Aus der Klinik für Pferde, allgemeine Chirurgie und Radiologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Influence of cycle stage, age and biopsy score on oxytocin receptor gene expression and distribution in the cervix and uterus of non-pregnant mares

Inaugural-Dissertation
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Doktors der Veterinärmedizin
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vorgelegt von
Ruth Ströhle
Tierärztin aus Ulm

Berlin 2018
Journal-Nr.: 4075

mbvberlin | mensch und buch verlag

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Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Johannes Handler
Zweiter Gutachter:	Univ.-Prof. Dr. Salah Amasheh
Dritter Gutachter:	Univ.-Prof. Dr. Robert Klopfleisch

Deskriptoren (nach CAB-Thesaurus):
mares, reproduction, biopsy, blood sampling, tissues, oxytocin, real time PCR, immunohistochemistry, progesterone

Tag der Promotion: 05.06.2018

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <https://dnb.de> abrufbar.

ISBN: 978-3-86387-923-5
Zugl.: Berlin, Freie Univ., Diss., 2018
Dissertation, Freie Universität Berlin
D188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex method for IHC detection
ART	Assisted reproductive techniques
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CH	Corpus haemorrhagicum
CL	Corpus luteum
C_T	Cycle threshold
DAB	3,3'-Diaminobenzidine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ds	Double strand
dsDNA	Double strand deoxyribonucleic acid
ET	Embryo transfer
F	Follicle
FSH	Follicle stimulating hormone
FVS-UP	Faculty of Veterinary Science, University of Pretoria, Onderstepoort
Gαq protein	Phospholipase C G protein subunit
Gαi protein	Adenylate cyclase inhibitor G protein subunit
GIFT	Gamete intrafallopian tube transfer
GnRH	Gonadotropin releasing hormone

GXR antibody	Polyclonal biotinylated goat anti-rabbit immunoglobulins (mainly IgG)
HE	Haematoxylin and eosin stain
ICSI	Intracytoplasmatic sperm injection
IgG	Immunoglobulin G
IH	Immunohistology
IHC	Immunohistochemistry
IVF	In vitro fertilisation
LH	Luteinising hormone
mRNA	Messenger-ribonucleic acid
NSS	Non-specific structures (includes follicles up to 1cm)
NT	Nuclear transfer
OR	Oxytocin receptor
OT	Oocyte transfer
PBS	Phosphate buffered saline
PBS-BSA	Phosphate buffered saline with bovine serum albumin
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2-*alpha*
PGFM	13, 14-dihydro-15-keto prostaglandin F2-*alpha*
PMN	Polymorphonuclear neutrophil

PPC	Plasma progesterone concentration
qPCR	Real-time polymerase chain reaction
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse-transcription polymerase chain reaction
RT-qPCR	Quantitative reverse-transcription polymerase chain reaction
sF	Small follicles (follicle size: 1-2 cm)
SOP	Standard operating procedure
[3H]oxytocin	[Tyrosyl-2, 6-^{3}H] oxytocin

CHAPTER 1: Introduction

Each year a significant proportion of mares fail to become pregnant despite optimised breeding management (no obvious reason of infertility in the mare, good semen quality of the stallion, correct timing of breeding, good insemination technique and post-breeding management; Da Silva 2008). These mares are considered subfertile (Da Silva 2008). Assisted reproductive techniques (ART) including embryo transfer (ET), oocyte transfer (OT), gamete intrafallopian tube transfer (GIFT), in vitro fertilisation (IVF), intracytoplasmatic sperm injection (ICSI) and nuclear transfer (NT; cloning) have been developed to achieve pregnancies in these problem mares (Hinrichs and Choi 2005; Da Silva 2008; Carnevale 2008). Different reproductive pathologies (ovulation failure, faulty oocyte transport, uterine inflammation, degenerative uterine changes) can cause a mare to not conceive or lose a pregnancy at an early stage with uterine pathologies like post-breeding endometritis being one of the most important reasons (Carnevale 2008).

Uterine clearance is one of the most important components of the uterine defence mechanism against infection (Troedsson 1999). Mares incapable of efficient uterine clearance post breeding and consequently incapable of resolving the transient physiological endometritis are likely to develop a persistent post-breeding endometritis leading to prolonged inflammation of the endometrium and consequently embryonic loss and subfertility (Troedsson 1999; Troedsson et al. 2001; Watson 2000). Different authors have suggested a significant role for oxytocin and its receptors in the pathogenesis of subfertility in mares. Nikolakopoulos et al. (2000) assumed a defective prostaglandin F2-*alpha* ($PGF_{2\alpha}$) release at the oxytocin receptor (OR) level, and Rigby et al. (2001) suspected a decreased level of oxytocin-stimulated myometrial $PGF_{2\alpha}$

production, were involved in the pathology of subfertility. Although defects at the OR level or a lack of receptors have been hypothesised as contributing to subfertility, the specific role of OR concentration and distribution in the pathogenesis of subfertility has not been reported.

Oxytocin is a neuropeptide hormone produced by the hypothalamus and endometrium of the mare (Behrendt–Adam et al. 1999; Handler 2001; Gimpl and Fahrenholz 2001; Bae and Watson 2003). In the equine reproductive tract, oxytocin is mainly responsible for contractions of smooth muscles in the oviduct, uterus and the myoepithelial cells of the mammary gland (Handler 2001). Myometrial contractility is not only important for foetal expulsion during parturition but also for mechanical drainage of uterine fluid and cellular debris especially at the time of oestrus (Nikolakopoulos and Watson, 1999). Oxytocin additionally causes the release of endometrial $PGF_{2\alpha}$ and is thus supposedly important in luteolysis (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993).

Oxytocin acts on target tissues by binding to its receptors. Oxytocin receptors have additionally been described in various tissues outside the genital tract (Gimpl and Fahrenholz 2001). In the equine reproductive tract, ORs could be demonstrated in the cytoplasm of various cells of endometrium, myometrium and the cervix (Stroehle et al. 2016). In previous studies, it has been reported that the concentration of ORs in the endometrium of mares fluctuates throughout the oestrous cycle showing a peak during the late phase of dioestrus (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). There are no reports of a detailed evaluation of OR density and distribution during the oestrous cycle stages in endometrium, myometrium and the cervix of mares. There are also no reports on the influence of age and biopsy score on OR density and distribution.

The objectives of the current study were to:

- Compare the levels of messenger-ribonucleic acid (mRNA) transcribed from the OR gene and distribution of ORs in mares in the luteal and non-luteal phase
- Compare the levels of mRNA transcribed from the OR gene and distribution of ORs in young and aged mares
- Compare the levels of mRNA transcribed from the OR gene and distribution of ORs with endometrial biopsy scores

CHAPTER 2: Literature review

2.1 Subfertility in mares

Each year numerous mares fail to produce offspring despite ideal breeding management (no obvious reason of infertility in mare, good semen quality of stallion, correct timing of breeding, good insemination technique and post-breeding management; Da Silva 2008). This is a significant problem in the equine industry as it implies substantial economic and genetic loss (Da Silva 2008). Mares, repeatedly failing to fall pregnant under optimal conditions, are considered subfertile (Da Silva 2008). Assisted reproductive techniques (ART) have been developed in equines over the last four decades to achieve pregnancies in these problem mares (Hinrichs and Choi 2005; Da Silva 2008; Carnevale 2008). Assisted reproductive techniques include embryo transfer (ET), oocyte transfer (OT), gamete intrafallopian tube transfer (GIFT), in vitro fertilisation (IVF), intracytoplasmatic sperm injection (ICSI) and nuclear transfer (NT; cloning) with ET and OT being the only two techniques of current clinical use (Hinrichs and Choi 2005; Da Silva 2008; Carnevale 2008). Several reproductive pathologies (ovulation failure, faulty oocyte transport, uterine inflammation, degenerative uterine changes) can cause a mare to not conceive or lose a pregnancy at an early stage with uterine pathologies like post-breeding endometritis being one of the most important causes (Carnevale 2008).

2.2 Post-breeding endometritis

Transient inflammatory reaction of the uterus is a physiological consequence of breeding and results from uterine contamination with both bacteria (Williamson et al. 1984; Troedsson et al. 1993b) and semen (Troedsson 1995; Katila 1995; Miragaya et al. 1997). The reproductively normal mare

efficiently resolves the inflammation within 24 - 36 hours of contamination (Katila 1995) providing an optimal intrauterine environment for embryo survival on entering the uterus 5 – 6 days after ovulation (Betteridge et al. 1982; Freeman et al. 1992). Mares incapable of resolving the transient physiological endometritis are likely to develop a persistent post-breeding endometritis leading to prolonged inflammation of the endometrium and consequently embryonic loss and subfertility (Troedsson 1999; Troedsson et al. 2001; Watson 2000). Persistent inflammation often results in premature luteolysis and embryonic loss because of increased prostaglandin F2-*alpha* ($PGF_{2\alpha}$) concentrations (Troedsson 1995). This inflammation could also interfere directly with the survival of an embryo (Troedsson 1995).

The mechanism of induced persistent post-breeding inflammation is similar to endometritis caused by bacteria, involving activation of the complement cascade (Troedsson et al. 1995a). The uterus responds quickly to an antigen with release of polymorphonuclear neutrophils (PMNs) and chemotactic mediators cause a rapid migration of PMNs into the uterine lumen (Liu and Troedsson, 2008).

Post-breeding endometritis is one of the major causes for subfertility and it develops as a consequence of defective uterine clearance (Pycock 1994; Rausch et al. 1996; LeBlanc and Causey, 2009). It is a manifestation of a subclinical endometritis with no obvious external clinical signs. In the endometrium, local degenerative changes are the most obvious microscopic signs revealing a predisposition to post-breeding endometritis (Bracher et al. 1992; Ferreira-Dias et al. 1994; Ferreira-Dias et al. 1999).

Older mares are more susceptible to persistent post-breeding endometritis (Carnevale and Ginther, 1992), as they tend to have delayed physical clearance of uterine contents (Evans et al. 1987; Troedsson and Liu, 1991; Neuwirth et al. 1995),

which is associated with dysfunctional uterine contractility (Carnevale and Ginther, 1992).

After intrauterine bacterial challenge, mares that were susceptible to persistent post-breeding endometritis showed reduced frequency, intensity, and duration of uterine myoelectrical activity *in vivo* compared to normal mares (Troedsson et al. 1993a). There are numerous causes for this decline in myometrial activity. Possible mechanisms include: 1) changes in the release, either systemically or locally, of uterotonins such as prostaglandins or oxytocin; 2) altered expression of receptors for uterotonins in the endometrium; and 3) an intrinsic change within the uterine muscle that delays uterine clearance (Rigby et al. 2001). Rigby et al. (2001) confirmed an intrinsic defect in myometrial function and also suggested a contribution of reduced oxytocin-stimulated myometrial $PGF_{2\alpha}$ production to the reduced contractile response of susceptible mares (Rigby et al. 2001).

Nikolakopoulos et al. (2000) suggested that decreased $PGF_{2\alpha}$ concentrations in a group of susceptible mares after oxytocin administration could be due to a possible defect at the oxytocin receptor (OR) or post-receptor level. Although the contribution of a defect at the OR level or even a lack of receptors has been hypothesised as a contributing factor to subfertility, there have been no further reports investigating the role of ORs in this regard.

The majority of mares showing intrauterine fluid accumulation after breeding seems to have negative culture results thus treatment of this condition is generally aimed at clearing contaminants and inflammatory products from the uterus rather than antibiotic treatment (Watson 2000). The use of ecbolics (oxytocin and prostaglandin) with or without conjunctive uterine lavage, has been shown to be effective in enhancing uterine clearance (LeBlanc et al. 1994; Troedsson et al. 1995a). Oxytocin (5 – 10 IU) is frequently administered $\geq$ 4 hours after

mating without fertility being compromised (Watson 2000; Hurtgen 2006). Troedsson (1997a) proposed the use of $PGF_{2\alpha}$ to be superior to oxytocin as it causes 5 hours of increased myoelectrical activity in comparison to only 1 hour with oxytocin. However, pregnancy failure might result from the administration of $PGF_{2\alpha}$ during the periovulatory period (2 – 4 days) after ovulation as it causes a transient decrease in plasma progesterone concentration (Troedsson et al. 2001; Brendemuehl 2002; Liu and Troedsson 2008).

2.3 Uterine clearance

Uterine clearance is one of the most important contributors to the uterine defence mechanism (Troedsson 1999). Mares with normal uterine clearance remove cellular and inflammatory by-products via coordinated contractions of the myometrial smooth muscles and by immune system phagocytosis within the first 24 - 36 hours after breeding (Katila 1995; Troedsson 1999; Katila et al. 2000).

Delayed or failed uterine clearance is the most critical factor in decreased uterine defence against infection (LeBlanc and Causey, 2009). Difficulty clearing debris from the uterus after mating can be due to anatomical and, or degenerative defects that interfere with uterine drainage, including poor perineal conformation, an incompetent cervix and periglandular fibrosis (Evans et al. 1987; Allen 1991; LeBlanc 2008). Another possible cause is a failure in the oxytocin system decreasing uterine motility and hence clearance of the uterine lumen especially at the time of oestrus (LeBlanc et al. 1994; Pycock and Newcombe, 1996a; Pycock and Newcombe, 1996b).

2.4 The effect of age on reproductive function in the mare

The effect of age on mare reproductive integrity and function has been extensively reported. Decreased fertility has been

documented in the older mare (≥ 14 years, Madill 2002). Conception and foaling rates were decreased significantly in older (31 % and 13 % per cycle respectively), compared to younger mares (57 % and 51 % per cycle respectively; Madill 2002). A similar outcome was reported by Carnevale and Ginther (1992), who investigated pregnancy rates and embryo loss rates in old (> 15 years) and young (7 - 15 years) pony mares. Old mares showed significantly lower pregnancy rates (38 % vs 100 % in young mares) and higher embryo loss rates (62.5 % vs 11 % in young mares). The uterus displays evidence of mucociliary dysfunction in older mares, which may contribute to the development of subfertility (Causey 2007). Disruptions of mucociliary clearance usually involve disruption of efficient mucociliary transport which requires mucus of appropriate composition for optimal flow and adequate numbers of functioning ciliated cells for propulsion which is suspected to play a role in the uterine clearance mechanism (Causey 2007). Furthermore, scarring and atrophy of uterine folds and damage to (or lack of) endometrial cilia, occurring mainly in older mares, have also been associated with subfertility (Bracher et al. 1992; Ferreira-Dias et al. 1994; Ferreira-Dias et al. 1999). Recent studies indicated that blood flow and tissue perfusion are diminished in some sub-fertile mares (Schoon et al. 1999; Esteller-Vico et al. 2007; Gastal et al. 2008). Liu et al. (2008) reported that old (> 15 years) pluriparous mares are more likely to have vascular degeneration which seems to reduce fertility indirectly through disturbance in uterine drainage.

Endometrosis, a degenerative uterine condition, may also play a role in the loss of uterine function and therefore in decreasing fertility (Evans et al. 1987). The term endometrosis is defined as “active or inactive periglandular and, or stromal endometrial fibrosis including glandular alterations within fibrotic foci” (Schoon et al. 1992; Kenney 1992; Hoffmann et al. 2009).

Different studies have shown an increasing degree of endometrosis with age of the mare (Ricketts and Alonso, 1991; Schoon et al. 1992; Hoffmann et al. 2009). The most important factor in uterine function leading to subfertility is a defect in uterine clearance after breeding or foaling (Evans et al. 1987). This defect can occur due to anatomical (poor perineal conformation, incompetent vagino-vestibular sphincter, vaginal stretching, an incompetent cervix or a pendulous uterus) and, or degenerative changes (lymphangiectasia and scarring and atrophy of endometrial folds) that influence uterine drainage (Evans et al. 1987). In older maiden mares decreased pregnancy rates can be considered a syndrome manifested by factors including accumulation of uterine fluid during oestrus, glandular dilatation, frequent anovulatory haemorrhagic follicles and cervical malfunction (failure to relax) due to fibrotic changes or adhesions (Pycock 2006; Samper 2008).

2.5 Equine reproductive physiology

2.5.1 Oestrous cycle

The mare is seasonally polyoestrous with ovulatory activity being related to long days during summer (Daels 1993; Aurich 2011). During the short days of winter cyclic activity is suppressed and mares are in anoestrus (Daels 1993). Transition phases from anoestrus to ovulatory season and back to non-cyclic season coincide with irregular oestrous cycles that vary considerably in length (Aurich 2011). During the breeding season in spring and summer the average length of the oestrous cycle is about 22 days (range 18 – 24 days; Day 1939; Ginther 1992; Aurich 2011). The oestrous cycle is described as the time frame from one ovulation to the following ovulation and consists of oestrus (follicular phase) and dioestrus (luteal phase; Blanchard and Schumacher 2003). Oestrus is highly

variable and may last three to ten days (average 5 – 7 days) with ovulation occurring in the last two days of oestrus (Day 1939; Aurich 2011). The dominant structures on the ovaries during oestrus are follicles which have an approximate size of 20 – 25 mm diameter at the onset of oestrus but usually only one of them grows to a pre-ovulatory size of 55 – 60 mm and ovulates (Irvine 1981; Aurich 2011). Dioestrus, on the other hand, remains constant at 14 - 15 days and is barely affected by seasonal changes unlike oestrus (Blanchard and Schumacher, 2003). Corpus luteum (CL) or/and corpus hemorrhagicum (CH) as dominant ovarian structure is considered indicative for dioestrus (Blanchard and Schumacher, 2003).

2.5.2 Endocrine regulation of the oestrous cycle in the mare

Different hormones influence the oestrous cycle of the mare. Gonadotropin releasing hormone (GnRH) is produced and pulsatile secreted by the hypothalamus during the breeding season (Aurich 2011). This is modulated by a steroid (oestrogen and progesterone) feedback mechanism. Gonadotropin releasing hormone has a stimulating effect on the anterior pituitary gland production of gonadotropins, follicle stimulating hormone (FSH) and luteinising hormone (LH; Ginther 1992; Aurich 2011). Follicle stimulating hormone stimulates follicular growth during oestrus as well as LH receptor development on follicular granulosa cells and LH plays an important role in follicle maturation, oestrogen production and ovulation (Ginther 1992). Oestrogen is produced by the follicles on the ovary as a response to LH stimulation during oestrus whereas progesterone is a product of the CL during dioestrus. High concentrations of oestrogen and low concentrations of progesterone facilitate oestrous behaviour of the mare (Crowell-Davis 2007; Aurich 2011). Increased levels of progesterone have a negative feedback effect on the pituitary gland causing a decrease in LH secretion

(Miller et al. 1980). Towards the end of dioestrus, $PGF_{2\alpha}$ causes luteolysis of the CL which causes the mare to return to oestrus in 1 - 3 days (Daels 1993).

2.6 Luteolysis

Functional luteolysis is characterised by a significant decrease of progesterone concentration in circulation at the end of dioestrus (day 15 – 17; Aurich 2011). Morphological regression of the CL, however, develops later (Ginther et al. 2005; Ginther et al. 2007). The initial signal for luteolysis in the mare is secretion of $PGF_{2\alpha}$ from the endometrium. Prostaglandin F2-*alpha* is released into the peripheral circulation and not in a local counter current system existing in cows, ewes and swine (Aurich 2011).

The control of luteolytic $PGF_{2\alpha}$ release is less clearly described in mares than in ruminants where the CL secretes oxytocin (Wathes and Swann, 1982; Wathes et al. 1983) which plays an important role in luteolysis because it stimulates the endometrial secretion of $PGF_{2\alpha}$ towards the end of dioestrus (Flint and Sheldrick, 1986). In ruminants, one principal factor for the onset of luteolysis is the increase of ORs in the endometrium (Mirando et al. 1993; Wathes et al. 1996).

In the mare, involvement of oxytocin in the events of luteolysis is also suspected as it was reported that administration of exogenous oxytocin in late dioestrus induces an immediate increase in peripheral plasma concentrations of 13, 14-dihydro-15-keto prostaglandin F2-*alpha* (PGFM), the circulating metabolite of $PGF_{2\alpha}$ (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). It was additionally shown that oxytocin stimulates the equine endometrium to produce $PGF_{2\alpha}$ *in vitro* (King 1984; Franklin et al. 1989). Furthermore, Stout et al. (1999) demonstrated that continuous, high-dose systemic oxytocin administration during days 8 - 20 after ovulation

prevents luteolysis. It is suspected that the continuous, high-dose administration of oxytocin causes suppression of the normal dioestrous rise in endometrial ORs due to downregulation of ORs which would, in turn, prevent oxytocin-induced $PGF_{2\alpha}$ secretion (Stout et al. 1999). In a study done by Shand et al. (1999) the occurrence of coincidental pulses of oxytocin and PGFM in the circulation of mares during days 13 – 16 after ovulation was demonstrated, supporting the assumption that oxytocin plays a role in luteolysis.

During late dioestrus, initial oxytocin secretion causing endometrial $PGF_{2\alpha}$ release most likely originates from the hypophysis (Tetzke et al. 1987; Aurich 2011). There is no significant oxytocin synthesis by the CL in the mare but oxytocin production has been demonstrated in the endometrium (Bae and Watson, 2003). This finding supports the hypothesis that oxytocin released by the endometrium acts in a paracrine manner to stimulate $PGF_{2\alpha}$ release (Aurich 2011).

2.7 Oxytocin system

2.7.1 Oxytocin

Oxytocin is an abundant neuropeptide hormone with two cysteine residues forming a disulfide bridge between positions 1 and 6 (Handler 2001; Gimpl and Fahrenholz 2001). It is mainly produced in the hypothalamus and it is stored in secretory vesicles of the posterior pituitary gland from where it is released into the bloodstream (Stull 1986). Additionally, local synthesis of oxytocin within the female reproductive tract is well described (Wathes and Swann, 1982; Fields et al. 1983; Kiehm et al. 1989). Oxytocin has been identified in the CL of several domestic species including cows and ewes but the equine CL is not a source of oxytocin at any stage of the oestrous cycle (Stevenson et al. 1991; Stock et al. 1995; Watson et al. 1999). However, the endometrium was shown to be a

source of oxytocin in the mare. Oxytocin messenger-ribonucleic acid (mRNA) has been demonstrated in the equine endometrium by Behrendt–Adam et al. (1999). Bae and Watson (2003) described specific secretory endometrial cells containing oxytocin, which are suspected to be part of a paracrine luteolytic system.

In the equine reproductive tract, oxytocin is mainly responsible for contractions of smooth muscles in the oviduct, uterus and the myoepithelial cells of the mammary gland which is especially important for mechanical drainage of cellular debris and uterine fluid (Nikolakopoulos and Watson, 1999; Handler 2001). Oxytocin additionally stimulates the release of endometrial $PGF_{2\alpha}$ and is thus purportedly important in luteolysis (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993).

Oxytocin plays an important role in the sexual function of the mare throughout the oestrous cycle. It is secreted in pulses with length and amplitude varying dependent on the cycle stage (Tetzke et al. 1987). During oestrus, oxytocin increases uterine motility to possibly enhance sperm cell transport to the oviduct as well as support uterine clearance (Pycock 1993; Pycock 1994; Handler 2001). Oxytocin released by the endometrium acts in a paracrine manner to stimulate $PGF_{2\alpha}$ release and thus luteolysis towards the end of dioestrus (Aurich 2011).

2.7.2 Oxytocin receptor

Oxytocin acts on target tissues by binding to its receptor (Gimpl and Fahrenholz, 2001). The OR belongs to the rhodopsin-type superfamily of G protein-coupled, seven transmembrane receptors (Gimpl et al. 2008). It couples to $G\alpha_q$ and $G\alpha_i$ proteins that activate the phospholipase C-ß isoforms which consequently leads to an increase of intracellular calcium and activation of several protein kinases (Strakova and Soloff 1997;

Gimpl and Fahrenholz, 2001; Gimpl et al. 2008). Oxytocin binding to the ORs finally induces secretion of prostaglandins in domestic ruminants and mares (Gimpl and Fahrenholz, 2001; Gimpl et al. 2008). The OR has two (mouse and rat) or three (human, sheep, pig, bovine, suspected equine) potential N-glycosylation sites in its extracellular NH_2-terminal domain (Gimpl and Fahrenholz 2001). It is suspected that different glycosylation patterns are responsible for different molecular masses of the OR in myometrium, mammary gland and amnion (Gimpl and Fahrenholz 2001).

Oxytocin receptors have also been described in various tissues outside the genital tract (Gimpl and Fahrenholz 2001). There is a widespread distribution of ORs in the brain, which has been established to be important for reproductive and social behaviour (Young et al. 1998). Additionally, OR expression has been shown in pituitary, kidney, thymus, heart, vascular endothelium, oesteoclasts, myoblasts, pancreatic islet cells, adipocytes and several types of cancer cells (Gimpl and Fahrenholz 2001).

For many years [Tyrosyl-2, 6-^{3}H] oxytocin ([3H]oxytocin) radioligand-binding assay was seen as the gold standard method in studies examining OR density in different tissues and species (Soloff and Swartz, 1974; Roberts et al. 1976; Soloff et al. 1977; Wathes and Swann, 1982; Fuchs et al. 1985; Stull 1986; Meyer et al. 1988; Fuchs et al. 1990; Jenner et al. 1991; Ayad et al. 1991; Stevenson et al. 1994; Okano et al. 1996; Sharp et al. 1997; Starbuck et al. 1998; Vanderwall et al. 2012).

In 1995, Ivell et al. started using the reverse-transcription polymerase chain reaction (RT-PCR) as a new method for detection of ORs in the bovine uterus. This method was adopted by other studies investigating OR gene expression in different domestic species including ovines, porcines and

equines (Bathgate et al. 1995; Feng et al. 2000; Beretsos et al. 2006; Villani et al. 2008; Siemieniuch et al. 2011).

Immunohistochemistry has been used for description of the OR localisation in a variety of animal species including the horse (Fuchs et al. 1990; Fuchs et al. 1996a; Fuchs et al. 1996b; Morel et al. 2001; Beretsos et al. 2006; Villani et al. 2008; Siemieniuch et al. 2011; Stroehle et al. 2016).

Distribution of ORs within the cervical and uterine tissue differs between species. In the sow (Soloff and Swartz, 1974) and rat (Soloff et al. 1977), binding-sites for oxytocin were only detected in the myometrium. In the ewe (Roberts et al. 1976), woman (Fuchs et al. 1985), cow (Meyer et al. 1988; Fuchs et al. 1990; Jenner et al. 1991; Robinson et al. 2001) and mare (Stull 1986, Stroehle et al. 2016) binding sites were demonstrated in the endometrium and myometrium. Furthermore, oxytocin receptors have been shown in the cervix of the ewe, cow and mare (Matthews and Ayad, 1994; Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013, Stroehle et al. 2016). In the equine uterus, ORs could be demonstrated in the cytoplasm of vascular endothelial cells, luminal and glandular epithelia, sub-epithelial and peri-glandular stromal cells of the endometrium and throughout the smooth muscle and stromal cells of the myometrium (Stroehle et al. 2016). A similar pattern of distribution of ORs in the uterus and cervix was found (Stroehle et al. 2016).

Different studies reported on the OR concentration in the uterus in cows, sows and ewes (Wathes and Swann, 1982; Meyer et al. 1988; Fuchs et al. 1990; Jenner et al. 1991; Ayad et al. 1991; Stevenson et al. 1994; Robinson et al. 2001). In the cow, the OR gene expression in endometrium and myometrium is regulated cyclically. It appears to be significantly increased at the time of oestrus (Meyer et al. 1988; Fuchs et al. 1990; Jenner et al. 1991; Robinson et al. 2001). A similar event was demonstrated in ewes (Wathes and Swann, 1982; Ayad et al.

1991; Stevenson et al. 1994) and sows (Okano et al. 1996). In the mare, the concentration of oxytocin binding-sites as well as the OR gene expression was shown to be lower in the endometrium than in the myometrium (Stull 1986; Stroehle et al. 2016). The OR density in the equine endometrium was observed to be the lowest on day 0 and 8 after ovulation (Sharp et al. 1997) and to be the highest in late dioestrus, between day 14 – 18 (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). Oxytocin receptors in the cow and ewe were mainly detected during oestrus whereas in the mare, ORs could be found in the cervix throughout the oestrous cycle (Stroehle et al. 2016).

Uterine and cervical ORs are influenced by the ovarian steroids oestrogen and progesterone in cattle and sheep (Fuchs et al. 1990; Robinson et al. 2001; Falchi and Scaramuzzi, 2013). Uterine OR concentration in the cow seems to be regulated differently to that in the ewe. In the ewe, OR levels in endometrial and myometrial tissues change simultaneously during the oestrous cycle, which is not the case in cows (Sheldrick and Flint, 1985; Fuchs et al. 1990). Oestrogen in the ewe causes upregulation of the OR in endo- and myometrium (Sheldrick and Flint, 1985; Hixon and Flint, 1987) whereas oestrogen does not appear to show an effect on OR expression in the uterine tissues of cows. Progesterone, on the other hand, is effective in the ewe and cow as it inhibits endo- and myometrial OR expression in both species (Wathes and Hamon, 1993; Robinson et al. 2001). Especially in sheep, this effect of progesterone seems to be only temporary. The inhibiting effect was shown to fade after 12 days of continuous exposure to progesterone (Vallet et al. 1990; Wathes et al. 1996). In the mare, an upregulating effect of oestrogen on ORs can also be suspected as studies have reported that the OR concentration is higher in late dioestrus (after luteolysis has taken place) than in any other stage of the oestrous cycle (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998).

2.8 Endometrial biopsy

Endometrial biopsy is a safe and effective technique for the evaluation of the mare's endometrium. It is possible to describe the histomorphological status of a specific uterus and, together with a detailed breeding history and pertinent clinical data, estimate the likelihood of a mare's ability to become pregnant and carry a foal to term (Kenney and Doig, 1986). Andrews (1941) was the first to describe histological changes in the endometrium throughout the oestrous cycle of mares, using endometrial biopsy specimens.

The junction of uterine horn to body is usually chosen as the preferred biopsy site if no abnormalities can be detected by palpation. This is the implantation site of the embryo and can thus be seen as the ideal location with which to assess endometrial histomorphology as a predictive indicator of a mare's ability to fall pregnant and sustain a pregnancy to term (Snider et al. 2011). The procedure may be performed during any stage of the oestrous cycle but the variability seems to be smaller if endometrial biopsies are obtained during dioestrus due to the absence of potentially confounding oedema (Kenney and Doig, 1986).

The histomorphological grading system for endometrial biopsy scoring that is considered to be the international standard is the modified Kenney-Doig system (Kenney and Doig, 1986). This system has four categories (I, IIA, IIB, III) principally based upon increasing severity of endometrial inflammation and fibrosis (see **Table 1**, summarised by Snider et al. (2011)). Category I contains all essentially normal uteri, whereas category III, includes those with severe, irreversible (chronic) inflammation and, or degenerative changes. Kenney also added expected foaling rates to each category (Kenney 1978; Kenney and Doig, 1986).

Table 1 Modification of the Snider *et al.* (2011) summary of the Kenney-Doig endometrial biopsy categories and prognoses for endometrial biopsies in mares

Category	Major findings[#]	Predicted foaling rate (%)
I	Essentially normal; inflammation or fibrosis mild (1-3 cell layers) and sparsely scattered	80–90
IIA	Mild, scattered inflammation and mild fibrosis (1-3 cell layers); endometrial atrophy in late breeding season	50–80
IIB	Moderate, scattered inflammation or moderate fibrosis (4-10 cell layers, glandular nests)	10–50
III	Moderate, scattered inflammation and moderate fibrosis (4-10 cell layers, glandular nests) or severe, irreversible changes including severe fibrosis (>11 cell layers) and inflammation	10

History of barrenness > 2 years increases the category assigned.

In order to better predict the likelihood of falling pregnant and carrying a foal to term, the following components were considered (see **Appendix 3**):

1. Adequacy of the tissue sample (quantity and quality):

An endometrial biopsy should possess an intact luminal epithelium, lamina propria and a portion of the myometrium. Sample processing artefacts such as cytoplasmatic blebbing of the luminal epithelium should be taken into consideration at the time of evaluation.

2. Assessment of endometrial histomorphology:

Depending on the stage of the oestrus cycle, the luminal and glandular epithelium comprises a single row of cells that vary from low cuboidal (anoestrus, late dioestrus) to tall columnar (oestrus, early dioestrus) in height (Van Camp 1988). A basement membrane separates the epithelium from the lamina propria. The lamina propria can be divided into a stratum compactum and stratum spongiosum. The stratum compactum consists of dense stromal tissue just below the epithelial basement membrane with subepithelial capillaries and the neck portion of the endometrial glands. The stratum spongiosum is the less dense tissue between stratum compactum and myometrium containing stromal cells, blood vessels, nerves, lymphatics and the major glandular component (Van Camp 1988).

(i) Endometrial histology in relation to stage of oestrus cycle:

The luminal and glandular epithelium consists of tall columnar cells during oestrus. The lamina propria is oedematous meaning that the stroma appears less dense and the glands are elongated. Neutrophils can be found within small blood vessels and on the surface of the endometrium (Snider et al. 2011). During dioestrus, the height of the epithelial cells is reduced from tall to low columnar. There is no evidence of oedema and hence the stroma appears more dense and the glands are closer together and more tortuous (Kenney 1978).

3. Characterisation of inflammation:

Kenney (1978) described in some detail the histopathology associated with inflammation and degeneration of the endometrium. Inflammation is characterised in terms of its severity (mild, moderate, severe), its longevity (acute, subacute or chronic), the nature of the exudate (presence of fibrin and, or predominant extravascular leucocyte/s in evidence e.g. neutrophils, lymphocytes, plasma cells, macrophages,

eosinophils, etc) and, finally, the distribution pattern of the inflammation (focal, multifocal, diffuse, perivascular, interstitial, etc) (Snider et al. 2011). Acute inflammation is generally characterised by a predominance of neutrophils (with or without fibrin exudation) whereas lymphocytes, plasma cells, eosinophils and macrophages, often together with fibrosis/scarring are more indicative of chronic inflammation (Van Camp 1988).

5. Assessment of the extent of endometrial degeneration including fibrosis, lymphatic lacunae and cystic endometrial glands

Endometrial fibrosis is graded as mild, moderate or severe if it is 1 - 3 cell layers, 4 - 10 cell layers or more than 11 cell layers thick around the endometrial glands, respectively. Periglandular fibrosis with subsequent cystic glandular dilatation and "nesting" of endometrial glands are the hallmark features of equine endometrial degeneration or endometrosis (Van Camp 1988).

2.9 Laboratory methodologies

2.9.1 Polymerase chain reaction (PCR)

Polymerase chain reaction was invented by Kary Mullis in 1983 at the Cetus Corporation. It is a simple process to multiply a targeted fragment of deoxyribonucleic acid (DNA) (Watson 2003). Polymerase chain reaction is an *in vitro* DNA amplification technique that involves a repeated cycling process of denaturation, annealing, and extension. The reagents required for the PCR include DNA polymerase, each of four nucleotides (dNTPs), a primer couple, magnesium source, buffer solution and DNA template (Rapley 1998).

When DNA is heated in excess of 94 – 95 ºC, the double strands come apart to produce two single strands (Rapley 1998, Watson

2003). This process is called denaturation. When the temperature is dropped (usually 50 – 60 °C), primers whose sequences are complementary to the DNA, bind to the DNA template (annealing). In the next stage, known as extension, the temperature is raised to approximately 72 °C, allowing the polymerase to make a complimentary copy of the template DNA starting from each primer, to create a double strand (ds) of the target region. In the next cycle, the double strand deoxyribonucleic acid (dsDNA) produced from the previous cycle becomes new template to produce a double new dsDNA.

Over the years, PCR methods were improved and real-time PCR (qPCR) was developed. Real-time PCR enabled the monitoring of the target DNA amplification progress in real time. The collection of the data takes place during the amplification process. Real-time PCR can detect and quantify DNA in samples to show the presence or abundance of a specific DNA sequence. It can also be applied to ribonucleic acid (RNA) quantification. The template used here is the so called complementary DNA (cDNA), which is obtained by reverse transcription of RNA. The technique used herefor is quantitative reverse-transcription PCR (RT-qPCR).

Real-time polymerase chain reaction has been used in previous studies to examine ORs in the uterine tissues of several species (Bathgate et al. 1995; Feng et al. 2000; Beretsos et al. 2006; Siemieniuch et al. 2011) including the endometrium of mares (Villani et al. 2008). In a recent comparative study, the expression of OR gene in endo-, myometrium and the cervix of mares was investigated (Stroehle et al. 2016). It was shown that relative to the expression of OR gene in the endometrium, the expression of the OR gene was four times higher in the myometrium but only one third in the cervix (Stroehle et al. 2016). No reports, however, are available investigating the influence of cycle stage, age and biopsy score on OR gene expression.

2.9.2 Immunohistochemistry (IHC)

Immunohistochemistry is a wide-used technique that combines biochemistry, anatomy, immunology and physiology. It is a method that visualises distribution and localisation of specific antigen or epitopes in separated tissues sections (Ramos-Vera 2011). Different detection systems (indirect and direct methods) are available with indirect methods being more sensitive than direct ones. One of the indirect methods is the avidin-biotin-peroxidase complex (ABC) method. Avidin is a large glycoprotein with four binding sites per molecule and high affinity for the vitamin biotin (Ramos-Vara 2005).

As the IHC method has developed, its use in diagnostic pathology has expanded to a routine procedure in surgical pathology, especially with respect to tumor diagnosis and classification. Immunohistochemistry has also been adapted to the identification and demonstration of both prognostic and predictive markers. The widespread use of IHC and the increasing demand for comparison of qualitative and semi-quantitative findings among laboratories have resulted in a growing focus on method reproducibility and have led to a new emphasis upon standardisation (Taylor et al. 2006).

Immunohistochemistry has been used in a variety of animal species including the horse to evaluate the OR (Fuchs et al. 1990; Fuchs et al. 1996a; Fuchs et al. 1996b; Morel et al. 2001; Beretsos et al. 2006; Villani et al. 2008; Siemieniuch et al. 2011). Budik et al. (2012) described ORs in the equine conceptuses between days 10 - 16 of pregnancy and Palm et al. (2013) identified ORs in the cytoplasm of endometrial luminal and deeper glandular epithelium in the endometrium of pony mares at parturition. Recently, a comparative study (Stroehle et al. 2016) investigated OR distribution in the endometrium, myometrium and cervix of mares. Oxytocin receptors were found in the cytoplasm of vascular endothelial cells

(throughout all layers of the uterine wall) especially associated with arterioles and capillaries, luminal and glandular epithelia, sub-epithelial and peri-glandular stromal cells of the endometrium and throughout the smooth muscle and stromal cells of the myometrium. The greatest intensity of labeling occurred consistently in the endothelium of blood vessels throughout the wall of the uterine samples. There was a similar pattern of distribution and intensity of staining of ORs in the uterus and cervix, with the exception of the glandular epithelium, which is absent in the cervix (Stroehle et al. 2016). However, no reports are available on the influence of cycle stage, age, biopsy score on the OR distribution in the uterine and cervical tissues.

CHAPTER 3: Materials and methods

3.1 Study population

Samples for this study were collected from twenty-seven mares of different breeds consigned for routine slaughter and grouped based both on their age category (2.5 – 9 years and ≥ 10 years) estimated via oral inspection of their dentition (Martin et al. 1999) and their cycle stage (luteal phase and non-luteal phase). Mares were routinely slaughtered using the stunning method according to international accepted standards at the Randfontein Abattoir (Plot 114 Middelvlei, Randfontein, 1764).

The Study was approved by the Animal Ethics Committee of the University of Pretoria (Study V005-12).

3.2 Collection and preparation of tissue samples

The whole reproductive tract of the mares (uterus, cervix, ovaries and associated broad ligament) was obtained at the slaughter processing line and taken for complete gross anatomical inspection of the internal reproductive organs as well as the collection of full-thickness tissue samples from the uterus and cervix. All samples were collected during the course of the physiological breeding season in the Southern hemisphere (October to April) to ensure cyclic status of the mares.

The reproductive tracts of the slaughtered mares were identified by plastic tags (cow ear tag marked with barcodes and RS identification numbers) that were attached to the broad ligament close to the tip of the right uterine horn at the time of sample retrieval at the abattoir to ensure correct identification.

The collection of all reproductive tracts took place within one hour of slaughter. The samples were placed on ice in a

styrofoam box and transported to the Faculty of Veterinary Science, University of Pretoria, Onderstepoort (FVS-UP) for further processing.

Entire uteri with their associated cervix, ovaries and oviducts were grossly inspected and macroscopically abnormal uteri (e.g. fluid-filled, adhesions, neoplasia) were excluded from the trial.

The size of both ovaries was determined with the help of a ruler and they were dissected using a scalpel blade (No 21). The investigation of ovarian size and ovarian structures were used as an additional method to confirm cyclic activity. The criteria of a minimum of 2 x 3 x 5 cm dimension of the ovary and, or follicles of > 2 cm diameter and, or the presence of corpora lutea (CLs) or corpora haemorrhagica (CHs) was used to define ovaries as active ovaries and those mares were considered to be actively cyclic (Daels et al. 1991; Evans et al. 1997; Stroehle et al. 2016).

Full-thickness tissue samples of a 20 x 20 x 5 mm were obtained using a scalpel blade (No 21) and surgical forceps from four standardised areas of the uterus. Samples were collected from the right and left base of the uterine horns, the uterine body and the cervix. These samples were used for endometrial biopsy scoring, polymerase chain reaction (PCR) and immunohistochemistry (IHC).

3.3. Collection and preparation of blood samples

Blood was collected from every mare during exsanguination at slaughter into 5 ml heparinised vacuum tubes (5 ml; Vacutainer). Blood samples were placed in a styrofoam box on ice and transported to the FVS-UP for further processing. At FVS-UP samples were immediately centrifuged at 3000 revolutions per minute (RPM) for 15 minutes at 25 °C and the serum was stored at -18 °C in barcode-labeled 5 ml plastic

sample tubes until analysis. As described in a previous study, samples were analysed at the Endocrine Laboratory, FVS-UP by radioimmunoassay (RIA) for plasma progesterone concentration (PPC; Coat-a-count RIA kit, Code No. TKPG1 - 1593 Diagnostic Product Corporation, Los Angeles, USA: Stroehle et al. 2016). Mares with PPC $\geq$ 3.18 nmol/l were defined as having active luteal tissue (Garcia 1990; Galvao et al. 2010; Szostek et al. 2014).

3.4 Endometrial biopsy

Endometrial biopsies were fixed in 10 % neutral buffered formalin for at least 24 hours, sectioned at a thickness of 4 - 6 µm by the laboratory staff, Section of Pathology, FVS-UP according to accredited standard operating procedures (SOPs) in the Histopathology Laboratory. Sections were routinely dewaxed and stained with haematoxylin and eosin (HE) according to standard techniques (Bancroft & Gamble 2002). Evaluation and grading of endometrial biopsies was performed by the primary investigator, together with an experienced veterinary pathologist, using an Olympus BX43 microscope. Slides were graded I, IIa, IIb, or III according to the Kenney and Doig classification system using a specifically designed evaluation sheet (Kenney and Doig, 1986; **Appendix 2**).

3.5 Immunohistochemistry

The IHC method used has been described in detail in a previous study done by Stroehle et al. (2016). Briefly, full-thickness samples taken from four separate regions of each uterus (including one from each horn, one from the uterine body and one sample of cervix) were fixed overnight in 10 % neutral buffered formalin, paraffin-embedded and sectioned at 4 µm according to accredited SOPs in the Histopathology Laboratory, Anatomical Pathology Section, FVS-UP.

We employed a polyclonal rabbit anti-oxytocin receptor (OR) primary antibody directed against the extracellular N-terminal component of the human OR (Code No. O4389, Sigma-Aldrich, Steinheim, Germany). Optimisation of the same anti-OR antibody has been previously reported in horse tissues (**Appendix 3**; Budik et al. 2012; Palm et al. 2013; Stroehle et al. 2016). The secondary antibody was a polyclonal goat anti-rabbit (GXR) IgG antibody (Code No. E0432, Dako, Denmark).

Prior to antigen retrieval and in order to block endogenous peroxidase activity, sections were incubated for 15 minutes with 3 % hydrogen peroxide. Thereafter, enzyme-induced epitope retrieval was performed on the tissue sections (Protease Type XIV in phosphate buffered saline/PBS-bovine serum albumin/BSA buffer, pH 7.6) in an oven at 38 - 40°C for 30 minutes. For the purpose of blocking non-specific protein binding, the sections were incubated with a 1:10 dilution of normal goat serum (Code No. G9023, Sigma-Aldrich, St Louis, Missouri, USA) with PBS-BSA buffer, whereafter sections were incubated overnight with the polyclonal rabbit anti-OR antibody (dilution 1:400). The biotinylated secondary GXR antibody (dilution1:500) was incubated with the sections for 30 minutes. Apart from the enzyme-induced epitope retrieval step, all reagent incubations were done at room temperature. An avidin-biotin complex immunoperoxidase-diaminobenzidine (DAB) detection system (Code No. K3468, Dako, Denmark) was used for development of colour (brown) in the tissue sections. In order to investigate potential primary antibody cross-reactivity and non-specific binding by the secondary antibody and ABC immunodetection system, an irrelevant polyclonal rabbit anti-S100 antibody (Code No. Z 0311, Dako, Denmark), at a dilution of 1:400 and with overnight incubation, was applied to additional designated uterine tissue sections per staining batch (Ramos-Vara et al. 2008; Stroehle et al. 2016).

The tissue sections were routinely mounted and coverslipped for light microscopic examination. Slide evaluation was performed by the primary investigator and an IHC-experienced veterinary pathologist, using an Olympus BX43 microscope and the cell Sens dimension software also from Olympus (Wirsam Scientific & Precision Equipment PTY LD, Johannesburg, South Africa). Oxytocin receptor-specific positive labeling consisted of brown cytoplasmic granular to diffuse staining in transmural vascular endothelium as well as uterine surface and superficial glandular epithelium (Palm et al. 2013, Stroehle et al. 2016).

Uterus from a mid to late dioestrus mare was used as the positive-tissue control due to the expectation of increased OR expression during this phase of the oestrus cycle (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). For negative tissue control purposes, the uterus of a 6 month-old foal was used due to the expectation that an immature uterus would express low levels of OR. On the other hand, according to Gimpl and Fahrenholz (2001), OR expression has been reported in a wide variety of different organs and tissues, namely heart, thymus brain, kidney, pituitary, testis, ovary, vascular endothelium, myoblasts, osteoclasts, adipocytes, pancreatic islet cells, and several types of cancer cells, making it difficult to select a true negative tissue control.

3.6 Quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

The RT-qPCR method used has been described into detail in a recent study done by Stroehle et al. (2016).

3.6.1 Sample collection

Samples were obtained from the left uterine horn and the cervix and used for quantitative analysis of the OR gene by RT-qPCR

assay. Cervical, endometrial- and myometrial samples were collected as described (see 3.2), cut into small blocks (0.5 cm^3) and placed in 1.5 ml Eppendorf containers (Eppendorf HQ, Hamburg, Germany) containing 5-10 µl of RNAlater (Lifetech, Carlsbad, USA) to preserve the ribonucleic acid (RNA). Samples were kept at 4 °C for 24 h and then stored at -20 °C until analysed at the Molecular Laboratory of the Department of Veterinary Tropical Diseases, FVS-UP.

3.6.2 Ribonucleic acid (RNA) extraction

The RNeasy® Plus Mini kit (Qiagen, Valencia, CA, USA) was used for the RNA extraction. Approximately 30 mg of previously thawed tissue material was transferred to RNase-free ceramic bead tubes containing 600 µl of Buffer RLT Plus from the RNeasy® Plus Mini kit andhomogenised in a bead-beater (Precellys 24, Bertin technologies, Paris, France) at 6500 rpm for 2 x 20 seconds with a 15 seconds break. The lysate was transferred into extraction columns and RNA extraction was performed according to the RNeasy® Plus Mini kit manufacturer's instructions. To minimise adverse effects of protein contamination, only the RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used for the analysis.

3.6.3 Quantitative reverse-transcription polymerase chain reaction

Oligonucleotide primers and probe sequences used for detection of OR and β-actin gene expression were obtained from three previous studies evaluating OR gene expression and distribution in the equine conceptus, in fetal membranes, in endometrium from pony mares at parturition and in endometrium, myometrium and cervix of non-pregnant mares (**Table 2**) (Budik et al. 2012; Palm et al. 2013, Stroehle et al.

2016). All primers and TaqMan probes used were synthesised by Life Technologies™ (Carlsbad, CA, USA). The RT-qPCR reaction mixture for a 25 µl reaction were prepared from the SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, United States of America). The RT-qPCR mixture contained 0.5 µl SuperScript III RT/Platinum®*Taq* Mix, 12.5 µl 2X Reaction Mix with ROX, 0.5 µl Forward primer (10 µM), 0.5 µl Reverse primer (10 µM), 0.25 µl of TaqMan probe (10 µM), 6.75 µl distilled water and 4 µl of 1 ng/µl RNA template. The RT-qPCR was performed following the instructions of Stroehle et al. (2016) and is shown in **Table 3.** In each RT-qPCR run the following control reactions were included: RNA extraction control, water control, no template control and RT-qPCR reagent control. The RT-qPCR experiments were performed employing ABI StepOnePlus™ system and software (Applied Biosystems, USA).

Table 2 Oligonucleotide primer and probe sequences for amplification of equine oxytocin receptor (OR) and endogenous control genes (Stroehle et al. 2016)

Primer/ probe	Sequence 5'–3'	Primer length (bp)	References
OR			Budik et al. 2012; Palm et al. 2013
Forward	TGCAACCCCTGGATCTACATG	21	
Reverse	GGAAGCGCTGCACGAGTT	18	
Probe	FAM-TGTTCACGGGCCACCTCTTCCAC-TAMRA	22	
ß-Actin			Budik et al. 2012; Palm et al. 2013
Forward	CCGGGACCTGACGGACTA	18	
Reverse	CCTTGATGTCACGCACGATT	20	
Probe	FAM-TACAGCTTCACCACCACGGCCG-TAMRA	22	

Table 3 Reverse-transcription PCR (RT-PCR) program and conditions used for amplification of oxytocin receptor (OR) and β-actin gene fragments (Stroehle et al. 2016)

RT-qPCR program		
Program name	**Cycle(s)**	**Analytical mode**
cDNA synthesis	1	None
Pre-incubation	1	None
Amplification	40	Quantitative
RT-qPCR conditions		
Program	**Temp (°C)**	**Hold time**
cDNA synthesis	50	15 minutes
Pre-incubation	95	2 minutes
Amplification (primer	95 60	15 seconds 30 seconds

3.6.4 Validation of the quantitative reverse-transcription polymerase chain reaction assay amplification efficiency

High PCR efficiency is usually correlated with high precision of RT-qPCR assays and it is particularly important for the evaluation of messenger-ribonucleic acid (mRNA) concentrations of target genes relative to those of endogenous control genes. An endogenous control gene is used to normalise the input amount of RNA added to the reactions. It is required that the assays of the target and endogenous control genes (OR

and β-actin, respectively) have similar amplification efficiency (90 - 110 %). The ß-actin gene was used as an endogenous control as in previous studies the expression of this gene has been shown to be the most stable of the housekeeping genes in equine tissues (Jischa et al. 2008; Palm et al. 2008; Willmann et al. 2011). This study relied on the validation experiment done by Stroehle et al. (2016) which demonstrated that the amplification efficiency for the ß-actin assay was 94,5 % (slope = -3,459; R^2 = 0,98; amplification = 1,95) and 93 % (slope = -3,501; R^2 = 0,97; amplification 1,93) for the OR assay respectively, as required.

3.6.5 Quantitative analysis of the oxytocin receptor gene expression

The quantitative analysis was based on a recent study by Stroehle et al. (2016) where the expression of the OR gene was determined in three tissue types (endometrium, myometrium and cervix). The β-actin assay was included in every RT-qPCR run for each tissue type; for normalisation of OR RT-qPCR data a single sample was run in triplicate. To standardise the results, the RT-qPCR assays for ß-actin and OR were performed using the same RNA concentration (1ng/µl) for all the samples investigated. For $\Delta\Delta C_T$ analysis, the ΔC_T values for each tissue type, cycle stage, age group and bopsy score were calculated from average cycle threshold (C_T) values of specific samples. The resulting ΔC_T values were used to calculate $\Delta\Delta C_T$ value determined from $\Delta C_{T\ (control)} - \Delta C_{T\ (target)}$. For this study, the luteal phase, young mares and biopsy score I were selected as controls and the fold difference in target (i.e. non-luteal phase, old mares and biopsy scores IIA and IIB) relative to the control was determined using the $2^{-\Delta\Delta Ct}$ method. The fold difference was used as a descriptive method to quantify different gene expressions between tissues.

3.7 Statistical analysis

For the statistical analysis the ΔC_T values were used and not the raw C_T values (Livak and Schmittgen 2001, Stroehle et al. 2016). The ΔC_T data were assessed for normality by calculating descriptive statistics, plotting histograms, and performing the Anderson-Darling test for normality using commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). A linear mixed model approach was used to estimate the effect of sample type (endometrium, myometrium and cervix), cycle stage (luteal and non-luteal phase), age and biopsy score on the RT-qPCR results. All models included a random effect term for horse to account for the repeated measurements. Bonferroni correction was used to adjust P values for multiple post-hoc comparisons. Statistical analyses were performed in commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and results were interpreted at the 5 % level of significance.

Biopsy scores summed for each mare were compared between old and young mares and luteal and non-luteal mares based on a Mann-Whitney U test.

CHAPTER 4: Results

4.1 Age estimation

Ten mares were found to be ≥ 10 years old (range 10 - 20) and seventeen mares were found to be < 10 years old (range 2.5 – 9).

4.2 Evaluation of cyclicity using macroscopic evaluation of ovaries and plasma progesterone concentration (PPC) values

The twenty-seven mares showed evidence of cyclic activity as confirmed by combination of macroscopic evaluation of ovaries to establish functional structures, size of ovaries and blood sample analysis for PPC. Fourteen (51.8 %; six old and eight young) of the mares were in the luteal phase and thirteen in non-luteal phase (48.2 %; three old and ten young) (**Appendix 1, Table 10**).

4.3 Endometrial biopsy

Endometrial biopsy sections were graded I, IIa, IIb, or III according to the Kenney and Doig classification system. Essentially, mares were placed into different biopsy score categories according to the extent of inflammation and endometrial glandular degeneration with fibrosis that was exhibited in the endometrial biopsy specimens (**Table 1**). Thus, in addition to the assessment of adequacy of the biopsy specimen, stage of cycle, the type, severity and duration of inflammation was characterised as lymphoid (**Figure 1 and 2**) or purulent (**Figure 3**). Furthermore, the extent of endometrial degeneration pertaining to fibrosis with associated onion skin-like layering of peri-glandular connective tissue (**Figure 4**) leading to glandular nesting (**Figure 5 and 6**), as well as

distended lymphatics (**Figure 7**) and cystically distended glands (**Figure 8**) was evaluated.

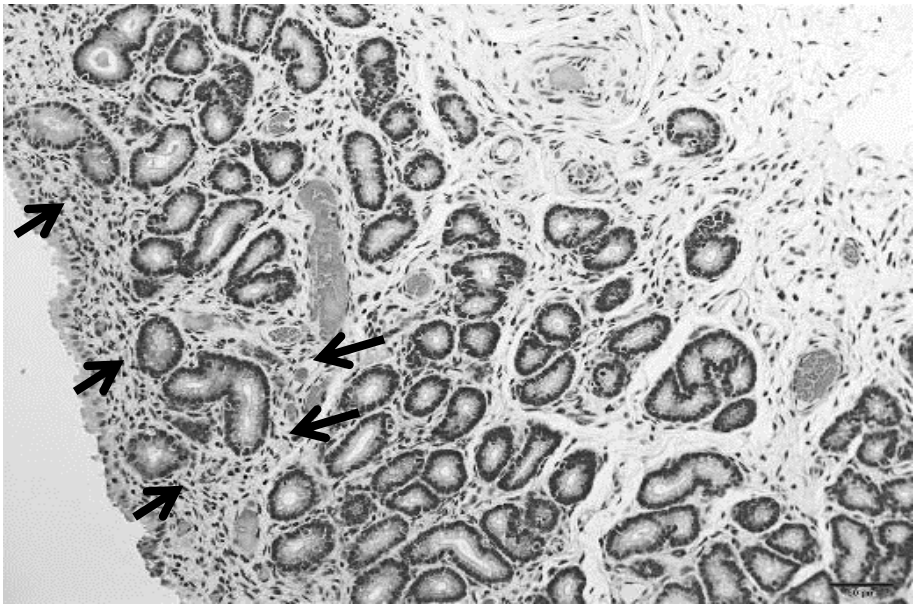


Figure 1 Endometrial biopsy showing inflammation (predominantly lymphoid cells) in the interstitium of both the stratum compactum and spongiosum (arrows; 50 μm).

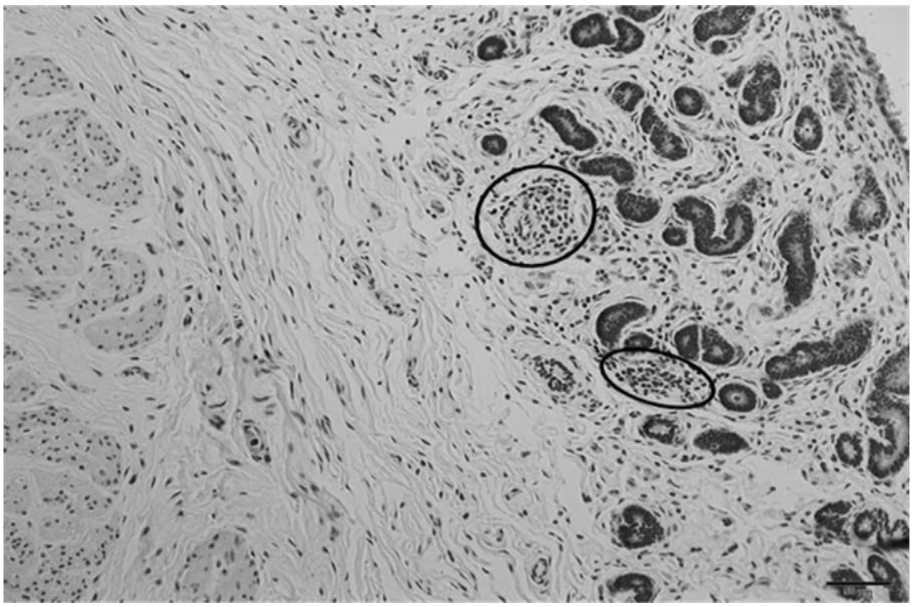


Figure 2 Endometrial biopsy showing small perivascular and periglandular lymphoid nodules in the stratum spongiosum (solid circles; 50 µm).

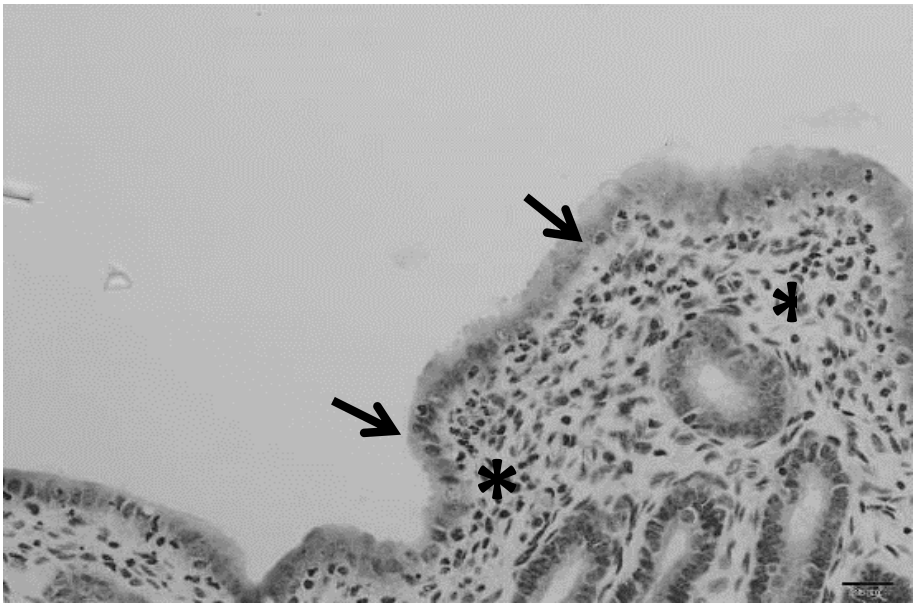


Figure 3 Endometrial biopsy exhibiting mild to moderate purulent inflammation in the stratum compactum (*) with exocytosis of neutrophils across the luminal epithelium (arrows; 50 µm).

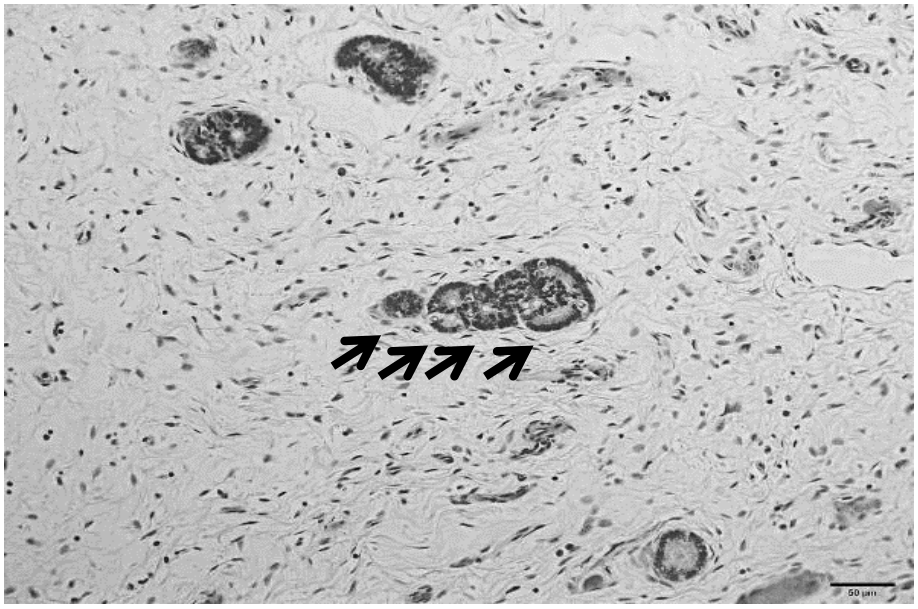


Figure 4 Endometrial biopsy showing onion skin-like layering of peri-glandular connective tissue with consequent formation of glandular "nests" within the deep stratum spongiosum (50 µm); 3-5 layers (arrows).

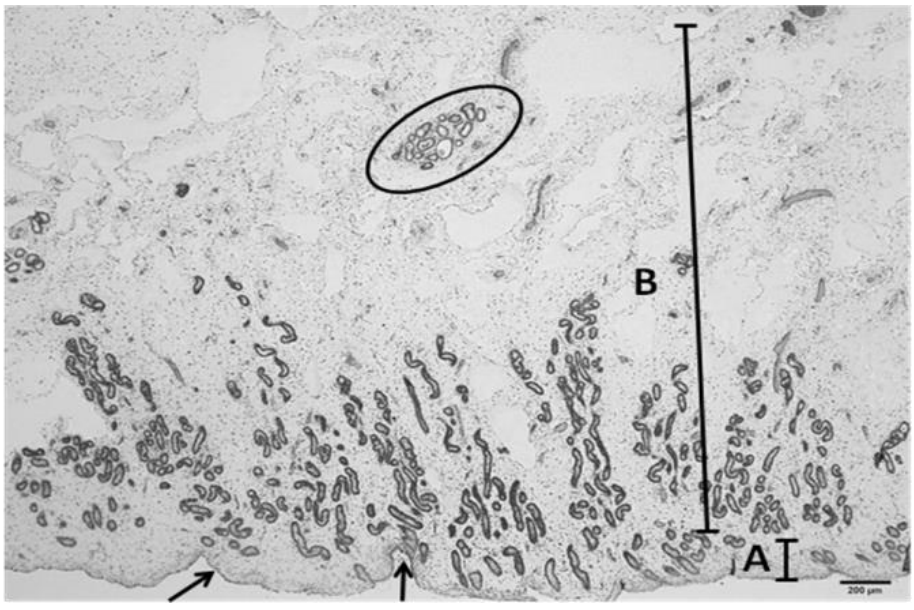


Figure 5 Equine endometrial biopsy assigned a Kenney-Doig grade of IIB (200 µm); overview of luminal epithelium (arrows), stratum compactum (A) and stratum spongiosum with endometrial glands (B) including an endometrial glandular nest (solid circle).

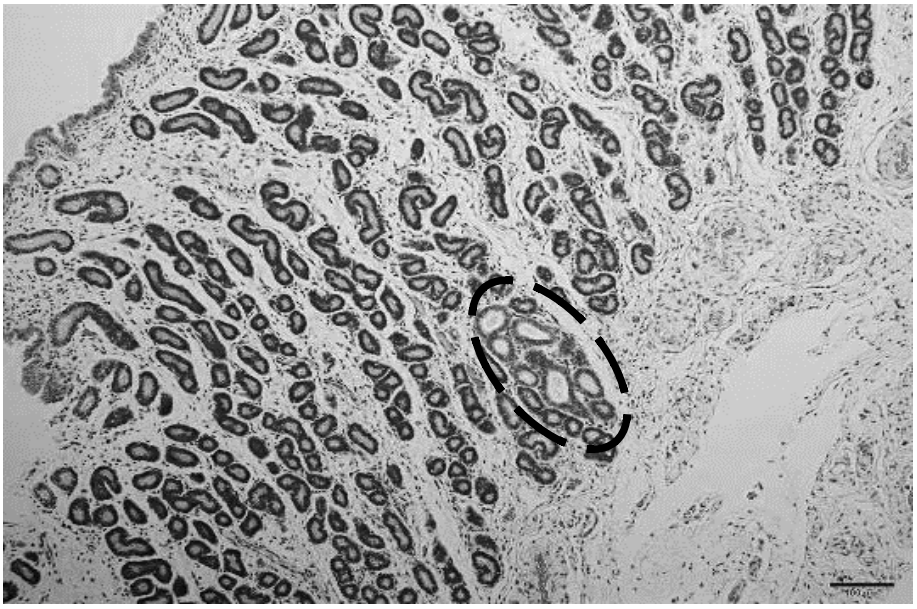


Figure 6 Endometrial biopsy exhibiting a small cluster of slightly distended glands in the stratum spongiosum (dotted circle; 100 µm).

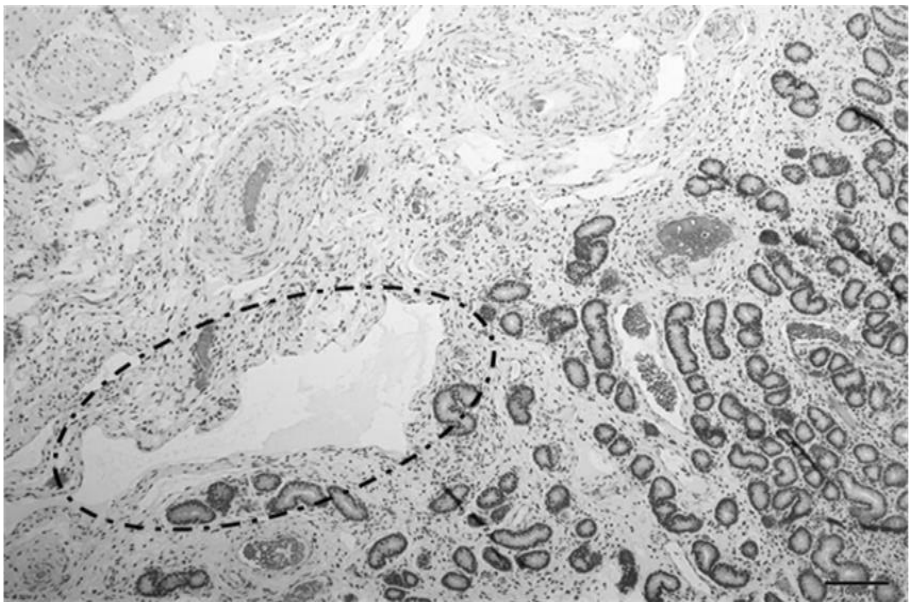


Figure 7 Endometrial biopsy exhibiting distended lymphatic vessels (dotted circle) within the stratum spongiosum **(**100 µm).

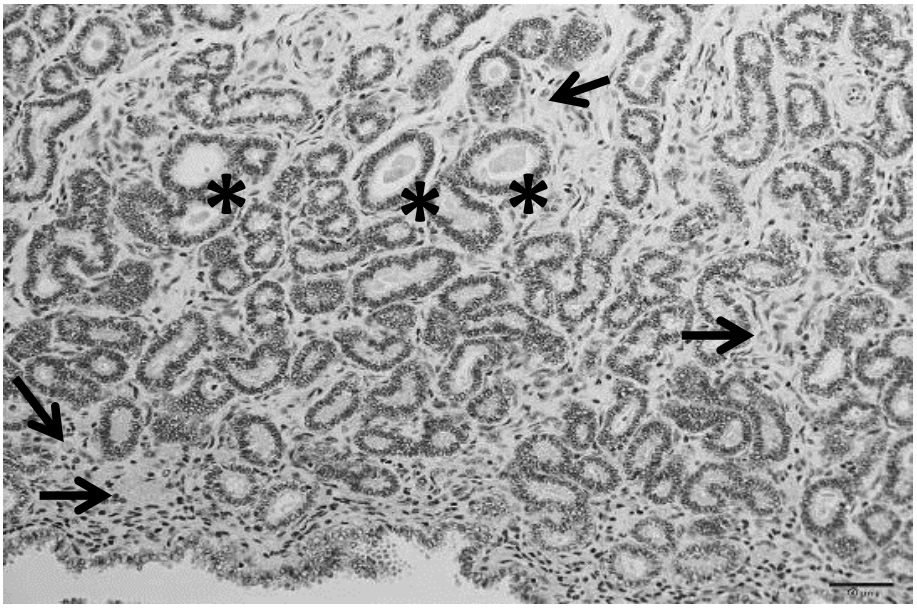
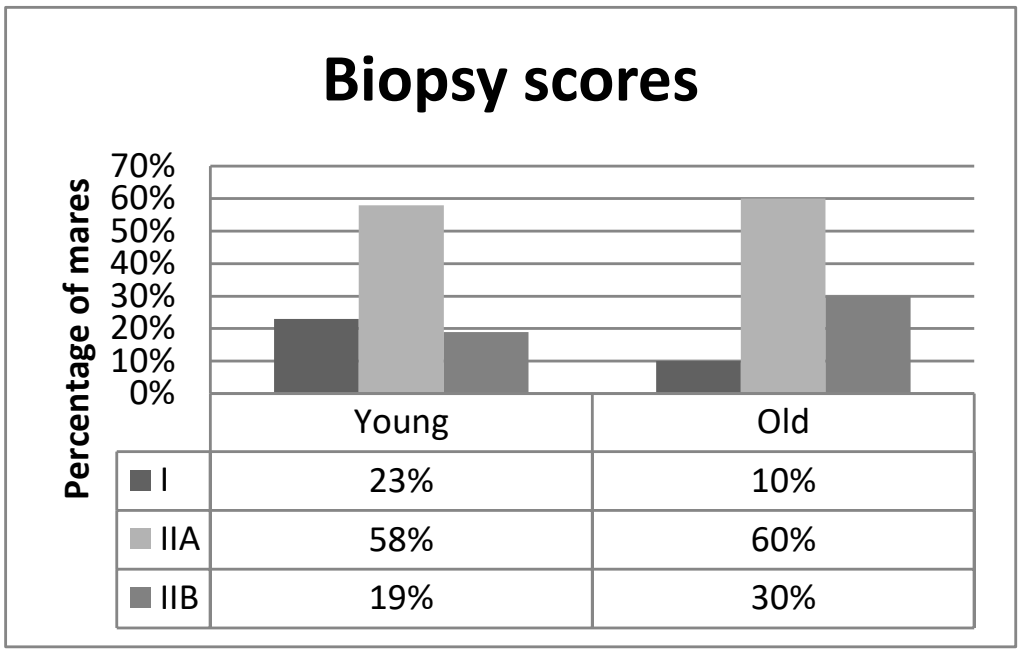


Figure 8 Endometrial biopsy showing mild endometrial glandular distention with intraluminal proteinaceous secretion within the stratum spongiosum (*; 50 μm).

Essentially normal endometrial biopsies were graded I (**Appendix 4**), endometrial biopsies showing mild inflammation and mild fibrosis were graded IIA (**Appendix 5**), endometrial biopsies showing moderate inflammation or moderate fibrosis were graded IIB (**Appendix 6**) and endometrial biopsies with moderate inflammation and moderate fibrosis or severe changes were graded III (Table 1). Five (18.52 %, one old mare and four young mares) of the mare group scored grade I on their endometrial biopsies, sixteen (59.26 %, six old mares and ten young mares) scored grade IIA and six (22.22 %) of the mares scored grade IIB. None of the mares in the study population scored grade III on their biopsies.

There was no significant difference in biopsy score between old and young mares (P=0.386). Mares in the non-luteal phase had significantly lower biopsy scores (I = highest score, IIB = lowest score) compared to horses during luteal phase (P = 0.003).



Young = 2.5 – 9 years, 17 mares; Old ≥ 10 years, 10 mares

Figure 9 Distribution of biopsy scores in old and young mares

All mares with biopsy score I were in the luteal phase and all mares with biopsy score IIB were in the non-luteal phase of their oestrus cycles.

4.4 Immunohistochemistry (IHC)

4.4.1 Analysis of immunohistochemical labeling

The initial intention to quantify oxytocin receptor (OR) - specific positive labeling in the uterine sections was soon abandoned due to the observation that there was fairly consistent positive labeling of the same cells and tissues across all uterine samples. The labeling intensity did vary, but due to the apparent variation between batches of slides stained on different days (manual staining versus autostainer) and the difference in labeling between different batches of the

polyclonal rabbit anti-human OR antibody, it was considered that quantification of labeling intensity would not be accurate and not add value to the study.

4.4.2 Negative reagent controls

The S100 polyclonal rabbit antibody labels a S100 protein isoform targeting mainly cells of neuroectodermal origin including neurons, melanocytes, adipocytes, myoepithelial cells, fibroblasts, etc. After applying the S100 antibody to our tissue sections S100-specific positive labeling of peripheral nerves within the uterine sections could be shown, as expected. There was no evidence of non-specific labeling by the S100 antibody in the sections of uterus and cervix.

4.4.3 Comparison of oxytocin receptor distribution between mares in different cycle stages, age groups and with different biopsy scores

By means of immunolabeling, ORs could be identified as fine brown granules in the cytoplasm of selected cells in both negative- and positive-tissue control tissues and in the test sections. Detailed description of OR distribution in uterine and cervical tissue was described in a recent study done by Stroehle et al. (2016).

In all endometrium and myometrium sections chosen, the immunolocalisation of ORs was demonstrated in the superficial luminal epithelium, glandular epithelium, smooth muscle fibres and vascular endothelium with the strongest staining intensity consistently found in the endothelium of the blood vessels. Immunolocalisation of the OR in the equine cervix was also found to be in the superficial luminal epithelium, the smooth muscle fibres and the vascular endothelium.

There was consistent labeling of superficial luminal epithelium and vascular endothelium in endometrial and myometrial

samples of mares during luteal (**Figure 10**) and non-luteal (**Figure 11**) phase of the oestrous cycle with additional pale glandular staining during non-luteal phase (**Figure 11**). The same consistency was found for the OR labeling of luminal epithelium and vascular endothelium in the cervix comparing the two different phases (**Figures 12 and 13**). No difference in OR distribution nor intensity of staining could be detected between mares of different cycle stages.

The comparison of immunohistochemically labeled uterine and cervical samples of old and young mares did not show any difference in OR distribution nor intensity. Uniform positive staining of luminal epithelium and vascular endothelium was demonstrated in endometrial (**Figures 14 and 15**) and cervical (**Figures 18 and 19**) sections of old (**Figures 14, 16 and 18**) and young (**Figures 15, 17 and 19**) mares as well as in myometrial sections where consistent OR labeling was found in smooth muscle fiber and vascular endothelia (**Figures 16 and 17**).

Endometrial sections of mares with different biopsy scores revealed the same immunohistochemical labeling distribution and intensity in all three groups (I, IIA and IIB).

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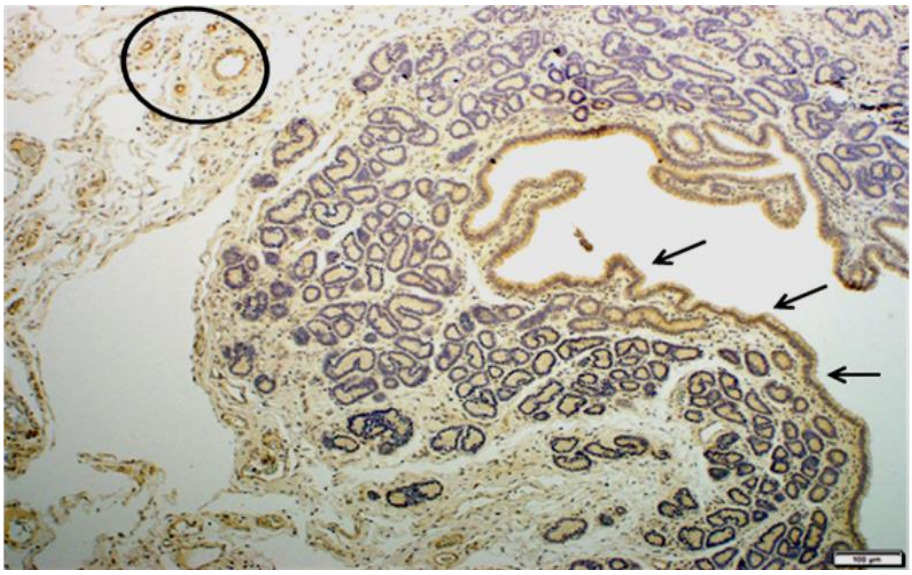

Figure 10 Immunolocalisation of ORs in horse endometrium during luteal phase (RS 26; 100 µm) endometrium with positive staining of the superficial luminal epithelium (arrows) and vascular endothelium (solid circle). Immunohistology (IH) with 3,3'-diaminobenzidine (DAB) substrate-chromogen and counterstained with Mayer's hematoxylin.

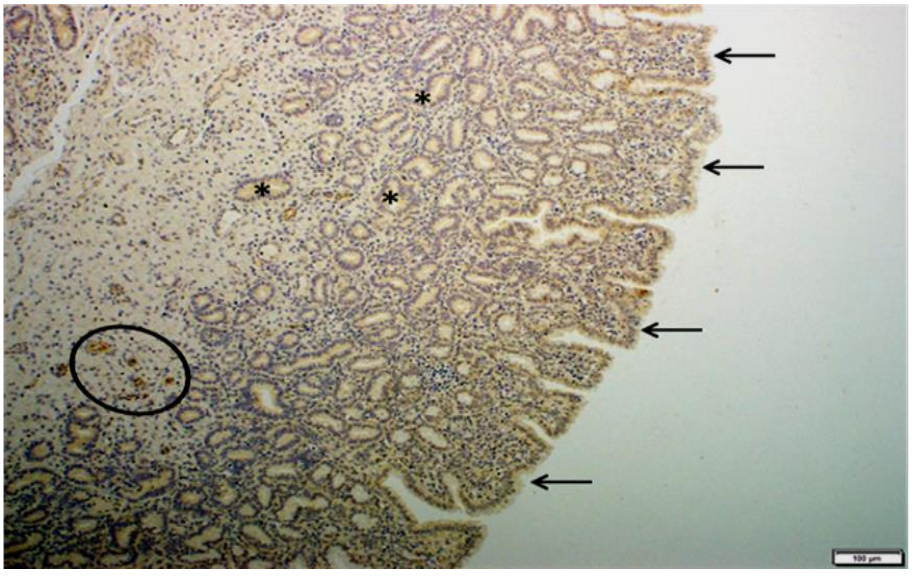

Figure 11 Immunolocalisation of ORs in horse endometrium during non-luteal phase (RS 50; 100 µm) with staining of the superficial luminal (arrows) and glandular (*) epithelium and blood vessel endothelium (solid circle). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

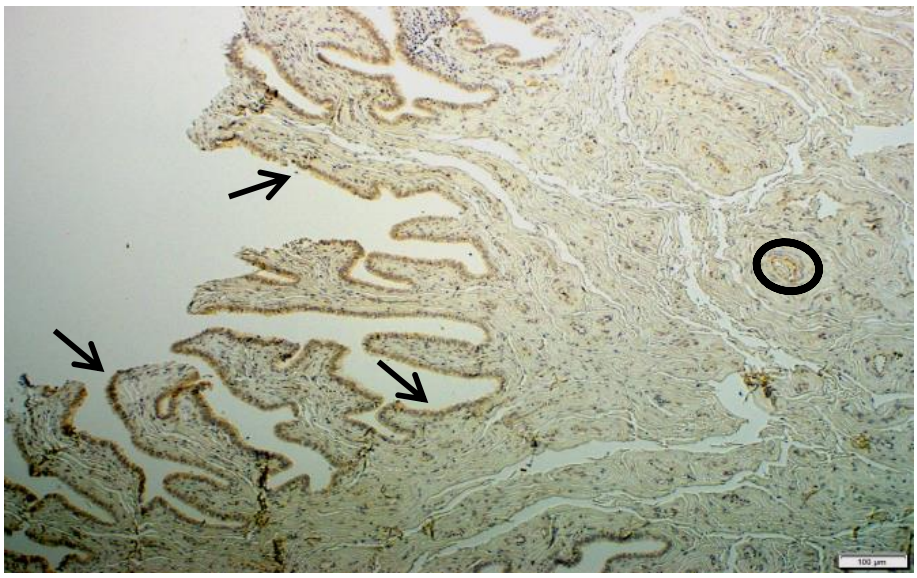

Figure 12 Immunolocalisation of ORs in the horse cervix during luteal phase (RS 26; 100 µm) with labeling of the apical cytoplasmic membrane of the luminal epithelium (arrows) and labeling of vascular endothelium (solid circle). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

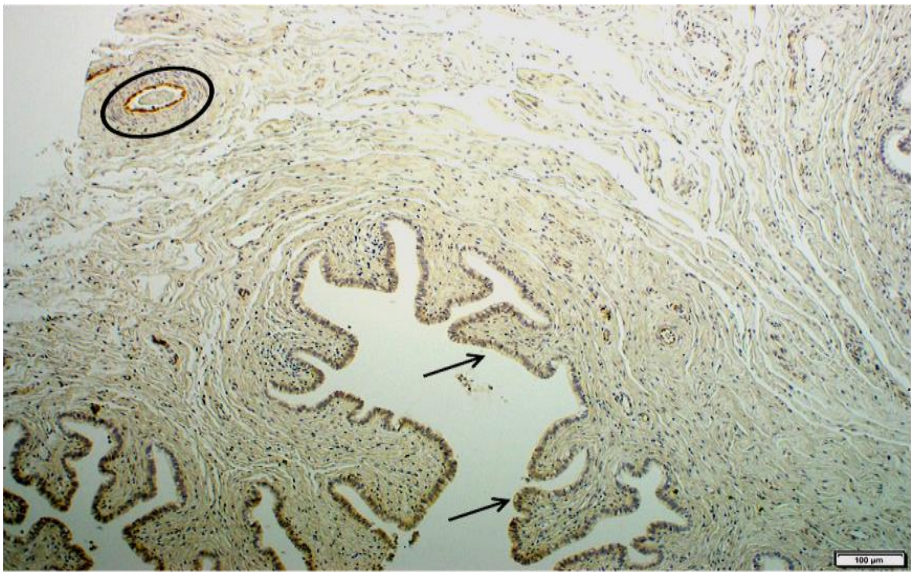

Figure 13 Immunolocalisation of ORs in the horse cervix during non-luteal phase (RS 50; 100 µm) with labeling of the apical cytoplasmic membrane of luminal epithelium (arrows) and labeling of vascular endothelium (solid circle). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

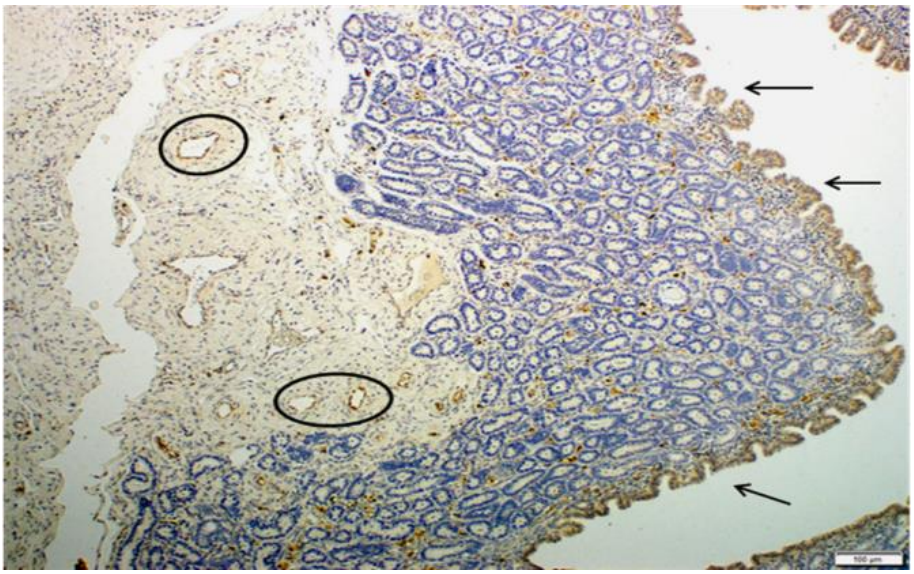

Figure 14 Immunolocalisation of ORs in the endometrium of an old horse during non-luteal phase (RS 57; 100 µm): There is intense positive staining of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles) IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

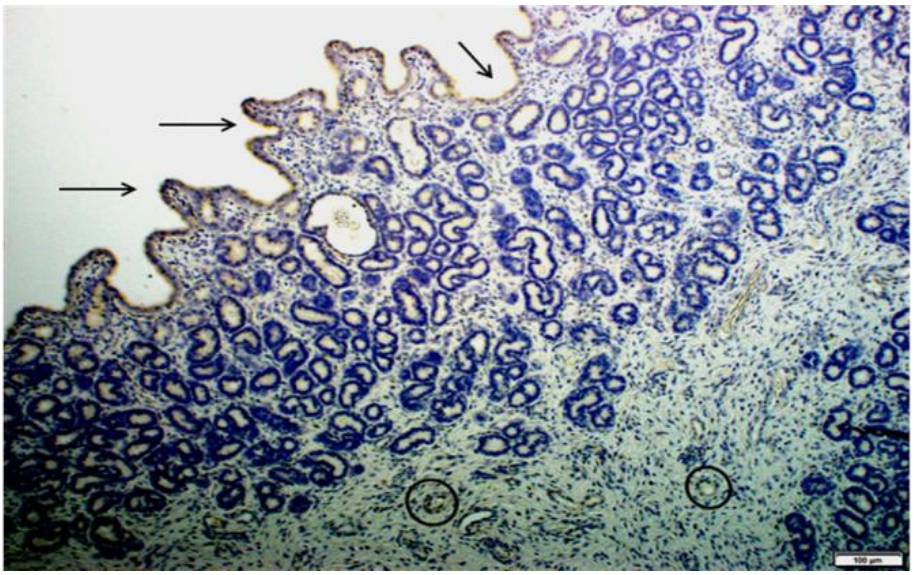

Figure 15 Immunolocalisation of ORs in the endometrium of a young horse during non-luteal phase (RS 42; 100 µm): Positive labeling of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

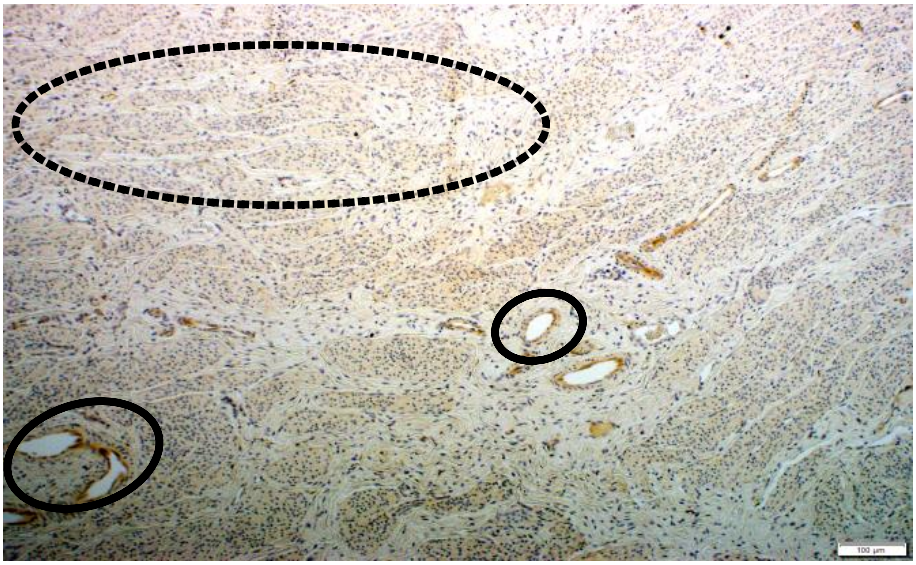

Figure 16 Immunolocalisation of ORs in the myometrium of an old horse in non-luteal phase (RS 57; 100 µm with intense positive staining of the vascular endothelium (solid circles) between the myofibres and paler but still specific staining of the smooth muscle fibres (dotted circle). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

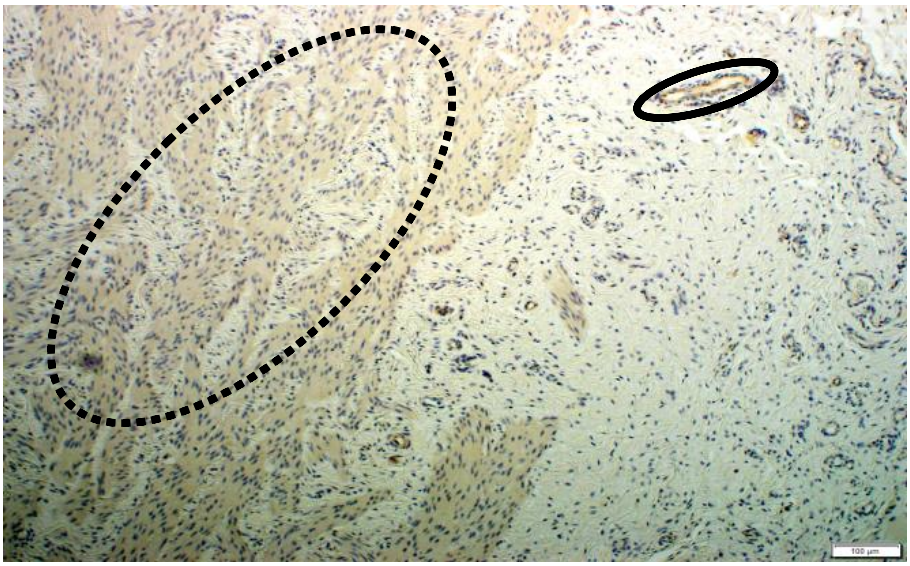

Figure 17 Immunolocalisation of ORs in the myometrium of a young horse in non-luteal phase (RS 42; 100 µm with intense staining of the vascular endothelium (solid circles) between myofibres and and paler but still specific staining of the smooth muscle fibres (dotted circle). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

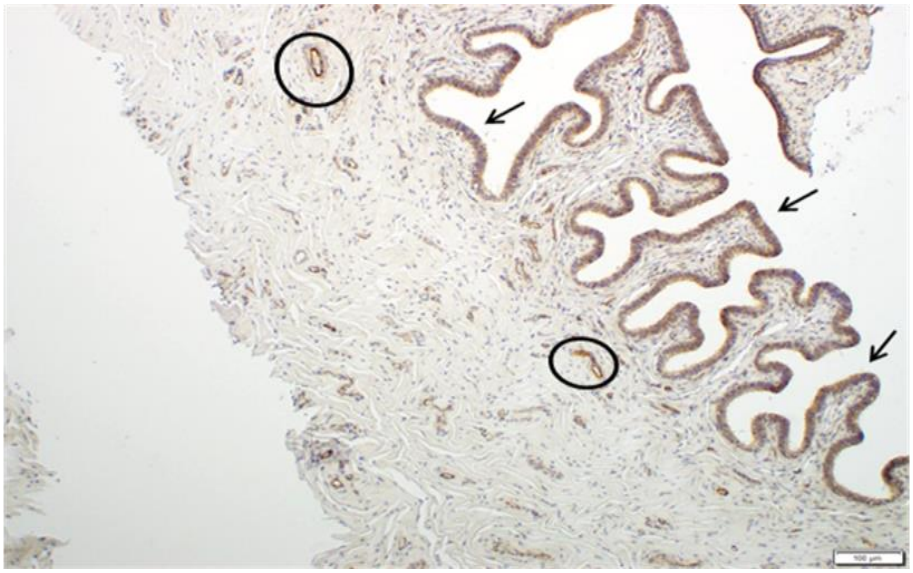

Figure 18 Immunolocalisation of ORs in the cervix of an old horse during non-luteal phase (RS 57; 100 µm) with labeling of the apical cytoplasmic membrane of the luminal epithelium (arrows) and of vascular endothelium (solid circles). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

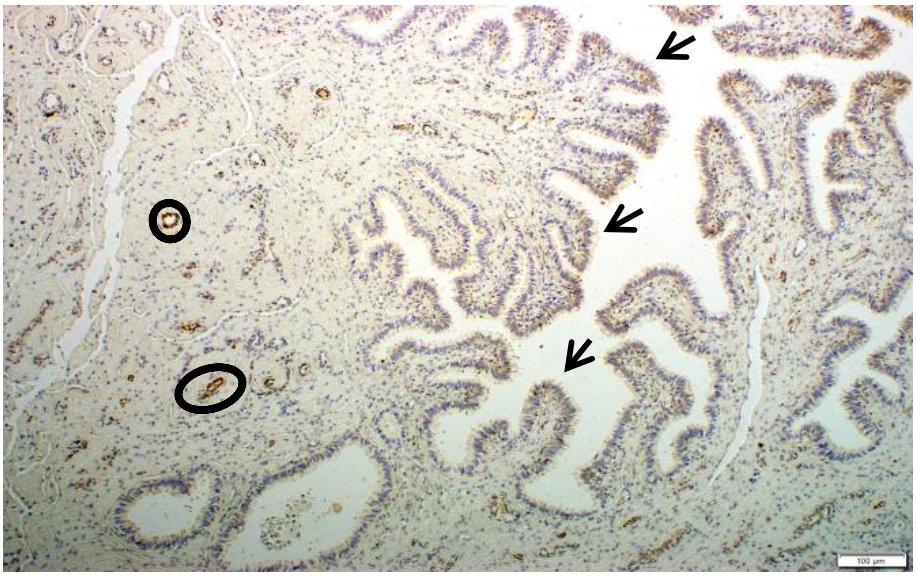

Figure 19 Immunolocalisation of ORs in the cervix of a young horse during non-luteal phase (RS 42; 100 µm) with labeling of the apical cytoplasmic membrane of the luminal epithelium (arrows) and of vascular endothelium (solid circles). IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

4.5 Oxytocin receptor gene expression analysis by reverse-transcription polymerase chain reaction (RT-PCR)

4.5.1 Validation experiment

Assay evaluation of the target and endogenous control genes for similar amplication to ensure robust- and preciseness of the quantitative RT-PCR (RT-qPCR) analysis efficiency was performed as described previously (Stroehle et al 2016) and gave the following results: the amplification efficiency for the ß-actin assay was 94,5 % (slope = -3,459; R^2 = 0,98; amplification = 1,95) and 93 % (slope = -3,501; R^2 = 0,97; amplification 1,93) for the OR assay respectively as required.

4.5.2 Quantification of oxytocin receptor gene expression using $\Delta\Delta C_T$ method

Detection of OR gene in the different samples, using RT-qPCR, was indicated by cycle threshold (C_T) values which were used to calculate the average C_T value for each group: tissue type and cycle stage (**Table 4)**, age (**Table 4**), biopsy score (**Table 7**). To determine the OR gene expression profile in the different tissues, the average C_T values for ß-actin C_T values were calculated from three replicates for each tissue type and found to be 23,65 for cervix, 19,84 for endometrium and 27,49 for myometrium. To normalise the detection of the target gene (OR), the average ß-actin C_T values were then applied to samples of specific tissue type and used to calculate the ΔC_T. ΔC_T was applied to determine $\Delta\Delta C_T$ and subsequently $2^{-\Delta\Delta Ct}$ values relative to a control (**Table 5, 6 and 8)**.

4.5.3 Oxytocin receptor gene expression during luteal and non-luteal phase

Relative to expression of OR gene during the luteal phase in endometrium, myometrium and cervix, RT-qPCR analysis showed higher expression of the OR gene using fold

differences during non-luteal phase in myometrium (26 times), endometrium (2 times) and cervix (3 times) (**Table 5)**. For the statistical analysis ΔC_T values were used. Values were normalised by taking the average ß-actin C_T values into consideration. The myometrium was found to have significantly higher OR gene expression during the non-luteal than during the luteal phase (P=0.004). There was no significant difference in the OR gene expression when comparing luteal and non-luteal phases in endometrium (P=0.574) and cervix (P=0.339) **(Table 5)**.

Table 4 Cycle treshold (C_T) values obtained from individual samples for each tissue type, cycle stage and age group

Cycle Stage	Age	Sample ID	Endometrium		Myometrium		Cervix		
			C_T Value	Av. C_T[a]	C_T Value	Av. C_T[a]	C_T Value	Av. C_T[a]	C_T Value[b]
Luteal phase	Old	RS 026	29,52		35,34		35,52		
		RS 029	29,75		33,57		31,25		
		RS 051	33,44		35,90		37,01		**33,01**
		RS 059	31,46		36,33		35,18		
		RS 063	25,22		32,95		32,77		
	Young	RS 025	24,82		30,72		31,11		
		RS 041	21,97		31,31		27,90		
		RS 042	21,44		37,47		34,89		
		RS 043	28,24	**28,06**	38,47	**35,19**	29,55	**32,85**	
		RS 047	31,46		36,33		33,31		
		RS 049	31,88		34,40		34,15		**31,61**
		RS 054	32,32		38,69		26,24		
		RS 055	31,50		37,12		36,40		
		RS 061	22,91		34,18		34,59		
Non-luteal phase	Old	RS 024	22,64		31,56		31,24		
		RS 030	36,67		35,45		37,37		
		RS 031	23,84		24,35		24,84		
		RS 060	31,88		34,40		*		
	Young	RS 032	30,11		33,19		30,11		
		RS 036	29,17		34,34		36,40		
		RS 038	33,64		36,94		38,68		
		RS 040	23,15	**27,09**	25,28	**30,48**	28,71	**31,20**	
		RS 045	30,36		31,58		36,71		
		RS 048	26,92		33,48		32,01		**29,62**
		RS 050	22,98		23,59		22,53		
		RS 056	20,51		21,62		26,11		
		RS 058	27,94		33,81		29,75		

Young = 2.5 – 9 years; Old ≥ 10 years

* too low for measurement

[a] Average C_T value of OR in the different tissues of all mares in luteal and non-luteal phase

[b] Average C_T value of OR in all three tissues combined of old and young mares in luteal and non-luteal phase

Table 5 Expression profiles of oxytocin receptor (OR) gene in the endometrium, myometrium and cervix at different cycle stages

Sample	Cycle stage	OR Av. C_T[a]	ß-actin Av. C_T[a]	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta Ct}$	P-value
Endometrium	**Luteal phase (control)**	28,06	19,84	8,22			
	Non-luteal phase (target)	27,09	19,84	7,25	-0,97	**1,96**	**0.574**
Myometrium	**Luteal phase (control)**	35,19	27,49	7,7			
	Non-luteal phase (target)	30,48	27,49	2,99	-4,71	**26,17**	**0.004**
Cervix	**Luteal phase (control)**	32,85	23,65	9,2			
	Non-luteal phase (target)	31,2	23,65	7,55	-1,65	**3,14**	**0.339**

C_T = cycle threshold
ΔC_T = OR average C_T - ß-actin average C_T
$\Delta\Delta C_T$ = Δ C_T target - Δ C_T control
[a] Average C_T value of OR in the different tissues of all mares in luteal and non-luteal phase

4.5.4 Oxytocin receptor expression during luteal and non-luteal phase in young and old mares

Relative to OR gene expression in young mares, RT-qPCR analysis showed lower (approximately half) expression of the OR gene in old mares during the luteal and the non-luteal phase (**Table 6**). These findings were based on the fold difference, which was calculated by using the average C_T values of specific samples, ΔC_T values (OR average C_T - ß-actin average C_T) and $\Delta\Delta C_T$ values ($\Delta C_{T\ (control)}$ - $\Delta C_{T\ (target)}$). For this part, the young mares were selected as a control and the fold difference in target (i.e. old mares) relative to the control was determined using $2^{-\Delta\Delta Ct}$.

Based on statistical analysis of ΔC_T values, the difference between old and young mares in luteal as well as non-luteal phase was not significant (P = 0.601).

Table 6 Expression profiles of oxytocin receptor (OR) gene in young and old mares at different cycle stages

Cycle Stage		OR Av. C_T[a]	ß-actin Av. C_T[a]	Δ C_T	ΔΔC_T	$2^{-\Delta\Delta Ct}$	P-value
Luteal phase	**Young mares (control)**	31,74	23,66	8,08			
	Old mares (target)	33.01	23,66	9.35	1.27	**0.41**	**0.601**
Non-luteal Phase	**Young mares (control)**	29,62	23,66	5,96			
	Old mares (target)	30.39	23,66	3.73	0.77	**0.58**	**0.901**

Young = 2.5 – 9 years; Old ≥ 10 years
C_T = cycle threshold
ΔC_T = OR average C_T - ß-actin average C_T
ΔΔC_T = Δ C_T target - Δ C_T control
[a] Average C_T value of OR in the different tissues of all mares in luteal and non-luteal phase

4.5.5 Oxytocin receptor gene expression in mares with different endometrial biopsy scores

Relative to OR gene expression in mares with endometrial biopsy score I, RT-qPCR analysis showed lower expression of the OR gene in mares with endometrial biopsy score IIA (1/6) and higher expression of the OR gene in mares with endometrial biopsy score IIB (4 times) based on usage of fold differences (**$2^{-\Delta\Delta Ct}$, Table 8)**. Neither of the differences using ΔC_T values was statistically significant.

Table 7 Oxytocin receptor cycle treshold (C_T) values obtained as an average of all three tissue samples from individual samples for each endometrial biopsy score

Biopsy score	Sample ID	Average C_T Value of Sample	Average C_T[a]
I	RS 041	27,06	
	RS 043	32,09	
	RS 047	33,70	**31,12**
	RS 054	32,41	
	RS 063	30,31	
IIA	RS 025	28,88	
	RS 026	33,46	
	RS 029	31,52	
	RS 030	36,49	
	RS 032	31,13	
	RS 036	33,31	
	RS 038	36,42	
	RS 040	25,71	**31,36**
	RS 042	31,27	
	RS 049	33,47	
	RS 050	23,03	
	RS 051	35,45	
	RS 055	35,01	
	RS 056	22,75	
	RS 059	33,27	
	RS 061	30,56	
IIB	RS 024	28,48	
	RS 031	24,34	
	RS 045	32,88	**29,11**
	RS 048	30,80	
	RS 058	30,50	
	RS 060	27,63	

C_T = cycle threshold

[a] Average OR C_T value of biopsy score groups

Table 8 Expression profiles of the oxytocin receptor (OR) gene in mares with endometrial biopsy scores I, IIA and IIB

Sample	OR Average C_T [a]	ß-actin Average C_T[a]	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta Ct}$	P-value
Biopsy score I (control)	31,12	23,66	7,46			
Biopsy score IIA (target)	31,36	23,66	7,7	0,24	**0,85**	**1.0**
Biopsy score IIB (target)	29,19	23,66	5,53	-1,93	**3,81**	**1.0**

C_T = cycle threshold
ΔC_T = OR average C_T - ß-actin average C_T
$\Delta\Delta C_T$ = ΔC_T target - ΔC_T control
[a] Combined C_T values for all tissues and all mares in biopsy score group

Table 9 Pairwise comparison of ΔC_T values ($\Delta C_T = C_T$ (OR) - C_T (ß-actin)) for biopsy score group I, IIA and IIB based on estimated marginal means

Biopsy Scores		Mean Diff.	Std. Error	df	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
I	IIA	-.243	1.963	23.968	1.000	-5.295	4.809
	IIB	1.985	2.328	24.298	1.000	-4.002	7.972
IIA	I	.243	1.963	23.968	1.000	-4.809	5.295
	IIB	2.228	1.845	24.496	.716	-2.513	6.969
IIB	I	-1.985	2.328	24.298	1.000	-7.972	4.002
	IIA	-2.228	1.845	24.496	.716	-6.969	2.513

* The mean difference is significant at the .05 level

[a] Adjustment for multiple comparisons: Bonferroni

CHAPTER 5: Discussion

5.1 Study population

Only cyclic mares were included in this study as it was unknown if oxytocin receptor (OR) distribution and gene expression is affected by seasonal changes which could bias the evaluation of the results (Snider et al. 2011).

Mares were assigned to either luteal or non-luteal phase by taking a combination of macroscopic evaluation of ovaries to establish functional structures, size of ovaries and blood sample analysis for plasma progesterone concentration (PPC) into consideration. Plasma progesterone concentration values $\geq$ 3.18 nmol/l with at least one corpus luteum (CL) or/and corpus hemorrhagicum (CH) as dominant ovarian structure was considered indicative for luteal phase, whereas mares with PPC values < 3.18 nmol/l generally in combination with follicles > 2 cm were consigned to the non-luteal phase (Garcia 1990; Daels et al. 1991; Evans et al. 1997; Galvao et al. 2010; Szostek et al. 2014). In our study, some mares did not display the typically expected combination of ovarian structures and PPC values (**Appendix 1**, **Table 10**). Mares RS 024, RS 031 and RS 038 showed PPC values < 3.18 nmol/l but CLs and, or CHs on the ovary. There are two possible explanations for this observation. The first is that although a CH forms immediately after a mare ovulates, progesterone production only starts 24 hours after ovulation and it reaches its peak 6 - 7 days post ovulation (Stabenfeldt et al. 1975; Daels et al. 1991).

This was however unlikely for the two mares RS 024 and RS 031 (CL sizes: 3.0 cm and 2.5cm, respectively) as a CH is usually bigger, similar to a pre-ovulatory follicle and would be expected to be 4.0 – 5.0 cm in diameter (Daels et al. 1991), in which case, a second scenario was more likely. The life span of CLs is normally 14 - 15 days in a non-pregnant mare; release

of prostaglandin F2-*alpha* ($PGF_{2\alpha}$) causes luteolysis and regression of the CL. Regressing CLs are not able to produce significant amounts of progesterone (Daels et al. 1991) and the mares are likely to be in the last two days of dioestrus approaching oestrus. The first explanation based on low PPC values would be applicable for classifying RS 038 (CH; PPC 1.19 nnmol/L) as being in the non-luteal phase.

Mares RS 030 and RS 032 were determined to have small follicles or non-significant structures on both ovaries and a low PPC value but the sizes of their ovaries were sufficiently large enough to consider them as cycling and were therefore allocated to the non-luteal group. Mares RS 025 and RS 029 showed PPC values $\geq$ 3.18 nmol/l, however no luteal tissue was noticed on the ovary. It was possible that the luteal tissue was missed during inspection of the ovaries. Mare RS 051 was found to have a large follicle (5 cm) on the left ovary as well as a CL on the right ovary and a PPC value $\geq$ 3.18 nmol/l. An impending asynchronous double ovulation was thought to be responsible for this observation (Bergfelt & Adams 2007). Due to the PPC value $\geq$ 3.18 nmol/l mare RS 051 was assigned to the luteal phase group.

5.2 Immunohistochemical labeling of oxytocin receptors in uterus and cervix at different cycle stages

No difference in distribution of ORs between the luteal and non-luteal phase was observed. Regardless of cycle stage, ORs were found in the cytoplasm of luminal and glandular epithelia, endothelium of blood vessels and sub-epithelial and periglandular stromal cells within the endometrium, as described by Stroehle (2016). Oxytocin receptor labeling was detected in smooth muscle cells and endothelium of blood vessels in the myometrium of mares in both the luteal and non-luteal phases. As described previously (Stroehle et al. 2016) there was a similar pattern of distribution and staining intensity

of ORs in the uterus (endometrium and myometrium) and cervix, with the exception of the glandular-epithelium, which was absent in the cervix. Oxytocin receptors are expected in glandular and luminal endometrial epithelium as there is reported evidence of oxytocin involvement in the events of luteolysis since administration of exogenous oxytocin stimulates the release of $PGF_{2\alpha}$ in late dioestrus (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993) and the equine endometrium produces $PGF_{2\alpha}$ in response to oxytocin *in vitro* (King 1984; Franklin et al. 1989).

Siemieniuch et al. (2011) investigated OR distribution in the endometrium of cats finding different expression based on intensity of immunohistochemical (IHC) labeling in superficial and glandular epithelium as well as the stromal cells when comparing different oestrous cycle stages. The comparison of staining intensity between slides and within individual samples was not considered feasible in the present study as too many variables influenced the staining intensity (Stroehle et al. 2016). A higher staining intensity could possibly be expected during the late luteal phase in mares with more stable staining methods, as higher concentrations of ORs in the endometrium and myometrium of mares at the time of luteolysis in late dioestrus have been described previously (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998).

5.3 Oxytocin receptor gene expression based on quantitative reverse-transcription polymerase chain reaction (RT-qPCR) results in the uterus (endo- and myometrium) of mares in luteal and non-luteal phase

Samples of mares in the non-luteal phase showed greater OR gene expression in the uterine tissues (endometrium and myometrium combined) than in the same samples of mares in the luteal phase**.** Looking at uterine tissues individually, only the myometrium was found to have a significantly higher OR

gene expression during the non-luteal phase in comparison to the luteal phase**.** There was no significant difference of OR gene expression in the endometrium comparing samples of mares in the luteal and non-luteal phase. These findings appear to contradict earlier studies in the mare where the highest concentrations of ORs in the endometrium and myometrium of mares was described at the time of luteolysis in late dioestrus (days 14 - 18) while the lowest receptor density was observed during oestrus and eight days after ovulation (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). The apparent disparity between our study and previous studies might be due to mares in the current study being assigned to either the luteal or non-luteal phase based on a single macroscopic evaluation of their ovaries and reproductive tracts and measured PPC values. Previous studies used repeated clinical monitoring of the oestrus cycle (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). As a consequence, luteal and non-luteal phase in this study were not equivalent to the oestrus and dioestrus classification used in other publications where repeated and daily rectal and ultrasonographic examinations were used to determine cycle stage. Therefore, the non-luteal phase defined in this study was likely to consist of 11 - 12 days instead of only the seven days of oestrus as the first 24 - 48 h post-ovulation as well as the last 2 - 3 days of dioestrus were included in the non-luteal phase. The luteal phase of this study was shortened to 9 - 10 days in comparison to the 14 - 16 days of dioestrus (Blanchard and Schumacher, 2003). Thus, the high OR density found by Stull (1986), Sharp et al. (1997), Starbuck et al. (1998) in late dioestrus was included in the non-luteal phase of this study which explained the higher OR gene expression during non-luteal phase in the myometrium in our study, which is in agreement with these previous studies.

Oxytocin is known to stimulate the synthesis and release of $PGF_{2\alpha}$ in uterine tissue in several species including rabbit, cattle and sheep causing luteolysis (Shemesh et al. 1997, Fuchs et al.

1982, Small et al. 1978). It is suspected to stimulate the release of equine endometrial $PGF_{2\alpha}$ and might therefore also be of importance in luteolysis in the mare (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). Its exact role in the mare, however, remains undefined. For oxytocin to be able to show its effect at the time of luteolysis, the gene expression of ORs has to increase which explains the described previous findings of Stull (1986), Sharp et al. (1997) and Starbuck et al. (1998). This study seems to confirm this hypothesis as greater OR gene expression was found in the uterine tissues (endometrium and myometrium) during the non-luteal phase which includes the time around luteolysis. However, the assignment of mares in this study to either the luteal or non-luteal phase in contrast to assignment of mares to oestrus and dioestrus in previous studies makes a direct comparison of results between the studies difficult.

Oxytocin additionally increases uterine motility and thus supports the uterine defence mechanisms by stimulating clearance of the uterine lumen especially at the time of oestrus in the mare (LeBlanc et al. 1994; Pycock and Newcombe, 1996a; Pycock and Newcombe, 1996b). A potential simultaneous rise of OR gene expression, especially in the myometrium, could be expected but so far has not been confirmed by earlier studies (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). Our results are the first to suggest an increased OR gene expression during the non-luteal phase in the myometrium. On the contrary, a recent study by Steckler et al. (2012) found that an increased amount of oxytocin is necessary to cause myometrial contractions in *vitro* in myometrial tissue during oestrus, which was hypothesised to be due to low concentrations of ORs. No information, however, exists on the effect of OR density on the degree of tissue response to oxytocin. Myometrial contractility in response to the long acting analogue of oxytocin, carbetocin, however, did not seem to be affected by cycle stage. Binding of carbetocin

to ORs and vasopressin receptors might explain this observation. Further studies are warranted in order to further investigate these findings.

5.4 Oxytocin receptor gene expression in the cervix of mares in luteal and non-luteal phase

Oxytocin receptors were detected in the cervix of the mare during the luteal and non-luteal phases. No significant difference in gene expression was found between the two phases. Oxytocin receptors also have been detected in the ovine and bovine cervix during oestrus (Matthews and Ayad, 1994; Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013). Finding of ORs in the cervix of cycling non-pregnant mares suggested that oxytocin plays a physiological role in cervical function during the oestrous cycle (Stroehle et al. 2016). In ewes and cows, oxytocin is suspected to stimulate prostaglandin E2 (PGE_2) release in the cervix of non-pregnant animals (Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013). Fuchs et al. (1995) demonstrated that oxytocin stimulates PGE_2 release from the bovine cervix *in vitro.* Prostaglandin E2 output was shown to be dependent on the cycle stage in ewes and cows, being the highest in oestrus (Matthews and Ayad, 1994; Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013). In the ewe, high expression of oestradiol receptor and OR gene and concomitantly PGE_2 synthesis can be found when the cervix spontaneously relaxes during the periovulatory period (Falchi and Scaramuzzi, 2013). Prostaglandin E2 has also been shown to cause dilation of the equine cervix *in vivo* when injected directly into cervical wall (Rigby et al. 1998). The equine cervix relaxes during the oestrous cycle when serum progesterone is low, starting at the beginning of oestrus and becoming fully relaxed at the time of ovulation (Carleton 1997). The expectation of describing concomitantly greater OR gene expression in the equine cervix during the non-luteal

phase was not met in this study as no significant difference was shown between the non-luteal and luteal phase. Further investigations on the possible effects of oxytocin on PGE_2 release in the equine cervix and the importance of cervical OR gene expression throughout the whole oestrous cycle are necessary.

5.5 Endometrial biopsy scores

5.5.1 Endometrial biopsy scores in different cycle stages and age groups

No mares from this study population were found to have a biopsy score III which may be due to the inclusion of a random abattoir study population without any clinical history.

Mares in the non-luteal phase had significantly lower biopsy scores (I = highest score, IIB = lowest score) compared to mares in the luteal phase. All mares with biopsy scores IIB were found to be in the non-luteal phase and all mares with biopsy score I in the luteal phase. Biopsies of mares with biopsy score IIB showed glandular nesting and fibrosis as the main pathological changes. There are numerous reports that outline the effects of normal oestrous cycle changes on endometrial architecture (Kenney 1978; Brandt 1970; Gordon and Sartin 1978; Samuel et al. 1979). During oestrus the epithelium consists of tall columnar cells. The lamina propria is oedematous meaning that the stroma appears less dense and the glands are elongated. Neutrophils can be found within small blood vessels and on the surface of the endometrium (Snider et al. 2011). During dioestrus the height of the epithelial cells is reduced from tall to low columnar. There is no evidence of oedema and hence the stroma appears more dense and the glands closer together and more tortuous (Kenney 1978). It was suggested that the increased finding of fibrosis and thus the lower biopsy scores during the non-luteal phase in our study

was influenced by the physiological oedema during oestrus (Van Camp 1988). No influence of the cycle stage on the biopsy score was described in previous studies. The age of the mares did not seem to influence the biopsy scoring in the luteal and non-luteal phase. Low and high biopsy grades were distributed relatively evenly amongst young and old mares in our study, with 23 %, 58 % and 19 % for biopsy scores I, IIA and IIB respectively in the young mare group; and 10 %, 60 % and 30 % for biopsy scores I, IIA and IIB respectively in the old mare group. No significant difference in biopsy scores between old and young mares could be found. This supported findings of Shideler et al. (1982) who also did not observe a significant relationship between biopsy grade and age. Several other studies, however, suggested an association between increasing age and endometrial fibrosis as well as increasing age and fertility (Gordon and Sartin 1978; Doig et al. 1981; Waelchi 1990; Ricketts and Alonso 1991). Gordon and Sartin (1978) described decreased fertility associated with advanced age, number of years barren, perineal confirmation, abortion history and degree of fibrosis, which was confirmed by Doig et al. (1981) in a subsequent study. Waelchi (1990) demonstrated that mares > 11 years old, on average, have a lower biopsy score and lower fertility than younger mares. Ricketts and Alonso (1991) found that maiden mares of increased age showed endometrial changes, indicating that age on its own can be a causative factor in the development of endometrosis. In contrast to this current study, these previous investigations all included mares that showed signs of prior clinical infertility whereas a randomly chosen study population with unknown breeding history was used in this study. That possibly influenced the observed outcomes and might be a more representative description for the general mare population and not necessarily for mares that have shown signs of infertility previously. No mares with severe endometrial changes were

identified in this study as none of the randomly chosen mares met the criteria to be classified as a biopsy score III.

5.5.2 Endometrial biopsy scores, immunohistochemical labeling of oxytocin receptors and oxytocin receptor gene expression

No difference was found in immunohistochemical labeling of ORs in samples with different biopsy scores. The biopsy score IIB samples had the greatest OR gene expression as shown by RT-qPCR results when compared to both graded IIA and I samples based on fold difference, but overall there was no significant difference between gene expression of ORs in samples with different biopsy scores. The highest OR receptor gene expression in biospy score IIB samples was an unexpected outcome. A possible explanation could be an upregulation of ORs as a compensation mechanism for pathological changes in uterine tissues. Further studies are necessary to investigate this hypothesis as the lower number of mares with biopsy scores IIB (six) in comparison to the higher number of mares with biopsy scores IIA (sixteen) might not be a representative overall sample and a limitation of the current study. Low biopsy scores (IIB and III) represent endometria that show pathological changes, which influence a mare's reproductive performance (Van Camp 1988). Post-breeding endometritis is one of the conditions that can be found in conjunction with low biopsy scores (Watson 2000). It is one of the major causes for subfertility and develops as a consequence of defective uterine clearance (Pycock 1994; Rausch et al. 1996; LeBlanc and Causey, 2009). Difficulty clearing debris from the uterus after mating may be due to anatomical and, or degenerative defects that interfere with uterine drainage. These defects include poor perineal conformation, an incompetent cervix and periglandular fibrosis (Evans et al. 1987; Allen 1991; LeBlanc 2008), as well as reduced frequency, intensity and duration of

myoelectrical myometrial activity *in vivo* compared to reproductively normal mares (Troedsson et al. 1993a). There are numerous hypothesised causes for the decline in myometrial activity. Possible mechanisms include: 1) changes in the release, either systemically or locally, of uterotonins such as prostaglandins or oxytocin; 2) altered expression of receptors for uterotonins in the endometrium; and 3) an intrinsic change within the uterine muscle that delays uterine clearance (Rigby et al. 2001). Rigby et al. (2001) confirmed an intrinsic defect in myometrial function and also suggested a contribution of reduced oxytocin-stimulated myometrial $PGF_{2\alpha}$ production to the reduced contractile response of susceptible mares. These findings suggest that failure of the oxytocin system plays a role in the compromised myometrial function during uterine clearance. This may simply be a lack of hormone and, or ORs in the target tissue. The findings of our study, however, did not support Rigby's hypothesis. A lack of ORs in the endometrium of mares with uterine pathology was not observed. On the contrary, the highest OR gene expression was found in the endometrium of mares with the lowest biopsy score (IIB) indicating a possible compensatory mechanism to a lack of response to uterotonins. Thus upregulation of the OR gene expression rather than downregulation as suspected by Rigby et al. (2001) in case of uterine pathology might be an explanation for this study's results.

5.6 Effect of age on oxytocin receptor gene expression and immunohistochemical labeling of oxytocin receptors

Young mares had a greater OR gene expression than did old mares during luteal and non-luteal phases based on fold difference; but the difference based on ΔC_T values was not statistically significant. In older mares, the uterus often displays evidence of mucociliary dysfunction, scarring and atrophy of uterine folds and damage to (or lack of) endometrial cilia,

which have been associated with subfertility (Bracher et al. 1992; Ferreira-Dias et al. 1994; Ferreira-Dias et al. 1999). The degenerative uterine condition, endometrosis may also play a role in loss of uterine function (Schoon et al. 1992; Kenney 1992; Hoffmann et al. 2009). Several studies have shown a significant association between the degree of endometrosis and the age of the mare (Ricketts and Alonso, 1991; Schoon et al. 1992; Hoffmann et al. 2009). It may be postulated that decreased OR gene expression and, or an alteration in OR distribution might be associated with this pathology complex. In a study reported by Grey and Dascanio (2006), OR gene expression in endometrial tissue of young and fertile, old and reproductively normal and old mares susceptible to delayed uterine clearance was evaluated. No significant differences were found between the groups. The authors suggested that instead of a change in OR gene expression, the oxytocin secretion or binding was impaired with increasing age or with disease, and that the receptors are constitutively expressed in aged or diseased uteri. Our results showed no influence of age on OR distribution and supported Grey and Dascanio's findings (Grey & Dascanio 2006).

5.7 Endometrial biopsy versus full thickness sample

Our study determined the sufficiency of an in vivo endometrial biopsy sample in comparison to a full thickness uterine sample. An in vivo endometrial biopsy sample contains endometrium and only occasionally small amounts of myometrium whereas a full thickness uterine sample includes all layers of the uterus. The OR distribution as determined by IHC did not vary between different cycle stages or age groups and the OR gene expression as determined by RT-qPCR was found to be equivalent (consistently lower but measurable) in endometrium in comparison to myometrium. Therefore, an in vivo endometrial biopsy sample was considered sufficient to

evaluate the OR gene expression in general and OR distribution in the endometrium. No statement can be made on OR distribution in deeper layers (especially myometrium) of the uterus. In cases with unusally low OR gene expression, inclusion of evaluation of the myometrium would be potentially beneficial due to significantly higher OR gene expression in the myometrium. In conclusion it may be stated, that an in vivo endometrial biopsy was considered sufficient for routine evaluation of OR gene expression and OR distribution in the uterus of the mare. The evaluation of OR gene expression by RT-qPCR assisted by IHC to show OR distribution provided no additional information to a routine biopsy for HE evaluation on likelihood of falling pregnant and carrying a foal to term (Kenney 1978; Kenney and Doig, 1986). Therefore, the *in vivo* endometrial biopsy used for HE staining and evaluation for grading seems sufficient for routine examinations of subfertile mares.

5.8 Analysis of study deficiencies

It is advisable for a standard RT-qPCR protocol to be performed in replicates (duplicates, triplicates) as it provides the preservation and monitoring data of the polymerase chain reaction (PCR) amplification precision data (Livak and Schmittgen 2001; Stahlberg et al. 2004). In this study, it was not possible to run replicates of all the samples due to financial constraints. However, by using an endogenous control gene (ß-actin; and thus normalising the amount of PCR added; Livak and Schmittgen 2001) and by ensuring optimisation of the validation experiment, the validity of the results in the present study were warranted. To fulfil the requirements of the comparative cycle threshold (C_T) method used to analyse the RT-qPCR data, assays of the target and endogenous control genes were evaluated to ensure that they have similar

amplification efficiency to guarantee a robust and precise RT-qPCR analysis.

CHAPTER 6: Conclusion

In conclusion, using Immunohistochemistry (IHC) in this study demonstrated a consistent distribution of oxytocin receptors (ORs) in several cell and tissue types in the endometrium, myometrium and cervix that was independent of mare age, cycle stage and endometrial biopsy score**.** There was no difference between OR gene expression based on quantitative reverse-transcription polymerase chain reaction (RT-qPCR) in the endometrium and cervix at different cycle stages, while the myometrium showed significantly higher OR gene expression during the non-luteal phase than during the luteal phase. A possible explanation was an increased requirement for oxytocin and its receptors at the time of oestrus for uterine clearance, one of the principal uterine defence mechanisms involving mainly the myometrium.

Young mares tended to have a greater OR gene expression than old mares during luteal and non-luteal phase based on fold difference. However, there was no statistical difference based on ΔCT values.

No significant difference in OR gene expression based on RT-qPCR was detected between mares with different biopsy scores in this study. Mares in the non-luteal phase had significantly lower biopsy scores (I = highest score, IIB = lowest score) compared to mares in the luteal phase using HE stained biopsies for evaluation.

Immunohistochemical labeling of ORs to evaluate OR distribution and RT-qPCR for OR gene expression were not good predictors of the prospective ability for mares to become pregnant and carry to term and have not been identified as value adding routine procedures for equine infertility work-ups.

CHAPTER 7: Summary

Each year numerous mares fail to fall pregnant despite optimised breeding management (no obvious reason of infertility in mare, good semen quality of stallion, correct timing of breeding, good insemination technique and post-breeding management; Da Silva 2008). These mares are considered subfertile (Da Silva 2008). Various reproductive pathologies may cause a mare to not conceive or to lose a pregnancy at an early stage, with uterine pathologies including post-breeding endometritis being one of the most important reasons (Carnevale 2008). Uterine clearance is one of the most important components of the uterine defence mechanism against infection (Troedsson 1999). Mares incapable of efficient uterine clearance and consequently incapable of resolving the transient physiological endometritis are likely to develop a persistent post-breeding endometritis leading to prolonged inflammation of the endometrium and consequently embryonic loss and subfertility (Troedsson 1999; Troedsson et al. 2001; Watson 2000).

Oxytocin, a neuropeptide hormone produced by the hypothalamus and endometrium of the mare (Behrendt–Adam et al. 1999, Handler 2001, Gimpl and Fahrenholz 2001, Bae and Watson 2003) is the main hormone responsible for contractions of smooth muscles in the oviduct, uterus and the myoepithelial cells of the mammary gland and is thus of importance for uterine clearance (Handler 2001). Oxytocin is additionally considered to be important in luteolysis (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). Oxytocin acts on target tissues by binding to its receptors. Oxytocin receptors (ORs) have been described in various tissues including tissues outside the genital tract (Gimpl and Fahrenholz 2001).

The important role of oxytocin in the equine oestrous cycle as well as in uterine clearance supports the need to investigate the location of ORs in the mare's reproductive tract (endometrium, myometrium and cervix). Previous studies have shown changes in the concentration of ORs in the equine endometrium throughout the oestrous cycle, with a reported peak during late dioestrus. There are, however, no reports comparing OR density and distribution in the myometrium and cervix of the non-pregnant mare during the different oestrous cycle stages. Despite considerable support for oxytocin playing an important role in the uterine defence mechanism and hence fertility, its exact significance is poorly defined. This study described the distribution and density of ORs in the mare's endometrium, myometrium and cervix during the luteal and non-luteal phases and additionally within different mare categories based on age and endometrial biopsy score using both immunohistochemistry (IHC) and quantitative reverse-transcription polymerase chain reaction (RT-qPCR).

Full-thickness uterine samples and endometrial biopsy samples were obtained from 27 routinely-slaughtered, cyclic mares of various breeds and ages (10 mares $\geq$ 10 years old, 17 mares < 10 years old) at three different uterine sites (uterine body, right and left horn) and one sample from the cervix. Endometrial biopsy sample sections were stained with haematoxylin and eosin by standard techniques and sections were graded I, IIa, IIb, or III according to the Kenney and Doig categorisation scheme. For IHC, all endometrial, myometrial and cervical samples were immunolabeled using an avidin-biotin-peroxidase complex (ABC) detection system and a polyclonal antibody against human ORs. Sections were evaluated using an Olympus light microscope and the Olympus cell sens dimension software. Additional samples obtained from the left uterine horn (endometrium and myometrium) and the cervix (luminal epithelium, propria-submucosa and muscularis layer) were used for the RT-PCR assay. Oligonucleotide primers and

probe sequences used for detection of OR and β-actin gene expression were obtained from three previous studies evaluating OR gene expression and distribution in the equine conceptus, fetal membranes, endometrium of pony mares at parturition and in endometrium, myometrium and cervix of non-pregnant mares. A RT-qPCR was used for detection of the OR messenger-ribonucleic acid (mRNA). The $\Delta\Delta C_t$ method using the StepOnePlus™ software was employed as a descriptive method to quantify different gene expression between tissues and ΔC_t values were used for statistical analyse of the data.

Five (18.52 %; one old mare and four young) mares showed a grade I biopsy score, sixteen (59.26 %, six old and 10 young) mares showed a grade IIA biopsy score and six (22.22 %) mares showed a grade IIB biopsy score. None of the mares in the study population showed a grade III biopsy score. All mares with biopsy score I were in the luteal phase and all mares with biopsy score IIB were in the non-luteal phase of their cycles.

There was no significant difference in biopsy scores between old and young mares ($P = 0.386$). Mares in the non-luteal phase had significantly lower biopsy scores (I = highest score, IIB = lowest score) compared to mares in the luteal phase ($P = 0.003$).

By means of immunolabeling, ORs could be identified as fine brown granules in the cytoplasm of selected cells in both negative- and positive-tissue control tissues and in the test sections. No difference in OR distribution nor intensity of staining could be detected between mares in luteal and non-luteal phases, between old and young mares and between mares with different biopsy scores.

Looking at fold differences, relative to expression of OR gene during luteal phase in endometrium, myometrium and cervix,

RT-qPCR analysis showed higher expression of the OR gene during the non-luteal phase in myometrium (26 times), endometrium (2 times) and cervix (3 times). Statistically the myometrium was found to have significantly higher OR gene expression during non-luteal than during luteal phases ($P = 0.004$). There was no significant difference in the gene expression of ORs when comparing luteal and non luteal-phases in endometrium ($P = 0.574$) and the cervix ($P = 0.339$).

Statistical analysis of the RT-qPCR data did not demonstrate a significant difference between OR gene expression in young and old mares or between mares with different endometrial biopsy scores ($P = 0.601$).

CHAPTER 8: Zusammenfassung

Der Einfluss des Sexualzykluses, des Alters und der Biopsie Kategorien auf die Genepression und Verteilung des Oxytocin Rezeptors in der Zervix und der Gebärmutter von nicht-tragenden Stuten

Jedes Jahr werden zahlreiche Stuten trotz optimalem Zuchtmanagement (kein offensichtlicher Grund für Unfruchtbarkeit bei der Stute, gute Samenqualität des Hengstes, korrektes Timing der Besamung, gute Besamungstechnik und Management nach der Besamung) nicht trächtig (Da Silva 2008). Diese Stuten werden als subfertil bezeichnet (Da Silva 2008). Verschiedene Fortpflanzungspathologien können dazu fuehren, dass eine Stute nicht trächtig wird oder in einem frühen Stadium Fruchtresorption erleidet. Pathologische Veraenderungen der Gebärmutter, wie Endometritis nach der Deckung, werden als einer der Hauptgründe dafür gesehen (Carnevale 2008).

Die uterine Säuberung ist einer der wichtigsten Bestandteile des Abwehrsystems gegen Infektionen (Troedsson 1999). Stuten, die unfähig zur effizienten uterinen Selbstreinigung sind und folglich unfähig sind, die vorübergehende physiologische Endometritis zu beheben, unterliegen einer großen Wahrscheinlichkeit eine chronische Endometritis nach der Deckung zu entwickeln. Diese führt zur längeren Entzündung der Gebärmutterschleimhaut und somit zum Verlust des Embryos und zur Unfruchtbarkeit (Troedsson 1999; Troedsson et al 2001;. Watson 2000). Oxytocin, ein Neuropeptidhormon, das vom Hypothalamus und Endometrium der Stute produziert wird (Behrendt-Adam et al. 1999; Handler 2001; Gimpl und Fahrenholz 2001; Bae und Watson 2003), ist hauptverantwortlich für die Kontraktionen der glatten Muskulatur im Eileiter, der Gebärmutter und der Myoepithelzellen der Brustdrüse im Pferdereproduktionstrakt

und damit wichtig fuer die uterine Säuberung (Handler 2001). Oxytocin wird zusätzlich bei der Luteolyse als wichtig erachtet (Betteridge et al 1985; Goff et al 1987; Goff et al., 1993). Oxytocin wirkt auf das Zielgewebe ein, indem es an seine Rezeptoren bindet. Oxytocin Rezeptoren (OR) wurden in verschiedenen Geweben auch außerhalb des Genitaltraktes (Gimpl und Fahrenholz 2001) beschrieben.

Die bedeutende Rolle von Oxytocin im Sexualzyklus der Stute sowie bei der uterinen Säuberung erklärt die Notwendigkeit, die Lokalisation von ORs im Reproduktionstrakt (Endometrium, Myometrium und Zervix) von Stuten zu untersuchen. Früheren Studien war es möglich Veränderungen in der Konzentration von ORs in Endometrium des Pferdes während des Sexualzyklus mit einem Höhepunkt im späten Diöstrus aufzuzeigen. Es gibt jedoch keine Analysen, welche die OR Dichte und Verteilung im Myometrium und Gebärmutterhals der nicht trächtigen Stute zwischen verschiedenen Zyklusstadien vergleichen. Trotz des begründeten Verdachts, dass Oxytocin eine wichtige Rolle im Gebärmutterabwehrmechanismus und damit für die Fruchtbarkeit der Stute spielt, gibt es keine Studie, die genauere Auswirkungen untersucht.

Die vorliegende Studie untersucht mit Hilfe von Immunohistochemie (IHC) und quantitativer reverse-transcription polymerase chain reaction (RT-qPCR) die OR Verteilung und Dichte im Endometrium, Myometrium und in der Zervix der Stute während der Gelbkörper und Nicht-Gelbkörperphase, sowie in verschiedenen Fruchtbarkeitsgruppen (basierend auf der Alter und Endometriumbiopsiekategorie).

Als Forschungsproben wurden transmurale Uterusbiopsien sowie eine Endometriumbiopsie von 27 routinemäßig geschlachteten, zyklischen Stuten verschiedener Rassen und Altersgruppen (10 Stuten> 10 Jahre alt, 17 Stuten <10 Jahre alt)

von 3 verschiedenen Stellen im Uterus (Corpus uteri, rechtes und linkes cornus uteri) und von der Zervix entnommen. Schnitte der Endometriumbioptate wurden mit Hämatoxylin und Eosin nach Standardtechniken gefärbt und anschließend entsprechend dem Kenney und Doig Kategorisierungsschema den Kategorien I, IIa, IIb oder III zugeordnet. Für die IHC wurden alle Endometrium-, Myometrium- und Zervixproben mit einem Immunperoxidase-Detektionssystem und einem polyklonalen Antikörper gegen humane ORs immunmarkiert. Die Schnitte wurden unter Verwendung eines Olympus Lichtmikroskop und der Olympus cell sens Dimension Software ausgewertet. Weitere Proben vom linken Uterushorn (Endometrium und Myometrium) und der Zervix wurden für einen RT-qPCR-Assay verwendet. Zum Nachweis von ORs und β-Actin Genexpression wurden Oligonukleotid-Primer und Sonden-Sequenzen verwendet, mit welchen bereits in drei früheren Studien ORs im Pferdekonzeptus, fötalen Membranen,im Endometrium von Ponystuten bei der Geburt und in Endometrium, Myometrium und Zervix von nicht-trächtigen Stuten nachgewiesen werden konnten. Mit Hilfe der RT-qPCR wurde die messenger-ribonucleic acid (mRNA) ermittelt und das ΔΔCt Verfahren unter Verwendung der StepOnePlus ™ Software zur Analyse der Daten herbeigezogen.

Fünf (18,52%, 1 alte Stute und 4 junge Stuten) Stuten konnten der Kategorie I, sechzehn (59,26%, 6 Stuten und 10 junge Stuten) der Kategorie IIA und sechs (22,22%) der Kategorie IIB des Endometriumbiopsiekategorieschemas zugeordnet werden. Keine der Stuten in der Studienpopulation wurde in die Kategorie III eingeteilt. Alle Stuten der Kategorie I befanden sich in der Gelbkörperphase und alle Stuten der Kategorie IIB in der Nicht-Gelbkörperphase ihrer Zyklen.

Es konnte kein signifikanter Unterschied zwischen den Biopsiekategorien zwischen alten und jungen Stuten

festgestellt werden (P = 0,386). Pferde in der Nicht-Gelbkörperphase hatten jedoch eine signifikant schlechtere Einordnung in Biopsiekategorien (I = beste Kategorie, III = schlechteste Kategorie) im Vergleich zu Pferden in der Gelbkörperphase (P = 0,003).

Oxytocin Rezeptoren konnten mittels Immunmarkierung als feine braune Granula im Zytoplasma von ausgewählten Zellen sowohl im Negativ- als auch im Positiv-Kontrollgewebe und in den Testabschnitten identifiziert werden. Zwischen Stuten in Gelbkörper- und Nicht-Gelbkörperphase, alten und jungen Stuten und zwischen Stuten mit verschiedenen Biopsiekategorien konnte weder ein Unterschied in der OR Verteilung, noch in der Intensität der Färbung nachgewiesen werden.

Die RT-qPCR Analyse zeigt höhere OR-Genexpressionen während der Nicht-Gelbkörperphase im Myometrium (26 mal), Endometrium (2 mal) und der Zervix (3 mal) in Bezug auf die Expression von OR-Genen während der Gelbkörperphase. Statistisch gesehen besaß das Myometrium signifikant mehr ORs während der Gelbkörperphase als während der Nicht-Gelbkörperphase (P = 0,004). Es gab keinen signifikanten Unterschied für die OR-Konzentrationen im Endometrium (P = 0,0574), und in der Zervix (P = 0,339) in unterschiedlichen Zyklusphasen.

Die statistische RT-qPCR Analyse wies weder einen signifikanten Unterschied zwischen der OR-Genexpression in jungen und alten Pferden nach, noch zwischen Pferden mit unterschiedlichen Biopsiekategorien.

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Appendix 1

Table 10 Summary of age, biopsy score, ovarian findings and plasma progesterone concentration (PPC) results for the study population (Stroehle et al. 2016)

ID Mare	Age (years)	Biopsy score	Ovaries	PPC (nmol/L)	Cycle Stage
RS 024	12	IIB	l: 6cm x 4,5cm x 3cm : NSS r: 5,5cm x 4,5cm x 3cm: 2 CL (3cm)	1,77	Non-luteal
RS 025	7	IIA	l: 7cm x 5,5cm x 3cm : NSS r: 9cm x 5,5cm x 4cm: F(3cm)	21,55	Luteal
RS 026	11	IIA	l: 6,5cm x 5cm x 3cm: CL (3cm) r: 7cm x 4cm x 2,5cm : NSS	29,52	Luteal
RS 029	15	IIA	l: 7cm x 4cm x 2cm : sF/ r: 7cm x 5cm x 2,5cm: sF	38,94	Luteal
RS 030	18	IIA	l: 5cm x 3cm x 2cm : sF / r: 5cm x 3cm x 3cm : sF	1,02	Non-luteal
RS 031	15	IIB	l: 5cm x 3cm x 2cm: CL (2,5cm) r: 4cm x 2cm x 2cm : NSS	2,45	Non-luteal
RS 032	1,5	IIA	l: 4cm x 2cm x 2cm : NSS r: 5cm x 2cm x 2cm : NSS	2,8	Non-luteal
RS 036	2,5	IIA	l: 5cm x 3cm x 4cm : NSS r: 5,5cm x 3cm x 3cm: F (2,5cm)	2,47	Non-luteal
RS 038	4,5	IIA	l: 6cm x 4cm x 4cm: CH r: 3,5cm x 3cm x 3cm: NSS	1,19	Non-luteal

RS 040	2	IIA	l: 6,5cm x 3,5cm x 2cm: sF r: 6,5cm x 4cm x 3cm: F(3cm)	0,56	Non-luteal
RS 041	2	I	l: 4cm x 3cm x 1,5cm: NSS r: 4cm x 2,5cm x 2,5cm: F(2,5cm)	4,38	Luteal
RS 042	2	IIA	l: 4,5cm x 3cm x 2cm: CL (1cm) r: 4cm x 3cm x 2cm: NSS	7,41	Luteal
RS 043	2	I	l: 6cm x 3cm x 3,5cm: CH r: 5cm x 3,5cm x 3cm: F(3cm)	45,56	Luteal
RS 045	4,5	IIB	l: 6cm x 6cm x 4cm: F (4cm) r: 4cm x 3cm x 3cm: NSS	1,27	Non-luteal
RS 047	4,5	I	l: 8cm x 6cm x 4cm: F (4,5cm) r: 5cm x 4cm x 3cm: CH (2cm)	23,31	Luteal
RS 048	4,5	IIB	l: 7cm x 5cm x 4cm: F (5cm) r: 3cm x 2cm x 2cm: NSS	1,31	Non-luteal
RS 049	5	IIA	l: 7cm x 4cm x 3cm: CL (2.5cm) r: 4cm x 3cm x 3cm NSS	26,12	Luteal
RS 050	1,5	IIA	l: 3.5cm x 2.5cm x 2.5cm NSS r: 5cm x 4cm x 3cm: F (3cm)	2,15	Non-luteal
RS 051	20	IIA	l: 7cm x 5cm x 4cm:F (5cm) r: 5cm x 3cm x 3cm: CL (2cm)	29,24	Luteal

RS 054	1,5	I	l: 2,5cm x 1,5cm x 1cm: NSS r: 4cm x 3cm x 3cm CH	51,85	Luteal
RS 055	3,5	IIA	l: 6cm x 4cm x 3cm: NSS r: 6cm x 5cm x 3cm: CL (2cm), F (2,5cm)	25,78	Luteal
RS 056	7	IIA	l: 8cm x 6cm x 4cm: F (6cm) r: 6cm x 4cm x 3cm: NSS	1,97	Non-luteal
RS 058	9	IIB	l: 5cm x 4cm x 3cm: NSS r: 7cm x 5cm x 4cm: F (4cm)	0,78	Non-luteal
RS 059	15	IIA	l: 5cm x 4cm x 2cm: CL (2,5cm) r: 4,5cm x 4cm x 2cm: NSS	8,84	Luteal
RS 060	15	IIB	l: 4cm x 3cm x 2cm F (2cm) r: 4cm x 3cm x 1,5cm: NSS	1,11	Non-luteal
RS 061	10	IIA	l: 5cm x 4cm x 3cm: NSS r: 5cm x 5cm x 4cm CL: (3cm)	29,09	Luteal
RS 063	15	I	l: 5cm x 4cm x 4cm: NSS r: 7cm x 4cm x 3cm: CL (2cm)	34,63	Luteal
Control C				1,67	Control

l= left ovary; r= right ovary; NSS= non significant structures (Follicles<1cm); CL= Corpus luteum; CH=Corpus hemorrhagicum; sF=small follicles (<2.5cm); F=follicles (≥2.5cm); Control C= control for PPC

Appendix 2

Equine Endometrial Biopsy Report

Mare: ***Section No:***

Quality of section: ☐ adequate ☐ non adequate

Artifact: cytoplasmatic blebbing: ☐ absent ☐ mild ☐ severe

Luminal contents: ☐ absent ☐ mild ☐ severe
☐ Neutrophils ☐ Eosinophils
☐ Mononuclear

comment

Luminal epithelium: ☐ cuboidal ☐ low columnar ☐ tall columnar

Exocitosis: ☐ absent ☐ mild ☐ severe
☐ Neutrophils ☐ Eosinophils
☐ Lymphocytes

comment

Str. Compactum:

Haemorrhage: ☐ absent ☐ mild ☐ severe
☐ focal ☐ multifocal ☐ diffuse

Oedema: ☐ absent ☐ mild ☐ severe

Inflammation: ☐ Neutrophils ☐ Eosinophils
☐ Lymphocytes Plasmacells
Macrophages

1. field: ***0 - absent*** ***1 - mild*** ***2 - severe***

2. field: ***1 - focal*** ***2 - multifocal*** ***3 – diffuse***

comment

Haemosiderin: ☐ absent ☐ mild ☐ severe

Str. Spongiosum:

Fibrosis: ☐ absent ☐ mild
☐ moderate ☐ severe
☐ focal ☐ multifocal ☐ diffuse

Inflammation: ☐ Neutrophils ☐ Eosinophils
☐ Lymphocytes ☐ Plasmacells
☐ Macrophages

1. field: ***0 - absent*** ***1 - mild*** ***2 - severe***

Eos clustered <15 normal

2. field: ***1 - focal*** ***2 - multifocal*** ***3 – diffuse***

comment

Congestion: ☐ absent ☐ mild ☐ severe

Haemorrhage: ☐ absent ☐ mild ☐ severe
☐ focal ☐ multifocal ☐ diffuse

Oedema: ☐ absent ☐ mild ☐ severe

Haemosiderin: ☐ absent ☐ mild ☐ severe

Glands:

Density: ☐ normal ☐ low

Nesting: ☐ absent ☐ mild nesting ☐ severe nesting

Branching: ☐ Yes ☐ No

Epithelium: ☐ cuboidal ☐ low columnar ☐ tall columnar

Exocitosis: ☐ absent ☐ mild ☐ severe ☐ Neutrophils ☐ Eosinophils ☐ Lymphocytes

comment

Ectasia/ cystic glands: ☐ absent ☐ mild ☐ severe ☐ focal ☐ multifocal ☐ diffuse

Content: ☐ absent ☐ mild ☐ severe ☐ Neutrophils ☐ Eosinophils ☐ Mononuclea***r***

Secretion: ☐ focal ☐ multifocal ☐ diffuse

comment

Blood vessels: ☐ normal ☐ mild thickened ☐ severe thickened

comment

☐ mild tortuous ☐ severe tortuous

Elastosis: ☐ absent ☐ mild ☐ severe
☐ Tunica intima ☐ Tunica adventitia
Sclerosis: ☐ absent ☐ mild ☐ severe
Leucostasis: ☐ absent ☐ mild ☐ severe
☐ focal ☐ multifocal ☐ diffuse
☐ Neutrophils ☐ Eosinophils
☐ Lymphocytes Monocytes

Lymphatics:

Dilatation: ☐ absent ☐ mild
☐ severe/lacuna
☐ focal ☐ multifoca ☐ diffuse

Stage of cycle:

Grade:

Appendix 3

Alignment of Equus caballus oxytocin receptor (OTXR), mRNA Sequence ID: ref|XM_001491665|and Homo sapiens oxytocin receptor (OXTR), mRNA Sequence ID: ref|NM_000916.3|

Range 1: 940 to 1792

Score	Expect	Identities	Gaps	Strand
1190 bits(644)	0.0	785/854(92%)	6/854(0%)	Plus/Plus

```
Equine 91    CGGG-CCGACCTGCTGTGCCGCCTGGTCAAGTACCTGCAGGTGGTGGGCATGTTCGCCTC  149
             ||||  ||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Human  940   CGGGCCCGACCTGCTGTGCCGCCTGGTCAAGTACTTGCAGGTGGTGGGCATGTTCGCCTC  999

Equine 150   CACCTACCTGCTGCTGCTCATGTCCCTCGACCGCTGCCTGGCCATCTGCCAGCCGCTGCG  209
             |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
Human  1000  CACCTACCTGCTGCTGCTCATGTCCCTGGACCGCTGCCTGGCCATCTGCCAGCCGCTGCG  1059

Equine 210   CGTGCTGCGCCGCCGCGTGGACCGCCTGGCCGTGCTCGCCACGTGGCTGGGCTGCCTGGT  269
             |  |||||||||||||   |||||||||||| |||||||||||||||||| ||||||||||
Human  1060  CTCGCTGCGCCGCCGCACCGACCGCCTGGCAGTGCTCGCCACGTGGCTCGGCTGCCTGGT  1119

Equine 270   GGCCAGCGCACCGCAGGTGCACATCTTCTCGCTGCGCGAGGTGGCCGAGGGCGTCTTTGA  329
             |||||||||  |||||||||||||||||||| ||||||||||||||| || |||||||| ||
Human  1120  GGCCAGCGCGCCGCAGGTGCACATCTTCTCTCTGCGCGAGGTGGCTGACGGCGTCTTCGA  1179

Equine 330   CTGCTGGGCTGTCTTCATCCAGCCCTGGGGGCCCAAGGCCTACGTCACGTGGATCACGCT  389
             |||||||||  |||||||||||||||||||| ||||||||||| |||| ||||||||||||
Human  1180  CTGCTGGGCCGTCTTCATCCAGCCCTGGGGACCCAAGGCCTACATCACATGGATCACGCT  1239

Equine 390   GTCCGTCTACATCGTGCCGGTCATCGTGCTCGCCGCCTGCTACGGCCTCATCAGCTTCAA  449
               | ||||||||||||||||||||||||||||| ||||||||||||||| ||||||||||||
Human  1240  AGCTGTCTACATCGTGCCGGTCATCGTGCTCGCTGCCTGCTACGGCCTTATCAGCTTCAA  1299

Equine 450   GATCTGGCAGAACTTGCGGCTCAAGAc-g--gcggcggcggccgctgaggggcccgaggg  506
             |||||||||||||||||||||||||||  |  || |||||||| || |||| ||| |||||
Human  1300  GATCTGGCAGAACTTGCGGCTCAAGACCGCTGCAGCGGCGGCGGCCGAGGCGCCAGAGGG  1359

Equine 507   cgcgtcggctggcagcgggggggcacgcggcTATGGCCCGCGTCAGCAGCGTCAAGCTCAT  566
             ||||  ||||||||   ||||||| || |||  |||| || ||||||||||||||||||||
Human  1360  CGCGGCGGCTGGCGATGGGGGGCGCGTGGCCCTGGCGCGTGTCAGCAGCGTCAAGCTCAT  1419

Equine 567   CTCCAAGGCCAAGATCCGCACGGTCAAGATGACCTTCATCATCGTGCTGGCCTTCATCGT  626
             |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Human  1420  CTCCAAGGCCAAGATCCGCACGGTCAAGATGACTTTCATCATCGTGCTGGCCTTCATCGT  1479

Equine 627   GTGCTGGACGCCATTCTTCTTCGTGCAGATGTGGAGCGTCTGGGACCCCAACGCGCCCAA  686
             ||||||||||| |||||||||||||||||||||||||||||  ||||||||||||||
Human  1480  GTGCTGGACGCCTTTCTTCTTCGTGCAGATGTGGAGCGTCTGGGATGCCAACGCGCCCAA  1539

Equine 687   GGAAGCCTCGGCTTTCATCATAGCCATGCTCCTGGCCAGCCTCAACAGCTGCTGCAACCC  746
             |||||||||||| |||||||| | ||||||||||||||||||||||||||||||||||||
Human  1540  GGAAGCCTCGGCCTTCATCATCGTCATGCTCCTGGCCAGCCTCAACAGCTGCTGCAACCC  1599

Equine  747   CTGGATCTACATGCTGTTCACGGGCCACCTCTTCCACGAACTCGTGCAGCGCTTCCTCTG  806
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Human  1600  CTGGATCTACATGCTGTTCACGGGCCACCTCTTCCACGAACTCGTGCAGCGCTTCCTGTG  1659

Equine 807   CTGCTCCTCCGGCTACCTGAAGGGCAGCCGGCCCGGG-GAGACGAGCGCCAGCAAGAAGA  865
             |||||||  || |||||||||||||||  || ||  ||| ||||||||  ||||||||  ||||
Human  1660  CTGCTCCGCCAGCTACCTGAAGGGCAGACG-CCTGGGAGAGACGAGTGCCAGCAAAAAGA  1718

Equine 866   GCAACTCGTCCACCTTTGTCCTGAGCGGGCACAGCTCCAGCCAGAAGAGCTGCTTGCAGC  925
             ||||||||||| |||||||||||||||    | |||||||||||||| ||||||||   ||||
Human  1719  GCAACTCGTCCTCCTTTGTCCTGAGCCATCGCAGCTCCAGCCAGAGGAGCTGCTCCCAGC  1778

Equine 926   CAGCCACTGTGTGA  939
             || |||| | ||||
Human  1779  CATCCACGGCGTGA  1792
```

Appendix 4

Equine endometrial biopsie report of mare with biopsy grade I

EQUINE ENDOMETRIAL BIOPSY REPORT

Mare: **S 1334.12** ***Section No: A***

Quality of section: adequate ☒ non adequate ☐

Artifact: cytoplasmatic blebbing: absent ☒ mild ☐ severe ☐

Luminal contents: absent ☒ mild ☐ severe ☐

Neutrophils ☐ Eosinophils ☐ Mononuclear ☐

comment

Luminal epithelium: cuboidal ☒ low columnar ☐ tall columnar ☐

Exocitosis: absent ☒ mild ☐ severe ☐

Neutrophils ☐ Eosinophils ☐ Lymphocytes ☐

comment

Str. Compactum:

Haemorrhage: absent ☒ mild ☐ severe ☐

focal ☐ multifocal ☐ diffuse ☐

Oedema: absent ☒ mild ☐ severe ☐

Inflammation: Neutrophils [1] [2] Eosinophils [ ] [ ] Lymphocytes [1] [3]

Plasmacells [ ] [ ] Macrophages [ ] [ ]

1.Feld: ***0 - absent 1 - mild 2 - severe***

2. Feld: ***1 - focal 2 - multifocal 3 – diffuse***

Comment

Haemosiderin: absent ☒ mild ☐ severe ☐

Str. Spongiosum:

Fibrosis:	absent	☒	mild	☐	moderate	☐	severe	☐
	focal	☐	multifocal	☐	diffuse	☐		
Inflammation:	Neutrophils	☐☐	Eosinophils	☐☐	Lymphocytes	☐☐		
	Plasmacells	☐☐	Macrophages	☐☐				
1.Feld:	***0 - absent***		***1 - mild***		***2 - severe***		***Eos clustered <15 normal***	
2. Feld:	***1 - focal***		***2 - multifocal***		***3 – diffuse***			

Comment
Multifocal nodules

Congestion:	absent	☐	mild	☒	severe	☐		
Haemorrhage:	absent	☒	mild	☐	severe	☐		
	focal	☐	multifocal	☐	diffuse	☐		
Oedema:	absent	☒	mild	☐	severe	☐		
Haemosiderin:	absent	☒	mild	☐	severe	☐		
Glands:								
Density:	normal	☐	low	☒				
Nesting:	absent	☒	mild nesting	☐	severe nesting	☐		
Branching:	Yes	☒	No	☐				
Epithelium:	cuboidal	☒	low columnar	☐	tall columnar	☐		
Exocitosis:	absent	☒	mild	☐	severe	☐		
	Neutrophils	☐	Eosinophils	☐	Lymphocytes	☐		

comment

Ectasia/ cystic glands:	absent	☐	mild	☒	severe	☐		
	focal	☐	multifocal	☐	diffuse	☒		
Content:	absent	☐	mild	☒	severe	☐		
	Neutrophils	☐	Eosinophils	☐	Mononuclear	☐		
	Secretion	☒						
	Focal	☐	Multifocal	☒	Diffuse	☐		

comment

Blood vessels: normal ☒ mild thickened ☐ severe thickened ☐

comment

mild tortuous ☐ severe tortuous ☐

Elastosis: absent ☒ mild ☐ severe ☐

Tunica intima ☐ Tunica media ☐ Tunica adventitia ☐

Sclerosis: absent ☒ mild ☐ severe ☐

Leucostasis: absent ☒ mild ☐ severe ☐

focal ☐ multifocal ☐ diffuse ☐

Neutrophils ☐ Eosinophils ☐ Lymphocytes ☐

Monocytes ☐

Lymphatics:

Dilatation: absent ☒ mild ☐ moderate ☐

focal ☐ multifocal ☐ diffuse ☐

Stage of cycle: early oestrus

Grade: I

Appendix 5

Equine endometrial biopsie report of mare with biopsy grade IIA

EQUINE ENDOMETRIAL BIOPSY REPORT

Mare: **S 1012.12** ***Section No: E2***

Quality of section: adequate ☒ non adequate ☐

Artifact: cytoplasmatic blebbing: absent ☐ mild ☒ severe ☐

Luminal contents: absent ☒ mild ☐ severe ☐

Neutrophils ☐ Eosinophils ☐ Mononuclear ☐

comment

Luminal epithelium: cuboidal ☒ low columnar ☒ tall columnar ☐

Exocitosis: absent ☐ mild ☒ severe ☐

Neutrophils ☒ Eosinophils ☐ Lymphocytes ☐

comment

Str. Compactum:

Haemorrhage: absent ☒ mild ☐ severe ☐

focal ☐ multifocal ☐ diffuse ☐

Oedema: absent ☒ mild ☐ severe ☐

Inflammation: Neutrophils [1 3] Eosinophils [ ] Lymphocytes [1 3]

Plasmacells [1 3] Macrophages [ ]

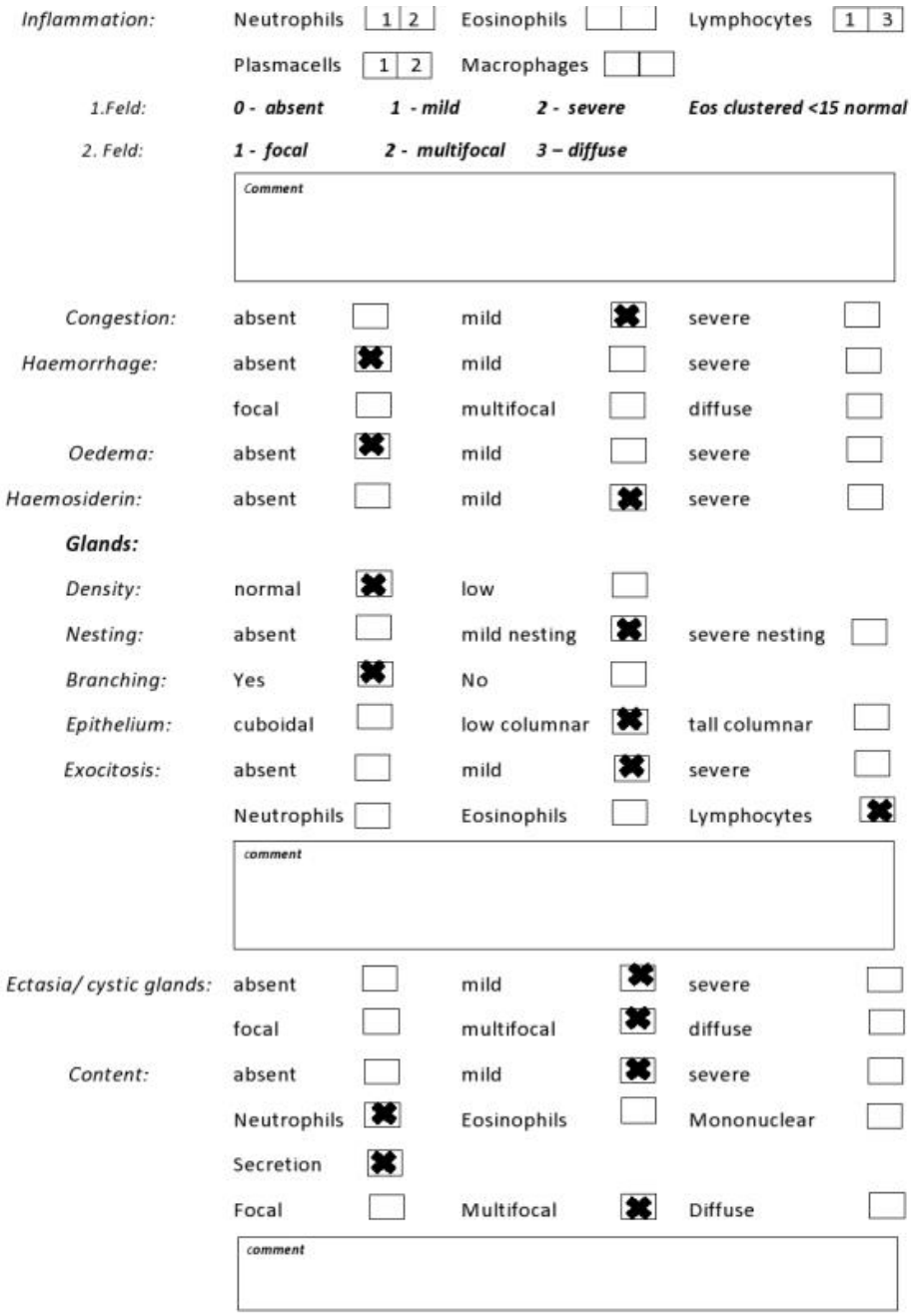
1.Feld: ***0 - absent 1 - mild 2 - severe***

2. Feld: ***1 - focal 2 - multifocal 3 – diffuse***

Comment

clustering

Haemosiderin: absent ☐ mild ☒ severe ☐


Inflammation:
Neutrophils 1 2
Eosinophils
Lymphocytes 1 3
Plasmacells 1 2
Macrophages
1.Feld: 0 - absent 1 - mild 2 - severe Eos clustered <15 normal
2. Feld: 1 - focal 2 - multifocal 3 – diffuse
Comment
Congestion: absent mild severe
Haemorrhage: absent mild severe
focal multifocal diffuse
Oedema: absent mild severe
Haemosiderin: absent mild severe
Glands:
Density: normal low
Nesting: absent mild nesting severe nesting
Branching: Yes No
Epithelium: cuboidal low columnar tall columnar
Exocitosis: absent mild severe
Neutrophils Eosinophils Lymphocytes
comment
Ectasia/ cystic glands: absent mild severe
focal multifocal diffuse
Content: absent mild severe
Neutrophils Eosinophils Mononuclear
Secretion
Focal Multifocal Diffuse
comment

Subintimal hyaline change, no thickening

	mild tortuous	☐	severe tortuous	☐		
Elastosis:	absent	☒	mild	☐	severe	☐
	Tunica intima	☐	Tunica adventitia	☐		
Sclerosis:	absent	☒	mild	☐	severe	☐
Leucostasis:	absent	☒	mild	☐	severe	☐
	focal	☐	multifocal	☐	diffuse	☐
	Neutrophils	☐	Eosinophils	☐	Lymphocytes	☐
	Monocytes	☐				

Lymphatics:

Dilatation:	absent	☐	mild	☒	moderate	☐
	focal	☐	multifocal	☒	diffuse	☐

small lacuni

Stage of cycle: late Dioestrus

Grade: *IIA*

Appendix 6

Equine endometrial biopsie report of mare with biopsy grade IIB

EQUINE ENDOMETRIAL BIOPSY REPORT

Mare: **S 1039.12** ***Section No: A1***

Quality of section: adequate ☒ non adequate ☐

Artifact: cytoplasmatic blebbing: absent ☐ mild ☒ severe ☐

Luminal contents: absent ☒ mild ☐ severe ☐

Neutrophils ☐ Eosinophils ☐ Mononuclear ☐

comment

Luminal epithelium: cuboidal ☒ low columnar ☒ tall columnar ☐

Exocitosis: absent ☐ mild ☒ severe ☐

Neutrophils ☒ Eosinophils ☐ Lymphocytes ☒

comment

Str. Compactum:

Haemorrhage: absent ☒ mild ☐ severe ☐

focal ☐ multifocal ☐ diffuse ☐

Oedema: absent ☒ mild ☐ severe ☐

Inflammation: Neutrophils 1 3 Eosinophils ☐☐ Lymphocytes 1 3

Plasmacells 1 3 Macrophages ☐☐

1.Feld: ***0 - absent 1 - mild 2 - severe***

2. Feld: ***1 - focal 2 - multifocal 3 – diffuse***

Comment

Haemosiderin: absent ☐ mild ☒ severe ☐

Inflammation:	Neutrophils	1 2	Eosinophils		Lymphocytes	1 2
	Plasmacells	1 2	Macrophages			
1.Feld:	***0 - absent***		***1 - mild***		***2 - severe***	***Eos clustered <15 normal***
2. Feld:	***1 - focal***		***2 - multifocal***		***3 – diffuse***	
	Comment Nodules of hypoplastic glands and Eosinophils					
Congestion:	absent	☐	mild	☒	severe	☐
Haemorrhage:	absent	☒	mild	☐	severe	☐
	focal	☐	multifocal	☐	diffuse	☐
Oedema:	absent	☒	mild	☐	severe	☐
Haemosiderin:	absent	☐	mild	☒	severe	☐
Glands:						
Density:	normal	☒	low	☐		
Nesting:	absent	☐	mild nesting	☒	severe nesting	☐
Branching:	Yes	☒	No	☐		
Epithelium:	cuboidal	☐	low columnar	☒	tall columnar	☐
Exocitosis:	absent	☐	mild	☒	severe	☐
	Neutrophils	☒	Eosinophils	☐	Lymphocytes	☒
	comment					
Ectasia/ cystic glands:	absent	☐	mild	☒	severe	☐
	focal	☐	multifocal	☒	diffuse	☐
Content:	absent	☐	mild	☒	severe	☐
	Neutrophils	☒	Eosinophils	☐	Mononuclear	☐
	Secretion	☒				
	Focal	☐	Multifocal	☒	Diffuse	☐
	comment					

Blood vessels: normal ☐ mild thickened ☒ severe thickened ☐

comment: Perivascular fibrosis

mild tortuous ☐ severe tortuous ☐

Elastosis: absent ☒ mild ☐ severe ☐

Tunica intima ☐ Tunica adventitia ☒

Sclerosis: absent ☒ mild ☐ severe ☐

Leucostasis: absent ☒ mild ☐ severe ☐

focal ☐ multifocal ☐ diffuse ☐

Neutrophils ☐ Eosinophils ☐ Lymphocytes ☐

Monocytes ☐

Lymphatics:

Dilatation: absent ☐ mild ☒ moderate ☐

focal ☐ multifocal ☒ diffuse ☐

Stage of cycle: late Dioestrus

Grade: *IIB*

LIST OF PUBLICATIONS

1. MSc Dissertation, 2016, Faculty of Veterinay Science, University of Pretoria.

ACKNOWLEDGEMENTS

I would like to take a moment to thank the people who have been instrumental in the completion of this research and thesis.

First and foremost, I want to thank my husband, Martino, for his support through thick and thin.

I want to thank my parents for their enduring support and encouragement for me from the very beginning.

I want to thank Dr. Annett Heise for her patience, guidance, and support for the past few years. She has been a great mentor.

I would like to thank Prof. Martin Schulman for his guidance and support throughout this research project.

I want to thank Prof. Johannes Handler for his support from the beginning and throughout the whole project.

I would like to thank Dr. Sarah Clift and her group at the Department of Pathology, FVS-UP.

I owe a huge debt of gratitude to Rephima Phaswane and Naomi Timmerman for their long hours and attention to detail in the processing of all of my samples.

I would like to thank Dr. Kgomotso Sibeko and her assitants Dr Mamohale Chaisi and Ms Thandeka Msezane at the Molecular Laboratory of the Department of Veterinary Tropical Diseases for their work with my samples.

I would like to thank Dr. Geoffrey Fosgate for his assistance with the statistical portion of this research and all his patience.

DECLARATION

I, Ruth Maria Ströhle, do hereby declare that the research presented in this dissertation was conceived and executed by myself and, apart from the normal guidance from my supervisors, I have received no assistance.

This dissertation is presented in fulfilment of the requirements for the degree Doctor of Veterinary Medicine (DVM).

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Ruth Maria Ströhle

Berlin

Date: 05.06.2018

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49,90 Euro | ISBN: 978-3-86387-923-5