Expression and distribution of oxytocin receptors in the cervix and uterus of the mare

by

Ruth Maria Stroehle

Submitted in partial fulfillment of the requirements for the degree of Magister Scientiae

in the

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Research conducted in the Section of Reproduction
Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Supervisor

Dr. Annett Heise
Section of Reproduction
Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Co-supervisors

Prof. Martin Schulman
Section of Reproduction
Department of Production Animal Studies
Faculty of Veterinary Science
University of Pretoria

Dr. Sarah Clift
Section of Pathology
Department of Paraclinical Sciences
Faculty of Veterinary Science
University of Pretoria

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LIST OF ABBREVIATIONS

ABC	Avidin-biotin-peroxidase complex method for IHC detection
BCS	Body condition score
BSA	Bovine serum albumin
cDNA	Complementary deoxyribose nucleic acid
CH	Corpus haemorrhagicum
CL	Corpus luteum
C_T	Cycle threshold
DAB	Diaminbenzidine
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
ds	Double strand
dsDNA	Double strand deoxyribonucleic acid
F	Follicle
FSH	Follicle stimulating hormone
FVS-UP	Faculty of Veterinary Science, University of Pretoria, Onderstepoort
GAPDH	Glyceraldehyde-triphosphate dehydrogenase
GnRH	Gonadotropin releasing hormone
GXR antibody	Polyclonal biotinylated goat anti-rabbit immunoglobulins (mainly IgG)
HE	Haematoxylin and eosin stain
IgG	Immunoglobulin G
IH	Immunohistology
IHC	Immunohistochemistry
LH	Luteinising hormone
mRNA	Messenger-ribonucleic acid
NSS	Non-specific structures (includes follicles up to 1cm)
OR	Oxytocin receptor
PBS	Phosphate buffered saline
PBS-BSA	Phosphate buffered saline with bovine serum albumin

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PCR	Polymerase chain reaction
$PGF_{2\alpha}$	Prostaglandin F2-*alpha*
PPC	Plasma progesterone concentration
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RPM	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
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

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SUMMARY

Expression and distribution of oxytocin receptors in the cervix and uterus of the mare

Ruth Maria Stroehle (rutistroehle@gmail.com)

Oxytocin is essential in the endocrine regulation of the oestrous cycle, during parturition, milk ejection and for myometrial contractility. It is well described that oxytocin acts on smooth muscles, including the myometrium and myoepithelia of mammary alveoli, stimulating contractions. Myometrial contractility is not only important for expulsion of the foetus during parturition in all species but also for the mechanical drainage of cellular debris and uterine fluid after mating especially in the mare. Failure to clear this intrauterine fluid post-breeding is a major cause of subfertility in the mare. Oxytocin furthermore stimulates the release of equine endometrial prostaglandin F2-*alpha* ($PGF_{2\alpha}$) and is thus proposed to be of importance in luteolysis.

The important role of oxytocin in both the oestrous cycle and in uterine contractility justifies the need to describe oxytocin receptors (ORs) in the endometrium, myometrium and cervix of mares. Studies have reported that the concentration of ORs in the equine endometrium changes throughout the oestrous cycle with a peak being reached during late dioestrus. There are, however, no reports comparing OR gene expression and distribution in the endometrium, myometrium and cervix of the non-pregnant mare. This study describes the distribution and density of ORs in the mare's endometrium, myometrium and cervix using immunohistochemistry (IHC) and quantitative reverse-transcription polymerase chain reaction (RT-qPCR), respectively.

Full-thickness uterine samples were obtained from 27 routinely-slaughtered, cyclic mares of various breeds and ages (range: 2 to 20 years) at three different uterine sites (uterine body, right and left horn) and one sample from the cervix. For IHC, all endometrial, myometrial and cervical samples were immunolabeled using an avidin-biotin-peroxidase complex (ABC) detection system and a polyclonal rabbit 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

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uterine horn (endometrium and myometrium) and the cervix (luminal epithelium, propria-submucosa and muscularis layer) were used for the RT-qPCR assay. Oligonucleotide primers and probe sequences used for detection of OR and β-actin gene expression were obtained from two previous studies evaluating ORs in the equine conceptus, foetal membranes and endometrium from pony mares at parturition. A RT-qPCR was used for detection of the 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 analysis of the data.

Immunohistochemistry showed ORs in both the uterus and cervix. Oxytocin receptors were specifically localised to the cytoplasm of the endometrial luminal and glandular epithelia, transmural vascular endothelium, sub-epithelial and peri-glandular stromal cells and smooth muscle cells of the myometrium. The greatest intensity of labeling occurred consistently in the vascular endothelium. There was a similar pattern of distribution and intensity of OR expression in the uterus and cervix, with the exception of the glandular epithelium which is absent in the cervix.

Relative to expression of OR gene in the endometrium, RT-qPCR analysis showed higher expression of the OR gene in the myometrium (4 times) and lower expression in the cervix (1/3). The statistical analysis demonstrated that the myometrium had significantly higher OR gene expression than both cervix ($P=0.001$) and endometrium ($P=0.009$). There was no significant difference in the gene expression of ORs when comparing cervix and endometrium ($P=1.0$)

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CHAPTER 1: Introduction

Oxytocin plays an essential role in the endocrine regulation of the oestrous cycle as well as in parturition and milk ejection in all domestic animals (Flint and Sheldrick, 1986; Ginther 1992). It is a neuropeptide hormone which is mainly produced in the hypothalamus. From there it arrives via neuroaxial transport at the posterior pituitary gland where it is stored in secretory vesicles (Stull 1986). In several species including domestic ruminants it is also synthesised in the corpus luteum (CL) but this has not been reported in mares (Stevenson et al. 1991; Stock et al. 1995; Watson et al. 1999). The endometrium was shown to be a source of oxytocin in the mare (Behrendt et al. 1997; Bae and Watson, 2003).

It is well described that oxytocin acts on smooth muscles, e.g. in the myometrium and in the myoepithelia of mammary alveoli, stimulating contractions (Gimpl and Fahrenholz, 2001). Myometrial contractility is not only important for expulsion of the foetus during parturition in all species but also for mechanical drainage of cellular debris and uterine fluid after mating especially in the mare (Nikolakopoulos and Watson, 1999). Failure to clear this intrauterine fluid post-breeding is a major cause of equine subfertility (Pycock and Newcombe, 1996b). Oxytocin furthermore stimulates the release of equine endometrial prostaglandin F2-*alpha* ($PGF_{2\alpha}$) and is thus purportedly important in luteolysis, however, its exact role is currently undefined (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993).

The important role of oxytocin in both the oestrous cycle and in uterine contractility justifies the need to describe oxytocin receptors (ORs) in the endometrium and myometrium of mares. Studies have reported that the gene expression of ORs in the equine endometrium changes throughout the oestrous cycle with a peak during late dioestrus (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). There are only reports describing OR quantity in the endometrium and myometrium; none are available comparing and describing receptor density and distribution in endometrium, myometrium and cervix.

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The objectives of the current study were to:

- Describe the distribution of ORs in the endometrium, myometrium and cervix of non-pregnant mares using Immunohistochemistry (IHC)
- Quantify levels of messenger-ribonucleic acid (mRNA) transcribed from the OR gene in the endometrium, myometrium and cervix of non-pregnant mares using quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

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CHAPTER 2: Literature Review

2.1 Equine uterine and cervical anatomy and histology

The equine uterus has three major parts: the uterine cervix, the uterine body and the uterine horns (Kainer 1993). The horns are approximately 20 - 25 cm in length, the uterine body 18 - 20 cm in length and the uterine cervix is 5 - 7 cm long (Sisson 1975).

The wall of the uterus consists of three tissue layers: endometrium, myometrium and serosa. Histologically, the endometrium contains simple columnar epithelium with cuboidal to tall columnar cells and tubular glands (Kenney 1978). In the myometrium, an inner circular layer of smooth muscle, a middle vascular layer and an outer longitudinal layer of smooth muscle can be found (Kainer 1993).

The microscopic anatomy of the uterus changes due to hormonal influences throughout the oestrous cycle (Kainer 1993). Diffuse epithelial hyperplasia in the endometrium was observed during oestrus, together with an increase in stromal oedema, while an apparent increase in the density of endometrial glands was described during dioestrus (Kenney 1978; Kenney and Doig, 1986).

The cervix is thick-walled and traversed by the cervical canal, which extends from the internal to the external cervical orifice. The wall contains collagenous connective tissue and a sphincter of smooth muscle derived from the inner layer of the myometrium and mucosa (Sisson 1975). The smooth muscle is formed by two layers: an inner circular and an outer longitudinal layer (Sertich 2011). The cervical canal of mares is lined with a simple columnar epithelium. The mucosa is branched to primary, secondary and tertiary folds and the glandless lamina propria mucosae contains vasculature (Katila 2012).

2.2 Oestrous cycle in the mare

Mares are seasonally polyoestrous with multiple oestrous cycles during the summer months and an anoestrous period of non-cyclicity in winter (Daels 1993). The beginning and the end of the anoestrus period is largely dependent upon photoperiod or day length. The effect of photoperiod occurs through release of melatonin during periods of darkness. Melatonin inhibits secretion of gonadotropin

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releasing hormone (GnRH) from the hypothalamus in the mare (Ginther 1992). As the day length increases, the duration of melatonin secretion decreases, thus leading to an increase in GnRH release. In response, the anterior pituitary gland increases secretion of gonadotropins and follicular activity begins (Paccamonti and Lyle, 2010).

The oestrous cycle is defined as the period from one ovulation to the next and consists of two phases in the mare: the follicular (oestrus) and luteal (dioestrus) phase (Blanchard and Schumacher, 2003). The length of the oestrous cycle ranges from 18 - 24 days (average = 21 - 22 days) although the length of oestrus is variable (2 - 12 days), averaging 7 days but is dependent on the time of year (Ginther 1992). The length of dioestrus remains relatively constant at 14 - 15 days and is less affected than oestrus by season (Blanchard and Schumacher, 2003).

The oestrous cycle of the mare is influenced by different hormones. The neurosecretory cells of the hypothalamus produce GnRH, which stimulates the anterior pituitary gland to produce gonadotropins: follicle stimulating hormone (FSH) and luteinising hormone (LH). During oestrus, FSH stimulates follicular growth and development of LH receptors on follicular granulosa cells. Luteinising hormone is of importance for follicle maturation, oestrogen production and ovulation (Ginther 1992). High concentrations of oestrogen and low concentrations of progesterone lead to increasing oedema in the uterine wall in combination with an opening of the cervix and flattening of the uterus and vagina (Aurich 2011). It also facilitates oestrous behaviour of the mare characterised by increased interest in stallions and proceptive behaviour in response to the sexual attractiveness of a stallion (Crowell-Davis 2007; Aurich 2011). Ovulation takes place 24 - 48 hours before the end of oestrus (Daels 1993). The corpus luteum (CL) that develops after ovulation starts secreting increased amounts of progesterone (Daels 1993). Increased levels of progesterone feed back negatively on the pituitary gland causing decreased LH secretion (Miller et al. 1980). During dioestrus, the uterine wall shows mild contractility and the cervix is firmly closed. The mare is behaviourally unreceptive to the stallion. Towards the end of dioestrus, the CL regresses due to luteolysis by prostaglandin F2-*alpha* ($PGF_{2\alpha}$), and the mare returns to oestrus in 1 - 3 days (Daels 1993).

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2.3 Oxytocin

Oxytocin is a neuropeptide hormone mainly synthesised in the hypothalamus and stored in secretory vesicles of the posterior pituitary gland (Stull 1986). Oxytocin is produced by the supraoptic, paraventricular and arcuate nuclei of the hypothalamus as part of a large precursor molecule. This molecule is split into neurophysin and oxytocin while being transported to the posterior pituitary gland from where it is released into the bloodstream (Gainer et al. 1985). There are many reports describing the local synthesis of oxytocin within the female reproductive tract, particularly the ovary (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 ovary of the mare does not seem to be a source of oxytocin (Stevenson et al. 1991; Stock et al. 1995; Watson et al. 1999).

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

Oxytocin causes contractions of smooth muscles in the oviduct, uterus and the myoepithelial cells of the mammary gland. Increased uterine contractility supports uterine defence by stimulating clearance of the uterine lumen especially during oestrus (Pycock and Newcombe, 1996a; Pycock and Newcombe, 1996b). Oxytocin additionally stimulates the release of equine endometrial $PGF_{2\alpha}$ and is therefore proposed to be of importance in luteolysis (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). Its exact role, however, remains undefined.

2.4 Oxytocin and the endocrine regulation of the oestrous cycle

The oestrous cycle of the mare relies on a delicate balance of hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries and the uterus (Evans et al. 1997). The neurosecretory cells of the hypothalamus produce GnRH, which stimulates the anterior pituitary gland to produce gonadotropins: FSH and LH. Follicle stimulating hormone and LH are released into the bloodstream and act at the level of the ovaries. Follicle stimulating hormone is responsible for follicular recruitment and growth and it stimulates development of LH receptors on granulosa

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cells within the dominant follicle. Luteinising hormone is of importance for follicle maturation, oestrogen production, ovulation, and luteinisation of the CL (Ginther 1992).

The CL secretes progesterone throughout dioestrus and during the early phase, approximately the first 75 days, of pregnancy (Morel 2008). Progesterone plays an important role during pregnancy in the uterus, preparing it to host a foetus (Squires 1993). This includes enabling uterine secretions important for the nutrition of the early conceptus (Squires 1993), an effect on mobility, fixation and orientation of the equine embryonic vesicle and causing uterine quiescence and tight closure of the cervix (Kastelic et al. 1987). During dioestrus its main role is to exert a negative feedback on the hypothalamus, preventing LH release in order to prevent ovulation (Miller et al. 1980). The CL in the horse is maintained until luteolysis occurs via endogenous release of $PGF_{2\alpha}$ from the uterine endometrium; normally between 13 - 16 days post-ovulation (Ginther 1992). Prostaglandin F2-*alpha* is a hormone synthesised in different organs and tissues, including the endometrium and has an important influence on the oestrous cycle (Poyser 1973). Release of $PGF_{2\alpha}$ from the endometrium results in luteolysis (Ginther 1992). Prostaglandin F2-*alpha* is thus known as the luteolytic agent in mares. In addition $PGF_{2\alpha}$ also acts as a potent myometrial stimulant causing myometrial contractions (Ginther 1992). Progesterone, oestrogen and oxytocin control the release of $PGF_{2\alpha}$ from the endometrium in mares (Ginther 1992).

The importance of oxytocin in the oestrous cycle can be summarised as follows:

1. it apparently stimulates the release of equine endometrial $PGF_{2\alpha}$ and might therefore be of importance in luteolysis (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). Its exact role remains undefined and
2. it increases uterine motility and thus supports uterine defence mechanisms by stimulating clearance of the uterine lumen especially at the time of oestrus (LeBlanc et al. 1994; Pycock and Newcombe, 1996a; Pycock and Newcombe, 1996b)

2.5 Oxytocin and luteolysis

The events that control luteolytic $PGF_{2\alpha}$ release are less clearly defined in mares than in ruminants. In ruminants, oxytocin secreted by the CL (Wathes and Swann,

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1982; Wathes et al. 1983) plays an important role in cyclical luteolysis because it stimulates the release of $PGF_{2\alpha}$ from the endometrium at the end of dioestrus (Flint and Sheldrick, 1986). Upregulation of oxytocin receptors (ORs) in the endometrium with a subsequent increase in uterine oxytocin responsiveness is a principal factor for the onset of luteolysis in ruminants (Mirando et al. 1993; Wathes et al. 1996). Oxytocin stimulates endometrial $PGF_{2\alpha}$ synthesis as well as release and, in turn $PGF_{2\alpha}$ induces further release of oxytocin from the CL (Flint and Sheldrick, 1982) and hypothalamus (Silvia et al. 1991).

In the mare, there is reported evidence that oxytocin is also involved 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). Stout et al. (1999) reported that continuous, high-dose systemic administration of oxytocin from 8 days after ovulation prevents luteolysis. This supports the previous hypothesis on involvement of oxytocin in luteolysis as it is likely that the treatment causes downregulation of ORs and thus suppression of the normal dioestrous rise in endometrial ORs which would have caused oxytocin-induced $PGF_{2\alpha}$ release.

Initial oxytocin secretion causing endometrial $PGF_{2\alpha}$ release probably originates from the hypophysis (Tetzke et al. 1987). There is no significant luteal oxytocin synthesis in the mare but the horse is the only domestic species where oxytocin production has been localised in the endometrium by immunostaining and immunogold labeling of endometrial biopsies (Bae and Watson, 2003). Thus the current assumption is that there is a paracrine-autocrine system involving endometrial oxytocin and $PGF_{2\alpha}$ release, which accelerates luteolysis in the non-pregnant mare (Aurich 2011).

2.6 Oxytocin receptors

Oxytocin needs specific receptors in different tissues to exert its effects (Gimpl and Fahrenholz, 2001). Morphologically, the OR is a seven domain trans-membrane molecule structurally integrated into the cell membrane. It belongs to the rhodopsin-like superfamily of G protein-coupled receptors (Gimpl et al. 2008). Oxytocin binding to the ORs leads to an increase in the intracellular calcium concentration, activates

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several protein kinases and also induces secretion of prostaglandins in domestic ruminants and mares (Gimpl and Fahrenholz, 2001; Gimpl et al. 2008).

Oxytocin receptors have been identified in the uterus of mares with the aid of modified radioligand-binding assays and reverse-transcription polymerase chain reaction (RT-PCR). Stull (1986), Sharp et al. (1997) and Vanderwall et al. (2012) used a modified Bio-Rad assay for characterisation of oxytocin binding sites previously used in the rat oviduct whereas Starbuck et al. (1998) made use of the sheep OR assay of Sheldrick and Flint (1985), which was modified and validated for use in equine oviducts by Benfield (Starbuck et al. 1998). Villani et al. (2008) and Palm et al. (2013) were the first to use RT-PCR for evaluation of ORs in the uterus of the mare.

Location of ORs within the uterine tissue is species-dependent. In the rat (Soloff et al. 1977) and sow (Soloff and Swartz, 1974), oxytocin binding-sites were only found in the myometrium, whereas in the ewe (Roberts et al. 1976) and woman (Fuchs et al. 1985), binding sites were found in the endometrium and myometrium. Evaluation of binding sites was done by using subcellular distribution of radioactivity from [Tyrosyl-2, 6-^{3}H] oxytocin ([3H]oxytocin). The mare is similar to both the ewe and woman whereby the concentration of the binding-sites is higher in the myometrium than in the endometrium and seems to fluctuate throughout the oestrous cycle (Stull 1986). The lowest OR density in the endometrium of mares was observed during oestrus and 8 days after ovulation (Sharp et al. 1997) and the highest concentration was found in late dioestrus, between day 14 - 18 (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). Neither Sharp (1997) nor Starbuck (1998) investigated the presence of ORs in the myometrium.

Several studies reported on the concentration of ORs in the uterus in other species including cows, sows and ewes. As in the mare, expression of OR mRNA in the endometrium and myometrium of cows is also regulated cyclically. The number of ORs seems to be significantly up-regulated around the time of oestrus (Meyer et al. 1988; Fuchs et al. 1990; Jenner et al. 1991; Robinson et al. 2001). A similar finding was reported in ewes (Wathes and Swann, 1982; Ayad et al. 1991; Stevenson et al. 1994) and sows using a radioreceptor assay (Okano et al. 1996). Oxytocin receptors have also been detected in the ovine and bovine cervix during oestrus (Matthews

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and Ayad, 1994; Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013). Oxytocin receptors in the equine cervix have not been described.

2.7 Oxytocin receptor detection assays

The OR was first identified using a radioligand-binding assay in the myometrium of the rat (Soloff and Swartz, 1973) where [3H]oxytocin was used as the radioligand and for many years studies on ORs in different tissues and species used this radioligand-binding assay or a modification thereof (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). The binding specificity of [3H]oxytocin is dependent on time, temperature and membrane protein concentration (Tahara et al. 2000) which renders this method as fairly unstable.

Ivell et al. (1995) established the reverse-transcription Polymerase chain reaction (RT-PCR) for ORs in the uterus of bovines. This method was used afterwards by many other studies investigating a variety of 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). Villani et al. (2008) were the first to use RT-PCR for evaluation of ORs in the equine uterus. Currently RT-PCR has been recognised as the method of choice for OR detection and evaluation of concentration being described as “the enabling technology par excellence of molecular diagnostics” (Bustin et al. 2009).

2.8 Laboratory Methodologies

2.8.1 Polymerase chain reaction (PCR)

Polymerase chain reaction, invented in 1983 by Kary Mullis at the Cetus Corporation, offers a simple process to massively enrich a targeted fragment of deoxyribonucleic acid (DNA) (Watson 2003).

Improvements in PCR methods led to the development of RT-PCR. In contrast to conventional PCR which measures the amount of accumulated PCR product at the end of the PCR cycle (Bustin 2000; Kubista et al. 2006), RT-PCR enables the

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monitoring of the progress of the amplification of the target DNA as it occurs (i.e. in real time). Data are collected throughout the amplification process, rather than just at the end. This completely revolutionised the approach to PCR-based quantification of DNA and ribonucleic acid (RNA). Reverse-transcription PCR can be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples. In the case of RNA quantification, the template is complementary DNA (cDNA), which is obtained by reverse transcription of RNA. In the latter instance the technique used is quantitative RT-PCR or RT-qPCR.

The RT-qPCR can be seen as a definitive technique for quantification of differences in gene expression levels between samples (Taylor et al. 2001). The quantification can be relative or absolute. Absolute quantification is required when a precise quantity of amplicon is desired, for example, calculation of viral load (Niesters 2001) and in relative quantification, the RT-qPCR data is presented relative to another gene often referred to as an internal control or reference gene. It is important that the reference is a housekeeping/endogenous control gene (such as glyceraldehyde-triphosphate dehydrogenase (GAPDH), β- actin, or a ribosomal ribonucleic acid (rRNA) gene) whose expression is not influenced by the experimental conditions (Muller et al. 2002). In experiments where the estimation of gene expression is crucial, it was recommended that the reverse transcription step be carried out in duplicate or triplicate and that the total RNA concentration be standardised for all samples to improve on the accuracy of measured results (Stahlberg et al. 2004) .

Reverse-transcription polymerase chain reaction has previously been used to investigate ORs in the uterus of several species (Bathgate et al. 1995; Feng et al. 2000; Beretsos et al. 2006; Siemieniuch et al. 2011) including the endometrium of horses (Villani et al. 2008). No comparative quantification of OR gene expression in the uterus and cervix in any species has been reported.

2.8.2 Immunohistochemistry (IHC)

Immunohistochemical techniques have been used since Coons et al. (1941) published their landmark paper describing the identification of tissue antigens using a direct fluorescence protocol. Immunohistochemistry enables the visualisation (using light or confocal microscopy) of the tissue distribution of specific antigens (or

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epitopes). The process localises protein targets of interest by applying specific monoclonal or polyclonal antibodies to tissue surfaces in a process called antibody incubation. There are different detection systems employing direct or indirect methods. Indirect methods are more sensitive than direct ones due to the incorporation of signal amplification techniques. One of the still commonly used indirect methods is the avidin-biotin-peroxidase complex (ABC) method. Avidin is a large glycoprotein extracted from egg white with four binding sites per molecule and it has a high affinity for the vitamin biotin. Biotin has one binding site for avidin and multiple binding sites for attachment to antibodies or other macromolecules (Ramos-Vara 2005). Multiple binding sites on the molecules result in the amplification of the tissue signal which is useful when trying to detect low copy number antigens (Haines and Chelack, 1991).

Immunohistochemistry used to be simply a “special stain” which was qualitatively evaluated by pathologists. With the use of IHC for demonstration of prognostic tumour markers in human and now also in veterinary pathology, the demand for quantification has rapidly increased. There are, however, still several significant limitations for the application of IHC as a quantitative assay (Taylor and Levenson, 2006). Standardisation of the total assay would be necessary in order to achieve properly quantifiable results (Grube 2004). This remains problematic, especially with regard to variables associated with sample preparation (in particular transportation, tissue fixation and sectioning) as the standardisation of these processes within and between IHC laboratories tends to be poor (Grube 2004; Taylor and Levenson, 2006). In addition, the methodology of IHC assays incorporates a number of steps which have not yet been uniformly standardised within and between veterinary and human IHC laboratories. These non-standardised components include: variable antigen retrieval methods, variable specificity of commercially available polyclonal primary antibodies, diverse selection of chromogens and detection systems. The degree to which any particular antigen is retrieved with reference to the absolute quantity of antigen, post-fixation, is entirely unknown (Taylor and Levenson, 2006). Polyclonal antibodies have different specificities between batches, but the quantity of antibody per batch is often limited to 200 – 400 µg, which contributes to variations in the assay, especially over a large number of test sections (Griffiths and Lucocq, 2014). Two chromogens that are commonly employed for IHC include 3,3'-

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Diaminobenzidine (DAB) and Vector Red. Important, but infrequently considered, is that the optical properties of different chromogens will affect the linearity and dynamic range of the assay, which in turn affects the accurate quantification of labeling intensity in test sections.

To date, IHC has been used in a variety of animal species including the horse to detect the whereabouts of the ORs (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) evaluated ORs in equine conceptuses between day 10 and 16 of pregnancy. Palm et al. (2013) observed ORs in the endometrium of pony mares at parturition, and the authors identified ORs in the cytoplasm of endometrial luminal and deeper glandular epithelium. No combined description of OR distribution in the equine endometrium, myometrium and cervix is currently reported, despite mare fertility being extensively researched over the past 40 years.

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CHAPTER 3: Materials and Methods

3.1 Study population and design

Twenty-seven cyclic mares of various breeds consigned for routine slaughter were included in this study and were selected based on their estimated age (> 2 years) and non-pregnant status. Mares are considered to be potentially cycling after passing puberty at 12 - 24 months of age (Evans et al. 1997). Mares were routinely slaughtered using the stunning method according to international accepted standards at the Randfontein Abattoir (Plot 114 Middelvlei, Randfontein, 1764).

The uterus, cervix, ovaries and associated broad ligament of all mares were retrieved at the slaughter processing line and used for further investigation. This enabled a complete gross anatomical inspection of the internal reproductive organs and retrieval of full-thickness tissue samples from the uterus and cervix for further evaluation and processing.

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

3.2 Collection and Preparation of Samples

3.2.1 Preliminary inspection of mares and blood sampling

Before slaughter, all the mares were individually identified by coat colour and their age was estimated via oral inspection of their dentition (Martin et al. 1999). The body condition score (BCS) of each mare was evaluated based on a 9 (1-9) point scale developed by Henneke et al. (1983; **Appendix 2**). A blood sample was obtained at slaughter during exsanguination. Heparin vacuum tubes (5 ml; Vacutainer) were used to collect 5 ml of blood per horse.

3.2.2 Uterine samples

The entire uterus, cervix, ovaries, oviducts and broad ligament were retrieved from each mare included in the study. Uteri of the slaughtered mares were identified individually by plastic tags (cow ear tag marked with barcodes) that were applied to

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the dorsal aspect of the tip of the right uterine horn at the processing line in the abattoir in order to ensure that each uterus corresponded with the correct mare.

All reproductive tracts were collected ≤ 1 hour after death and placed on ice in a cooler box. Cooler boxes were transported to the Faculty of Veterinary Science, University of Pretoria, Onderstepoort (FVS-UP) for further processing within 2 hours following slaughter.

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

Both ovaries were measured using a ruler, dissected using a scalpel blade (No 21) and inspected to describe ovarian size and ovarian structures 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).

A scalpel blade (No 21) and surgical forceps were used to obtain full-thickness tissue samples of a predetermined size (base of both uterine horns, middle of uterine body and cervix as described below) for polymerase chain reaction (PCR) and immunohistochemistry (IHC) from four standardised areas of the uterus. Samples were obtained from the left and right base of the uterine horns, the uterine body and the cervix, respectively, as shown in **Figure 1**.

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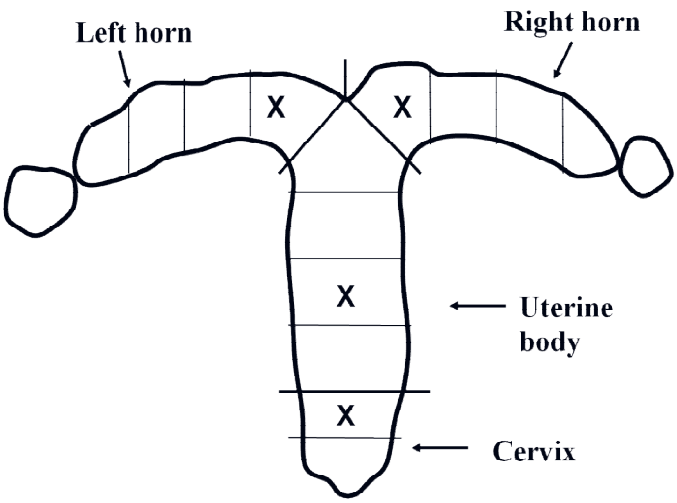

Figure 1 Diagrammatic representation of sample collection areas in the uterus and cervix

3.3 Laboratory analysis

3.3.1 Blood samples

Blood samples were placed in a cooler box on ice and transported to the FVS-UP for analysis. On arrival, samples were centrifuged at 3000 revolutions per minute (RPM) for 15 minutes at 25°C and the serum stored at -18°C in barcode-labeled 5 ml plastic sample tubes until analysis. 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) according to the manufacturer's instructions. All samples were run in one batch on one day. The Coat-a count kit included seven vials of progesterone calibrators which were used to determine a standart curve. All samples were read against this curve and calibrator sample C was used as a standard control for assay validation purposes. 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).

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3.3.2 Immunohistochemistry (IHC)

3.3.2.1 Test samples

The samples utilised for IHC were full-thickness samples of 20 × 20 x 5 mm that were fixed in 10% neutral buffered formalin overnight, embedded in paraffin and sectioned at a thickness of 4 µm by the laboratory staff, Section of Pathology, FVS-UP according to the in-house DAFF-accredited standard operating procedures (SOPs) in the Histopathology Laboratory.

3.3.2.2 Primary and secondary antibodies

The polyclonal rabbit oxytocin receptor (OR) antibody directed against the N-terminal extracellular domain of the human OR (Code No. O4389, Sigma-Aldrich, Steinheim, Germany) was used as the primary antibody. It was successfully used in two previous studies on horse tissues (Budik et al. 2012, Palm et al. 2013). In addition, the sequences of the human and horse ORs obtained by PubMed nucleotide database search were compared (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi, accessed 20 June 2012; see accession numbers in **Appendix 3**). They were found to be quite substantially (92 %) homologous (see **Appendix 3**). The secondary antibody, used for immunolabeling, was a polyclonal biotinylated goat anti-rabbit (GXR) predominantly IgG antibody (Code No. E0432, Dako, Denmark).

3.3.2.3 Immunoperoxidase labeling procedure

Tissue sections were subjected to the avidin-biotin-peroxidase complex (ABC) detection system (Vectastain Elite ABC standard kit, Code No. PK-6100, Vector Laboratories, Burlingame, CA, USA) routinely employed by the Immunohistochemistry Laboratory in the Section of Pathology at FVS-UP, adapted from the methodology reported by Haines and Chelack (1991). For each uterus, four sections, consisting of three full-thickness samples of different regions in the uterus (see **Figure 1**) and one sample of cervix were labeled using the abovementioned ABC system. The 4 µm-thick tissue sections were mounted on positively-charged microscope glass slides (SuperFrost® Plus, Menzel-Glaser, Portsmouth, New Hampshire, USA) to enhance tissue adhesion. Sections were then dried overnight in an oven at 38 - 40°C to further enhance tissue adhesion. The IHC was performed

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manually by the same properly trained and highly experienced technologist in different staining batches over the period of a month. The IHC technique was performed strictly in accordance with DAFF-accredited SOPs in the IHC laboratory, Section of Pathology, Department of Paraclinical Sciences, FVS-UP, which were developed according to the most recent guidelines employed by the American Association of Veterinary Laboratory Diagnosticians (Ramos-Vara et al. 2008). Routine dewaxing and rehydration was done according to prescribed methods (Bancroft and Gamble, 2002). Thereafter, sections were incubated with 3 % hydrogen peroxide for 15 minutes in order to quench endogenous peroxidase activity. For antigen retrieval purposes the samples were placed in a Protease solution consisting of 0,05 mg Protease Type XIV powder in 100 ml PBS-BSA buffer (0.1 molar phosphate buffered saline/PBS, pH 7.6 containing 0.1 % bovine serum albumin/BSA) for 30 minutes in an oven at 38 - 40°C (Protease Type XIV isolated from *Streptomyces griseus*, Code No. P5147-5G, Sigma-Aldrich, St. Louis, Missouri, USA). After washing with PBS-BSA buffer (pH 7.6) for 10 minutes at room temperature, normal goat serum (Code No. G9023, Sigma-Aldrich, St Louis, Missouri, USA), diluted 1:10 with PBS-BSA buffer was incubated with each tissue section in a humidified chamber at room temperature for 20 minutes in order to block non-specific protein binding. Thereafter sections were incubated with the polyclonal rabbit OR antibody (Code No. O4389, Sigma-Aldrich, Steinheim, Germany), diluted 1:400 overnight. Post-incubation with the primary antibody, and following immersion in PBS-BSA buffer for 10 minutes, sections were incubated with the biotinylated secondary polyclonal GXR antibody (Code No. E0432, Dako, Denmark) at a dilution of 1:500 for 30 minutes at room temperature. After an additional 10 minutes in PBS-BSA buffer, sections were incubated with the AB Complex Elite kit (per manufacturer's instructions) for 30 minutes. The Vector Nova-Red substrate (Code No. SK4800, Burlingame, Canada) was first attempted for development of colour during the optimisation process but the labeling proved to be overly sensitive to laboratory variables (introduced during the manual staining process) and consistent staining could not be achieved during the optimisation process. Therefore, the brownish colour visualised in tissue sections was developed via incubation of the sections with the liquid DAB+ Substrate Chromogen System (Code No. K3468, Dako, Denmark) for approximately 3 minutes, before the sections were counterstained with Mayer's hematoxylin for 20 seconds, rinsed with water for 10

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minutes, routinely dehydrated through increasing alcohol concentrations and xylol, mounted using Entellan™ new (Code No. 10796, Merck Millipore, Darmstadt, Germany) and coverslipped for examination under the light microscope. Evaluation of the slides was done by the primary investigator, together with an IHC-experienced veterinary pathologist, using an Olympus BX43 microscope and the Olympus cell sens dimension software (Wirsam Scientific & Precision Equipment PTY LD, Johannesburg, South Africa). The purpose of the software was to enable quantification of positive labeling in sections. Positive labeling was identified as being brown in colour and occurring in a finely cytoplasmic granular to cytoplasmic diffuse pattern in reported target cells, namely endometrial luminal and superficial glandular epithelium and vascular endothelium (Palm et al. 2013). Labeling was expected to be especially strong towards the apical (secretory) surface of the epithelial cells (Palm et al. 2013).

3.3.2.4 Controls

3.3.2.4.1 Negative reagent controls

For negative reagent control purposes, per entire batch of sections stained per day, two additional sections of uterine horn or body were cut from one test case (selected at random) and these sections were treated in exactly the same way as the other tissue sections. However, instead of applying the anti-human OR affinity purified polyclonal primary antibody (produced in rabbit), an irrelevant polyclonal rabbit antibody (S100 at a dilution of 1:400, incubated overnight) and phosphate buffered saline with bovine serum albumin (PBS-BSA) buffer solution were applied to the two designated tissue sections in order to investigate the degree of cross-reactivity of the primary antibody and the extent of non-specific binding by the link (secondary) antibody and detection system (Ramos-Vara et al. 2008). The S100 polyclonal rabbit (irrelevant) antibody (Code No. Z 0311, Dako, Denmark) used in the present study labels an isoform of an S100 protein (of which there are at least 21 different types) that is largely restricted to cells of neuroectodermal origin. According to the data specification sheet (specifically pertinent to human tissues), target cells include melanocytes, neurons, glial cells of the central and peripheral nervous systems, schwann cells, as well as dendritic macrophages, chondrocytes, adipocytes, fibroblasts, myoepithelial cells, keratinocytes, etc. In veterinary diagnostic

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immunohistochemistry laboratories, S100 is most often used together with a number of other antibodies in order to try to confirm or exclude a diagnosis of melanocytic neoplasia in domestic species, including horses, especially the amelanotic or poorly melanised ones, and it is also frequently used as part of a panel to try to detect a variety of neural tumours in domestic species, e.g. peripheral nerve sheath tumours in dogs and horses (Ramos-Vara et al. 2014; Bogaert et al. 2011).

3.3.2.4.2 Tissue controls

The positive-tissue control was a section of the uterus from a mare in mid to late dioestrus which was therefore expected to have an increased expression of ORs according to a number of investigators (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). Optimisation of immunolabeling was performed on this tissue until the labeling was considered to be specific and reasonably strong with minimal background labeling**.** Positive internal tissue controls included vascular endothelial cells and endometrial luminal and glandular epithelial cells (Palm et al. 2013). Sections of the uterus of a 6 month-old foal were used for negative tissue control purposes as an immature uterus was expected to have a very low concentration of ORs. Gimpl and Fahrenholz (2001) reported OR expression in brain, pituitary, kidney, ovary, testis, thymus, heart, vascular endothelium, osteoclasts, myoblasts, pancreatic islet cells, adipocytes and several types of cancer cells making the search for OR-free tissue almost impossible.

3.3.3 Quantification of the oxytocin receptor gene expression using quantitative reverse-transcription polymerase chain reaction (RT-qPCR)

3.3.3.1 Sample collection

Samples obtained from the left uterine horn and the cervix were used for quantitative analysis of the OR gene by RT-qPCR assay. Cervical, endo- and myometrial samples were collected as described under point 3.2.2, 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 hours and then stored at -20°C until analysed at the Molecular Laboratory of the Department of Veterinary Tropical Diseases, FVS-UP.

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3.3.3.2 RNA extraction

Frozen tissue samples preserved in RNAlater were thawed on ice for one hour before RNA extraction was performed. Approximately 30 mg of frozen tissue was used for RNA extractions using RNeasy® Plus Mini kit (Qiagen, Valencia, CA, USA). The tissue material was transferred to RNase-free ceramic bead tubes and 600 µl of Buffer RLT Plus from the RNeasy® Plus Mini kit was added. Subsequently the tissue samples were homogenised 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.

3.3.3.3 Quantitative reverse-transcription polymerase chain reaction

Oligonucleotide primers and probe sequences used for detection of OR and β-actin gene expression were obtained from two previous studies evaluating OR gene expression and distribution in the equine conceptus, in fetal membranes and endometrium from pony mares at parturition (**Table 1;** Budik et al. 2012; Palm et al. 2013). All primers and TaqMan probes used were synthesised by Life Technologies™ (Carlsbad, CA, USA). The primers and probes were each prepared to a stock of 100 µM (100 pmol/µl) from which a working stock of 10 µM was prepared. 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 conditions followed are shown in **Table 2**; briefly, the PCR conditions included one cycle of complementary deoxyribose nucleic acid (cDNA) synthesis, one cycle of pre-incubation and 40 cycles of amplification consisting of denaturing and primer annealing/elongation. In each RT-qPCR run the following control reactions were included: RNA extraction control, water control, no template control and RT-qPCR

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reagent control. The RT-qPCR experiments were performed employing ABI StepOnePlus™ system and software (Applied Biosystems, USA).

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Table 1 Oligonucleotide primer and probe sequences for amplification of equine oxytocin receptor and endogenous control genes

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 2 The RT-qPCR program and conditions used for amplification of OR and β-actin gene fragments

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**
cDNA synthesis	50	15 minutes
Pre-incubation	95	2 minutes
Amplification (primer annealing/elongation)	95 60	15 seconds 30 seconds

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3.3.3.4 Validation experiment for evaluation of assay amplification efficiency

Robust and precise RT-qPCR assays are usually correlated with high PCR efficiency. Polymerase chain reaction efficiency is particularly important when reporting messenger-ribonucleic acid (mRNA) concentrations for 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; 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). Thus, to determine the amplification efficiencies of the two assays, a set of 10x dilution series, ranging from 485.5 ng/µl to 0.0048 ng/µl, was prepared from RNA of sample RS 042G (G = sample from endometrium of uterine body) and used to generate standard curves for each gene. For the RT-qPCR reaction, 4 µl of each RNA dilution was used as a template and all dilutions were run in triplicates. The mean cycle threshold (C_T) values generated from equivalent standard curve mass points (target gene vs endogenous control gene) were then used to calculate the ΔC_T (C_T target gene- C_T endogenous control gene). These ΔC_T values were plotted against a log input amount of cDNA to create a semi-log regression line (i.e. the validation plot). The slope of the semi-log regression line was used as a general criterion for passing the validation experiment. The absolute value of the slope of the ΔC_T vs log input plot is required to be < 0.1 for the experiment to be considered valid (Livak and Schmittgen, 2001; Pfaffl 2001).

3.3.3.5 Quantitative analysis of the oxytocin receptor gene expression by quantitative reverse-transcription polymerase chain reaction

The expression of the OR gene was determined in three tissue types (endometrium, myometrium and cervix), each group consisting of 27 samples. The ß-actin C_T values were determined for all three tissues (endometrium, myometrium and cervix) using RNA of sample RS 042; for normalisation of OR RT-qPCR data a single sample was run in triplicate. To standardise the results for accurate comparison of OR gene expression between tissue samples, the same RNA concentration (1 ng/µl) was used for all the samples investigated. For $\Delta\Delta C_T$ analysis, the ΔC_T values for each tissue

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type were calculated from average C_T values of specific samples. The resulting ΔC_T values were used to calculate $\Delta\Delta C_T$ values determined from $\Delta C_{T\ (control)}$ - $\Delta C_{T\ (target)}$. For this study, the endometrial tissue was selected as a control tissue and the fold difference in target (i.e. myometrium and cervix) relative to the control was determined using $2^{-\Delta\Delta Ct}$. The fold difference was used as a descriptive method to quantify different gene expressions between tissues.

3.3.4 Statistical analysis of results

The ΔC_T values were used for statistical analysis, not the raw C_T values because the latter are exponential, not linear values (Livak and Schmittgen 2001). The raw CT values were normalised by taking the average ß-actin C_T values into consideration. Additionally, 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) 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.

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CHAPTER 4: Results

4.1 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 macroscopic evaluation of the ovaries to establish size of ovaries, functional structures as well as blood sample analysis for PPC (**Appendix 1**).

4.2 Body condition score (BCS)

All the mares had a BCS > 3 (**Appendix 1**).

4.3 Immunohistochemistry (IHC)

4.3.1 Analysis of immunohistochemical labeling

Although it was the initial intention to quantify positive labeling, it was found that the same cells and tissues appeared to stain fairly consistently across all uterine samples, rendering quantification via IHC irrelevant, especially considering the quantitative reverse-transcription polymerase chain reaction (RT-qPCR) aspect of the project. Additionally, it was found that the intensity of staining varied quite considerably between different cell and tissue types within a section, also between staining batches and between sections stained with different batches of the same commercial polyclonal rabbit anti-human oxytocin receptor (OR) antibody.

4.3.2 Distribution of oxytocin receptors

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 (**Figures 2, 3 and 4**). In the uterus, in order of decreasing labeling intensity, ORs 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 (**Figures 5, 6 and 7**) and throughout the smooth muscle and stromal cells of the myometrium (**Figures 8 and 9**). The greatest intensity of labeling occurred consistently in the endothelium of blood vessels throughout the

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wall of the uterine samples (**Figures 6, 7 and 9**). 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 (**Figures 10 and 11**).

4.3.3 Negative reagent controls

Where phosphate buffered saline with bovine serum albumin (PBS-BSA) buffer solution was used in a subset of horse uterine sections instead of the polyclonal rabbit anti-human OR antibody, but the sections were otherwise treated in exactly the same manner as the positive- and negative-tissue controls and the other test sections (viz. same antigen retrieval methods, incubation times, etc.), there was no evidence of potentially confusing non-specific labeling of uterine tissues (**Figures 12, 13, 14 and 15**). Where the irrelevant S100 polyclonal rabbit antibody was applied to selected tissue sections instead of the polyclonal rabbit anti-human OR antibody (keeping the antigen retrieval method, antibody dilution, incubation time, etc. constant), the only S100-specific positive labeling was identified in association with nerves transmurally in the sections of uterus, as expected (**Figures 16, 17 and 18**). No other, potentially confusing non-specific labeling could be identified in the irrelevant antibody control sections (**Figures 16, 17 and 18**).

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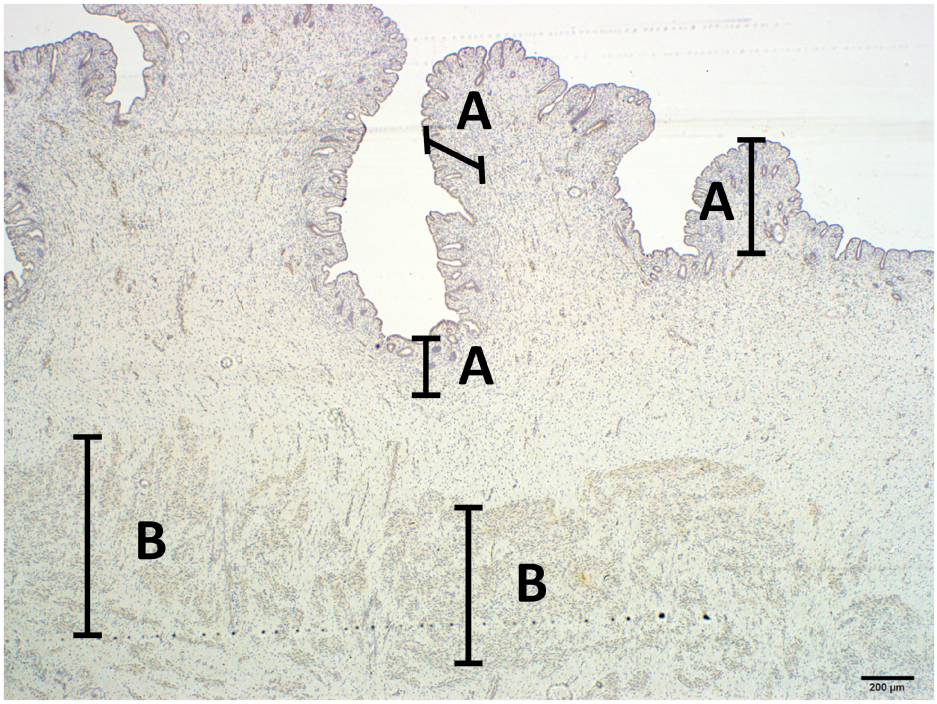

Figure 2 Negative tissue control (6-month-old-foal) showing immunolocalisation of ORs in uterine tissue (200 µm): There was weak positive labeling of luminal epithelium and smooth muscle fibres. **A** delineates the endometrium with superficial luminal epithelium and a small number of glands and blood vessels; **B** delineates the myometrium with smooth muscle fibres and blood vessels. Immunohistology (IH) with diaminbenzidine **(**DAB) substate-chromogen and counterstained with Mayer's hematoxylin.

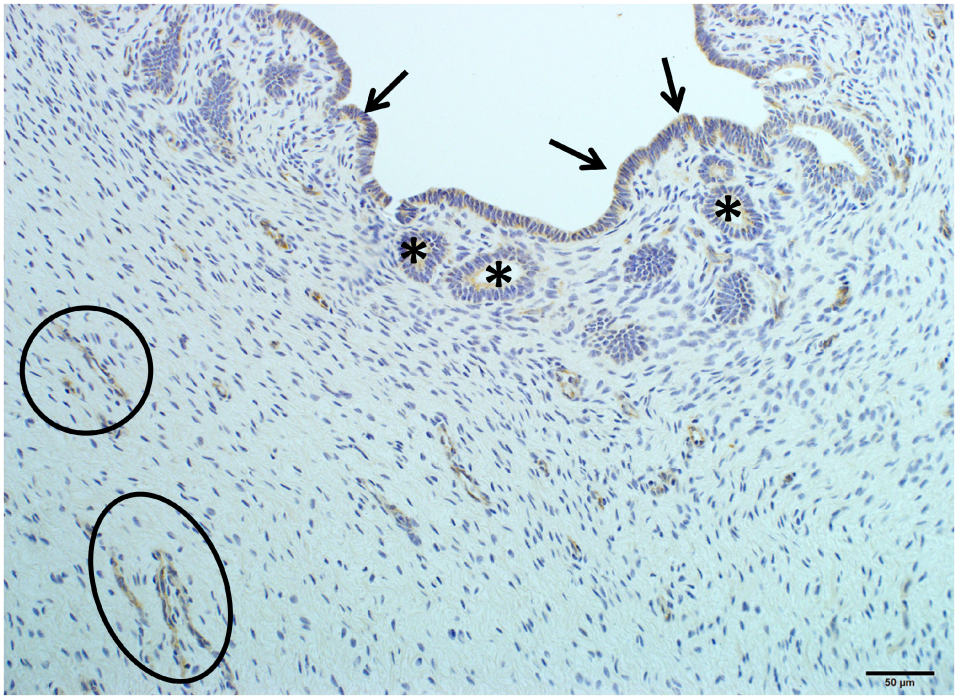

Figure 3 Negative tissue control (6 month-old-foal) showing immunolocalisation of ORs in uterine tissue (50 µm): There was weak positive cytoplasmic labeling in superficial luminal endometrial epithelium (arrows) with very weakly-labeled glandular epithelium (*) and slightly more intense labeling of vascular endothelium (solid circles). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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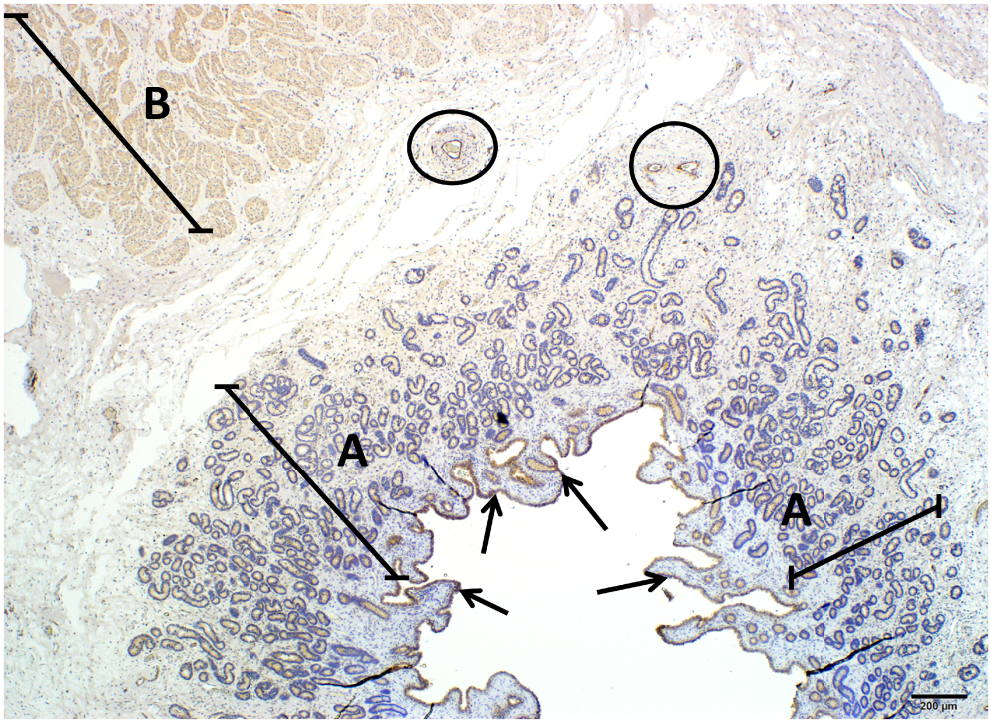

Figure 4 Positive tissue control (RS 24) with immunolocalisation of ORs in horse uterine tissue (200 µm): **A** delineates the endometrium with intense positive cytoplasmic labeling of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles); **B** delineates far less intense labeling of myometrial smooth muscle. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

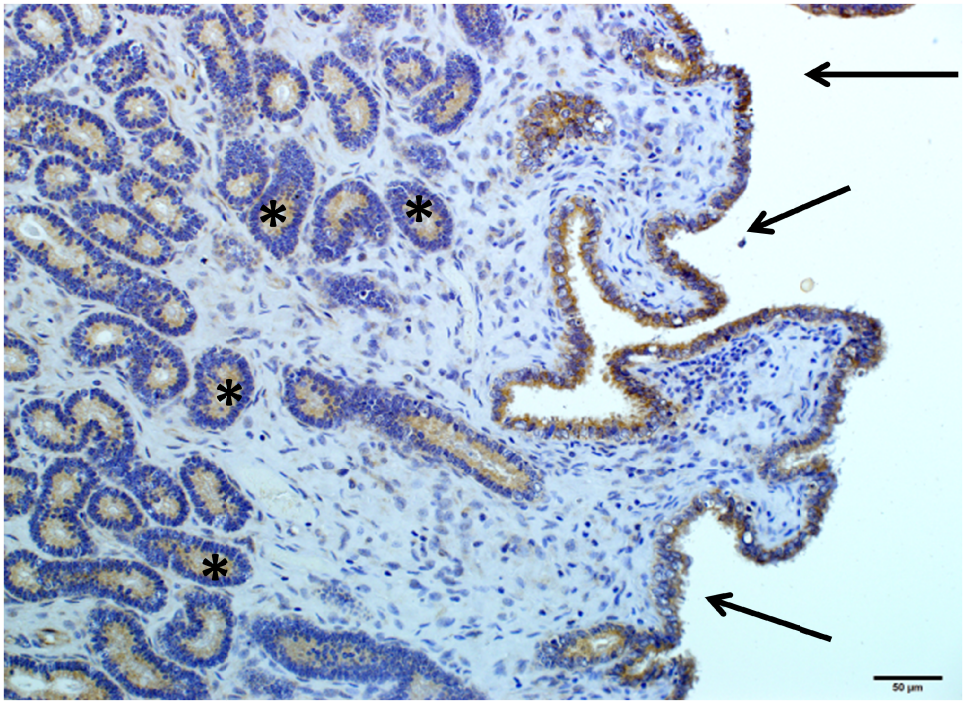

Figure 5 Positive tissue control (RS 24) showing immunolocalisation of ORs in horse endometrium (50 µm): There is positive cytoplasmic labeling of superficial luminal epithelium (arrows) with much paler, but still specific positive labeling of the cytoplasm of glandular epithelial cells (*). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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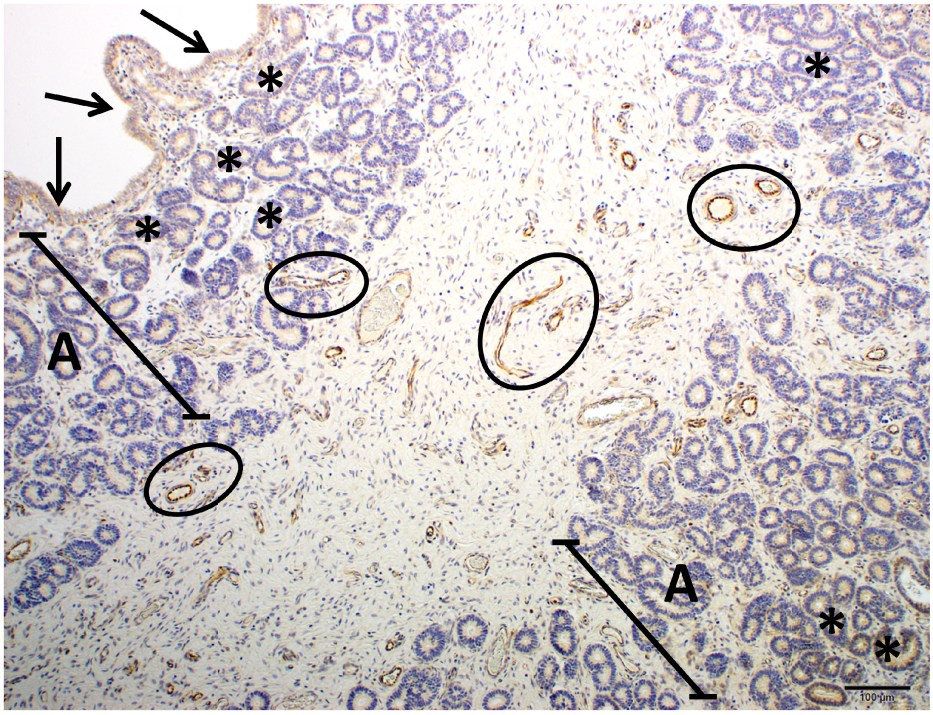

Figure 6 Immunolocalisation of ORs in horse uterine tissue (RS 42; 100 µm): **A** refers to the endometrium with positive labeling of the superficial luminal epithelium (arrows) and vascular endothelium (solid circles) and also the more superficial glandular epithelium (*). The strongest labeling intensity was consistently found in association with the vascular endothelium transmurally in the full-thickness uterine sections. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

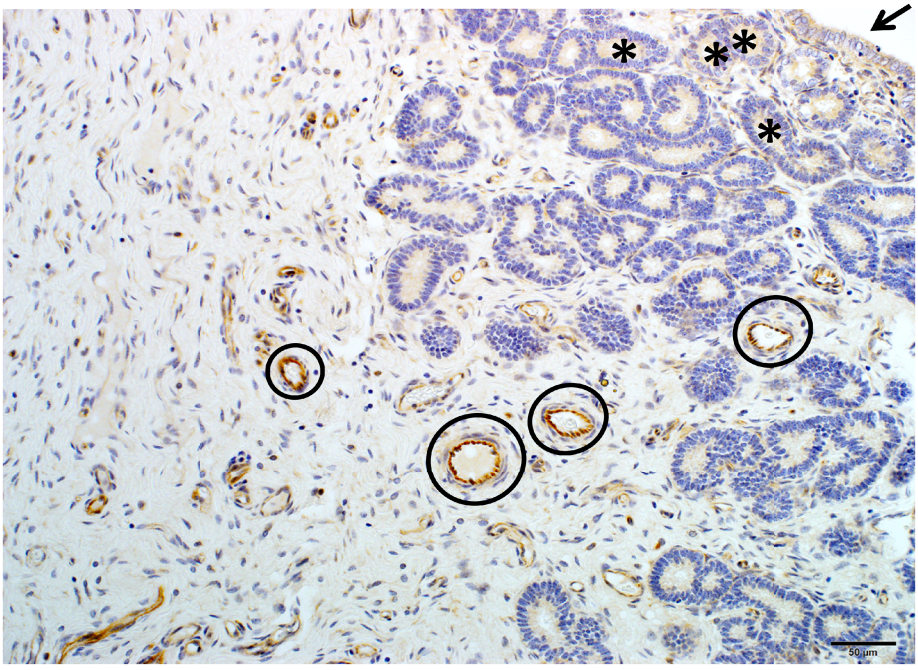

Figure 7 Immunolocalisation of ORs in horse endometrium (RS 42; 50 µm): With pale/weak staining of the superficial luminal (arrow) and glandular epithelium (*) and vascular endothelium (solid circles). The strongest labeling intensity was consistently found in association with vascular endothelium throughout the uterine wall. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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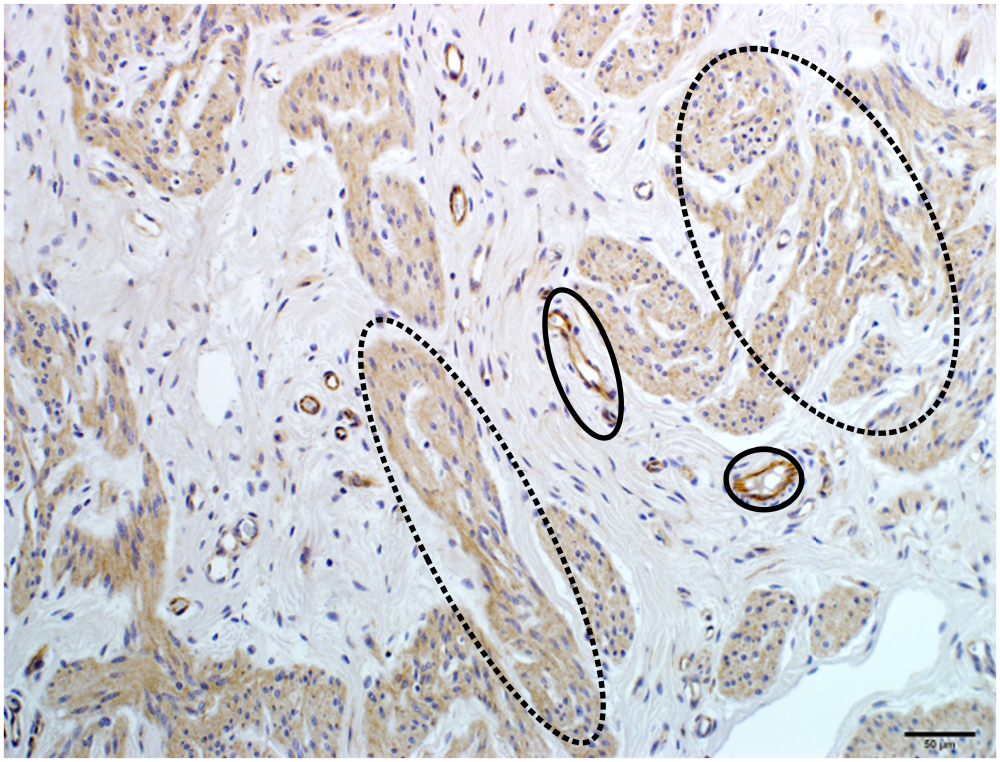

Figure 8 Positive tissue control (RS 24) showing immunolocalisation of ORs in horse myometrium (50 µm): Positive labeling is demonstrated in myometrial smooth muscle (dotted circles) with more intense labeling of vascular endothelium (solid circles) between the myofibres. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

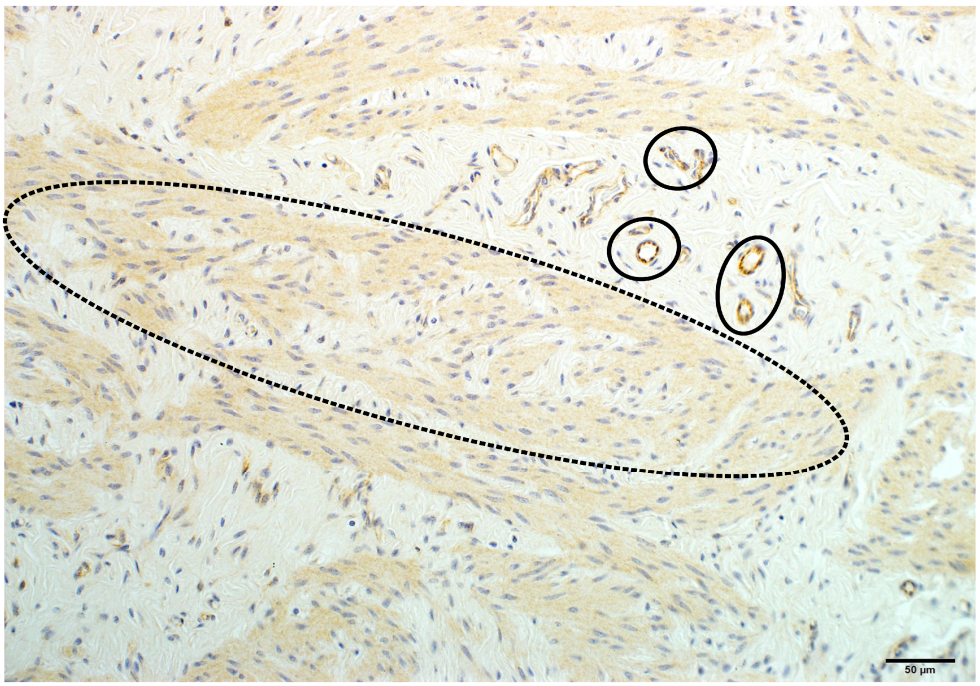

Figure 9 Immunolocalisation of ORs in horse myometrium (RS 42; 50 µm): With intense labeling of vascular endothelium (solid circles) between myofibres compared to the pale but specific labeling of myometrial smooth muscle fibres (dotted circle). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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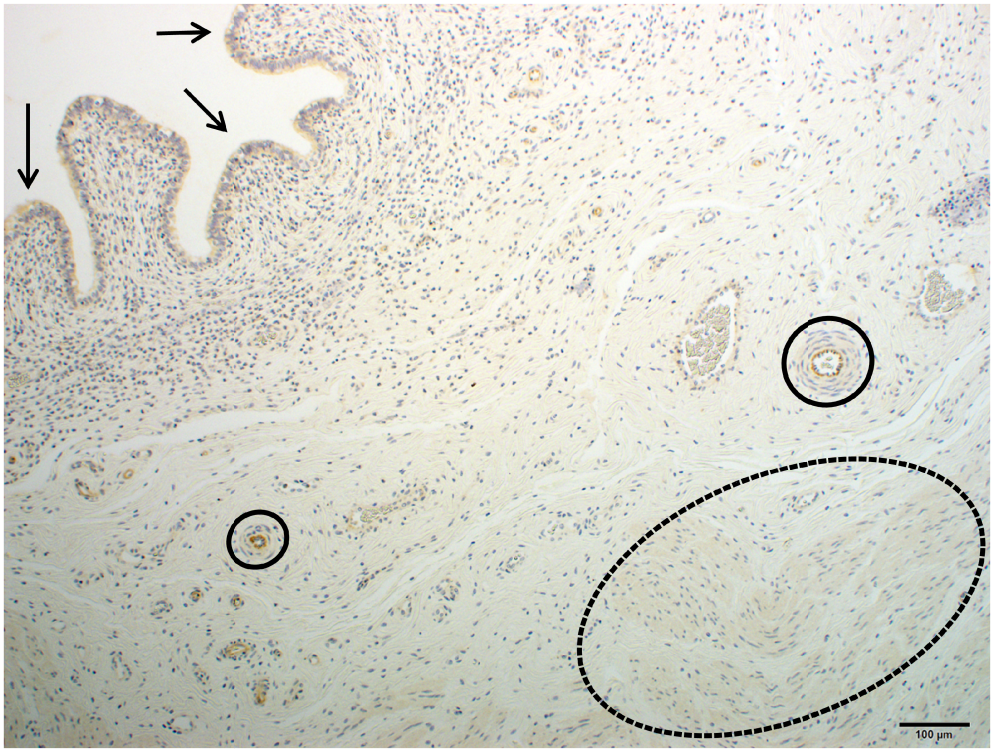

Figure 10 Immunolocalisation of ORs in the horse cervix (RS 45; 100 µm): With pale labeling of the superficial luminal epithelium (arrows), far more intense labeling of vascular endothelium (solid circles) and less intense labeling of smooth muscle myofibres (dotted circle). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

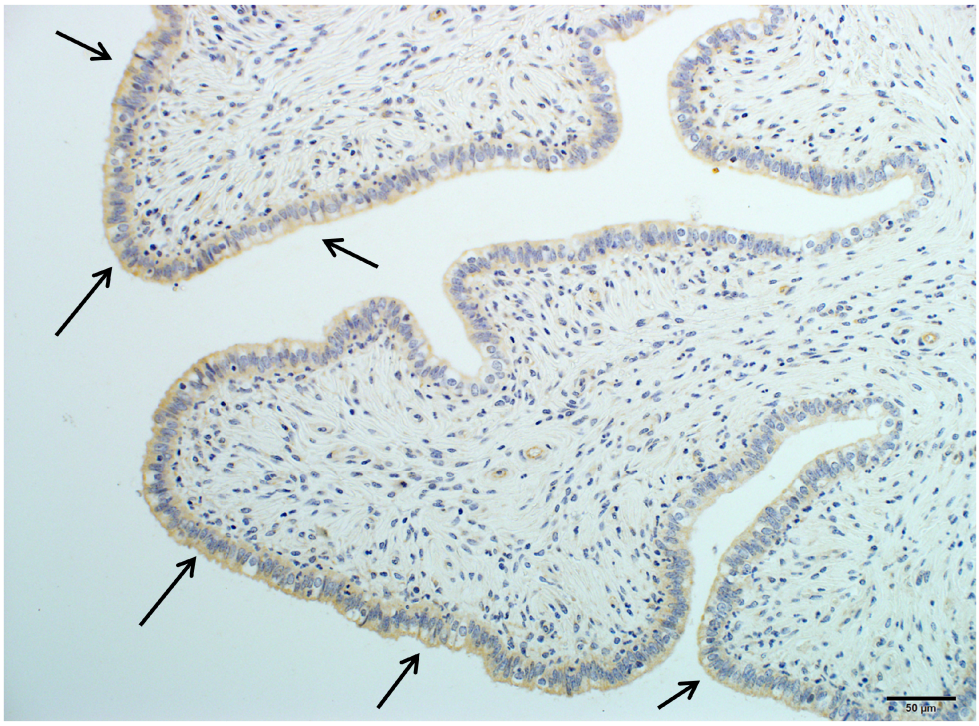

Figure 11 Immunolocalisation of ORs in the horse cervix (RS 45; 50 µm): With clear labeling of the apical cytoplasmic membrane of the luminal epithelium (arrows). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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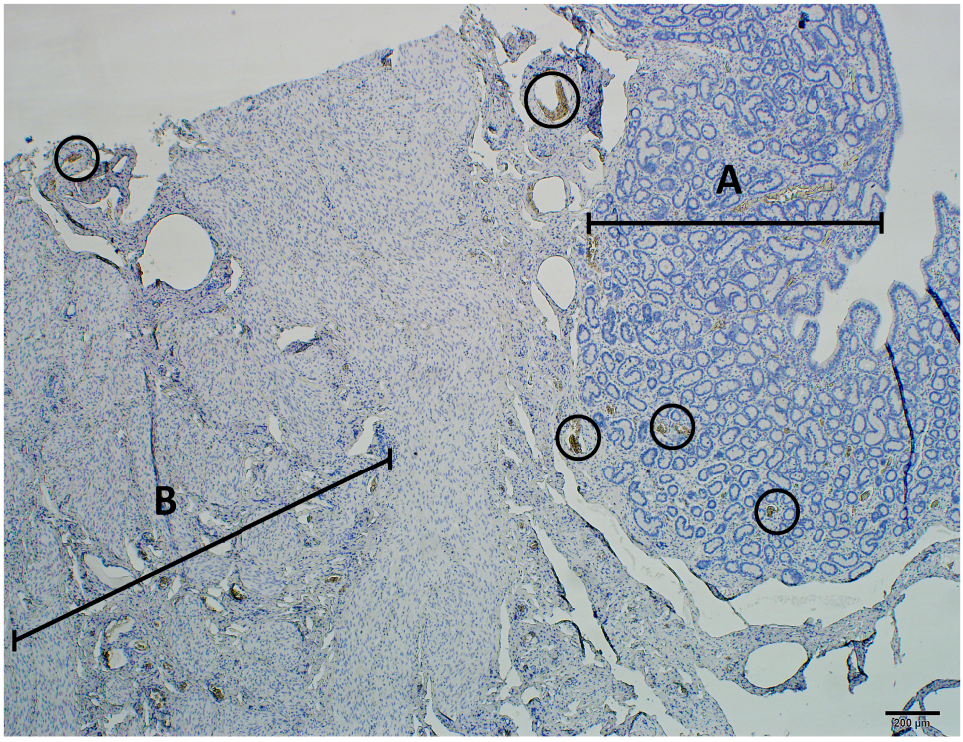

Figure 12 Negative reagent control using PBS-BSA buffer solution instead of the primary polyclonal rabbit anti-human OR antibody in horse uterine tissue (200 µm)**:** No positive labeling was visible. A delineates endometrium with superficial luminal epithelium, endometrial glands and vasculature with intravascular deposit of erythrocytes (solid circles); B delineates myometrium with smooth muscle and vasculature. IH with DAB substrate-chromogen and counterstained with Mayer's hematoxylin.

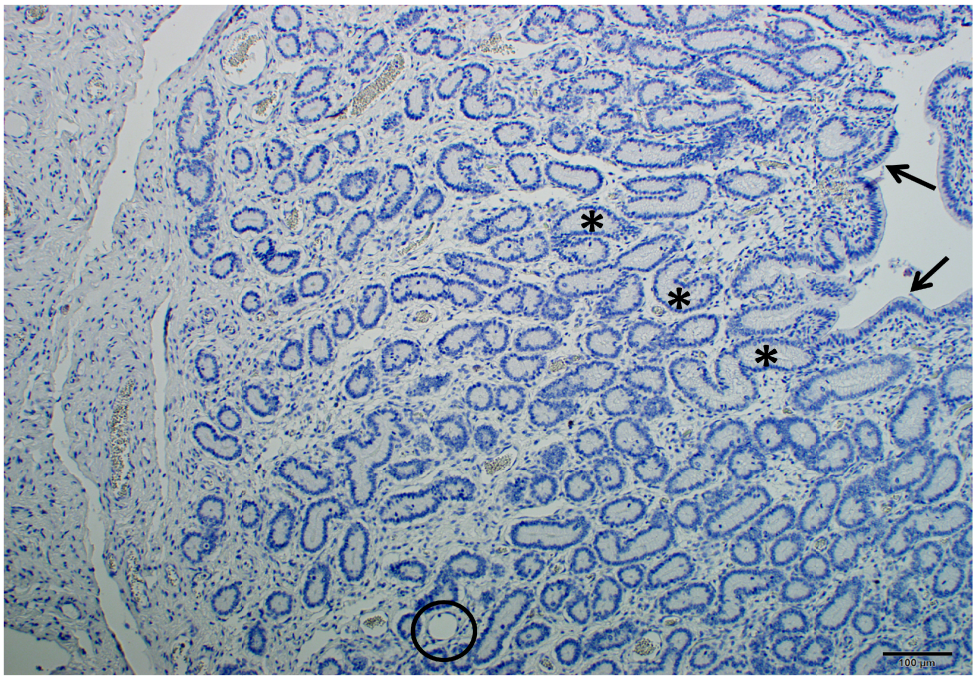

Figure 13 Negative reagent control using PBS-BSA buffer solution instead of the primary polyclonal rabbit anti-human OR antibody in horse endometrium (100 µm): No positive labeling could be identified in the luminal epithelium (arrows), glandular epithelium (*) or vascular endothelium (solid circles). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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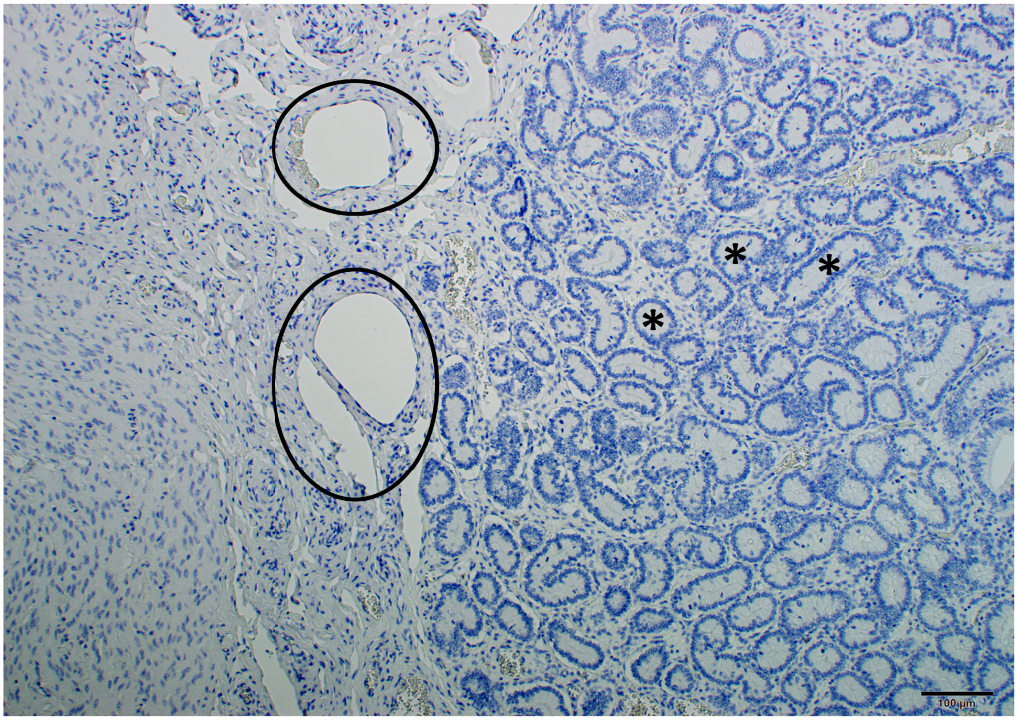

Figure 14 Negative reagent control using PBS-BSA buffer solution instead of the primary polyclonal rabbit anti-human OR antibody in horse uterine tissue (100 µm): No positive labeling was visible in glandular epithelium (*) and vascular endothelium (solid circles). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

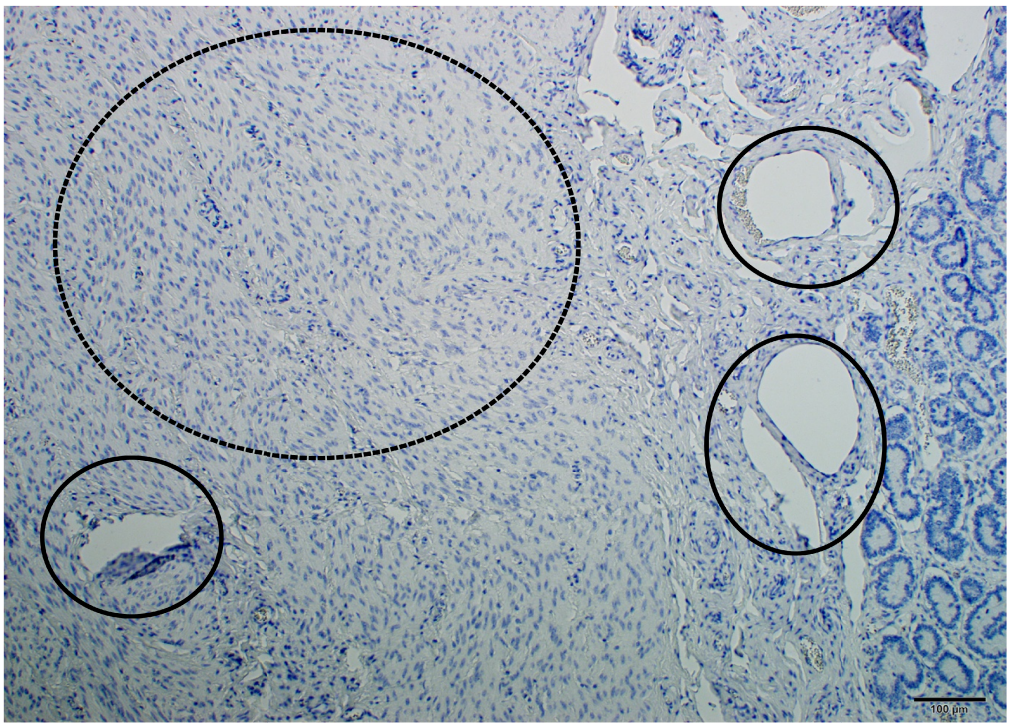

Figure 15 Negative reagent control using PBS-BSA buffer solution instead of the primary polyclonal rabbit anti-human OR antibody in horse myometrium (100 µm): No positive labelling was visible in vascular endothelium (solid circles) or myometrial smooth muscle (dotted circle). IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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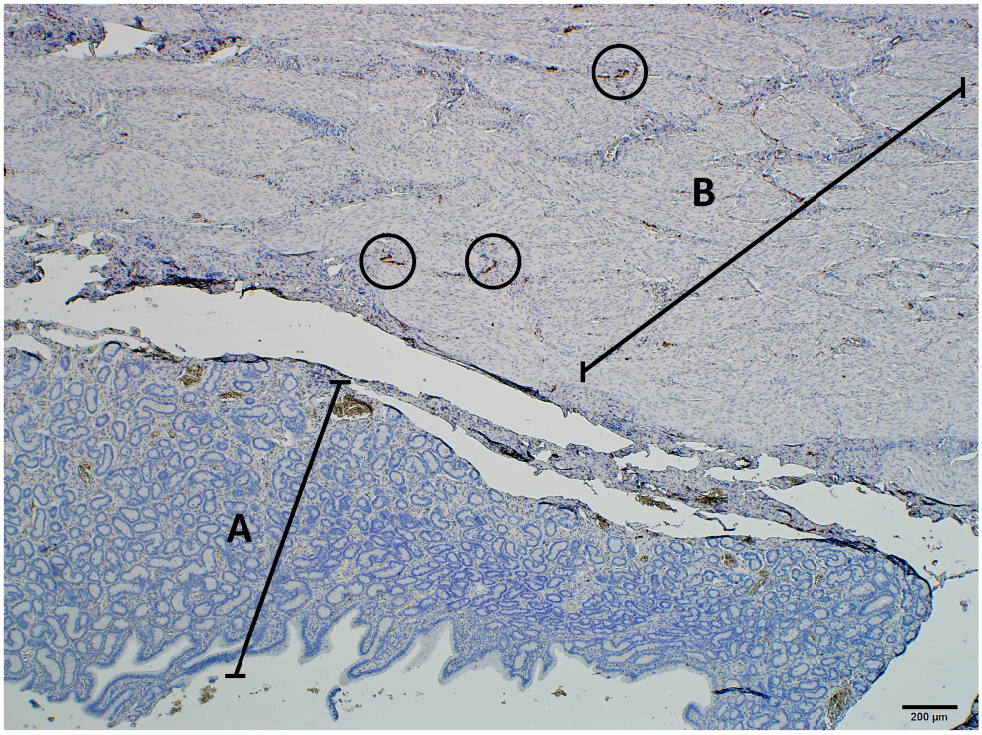

Figure 16 Irrelevant antibody control using an S100 polyclonal rabbit antibody instead of the primary polyclonal rabbit anti-human OR antibody in horse uterine tissue (200 µm): No positive labeling was visible in epithelium, smooth muscle or endothelial cells lining microvessels. However, peripheral nerves within the uterus (solid circles) did label specifically with the S100 antibody, as expected. **A** delineates the endometrium with superficial luminal epithelium, endometrial glands and vasculature; **B** delineates the myometrium with smooth muscle and vasculature. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

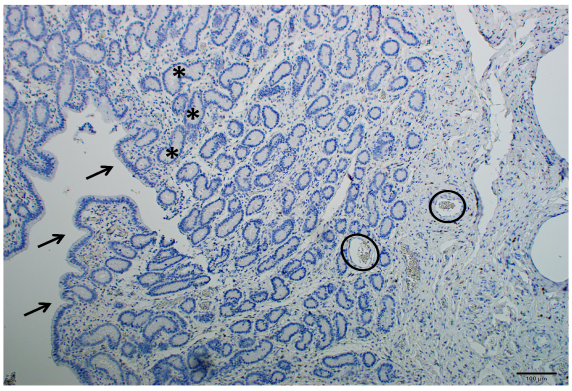

Figure 17 Irrelevant antibody control using an S100 polyclonal rabbit antibody instead of the primary polyclonal rabbit anti-human OR antibody in horse endometrium (100 µm): No positive labeling was visible in luminal epithelium (arrows), glandular epithelium (*) or vascular endothelium (solid circles) of the endometrium. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

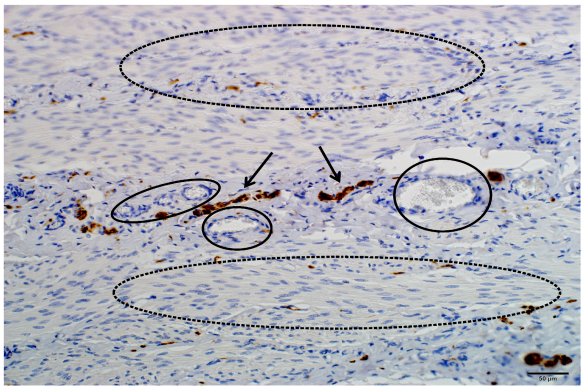

Figure 18 Irrelevant antibody control using an S100 polyclonal rabbit antibody instead of the primary polyclonal rabbit anti-human OR antibody in horse myometrium (50 µm): No positive labeling was visible in association with vascular endothelium (solid circles) or in myometrial smooth muscle (dotted circle). However, there was S100-specific positive labeling of peripheral nerves (arrows), as expected. IH with DAB substate-chromogen and counterstained with Mayer's hematoxylin.

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4.4 Oxytocin receptor gene expression analysis by reverse-transcription polymerase chain reaction

4.4.1 The generation of a validation plot for evaluation of the amplification efficiencies of the target gene (oxytocin receptor) and endogenous control gene (β-actin) assays

To fulfil the requirements of a robust and precise RT-qPCR analysis, the assays of the target and endogenous control genes were evaluated to ensure that they have a similar amplification efficiency. Standard curves for the OR and β-actin assays were generated from a 10-fold dilution series (485.0 ng/µl to 0.00485 ng/µl) and analysed in triplicate per dilution. 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 (**Figure 19**). The slopes obtained for the two standard curves were between the acceptable range of -3,1 and -3,6, which gives reaction efficiencies of between 90 and 110 %.

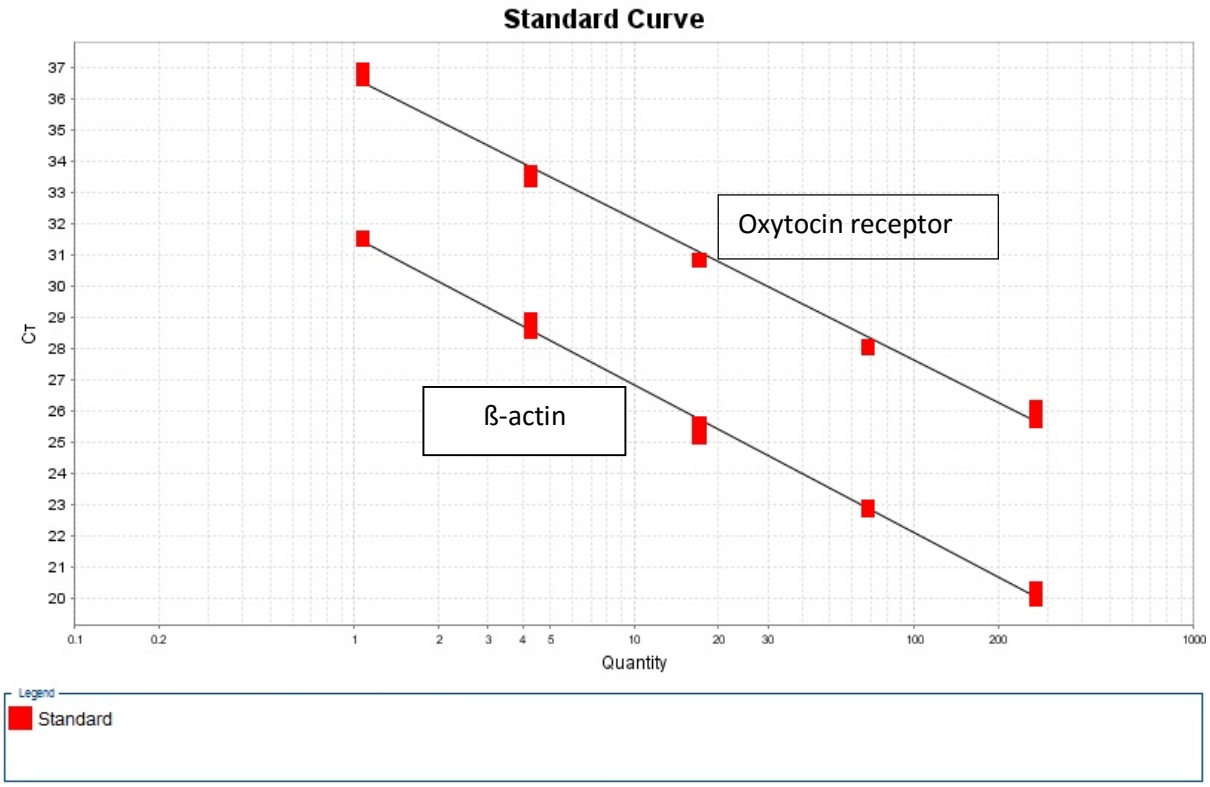

Figure 19 The RT-qPCR standard curves of OR and β-actin assays: Generated for construction of the validation plot used for evaluation of the amplification efficiencies of the two assays.

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The mean cycle threshold (C_T) values generated from equivalent standard curve mass points (target gene vs endogenous control gene) were then used for calculating the ΔC_T values (C_T target gene - C_T endogenous control gene). These ΔC_T values were plotted against a log input amount of complementary deoxyribose nucleic acid (cDNA) to create a validation plot. The absolute value of the slope of ΔC_T vs log input was 0.0412, which is below 0.1 as required for assays with similar amplification efficiency (**Figure 20**).

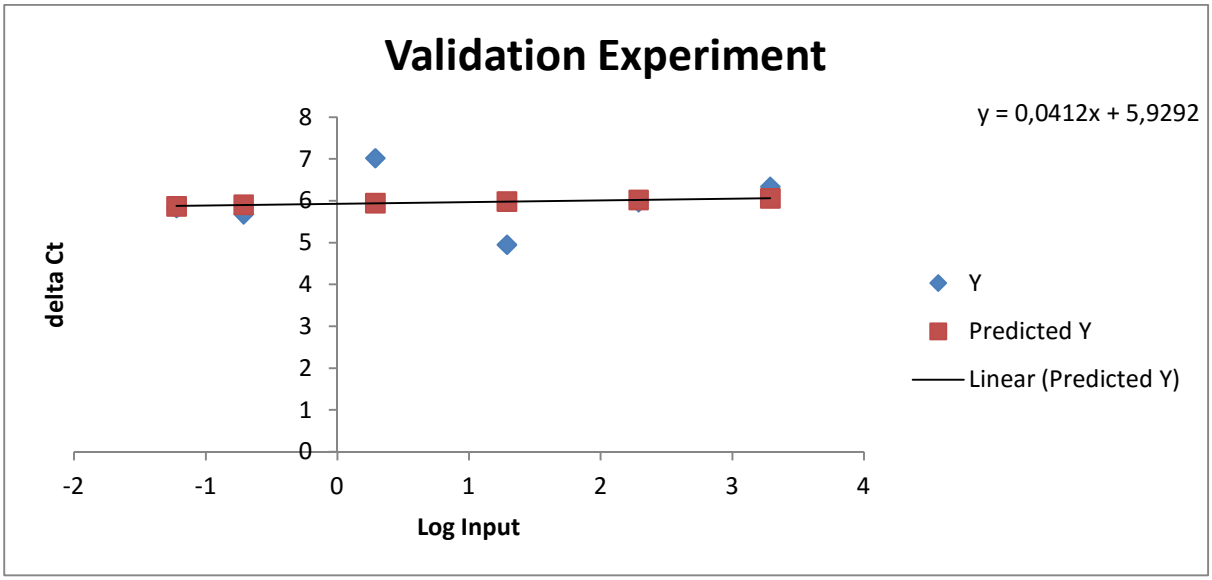

Figure 20 The validation plot generated from standard curves of the OR and β-actin assays (**Figure 19**). The validation experiment is conducted to evaluate the compatibility of the amplification efficiencies of two assays to allow analysis of RT-qPCR data using the comparative C_T method.

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

Detection of the OR gene in the different samples per tissue group, i.e. cervix, endometrium and myometrium, using RT-qPCR, was indicated by C_T values which were used to calculate the average C_T value for each tissue (**Table 3**). 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 were found to be 23,65; 19,84 and 27,49 for cervix, endometrium and myometrium,

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respectively. To normalise the detection of the target gene (OR), the average ß-actin C_T values were then applied to samples of the specific tissue type and used to calculate the ΔC_T which was applied to determine $\Delta\Delta C_T$ and subsequently $2^{-\Delta\Delta Ct}$ values (**Table 4**). Relative to expression of the OR gene in the endometrium, RT-qPCR analysis showed four times higher expression of the OR gene in the myometrium but only one third of the OR expression in the cervix. For 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 than both cervix (P=0.001) and endometrium (P=0.009). There was no significant difference in the gene expression of ORs when comparing the cervix and endometrium (P=1.0; **Table 5**).

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Table 3 C_T values obtained from individual samples for each tissue type

Sample ID (n=27)	Cervix		Myometrium		Endometrium	
	C_T Value	Average C_T	C_T Value	Average C_T	C_T Value	Average C_T
RS 024	31,24		31,56		22,64	
RS 025	31,11		30,72		24,82	
RS 026	35,52		35,34		29,52	
RS 029	31,25		33,57		29,75	
RS 030	37,37		35,45		36,67	
RS 031	24,84		24,35		23,84	
RS 032	30,11		33,19		30,11	
RS 036	36,40		34,34		29,17	
RS 038	38,68		36,94		33,64	
RS 040	28,71		25,28		23,15	
RS 041	27,90		31,31		21,97	
RS 042	34,89		37,47		21,44	
RS 043	29,55	32,09	38,47	32,92	28,24	27,59
RS 045	36,71		31,58		30,36	
RS 047	33,31		36,33		31,46	
RS 048	32,01		33,48		26,92	
RS 049	34,15		34,40		31,88	
RS 050	22,53		23,59		22,98	
RS 051	37,01		35,90		33,44	
RS 054	26,24		38,69		32,32	
RS 055	36,40		37,12		31,50	
RS 056	26,11		21,62		20,51	
RS 058	29,75		33,81		27,94	
RS 059	35,18		36,26		28,37	
RS 060	*		31,03		24,24	
RS 061	34,59		34,18		22,91	
RS 063	32,77		32,95		25,22	

* too low for measurement

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Table 4 Expression profiles of OR gene in cervix, myometrium and endometrium

Sample	OR Average C_T	ß-actin Average C_T	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta Ct}$	P-value
Endometrium (control)	27,59	19,84	7,74			
Myometrium (target)	32,92	27,49	5,43	-2,32	**4,99**	**0.009**
Cervix (target)	32,09	23,65	8.43	0,69	**0,62**	**1.0**

$\Delta C_T = C_T$ (OR) - C_T (ß-actin)

$\Delta\Delta C_T = \Delta C_T$(target)- ΔC_T(control)

Table 5 Pairwise comparison of $\Delta C_T = C_T$ (OR) - C_T (ß-actin) of the endometrium, myometrium and cervix based on estimated marginal means

Tissue samples		Mean Difference	Std. Error	Df	Sig.[a]	95% Confidence Interval for Difference[a]	
						Lower Bound	Upper Bound
Cervix	**Endometrium**	.611	.752	51.297	**1.00**	-1.250	2.471
	Myometrium	2.923*	.752	51.297	**.001**	1.063	4.784
Endometrium	**Cervix**	-.611	.752	51.297	**1.00**	-2.471	1.250
	Myometrium	2.313*	.742	51.085	**.009**	.476	4.149
Myometrium	**Cervix**	-2.923*	.752	51.297	**.001**	-4.784	-1.063
	Endometrium	-2.313*	.742	51.085	**.009**	-4.149	-.476

* The mean difference is significant at the .05 level

[a] Adjustment for multiple comparisons: Bonferroni

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CHAPTER 5: Discussion

Oxytocin receptors (ORs) were identified in the three tissues: endometrium, myometrium and cervix. The precise histological distribution of ORs in the myometrium and cervix in the mare was a novel demonstration.

In the endometrium, ORs were found in the cytoplasm of luminal and glandular epithelia, the endothelium of blood vessels and sub-epithelial and periglandular stromal cells. This supports the findings of Palm et al. (2013) who investigated OR location specifically within the mare endometrium. They described positive labeling in the endometrial luminal epithelium, mainly associated with the apical margin of epithelial cells, as well as diffusely distributed OR granules within the cytoplasm of glandular epithelial cells, especially in the deeper uterine glands. Siemieniuch et al. (2011) investigated OR distribution in the endometrium of cats and they found differing intensity of expression in superficial and glandular epithelia as well as in stromal cells when comparing different stages of the oestrus cycle. The comparison of labeling intensity between slides and within individual samples was not considered feasible in the present study as too many variables had an influence on labeling intensity. Changes in environmental temperature, employment of a manual immunodetection technique, the purchase of multiple batches of polyclonal rabbit anti-human OR antibodies (albeit the same one each time), the choice of diaminbenzidine (DAB) as substrate-chromogen and additional potentially innumerable factors undoubtedly influenced the intensity of the labeling in different sections. Polyclonal antibodies have different specificities between batches. Because of the limited quantity of antibody per batch, three different batches had to be used in the present study, which, in all likelihood, had a significant effect on the labeling intensity. Two of the most commonly employed chromogens that are used in Immunohistochemistry (IHC) include DAB and Vector Nova-Red. The optical properties of the chosen chromogen affected the linearity and dynamic range of the assay. Only pure absorbers follow the Lambert-Beer law which describes the linear relationship between the concentration of a compound and its absorbance (Taylor and Levenson, 2006). Diaminbenzidine exihibits scattering behaviour and there is only a linear relationship between the amount of antigen and labeling intensity at low levels of the antigen, resulting in inaccurate interpretation of intense labeling (Fritz et al. 1995; Walker 2006). It is for this reason that the Vector Nova-Red substrate was

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first attempted for the development of colour during the optimisation process, but consistent labeling could not be achieved using the Nova-Red substrate on the uterine samples. Automated IHC is far superior to manual staining as it can overcome some of the variables due to technical factors, but mainly for economic reasons, automated IHC is not yet routinely employed in veterinary diagnostic laboratories worldwide, although it is becoming far more commonplace in veterinary laboratories in the first world (Walker 2006). Oxytocin receptors are to be expected in endometrial glandular and luminal epithelium as there is evidence of oxytocin involvement in the events of luteolysis based on the administration of exogenous oxytocin, which then stimulates the release of prostaglandin F2-*alpha* ($PGF_{2\alpha}$) in late dioestrus (Betteridge et al. 1985; Goff et al. 1987; Goff et al. 1993). Furthermore, the equine endometrium produces $PGF_{2\alpha}$ in response to oxytocin *in vitro* (King 1984; Franklin et al. 1989).

Oxytocin receptor labeling was also detected in smooth muscle cells and in the endothelium of blood vessels in the myometrium of the mare similar to the reported findings of Perumamthadathil et al. (2014), who reported a similar location of ORs in the cow, describing ORs in smooth muscle cells, vascular endothelium, especially associated with arterioles and capillaries in the bovine myometrium. The location of ORs in the myometrium, especially in smooth muscle cells might be explained by oxytocin function. Oxytocin causes the myometrial contractions that are important for the expulsion of the foetus during parturition and also for the mechanical drainage of cellular debris and uterine fluid post-mating in the mare (Nikolakopoulos and Watson, 1999).

There was a similar pattern of distribution and labeling intensity of ORs in the uterus (endometrium and myometrium) and cervix, with the exception of the glandular-epithelium which is absent in the cervix. This is in accordance with the findings emanating from two earlier studies on ORs in the cervix of pregnant and non-pregnant cows, where ORs were present in epithelial cells at the luminal surface of the mucosa and in smooth muscle cells of the myometrium (Fuchs et al. 1996b). Epithelial cells at the luminal surface of the mucosa were the principal site for OR detection in the aforementioned studies. Understanding the cellular and tissue distribution of ORs potentially provides valuable information regarding the underlying mechanism of both the regulation of ORs and oxytocin action within the cervix. The

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discovery of ORs in the equine cervix of cycling non-pregnant mares suggests that oxytocin plays a physiological role in cervical function during the oestrous cycle. 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.* PGE_2 output was shown to be dependent on the cycle stage in ewes and cows (Matthews and Ayad, 1994; Fuchs et al. 1996b; Falchi and Scaramuzzi, 2013). Prostaglandin E2 has also been shown to cause dilation of the equine cervix (Rigby et al. 1998). Further investigation however, is necessary to investigate the possible effects of oxytocin on PGE_2 release in the equine cervix.

The greatest intensity of IHC labeling occurred consistently in microvascular endothelial cells throughout the entire uterine wall which implies a role for oxytocin in the regulation of uterine vasculature. Previously, ORs were reported in both bovine and human myometrial vascular endothelium (Weston et al. 2003; Perumamthadathil et al. 2014). The precise action of oxytocin on vascular endothelium and whether or not it causes vasodilation or vasoconstriction remains undefined. It was proposed to cause necrosis and ischemia of the caruncles by vasoconstriction in cows during the post-partum period (Perumamthadathil et al. 2014). Another peptide hormone, vasopressin, has a structure similar to that of oxytocin and is able to cross-react with the OR. Vasopressin causes vasoconstriction and may play a role in uterine involution (Perumamthadathil et al. 2014). Additional studies are required in order to determine the physiological role of ORs in microvascular endothelial cells in the equine uterus.

Quantitative reverse-transcription polymerase chain reaction was used in order to quantify OR gene expression. The myometrium had the greatest OR gene expression when compared to both the endometrium and cervix. This supports the findings of Stull (1986) who found a threefold higher concentration of ORs in myometrium compared to endometrium throughout the oestrous cycle using the radioligand-binding assay. In the present study there was also a significant difference in OR gene expressions between the endometrium and myometrium. In the cow, OR concentration in the endometrium exceeded the OR density in the myometrium severalfold during oestrus, whereas the myometrial receptor concentration exceeded the endometrial concentration two to three fold during the

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luteal phase (Fuchs et al. 1990). The opposite would be expected in the mare, as oxytocin increases uterine motility during oestrus to enhance the transport of sperm cells to the oviduct on the one hand, and on the other hand also to support uterine defence mechanisms by stimulating the clearance of uterine fluid post-mating (Pycock 1993; LeBlanc et al. 1994). According to Stull (1986), however, the myometrium contained a consistently higher density of ORs throughout the oestrous cycle of the mare.

The results of the present study also showed a significant difference between the OR gene expression in the myometrium and cervix but no significant difference between the OR density in the endometrium and cervix. This was surprising as the histomorphological structure of the cervix (consisting largely of smooth muscle tissue and no glandular tissue), seemed ostensibly to be more similar to myometrium than endometrium.

Endometrial, myometrial and cervical ORs are influenced by oestrogen and progesterone in cattle and sheep (Fuchs et al. 1990; Robinson et al. 2001; Falchi and Scaramuzzi, 2013). In the cow, endo- and myometrial OR concentration seems to be regulated differently by ovarian steroids than is the case in ewes. The steroid hormone influence on bovine OR concentration stands in contrast to findings in the ewe where OR levels in both tissues change simultaneously during the oestrous cycle (Sheldrick and Flint, 1985; Fuchs et al. 1990). In ewes, oestrogen causes OR upregulation in the endo- and myometrium (Sheldrick and Flint, 1985; Hixon and Flint, 1987) whereas oestrogen does not seem to have an effect on OR expression in the endometrium and myometrium of cows. Progesterone, on the other hand, inhibits OR gene expression in the ewe and cow (Wathes and Hamon, 1993; Robinson et al. 2001). This effect of progesterone, however, seems to be only temporary. In sheep, the blocking effect faded after 12 days of continuous exposure to progesterone (Vallet et al. 1990; Wathes et al. 1996). In the cow, endometrial ORs seem to be more responsive to altered progesterone levels than myometrial ORs. Oxytocin receptor concentration was reported to be higher in late dioestrus than in any other stage of the equine oestrous cycle (Stull 1986; Sharp et al. 1997; Starbuck et al. 1998). This may be indicative of an upregulating effect of oestrogen on ORs but the real effect of ovarian steroids on ORs has not yet been investigated in the mare.

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It is recommended that a standard RT-qPCR protocol should be performed in replicates (duplicates, triplicates) because it provides data preservation and monitoring of the polymerase chain reaction (PCR) amplification precision (Livak and Schmittgen 2001, Stahlberg et al. 2004). Due to financial constraints it was not possible to run all of the samples in replicates. However, by using ß-actin as an endogenous control gene (and thus normalising the PCR for the amount of ribonucleic acid (RNA) added; Livak and Schmittgen 2001) as well as by ensuring proper optimisation of the validation experiment, there was a clear attempt to ensure the validity of the results in the present study. To fulfil the requirements of the comparative 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 efficiencies. The amplification efficiency for the ß-actin assay was 94,5 % and 93 % for the OR assay, respectively. The slope of the semi-log regression line was used as a general criterion for passing the validation experiment. The absolute value of the slope of the ΔC_T vs log input plot is required to be < 0.1 for the experiment to be considered valid (Livak and Schmittgen, 2001; Pfaffl 2001). The slopes in the present study were between -3,1 and -3,6. Additionally, individual samples within tissue groups were used as replicates. The average C_T value for each tissue type was utilised to calculate the fold difference which was used to describe the OR gene expression between the different tissue types.

The fold difference could not be used for statistical analysis as the average cycle threshold (C_T) values for each tissue type were used to calculate the fold difference, resulting in one value per taget group and rendering it unusable for statistical calculations. Therefore, ΔC_T values were used instead. Another reason for using ΔC_T values instead of $\Delta\Delta C_T$ or $2^{-\Delta\Delta Ct}$ values for statistical calculations was that it enabled comparison of all tissue types with each other instead of comparing myometrium and cervix (as targets) to endometrium (control) only. According to Livak and Schmittgen (2001) the raw C_T data should not be used because C_T is an exponential and not a linear term. The C_T values in the present study, however, were normalised by taking the average ß-actin C_T values into consideration which facilitated using the ΔC_T values for the statistical analysis.

Only cyclic mares were used in the present study. Macroscopically evident thinning of the uterine wall can be observed during anoestrus, as well as histomorphological

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changes consistent with physiological endometrial atrophy - characterised by cuboidal luminal and glandular epithelia and straight glands (Kenney and Doig, 1986). It is not known whether OR distribution is affected by these seasonal changes, which could in turn lead to an inaccurate description of OR distribution. It was for this reason that only cyclic mares were included in the present study - to avoid this possible confounding effect.

Several studies have documented that a low body condition score (BCS) can have a negative effect on reproductive efficiency (Henneke et al. 1984, Cavinder et al. 2009, Morley and Murray 2014). A BCS < 4 was shown to have an influence on ovulation and conception rate (Henneke et al. 1984). In this study several mares were found to have a BCS of 3 but they did not show any signs of negatively affected cyclicity. One explanation for this finding might be that Henneke's system was originally developed for use in Quarter Horse mares and it is therefore most appropriate for use in horses with a similar body type and pattern of fat deposition, whereas the mares used in this study were of various sizes and were derived from a variety of different breeds (Henneke et al. 1983).

In conclusion, this study demonstrated the presence of ORs in several cell- and tissue-types in the endometrium, myometrium and cervix of the mare. The indication is that these cells are the targets for oxytocin action such as the stimulation of $PGF_{2\alpha}$ release during luteolysis, uterine contraction and possible softening of the cervix through oxytocin induced PGE_2 release. The distribution of ORs was consistent across the different cell- and tissue-types studied. Based on the RT-qPCR results, the greatest gene expression of ORs was consistently found in the myometrium whereas there was no significant difference between OR gene expression in the endometrium and cervix. Immunohistochemical demonstration of ORs throughout the myometrium was generally pale, but there was diffuse specific labeling of proportionally larger areas of smooth muscle in the tissue sections, as compared to the slightly more intense, but multifocal labelling of thinner segments of luminal and glandular epithelium. Microvascular endothelial cells throughout the wall, but especially numerous between smooth muscle fibres in the myometrium, would also have contributed to increased OR gene expression as shown by the RT-qPCR results.

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Appendix 1

Table 6 Summary of BCS, age, ovarian findings and PPC results for the study population

ID Horse	BCS	Age	Ovaries	PPC (nmol/L)	Cyclicity
RS 024	4	12	l: 6cm x 4,5cm x 3cm : NSS / r: 5,5cm x 4,5cm x 3cm: 2 CL (3 cm)	1,77	Cycling
RS 030	3	18	l: 5cm x 3cm x 2cm : sF / r: 5cm x 3cm x 3cm : sF	1,02	Cycling
RS 031	4	15	l: 5cm x 3cm x 2cm: CL (2,5cm) / r: 4cm x 2cm x 2cm : NSS	2,45	Cycling
RS 060	3	15	l: 4cm x 3cm x 2cm F (2 cm) / r: 4cm x 3cm x 1,5cm: NSS	1,11	Cycling
RS 026	6	11	l: 6,5cm x 5cm x 3cm: CL (3cm) / r: 7cm x 4cm x 2,5cm : NSS	29,52	Cycling
RS 029	5	15	l: 7cm x 4cm x 2cm : sF / r: 7cm x 5cm x 2,5cm: sF	38,94	Cycling
RS 051	4	20	l: 7cm x 5cm x 4cm:F (5 cm) / r: 5cm x 3cm x 3cm: CL (2 cm)	29,24	Cycling
RS 059	3	15	l: 5cm x 4cm x 2cm: CL (2,5cm) / r: 4,5cm x 4cm x 2cm: NSS	8,84	Cycling
RS 063	5	15	l: 5cm x 4cm x 4cm: NSS / r: 7cm x 4cm x 3cm: CL (2 cm)	34,63	Cycling
RS 032	4	2	l: 4cm x 2cm x 2cm : NSS / r: 5cm x 2cm x 2cm : NSS	2,8	Cycling
RS 036	5	2,5	l: 5cm x 3cm x 4cm : NSS / r: 5,5cm x 3cm x 3cm: F (2,5 cm)	2,47	Cycling
RS 038	7	4,5	l: 6cm x 4cm x 4cm: CH / r: 3,5cm x 3cm x 3cm: NSS	1,19	Cycling
RS 040	3	2	l: 6,5cm x 3,5cm x 2cm: sF / r: 6,5cm x 4cm x 3cm: F(3 cm)	0,56	Cycling
RS 045	6	4,5	l: 6cm x 6cm x 4cm: F (4 cm) / r: 4cm x 3cm x 3cm: NSS	1,27	Cycling

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RS 048	6	4,5	l: 7cm x 5cm x 4cm: F (5 cm) / r: 3cm x 2cm x 2cm: NSS	1,31	Cycling
RS 050	4	2	l: 3.5cm x 2.5cm x 2.5cm NSS / r: 5cm x 4cm x 3cm: F (3 cm)	2,15	Cycling
RS 056	5	7	l: 8cm x 6cm x 4cm: F (6 cm) / r: 6cm x 4cm x 3cm: NSS	1,97	Cycling
RS 058	4	9	l: 5cm x 4cm x 3cm: NSS / r: 7cm x 5cm x 4cm: F (4 cm)	0,78	Cycling
RS 025	8	7	l: 7cm x 5,5cm x 3cm : NSS / r: 9cm x 5,5cm x 4cm: F(3 cm)	21,55	Cycling
RS 041	5	2	l: 4cm x 3cm x 1,5cm: NSS / r: 4cm x 2,5cm x 2,5cm: F(2,5 cm)	4,38	Cycling
RS 042	5	2	l: 4,5cm x 3cm x 2cm: CL (1cm) / r: 4cm x 3cm x 2cm: NSS	7,41	Cycling
RS 043	4	2	l: 6cm x 3cm x 3,5cm :CH / r: 5cm x 3,5cm x 3cm: F(3 cm)	45,56	Cycling
RS 047	5	4,5	l: 8cm x 6cm x 4cm: F (4,5 cm) / r: 5cm x 4cm x 3cm: CH(2 cm)	23,31	Cycling
RS 049	4	5	l: 7cm x 4cm x 3cm: CL (2.5 cm) / r: 4cm x 3cm x 3cm NSS	26,12	Cycling
RS 054	4	2	l: 2,5cm x 1,5cm x 1cm: NSS / r: 4cm x 3cm x 3cm CH	51,85	Cycling
RS 055	6	3,5	l: 6cm x 4cm x 3cm: NSS / r: 6cm x 5cm x 3cm: CL(2 cm), F (2,5cm)	25,78	Cycling
RS 061	4	10	l: 5cm x 4cm x 3cm: NSS / r: 5cm x 5cm x 4cm CL: (3 cm)	29,09	Cycling
Control	**C**			1,67	

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

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Appendix 2

Table 7 Body condition scoring system for horses by Henneke et al. (1983)

Score Name	Description
1. Poor	Animal is extremely emaciated. Spinous processes (portion of the vertebra of the backbone which project upward), ribs, tail-head, and bony protrusions of the pelvic girdle (hooks and pins) are prominent. Bone structure of, shoulders, and neck are easily noticeable. No fatty tissues can be felt.
2. Very thin	Animal is emaciated. Slight fat covering over base of the spinous processes. Transverse processes (portion of vertebrae which project outward) of lumbar (loin area) vertebrae feel rounded. Spinous processes, ribs, shoulders, and neck structures are faintly discernible.
3. Thin	Fat is built up about halfway on spinous processes. Transverse processes cannot be felt. Slight fat cover over ribs. Spinous processes and ribs are easily discernible. Tail-head is prominent, but individual vertebrae cannot be visually identified. Hook bones (protrusion of pelvic girdle appearing in upper and forward part of the hip) appear rounded but are easily discernible. Pin bones (bony projections of pelvic girdle located toward rear and midsection of the hip) are not distinguishable. Accentuation of withers shoulders and neck.
4. Moderately thin	Negative crease along back (spinous processes of vertebrae protrude slightly above surrounding tissue). Faint outline of ribs is discernible. Fat can be felt around tail-head. Hook bones are not discernible. Withers, shoulders, and neck are not obviously thin.
5. Moderate	Back is level. Ribs cannot be visually distinguished but can be easily felt. Fat around tail-head begins to feel spongy. Withers appear rounded over spinous processes. Shoulders and neck blend smoothly into body.
6. Moderately fleshy	May have slight crease down back. Fat over ribs feels spongy. Fat around tail-head feels soft. Fat begins to be deposited along the sides of the withers, behind shoulders and along neck.
7. Fleshy	May have crease down back. Individual ribs can be felt but with noticeable filling of fat between ribs. Fat around tail-head is soft. Fat is deposited along withers, behind shoulders, and along neck.
8. Fat	Crease down back. Difficult to feel ribs. Fat around tail-head is very soft. Area along withers is filled with fat. Area behind shoulder is filled in flush with rest of the body. Noticeable thickening of neck. Fat is deposited along inner buttocks.
9. Extremely Fat	Obvious crease down back. Patchy fat appears over ribs. Bulging fat around tail-head, along withers, behind shoulders, and along neck. Fat along inner buttocks may rub together. Flank is filled in flush with rest of the body.

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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   cgcgtcggctggcagcgggggcacgcggcTATGGCCCGCGTCAGCAGCGTCAAGCTCAT  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
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Appendix 4

Table 8 Data obtained from a 10x dilution series of RNA from an endometrial uterine body sample (RS042G) used to generate standard curves for ß-actin and OR gene expression as part of the assay efficiency evaluation

Concentration	ß-actin Average C_T	Oxytocin receptor (OR) Average C_T	Δ C_T (ß-actin-OR)
485,5	15,04±0,11	21,38±0,12	6,34±0,16
48,55	16,03±0,29	21,98±0,08	5,95±0,3
4,85	20,36±0,75	25,32±0,24	4,96±0,79
0,485	24,45±0,21	31,47±2,35	7,02±2,36
0,0485	28,7±0,28	34,38±0,14	5,68±0,31
0,016	32,57±0,32	38,43±0,31	5,86±0,45

Table 9 The Log input RNA and Δ C_T values used to create the RT-qPCR validation plot

Input amount of total RNA (ng/µl)	Log of Input Amount: ng Total RNA	ΔC_T target-reference
1942	3,29	6,34±0,16
194,2	2,29	5,95±0,3
19,4	1,29	4,96±0,79
1,942	0,29	7,02±2,36
0,19	-0,72	5,68±0,31
0,06	-1,22	5,86±0,45

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