993 Developmental Analysis of Tropomyosin Gene-Expression in Embryonic Stem-Cells and Mouse Embryos. *Molecular and Cellular Biology* **13** 3311-3323.

Nagase, H, R Visse, and G Murphy 2006 Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research* **69** 562-573.

Nakahari, T, A Nishimura, C Shimamoto, A Sakai, H Kuwabara, T Nakano, S Tanaka, Y Kohda, H Matsumura, and H Mori 2011 The regulation of ciliary beat frequency by ovarian steroids in the guinea pig Fallopian tube: interactions between oestradiol and progesterone. *Biomed Res* **32** 321-328.

Namura, S, JM Zhu, K Fink, M Endres, A Srinivasan, KJ Tomaselli, JY Yuan, and MA Moskowitz 1998 Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *Journal of Neuroscience* **18** 3659-3668.

Nelis, H, K D'Herde, K Goossens, L Vandenberghe, B Leemans, K Forier, K Smits, K Braeckmans, L Peelman, and A Van Soom 2014 Equine oviduct explant culture: a basic model to decipher embryo–maternal communication. *Reproduction, Fertility and Development* **26** 954-966.

Nelis, H, J Vanden Bussche, B Wojciechowicz, A Franczak, B Leemans, L Vanhaecke, P Cornillie, L Peelman, A Van Soom, and K Smits 2015a Steroids in the equine oviduct: synthesis, local concentrations and receptor expression. *Reproduction, Fertility and Development*.

Nelis, H, B Wojciechowicz, A Franczak, B Leemans, K D'Herde, K Goossens, P Cornillie, L Peelman, A Van Soom, and K Smits 2015b Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and immunoreactive steroidogenic protein expression in equine oviduct explants in vitro. *Reprod Fertil Dev*.

Nelis, HM, K Goossens, B Leemans, L Peelman, and A Van Soom 2013 Steroid-Regulated Mrna Expression in Oviduct Epithelial Cells in the Mare. *Reproduction Fertility and Development* **25** 258-258.

Nelis, HM, L Vanhaecke, K Roels, and A Van Soom 2011 Serum steroid fluctuations during early pregnancy in mares, measured by ultra-high performance liquid chromatography coupled to tandem mass spectrometry. *Reproduction in Domestic Animals* **46 (s3)** 134.

Nguyen, DH, T Zhou, J Shu, and J-H Mao 2013 Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity., Cancer InCytes, edn Summer 2013. Santa Monica.

Nielsen, OB, F de Paoli, and K Overgaard 2001 Protective effects of lactic acid on force production in rat skeletal muscle. *Journal of Physiology-London* **536** 161-166.

Nishimura, A, K Sakuma, C Shimamoto, S Ito, T Nakano, E Daikoku, M Ohmichi, T Ushiroyama, M Ueki, H Kuwabara, H Mori, and T Nakahari 2010 Ciliary beat frequency controlled by oestradiol and progesterone during ovarian cycle in guinea-pig Fallopian tube. *Experimental Physiology* **95** 819-828.

Noiva, R 1999 Protein disulfide isomerase: The multifunctional redox chaperone of the endoplasmic reticulum. *Seminars in Cell & Developmental Biology* **10** 481-493.

Nothnick, WB 2001 Reduction in reproductive lifespan of tissue inhibitor of metalloproteinase 1 (TIMP-1)-deficient female mice. *Reproduction* **122** 923-927.

Ochieng, J, V Furtak, and P Lukyanov 2002 Extracellular functions of galectin-3. *Glycoconjugate Journal* **19** 527-535.

Ohe, T, M Hirobe, and T Mashino 2000 Novel metabolic pathway of estrone and 17 beta-estradiol catalyzed by cytochrome P-450. *Drug Metabolism and Disposition* **28** 110-112.

Oliveros, JC 2007-2015, posting date. enny. An interactive tool for comparing lists with Venn's diagrams. . [Online.]

Olson, SH, EV Bandera, and I Orlow 2007 Variants in estrogen biosynthesis genes, sex steroid hormone levels, and endometrial cancer: A HuGE review. *American Journal of Epidemiology* **165** 235-245.

Orihuela, PA, A Parada, and HB Croxatto 2001 Estradiol-induced oocyte transport acceleration is mediated by estrogen receptor and nongenomic activation of protein kinase-A signaling pathway in the rat oviduct. *Biology of Reproduction* **64** 317-317.

Orsi, NM, and JB Reischl 2007 Mammalian embryo co-culture: Trials and tribulations of a misunderstood method. *Theriogenology* **67** 441-458.

Page-McCaw, A, AJ Ewald, and Z Werb 2007 Matrix metalloproteinases and the regulation of tissue remodelling. *Nature Reviews Molecular Cell Biology* **8** 221-233.

Pampfer, S, RJ Arceci, and JW Pollard 1991a Role of Colony Stimulating Factor-I (Csf-1) and Other Lympho-Hematopoietic Growth-Factors in Mouse Preimplantation Development. *Bioessays* **13** 535-540.

Pampfer, S, S Tabibzadeh, FC Chuan, and JW Pollard 1991b Expression of Colony-Stimulating Factor-I (Csf-1) Messenger-Rna in Human Endometrial Glands during the Menstrual-Cycle - Molecular-Cloning of a Novel Transcript That Predicts a Cell-Surface Form of Csf-1. *Molecular Endocrinology* **5** 1931-1938.

Paria, BC, C Chakraborty, and SK Dey 1990 Catechol estrogen formation in the mouse uterus and its role in implantation. *Molecular and Cellular Endocrinology* **69** 25-32.

Paria, BC, WG Ma, J Tan, S Raja, SK Das, SK Dey, and BLM Hogan 2001 Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally

applied growth factors. *Proceedings of the National Academy of Sciences of the United States of America* **98** 1047-1052.

Paria, BC, J Reese, SK Das, and SK Dey 2002 Deciphering the cross-talk of implantation: Advances and challenges. *Science* **296** 2185-2188.

Patek, E, Johannis.E, and L Nilsson 1972 Scanning Electron-Microscopic Study of Human Fallopian Tube .1. Proliferative and Secretory Stages. *Fertility and Sterility* **23** 459-&.

Pattison, ML, CL Chen, ST Kelley, and GW Brandt 1974 Luteinizing-Hormone and Estradiol in Peripheral-Blood of Mares during Estrous-Cycle. *Biology of Reproduction* **11** 245-250.

Pepper, MS, D Belin, R Montesano, L Orci, and JD Vassalli 1990 Transforming Growth Factor-Beta-1 Modulates Basic Fibroblast Growth-Factor Induced Proteolytic and Angiogenic Properties of Endothelial-Cells Invitro. *Journal of Cell Biology* **111** 743-755.

Petroski, MD 2008 The ubiquitin system, disease, and drug discovery. *Bmc Biochemistry* **9**.

Pfeifer, TL, and N Chegini 1994 Immunohistochemical Localization of Insulin-Like Growth-Factor (Igf-I), Igf-I Receptor, and Igf Binding-Proteins-1-4 in Human Fallopian-Tube at Various Reproductive Stages. *Biology of Reproduction* **50** 281-289.

Pierson, RA 1993 Folliculogenesis and ovulation. . *In* VJL McKinnon A.O. (ed.), Equine reproduction. , pp. 151-161. Philadelphia: Lea and Febiger.

Pierson, RA, and OJ Ginther 1985 Ultrasonic evaluation of the corpus luteum of the mare. *Theriogenology* **23** 795-806.

Pines, J 2006 Mitosis: a matter of getting rid of the right protein at the right time. *Trends Cell Biol* **16** 55-63.

Pope, WF, RR Maurer, and F Stormshak 1982 Distribution of progesterone in the uterus, broad ligament, and uterine arteries of beef cows. *The Anatomical Record* **203** 245-249.

Purcell, SH, and KH Moley 2009 Glucose transporters in gametes and preimplantation embryos. *Trends Endocrinol Metab* **20** 483-489.

Rabbani, MLM, and PAW Rogers 2001 Role of vascular endothelial growth factor in endometrial vascular events before implantation in rats. *Reproduction* **122** 85-90.

Rajagopal, M, TL Tollner, WE Finkbeiner, GN Cherr, and JH Widdicombe 2006 Differentiated structure and function of primary cultures of monkey oviductal epithelium. *In Vitro Cellular & Developmental Biology-Animal* **42** 248-254.

Raleigh, JA, DP Calkins-Adams, LH Rinker, CA Ballenger, MC Weissler, WC Fowler, DB Novotny, and MA Varia 1998 Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. *Cancer Research* **58** 3765-3768.

Rambags, BPB, HTA van Tol, MM van den Eng, B Colenbrander, and TAE Stout 2008 Expression of progesterone and oestrogen receptors by early intrauterine equine conceptuses. *Theriogenology* **69** 366-375.

Reischl, J, K Prelle, H Schol, C Neumuller, R Einspanier, F Sinowatz, and E Wolf 1999 Factors affecting proliferation and dedifferentiation of primary bovine oviduct epithelial cells in vitro. *Cell and Tissue Research* **296** 371-383.

Reischl, J, K Prelle, and E Wolf 1997 Physiological characteristics of bovine oviduct epithelial cells are maintained in perfusion culture. *European Journal of Cell Biology* **74** 132-132.

Reneker, LXW, DW Silversides, K Patel, and PA Overbeek 1995 Tgf-Alpha Can Act as a Chemoattractant to Perioptic Mesenchymal Cells in Developing Mouse Eyes. *Development* **121** 1669-1680.

Reverchon, M, M Cornuau, C Rame, F Guerif, D Royere, and J Dupont 2012 Chemerin inhibits IGF-1-induced progesterone and estradiol secretion in human granulosa cells. *Human Reproduction* **27** 1790-1800.

Rexroad, CE 1989 Co-Culture of Domestic-Animal Embryos. *Theriogenology* **31** 105-114.

Rexroad, CE, and AM Powell 1993 Development of Ovine Embryos Cocultured on Oviductal Cells, Embryonic Fibroblasts, or Sto Cell Monolayers. *Biology of Reproduction* **49** 789-793.

Richardson, LL, and G Oliphant 1981 Steroid Concentrations in Rabbit Oviducal Fluid during Estrus and Pseudopregnancy. *Journal of Reproduction and Fertility* **62** 427-431.

Richter, KS 2008 The importance of growth factors for preimplantation embryo development and in-vitro culture. *Current Opinion in Obstetrics & Gynecology* **20** 292-304.

Rief, S, F Sinowatz, M Stojkovic, R Einspanier, E Wolf, and K Prelle 2002 Effects of a novel co-culture system on development, metabolism and gene expression of bovine embryos produced in vitro. *Reproduction* **124** 543-556.

Rieger, D, NM Loskutoff, and KJ Betteridge 1992 Developmentally Related Changes in the Metabolism of Glucose and Glutamine by Cattle Embryos Produced and Cocultured Invitro. *Journal of Reproduction and Fertility* **95** 585-595.

Risau, W 1997 Mechanisms of angiogenesis. *Nature* **386** 671-674.

Rizos, D, P Lonergan, MP Boland, R Arroyo-Garcia, B Pintado, J de la Fuente, and A Gutierrez-Adan 2002 Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: Implications for blastocyst quality. *Biology of Reproduction* **66** 589-595.

Rizos, D, MA Ramirez, B Pintado, P Lonergan, and A Gutierrez-Adan 2010 Culture of bovine embryos in intermediate host oviducts with emphasis on the isolated mouse oviduct. *Theriogenology* **73** 777-785.

Roach, HI, and NMP Clarke 2000 Physiological cell death of chondrocytes in vivo is not confined to apoptosis - New observations on the mammalian growth plate. *Journal of Bone and Joint Surgery-British Volume* **82B** 601-613.

Roberts, AJ, and MK Skinner 1991 Transforming Growth Factor-Alpha and Factor-Beta Differentially Regulate Growth and Steroidogenesis of Bovine Thecal Cells during Antral Follicle Development. *Endocrinology* **129** 2041-2048.

Rockwell, LC, S Pillai, CE Olson, and RD Koos 2002 Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. *Biology of Reproduction* **67** 1804-1810.

Rodgers, WH, LM Matrisian, LC Giudice, B Dsupin, P Cannon, C Svitek, F Gorstein, and KG Osteen 1994 Patterns of Matrix Metalloproteinase Expression in Cycling Endometrium Imply Differential Functions and Regulation by Steroid-Hormones. *Journal of Clinical Investigation* **94** 946-953.

Roemer, SC, J Adelman, MEA Churchill, and DP Edwards 2008 Mechanism of high-mobility group protein B enhancement of progesterone receptor sequence-specific DNA binding. *Nucleic Acids Research* **36** 3655-3666.

Rogalla, T, M Ehrnsperger, X Preville, A Kotlyarov, G Lutsch, C Ducasse, C Paul, M Wieske, AP Arrigo, J Buchner, and M Gaestel 1999 Regulation of Hsp27 oligomerization, chaperone function, and protective activity against oxidative stress tumor necrosis factor alpha by phosphorylation. *Journal of Biological Chemistry* **274** 18947-18956.

Rognan, D 2015 Rational design of protein-protein interaction inhibitors. *Medchemcomm* **6** 51-60.

Roldan-Olarte, M, DC Garcia, M Jimenez-Diaz, PA Valdecantos, and DC Miceli 2012 In vivo and in vitro expression of the plasminogen activators and urokinase type

plasminogen activator receptor (u-PAR) in the pig oviduct. *Animal Reproduction Science* **136** 90-99.

Roldan-Olarte, M, M Jimenez-Diaz, and DC Miceli 2005 Plasminogen detection in oocytes and plasminogen activator activities in the porcine oviduct during the estrous cycle. *Zygote* **13** 115-123.

Rose, IA, and JVB Warms 1966 Control of Glycolysis in Human Red Blood Cell. *Journal of Biological Chemistry* **241** 4848-&.

Rottmayer, R, SE Ulbrich, S Kolle, K Prelle, C Neumueller, F Sinowatz, HHD Meyer, E Wolf, and S Hiendleder 2006 A bovine oviduct epithelial cell suspension culture system suitable for studying embryo-maternal interactions: morphological and functional characterization. *Reproduction* **132** 637-648.

Roudebush, WE, JB Massey, HI Kort, CW Elsner, AA Toledo, D Mitchell-Leef, and DB Shapiro 2004 Exposure of preimplantation embryos to platelet-activating factor increases birth rate. *Journal of Assisted Reproduction and Genetics* **21** 297-300.

Ruckebusch, Y, and F Bayard 1975 Motility of Oviduct and Uterus of Cow during Estrous-Cycle. *Journal of Reproduction and Fertility* **43** 23-32.

Rudolf, E, K Rudolf, and M Cervinka 2008 Selenium activates p53 and p38 pathways and induces caspase-independent cell death in cervical cancer cells. *Cell Biol Toxicol* **24** 123-141.

Sakkas, D, AO Trounson, and I Kola 1989 Invivo Cleavage Rates and Viability Obtained for Early Cleavage Mouse Embryos in Co-Culture with Oviduct Cells. *Reproduction Fertility and Development* **1** 127-136.

Santiago, AR, AJ Cristovao, PF Santos, CM Carvalho, and AF Ambrosio 2007 High glucose induces caspase-independent cell death in retinal neural cells. *Neurobiol Dis* **25** 464-472.

Schafer, DA, PB Jennings, and JA Cooper 1996 Dynamics of capping protein and actin assembly in vitro: Uncapping barbed ends by polyphosphoinositides. *Journal of Cell Biology* **135** 169-179.

Schell, DL, PA Mavrogianis, AT Fazleabas, and HG Verhage 1994 Epidermal Growth-Factor, Transforming Growth-Factor-Alpha, and Epidermal Growth-Factor Receptor Localization in the Baboon (Papio-Anubis) Oviduct during Steroid Treatment and the Menstrual-Cycle. *Journal of the Society for Gynecologic Investigation* **1** 269-276.

Schirenbeck, A, R Arasada, T Bretschneider, M Schleicher, and J Faix 2005 Formins and VASPs may co-operate in the formation of filopodia. *Biochemical Society Transactions* **33** 1256-1259.

Schmaltz-Panneau, B, A Cordova, S Dhorne-Pollet, C Hennequet-Antier, S Uzbekova, E Martinot, S Doret, P Martin, P Mermillod, and Y Locatelli 2014 Early bovine embryos regulate oviduct epithelial cell gene expression during in vitro co-culture. *Animal Reproduction Science* **149** 103-116.

Schmidt, A, R Einspanier, W Amselgruber, F Sinowatz, and D Schams 1994 Expression of Insulin-Like Growth-Factor-1 (Igf-1) in the Bovine Oviduct during the Estrous-Cycle. *Experimental and Clinical Endocrinology* **102** 364-369.

Schneider, CA, WS Rasband, and KW Eliceiri 2012 NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9** 671-675.

Schroeder, AC, RM Schultz, GS Kopf, FR Taylor, RB Becker, and JJ Eppig 1990 Fetuin Inhibits Zona-Pellucida Hardening and Conversion of Zp2 to Zp2f during Spontaneous Mouse Oocyte Maturation Invitro in the Absence of Serum. *Biology of Reproduction* **43** 891-897.

Schuetz, AW, and NH Dubin 1981 Progesterone and Prostaglandin Secretion by Ovulated Rat Cumulus Cell-Oocyte Complexes. *Endocrinology* **108** 457-463.

Sciandra, JJ, JR Subjeck, and CS Hughes 1984 Induction of Glucose-Regulated Proteins during Anaerobic Exposure and of Heat-Shock Proteins after Reoxygenation. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **81** 4843-4847.

Seytanoglu, A, AS Georgiou, E Sostaric, PF Watson, WV Holt, and A Fazeli 2008 Oviductal cell proteome alterations during the reproductive cycle in pigs. *Journal of Proteome Research* **7** 2825-2833.

Shamsuddin, M, and H Rodriguezmartinez 1994 Fine-Structure of Bovine Blastocysts Developed Either in Serum-Free Medium or in Conventional Coculture with Oviduct Epithelial-Cells. *Journal of Veterinary Medicine Series a-Zentralblatt Fur Veterinarmedizin Reihe a-Physiology Pathology Clinical Medicine* **41** 307-316.

Shao, R, E Egecioglu, B Weijdegard, JJ Kopchick, J Fernandez-Rodriguez, N Andersson, and H Billig 2007a Dynamic regulation of estrogen receptor-alpha isoform expression in the mouse fallopian tube: mechanistic insight into estrogen-

dependent production and secretion of insulin-like growth factors. *American Journal of Physiology-Endocrinology and Metabolism* **293** E1430-E1442.

Shao, RJ, K Ljungstrom, B Weijdegard, E Egecioglu, J Fernandez-Rodriguez, FP Zhang, A Thurin-Kjellberg, C Bergh, and H Billig 2007b Estrogen-induced upregulation of AR expression and enhancement of AR nuclear translocation in mouse fallopian tubes in vivo. *American Journal of Physiology-Endocrinology and Metabolism* **292** E604-E614.

Sharkey, A 1998 Cytokines and implantation. *Reviews of Reproduction* **3** 52-61.

Shweiki, D, A Itin, D Soffer, and E Keshet 1992 Vascular Endothelial Growth-Factor Induced by Hypoxia May Mediate Hypoxia-Initiated Angiogenesis. *Nature* **359** 843-845.

Silva, ES, KE Scoggin, IF Canisso, MHT Troedsson, EL Squires, and BA Ball 2014 Expression of receptors for ovarian steroids and prostaglandin E2 in the endometrium and myometrium of mares during estrus, diestrus and early pregnancy. *Animal Reproduction Science* **151** 169-181.

Simard, J, ML Ricketts, B Gingras, P Soucy, FA Feltus, and MH Melner 2005 Molecular biology of the 3 beta-hydroxysteroid dehydrogenase/Delta(5)-Delta(4) isomerase gene family. *Endocrine Reviews* **26** 525-582.

Smith, OF, AA Ogunsola, AO Ladokun, and A Ajadi 2011 Synergistic effects of insulin-like growth factor ii (IGF-II) and leutinizing hormone (LH) on steroid hormone secretion by cultured bovine granulosa cells. *Indian Journal of Animal Sciences* **81** 1116-1120.

Smith, S, M Schmidt, B Purwantara, and T Greve 1992 Oviduct Epithelial-Cell Coculture of Early Porcine Embryos. *Acta Veterinaria Scandinavica* **33** 349-355.

Smits, K 2010 Equine embryos produced in vitro: How much do they miss a mare?, Faculty of Veterinary Medicine, Department of Reproduction, Obstetrics and Herd Health. Merelbeke: Ghent University.

Smits, K, K Goossens, A Van Soom, J Govaere, M Hoogewijs, and LJ Peelman 2011 In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reproduction Fertility and Development* **23** 364-375.

Smits, K, J Govaere, M Hoogewijs, C De Schauwer, E Van Haesebrouck, M Van Poucke, L Peelman, M van den Berg, T Vullers, and A Van Soom 2010 Birth of the first ICSI foal in the Benelux. *Vlaams Diergeneeskundig Tijdschrift* **79** 134-138.

Smits, K, J Govaere, M Hoogewijs, S Piepers, and A Van Soom 2012a A Pilot Comparison of Laser-Assisted vs Piezo Drill ICSI for the In Vitro Production of Horse Embryos. *Reproduction in Domestic Animals* **47** e1-e3.

Smits, K, J Govaere, LJ Peelman, K Goossens, DC de Graaf, D Vercauteren, L Vandaele, M Hoogewijs, E Wydooghe, T Stout, and A Van Soom 2012b Influence of the uterine environment on the development of in vitro-produced equine embryos. *Reproduction* **143** 173-181.

Smits, KG, K.; Van Soom, A.; Govaere, J.; Hoogewijs, M.; Vanhaesebrouck, E.; Galli, C.; Colleoni, S.; Vandesompele, J.; Peelman, L. 2009 Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes* **2** 246.

Spilman, CH, and JW Wilks 1976 Peripheral Plasma Progesterone during Egg Transport in Rabbit. *Proceedings of the Society for Experimental Biology and Medicine* **151** 726-729.

Squires, EL, and OJ Ginther 1975 Collection Technique and Progesterone Concentration of Ovarian and Uterine Venous-Blood in Mares. *Journal of Animal Science* **40** 275-281.

Stadelmann, C, and H Lassmann 2000 Detection of apoptosis in tissue sections. *Cell and Tissue Research* **301** 19-31.

Staples, LD, IR Fleet, and RB Heap 1982 Anatomy of the Utero-Ovarian Lymphatic Network and the Composition of Afferent Lymph in Relation to the Establishment of Pregnancy in the Sheep and Goat. *Journal of Reproduction and Fertility* **64** 409-420.

Stefanczyk-Krzymowska, S, T Krzymowski, B Wasowska, and J Chlopek 2002 Retrograde transfer of ovarian steroid hormones to the ovary in the porcine periovarian vascular complex. *Experimental Physiology* **87** 361-371.

Stevenson, KR, and DC Wathes 1996 Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *Journal of Reproduction and Fertility* **108** 31-40.

Stocco, DM 2001 StAR protein and the regulation of steroid hormone biosynthesis. *Annual Review of Physiology* **63** 193-213.

Stocco, DM, XJ Wang, Y Jo, and PR Manna 2005 Multiple signaling pathways regulating steroidogenesis and steroidogenic acute regulatory protein expression: More complicated than we thought. *Molecular Endocrinology* **19** 2647-2659.

Stoklosowa, S, E Gregoraszczuk, and CP Channing 1982 Estrogen and Progesterone Secretion by Isolated Cultured Porcine Thecal and Granulosa-Cells. *Biology of Reproduction* **26** 943-952.

Suarez, S, K Redfern, P Raynor, F Martin, and DM Phillips 1991 Attachment of Boar Sperm to Mucosal Explants of Oviduct Invitro - Possible Role in Formation of a Sperm Reservoir. *Biology of Reproduction* **44** 998-1004.

Suarez, SS 2008 Regulation of sperm storage and movement in the mammalian oviduct. *International Journal of Developmental Biology* **52** 455-462.

Suarez, SS, and HC Ho 2003 Hyperactivated motility in sperm. *Reproduction in Domestic Animals* **38** 119-124.

Sukerkar, PA, KW MacRenaris, TR Townsend, RA Ahmed, JE Burdette, and TJ Meade 2011 Synthesis and Biological Evaluation of Water-Soluble Progesterone-Conjugated Probes for Magnetic Resonance Imaging of Hormone Related Cancers. *Bioconjugate Chemistry* **22** 2304-2316.

Swan, CL, MC Agostini, PM Bartlewski, V Feyles, RJ Urban, and PJ Chedrese 2002 Effects of progestins on progesterone synthesis in a stable porcine granulosa cell line: Control of transcriptional activity of the cytochrome P450 side-chain cleavage gene. *Biology of Reproduction* **66** 959-965.

Swangchan-Uthai, T, SW Walsh, SLH Alexander, ZR Cheng, MA Crowe, ACO Evans, and DC Wathes 2011 Comparison of mRNA for IGFs and their binding proteins in the oviduct during the peri-oestrous period between dairy heifers and lactating cows. *Reproduction* **142** 457-465.

Swiatkowska, M, A Cierniewska-Cieslak, Z Pawlowska, and CS Cierniewski 2000 Dual regulatory effects of nitric oxide on plasminogen activator inhibitor type 1 expression in endothelial cells. *European Journal of Biochemistry* **267** 1001-1007.

Syvala, H, A Vienonen, YH Zhuang, M Kivineva, T Ylikomi, and P Tuohimaa 1998 Evidence for enhanced ubiquitin-mediated proteolysis of the chicken progesterone receptor by progesterone. *Life Sciences* **63** 1505-1512.

Szafranska, B, A Ziecik, and S Okrasa 2002 Primary antisera against selected steroids or proteins and secondary antisera against gamma-globulins--an available tool for studies of reproductive processes. *Reproductive Biology* **2** 187-204.

Tadokoro, C, Y Yoshimoto, M Sakata, T Imai, M Yamaguchi, H Kurachi, Y Oka, T Maeda, and A Miyake 1995 Expression and Localization of Glucose-Transporter-1 (Glut1) in the Rat Oviduct - a Possible Supplier of Glucose to Embryo during Early Embryonic-Development. *Biochem Biophys Res Commun* **214** 1211-1218.

Taipale, J, and J KeskiOja 1997 Growth factors in the extracellular matrix. *Faseb Journal* **11** 51-59.

Takeda, T, Y Tsutsumi, S Hara, and M Ida 1978 Effects of Prostaglandin-F2-Alpha on Egg Transport and Invivo Egg Recovery from Vaginas of Rabbits. *Fertility and Sterility* **30** 79-85.

Tetsuka, M, M Milne, and SG Hillier 1998 Expression of oestrogen receptor isoforms in relation to enzymes of oestrogen synthesis in rat ovary. *Molecular and Cellular Endocrinology* **141** 29-35.

Teves, ME, F Barbano, HA Guidobaldi, R Sanchez, W Miska, and LC Giojalas 2006 Progesterone at the picomolar range is a chemoattractant for mammalian spermatozoa. *Fertility and Sterility* **86** 745-749.

Thibodeaux, JK, and RA Godke 1992 Invitro Enhancement of Early-Stage Embryos with Coculture. *Archives of Pathology & Laboratory Medicine* **116** 364-372.

Thibodeaux, JK, Y Menezo, JD Roussel, W Hansel, LL Goodeaux, DL Thompson, and RA Godke 1992a Coculture of Invitro Fertilized Bovine Embryos with Oviductal Epithelial-Cells Originating from Different Stages of the Estrous-Cycle. *Journal of Dairy Science* **75** 1448-1455.

Thibodeaux, JK, MW Myers, LL Goodeaux, Y Menezo, JD Roussel, JR Broussard, and RA Godke 1992b Evaluating an in vitro culture system of bovine uterine and oviduct epithelial cells for subsequent embryo co-culture. *Reproduction, Fertility and Development* **4** 573-583.

Thomas, PGA, BA Ball, and SP Brinsko 1994 Interaction of Equine Spermatozoa with Oviduct Epithelial-Cell Explants Is Affected by Estrous-Cycle and Anatomic Origin of Explant. *Biology of Reproduction* **51** 222-228.

Thomas, PGA, GG Ignotz, BA Ball, PG Miller, SP Brinsko, and B Currie 1995a Isolation, Culture, and Characterization of Equine Oviduct Epithelial-Cells in-Vitro. *Molecular Reproduction and Development* **41** 468-478.

Thomas, PGA, GG Ignotz, BA Ball, PG Miller, SP Brinsko, and B Currie 1995b Isolation, culture, and characterization of equine oviduct epithelial cells in vitro. *Molecular Reproduction and Development* **41** 468-478.

Thompson, JG, NW Allen, LT McGowan, ACS Bell, MG Lambert, and HR Tervit 1998 Effect of delayed supplementation of fetal calf serum to culture medium on bovine embryo development in vitro and following transfer. *Theriogenology* **49** 1239-1249.

Thompson, JG, DK Gardner, PA Pugh, WH Mcmillan, and HR Tervit 1995 Lamb Birth-Weight Is Affected by Culture System Utilized during in-Vitro Pre-Elongation Development of Ovine Embryos. *Biology of Reproduction* **53** 1385-1391.

Tibbetts, TA, M Mendoza-Meneses, BW O'Malley, and OM Conneely 1998 Mutual and intercompartmental regulation of estrogen receptor and progesterone receptor expression in the mouse uterus. *Biology of Reproduction* **59** 1143-1152.

Timmons, L, H Tabara, CC Mello, and AZ Fire 2003 Inducible systemic RNA silencing in Caenorhabditis elegans. *Molecular Biology of the Cell* **14** 2972-2983.

Totary-Jain, H, T Naveh-Many, Y Riahi, N Kaiser, J Eckel, and S Sasson 2005 Calreticulin destabilizes glucose transporter-1 mRNA in vascular endothelial and smooth muscle cells under high-glucose conditions. *Circulation Research* **97** 1001-1008.

Tremoleda, JL, TAE Stout, I Lagutina, G Lazzari, MM Bevers, B Colenbrander, and C Galli 2003 Effects of in vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. *Biology of Reproduction* **69** 1895-1906.

Ulbrich, SE, AE Groebner, and S Bauersachs 2013a Transcriptional profiling to address molecular determinants of endometrial receptivity - Lessons from studies in livestock species. *Methods* **59** 108-115.

Ulbrich, SE, A Kettler, and R Einspanier 2003 Expression and localization of estrogen receptor α, estrogen receptor β and progesterone receptor in the bovine oviduct in vivo and in vitro. *The Journal of Steroid Biochemistry and Molecular Biology* **84** 279-289.

Ulbrich, SE, SU Meyer, K Zitta, S Hiendleder, F Sinowatz, S Bauersachs, M Buttner, T Frohlich, GJ Arnold, HD Reichenbach, E Wolf, and HHD Meyer 2011 Bovine

endometrial metallopeptidases MMP14 and MMP2 and the metallopeptidase inhibitor TIMP2 participate in maternal preparation of pregnancy. *Molecular and Cellular Endocrinology* **332** 48-57.

Ulbrich, SE, S Rehfeld, S Bauersachs, E Wolf, R Rottmayer, S Hiendleder, M Vermehren, F Sinowatz, HHD Meyer, and R Einspanier 2006 Region-specific expression of nitric oxide synthases in the bovine oviduct during the oestrous cycle and in vitro. *Journal of Endocrinology* **188** 205-213.

Ulbrich, SE, E Wolf, and S Bauersachs 2013b Hosting the preimplantation embryo: potentials and limitations of different approaches for analysing embryo-endometrium interactions in cattle. *Reproduction Fertility and Development* **25** 62-70.

Ullrich, A, A Gray, AW Tam, T Yangfeng, M Tsubokawa, C Collins, W Henzel, T Lebon, S Kathuria, E Chen, S Jacobs, U Francke, J Ramachandran, and Y Fujitayamaguchi 1986 Insulin-Like Growth Factor-I Receptor Primary Structure - Comparison with Insulin-Receptor Suggests Structural Determinants That Define Functional Specificity. *Embo Journal* **5** 2503-2512.

Urban, RJ, JC Garmey, MA Shupnik, and JD Veldhuis 1991 Follicle-Stimulating-Hormone Increases Concentrations of Messenger-Ribonucleic-Acid Encoding Cytochrome-P450 Cholesterol Side-Chain Cleavage Enzyme in Primary Cultures of Porcine Granulosa-Cells. *Endocrinology* **128** 2000-2007.

Van den Broeck, W, M D'haeseleer, M Coryn, and P Simoens 2002 Cell-specific distribution of progesterone receptors in the bovine ovary. *Reproduction in Domestic Animals* **37** 164-170.

van den Driesche, S, M Myers, E Gay, KJ Thong, and WC Duncan 2008 HCG up-regulates hypoxia inducible factor-1 alpha in luteinized granulosa cells: implications for the hormonal regulation of vascular endothelial growth factor A in the human corpus luteum. *Molecular Human Reproduction* **14** 455-464.

Vandaele, L, B Mateusen, DGD Maes, A de Kruif, and A van Soom 2007 Temporal detection of caspase-3 and-7 in bovine in vitro produced embryos of different developmental capacity. *Reproduction* **133** 709-718.

Vandesompele, J, K De Preter, F Pattyn, B Poppe, N Van Roy, A De Paepe, and F Speleman 2002 Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**.

Vanhaecke, L, JV Bussche, K Wille, K Bekaert, and HF De Brabander 2011 Ultra-high performance liquid chromatography-tandem mass spectrometry in high-throughput confirmation and quantification of 34 anabolic steroids in bovine muscle. *Analytica Chimica Acta* **700** 70-77.

Vansoom, A, I Vanvlaenderen, AR Mahmoudzadeh, H Deluyker, and A Dekruif 1992 Compaction Rate of Invitro Fertilized Bovine Embryos Related to the Interval from Insemination to 1st Cleavage. *Theriogenology* **38** 905-919.

Verdugo, P, RE Rumery, and PY Tam 1980 Hormonal-Control of Oviductal Ciliary Activity - Effect of Prostaglandins. *Fertility and Sterility* **33** 193-196.

Verhage, HG, ML Bareither, RC Jaffe, and M Akbar 1979 Cyclic Changes in Ciliation, Secretion and Cell Height of the Oviductal Epithelium in Women. *American Journal of Anatomy* **156** 505-521.

Visse, R, and H Nagase 2003 Matrix metalloproteinases and tissue inhibitors of metalloproteinases - Structure, function, and biochemistry. *Circulation Research* **92** 827-839.

Wagh, PV, and J Lippes 1989 Human Oviductal Fluid Proteins .3. Identification and Partial-Purification. *Fertility and Sterility* **51** 81-88.

Walter, I 1995 Culture of Bovine Oviduct Epithelial-Cells (Boec). *Anatomical Record* **243** 347-356.

Walters, KW, JF Roser, and GB Anderson 2001 Maternal-conceptus signalling during early pregnancy in mares: oestrogen and insulin-like growth factor I. *Reproduction* **121** 331-338.

Wang, GL, BH Jiang, EA Rue, and GL Semenza 1995 Hypoxia-Inducible Factor-1 Is a Basic-Helix-Loop-Helix-Pas Heterodimer Regulated by Cellular O-2 Tension. *Proceedings of the National Academy of Sciences of the United States of America* **92** 5510-5514.

Wang, JZ, HS Sui, DQ Miao, N Liu, P Zhou, L Ge, and JH Tan 2009 Effects of heat stress during in vitro maturation on cytoplasmic versus nuclear components of mouse oocytes. *Reproduction* **137** 181-189.

Wathes, DC, TS Reynolds, RS Robinson, and KR Stevenson 1998 Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of Dairy Science* **81** 1778-1789.

Watson, AJ, LC Barcroft, DH Bets, PA DeSousa, E Gilfoyle, J Looye, J PierreLouis, and QA Winger 1996 Maternal and embryonic control of bovine pre-attachment development: Expression of oviductal and embryonic genes. *Archiv Fur Tierzucht-Archives of Animal Breeding* **39** 55-69.

Watson, ED, SB Skolnik, and HG Zanecosky 1992 Progesterone and Estrogen-Receptor Distribution in the Endometrium of the Mare. *Theriogenology* **38** 575-580.

Watson, ED, R Thomassen, M Steele, M Heald, R Leask, NP Groome, and SC Riley 2002 Concentrations of inhibin, progesterone and oestradiol in fluid from dominant and subordinate follicles from mares during spring transition and the breeding season. *Animal Reproduction Science* **74** 55-67.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991a Prostaglandin E2 hastens oviductal transport of equine embryos. *Biol Reprod* **45** 544-546.

Weber, JA, DA Freeman, DK Vanderwall, and GL Woods 1991b Prostaglandin E2 secretion by oviductal transport-stage equine embryos. *Biol Reprod* **45** 540-543.

Weber, JA, GL Woods, DA Freeman, and DK Vanderwall 1993 Oviductal and uterine influence on the development of Day-2 equine embryos in vivo and in vitro. *Theriogenology* **40** 689-698.

Weems, CW, YS Weems, CN Lee, and DL Vincent 1989 Progesterone in uterine and arterial tissue and in jugular and uterine venous plasma of sheep. *Biology of Reproduction* **41** 1-6.

Wessel, T, U Schuchter, and H Walt 2004 Ciliary motility in bovine oviducts for sensing rapid non-genomic reactions upon exposure to progesterone. *Hormone and Metabolic Research* **36** 136-141.

West, NB, and RM Brenner 1985 Progesterone-Mediated Suppression of Estradiol Receptors in Cynomolgus Macaque Cervix, Endometrium and Oviduct during Sequential Estradiol-Progesterone Treatment. *Journal of Steroid Biochemistry and Molecular Biology* **22** 29-37.

West, NB, HG Verhage, and RM Brenner 1977 Changes in Nuclear Estradiol Receptor and Cell Structure during Estrous Cycles and Pregnancy in Oviduct and Uterus of Cats. *Biology of Reproduction* **17** 138-143.

Whiteside, EJ, M Kan, MM Jackson, JG Thompson, C McNaughton, AC Herington, and MB Harvey 2001 Urokinase-type plasminogen activator (uPA) and matrix

metalloproteinase-9 (MMP-9) expression and activity during early embryo development in the cow. *Anatomy and Embryology* **204** 477-483.

Wijayagunawardane, MPB, WA Cerbito, A Miyamoto, TJ Acosta, M Takagi, K Miyazawa, and K Sato 1996 Oviductal progesterone concentration and its spatial distribution in cyclic and early pregnant cows. *Theriogenology* **46** 1149-1158.

Wijayagunawardane, MPB, YH Choi, A Miyamoto, H Kamishita, S Fujimoto, M Takagi, and K Sato 1999 Effect of ovarian steroids and oxytocin on the production of prostaglandin E-2, prostaglandin F-2 alpha and endothelin-1 from cow oviductal epithelial cell monolayers in vitro. *Animal Reproduction Science* **56** 11-17.

Wijayagunawardane, MPB, SP Kodithuwakku, D Yamamoto, and A Miyamoto 2005 Vascular endothelial growth factor system in the cow oviduct: A possible involvement in the regulation of oviductal motility and embryo transport. *Molecular Reproduction and Development* **72** 511-520.

Wijayagunawardane, MPB, A Miyamoto, WA Cerbito, TJ Acosta, M Takagi, and K Sato 1998 Local distributions of oviductal estradiol, progesterone, prostaglandins, oxytocin and endothelin-1 in the cyclic cow. *Theriogenology* **49** 607-618.

Wildt, L, S Kissler, P Licht, and W Becker 1998 Sperm transport in the human female genital tract and its modulation by oxytocin as assessed by hysterosalpingoscintigraphy, hysterotonography, electrohysterography and Doppler sonography. *Human Reproduction Update* **4** 655-666.

Wilsher, S, S Gower, and WR Allen 2011 Immunohistochemical localisation of progesterone and oestrogen receptors at the placental interface in mares during early pregnancy. *Animal Reproduction Science* **129** 200-208.

Wolf, E, GJ Arnold, S Bauersachs, HM Beier, H Blum, R Einspanier, T Frohlich, A Herrler, S Hiendleder, S Kolle, K Prelle, HD Reichenbach, M Stojkovic, H Wenigerkind, and F Sinowatz 2003a Embryo-maternal communication in bovine - Strategies for deciphering a complex cross-talk. *Reproduction in Domestic Animals* **38** 276-289.

Wolf, E, GJ Arnold, S Bauersachs, HM Beier, H Blum, R Einspanier, T Fröhlich, A Herrler, S Hiendleder, S Kölle, K Prelle, HD Reichenbach, M Stojkovic, H Wenigerkind, and F Sinowatz 2003b Embryo-Maternal Communication in Bovine – Strategies for Deciphering a Complex Cross-Talk. *Reproduction in Domestic Animals* **38** 276-289.

Wollenhaupt, K, A Kettler, Br, K-P ssow, F Schneider, W Kanitz, and R Einspanier 2001 Regulation of the expression and bioactivation of the epidermal growth factor receptor system by estradiol in pig oviduct and endometrium. *Reproduction, Fertility and Development* **13** 167-176.

Wollenhaupt, K, U Tiemann, R Einspanier, F Schneider, W Kanitz, and KP Brussow 1997 Characterization of the epidermal growth factor receptor in pig oviduct and endometrium. *Journal of Reproduction and Fertility* **111** 173-181.

Wrenzycki, C, D Herrmann, JW Carnwath, and H Niemann 1998a Expression of RNA from developmentally important genes in preimplantation bovine embryos produced in TCM supplemented with BSA. *Journal of Reproduction and Fertility* **112** 387-398.

Wrenzycki, C, D Herrmann, L Keskintepe, A Martins, S Sirisathien, B Brackett, and H Niemann 2001a Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Human Reproduction* **16** 893-901.

Wrenzycki, C, D Herrmann, E Lemme, K Korsawe, JW Carnwath, and H Niemann 1998b Transcriptional level of developmentally important genes in bovine preimplantation embryos generated in vitro. *Theriogenology* **49** 191-191.

Wrenzycki, C, D Wells, D Herrmann, A Miller, J Oliver, R Tervit, and H Niemann 2001b Nuclear transfer protocol affects messenger RNA expression patterns in cloned bovine blastocysts. *Biology of Reproduction* **65** 309-317.

Wydooghe, E, S Heras, J Dewulf, S Piepers, E Van den Abbeel, P De Sutter, L Vandaele, and A Van Soom 2014 Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture. *Reprod Fertil Dev* **26** 717-724.

Wydooghe, E, L Vandaele, J Beek, H Favoreel, B Heindryckx, P De Sutter, and A Van Soom 2011 Differential apoptotic staining of mammalian blastocysts based on double immunofluorescent CDX2 and active caspase-3 staining. *Analytical Biochemistry* **416** 228-230.

Xu, J, TM Cheung, ST-h Chan, PC Ho, and WSB Yeung 2000 Human oviductal cells reduce the incidence of apoptosis in cocultured mouse embryos. *Fertility and Sterility* **74** 1215-1219.

Xu, JS, STH Chan, WWM Lee, KF Lee, and WSB Yeung 2004 Differential growth, cell proliferation, and apoptosis of mouse embryo in various culture media and in coculture. *Molecular Reproduction and Development* **68** 72-80.

Yadav, PS, A Saini, A Kumar, and GC Jain 1998 Effect of oviductal cell co-culture on cleavage and development of goat IVF embryos. *Animal Reproduction Science* **51** 301-306.

Yamada, M, T Horiuchi, T Oribe, S Yamamoto, H Matsushita, and PA Gentry 1996 Plasminogen activator activity in the bovine oocyte-cumulus complex and early embryo. *Journal of Veterinary Medical Science* **58** 317-322.

Yan, L, N Borregaard, L Kjeldsen, and MA Moses 2001 The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil gelatinase-associated lipocalin (NGAL) - Modulation of MMP-9 activity by NGAL. *Journal of Biological Chemistry* **276** 37258-37265.

Yang, J, A Blum, T Novak, R Levinson, ES Lai, and J Barasch 2002 An epithelial precursor is regulated by the ureteric bud and by the renal stroma. *Developmental Biology* **246** 296-310.

Yaniz, JL, M Lopez-Bejar, P Santolaria, J Rutllant, and F Lopez-Gatius 2002 Intraperitoneal insemination in mammals: A review. *Reproduction in Domestic Animals* **37** 75-80.

Yuan, YQ, A Van Soom, FOJ Coopman, K Mintiens, ML Boerjan, A Van Zeveren, A de Kruif, and LJ Peelman 2003 Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology* **59** 1585-1596.

Zavy, JT, R Mayer, MW Vernon, FW Bazer, and DC Sharp 1979 An investigation of the uterine luminal environment of non-pregnant and pregnant pony mares. *J Reprod Fertil Suppl* 403-411.

Zhang, SD, and TW Gant 2005 Effect of pooling samples on the efficiency of comparative studies using microarrays. *Bioinformatics* **21** 4378-4383.

Zhang, X, GM Kidder, C Zhang, F Khamsi, and DT Armstrong 1994 Expression of Plasminogen-Activator Genes and Enzymatic-Activities in Rat Preimplantation Embryos. *Journal of Reproduction and Fertility* **101** 235-240.

Zhao, Y, N Chegini, and KC Flanders 1994 Human Fallopian-Tube Expresses Transforming Growth-Factor (Tgf-Beta) Isoforms, Tgf-Beta Type I-Iii Receptor Messenger-Ribonucleic-Acid and Protein, and Contains [I-125] Tgf-Beta-Binding Sites. *Journal of Clinical Endocrinology & Metabolism* **79** 1177-1184.

Zhao, YG, AZ Xiao, XM Cao, and C Zhu 2002 Expression of matrix metalloproteinase-2,-9 and tissue inhibitors of metalloproteinase-1,-2,-3 mRNAs in rat uterus during early pregnancy. *Molecular Reproduction and Development* **62** 149-158.

Zuker, M 2003 Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Research* **31** 3406-3415.

SUMMARY

Science never solves a problem without creating ten more.

George Bernard Shaw, playwriter

SUMMARY

To date, *in vivo* derived equine embryos are of superior quality compared to those produced *in vitro,* in terms of morphology and ultrastructure, gene expression and developmental competence. This phenomenon confirms that current *in vitro* systems are deprived of particular essential maternal factors or signals and illustrates the importance of embryo-maternal interplay. So far, only very few signals involved in the embryo-maternal dialogue have been identified in the horse. Although *in vivo* models are the gold standard, it is difficult to investigate molecular processes within a relatively large space like the equine oviduct. It is for instance difficult to locate gametes and the embryo in the oviduct and to unravel local paracrine and autocrine events, which are essential in elucidating intra- and extracellular molecular pathways and processes. The general goal of this thesis was to gain insight in the embryo-maternal communication in the horse. To achieve this aim, the influence of steroids on the oviduct was *in vivo* investigated (CHAPTER 4). These effects were simulated *in vitro* (CHAPTER 5.1) by means of an optimized *in vitro* oviduct explant model (CHAPTER 3). The effect of steroids (CHAPTER 5.1) and embryos (CHAPTER 5.2) on oviduct explant's gene expression was assessed. Next, as a step forward to the improvement of *in vitro* equine embryo culture, it was also investigated whether embryos *in vitro* benefit from culture at the mare's body temperature (CHAPTER 6).

In **CHAPTER 3.1**, a culture system which sustains equine oviduct explants bordered by highly differentiated, glucose consuming, functional and intact epithelial cells showing vigorous ciliary activity during 6 days of culture was optimized. Only a negligible percentage (1–2%) of the cells in the explants showed features of apoptosis or necrosis and therefore, it could be concluded that the explants mimic the *in vivo* situation very closely. Although dark-cell degeneration, a hypoxia related type of cell death, was detected using TEM, the hypoxia marker genes *HIF1A, GLUT1* and *VEGFA* were not upregulated in the culture system, even in culture at high oxygen concentrations. In an attempt to further unravel the origin of the dark cell degeneration, explants were cultured with and without foetal calf serum (FCS) or supplemented with serum replacement insulin-transferrin-selenium (ITS) in **CHAPTER 3.2**. It turned out that selenium increases while FCS decreases to some extent the incidence of explants showing dark cell degeneration.

In the horse no information is available concerning local steroid concentrations in the oviduct and their fluctuations during the oestrous cycle. Therefore in **CHAPTER 4** the concentrations of progesterone, oestradiol, testosterone and 17-hydroxyprogesterone were determined in equine oviductal tissue by the highly powerful technique UHPLC-MS/MS whereas RIA was applied to measure steroids in oviductal fluid. Progesterone concentrations were high in oviductal tissue and fluid ipsilateral to the ovulation side during diestrus, whereas testosterone, and 17α-testosterone and 17-hydroxyprogesterone other steroid hormone concentrations were not influenced by the side of ovulation. The most plausible explanation for the elevated progesterone concentration in the ipsilateral oviduct of the mare is a combination of 1) the contribution from follicular fluid in the oviduct and the diffusion of follicular fluid steroids after ovulation; 2) a local transfer of steroids via blood or lymph, 3) local synthesis of progesterone in the oviduct which is confirmed by the expression of StAR, cytochrome P450scc and 3-beta-HSD, key enzymes in the progesterone synthesis, as well as aromatase, which is suggestive of local steroidogenesis; and, 4) the paracrine contribution from follicular cells.

In **CHAPTER 5.1**, preovulatory explants were stimulated with hormone concentrations as they prevail in the postovulatory stage and vice versa. The influence of these steroid hormones on the function (ciliary activity, glucose consumption and lactate production), the ultrastructure, the mRNA expression of a set of embryotrophic genes, the steroidogenic capacities and the progesterone receptor expression in the equine oviduct was assessed. Progesterone and 17β-oestradiol were able to modify ciliary activity, energy metabolism, gene expression, immunoreactive steroidogenic enzyme expression and progesterone receptor expression in oviductal explants *in vitro*. Furthermore, *PAI1*, *PLAU*, *GLUT1, CSF1, TGFA* and *MMP2* were shown to be upregulated in the oviductal epithelium originating from mares in the postovulatory cycle stage. Moreover, preovulatory oviduct explants, primed by steroids *in vivo*, are responsive to *in vitro* stimulation with postovulatory oviductal progesterone and 17β-oestradiol concentrations and approach the *in vivo* condition at the level of functionality and gene expression. This endorses that our explant model remains functional and responsive for at least three days (**CHAPTER 3**). In addition, it turned out that oviduct explants are capable of producing large amounts of progesterone *in vitro* and are able to remove considerable amounts of oestrone, 17β-oestradiol and testosterone from the culture medium. This confirms again the functional integrity of the culture system.

The oviductal environment represents the optimal environment for early embryo development. Supposing that oviductal cells provide specific mitogenic factors that would normally be present in the oviduct, or non-specific factors that improve the culture environment such as reduction of oxygen tension, removal of waste products or provision of substrates and co-factors, we cultured equine zygotes, obtained by ICSI, with and without equine oviduct explants in **CHAPTER 5.2**. To elucidate the role of developing embryos on the modulation of gene expression in the oviduct, we unraveled the response of the same set of embryotrophic genes as used in **CHAPTER 5.1** in oviductal cells cultured together with equine putative zygotes (**CHAPTER 3**). Co-culture with equine embryos stimulated the expression of the embryotrophic genes *TIMP1, PTGER2, TGFA, MMP2, CSF1* and *PAI1* in the oviduct explant, which have been described to be involved in embryo transport, and in stimulating embryonic development and quality, and they modulate oviductal matrix turnover. Co-culture did not affect ciliary activity or viability of oviduct explants.

In an attempt to further improve embryo culture conditions, it was investigated in **CHAPTER 6** if oocyte maturation, cleavage, blastocyst rate and blastocyst diameter could be improved when applying the physiological body temperature of the mare (37.3 °C) rather than the conventional 38.5°C. Cytoplasmic maturation does not differ in both groups. The size of blastocysts was smaller in the oocytes matured and embryos cultured at 37.3°C compared with the matured and cultured at 38.5°C. Since blastocyst size is a parameter of embryo viability, culture at 38.5°C may be recommended rather than culture at 37.3°C.

In the final **CHAPTER 7** the general discussion and conclusions are presented. Our findings indisputably demonstrate that the equine oviduct is able to respond to both steroids and embryonic signals *in vivo* and *in vitro* and that our oviduct explant model is an excellent model to further unravel the embryo-maternal interplay in the horse.

SAMENVATTING

Facts are the air of scientists; without them you cannot fly

Linus Pauling, biochemist

SAMENVATTING

Tot nu toe zijn *in vivo* paardenembryo's van betere kwaliteit op vlak van morfologie, ultrastructuur en genexpressie en ontwikkelen ze zich bovendien sneller tot blastocyst in vergelijking met *in vitro* geproduceerde embryo's. Dit bevestigt dat onder andere in de huidige *in vitro* systemen bepaalde essentiële maternale factoren ontbreken en benadrukt het belang van embryo-maternale interactie. Tot nu toe zijn er slechts een paar signalen van de embryo-maternale dialoog geïdentificeerd bij het paard. Alhoewel *in vivo* modellen de gouden standaard zijn, is het zeer moeilijk om processen op het moleculaire niveau te ontrafelen in een relatief grote ruimte zoals het paardenoviduct. Het is bijvoorbeeld moeilijk om de gameten en het embryo te lokaliseren en om lokale paracriene en autocriene signalen in het oviduct op te vangen die essentieel zijn voor intra- en extracellulaire moleculaire signaaltransducties en processen. Het algemene doel van deze thesis was inzichten verwerven in de embryo-maternale communicatie bij het paard. Daarom werd *in vivo* de invloed van steroïden op het oviduct nagegaan (HOOFDSTUK 4). Dit werd gesimuleerd *in vitro* (HOOFDSTUK 5.1) met een geoptimaliseerd *in vitro* oviductexplantmodel (HOOFDSTUK 3). Het effect van steroïden (HOOFDSTUK 5.1) en embryo's (HOOFDSTUK 5.2) op de genexpressie van oviductexplanten werd beoordeeld. In een volgende stap, met als doel de *in vitro* cultuur van paardenembryo's verder te verbeteren, werd nagegaan of embryo's *in vitro* baat hebben bij cultuur aan de lichaamstemperatuur van de merrie (HOOFDSTUK 6).

In HOOFDSTUK 3.1 werd een cultuursysteem geoptimalizeerd voor oviductexplanten. Die explanten zijn afgelijnd door hoog gedifferentieerde, glucoseconsumerende, functionele en intacte epitheliale cellen die uitgesproken ciliënbeweging vertonen gedurende een cultuurperiode van 6 dagen. Slechts een verwaarloosbaar percentage (1-2%) van de cellen in de explanten vertoonde tekenen van apoptose of necrose. Deze explanten bootsen dus zeer sterk de *in vivo* situatie na. Hoewel "dark-cell degeneration" (DCD), een hypoxie-gerelateerde vorm van celdood, door middel van TEM werd gedetecteerd in de explanten, waren de markers voor hypoxie *HIF1A*, *GLUT1* en *VEGFA* niet opgereguleerd, zelfs niet in cultuur bij lage zuurstofconcentratie. In een poging om de oorzaak van de DCD te achterhalen werden explanten op cultuur gezet al of niet met foetaal kalfserum en al of niet gesupplementeerd met de serumvervanger insuline-transferrine-selenium (HOOFDSTUK 3.2). Blijkbaar verhoogde

selenium de incidentie van DCD terwijl serum, tot op zekere hoogte, de incidentie van DCD verlaagde.

Bij het paard zijn er geen gegevens beschikbaar betreffende lokale concentraties en fluctuaties van steroïden in het oviduct tijdens de voortplantingscyclus. Daarom werden (HOOFDSTUK 4) de concentraties van progesteron, oestradiol, testosteron en 17-hydroxyprogesterone bepaald in oviductweefsel met de zeer krachtige techniek U-HPLC-MS/MS, terwijl met RIA de steroïdconcentraties in oviductvocht werden gemeten. De progesteronconcentratie was zeer hoog in zowel weefsel als vocht tijdens dioestrus in de oviducten ipsilateraal van de kant van ovulatie. De meest aannemelijke verklaring voor de verhoogde progesteronconcentraties in de ipsilaterale oviducten is een combinatie van 1) een bijdrage van follikelvocht en de diffusie van steroïden uit het follikelvocht na ovulatie; 2) een lokale transfer van steroïden via het bloed of lymfe; 3) lokale synthese van progesteron in het oviduct. Dit werd bevestigd door de expressie van immunoreactieve proteïnen van StAR, cytochroom P450scc en 3-beta-HSD, enzymen die een cruciale rol spelen in de progesteronsynthese. Aromatase werd ook gelokaliseerd; 4) de paracriene bijdrage van follikelcellen.

In HOOFDSTUK 5.1 werden preovulatoire explanten gestimuleerd met hormoonconcentraties zoals ze voorkomen in postovulatoire oviducten (HOOFDSTUK 4) en vice versa. De invloed van deze steroïden op de functie (ciliënbeweging, glucoseverbruik en lactaat productie), de ultrastructuur, de mRNA expressie van een set embryotrofe genen, de steroidogene capaciteit en de expressie van progesteronreceptoren in het paardenoviduct werden beoordeeld. Progesteron en 17β-oestradiol waren in staat om *in vitro* ciliënbeweging, energiemetabolisme, genexpressie, expressie van immunoreactieve proteïnen van steroidogene enzymen en progesteronreceptorexpressie in oviductexplanten te moduleren. *PAI1*, *PLAU*, *GLUT1, CSF1, TGFA* en *MMP2* waren opgereguleerd in het oviduct epithelium afkomstig van merries in het postovulatoire cyclusstadium. Bovendien bleken preovulatoire explanten, geïnitieerd door steroïden *in vivo*, te reageren op *in vitro* stimulatie met postovulatoire progesteron en 17 β-oestradiol concentraties en benaderden zij de *in vivo* status op vlak van functionaliteit en genexpressie. Dit bevestigt dat ons explantmodel functioneel en responsief blijft gedurende minstens 3 dagen. Bovendien is gebleken dat oviduct explanten *in vitro* grote hoeveelheden progesteron kunnen produceren en in staat zijn aanzienlijke hoeveelheden oestrone, 17β-oestradiol en testosteron uit het cultuurmedium te verwijderen. Dit bevestigt opnieuw de functionele integriteit van de explanten.

Het oviduct is de meest optimale omgeving voor de vroege embryonale ontwikkeling. Oviductcellen *in vitro* produceren specifieke mitogene factoren die normaal gezien in het oviduct voorkomen, alsook niet-specifieke factoren die de cultuurparameters, zoals de zuurstofspanning of het neutraliseren van afvalproducten verbeteren of de aanvoer van substraten en cofactoren bevorderen. Daarom werden paardenzygoten, verkregen door ICSI, met of zonder oviduct explanten op cultuur gezet (HOOFDSTUK 5.2). Om het effect van de ontwikkelende embryo's op de genexpressie van het oviduct na te gaan werd de expressie van dezelfde set embryotrofe genen als in HOOFDSTUK 5.1 vergeleken in oviduct explanten met en zonder co-cultuur. Co-cultuur met paardenembryo's verhoogde de expressie van de embryotrofe genen *TIMP1, PTGER2, TGFA, MMP2, CSF1* and *PAI1*, dewelke allen betrokken zijn bij embryotransport en het ondersteunen van de embryonale ontwikkeling en kwaliteit alsook bij het vernieuwen van de extracellulaire matrix. Co-cultuur had geen effect op de ciliënbeweging of vitaliteit van de explants.

In een poging om de cultuuromstandigheden voor het embryo verder te verbeteren werd (HOOFDSTUK 6) nagegaan of eicelmaturatie, deling, blastocystpercentage en -diameter konden verbeterd worden door de conventionele cultuurtemperatuur van 38.5 °C te verlagen naar de fysiologische lichaamstemperatuur van de merrie (37.3 °C). Cytoplasmatische maturatie was niet verschillend in beide groepen. De diameter van de blastocysten waren kleiner in de groep gecultiveerd bij 37.3 °C vergeleken met de groep bij 38.5 °C. Aangezien de blastocystdiameter een parameter is die de viabiliteit van een embryo karakteriseert, geniet cultuur bij 38.5°C de voorkeur boven 37.3 °C.

De algemene discussie, de conclusies en toekomstperspectieven zijn weergegeven in het laatste HOOFDSTUK 7. Onze bevindingen tonen onweerlegbaar aan dat het paardenoviduct in staat is om te reageren op zowel steroïden als embryonale signalen *in vivo* en *in vitro* en dat ons explantcultuursysteem het model bij uitstek is om de embryo-maternale dialoog bij het paard verder te ontrafelen.

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Do not kick away the canoe which helped you

to cross the river

Madagascan Proverb

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De zonne zonk,
het duister klom,
en verre sterren staan alom.
De lange tocht loopt ten einde,
de paarden mogen nu op stal...

“*Dankbaarheid is een bloem die in weinig hoven bloeit*”, dichtte Guido Gezelle, maar toch wisten wij gedurende deze zes jaar durende marathon, een omvangrijk boeket van deze zeldzame bloemen te plukken en te verzamelen om ze nu op het einde van deze rit met gulle hand te kunnen uitdelen. Nu is immers de tijd aangebroken om bloemen van dankbaarheid te schenken en gouden pluimen uit te delen.
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"Wie de titel van Doctor krijgen wil,

moet werken als een paard,

vlijtig zijn als een bij,

moedig zijn als een leeuw,

taai zijn als een ezel,

en als 't op rust aankomt,

moedig zijn om op te staan…

…en door te gaan."

(Uit: "Mensen en dieren" door J. Apostel)

Hilde Nelis, mei 2015

CURRICULUM VITAE

The only source of knowledge is experience

Albert Einstein, physicist

CURRICULUM VITAE

Hilde Nelis werd geboren op 19 augustus 1985 te Deinze. In 2003 behaalde zij het diploma hoger secundair onderwijs met onderscheiding aan het Sint-Barbaracollege te Gent (Grieks-Wetenschappen). Zij werd bekroond als laureaat in de Chemie. In 2003 startte zij de studie Diergeneeskunde aan de Universiteit Gent. Zij behaalde het diploma van zowel kandidaat Dierenarts als Dierenarts (optie paard) met grote onderscheiding. Als beste student optie Paard ontving zij de prijs van de Wetenschappelijke Vereniging voor de Gezondheid van het Paard. Haar afstudeerwerk met als titel "*Evaluatie van een ELISA-test voor de serologische diagnostiek van Fasciola bij het paard*" werd bekroond met een prijs geschonken door Vétoquinol, als beste scriptie over paarden. Bovendien ontving zij de prijs van het Vlaams Diergeneeskundig Tijdschrift voor het overzichtsartikel "*Fasciola heptica bij het paard*" dat zij schreef gebaseerd op haar scriptie.

Van jongs af aan geïnteresseerd in paarden en gefascineerd door wetenschappelijk onderzoek, trad zij op 1 oktober 2009 in dienst als aspirant voor het Fonds voor Wetenschappelijk Onderzoek Vlaanderen (FWO) aan de vakgroep Voortplanting, Verloskunde en Bedrijfsdiergeneeskunde, waar zij tot 30 september 2013 onderzoek verricht naar de embryo-maternale interacties bij het paard. Naast haar onderzoek was zij promotor van 13 masterproeven en was zij werkzaam in de kliniek verloskunde en voortplanting herkauwer en paard waar zij participeerde in de nacht- en weekenddiensten. In 2015 voltooide zij de doctoraatsopleiding van de Doctoral School of Life Sciences and Medicines.

Op 1 oktober 2013 vervoegde zij de afdeling Wetenschappelijke Evaluatoren van het Federaal Agentschap voor Geneesmiddelen en Gezondheidsproducten (FAGG).

Hilde Nelis is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften, gaf verschillende presentaties op meerdere internationale congressen en is reviewer voor diverse tijdschriften in haar vakgebied.

BIBLIOGRAPHY - BIBLIOGRAFIE

No amount of experimentation can ever prove me right;

a single experiment can prove me wrong

Albert Einstein, physicist

SCIENTIFIC PUBLICATIONS WITH PEER REVIEW

Nelis H., Geurden T., Deprez P. (2010). *Fasciola hepatica* bij het paard. *Vlaams Diergeneeskundig Tijdschrift 79*, 436-444.

Nelis H. M., Leemans B., de Vries C., Hoogewijs M., Chiers K., Van Soom A., Govaere J. (2012) Oviductal and uterine leiomyomata in the mare. *Vlaams Diergeneeskundig Tijdschrift, 82,* 75-80.

Leemans B., Vandenberghe L., Smits K., Van Soom A., **Nelis H. M**. (2012) Nieuwe fertiliteitsbehandelingen bij het paard: toepassingsmogelijkheden en beperkingen. *Vlaams Diergeneeskundig Tijdschrift 81*, 329-340.

Nelis H. M., D'Herde K., Goossens K., Vandenberghe L., Leemans B., Smits K., Forier K., Braeckmans K., Peelman L., Van Soom A. (2014) Morphology, ultrastructure and gene expression of equine oviduct epithelial explants during culture. *Reproduction, Fertility and Development* 26, 954–966.

Nelis H. M., Vanden Bussche J., Goossens K., Van Haecke L., Leemans B., Peelman L., Van Soom A., Smits K. (2015). Steroids in the equine oviduct: local concentration, synthesis and progesterone receptor expression. Accepted *Reproduction, Fertility and Development*, Epub March 10th http://dx.doi.org/10.1071/RD14483.

Nelis H. M., Wojciechowicz B., Franczak A., Goossens K., Leemans B., Cornillie P., Peelman L., Van Soom A., Smits K. (2015) Steroids affect gene expression, ciliary activity, glucose uptake, progesterone receptor expression and immunoreactive steroidogenic protein expression in equine oviduct explants *in vitro*. *Reproduction, Fertility and Development*, Epub June 18th http://dx.doi.org/10.1071/RD15044.

Nelis H. M., Van Steendam K., Deforce D., Govaere J., Bienzle D., Leemans B., Van Soom A., Smits K. The proteome of equine oviductal fluid: influence of ovulation and pregnancy. Submitted.

Nelis H. M., Leemans B., Goossens K., Peelman L., Van Soom A., Smits K. Equine embryos affect equine oviductal gene expression *in vitro*. In revision.

Nelis H. M., Leemans B., Van Soom A., Smits K. The steeple chase of *in vitro* equine embryo culture: an overview of the crossed hurdles up to now. Submitted.

Vandenberghe L.T.M., Govaere J., **Nelis H. M.,** Hoogewijs M., Daels P., Van Soom A. (2012). Embryotransplantatie bij het paard: onmisbaar in de moderne fokkerij. *Vlaams Diergeneeskundig Tijdschrift 81*, 274-282.

Bustin S., Benes V., Garson J., Jan Hellemans J., Huggett J., Mikael Kubista M., Mueller R., Nolan T., Pfaffl M.W., Shipley G., Wittwer C.T., Schjerling P., Day P.J., Abreu M., guado B., Beaulieu J-F., Beckers A., Bogaert S., Browne J.A., Carrasco-Ramiro F., Ceelen L., Ciborowski K., Cornillie P., Coulon S., Cuypers A., De Brouwer S., De Ceuninck L., De Craene J., De Naeyer H., De Spiegelaere W., Deckers K., Dheedene A., Durinck K., Ferreira-Teixeira M., Fieuw A., Gallup J.M., Gonzalo-Flores S., Goossens K., Heindryckx F., Herring E., Hoenicka H., Icardi L., Jaggi R., Javad F., Karampelias M., Kibenge F., Kibenge M., Kumps C., Lambertz I., Lammens T., Markey A., Messiaen P., Mets E., Morais S., Mudarra-Rubio A., Nakiwala J., **Nelis H. M,** Olsvik P.A., Pérez-Novo C., Plusquin M., Remans T., Rihani A., Rodrigues-Santos P., Rondou P., Sanders R., Schmidt-Bleek K., Skovgaard K., Smeets K., Tabera L., Toegel S., Van Acker T., Van den Broeck W., Van der Meulen J., Van Gele M., Van Peer G., Van Poucke M., Van Roy N., Vergult S., Wauman J., Tshuikina-Wiklander M., Willems E., Zaccara S., Zeka F. & Vandesompele J. 2013 An assessment of the reliability of RT-qPCR data published in the peer-reviewed literature. *Nature Methods 10 (11),* 1063-1067.

Leemans B., Gadella B. M., Sostaric E., **Nelis H. M,** Stout T. A. E., Hoogewijs M., Van Soom A. Oviduct Binding and Elevated Environmental pH Induce Protein Tyrosine Phosphorylation in Stallion Spermatozoa. *Biology of Reproduction 91(1):13*, 1–12.

Leemans B., Gadella B., Stout T., Heras S., Smits K., Ferrer-Buitrago M., Claes E., Heindryckx B., De Vos W., **Nelis H. M.,** Hoogewijs M., Van Soom A. 2015. Procaine induces parthenogenetic cytokinesis in horse oocytes via a pH dependent mechanism. Accepted *Biology of Reproduction, Epub June 17th* http://dx.doi.org/10.1095/biolreprod.114.127423.

Leemans B., Gadella B., Stout T., **Nelis H. M.,** Hoogewijs M., Van Soom A. 2015. An alkaline follicular fluid fraction induces capacitation and limited sperm-oviduct release. *Reproduction 150(3): 193-208.*

Leemans B., Gadella B., Sostaric E., Stout T., **Nelis H. M.,** Hoogewijs M., Van Soom A. 2015. Equine sperm affinity for the oviduct is depending on the combined effect of HCO_3^- and albumin but Ca^{2+}, carbohydrate, lectin, penicillamine independent. Submitted to *Biology of Reproduction*.

Leemans B., Gadella B., Stout T., **Nelis H. M.,** Hoogewijs M., Van Soom A. 2015. New insights in capacitation of stallion spermatozoa: What is the role of the oviduct? A review. In preparation.

POSTERS ON INTERNATIONAL CONFERENCES

Nelis H., Cornillie P., Dewulf J., Van Soom A. (2010). Morphological evaluation of three equine oviduct epithelial cell culture systems. COST action FA0702 GEMINI: General Conference, 3rd, Soustons, France. Proceedings p103.

Nelis H.M., Cornillie P., De Wulf J., Piepers S., Van Soom A. (2010). Influence of cooling during oviduct transportation on the establishment of equine oviduct epithelial cell vesicle culture. 26th Scientific Meeting of the AETE, Kuopio, Finland, 10th-11th September. Proceedings p 214.

Nelis H. M., Vanhaecke L., Roels K., Van Soom A. (2011). Serum a-and b- estradiol, a-and b-testosterone and 17-OH-progesterone concentration during the first seven days of pregnancy in mares. Proceedings ESDAR conference, September 15th-17th,2011; Antalya, Turkey.

Nelis H. M., Leemans B., D’Herde K., Cornillie P., Van Soom A. (2011) Histological and ultrastructural characterization of equine oviduct explants. COST action FA0702 GEMINI: General Conference, 4rd, Gijon, Spain., p. 69.

Nelis H. M., D’Herde K., Goossens K., Vandenberghe L., Leemans B., Forier K., Peelman L., Van Soom A. (2012). Optimization of an equine oviduct explant culture system:

ultrastructural features and gene expression. AETE conference, 6-8th September, Saint-Malo, France, 192.

Nelis H. M., Goossens K., Leemans B., Peelman L., Van Soom A. (2013). Steroid regulated mRNA expression in oviduct epithelial cells in the mare. *Reproduction, Fertility and Development* **25** (1), abstract 220.

Nelis H. M., Vanden Bussche J. , Goossens K. , L. Vanhaecke L, Vandenberghe L., Leemans B., L. Peelman L., Van Soom A. (2013) Postovulatory concentrations of progesterone and β-oestradiol in the equine oviduct are upregulating mRNA expression of *PAI1, CSF* and *MMP1* both *in vivo* and in an explant model *in vitro.* COST action Epiconcept, First General meeting, Antalya, Turkey, 24th-25th april, proceedings p. 56.

Nelis H. M., Vandenberghe L., D'Herde K., Leemans B., Van Soom, A., Smits, K. (2015) Supplementation of culture medium with foetal calf serum or insulin – transferrin – selenium affects the integrity of equine oviduct explants. AETE conference 2015, Ghent, Belgium, proceedings p. 158.

Leemans B. , **Nelis H. M.**, Vandaele L., and Van Soom A. (2011). Procaine does not activate equine oocytes. Proceedings ESDAR conference, September 15th-17th,2011; Antalya, Turkey.

Leemans B, Gadella B.M. , Sostaric E., **Nelis H. M.,** Hoogewijs M., Van Soom A. (2011). Whittens medium maintains oviduct cell viability and facilitates oviduct-sperm binding in the horse. COST action FA0702 GEMINI: General Conference, 4th, Gijon, Spain, p. 61.

Leemans B., Gadella B.M. , Sostaric E., **Nelis H. M.,** Hoogewijs M., Van Soom A. (2012). Increased pH induces tyrosine phosporylation without activation of acrosome reaction in stallion semen. COST action Epiconcept, First General meeting, Antalya, Turkey, 24th-25th april, proceedings p. 95.

Smits K., **Nelis H. M.,** Leemans B., Van Soom A. (2014). Co-culture with equine embryos affects gene expression in equine oviduct epithelial explants. EPICONCEPT Workshop 2014 Epigenomic Toolbox: from Methods to Models, Las Palmas, Spain, 7-9th May , proceedings p. 56.

Leemans B., Gadella B.M., Stout T.A.E., Heras S., Smits S., Ferrer-Buitrago M., Claes E., Heindryckx B., De Vos W.H., **Nelis H. M.,** Hoogewijs M., Van Soom A. (2015). Procaine induces parthenogenetic cytokinesis in horse oocytes via a pH dependent mechanism 48th Annual Meeting of the Society for the Study of Reproduction, 18 - 22 June, San Juan, Puerto Rico.

ORAL PRESENTATIONS ON INTERNATIONAL CONFERENCES

Nelis H.M., Cornillie P. , Dewulf J., Van Soom A. (2010). Optimalizatie van een oviductcelcultuur bij het paard: invloed van vesikelgrootte en cultuursysteem op overleving van explanten. Najaarsvergadering Vereniging voor Fertiliteitsstudie, October 29th, Nijmegen, The Netherlands.

Nelis H.M., Leemans B., Van Soom A. (2012). Culturing horse oocytes and embryos at the physiological temperature of the mare. *Journal of Equine Veterinary Science 32(7),* 410**.** Presented at the International Equine Embryo Transfer Symposium. 26-29 th July, Vancouver, Canada.

Nelis H.M., Leemans B., Van Soom A. (2012). Effect van temperatuur op de eicelmaturatie en de embryocultuur bij het paard. Najaarsvergadering Vereniging voor Fertiliteitsstudie, 5 oktober, Rotterdam, The Netherlands.

Leemans B, Gadella B.M. , Sostaric E., **Nelis H.M.,** Hoogewijs M., Van Soom A. (2011). Spermacapacitatie bij het paard: een rol voor het oviduct? Najaarsvergadering Vereniging voor Fertiliteitsstudie, November 18th, Utrecht, The Nederlands.

Leemans B., Gadella B. M., Sostaric E., **Nelis H. M**., Hoogewijs M., Van Soom A. (2012). How can oviduct-explants influence capacitation of equine sperm cells? International Symposium on Stallion Reproduction. 6-8th September 2012, Vienna, Austria.

Leemans B., Gadella B., Stout T., **Nelis H. M.,** Hoogewijs M., Van Soom A. 2013. Hitte geïnactiveerd follikelvocht veroorzaakt loslating van gecapaciteerde spermacellen van het oviductepitheel van de merrie. Najaarsvergadering Vereniging voor Fertiliteitsstudie (VFS), 29 November, Maastricht, The Netherlands.

Smits K., **Nelis H. M.,** Van Steendam K., Govaere J., Hoogewijs M., Deforce D., Van Soom A. (2013) Havemeyer Foundation Workshop on Equine Implantation, Savannah, Georgia, USA, 27th-29th October, proceedings p5-6.

Leemans B., Gadella B.M., Stout T.A.E., **Nelis H.M.,** Hoogewijs M., Van Soom A. (2014) Heat inactivated follicular fluid induces release from oviduct explants of stallion sperm primed for fertilization. XIth International Symposium on Equine Reproduction (ISER), Hamilton, New Zealand, 26th -31st January, abstract 86.

POPULAR SCIENTIFIC WRITING

Nelis H.M. Schijndracht bij de geit. Landbouw en Techniek. Februari 2011.

Nelis H.M. Mijn merrie blijft aan de nageboorte staan; wat nu? Landbouwleven, Juli 2011.

Nelis H. M. De meest beruchte giftige planten, struiken, bomen en groenten voor schapen en geiten. Management & Techniek, Februari 2012.

Nelis H.M. Welke planten zijn giftig voor schapen en geiten? De Schapenhouder, April 2012.

Nelis H.M. De meest beruchte giftige planten, struiken en bomen voor paarden. Landbouwleven, Juni 2012.

Nelis H. M. Giftige planten in en rond de weide. Hippo Revue, oktober 2012 en Vlaams Paardenloket http://www.vlaamspaardenloket.be/nl/themas/Paardenwelzijn-gezondheid-en-verzorging/De-meest-beruchte-giftige-planten-struiken-en-bomen-voor-paarden-Brochure-Hilde-Nelis-UGent. Laats geraadpleegd online: september 2015.

Nelis H.M. De meest beruchte huidproblemen bij schapen en geiten. Management en techniek, September 2012.

Nelis H.M. Huidproblemen bij schapen en geiten. De Schapenhouder, Oktober 2012.

Nelis H.M. Help! Mijn paard heeft jeuk, wat nu? Landbouwleven, Januari 2013.

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Nelis H.M. Wat u zeker moet weten vóór het lammerseizoen aanbreekt. Landbouwleven, Februari 2014.

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Nelis H.M. Problemen bij de merrie kort na het veulenen. Landbouwleven, Maart 2015.

Nelis H.M. Jacobskruiskruid en Sint-Janskruid. Haflinger Revue, Juni 2015

Nelis H.M. Koliek bij paarden: wat kan u doen om het te voorkomen? Landbouwleven, September 2015.

Schulpzand B. en **Nelis H.M**. Giftige planten in de herfst. EquiTime, Oktober 2015.

Every new beginning comes from some other beginning's end

Seneca, Roman statesman and philosopher