Embryonic quality and survival in the horse

maternal and intrinsic aspects

Björn Rambags

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Maternale en intrinsieke aspecten

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Chapter 1

General Introduction

INTRODUCTION

During the last 20 years, advances in equine reproductive medicine have led to appreciable improvements in the foaling rate and in the efficiency of the average breeding programme. In particular, the introduction of ultrasonography to mare fertility management during the 1980's has enabled more precise monitoring of follicle development, ovulation and intra-uterine fluid accumulation, and early and accurate detection of pregnancy and pregnancy loss. These improvements in our ability to monitor events during both the cycle and early pregnancy have led to significant increases in both the mean per cycle pregnancy rate (52%→60%) and the foaling percentage (77%→83%; Morris and Allen 2002). Moreover, the incidence of abortion due to twin pregnancy has dropped dramatically, from 22-29% (Whitwell 1980) to 6% (Giles *et al.* 1993; Hong *et al.* 1993; Smith *et al.* 2003) of all abortions, because ultrasonography has made it possible to diagnose a twin pregnancy before the end of the conceptus mobile phase (day 16-17: Ginther 1984). During this period, a twin can easily and successfully be reduced to a singleton by manual crushing of one of the conceptus vesicles.

More recently, the large-scale, commercial exploitation of artificial insemination and, to a lesser extent, embryo transfer (ET) has further improved both the efficiency and the rate of genetic improvement within the sport horse breeding industry. Nowadays, more offspring can be produced per year from genetically valuable stallions and mares, and their genetic material (semen and embryos, respectively) can be cooled or frozen for storage or for transport within or between countries. The most recent developments in equine assisted reproduction include *in vitro* production of embryos using intracytoplasmic sperm injection (ICSI) or nuclear transfer (NT: 'cloning'). The first ICSI horse (Squires et al. 1996; Cochran et al. 1998; McKinnon et al. 2000; Li et al. 2001; Galli et al. 2002), cloned horse (Galli *et al.* 2003) and cloned mule (Woods *et al.* 2003) foals were produced at the turn of the millennium, and both ICSI and cloning are now offered as commercial services, albeit for a select group of sport horses and owners (Palmer 2003; Hinrichs 2005).

Despite these advances, the incidence of early pregnancy loss (EPL) has altered little during the last 20 years. Indeed, EPL remains a significant source of economic loss to the horse breeding industry, and one for which the underlying causes are poorly understood. Nevertheless, it is clear that the majority of equine pregnancy losses occur during the first 5 weeks of gestation (Ball 1988; Morris and Allen 2002), and that advancing maternal age is an important predisposing factor. In this latter respect, although fertilisation rates are similarly high in both aged (≥18 years) and young (≤8 years) mares (approaching 90%: Ball et al. 1986; Ball et al. 1989), embryo recovery and pregnancy rates at days 4, 7 and 15 after ovulation are significantly lower in aged than in younger animals (Ball et al. 1986; Ball et al. 1989; Vogelsang and Vogelsang 1989; Morris and Allen 2002). In addition, more than 20% of day 15 pregnancies confirmed by ultrasonography in aged mares are lost during the following 20 days, as compared to only 5% in younger animals (Morris and Allen 2002). When embryos are produced *in vitro*, reproductive efficiency is lower at every stage of the

process (i.e. fertilisation, embryo development and the establishment and maintenance of pregnancy: Squires et al. 2003; Hinrichs 2005).
This chapter reviews equine embryonic development during the first two weeks of pregnancy, with the aim of highlighting possible sources of compromised developmental competence for embryos generated either *in vivo* or *in vitro*, with particular emphasis on the roles of maternal age and fetal-maternal interaction.

EARLY DEVELOPMENT OF HORSE EMBRYOS *IN VIVO*

Final oocyte maturation

As in other mammalian species (Paulson 1991; Bevers et al. 1997; Bevers and Izadyar 2002), final oocyte maturation in the horse is characterised by two 'resting phases' (Ginther 1992c). At birth, oocytes in the developing ovaries have duplicated their DNA and progressed to diplotene of the first meiotic prophase; they now enter a prolonged resting phase known as the germinal vesicle (GV) stage. The GV stage can last anything from years to decennia, and does not end until the follicle that contains the oocyte has been recruited to the 'growing pool' and is subsequently exposed to the rapid rise in maternal LH concentrations characteristic of oestrus. Rising LH stimulates the follicle to develop further and triggers the oocyte to undergo germinal vesicle breakdown (GVBD) and complete the first meiotic division. At the end of meiosis I, the homologous chromosomes are separated and one set is 'discarded' by extrusion, together with a minimum of cytoplasm, as the first polar body. The oocyte then embarks on the second meiotic division but again arrests, this time at the metaphase-II (M-II) stage with the sister chromatids aligned along the metaphase plate in preparation for the second reducing division that will result in a gamete with a haploid complement of chromosomes (32 in the horse). By this time, the antral follicle should have developed to the point of ovulation.

Oviductal development of the horse embryo

Following ovulation, the oocyte arrives in the ampulla of the oviduct still surrounded by its protective coating of cumulus cells. The cumulus oocyte complex (COC) then passes rapidly through the ampulla to the ampullary-isthmic junction where it lodges and, assuming mating/insemination has already taken place, is fertilised by one of the spermatozoa already present (Boyle et al. 1987; Ginther 1992c; Hunter 2002; Hunter 2005). After penetration of the oocyte by a spermatozoon, the second meiotic division restarts, the second polar body is extruded and the remaining chromosomes are organised into the female pronucleus. This haploid female pronucleus then fuses with the sperm-derived male pronucleus to give rise to the diploid nucleus of the zygote (Ginther 1992a). Two days later the developing conceptus, still lodged at the ampullary-isthmic junction (Ginther 1992c), contains approximately 4 blastomeres, and the embryonic genome is activated (Betteridge et al. 1982; Betteridge 2000). Embryonic development continues within the oviduct for another 4 days until the compact morula begins to secrete the PGE_2 (Weber et al. 1991b)

which induces relaxation of the ampullary-isthmic 'sphincter' (Weber et al. 1991a) and enables the embryo to pass rapidly through the isthmus and uterotubal junction to enter the uterine lumen at around day 6-6.5 after ovulation (Freeman *et al.* 1991; Battut *et al.* 1997). At the time of uterine entry, the embryo is at the late morula or early blastocyst stage of development, contains around 600 cells and is still surrounded by the zona pellucida (Betteridge et al. 1982; Freeman et al. 1991; Battut et al. 1997; Rambags et al. 2005).

Early intrauterine embryo development

Soon after its arrival in the uterus, the developing embryo begins to form a unique tertiary embryo coat, the acellular glycoprotein capsule (Betteridge *et al.* 1982). The capsule first becomes visible at around the time of uterine entry as patches of iridescent material between the trophectoderm and the zona pellucida (Stout et al. 2005); by day 7 after ovulation the primordial capsular material has coalesced into a confluent layer, the embryo has formed a blastocyst cavity and embarked on expansion and, soon after, the embryo hatches from its zona pellucida fully enveloped by its new protective coat. The newly hatched early blastocyst has a diameter of approximately 200-250 μm (Betteridge *et al.* 1982) and contains a few thousand cells (Tremoleda et al. 2003a; Rambags et al. 2005). During the following week of development, the conceptus remains spherical, surrounded by the capsule and continues to expand extremely rapidly, reaching 3-5 mm in diameter on day 10 after ovulation and 15-20 mm by day 14, predominantly as a result of blastocoel/yolk sac fluid accumulation (Betteridge *et al.* 1982). During the same time period, the embryo proper develops from an extremely small, relatively undifferentiated inner cell mass on day 7, to a more extensive, but still microscopic, embryonic disc consisting of (troph)ectoderm and yolk-sac endoderm on day 10; by day 14, the embryonic disc is visible macroscopically and contains a clear primitive streak through which cells have begun to migrate and differentiate to create mesoderm, in a process known as gastrulation (Ginther 1992a; Enders et al. 1993; Gilbert 1997a; Betteridge 2000).

Fetal-maternal interaction

Following its arrival in the uterus, the equine conceptus neither invades nor makes a firm attachment to the endometrium (implantation) until surprisingly late in gestation (approximately day 40; for review see Allen and Stewart 2001). Instead, the spherical vesicle migrates throughout the uterine lumen propelled by myometrial contractions (Stout and Allen 2001), until it becomes fixed at the base of one of the uterine horns on around day 16-17 after ovulation (Ginther 1984) as a result of increasing embryonic size combined with increasing uterine tone (Ginther 1983). As will be discussed later, the so-called mobile phase appears to play a vital role in fetal-maternal interaction, including the maternal recognition of pregnancy, and may also help the conceptus to more efficiently harvest uterine nutrients.

Histotrophe production by the endometrium

The suitability of the uterine environment for supporting conceptus growth and development depends critically on maternal circulating reproductive steroid concentrations. In particular, progesterone is essential for the maintenance of pregnancy because it prepares the uterus for the arrival of the conceptus by stimulating proliferation of the endometrial glands (Kenney 1978; Clarke and Sutherland 1990) that produce the protein-rich histotrophe ('uterine milk'), the only source of nutrient for the pre-implantation conceptus (Allen 2001). Moreover, the precise composition of histotrophe alters during the course of early pregnancy under the control of maternal and conceptus progesterone and oestrogen production (Zavy et al. 1979a; Zavy et al. 1982; McDowell et al. 1990). Histotrophe consists of an array of reproductive-steroid inducible proteins thought to be involved in the transport of essential nutrients to the conceptus, such as uteroferrin (Zavy *et al.* 1982), uteroglobin (Muller-Schottle *et al.* 2002) and lipocalin (P19: Crossett et al. 1996; Crossett et al. 1998).

Maternal recognition of pregnancy

A continuing supply of (maternal) progesterone is the single most important condition necessary for the maintenance of pregnancy in eutherian mammals. In polyoestrous species like the horse, continued progesterone provision is initially achieved by a conceptus-directed prolongation of the lifespan of the primary corpus luteum (CL), in a process known as the maternal recognition of pregnancy (MRP: Short 1969; Allen 2001). In the case of the horse, it appears that conceptus signalling for MRP must start shortly before day 10 after ovulation (Goff et al. 1987; Stout et al. 1999) and be completed before days 14-16 (Hershman and Douglas 1979; Sharp et al. 1989b). Moreover, luteal prolongation in the mare appears to be achieved by an antiluteolytic mechanism in which the conceptus must contact the entire endometrium and suppress the cyclical release of the uterine luteolysin, prostaglandin $F_{2\alpha}$ (Sharp et al. 1989b). Since the horse is monotocous and the single conceptus remains enveloped by the tough glycoprotein capsule and, probably as a result, completely spherical throughout the MRP period (Allen 2001), it achieves its goal of interacting with as much of the endometrium as possible by migrating throughout the uterine lumen, driven by myometrial contractions (Stout and Allen 2001). In this respect, conceptus mobility in the equids is functionally analogous to the dramatic elongation of the trophoblast that is such a marked feature of the MRP phase in the other domestic ungulate species (Amoroso 1952), but is so obviously absent in the horse. That conceptus mobility is essential to MRP in the horse was proven by McDowell et al. (1988) who found that surgically restricting conceptuses to a single uterine horn resulted in a failure to prolong CL lifespan. Although the precise nature of the MRP signal in the horse is not known, recent studies have demonstrated that the antiluteolytic mechanism is based on a conceptus-directed suppression of the cyclical up-regulation of oxytocin receptors in the endometrium (Stout *et al.* 2000). During days 10 to 16 after ovulation, the increases in endometrial oxytocin receptor number (Starbuck *et al.* 1998) and the $PGF_{2\alpha}$ release response to oxytocin challenge that underlie cyclical luteolysis are completely abolished in pregnant mares (Goff *et al.* 1987; Starbuck *et al.* 1998). Furthermore, equine conceptuses are known to secrete a variety of substances like PGE_2 and $PGF_{2\alpha}$ (Stout and Allen 2002), IGF-1 (Walters *et al.*

2001), and oestrogens (Zavy et al. 1979b; Marsan et al. 1987; Heap et al. 1991; Walters et al. 2001) that, even if they are not directly involved in MRP, play a role in processes such as uterine migration of the conceptus (Freeman et al. 1991; Allen 2001), increased uterine vascularity, and the establishment and maintenance of an environment capable of supporting pregnancy (Simmen *et al.* 1993; Walters *et al.* 2001).

PROBABLE CAUSES OF EARLY PREGNANCY LOSS *IN VIVO*

The underlying causes of early pregnancy loss in the mare are likely to include abnormalities of the oviductal or uterine environments in which the embryo must develop (maternal factors), aberrations within the preovulatory oocyte and/or embryo itself (intrinsic factors), and failures of fetal-maternal communication (e.g. failed MRP).

Maternal factors

Since equine embryos spend an unusually long part of their early development within the oviduct, it is likely that an inadequate oviductal microenvironment, e.g. due to impaired oviduct secretory activity or salpingitis (which is more common in aged mares: Henry and Vandeplassche 1981), could adversely affect subsequent embryonic development. Similarly, a suboptimal uterine environment as a result of either an acute inflammatory process or a more chronic endometrial degenerative condition (e.g. endometrosis) will be detrimental to conceptus development. In this respect, endometrosis is an age-related problem characterised by endometrial fibrosis, dilation of glands and the formation of both glandular and lymphatic cysts, and is associated with higher rates of pregnancy loss, possibly because of impaired histotrophe production or physical obstruction to conceptus mobility (Kenney and Ganjam 1975; Kenney 1978; Kenney and Doig 1986; Adams et al. 1987; Bracher et al. 1992; Tannus and Thun 1995; Rambags and Stout 2005). The incidence of post-breeding endometritis is also higher in aged mares because they are more often incapable of adequately resolving the physiological inflammatory response to mating or insemination because, for example, of deficiencies in uterine contractility, lymphatic drainage and cervical and/or vulval conformation (Rambags *et al.* 2003); if the inflammation is not resolved by the time the embryo enters the uterus, EPL will result from infection of the embryo or premature luteolysis (Adams *et al.* 1987; Katila 1996; Troedsson 1999).

Even when possible oviductal and/or uterine deficiencies are circumvented by recovering oocytes, day 4 or day 7 embryos from aged mares and transferring them to the oviduct or uterus of a young mare, pregnancy rates are significantly lower than if the oocyte or embryo was derived from a younger mare (Ball et al. 1989; Vogelsang and Vogelsang 1989; Carnevale and Ginther 1995). A similar effect of maternal age has been noted in human oocyte donation programmes (Templeton et al. 1996; Sauer 1997). Overall, it appears that the age-related increase in EPL in mares is more likely to be related to defects

within the oocyte and/or embryo *per se*, than to a suboptimal oviductal or endometrial environment.

Intrinsic factors

Of the many theoretically possible causes of impaired oocyte and embryo developmental competence, the two thought to be most closely associated to the age-related decline in female fertility are a decline in mitochondrial function and an increased incidence of chromosomal abnormalities.

Mitochondrial quality and quantity

Mitochondria are classically the site of intracellular energy generation via oxidative phosphorylation (OXPHOS). However, they are also involved in other metabolic processes, such as steroid production, β-oxidation and calcium homeostasis, and in various processes associated with cell deterioration, such as the production of reactive oxygen species (ROS: Wallace 1994; Scheffler 2000). In this respect, ROS are known to be harmful to intracellular structures such as lipids, proteins and DNA, and the damage they cause has been shown to accumulate with age, particularly in energy demanding post-mitotic tissues such as brain and muscle; indeed, age-related increases in mitochondrial DNA (mtDNA) mutations have been described in these tissues (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Melov et al. 1995; Jazin et al. 1996; Nagley and Wei 1998). Although their energy demands are modest, mammalian oocytes in the 'resting pool' are also non-dividing, post-mitotic cells. Moreover, the long period between birth and final oocyte maturation may allow ROS induced damage to accumulate. Indeed, minor structural damage has been described in the mitochondria of resting pool oocytes from aged women (de Bruin *et al.* 2004). The effects of such damage may go essentially unnoticed until the oocyte embarks on its final maturation, and its energy demands rise. An inability to respond to the sudden rise in energy requirements may initiate a vicious circle of further ROS production, damage and loss of mitochondria and, as a result, apoptosis or reduced oocyte developmental competence and fertilisability. However, the adverse consequences need not be restricted to the oocyte. Embryonic mitochondria are exclusively maternally inherited, i.e. oocyte derived (Hutchison et al. 1974; Giles et al. 1980; Kaneda et al. 1995) because the few mitochondria introduced by the sperm during fertilisation are subsequently eliminated by ubiquitination and proteolysis (Sutovsky *et al.* 1999). Moreover, at least in the mouse (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005), mitochondrial replication is arrested between fertilisation and gastrulation, and a finite pool of oocyte-derived mitochondria is therefore divided over an ever increasing number of blastomeres. Any reduction in oocyte mitochondrial quantity or quality may, thus, also affect embryonic development and survival up to the gastrula stage. However, while there is some evidence to support the hypothesis that reduced fertility in aging female mammals is due to mitochondrial degeneration (Keefe et al. 1995; Larsson et al. 1998; Barritt et al. 2000; Thouas et al. 2004; Takeuchi et al. 2005; Thouas et al. 2005; El Shourbagy et al. 2006), it is far from proven. Moreover, the possible role of mitochondrial degeneration in the age-related decline in fertility in mares has not been examined.

Chromosomal abnormalities

Most chromosomal abnormalities are numerical aberrations that arise spontaneously during gametogenesis, at fertilisation or during early embryonic development (King 1990). These numerical chromosomal abnormalities are a relatively common finding in oocytes and embryos of many mammalian species (McFeely 1967; Fechheimer and Beatty 1974; Hare et al. 1980; Long and Williams 1980; Munne et al. 1994; Villamediana et al. 2001) and will almost certainly compromise the viability of a developing conceptus (King 1990). Moreover, the incidence of chromosomal aberrations in human oocytes (Pellestor et al. 2003; Pellestor et al. 2005) and embryos (Hook *et al.* 1983) increases with maternal age, coincident with a decrease in pregnancy rate and an increase in the incidence of spontaneous abortion (Menken et al. 1986; Nybo Andersen et al. 2000; Heffner 2004). As a result, chromosomal abnormalities are considered an important cause of reduced fertility in older women.

Chromosomal abnormalities have also been proposed as an important cause of early embryonic loss in aged mares (Ball 1988; King 1990). However, while previous studies have revealed a low incidence of chromosomal abnormalities in horse oocytes (King *et al.* 1990; Lechniak *et al.* 2002), spermatogonia (Scott and Long 1980) and adults (Long 1988; Lear et al. 1999), their presence in equine conceptuses, or role in age-related mare infertility, has not yet been confirmed (Blue 1981; Haynes and Reisner 1982; Romagnano *et al.* 1987).

Impaired fetal-maternal interaction

Pregnancy maintenance depends critically on an adequate and properly timed exchange of signals between a conceptus and its dam. This is illustrated clearly by the fact that for successful embryo transfer (ET) the cycle stage of the recipient mare must be synchronised with the developmental stage of the transferred embryo. The highest pregnancy rates are achieved when embryos are transferred to recipients that ovulated not more than one day before, or up to 3 days after, the donor (Squires et al. 1982; McCue and Troedsson 2003; Stout 2003). Failure of embryo survival when recipient synchrony is inadequate is most probably for one of two reasons. On the one hand, the conceptus will be exposed to an asynchronous endometrial environment with ‘out of phase’ levels of growth factors and other proteins, which could result in growth retardation or embryonic death (Barnes 2000). On the other hand, the conceptus may not be able communicate its presence to its dam in time to ensure MRP or to promote the changes in endometrial secretion that it requires for survival.

A comparable impairment of embryonic-maternal communication could also occur during a conventional (i.e. not ET) pregnancy and thereby result in embryonic loss in the period soon after the embryo’s arrival in the uterus. Molecules likely to play important roles in embryo-maternal communication in the mare include the MRP signal and the reproductive steroids, progesterone and oestrogens.

Reproductive steroids and EPL

As mentioned previously, sufficient levels of the reproductive steroid hormones, in particular progesterone, are essential for pregnancy maintenance in mammals. Moreover, it has long been believed that the stimulatory effects of progesterone and oestrogens on conceptus development are indirect, and mediated primarily via the endometrium (Wu et al. 1971; Baulieu 1989; Clarke and Sutherland 1990; Singh et al. 1996). In this respect, the steroid hormones classically bind to intracellular (and sometimes nuclear) receptors and exert their effects via a relatively slow genomic pathway that involves the migration of the steroid-receptor complex to the nucleus where it influences both the transcription of genes into mRNA and the subsequent translation of mRNA into the proteins that exert the appropriate biological effects (Jensen et al. 1968; Beato 1989; Tuohimaa et al. 1996). However, in recent years these concepts have been challenged in two ways. First, progesterone (PR) and oestrogen (ERα and ERβ) receptor mRNA and protein have been detected in the mature cumulus oocyte complex and embryonic cells of several species (Hou and Gorski 1993; Hou et al. 1996; Ying et al. 2000; Kowalski et al. 2002; Hong et al. 2004; Hasegawa et al. 2005), suggesting that reproductive steroids may have direct effects on the conceptus and its pre-implantation development. Second, it has become increasingly obvious that steroid hormones sometimes induce their effects rapidly, even in cells that lack a (functioning) nucleus and are therefore incapable of employing genomic pathways (Losel and Wehling 2003). In spermatozoa for example, binding of progesterone to the cell membrane can induce the acrosome reaction in a matter of seconds (Meizel and Turner 1991; Roldan et al. 1994; Sabeur et al. 1996; Cheng et al. 1998a; Cheng et al. 1998b), even though the sperm nuclear DNA is transcriptionally inactive (Gilbert 1997b). In addition, two novel membrane associated progesterone receptors, PGRMC1 (Falkenstein et al. 1996; Meyer et al. 1996; Selmin et al. 1996; Falkenstein et al. 1998; Gerdes et al. 1998; Losel et al. 2004; Losel et al. 2005) and mPR (Zhu *et al.* 2003), have now been characterised in a number of different cell types, including sperm, of several mammalian species (Losel et al. 2004; Losel et al. 2005). It thus appears increasingly likely that reproductive steroids may affect conceptus development directly, rather than only by modulating endometrial function. In fact, it is not only maternally-derived steroids that may influence conceptus development, it is also possible that there are autocrine or paracrine effects since mammalian conceptuses from a range of species, including the horse (Zavy et al. 1979b; Marsan et al. 1987; Walters et al. 2001), produce both oestrogens and, to a lesser extent, progesterone (Perry et al. 1973; Dickmann and Dey 1974; Dey and Dickmann 1974; Gadsby et al. 1980; Hoversland et al. 1982; Eley et al. 1983; Sholl et al. 1983; Heap et al. 1991; Edgar et al. 1993; Skidmore et al. 1994). Nevertheless, it is currently not known whether progesterone and oestrogens have direct effects (autocrine, paracrine or endocrine) on early equine conceptus development and, indeed, whether the conceptus possesses receptors that would allow it to respond to these hormones via either the genomic or alternative pathways. Identification and localisation of the receptors, followed by investigations into the possible direct roles of the reproductive steroids in conceptus development, are important initial steps in determining whether deficiencies in reproductive steroid stimulation could contribute to early embryonic death.

Maternal recognition of pregnancy and EPL

The most critical physiological aspect of fetal-maternal communication during the second week of pregnancy in the mare is the maternal recognition of pregnancy. If the conceptus fails to adequately signal its presence to its dam, or she fails to adequately detect and respond to that signal, luteolysis will occur at around days 14-16 after ovulation, and pregnancy loss will follow shortly as a result of the fall in circulating progesterone concentrations (Ginther 1992b). Since pregnancy loss occurs most frequently between the time of initial pregnancy detection (day 15) and day 35 after ovulation (Morris and Allen 2002), it is tempting to speculate that aberrations in MRP, for example due to retarded embryo development, may be a significant cause of pregnancy loss in the horse. If that were the case, parenteral administration of the MRP signal would be expected to reduce the incidence of early pregnancy loss by prolonging the lifespan of the primary corpus luteum, as oestrogens do in sows (Pusateri *et al.* 1996). Unfortunately, while the MRP signal is known for most large domestic ungulates (oestrogens in pigs and interferon τ in ruminants: Spencer et al. 2004), the identity of the equine anti-luteolytic factor remains a mystery. Previous studies have, for various reasons, ruled out several potential equine MRP signals such as oestrogens (Woodley et al. 1979; Goff et al. 1993; Vanderwall et al. 1994), interferons (Sharp et al. 1989a; McDowell et al. 1990; Baker et al. 1991), and PGE_2 (Vanderwall et al. 1994; Ababneh et al. 2000). On the other hand, experiments involving fractionation of conceptus products have indicated that the equine MRP signal has a molecular weight between 3 and 10 kDa (Sharp et al. 1989b; Ababneh et al. 2000). In this latter respect, Stout *et al.* (2004) recently proposed insulin, a 6kDa protein, as a candidate equine MRP signal; this hypothesis has still to be tested.

IN VITRO PRODUCTION OF EQUINE EMBRYOS

To date, and despite numerous attempts, only two foals have been produced by conventional *in vitro* fertilization (IVF: Palmer et al. 1991; Bezard 1992). The main barrier to successful IVF appears to be zona pellucida penetration by the sperm, since zona dissection (Choi *et al.* 1994) or drilling (Li *et al.* 1995) both increase fertilisation rates. For this reason, intracytoplasmic sperm injection (ICSI) has become the method of choice for *in vitro* fertilisation in horses.

ICSI

ICSI involves the injection of a single sperm into an M-II oocyte. In general, a single motile sperm is selected and immobilised by crushing its tail between the injection pipette and the bottom of the petri dish; the sperm is then aspirated into the injection pipette and injected into the cytoplasm of the matured oocyte at 3 o'clock, while the first polar body is held at either 6 or 12 o'clock; this minimises the risk of damaging the meiotic spindle (metaphase plate). The zygote can subsequently either be transferred to the oviduct of a recipient mare or cultured further, either in the oviduct of a progesterone-treated ewe or *in vitro*, before transfer to the uterus of a recipient (Squires et al. 2003; Hinrichs 2005; Galli et al. 2006). In

1996, Squires *et al.* reported the first ICSI horse pregnancy; subsequently, a number of ICSI foals have been born at various clinics (Cochran *et al.* 1998; McKinnon *et al.* 2000; Li *et al.* 2001; Galli *et al.* 2002), some of which now offer ICSI as a commercial service (Hinrichs et al. 2005).

Impaired developmental competence of equine ICSI embryos

Compared to the *in vivo* generation of pregnancies/foals, the *in vitro* production (IVP) of equine embryos to produce foals suffers from significant reductions in the success rate at almost every stage of the process. For example, although a high percentage of *in vitro* matured oocytes reach the M-II stage (58-84%: Choi et al. 2002; Tremoleda et al. 2003b) and the majority of these M-II oocytes cleave after ICSI (60-94%: Choi et al. 2002; Choi et al. 2004a), only a small percentage of cleaved ICSI embryos reach the blastocyst stage during subsequent *in vitro* culture (2-27%: Choi et al. 2004b; Choi et al. 2006). Furthermore, transcervical transfer of IVP blastocysts to recipient mares appears to result in a slightly reduced pregnancy rate (46% [6/13]: Hinrichs et al. 2005; 66% [16/24]: Galli et al. 2007) when compared to transfer of *in vivo* generated embryos in a commercial embryo transfer programme (70-75%: Squires et al. 2003), and preliminary evidence suggests that the likelihood of early pregnancy loss may be increased (33%, Hinrichs et al. 2005; 53%, Galli et al. 2007). Causes for the apparent reduction in the developmental competence of ICSI embryos are likely to include the same intrinsic factors as described for *in vivo* embryos, e.g. impaired mitochondrial function and chromosomal abnormalities. Indeed, suboptimal *in vitro* culture conditions may predispose to these abnormalities.

SCOPE OF THE THESIS

The overall aim of this thesis was to examine intrinsic (oocyte and embryo) and/or maternal factors likely to play important roles in the establishment, maintenance or loss of pregnancy. The period of interest would span from final oocyte maturation up to the completion of maternal recognition of pregnancy (i.e. prolongation of CL activity beyond day 16 after ovulation). A better understanding of embryo development and embryo-maternal interaction during the first two weeks of pregnancy should help to define critical processes or events that, if they fail, might lead to early pregnancy loss. Moreover, if (potential) causes of early pregnancy loss could be defined, it should become possible to design treatments to reduce its incidence (i.e. prevent pregnancy losses resulting from defined deficiencies).

The first specific aim was to investigate the effects of maternal age and *in vitro* culture on mitochondrial quantity and quality in oocytes, and in embryos during their development prior to the onset of mitochondrial replication. To this end, in chapter 2 the influence of maternal age on oocyte mitochondrial quantity and quality before and after maturation of oocytes *in vitro* was studied by examining mtDNA copy number, mtDNA sequence deletions or mutations, and the normality of mitochondrial morphology. Subsequently, the

time course of mitochondrial replication during early embryonic development, and the possible negative effects of maternal aging or *in vitro* embryo production on mitochondrial number were investigated (chapter 3).

One of the proposed consequences of inadequate oocyte / early embryo mitochondrial dysfunction is aberrant meiotic spindle function, abnormal chromosome disjunction and an increased incidence of maternally-inherited chromosomal abnormalities (Eichenlaub-Ritter et al. 2004). In this respect, chromosomal abnormalities have frequently been proposed as one of the major causes of early pregnancy loss in the mare; however, their occurrence has not yet been conclusively demonstrated. This may, in part, be because previous studies have relied on conventional karyotypic analysis, which can only be performed on the small proportion of nuclei in metaphase or those forced into metaphase by a short period in culture. In chapter 4, we validated fluorescent in situ hybridization (FISH) probes to enable us to identify specific equine chromosomes in non-dividing nuclei and thereby to determine whether (gross) numerical chromosomal abnormalities occur in equine embryos and whether their incidence is increased by *in vitro* production.

Chapters 5 and 6 examine two specific aspects of fetal-maternal communication, the one focussing on how the embryo might direct MRP, and the other on a novel route by which the maternal organism might influence conceptus development during the second week of pregnancy. Preliminary data from other mammalian species suggests that the reproductive steroids may influence conceptus development directly, and not only via their effects on endometrial 'receptivity'. To determine whether early uterine stage horse conceptuses may also be amenable to direct modulation by maternal progesterone and/or oestrogens, the expression and localisation of steroid receptors in day 7-16 equine embryos was examined (chapter 5). Chapter 6 focussed on how the conceptus signals its presence to its dam to prevent luteolysis and maintain the high progesterone levels required for the continuation of pregnancy. Although the identity of the equine MRP signal is still not known, it has recently been suggested that conceptus-produced insulin may fulfil this role, both because the molecule is in the appropriate molecular size range for the putative MRP signal and because insulin has been shown to have myriad effects on reproductive cyclicity and CL activity in other species. In chapter 6, the effects of parenteral insulin administration on luteolysis and reproductive cyclicity in the mare were investigated to determine whether insulin is capable of influencing CL lifespan or activity in the way that would be expected of a putative MRP signal.

Finally, the results of all of these studies and their relevance to the developmental competence and survival of horse oocytes and early embryos are summarised and discussed in chapter 7.

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Chapter 2

Maternal ageing predisposes to mitochondrial damage and loss during *in vitro* maturation of equine oocytes

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ABSTRACT

In many mammalian species, reproductive success decreases with maternal age. This decrease has been proposed to result, at least in part, from an age-related reduction in the mitochondrial quantity and/or quality in oocytes. The aim of this study was to determine whether maternal age and *in vitro* maturation (IVM) affect the quantity and quality of mitochondria in equine oocytes. Oocytes were collected from the ovaries of slaughtered mares categorized as young (<12 years) or aged (≥12 years). The mean mitochondrial DNA copy number was estimated by quantitative PCR and found to be significantly lower in oocytes from aged mares after IVM than in any other group. No evidence of age-related deletions or mutations in the mitochondrial genome was found. Transmission electron microscopy demonstrated that mitochondria in aged mare oocytes subjected to IVM experienced significantly more swelling and damage to the cristae than those in other groups. In conclusion, maternal ageing is associated with a heightened susceptibility to mitochondrial damage and loss in equine oocytes, which manifests during maturation *in vitro*. This predisposition to mitochondrial degeneration probably contributes to the age-related reduction in reproductive success in mares and other female mammals.

INTRODUCTION

In many mammalian species, there is a threshold maternal age beyond which reproductive success decreases markedly (Morris and Allen 2002; Heffner 2004). In women, fertility declines after 35 years of age (Menken et al. 1986), largely as a result of an associated increase in the incidence of spontaneous abortion (Nybo Andersen et al. 2000). The decline culminates in the cessation of fertility in the majority of women at around 41 years of age, even though the menopause does not onset until a mean age of 51 years (te Velde and Pearson 2002). Similarly, the likelihood of a live birth per embryo transferred in a human IVF-program decreases from 24% at maternal ages below 30 years, to 8% at 42 years and 4% at 45 years or more (Templeton et al. 1996). The underlying causes of reproductive senescence have been proposed to include a decrease in the size of the resting follicle pool, a decrease in oocyte quality, and impaired endometrial receptivity. The size of the resting follicle pool is indeed closely related to fertility in women, especially just prior to the menopause (Faddy et al. 1992; Faddy and Gosden 1995). However, a reduction in oocyte number does not explain why the majority of women are cyclic but unable to conceive in the last ten years before the menopause (te Velde and Pearson 2002). Similarly, because the negative effects of advanced maternal age on IVF success can largely be overcome by using an oocyte donated by a younger woman (≤35 years), impaired endometrial receptivity seems a relatively minor contributor to the pre-menopausal decline in female fertility (Templeton et al. 1996; Sauer 1997). Instead, it has been suggested that the primary contributor to reduced fertility is reduced oocyte quality; previous studies have reported a relatively low expression of spindle assembly checkpoint mRNA (Steuerwald et al. 2001), high incidence of spindle aberrations (Battaglia et al. 1996) and high incidence of chromosomal abnormalities (Pellestor et al. 2003; Pellestor et al. 2005) in oocytes from aged women. Similarly, intrafallopian transfer of oocytes from aged mares (20–26 years old) produced significantly fewer embryonic vesicles (31%) in inseminated young mare recipients than when the transferred oocytes were recovered from young (6-10 years old) mares (92%: Carnevale and Ginther 1995).

One of the postulated underlying causes of the age-dependent decrease in oocyte quality is a decline in mitochondrial function (Nagley and Wei 1998; Tilly 2001) similar to that observed during apoptosis. Mitochondria play several important metabolic roles in eukaryotic cells, including energy generation by oxidative phosphorylation (OXPHOS), steroid production, β-oxidation and calcium homeostasis. However, mitochondria are also implicated in processes associated with cell deterioration, such as the production of potentially harmful reactive oxygen species (ROS: Wallace 1994; Scheffler 2000). Since mtDNA is located close to the site of ROS generation, it may be particularly prone to the accumulation of oxidative damage over time. Moreover, mtDNA is more sensitive to mutagens than nuclear DNA because it lacks introns, protective histones (Wallace et al. 1987) and DNA repair mechanisms. Indeed, the mutation rate of mtDNA is more than 10 times higher than that of nuclear DNA (Wallace et al. 1987), and point mutations, deletions and duplications have been reported to accumulate in mtDNA over time, particularly in slowly or non-dividing, post-mitotic tissues with high energy demands such as brain and muscle (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Melov et al. 1995; Jazin et

al. 1996; Nagley and Wei 1998). Mammalian oocytes are also non-dividing, post-mitotic cells; however, their energy demands should be modest since they arrest after entering meiosis and remain in a resting phase until reactivation during final follicle development prior to ovulation. Nevertheless, because this resumption of meiosis may occur many years later, oocytes may, like post-mitotic somatic cells, accumulate mitochondrial DNA damage with increasing host age. However, studies that examined a possible age-related decline in oocyte mtDNA quality have produced conflicting results (Chen et al. 1995; Keefe et al. 1995; Muller-Hocker et al. 1996; Blok et al. 1997; Brenner et al. 1998; Barritt et al. 1999; Barritt et al. 2000). In particular, it has yet to be demonstrated conclusively that maternal age is related to a decrease in oocyte mtDNA quantity (Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002). Critically, several of the earlier studies were hampered by the use of relatively small and biased populations of oocytes obtained from aged women attending IVF clinics because of fertility problems. Furthermore, the oocytes available for analysis were predominantly those considered unsuitable for transfer because of fertilisation failure.

The aim of the present study was to determine whether maternal age significantly affects the quality of equine oocytes before and/or after maturation *in vitro*, in terms of mtDNA copy number, mtDNA sequence deletions or mutations, and the normality of mitochondrial morphology. The mare is an attractive animal model, because the horse is a monovulatory species in which fertility decreases markedly with advancing maternal age (Morris and Allen 2002), the time interval to reproductive senescence is more comparable to women than in laboratory species (e.g. mouse), and oocytes can be obtained from slaughtered animals.

MATERIALS AND METHODS

Collection and culture of cumulus oocyte complexes

Immediately after slaughter, the ovaries were recovered from 268 mares. Age was estimated on the basis of standard parameters for dental eruption and wear described for horses (Muylle et al. 1996; Lowder and Mueller 1998). Animals for which the age could not be estimated reliably, due for example to missing dental elements, dental malformation, abnormal attrition or dental disease, were excluded from the study. After recovery, the ovaries of young mares (<12 years old) and aged mares (≥12 years) were transported separately to the laboratory in thermos flasks at approximately 30°C. Upon arrival at the laboratory within 4 hours of slaughter, the ovaries were washed with tap water (30°C) and any extraneous tissue was removed. The ovaries were subsequently maintained at 30°C in a 0.9% (w/v) saline solution supplemented with 0.1% (v/v) penicillin/streptomycin (Gibco BRL, Life Technologies, Paisley, UK). Cumulus oocyte complexes (COCs) were then recovered and, either immediately prepared for storage, or cultured as previously described by Tremoleda *et al.* (2001), with minor modifications. COCs were collected by aspirating the contents of 5-30 mm follicles using a 16 gauge short-bevel needle attached via an infusion set to a 250 ml polypropylene collection tube (Corning Incorporated–Life

Sciences, Big Flats, New York, USA) which was, in turn, connected to an adjustable vacuum pump. To increase the likelihood of COC recovery, after aspiration the follicle lumen was flushed 2-3 times in rapid succession with 0.9% (w/v) saline solution supplemented with 0.1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Chemicals BV, Zwijndrecht, the Netherlands) and 25 IU/ml heparin (Leo Pharmaceutical, Weesp, The Netherlands) while the follicle wall was scraped vigorously with the bevel of the needle. The recovered fluid was then allowed to stand for approximately 10 minutes at room temperature so that the COCs could settle to the bottom of the tube. The sediment containing the COCs was then recovered and washed twice with HEPES-buffered synthetic Human Tubal Fluid (Q-HTF; BioWhittaker, Verviers, Belgium) supplemented with 0.4% BSA, after which the COCs were isolated by searching the sediment with a stereomicroscope. For each mare age group, recovered oocytes were divided randomly into 2 groups. In the first group (young, n = 127; aged, n = 106), oocytes were denuded of their cumulus cells (not-IVM) by vortexing them for 4 min in calcium and magnesium free Earles's Balanced Salt Solution (EBSS) containing 0.25% (v/v) trypsin-EDTA (Gibco BRL, Life Technologies, Paisley, UK). After vortexing, oocytes were examined under an inverted stereomicroscope to confirm that there were no remaining cumulus cells; incompletely denuded oocytes were discarded. In the second group (young, n = 114; aged, n= 154), COCs were matured *in vitro* (IVM) before denudation and storage. Prior to IVM, these COCs were washed twice in 38ºC prewarmed HEPES-M199 and once in a maturation medium consisting of M199 tissue culture medium supplemented with 10% FCS, 0.01 units/ml porcine FSH and 0.01 units/ml equine LH (Sigma-Aldrich Chemicals BV). Oocytes were then matured by incubating them in groups of 10-25 in 500 µl of maturation medium in 4-well plates (Nunc A/S, Roskilde, Denmark) for 30 h at 38.7°C in a humidified atmosphere of 5% CO_2-in-air. After denudation, IVM oocytes were examined with an inverted stereomicroscope to confirm that cumulus removal was complete and to determine whether a first polar body was visible. The IVM oocytes were then divided into those that had clearly reached the M-II stage, i.e. they had a visible first polar body (M-II), and those that had not (not-M-II). In all cases, after denudation and examination, oocytes were washed three times in PBS containing 0.1% (w/v) PVA (polyvinyl alcohol; Sigma-Aldrich Chemicals BV) and three times in TE, consisting of 10 mM Tris (MP Biomedicals Inc., Eschwege, Germany) and 0.1 mM EDTA (BDH Ltd., Poole, UK) in double distilled water, before they were placed individually in eppendorf tubes in 5 µl of TE and stored at –20°C until further analysis.

DNA extraction

Oocytes were lysed by adding 5 µl of an alkaline lysis buffer (200 mM KOH and 50 mM dithiothreitol), incubating them at 65˚C for 10 minutes and then vortexing. After addition of 5 µl of neutralisation buffer (0.9 M Tris-HCl, 0.3 M KCl and 0.2 M HCl), the lysates were further diluted to a total volume of 150 µl and stored at -20°C.

Preparation of reference samples and internal controls

Two series of reference samples were produced to ensure that values obtained in different quantitative PCR (QPCR) plates were comparable. First, a DNA sequence of 428 bp spanning the fragment used for QPCR was amplified (Table 1: #1) and purified using the Qiaquick Purification Kit (Qiagen, Venlo, the Netherlands). The copy number of the PCR was determined by measuring absorbance at 260 nm. Although after quantitative PCR, serial dilutions gave a linear standard curve, results for the lower concentrations became less reliable after storage at -20ºC. Therefore, a stock of lysed oocytes enriched with purified genomic DNA isolated from peripheral equine lymphocytes was prepared. Tenfold serial dilutions were amplified by QPCR and calibrated using freshly prepared dilutions of the PCR product described above and analyzed on the same microtiter plate. This reference series was reproducible after a period of storage at -20°C and was aliquoted, stored at -20°C and subsequently used on all microtitre plates. Internal assay controls were prepared by pooling a number of lysed denuded equine oocytes and storing aliquots at -20ºC either undiluted (IC-high) or after 10-fold dilution (IC-low).

Determining oocyte mtDNA copy number by quantitative PCR

Quantitative PCR (QPCR) was performed in 96-well plates using a real-time PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, the Netherlands). To minimize plate dependent effects, 96-well plates included triplicate samples of the 2 negative controls (water), the 2 internal controls (IC-high and IC-low), the 6 reference samples, and 11 not-IVM oocytes and 11 IVM oocytes. The location of the not-IVM (n = 233) and IVM samples (n = 268) was alternated in successive plates.
PCR primers (Table 1: #2) were designed for the quantification of oocyte mtDNA content. The PCR reaction mixture contained 1 µl of 1:150 diluted oocyte lysate, 2.5 µl GeneAmp® 10x PCR Gold Buffer (Applied Biosystems, Nieuwerkerk a/s IJssel, the Netherlands), 2mM $MgCl_2$ (Applied Biosystems), 10pM fluorescein (Bio-Rad), 5 µl of a 1:20,000 dilution of SyberGreen (Bio Wittaker, Inc., Walkersville, MD, USA), 0.2mM of each dNTP (Promega Benelux BV, Leiden, the Netherlands), 0.4mM of each primer (Isogen Bioscience BV, Maarsen, the Netherlands) and 0.625 IU Amplitaq Gold (Applied Biosystems) made up to a total volume of 25 µl with double distilled water. After denaturation by heating to 95˚C for 5 min, 40 PCR cycles consisting of incubation at 95˚C for 20 s, 67.7˚C for 30 s and 72˚C for 30 s, were followed by a further 5 min at 72˚C. The PCR product was checked by melting curve analysis, gel electrophoresis and sequencing of the amplicons. To determine mtDNA copy number in each oocyte, sample threshold cycles were plotted against DNA concentration. The internal control samples (IH-low and IC-high) were used to monitor the intra- and inter-assay coefficients of variation which were 13.1% and 26.7%, respectively; i.e., similar to those reported previously (Reynier et al. 2001; Bhat and Epelboym 2004).

TABLE 1. Primer pairs used for mtDNA quantification, detection of deletions and mutations, and the production of a reference PCR product.

ID	Forward primer (5'-3')	Position*	Reverse Primer (5'-3')	Position*	T_a	Purpose
#1	AAGAAAACCCCACAAAACTA	14051	GTGAATGAAGAGGCAGATAAAA	14478	55°C	Reference PCR product
#2	CATGATGAAACTTCGGCTCCCT	14273	TGAGTGACGGATGAGAAGGCAG	14390	67.7˚C	mtDNA quantification
#3	GTTCAGACCGGAGTAATCCAGG	2528	AGGATTGGTGCGATGATGAATA	3000	62˚C	Detection of deletions and mutations
#4	TCCAATCCTTTATCAACACC	6042	AGGTTTGGTTGAGTGTGTA	6586	58°C	Detection of deletions
#5	CACCATCAACACCCAAAGCT	15424	CCTGAAGAAAGAACCAGATGC	15862	58°C	Detection of mutations

*bp position in equine mtDNA (GenBank entry: NC_001640); T_a: Annealing temperature.

Other PCR reactions and sequencing

Two primer pairs (Table 1: #3 and #4) enclosed a DNA region with two direct sequence repeats (12 and 13bp, respectively), since such repeats are known to predispose to mtDNA deletions (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996). A third primer pair (Table 1: #5) amplified a sequence within the D-loop, the most variable region of the mtDNA; this amplicon did not include any >10bp repeats. PCR amplification was performed in standard conditions using the following protocol: denaturation at 94˚C for 2 min, 35 cycles of 94˚C for 30 s, annealing at the appropriate temperature (Table 1) for 45 s and 72˚C for 30 seconds, followed by 72˚C for 2 min. The PCR product sizes were analyzed by agarose gel electrophoresis and sequencing from both ends using the Cy5 Big Dye terminator kit (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands) and the ABI Prism 3100 sequence apparatus, as described by the manufacturer. Only sequences without background noise were examined for the presence of mutations and/or mutation heteroplasmy.

Electron microscopic assessment of mitochondrial morphology

Oocyte mitochondrial morphology was examined by transmission electron microscopy (TEM). Directly after collection or after *in vitro* maturation, COCs were fixed for 35-65 h in Karnovsky fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 0.08 M Na-cacodylate buffer (pH 7.4), 0.25 mM $CaCl_2$ and 0.5 mM $MgCl_2$) without prior denudation. After fixation, the oocytes were washed for 10 min in 0.1 M Na-cacodylate buffer (pH 7.4) and immersed for 2 periods of 1 h in 2% osmium tetroxide in the same buffer. Subsequently, the samples were stained for 1 h with 2% aqueous uranyl, before being dehydrated by passage through a graded series of acetone solutions, and then embedded in Durcupan ACM (Fluka, Buchs, Switzerland). At intervals of 50 µm throughout each block, exploratory semithin sections (1 µm) were cut using a glass knife on a Reichert Ultracut S microtome (Leica Microsystems B.V., Rijswijk, the Netherlands). These sections were stained with toluidine blue and screened for the presence of the COC by light microscopy. If the COC was detected, further semithin sections were cut at 10 µm intervals until the maximum diameter of the COC was reached. Subsequently, ultrathin sections (50 nm) were cut with a diamond knife and mounted on single-slot formvar-carbon-coated copper grids. After staining with lead citrate for 2 min, the sections were examined and electron micrographs were taken at random locations within the equatorial plane of the oocyte via an electron microscope (Philips CM 10; Philips, Eindhoven, the Netherlands) using 80 kV and magnifications of approximately 3,000x for general overviews and 30,000x for more detailed morphological analysis. Negatives were scanned with a Linoscan 1450 scanner (Heidelberger Druckmaschinen AG, Heidelberg, Germany) at a resolution of 600 dpi. The micrographs were subsequently used to examine and describe mitochondrial morphology, while mitochondrial size was measured by hit point analysis (Griffiths 1993).

Statistical analysis

Statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). The influence of maternal age, oocyte maturation stage and the interaction between these two variables on the (natural logarithm of the) mtDNA copy number was examined by ANOVA. A post-hoc Bonferroni test was used to determine which of the (maternal age x maturation stage) groups had significantly different mtDNA copy numbers. Differences in mitochondrial diameter between groups were also analysed using ANOVA and a post-hoc Bonferroni test. Normality of distribution for the mitochondrial diameter and for the natural logarithms of mtDNA copy number was confirmed in a quantile-quantile (qq) plot; in a normally distributed data set, this plot tends to a straight line. Differences between groups were considered statistically significant if $P < 0.05$.

RESULTS

Quantity of oocyte mtDNA

In total, 501 oocytes were analyzed for mtDNA quantity (Fig 1). The mean mtDNA copy number per oocyte was 1.13×10^6, but variation between individual oocytes was considerable (sem = 3.53×10^4: range = 4.68×10^3 to 3.82×10^6).

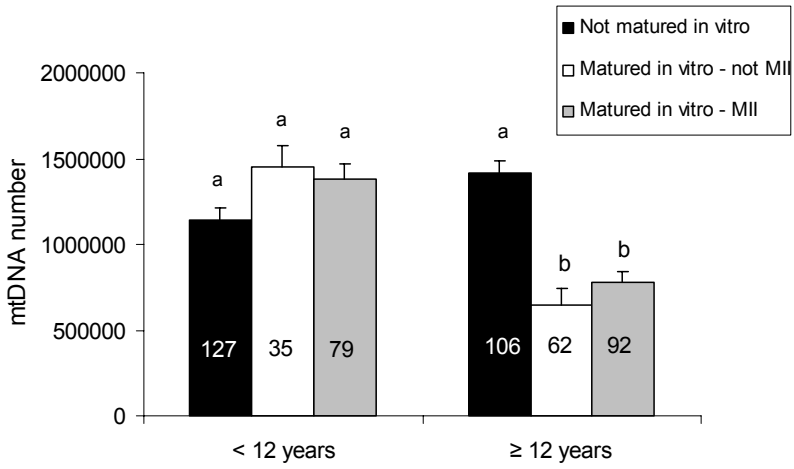

FIG. 1. Mean (± sem) mtDNA copy number in oocytes recovered from young (<12 years) and aged (≥12 years) mares, as determined by quantitative PCR. Within age classes**,** the oocytes are divided into three groups: (i) those that were not matured *in vitro*, (ii) those that were matured *in vitro* but did not reach M-II and (iii) those that were matured *in vitro* and did reach the M-II stage. The number of oocytes per group is indicated on the column. [a,b]Groups with different superscripts had significantly different mtDNA copy numbers ($P < 0.001$).

No significant differences in mtDNA copy number were found between oocytes recovered from young mares irrespective of whether they had been processed immediately after recovery (not-IVM: 1.15 x 10^6) or had been matured *in vitro* and had either reached the M-II stage (IVM/M-II: 1.38 x 10^6) or not (IVM/not-M-II: 1.46 x 10^6). Oocytes recovered from aged mares and not subjected to maturation *in vitro* contained similar numbers of mtDNA copies (1.41 x 10^6) as oocytes from young mares. By contrast, mtDNA copy numbers in both groups of oocytes recovered from aged mares and incubated *in vitro* (IVM/not-M-II: 0.65 x 10^6; IVM/M-II: 0.78 x 10^6) were significantly lower than in all other groups ($P<0.001$).

Deletions in oocyte mtDNA

In total, 347 oocytes were analysed for the presence of deletions; these included 162 oocytes recovered from young mares (79 not-IVM and 83 IVM oocytes) and 185 from aged mares (103 not-IVM and 82 IVM oocytes). Since most deletions described for mtDNA have been flanked by direct repeat sequences (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996), we attempted to detect deletions in horse mtDNA by amplifying DNA segments containing two copies of a direct repeat in close proximity: a 12 bp sequence found at mtDNA positions 2622-2633 (GenBank entry NC_001640) and 2924-2935, and a 13-bp sequence located at positions 6207-6219 and 6384-6386. In addition, a segment of the control region was amplified. Despite amplification of DNA extracted from 347 oocytes, no deletions were detected in any of these regions. Furthermore, sequencing the mtDNA regions 2550-2978 and 15444-15841 in the mtDNA of 48 oocytes (12 not-IVM oocytes from young mares, 12 not-IVM oocytes from aged mares, 12 IVM oocytes from young mares and 12 IVM oocytes from aged mares) did not indicate any heteroplasmy as a result of somatic mutations (Jazin et al. 1996).

Oocyte mitochondrial morphology

Oocyte mitochondrial morphology was examined in 20 oocytes (5 per group for young versus aged combined with not-IVM versus IVM). Mitochondria in oocytes from young mares and in aged mare oocytes that had not been matured *in vitro* were comparable in size and morphology (Fig. 2A-C) with intact mitochondrial membranes, clearly appreciable transverse cristae and a mitochondrial matrix of moderate electron density. By contrast, mitochondria in IVM oocytes from aged mares had a swollen appearance (Fig. 2D). This swelling was confirmed by hit point analysis, which revealed a significantly larger mean surface area than found for mitochondria in not-IVM oocytes from both young and aged mares (Table 2). In addition, the internal architecture of the mitochondria in three out of five aged mare IVM oocytes was severely disrupted with hardly any transverse cristae visible and a relatively low mitochondrial matrix density (Fig. 2D).

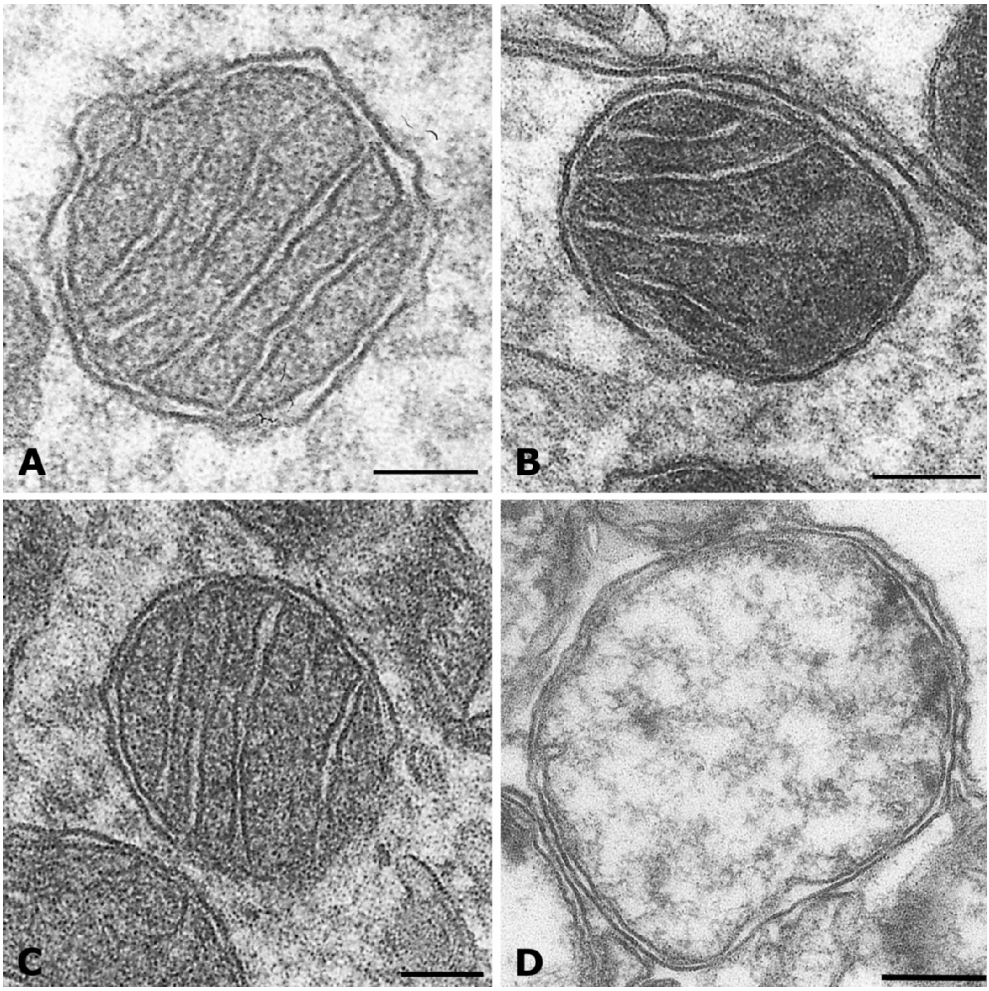

FIG. 2. Transmission electron micrographs of mitochondria in equine oocytes. **A** and **B)** Mitochondria in young mare oocytes that were not (A) or were (B) matured *in vitro*. The mitochondria have intact membranes and transverse cristae. **C)** A mitochondrion in an aged mare's oocyte that had not been matured *in vitro*. Its appearance is comparable to that of mitochondria in the oocytes of young mares. **D)** A mitochondrion in an aged mare's oocyte that had been matured *in vitro*. The mitochondrion is grossly swollen, has lost its transverse cristae and has a low mitochondrial matrix density. (Scale bar = 200nm).

TABLE 2. Relative size of mitochondria in equine oocytes, as determined by hit point analysis.

Maternal age	Oocyte maturation stage	Mitochondrial size*
<12 years	Not-IVM	40.6[a]
<12 years	IVM	61.3[a,b]
≥ 12 years	Not-IVM	53.1[a]
≥ 12 years	IVM	99.7[b]

*Relative mitochondrial size in hit points per mitochondrion (n = 5 COCs/group).
[a,b]Values with different superscripts differ significantly ($P < 0.05$).
IVM = *in vitro* matured.

DISCUSSION

In this study, we used quantitative PCR and TEM to demonstrate that maternal age predisposes to mitochondrial degeneration in equine oocytes. In fact, oocyte mitochondrial degeneration only manifested after *in vitro* maturation of the oocytes from aged mares, and it was characterized by damage, swelling and loss of the mitochondria. In essence, these results echo the previous finding that the mitochondria in matured oocytes from aged mice are more sensitive to experimentally induced damage than those in M-II oocytes from prepubertal animals (Thouas et al. 2005). The cause of the increased susceptibility to oocyte mitochondrial damage with increasing maternal age may include a role for ROS produced by the mitochondria during OXPHOS. ROS are able to damage various cellular components including proteins, lipid membranes and DNA, and the damage inflicted on cellular structures can accumulate during an individual's lifetime (Lee and Wei 2001). Indeed, mitochondria in somatic cells of aged rats have been reported to exhibit increased oxidative stress and to allow more rapid accumulation of oxidant-induced damage to proteins and lipids than mitochondria from younger individuals (Judge et al. 2005). On the basis of the current findings and previous studies in aged women (de Bruin et al. 2004) and mice (Thouas et al. 2005), it appears that mitochondria in oocytes in the resting ('non-recruited') pool may accumulate ROS-induced damage during a female's lifetime as a result of constitutive OXPHOS. This damage may be subtle or 'subclinical' and only become apparent when the metabolic demands of the oocyte increase after the onset of follicle growth and oocyte maturation (Zeilmaker and Verhamme 1974; Magnusson et al. 1977; Magnusson and LeMaire 1981; Sutton et al. 2003). The damaged mitochondria with an impaired OXPHOS pathway may not be able to meet the increased demands for energy, leading the oocyte to enter a vicious circle of spiraling ROS production and mitochondrial damage that culminates in acute severe oxidative stress characterized by swelling, rupture and loss of mitochondria (Takeyama et al. 1993; Bernardi et al. 1999; Sastre et al. 2000).
We did not find any evidence of mtDNA damage in DNA regions predisposed to deletions by the presence of direct repeats (Johns et al. 1989; Schon et al. 1989; Shoffner et al. 1989; Van Tuyle et al. 1996). By contrast, accumulation of several aberrations, including the large so-called 'common deletion' and several point mutations, have been described to

accumulate in human mtDNA in various cell types (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Melov et al. 1995; Jazin et al. 1996; Nagley and Wei 1998) including oocytes (Chen et al. 1995; Keefe et al. 1995; Muller-Hocker et al. 1996; Blok et al. 1997; Brenner et al. 1998; Barritt et al. 1999; Barritt et al. 2000).

In the current study, mtDNA copy number did not differ between M-II and not-M-II oocytes in either young or aged mares. A similar finding was reported for human oocytes (Barritt et al. 2002), and it suggests strongly that mitochondrial replication in the female germ cell line (Jansen and de Boer 1998) is completed before the oocyte embarks on its final maturation. Furthermore, since ATP content does not differ between *in vitro* matured mouse oocytes at the GVB (germinal vesicle break-down), M-I and M-II stages (Van Blerkom et al. 1995), it appears that neither an increase in mitochondrial number nor activity is required for final oocyte maturation.

The significance of the predisposition to mitochondrial damage and loss in the oocytes of aged mares relates to the likely consequences for oocyte developmental competence, i.e. the ability to yield a viable embryo/pregnancy. For example, low mtDNA copy numbers have been associated with reduced oocyte fertilisability in women (Reynier et al. 2001) and sows (El Shourbagy et al. 2006), while extensive mitochondrial damage has been shown to impair normal germinal vesicle break down, meiotic spindle formation and chromosome segregation (Takeuchi et al. 2005). In addition, oocyte mitochondrial dysfunction has been linked to aberrant meiotic spindle function, abnormal chromosome disjunction and an increased incidence of maternally-inherited chromosomal abnormalities (Eichenlaub-Ritter et al. 2004). Low energy production (Van Blerkom et al. 1995) and mitochondrial damage (Thouas et al. 2004; Thouas et al. 2005;) in pre-ovulatory oocytes have also been associated with poor embryo development and increased pre-blastulation embryonic death. Moreover, the incidence of meiotic spindle aberrations (Eichenlaub-Ritter 1989; Battaglia et al. 1996; Liu and Keefe 2002) and chromosomal abnormalities in both oocytes (Pellestor et al. 2003; Pellestor et al. 2005;) and blastocysts (Munne et al. 1995) are higher in aged than younger females.

Critically, an embryo's mitochondria are almost exclusively maternally inherited (i.e. oocyte derived: Hutchison et al. 1974; Giles et al. 1980; Kaneda et al. 1995; Sutovsky et al. 1999) and, post-fertilisation, it is thought that embryonic mitochondrial replication is not initiated until the early gastrula stage (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005). Larsson *et al.* (1998) illustrated this concept by disrupting Tfam, a gene critical for mitochondrial replication, in mice; although embryos homozygous for the disrupted gene reached gastrulation, they died soon afterwards. Given the late onset of mitochondrial replication, early embryonic cell divisions must involve the partitioning of a finite number of oocyte-derived mitochondria over an exponentially increasing number of blastomeres. Furthermore, all of the energy requirements during early embryonic development up to gastrulation must be met by this increasingly sub-divided pool of mitochondria. The decrease in mitochondrial quantity detected in the current study in aged mares oocytes subjected to IVM could easily result in insufficient mitochondria per embryonic cell to support development up to gastrulation, and thereby predispose to early embryonic loss. Indeed, aged mares are known to suffer a higher incidence of early pregnancy loss than younger individuals (Morris and Allen 2002). Reduction of the number

of mitochondria per cell before gastrulation may also explain aberrant chromosomal segregation during mitosis (Eichenlaub-Ritter et al. 2004) and karyotypic abnormalities (Hassold and Hunt 2001). Such aneuploid and mixoploid embryo mosaics have been described for many mammalian species, including man (Munne et al. 1994; Bielanska et al. 2002), rabbit (Fechheimer and Beatty 1974), pig (McFeely 1967; Long and Williams 1982), sheep (Murray et al. 1986), cow (Hare et al. 1980; Viuff et al. 1999) and horse (Rambags et al. 2005).

In conclusion, the present study demonstrates conclusively that maternal ageing predisposes to mitochondrial damage and loss, at least during equine oocyte maturation *in vitro*. It appears that when an aged oocyte reactivates, damage to the mitochondrial membranes triggers the cascade leading to swelling and loss of the mitochondrion; however, the process appears to be independent of pre-existing mtDNA sequence damage. This predisposition to mitochondrial damage may contribute to the age-dependent decrease in fertility in female mammals.

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Chapter 3

Influence of developmental stage, maternal age and *in vitro* production on mitochondrial DNA number in equine embryos

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ABSTRACT

Pregnancy loss is a common but poorly understood phenomenon in the mare. Known predispositions include advanced maternal age and *in vitro* embryo production (IVP). An inadequate number of mitochondria has been proposed to contribute to both of these potential causes of impaired developmental competence. This study aimed to determine whether, as in the mouse, mitochondrial replication is initiated at the time of gastrulation in equine embryos, and whether maternal age (<12 versus >15 years) or IVP affect mitochondrial number in early (day 7.5) embryos. Histological examination of day 10.5-13.5 horse embryos demonstrated that gastrulation begins around day 12 after ovulation. Quantitative rtPCR to examine the expression of genes involved in mitochondrial replication combined with quantitative PCR to determine mitochondrial DNA (mtDNA) copy number demonstrated that mtDNA replication starts well before gastrulation. Indeed, normal day 7.5 embryos already contained significantly ($P<0.01$) more mtDNA copies than oocytes. By contrast, day 7.5 embryos recovered from aged mares, and those produced *in vitro,* exhibited mitochondrial numbers more comparable to oocytes, and the embryos were less well developed than *in vivo* embryos from young mares. The apparent delay in the onset of mtDNA replication in aged mare and IVF embryos may be either a cause or an effect of the associated developmental retardation; in either case it is likely to adversely influence further development and survival of the affected embryos.

INTRODUCTION

Despite considerable advances in equine reproductive medicine during the last 20 years, early embryonic death remains poorly understood and is a significant cause of economic loss to the horse breeding industry (Morris and Allen 2002). Typically, 8-9 % of pregnancies diagnosed at day 14-16 after ovulation are lost during the subsequent 4 weeks of gestation, and the likelihood of pregnancy loss increases markedly as maternal age exceeds 14 years (Ginther 1992b; Morris and Allen 2002; Allen et al. 2007). Furthermore, while fertilization rates in the mare have been shown to approach 90% irrespective of maternal age (Ball et al. 1986; Ball et al. 1989; Ginther 1992b), both the recovery rate of day 7 embryos (30% versus 61%: Vogelsang and Vogelsang 1989) and day 15 pregnancy rates (50% versus 63%: Morris and Allen 2002) are significantly lower, and the incidence of pregnancy loss between days 15 and 42 after ovulation is significantly higher (23% versus 5%), in aged compared to younger mares (Morris and Allen 2002; Allen et al. 2007). Although impaired oviductal and endometrial environments may contribute to the maternal age-related reduction in embryo survival, the finding by Carnevale and Ginther (1995) that intrafallopian transfer of oocytes recovered from aged versus young mares resulted in fewer pregnancies (31% versus 92%, respectively) suggests that intrinsic oocyte and/or embryonic defects contribute significantly to the age-related decline in embryonic survival.
Equine embryos produced *in vitro* by intra-cytoplasmic sperm injection (ICSI) also appear to suffer from reduced developmental competence. Indeed, although a high percentage of oocytes matured *in vitro* reach the M-II stage (58-84%: Choi et al. 2002; Tremoleda et al. 2003b) and the majority of M-II oocytes cleave after ICSI (60-94%; Choi et al. 2002; Choi et al. 2004a), relatively few of the cleaved zygotes reach the blastocyst stage during subsequent culture *in vitro* (2-27%:(Choi et al. 2004b; Choi et al. 2006). Furthermore, while transcervical transfer of IVP blastocysts to recipient mares results in only a slightly reduced pregnancy rate (46% [6/13]: Hinrichs et al. 2005; 66% [16/24]: Galli et al. 2007) compared to transfer of *in vivo* generated embryos in a commercial embryo transfer programme (70-75%: Squires et al. 2003), preliminary evidence suggests that the likelihood of early pregnancy loss may be increased (33%, Hinrichs et al. 2005; 53%, Galli et al. 2007).
One factor that may contribute to both maternal age and IVP related increased susceptibility to pregnancy loss is mitochondrial insufficiency. Early embryonic development is characterised by a dramatic increase in cell number accompanied by differentiation of the cells into the three primitive germ layers, endoderm, ectoderm and mesoderm, and complex rearrangements of these layers to establish a multilayered embryo and placenta; these changes are broadly described by the term gastrulation (Gilbert 1997). Such developmental processes depend critically on energy (ATP) produced by mitochondria, which are inherited exclusively from the mother, i.e. oocyte derived (Hutchison et al. 1974; Giles et al. 1980; Kaneda et al. 1995; Sutovsky et al. 1999). Interestingly, while mitochondrial number increases dramatically during oogenesis, from tens in a primordial germ cell to hundreds of thousands in a preovulatory oocyte (Jansen and de Boer 1998), mitochondrial replication arrests shortly prior to fertilisation and, in the mouse, does not restart until the resulting embryo has begun to gastrulate (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005). Hence, embryo survival depends critically on the fertilised

oocyte containing sufficient functional mitochondria to support normal development until the onset of mitochondrial replication at around the time of gastrulation, despite the subdivision of those mitochondria over a rapidly increasing number of blastomeres. In this respect, we recently reported that oocytes from aged (≥ 12 years) mares are more susceptible to mitochondrial damage and loss during maturation *in vitro* than those from younger mares (Rambags et al. 2006).
The aim of the current study was to determine whether maternal age or IVP negatively affect mitochondrial numbers in horse embryos prior to the anticipated onset of mitochondrial replication. In addition, we aimed to determine whether the onset of mitochondrial replication in the horse embryo coincides with gastrulation, as it does in the mouse (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005). Finally, since gastrulation in equine embryos has not been described in detail, we aimed to determine if this process really does begin at around day 12 after ovulation, as suggested by Enders *et al.* (1993).

MATERIALS AND METHODS

Experimental protocol

The study comprised 4 experiments. In Experiment 1, the timing of gastrulation was investigated by histological examination of day 10-13 embryos. In Experiment 2, quantitative rtPCR (Q-rtPCR) was used to examine the expression of genes required for mtDNA replication by day 7.5, 10.5 and 14.5 embryos. Experiment 3 investigated the onset of embryonic mtDNA replication by comparing mtDNA copy number in oocytes and day 7.5, 10.5 and 14.5 embryos by quantitative PCR (QPCR). Finally, experiment 4 examined the influence of maternal age and IVP on mitochondrial number by comparing mtDNA copy number in day 7.5 embryos produced *in vitro* with those of embryos recovered from young (<12 years old) or aged (>15 years old) mares.

Collection and preparation of oocytes

Cumulus oocyte complexes (COCs) were recovered from the ovaries of slaughtered mares, as described previously by Tharasanit *et al.* (2006). The COCs were aspirated from 5-30 mm follicles using a 16 gauge needle attached via an infusion set and a 250 ml polypropylene collection tube (Corning Incorporated – Life Sciences, Big Flats, New York, USA) to a vacuum pump. Following aspiration of the fluid, the follicle lumen was flushed 2-3 times with 0.9% (w/v) saline solution supplemented with 0.1% w/v bovine serum albumin (BSA; Sigma-Aldrich Chemicals BV, Zwijndrecht, the Netherlands) and 25 IU/ml heparin (Leo Pharmaceuticals, Weesp, the Netherlands), while scraping the follicle wall vigorously with the bevel of the needle. The recovered fluid was allowed to stand at room temperature for 10 min after which the resulting sediment containing the COCs was washed twice in HEPES-buffered synthetic Human Tubal Fluid (Q-HTF; BioWhittaker, Verviers, Belgium) supplemented with 0.4 % BSA. Next, the COCs were isolated using a

stereomicroscope, and denuded of their cumulus investment by vortexing for 4 min after incubation in calcium and magnesium free Earles's Balanced Salt Solution (EBSS) containing 0.25% v/v trypsin-EDTA (Gibco BRL, Life Technologies, Paisley, UK). The oocytes were then examined under an inverted stereomicroscope to ensure complete removal of all cumulus cells; incompletely denuded oocytes were discarded. Subsequently, suitable oocytes (n = 24) were washed three times in PBS containing 0.1% w/v PVA (polyvinyl alcohol; Sigma-Aldrich Chemicals BV) and three times in TE (10mM Tris [MP Biomedicals Inc., Eschwege, Germany] and 0.1 mM EDTA [BDH Ltd., Poole, UK] in double distilled water), before being transferred individually to eppendorf tubes in 5 µl TE. Next, the oocytes were lysed by incubating them at 65˚C for 10 min in 5 µl of an alkaline lysis buffer (200mM KOH and 50mM dithiothreitol) followed by vortexing. After adding 5 µl of a neutralisation buffer containing 0.9M Tris-HCl, 0.3M KCl and 0.2M HCl, the lysates were diluted with double distilled water to a total volume of 150 µl and stored at –20°C.

Collection of *in vivo* embryos

The majority of embryos used in this study were recovered from a herd of 13 young Warmblood mares (< 12 y.o.) flushed repeatedly. During oestrus, their uterus and ovaries were examined thrice weekly by transrectal palpation and ultrasonography. Once the dominant ovarian follicle(s) exceeded 35 mm in diameter, the mare was inseminated with >300 x 10^6 morphologically normal, progressively motile sperm from one of two fertile stallions. Thereafter, the mares were examined daily for ovulation and re-inseminated every 48 h until ovulation had occurred. In total, 59 embryos were collected on days 7 (n=16), 10 (n=18), 11, (n=3), 12 (n=3), 13 (n=2) or 14 (n=16) after ovulation (day 0). Therefore, at the time of collection, the embryos were classed as being 7.5, 10.5, 11.5, 12.5, 13.5, or 14.5 ± 0.5 days old.
Day 7.5 embryos were collected by non-surgical uterine lavage using Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.5% v/v fetal calf serum (FCS; Sigma-Aldrich-Chemicals) introduced into, and recovered from, the uterus using a standard cuffed embryo flushing cathether with an internal diameter of 8 mm (Bivona, Gary, Indiana, USA). Day 10.5 and older embryos were recovered using Ringer's solution and a 24 mm endotracheal tube, in a modification of the technique described by Sirois and Betteridge (1988). After recovery, each embryo was washed in fresh DPBS (day 7.5) or Ringer's solution (day 10.5-14.5) to remove any FCS (day 7.5 embryos) and/or possible contaminating maternal tissue.
A further seven day 7.5 embryos were recovered from a group of 5 aged (15 – 20 years old) Thoroughbred mares that had been inseminated with semen from a fertile Thoroughbred stallion, by uterine lavage using Emcare Flushing Solution (ICP, Auckland, New Zealand).

***In vitro* embryo production**

The final eight embryos used in the study were produced *in vitro*, as described previously (Tremoleda et al. 2003a; Rambags et al. 2005). Briefly, compact COCs recovered from the ovaries of slaughtered mares were matured *in vitro* for 30 h after which the oocytes were

denuded of cumulus cells. Those that had reached the MII stage of development were fertilised by ICSI and the resulting presumptive zygotes were cultured for 2 days. On day 2 after ICSI, morphologically normal 2 and 4-cell embryos were selected, embedded in agar chips and transferred surgically to the ligated oviduct of a progesterone-treated ewe. Five days later, the embryos were harvested by surgical oviduct lavage. For transport to the QPCR laboratory, IVP embryos were cryopreserved in modified synthetic oviductal fluid (Hepes-SOF; Gardner et al. 1994) containing 10% glycerol as cryoprotectant. In preparation for freezing, the embryos were equilibrated for 5 min in SOF containing 5% glycerol and then for 20 min in SOF containing 10% glycerol; they were then loaded into 0.5 ml straws and frozen using a programmable freezing machine (Bio-cool IV, FTS System, Stone Ridge, NY, USA). Initial cooling involved plunging the straws into a -6°C methanol bath, in which they were held for 10 min and seeded. Thereafter, the embryos were cooled at 0.5°C/min down to -32°C, before being plunged into liquid nitrogen. Immediately prior to QPCR, the embryos were thawed by holding the straw in air for 5 sec and then immersing it in water at 25°C for 1 min. After thawing, the cryoprotectant was removed by washing the embryos for 5 min each in solutions of OCM (Ovum Culture Medium: ICN Biomedicals, Zoetermeer, the Netherlands) containing declining concentrations (8%, 6%, 4%, 2% and 0%) of glycerol. The embryos were further washed for 5 min in each of OCM and a 1:1 OCM:DPBS solution, and then twice in DPBS before being prepared for QPCR.

Primer design

The primer pairs used for Q-rtPCR and QPCR are summarized in Table 1. Q-rtPCR was performed to assess the relative expression of mitochondrial transcription factor A (Tfam), mtDNA polymerase γ subunit B (PolgB) and mitochondrial single stranded DNA binding protein (SSB) with respect to a reference gene (β-actin); QPCR was used to calculate absolute mitochondrial DNA (mtDNA) copy numbers. Primers were designed for the equine coding sequence (β-actin, mtDNA) or, if this was not available (Tfam, PolgB, SSB), coding sequences within the target gene conserved well across other mammalian species, using Primer Designer version 2.0 (Scientific & Educational Software, Cary, NC, USA). Preferably, primers of a pair were located on different gene exons. All the primer pairs were validated using qualitative PCR on an equine embryo cDNA panel. After the PCR reaction, 10 µl of the product was resolved on a 1% agarose gel containing 0.4µg/ml ethidium bromide to visualize the PCR product. A 100 basepair (bp) ladder was included as a reference for fragment size and a standard sequencing procedure was used to verify the identity of the PCR products.

Experiment 1: Onset of gastrulation

Immediately after conceptus recovery, the embryonic discs (ED) of day 10.5 (n=3), 11.5 (n=3), 12.5 (n=3) and 13.5 (n=2) embryos were examined using a dissecting microscope for evidence of a visible thickening of the posterior epiblast, or a developing primitive streak. Subsequently, the ED area was isolated and prepared for histological analysis. In each case,

the ED was excised together with a substantial margin of surrounding trophoblast using micro-surgical scissors, and then fixed overnight in 4% formaldehyde. Next day the conceptus tissue was dehydrated for 30 min in 70% ethanol, stained for 2 min with eosin and then further dehydrated by immersion for 3 x 15 min periods in 96% and 100% ethanol, followed by 2 x 15 min in xylene, before being embedded in paraffin wax. Once the wax had solidified, the eosin stained tissue was located, excised and reembedded in a regular paraffin block such that the ED could be sectioned transversely starting from its posterior end. Next, 5µm sections were cut with a microtome and attached to coated glass slides (Superfrost® Microscope Slides: Menzel-Gläser, Braunschweig, Germany) by incubation overnight at 55˚C. Next day, every tenth slide was stained with haematoxylin and eosin (H&E) and examined microscopically for signs of gastrulation, i.e. the appearance of a third germlayer, mesoderm, between the ectoderm and the endoderm.

TABLE 1. Primers used for gene amplification in the PCR experiments.

Gene	Genbank accession number	Primer sequence (5'→3')	T_a (ºC)	Product size (bp)
Tfam	NM_001034016	s: GGCAGGTATACAAGGAAGAG as: GTTATAAGCTGAGCGAGGTC	58ºC	170
PolgB	NM_015810	s: CCGAGTAAGGAACAGCTAGT as: ACTCCAATCTGAGCAAGACC	58ºC	155
SSB	XM_133058	s: CATGAGACAGGTGGAAGGAA as: GATATGCCACATCTCTGAGG	58ºC	167
β-actin	AF035774	s: CCAACCGCGAGAAGATGACC as: ACCGGAGTCCATCACGATGC	60˚C	128
mtDNA	NC_001640	s: CATGATGAAACTTCGGCTCC as: TGAGTGACGGATGAGAAGGCAG	67.7˚C	118

T_a = annealing temperature; s = sense; as = anti-sense.

Experiment 2: Expression of genes involved in mtDNA replication

RNA extraction and cDNA synthesis

Eight embryos recovered from young (<12 y.o.) mares at each of days 7.5, 10.5 and 14.5 after ovulation were used to examine the expression of genes critical for mtDNA replication. After removing the yolk sac fluid and capsule, the entire choriovitelline membrane of day 10.5 and 14.5 embryos was snap-frozen in liquid nitrogen and stored at -80°C; day 7.5 embryos were snap-frozen intact. Isolation of total RNA and on-column DNAse digestion was performed using the Invisorb® Spin Cell RNA Mini Kit (Invitek GmbH, Berlin, Germany) and the RNAse-free DNAse Set (Qiagen, Valencia, CA, USA). Briefly, each embryo was lysed in 700 µl lysis buffer. Next, 350 µl, 50 µl or 10 µl of the lysate (for day 7.5, 10.5 and 14.5 embryos, respectively), made up to 350 µl with fresh lysis buffer, was applied to the DNA-binding spin filter. After incubation for 2 min at room temperature and centrifugation for 2 min at 11,000g, the binding filter containing the DNA was discarded, and the remaining lysate was diluted (1:1) with 70% ethanol and applied

directly onto a RNA-binding filter. Next, the column was washed with 300 µl of wash buffer before RNAse-free DNAse was applied to the RNA-binding filter to remove any remaining DNA during a 15 min incubation at room temperature. After three further washes with buffer, the RNA was eluted from the RNA-binding filter using 33 µl RNAse-free water.
Reverse transcription was performed in a total volume of 20 µl, made up of 10 µl sample RNA, 4 µl of 5xRT buffer (Invitrogen, Breda, The Netherlands), 8 units RNAsin (Promega, Leiden, The Netherlands), 150 units Superscript II reverse transcriptase (Invitrogen), 0.036 units of random primers (Invitrogen), 10 mM DTT (Invitrogen) and 0.5 mM of each dNTP (Promega). The mixture was incubated for 1 h at 42°C, followed by 5 min at 80°C to inactivate the reverse transcriptase, before being stored at –20°C. Minus RT blanks were prepared similarly but in the absence of reverse transcriptase. RNA extraction and cDNA synthesis were then checked by rtPCR with primers for β-actin; samples that failed to yield cDNA or those for which the minus RT blank was positive, were excluded from further analysis.

Q-rtPCR
Quantification of cDNA product was performed using a real-time PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, The Netherlands). For each target gene, a 96-well plate was loaded with 24 experimental samples, 1 negative control (double distilled water) and 7 standards, all in triplicate. The standard curve was constructed from 10-fold serial dilutions of known amounts of target gene PCR product. The 25µl Q-rtPCR reaction mixture contained 1 µl cDNA, 0.5mM of each primer (Isogen Bioscience BV, Maarssen, The Netherlands) and 12.5 µl of IQ™ Sybr® Green Supermix (Bio-Rad Laboratories). Initial DNA denaturation at 95˚C for 5 min was followed by 40 cycles of 15 sec at 95˚C, 30 sec at the primer specific annealing temperature (see Table 1) and 45 sec at 72˚C; the reaction was completed during a 7 min period at 72˚C. The purity of the amplified product was checked by examining the melting curves. Standard curves were produced by plotting the log of the starting amount of product against the threshold cycle for detection, and gene expression was expressed in terms of the relative expression level (REL), i.e. the ratio of the calculated quantity of a target gene to that of β-actin in the same sample.

Experiment 3: mtDNA copy numbers during early conceptus development

This experiment compared mtDNA copy numbers between oocytes (n=24) and embryos recovered at 7.5, 10.5 and 14.5 days after ovulation (n=8 in each group).

DNA isolation
The isolation of DNA from oocytes is described above. To recover DNA from embryos, the membranes were first lysed by incubation for 1 h at 37ºC in 200 µl (day 7.5) or 1800 µl (days 10.5 and 14.5) lysis buffer (LB: 50mM Tris-HCl [pH 8.0], 100mM EDTA [pH 8.0], 100 mM NaCL, 1% w/v sodium dodecyl sulphate [SDS] and 20µg/ml DNase free RNase [Roche Diagnostics GmbH, Mannheim, Germany] in double distilled water). For day 7.5

embryos, the lysate was then thoroughly mixed and the entire 200 µl was transferred to a fresh eppendorf tube along with a further 100 µl LB used to rinse remaining DNA from the initial tube. For day 10.5 and 14.5 conceptuses, 300 µl of well mixed lysate was transferred to a new tube. After adding 1.5 µl proteinase K (20 mg/ml; Invitrogen, Carlsbad, CA, USA), the 300 µl lysate was again mixed then incubated for 3 h in a 50ºC water bath. Once the reaction mixture had cooled to room temperature (RT), 300 µl of water-saturated phenol (Serva Electrophoresis GmbH, Heidelberg, Germany) was added, and the two phases were mixed by vortexing slowly for 30 sec and then reseparated by centrifuging at 11,000g for 2 min. The viscous aqueous phase containing the DNA was transferred to a fresh eppendorf tube together with an equal volume (300 µl) of chloroform (Sigma-Aldrich Chemicals). Again, the two phases were mixed by vortexing and separated by centrifugation before the aqueous phase containing the DNA was transferred to a fresh tube. To minimise DNA loss, 50 µl of double distilled water was used to recover remaining DNA from first the phenol phase and then the chloroform phase by mixing and centrifugation, and was added to the previously recovered DNA solution.

The DNA in aqueous solution was precipitated with 350 µl iso-propanol (Sigma-Aldrich Chemicals) and the resulting emulsion was mixed thoroughly and left to stand overnight at RT. Next day, the tubes were centrifuged at 11,000g for 20 min at RT and the supernatant was carefully aspirated and discarded. The tubes were then rinsed with 200 µl 70% ethanol (-20ºC) and centrifuged at 11,000g at 4ºC before the supernatant was again aspirated and discarded. Finally, the DNA was dried by placing the tubes in a heating block at 40ºC, after which 50 µl of DNA buffer (10mM Tris-HCL [pH 8.0] and 0.1 mM EDTA) was added and the samples were incubated overnight in a 40ºC water bath to ensure complete DNA dissolution. The DNA solutions were stored at -20ºC.

QPCR

QPCR was essentially performed as described in Experiment 2. Minor modifications included a different primer pair (mtDNA) and annealing temperature (see Table 1). Each 96-well assay plate included 24 samples in triplicate, together with 8 standards and a negative control sample in duplicate. In addition, because Experiments 3 (and 4) involved the determination of absolute numbers rather than ratios, triplicate samples of two internal controls were included to monitor intra- and inter-assay variation. The resulting intra- and inter-assay coefficients of variation were 12.8% and 15.3% respectively, and were thus within the normal range for this technique (Reynier et al. 2001; Bhat and Epelboym 2004).

Experiment 4: Influence of maternal age and IVP on embryonic mtDNA copy number

DNA isolation and QPCR were performed as described for Experiment 3 on eight day 7.5 embryos recovered from young (<12 years old) mares, 7 day 7.5 embryos collected from aged (>15 years old) mares and 8 day 7.5 IVP embryos.

Statistical analysis

Statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). In the case of RELs and mtDNA copy numbers, natural logarithmic transformation of the data was used to obtain a more continuous and normally distributed data set; the normality of distribution was confirmed by plotting the quantiles of the residuals against the expected normal residuals (quantile-quantile or qq plot). Differences in RELs or mtDNA copy numbers between groups of oocyte and embryos were analysed using a general linear model analysis of variance (ANOVA) combined with a post-hoc Bonferroni test. In all cases, differences between groups were considered statistically significant if $P<0.05$.

RESULTS

Experiment 1

Thickening of the posterior epiblast or presence of a primitive streak were taken to indicate the onset of gastrulation (Gilbert 1997). While these two anatomical features were present in all the day 12.5 and 13.5 embryos examined (Table 2; Figs 1E and 1G), they were absent in younger embryos (Fig 1A) with the exception of a single day 11.5 embryo which exhibited marginal posterior epiblast thickening (Fig 1C). Similarly, examination of H&E-stained sections of the embryonic disc region showed that younger embryos (day 10.5 and 11.5) were bilaminar (Figs 1B and 1D) and that a third layer of presumptive mesoderm cells first appeared at day 12.5 (Fig 1F). After this time, the membrane in the ED area was clearly trilaminar (day 13.5: Fig 1H).

TABLE 2. Onset of gastrulation in equine embryos, as evidenced by thickening of the posterior epiblast and development of a primitive streak during days 10.5-13.5 after ovulation.

Conceptus age (± 0.5 days)	Number of embryos	Thickened posterior epiblast	Primitive streak
10.5 days	3	0/3	0/3
11.5 days	3	1/3	0/3
12.5 days	3	3/3	3/3
13.5 days	2	2/2	2/2

Experiment 2

PolgB, Tfam and SSB were expressed at all of the developmental stages examined. Expression of PolgB and Tfam was lower in day 14.5 than in younger embryos ($P<0.01$; Figs 2A and 2B), whereas SSB expression was constant throughout the period studied (Fig 2C).

Experiment 3

Oocytes collected from antral follicles contained a mean (± sem) of $2.7x10^6$ (± $4.0x10^5$) mtDNA copies, whereas embryos collected at 7.5, 10.5 and 14.5 days after ovulation, contained $5.4x10^7$ (± $2.9x10^7$), $1.1x10^{10}$ (± $1.9x10^9$) and $1.5x10^{11}$ (± $1.4x10^{10}$) mtDNA copies, respectively. Thus, mtDNA copy number increased significantly throughout the period of development examined ($P<0.01$; Fig 3).

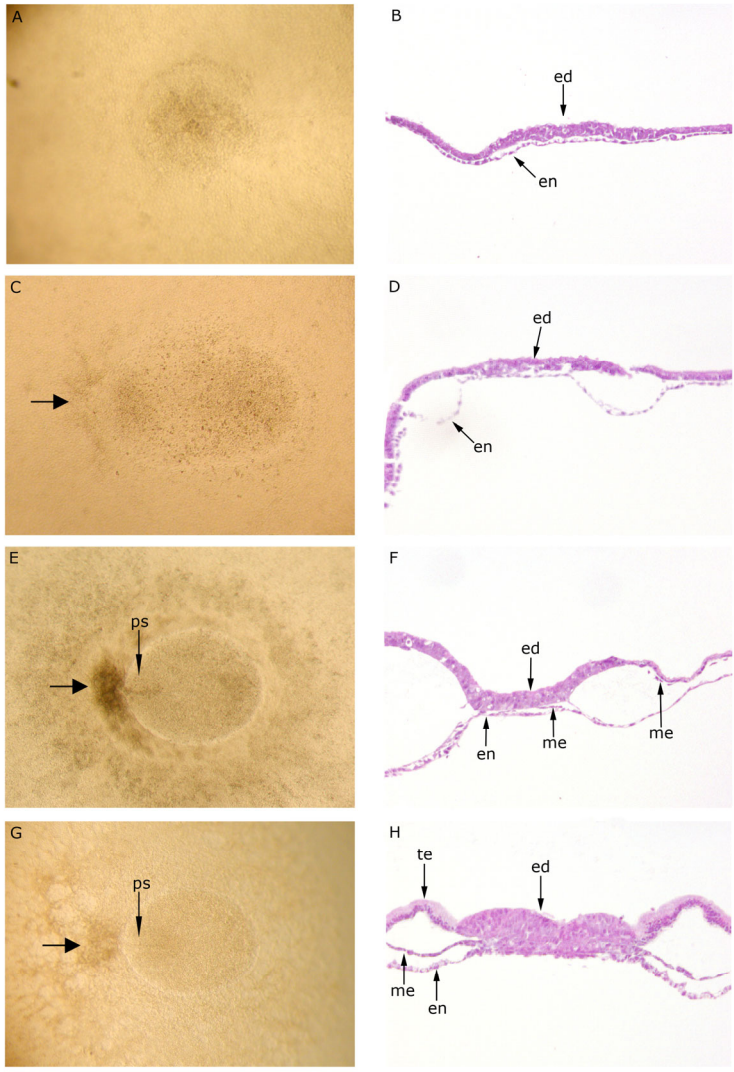

FIG. 1. Representative micrographs used for detecting the onset of gastrulation in day 10.5-13.5 horse embryos.
A, C, E, G: Dissecting microscope images of the embryonic disc area.
B, D, F, H: Haematoxylin and eosin stained histological sections of the embryonic disc.
On days 10.5 (A, B) and 11.5 (C, D), the embryonic disc (ed) was entirely bilaminar. In one day 11.5 embryo (C) and all day 12.5 (E) and day 13.5 (G) embryos, a visible thickening of the epiblast was observed at the posterior end of the ed (bold arrow); the primitive streak (ps) was visible from day 12.5. Presumptive mesoderm cells (me) were first observed on day 12.5 (F), and the embryo was clearly trilaminar (H) by day 13.5, with a distinct mesoderm (me) interposed between the trophectoderm (te) and endoderm (en).

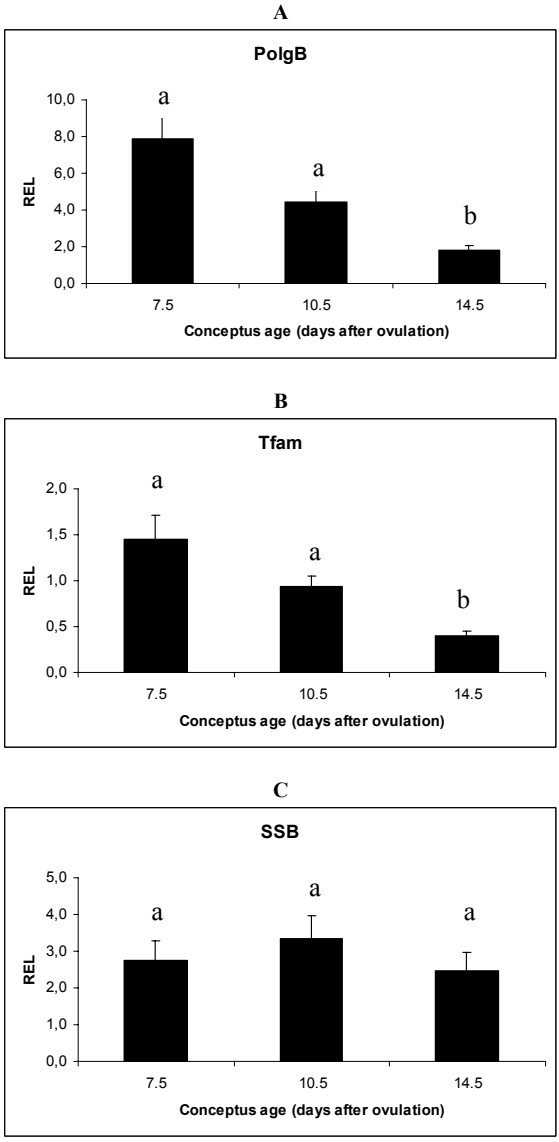

FIG. 2. Expression of genes involved in mitochondrial DNA (mtDNA) replication in equine conceptuses recovered 7.5, 10.5 or 14.5 days after ovulation. Expression of PolgB (A), Tfam (B) and SSB (C) was detected at all developmental stages. Values are the mean (± sem) relative gene expression level (REL) for 8 conceptuses, i.e. the ratio between the amount of mRNA for the target gene (PolgB, Tfam or SSB) and that for the reference gene (β-actin).
a,b: Columns with different labels differ significantly ($P<0.01$).

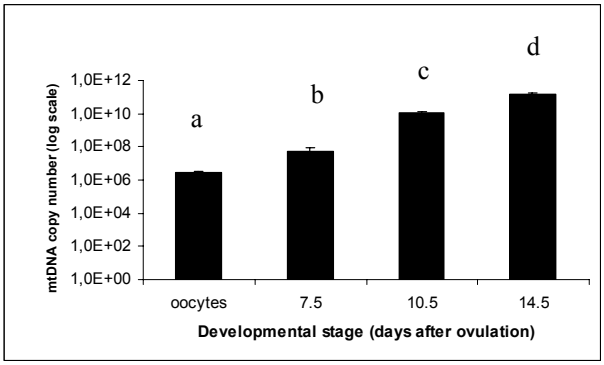

FIG. 3. Mitochondrial DNA (mtDNA) copy number in equine oocytes and embryos determined by quantitative PCR analysis. The oocytes (n=24) were recovered from antral follicles while the embryos were collected 7.5, 10.5 and 14.5 days after ovulation (for all groups: n = 8). a-d: Columns with different labels differ significantly (P<0.01).

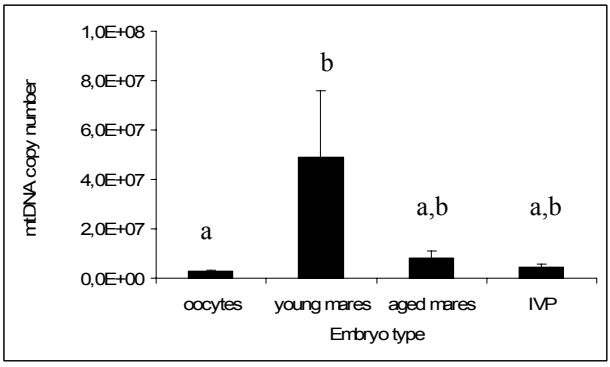

FIG. 4. Mitochondrial DNA (mtDNA) copy number in equine oocytes (n=24) and day 7.5 embryos produced *in vitro* (IVP: n=8) or recovered from young (<12 years old: n=8) or aged (>15 years old: n=7) mares, as determined by quantitative PCR. Although differences between groups of embryos were not significant, only embryos from young mares contained significantly more mtDNA copies than oocytes from antral follicles (P<0.01).
a,b: Columns with different labels differ significantly (P<0.01).

Experiment 4

There were no statistically significant differences in the number of mtDNA copies between day 7.5 embryos collected from young or aged mares, or those produced *in vitro* (P>0.2; Fig 4). However, only the *in vivo* generated embryos recovered from young mares contained significantly more mtDNA copies than oocytes from antral follicles ($4.9x10^7$ ± $2.7x10^7$ versus $2.7x10^6$ ± $4.0x10^5$; P=0.003); IVP embryos ($4.5x10^6$ ±$1.3x10^6$) and *in vivo* generated embryos collected from aged mares ($8.2x10^6$ ± $2.8x10^6$) contained similar mtDNA copy numbers to oocytes (P>0.2).

DISCUSSION

Early embryonic development is characterised by processes like rapid cell division and extensive tissue differentiation that require copious amounts of energy in the form of mitochondrion-derived ATP. Despite a dramatic increase in cell number, however, the total number of mitochondria does not increase in mouse embryos until the gastrula stage of development (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005); the stage of embryonic development at which mitochondrial replication is initiated in other mammalian species has yet to be reported. To determine whether mitochondrial replication is initiated at or before gastrulation in the horse embryo, the present study initially examined when signs of gastrulation first become apparent. A thickening of the posterior epiblast was seen as early as day 11.5 in one embryo but, in general, clear signs of gastrulation in equine embryos first became apparent on day 12 after ovulation, as reported by Enders *et al.* (1993) rather than day 14 as proposed by Ginther (1992a).

To determine whether the conditions for mitochondrial replication were present, the expression of genes for enzymes involved in mtDNA replication was examined in day 7.5-14.5 horse embryos. The genes examined included PolgB, a subunit of the mtDNA polymerase γ enzyme responsible for mtDNA multiplication; PolgB expression has previously been reported to correlate well with mtDNA replication (Moraes 2001). The other genes examined were Tfam which plays an important role in the formation of an RNA primer required for initiating mtDNA polymerase activity, and SSB which supports mtDNA polymerase γ function (Moraes 2001). The fact that PolgB, Tfam and SSB were all expressed by day 7.5 and 10.5 embryos demonstrated that the machinery for mtDNA replication is present in the horse embryo considerably before the onset of gastrulation. The suspicion that mitochondrial replication in the developing horse embryo is initiated at least as early as the blastocyst stage was confirmed by QPCR measurement of significantly higher mtDNA numbers in day 7.5 blastocysts than in oocytes, and the subsequent continuing increase in mitochondrial number with developmental stage. This contrasts markedly with the finding that mtDNA copy number does not increase until after the onset of gastrulation in the mouse embryo (Piko and Taylor 1987; Ebert et al. 1988; Thundathil et al. 2005). Although the reason for this marked between-species difference is not known, it may be driven by a need to maintain a minimum number of mitochondria per cell. Murine blastocysts and pre-gastrulation day 6.5 mouse embryos have been reported to contain, respectively, 32 (Piko and Taylor 1987) and approximately 566 cells (Power and Tam 1993). Since other studies suggest that the mouse embryo contains around $1.8x10^5$ mtDNA copies (Piko and Taylor 1987; Ebert et al. 1988; Thundathil et al. 2005), these figures equate to approximately 5600 and 300 mtDNA copies per blastomere, at the respective stages. By comparison, a day 7.5 equine blastocyst contains approximately 1,700 cells (Rambags et al. 2005) and a day 12.5 embryo has in excess of 44,500 (Tremoleda et al. 2003a) cells. If an equine oocyte's content of $2.7x10^6$ mtDNA copies were to be divided amongst all these cells, mtDNA number per cell would be as low as 1600 and 60, where the latter figure might well be insufficient to support cellular metabolism (Moraes 2001).

With regard to maternal age and IVP-related reductions in oocyte/embryo developmental competence, although total mtDNA copy number did not differ significantly between day

7.5 embryos produced *in vitro* and those recovered from young (<12 years) or aged (>15 years) mares, only embryos recovered from the young mares contained significantly more mtDNA copies than oocytes (P=0.003). The failure of differences between embryo groups to reach statistical significance may have been a product of the relatively low numbers of embryos (seven or eight per group) and the large inter-embryo differences in absolute mtDNA copy number. Any reduction in mtDNA number in aged mare or IVP embryos may be a product of either a low initial mtDNA number, or a delay in the onset of mitochondrial replication or gene expression in general. In the former respect, we recently demonstrated that oocytes from aged mares are more susceptible to mitochondrial damage and loss during maturation *in vitro* than those from young mares (Rambags et al. 2006). On the other hand, any delay in the onset of mitochondrial replication in aged mare or IVP embryos could either result from a primary delay in activation of the embryonic genome or be a function of a general retardation in embryo development. In respect of the latter, day 7 IVP horse embryos are less well developed and contain fewer cells than *in vivo* generated embryos at the same stage (Tremoleda et al. 2003a; Rambags et al. 2005), while there are indications that maternal age predisposes to delayed embryo development in rodents (Sopelak and Butcher 1982; Ishikawa and Yamauchi 2003; Trejo et al. 2005). Indeed, Hamatani *et al.* (2004b) have described an age-related decrease in mRNA for genes involved in transcription, cell cycle regulation, preimplantation development and mitochondrial function in mouse oocytes that could delay activation of the embryonic genome (Hamatani et al. 2004a). In the present study, both IVP and aged mare day 7.5 embryos tended to be less well developed than *in vivo* generated embryos from young mares (Table 3), and it is therefore quite possible that they had simply not yet reached the developmental stage associated with the onset of mtDNA replication.

TABLE 3. Developmental stage of day 7.5 equine embryos produced *in vivo* or *in vitro*.

Type of embryo	Number of embryos	Developmental stage	Number of embryos
in vivo - young mare (<12 years old)	8	Morula	-
		Early blastocyst	2
		Expanded blastocyst	6
in vivo - aged mare (>15 years old)	7	Morula	-
		Early blastocyst	4
		Expanded blastocyst	3
in vitro - ICSI/ sheep oviduct	8	Morula	6
		Early blastocyst	2
		Expanded blastocyst	-

ICSI = intra-cytoplasmic sperm injection; IVC = *in vitro* embryo culture

In conclusion, the present study demonstrated that gastrulation in the horse embryo begins between days 11.5 and 12.5 after ovulation, while mitochondrial DNA replication begins considerably earlier. Indeed, it appears that mitochondrial replication starts at around the time of blastulation because, whereas day 7.5 expanded blastocysts from young mares had significantly higher mtDNA copy numbers than oocytes, developmentally retarded day 7.5 morulae or early blastocysts recovered from aged mares, or produced *in vitro,* had yet to undergo significant mtDNA multiplication. Finally, we speculate that either an oocyte-derived reduction in mtDNA content, or a delay in the onset of mitochondrial replication, contributes to the maternal age and IVP-related reductions in equine embryonic developmental competence and survival observed in the field.

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Chapter 4

Numerical chromosomal abnormalities in equine embryos produced *in vivo* and *in vitro*

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ABSTRACT

Chromosomal aberrations are often listed as a significant cause of early embryonic death in the mare, despite the absence of any concrete evidence for their involvement. The current study aimed to validate FISH probes to label specific equine chromosomes (ECA2 and ECA4) in interphase nuclei and thereby determine whether numerical chromosome abnormalities occur in horse embryos produced either *in vivo* (n=22) or *in vitro* (IVP: n=20). Overall, 75% of 36,720 and 88% of 2978 nuclei in the *in vivo* developed and IVP embryos were analysable. Using a scoring system in which extra FISH signals were taken to indicate increases in ploidy and 'missing' signals were assumed to be 'false negatives', 98% of the cells were scored as diploid and the majority of embryos (30/42: 71%) were classified as exclusively diploid. However, one IVP embryo was recorded as entirely triploid and a further 7 IVP and 4 *in vivo* embryos were classified as mosaics containing diploid and polyploid cells, such that the incidence of apparently mixoploid embryos tended to be higher for IVP than *in vivo* embryos ($p = 0.118$). When the number of FISH signals per nucleus was examined in more detail for 11 of the embryos, the classification as diploid or polyploid was largely supported because 2174 of 2274 nuclei (95.6%) contained equal numbers of signals for the two chromosomes. However, the remaining 100 cells (4.4%) had an uneven number of chromosomes and, while it is probable that many were artefacts of the FISH procedure, it is also likely that a proportion were the result of other types of aneuploidy (e.g. trisomy, monosomy or nullisomy). These results demonstrate that chromosomally abnormal cells are present in morphologically normal equine conceptuses and suggest that IVP may increase their likelihood. Definitive distinction between polyploidy, aneuploidy and FISH artefacts would require the use of more than one probe per chromosome and/or probes for more than two chromosomes.

INTRODUCTION

Resumption of meiosis by the preovulatory oocyte, fusion of the (haploid) sperm and oocyte pronuclei, and subsequent mitotic cell divisions within the zygote/embryo are all fundamental steps in the development of new mammalian life. So much so, that even a relatively minor disturbance in one of these processes can lead to an abnormality of chromosome number that will almost certainly interfere with the viability of the developing conceptus. Numerical chromosomal abnormalities are a fairly common finding in both macroscopically abnormal and normal conceptuses in a large number of mammalian species, including man (Munne et al. 1994; Bielanska et al. 2002a, 2002b), pig (McFeely 1967; McCauley et al. 2003), cow (Hare et al. 1980; Viuff et al. 1999, 2002), sheep (Long and Williams 1980; Murray et al. 1986a, 1986b), goat (Villamediana et al. 2001) and rabbit (Fechheimer and Beatty 1974), and have been proposed to be a major cause of early embryonic loss (King 1990; Munne et al. 1995). Embryonic chromosomal aberrations arise in two broadly different ways, either as a result of a pre-existing parental chromosome abnormality being transmitted to the gamete in an unbalanced form, such as a translocation, or much more commonly, as a result of a spontaneous numerical aberration arising either during gametogenesis, at fertilisation, or during early embryonic development (King 1990). Conventional karyotypic analysis of metaphase spreads has suggested that most of the spontaneously generated aberrations in cattle (Hare et al. 1980), sheep (Murray et al. 1986b) and pig (Long and Williams 1982; McCauley et al. 2003) conceptuses result in various types and degrees of polyploidy or mixoploidy (i.e. diploid-polyploid mosaics).

The development of DNA probes that can be used, via fluorescent *in situ* hybridisation (FISH), to label specific chromosomes in interphase nuclei has considerably enhanced our ability to analyse chromosome number in a large proportion of the cells in a given tissue, without the need for a preparatory cell culture to drive those cells into metaphase (Viuff et al, 1999). Performing FISH with chromosome specific probes on interphase cells therefore allows a more complete and unbiased estimation of the incidence of abnormalities such as polyploidy, mixoploidy or aneuploidy. To date, FISH has been used to show that a high proportion (>70%) of IVP bovine (Viuff et al. 1999, 2002) and human (Bielanska et al. 2002a, 2002b) blastocysts are mixoploid, or aneuploid mosaics (Daphnis *et al.*, 2005). Moreover, using FISH probes for two chromosomes and a scoring system that counted extra FISH signals as increases in ploidy and 'missing' signals as false negatives, Viuff *et al.* (1999) reported that IVP bovine embryos have a higher rate of mixoploidy than their *in vivo* counterparts.

Chromosomal abnormalities have also been proposed as an important cause of early embryonic loss in the horse (Ball 1988; Ginther 1992), although their occurrence has yet to be demonstrated. To date, the studies performed to examine the chromosomal constitution of equine gametes or embryos have used conventional karyotypic analysis, and revealed a low incidence of chromosomal abnormalities in spermatogonia (Scott and Long 1980) and oocytes (King et al. 1990; Lechniak et al. 2002), but none in conceptuses (Blue 1981; Haynes and Reisner 1982; Romagnano et al. 1987). The aim of the current study was to validate FISH probes for identifying specific equine chromosomes in non-dividing

(interphase) nuclei and to use these probes to examine the incidence of chromosomal aberrations in *in vivo* developed and IVP horse embryos.

MATERIALS AND METHODS

Preparation of FISH probes

The FISH probes were produced by isolating and fluorescently labelling DNA sequences derived from equine chromosome specific Bacterial Artificial Chromosomes (BACs) that were cloned in a chloramphenicol resistant E. Coli strain. The BAC clones chosen for this study were located on equine chromosomes 2 (Solute carrier family 2 member 1, SLC2A1; chromosome location 2p16) and 4 (Aldehyde reductase, AKR1B1; chromosome location 4q23-q24; Milenkovic et al. 2002) and were a gift from the INRA horse BAC library (Institut Nationale de Recherche Agronomique, Jouy-en-Josas, France). The BAC clones were cultured in Luria-Bertani-medium (LB Broth, Miller; Brunschwig Chemie, Amsterdam, the Netherlands) containing 20 µg/l chloramphenicol (Roche Diagnostics GmbH, Mannheim, Germany), and the BAC DNA was isolated using Plasmid Midi Kits (Qiagen, Hilden, Germany). Once isolated, the BAC DNA concentration and quality were estimated by spectrophotometry at wavelengths of 260 and 280 nm, and agarose gel electrophoresis alongside standard molecular weight markers (Lambda DNA/ Eco RI + HindIII Marker, 3: MBI Fermentas GmbH, St. Leon-Rot, Germany). The BAC DNAs for chromosomes 4 (ECA 4) and 2 (ECA 2) were then labelled by nick translation with, respectively, digoxigenin (DIG) and biotin (DIG–Nick Translation Mix; Biotin–Nick Translation Mix; both from Roche Diagnostics GmbH, Mannheim, Germany). In short, 1 µg of BAC DNA was incubated for 90 minutes at 15ºC with 4 µl of the appropriate Nick Translation Mix in a total reaction volume of 20 µl, made up with distilled water. The reaction was stopped by adding 1 µl 0.5M EDTA (pH 8.0) and heating to 65°C for 10 minutes. After adding 10 µl of herring sperm DNA (10 µg/µl), 3 µl 3M sodium acetate and 600 µl isopropanol, the incubants were centrifuged (10 minutes; 16,000g; 4ºC) to precipitate the labelled probes. Next, the probes were washed with 600 µl 70% ethanol, centrifuged for 10 minutes at 16,000g and 4ºC, and the supernatant was discarded. The pellets were then air dried and dissolved in 100 µl Tris-EDTA to a final concentration of 10 ng/µl; the FISH probes were stored in this form at 4ºC.

Validation of the FISH probes

Lymphocyte culture

The efficacy and specificity of the FISH probes was validated using equine lymphocytes in both metaphase and interphase. The lymphocytes were recovered from the peripheral blood of a single stallion, and cultured to increase the number of cells in metaphase. Briefly, 10 ml of jugular vein blood was collected in a sodium-heparinised vacutainer tube (Vacutainer®, Becton Dickinson, Drogheda, Ireland) and centrifuged at 390g for 15 minutes at room temperature. After centrifugation, the white blood cells in the buffy coat

and 2 ml of the autologous plasma were transferred to a culture flask containing 10 ml of culture medium (RPMI 1640 Medium with Glutamax-I; Gibco, Paisley, Scotland, UK) supplemented with penicillin-streptomycin (100 IU/ml penicillin G sodium and 100 μg/ml streptomycin sulphate; Gibco, Paisley, Scotland, UK), 20 % (v:v) fetal calf serum (FBS, Bio Whittaker Europe, Verviers, Belgium) and 0.35 mg of the mitogen, pokeweed (Pokeweed, PWM; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). These cells were incubated at 37ºC for 95 h, during which period the flasks were inverted twice daily. After 72 h of the incubation, 1 μg methotrexate (Emthexate PF; Pharmachemie, Haarlem, the Netherlands) was added to each culture flask to stop the cell cycle in the S-phase, and thereby synchronise the dividing cells. A further 17 hours later, the contents of the culture flask were centrifuged at 180g for 10 minutes at room temperature to remove the methotrexate. The lymphocytes were resuspended in 5 ml of culture medium without pokeweed mitogen but containing 0.2 mg 5-bromo-2'-deoxyuridine (Br-dU; Sigma, Steinheim, Germany), to restart the cell cycle. After 5 hours of culture, 0.5 μg colchicine (Colcemid®; Gibco, Paisley, Scotland, UK) was added to arrest dividing cells at metaphase. After a further 25 minutes, the contents of the culture flask were centrifuged at 180g for 10 minutes at room temperature, the supernatant was removed and the pellet was resuspended in 10 ml 0.075 M KCl at 37ºC. Finally, the lymphocytes were fixed by washing four times in a 3:1 methanol:glacial acetic acid solution (fixative) and stored at -20ºC.

Lymphocyte chromosome preparations

Lymphocyte chromosome preparations were made by dropping cell suspension onto a microscope slide covered in fixative, followed by air-drying and storage for 2 days at room temperature. Subsequently, the slides were incubated for 7 minutes at 37ºC in a 0.01% pepsin (25.9 mA/mg porcine pepsin; Serva Electrophoresis GmbH, Heidelberg, Germany)/10 mM HCl solution, to partially digest the nuclear proteins and thereby allow the FISH probes better access to their hybridization targets. The preparations were then washed twice for 5 minutes with phosphate buffered saline (PBS), incubated in a 1% formaldehyde/0.05M $MgCl_2$/PBS solution for 10 minutes, and washed twice more in PBS for 5 minutes. Next, the preparations were dehydrated by immersing them for 2 minutes each in 70%, 85% and 100% ethanol, before being air-dried. Finally, the preparations were incubated with 100 μl of a 70% formamide/2xSSC (300 mM sodium chloride and 30 mM sodium citrate solution, pH 7.0) denaturation solution under a cover slip at 80ºC for 5 minutes. Directly thereafter, the preparations were again dehydrated in 70%, 85% and 100% ethanol solutions (-20ºC) before being allowed to air dry.

Suppression of repetitive DNA in FISH probes

Because repetitive DNA sequences in the FISH probes can lead to binding of the probes to other chromosome locations, these sequences were blocked by mixing the FISH probe DNA with sonicated equine DNA at a ratio of 75 : 1, a process known as 'competition'. Prior to the FISH procedure, the probe/sonicated equine DNA mixture was precipitated in sodium acetate and isopropanol, and washed in 70% ethanol, as described above. The pellet was left to air dry and was then redissolved by incubation in hybridization mix (50%

formamide/2xSSC/0.1% Tween-20) for 30 minutes at 60ºC. Next, the probe DNA was denatured by incubation at 80ºC for 5 minutes. Denaturation was stopped by placing the eppendorf tubes on ice for 3 minutes, after which competition was induced by incubating at 37ºC for 30 minutes. The resulting FISH probes were kept at 4ºC until further use.

In situ hybridisation and detection of FISH probes

Ten µl of a solution containing 100 ng of each FISH probe was applied to each preparation, which was then covered with a cover slip, sealed with glue and incubated in a humid box at 37ºC for approximately 12 hours. The cover slips were removed and the slides washed twice in 0.4xSSC/0.05% Tween-20 at 72ºC for 5 minutes, once in 2xSSC/0.05% Tween-20 at room temperature for 5 minutes, and once in 4xSSC/0.05% Tween-20 at room temperature for 5 minutes, to remove any remaining non-hybridised probe material. Next, the slides were incubated with 100 µl non-fat dry milk solution (NFDM: 0.25 gram non-fat dry milk in 5 ml 4xSSC/0.05% Tween-20) under a cover slip and placed in a humid incubation box at room temperature for 10 minutes. Fluorescent labelling was performed by incubating the preparations with 100 µl of an antibody solution containing 5µg/ml streptavidin-CY3 (Amersham Biosciences, Uppsala, Sweden) and 10µg/ml anti-digoxigenin-fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) dissolved in NFDM. The slides were covered with a cover slip and placed in a humid incubation box at 37ºC for 30 minutes to allow the antibodies to bind to the biotin or DIG incorporated in the FISH probes and, thereby, label the probes with the fluorescent dyes CY3 and fluorescein, respectively. Once labelling was complete, the slides were washed once in 4xSSC/0.05% Tween-20 for 5 minutes and twice in PBS for 3 minutes, to remove any remaining antibody mix, and then dehydrated by incubation for two minutes each in 70%, 85% and 100% ethanol. Finally, the preparations were air dried and the nuclear DNA was counterstained by applying 15 µl of mounting medium containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Vectashield® Mounting Medium with DAPI: Vector Laboratories Inc., Burlingame, CA, USA).

Microscopic analysis

FISH probe signals within individual nuclei were detected using an epifluorescence microscope (Axioplan®-2: Carl Zeiss, Oberkochen, Germany). Three different filter combinations were used to separately visualise CY3 staining of ECA 2 (red), fluorescein staining of ECA 4 (green) and DAPI staining of nuclear DNA (blue). The images were subsequently recorded, intensified, merged and stored digitally (Cytovision 3.0, Applied Imaging International Ltd, Newcastle upon Tyne, UK).

Collection of *in vivo* embryos

In vivo embryos (n = 22) were recovered during the physiological breeding season (April-September) from 13 Warmblood mares ranging from 3-18 years of age. To determine when they were in oestrus, the mares were teased using a vigorous stallion and their reproductive organs were examined by *per rectum* palpation and ultrasonography thrice weekly. When a

mare was in heat with one or more ovarian follicles of at least 35 mm in diameter, she was inseminated with semen from one of two stallions of proven good fertility (>300 x 10^6 morphologically normal, progressively motile spermatozoa per insemination dose). Following insemination, the mare's ovaries were examined daily to determine the time of ovulation. Six or seven days after ovulation, the embryos were recovered by uterine lavage with 3L Dulbecco's Phosphate Buffered Saline (DPBS) supplemented with 0.5% (v:v) fetal calf serum (FCS; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). At the time of collection, the embryos were thus 6.5 (n = 8) or 7.5 (n = 13) ± 0.5 days old; one embryo resulting from an asynchronous double ovulation was recovered 5.5 ± 0.5 days after ovulation. After recovery, embryos were washed four times in sterile DPBS supplemented with 0.5% (v:v) FCS.

Production and collection of IVP embryos

Day 7 IVP embryos were produced as described previously (Tremoleda et al. 2003a). In short, compact cumulus oocyte complexes recovered from the ovaries of slaughtered mares were matured *in vitro* for 30 h, after which the oocytes were denuded and those that had reached the MII stage were fertilised by intracytoplasmic sperm injection (ICSI). The resulting presumptive zygotes were cultured *in vitro* for 2 days. On day 2 after ICSI, morphologically normal two and four-cell embryos were selected for further culture, embedded in agar chips and transferred surgically to the ligated oviduct of a progesterone-treated ewe. Five days later, the embryos were harvested by surgical oviduct lavage. For transport to the FISH laboratory, recovered IVP embryos were cryopreserved in modified synthetic oviductal fluid (Hepes-SOF; Gardner et al. 1994) containing 10% glycerol as cryoprotectant. In preparation for freezing, the embryos were incubated first for 5 minutes in SOF containing 5% glycerol and then for 20 minutes in SOF containing 10% glycerol. The embryos were then loaded into 0.5 ml straws and frozen using a programmable freezing machine (Bio-cool IV, FTS System, Stone Ridge, NY, USA). Initially, they were cooled by plunging them into a -6°C methanol bath, in which they were held for ten minutes and seeded. Thereafter, the embryos were cooled at 0.5°C /minute down to -32°C and, finally, they were plunged into and stored in liquid nitrogen. After transport to the FISH laboratory, the embryos were thawed by removing the straw from the liquid nitrogen and holding it in the air for 5 seconds before immersing it in 25°C water for 1 minute. After thawing, the embryos were expelled from the straws and washed for 5 minutes each in solutions of SOF containing 8%, 6%, 4%, 2% and 0% glycerol, to remove the potentially toxic cryoprotectant.

Embryonic spread preparations

Prior to spreading, the embryos were washed twice in PBS containing 0.1% PVA (polyvinyl alcohol; Sigma-Aldrich Chemie BV, Zwijndrecht, the Netherlands). Next, the embryo was immersed in a 15 µl drop of lysis buffer (16 µl 5M HCl and 8 µl Tween-20 in 10 ml dH_2O) on a glass microscope slide. Thereafter, the embryo was monitored continuously using an inverted phase-contrast microscope until cell lysis occurred; this took

approximately 15 minutes. If the buffer evaporated prior to completion of cell lysis, a further 5 µl was added. In most cases, the blastocyst capsule was not lysed by the lysis buffer and was instead ruptured using a 28 gauge needle. This allowed the liberated nuclei to exit the capsule and spread out over the slide. The dispersed nuclei were then fixed by immersing the slide in fixative (3:1 methanol:glacial acetic acid) at room temperature for at least 24 hrs. After fixation, the slides were air dried, incubated overnight at 60ºC and stored at -80ºC until staining.

FISH and microscopic analysis

Prior to FISH analysis, the embryonic spread preparations were removed from the freezer and maintained for approximately 1 hour at room temperature to air dry. Subsequently, the slides were incubated for 7 minutes at 37 ºC in a 0.01% pepsin solution (25.9 mA/mg porcine pepsin/10 mM HCl). Thereafter, FISH and microscopic analysis was performed as described previously for lymphocyte spread preparations.

Analytical criteria

Embryonic nuclei were scored only if they were intact and non-overlapping. Fluorescent signals detected within a nucleus were considered to reflect the presence of a single chromosome if the signal was of the appropriate size (~0.5 µm) and shape, and was separated from other signals by more than the diameter of a single signal. Because of the large numbers of nuclei per embryo (sometimes several thousand), counting of signal numbers and interpretation with respect to abnormalities of chromosome number was performed in accordance with the system described by Viuff *et al.* (1999, 2002), who also used 2 chromosome specific probes to study abnormalities of ploidy in embryos. In short, a nucleus was considered to be diploid if either 2+2 (Fig 1D), 2+1 or 2+0 signals were recorded, triploid if 3+3 (Fig 1E), 3+2, 3+1, or 3+0 signals were found, and tetraploid if 4+4 (Fig 1F), 4+3, 4+2, 4+1, or 4+0 signals were counted. Thus, when nuclei had fewer fluorescent signals for one probe than the other the deficit was considered to be a false negative, unless many nuclei in the same embryo displayed the same pattern. Similarly, when nuclei had fewer than the expected number of signals for both probes (e.g. 0+0, 0+1, 1+1) they were considered to be false negatives, and the nuclei were classed as unscorable and excluded from further analysis. As a consequence, nuclei with nullisomy or monosomy of either ECA 2 or 4 would have been scored as diploid, while trisomy of either ECA 2 or 4 would have been interpreted as triploidy. Embryos that contained a mixture of diploid and polyploid nuclei were classified as mixoploid. To examine the validity of the analytical criteria, complete analysis of FISH signal numbers was performed in 11 of the 42 embryos (4 *in vivo* and 7 IVP). Finally, only embryos in which more than 50% of at least 30 cells could be scored were included in the final analysis.

Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA). The incidence of mixoploidy in *in vivo* versus IVP embryos was compared using the Chi squared test. In addition, the effects of production method and embryo developmental stage on total cell number were examined using an unpaired Students *t*-test, and a one-way ANOVA followed by pairwise multiple comparisons (Bonferroni *t*-test) of the natural logarithm of the cell number. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Validation of FISH probes

The FISH probes for ECA 2 and ECA 4 produced small, but distinct and clear signals on the expected regions of the appropriate chromosomes in metaphase lymphocyte nuclei (see Fig 1A for the probe for ECA 4), with little or no non-specific staining. Both probes also produced easily identifiable signals in interphase lymphocyte nuclei, when used independently (See Fig 1B for the probe for ECA 2) or in combination (Fig 1C). It was anticipated that the probes might occasionally give rise to double signals per chromosome as a result of DNA replication, and separation into sister chromatids, prior to cell division. These 'double chromatid signals' were indeed found in many metaphase (Fig 1A), and occasional interphase, lymphocyte nuclei (Fig. 1C) but since the sister chromatid signals were always less than the diameter of a single signal apart, they would always have been scored as a single signal.

In vivo developed equine embryos

Following validation on lymphocytes, the FISH probes were used in combination to stain the nuclei in the embryonic spread preparations (Figs. 1D-F). Eleven of the 22 *in vivo* embryos were at the morula or early blastocyst stage of development, while the remainder were expanded blastocysts (Table 1); the mean embryonic cell number was 1669 (sem: 446; range: 90–7694). In total, 27,712 of the 36,720 (75%) nuclei could be scored for chromosome copy number, and only 435 (1.6%) were scored as polyploid. Moreover, the 'polyploid' cells were restricted to 4 of the 22 embryos (18%), all of which also contained diploid cells and were therefore classified as mixoploid (Table 2). The mixoploid embryos fell into two different categories, namely diploid-triploid (n=2) and diploid-triploid-tetraploid (n=2) mosaics (Table 2). One of the former had a high percentage of triploid cells (69%), whereas the others contained less than 20% polyploid cells.

IVP embryos

Two of the 22 IVP embryos had such low cell numbers (4 and 12 cells respectively, of which all analysable nuclei were scored as diploid) that they were considered grossly abnormal and excluded from further analysis. The remaining 20 embryos had a mean of 149 cells (sem: 16; range: 57 – 390) and, with the exception of one early blastocyst, were at the morula stage of development (Table 1). A total of 2634 of the 2978 (88%) nuclei were scored for chromosome copy number, and 147 (5.6%) were recorded as polyploid. Polyploid cells were detected in 8 of the 20 embryos (40%: Table 2); one of the chromosomally abnormal embryos was scored as entirely triploid while the other 7 were classified as mixoploids containing less than 30% polyploid cells. The mixoploids included diploid-triploid (n=2), diploid-tetraploid (n=4) and diploid-triploid-tetraploid (n=1) mosaics (Table 2).

Validity of the scoring system

Of the eleven embryos (4 *in vivo*, 7 IVP) for which complete analysis of chromosome copy numbers was performed, 1 had been classified as triploid, 5 as mixoploid and 5 as exclusively diploid using the scoring system described previously. In most cases, more detailed analysis supported the simplified scoring system because most cells scored as diploid, triploid or tetraploid did indeed exhibit an equal number of FISH signals for chromosomes 2 and 4 (>95%: Table 3). However, there were 100 cells (4.4% of all cells examined) in which 'missing' chromosomes were assumed to be false negatives, but could just have easily have been evidence of true but sporadic nullisomies, monosomies, trisomies or haploidies. Indeed, if all of these missing signals were accurate reflections of chromosome constitution, then all 11 embryos would have to be reclassified as mosaics containing chromosomally abnormal cells of various types.

FIG. 1. Fluorescence photomicrographs to demonstrate the typical staining patterns of two colour FISH with probes for equine chromosomes 2 and 4 on metaphase and interphase nuclei in equine lymphocytes, and interphase nuclei from early equine embryos produced *in vivo* or *in vitro*. In all cases, nuclear DNA is coloured blue and the FISH probes specific for equine chromosomes 2 and 4 are red and green, respectively. After preparation of the FISH probes, their binding to the expected regions of the appropriate chromosomes was verified by labelling lymphocytes arrested in metaphase (**A**: FISH probe specific for equine chromosome 4). The utility of these probes for labelling interphase nuclei was then validated by staining lymphocytes in interphase (**B**: FISH probe specific for equine chromosome 2). In dividing cells, the chromosomes consist of two sister chromatids and, since the FISH probes labelled regions at a relatively large distance from the centromere, binding of the probes to both chromatids sometimes gave rise to two signals per chromosome (**A**). To avoid misinterpretation of such a double signal in an interphase cell, 'double chromatid signals' separated by less than the diameter of a single signal were recorded as a single chromosome (**C**: a diploid interphase nucleus from an equine lymphocyte). In the equine embryos examined, more than 98% of the nuclei scored were diploid (**D**: a diploid nucleus from an *in vivo* produced embryo). However, triploid (**E**: a triploid nucleus from an *in vivo* embryo) and tetraploid nuclei (**F**: a tetraploid nucleus from an IVP embryo) were also found.

FIGURE 1

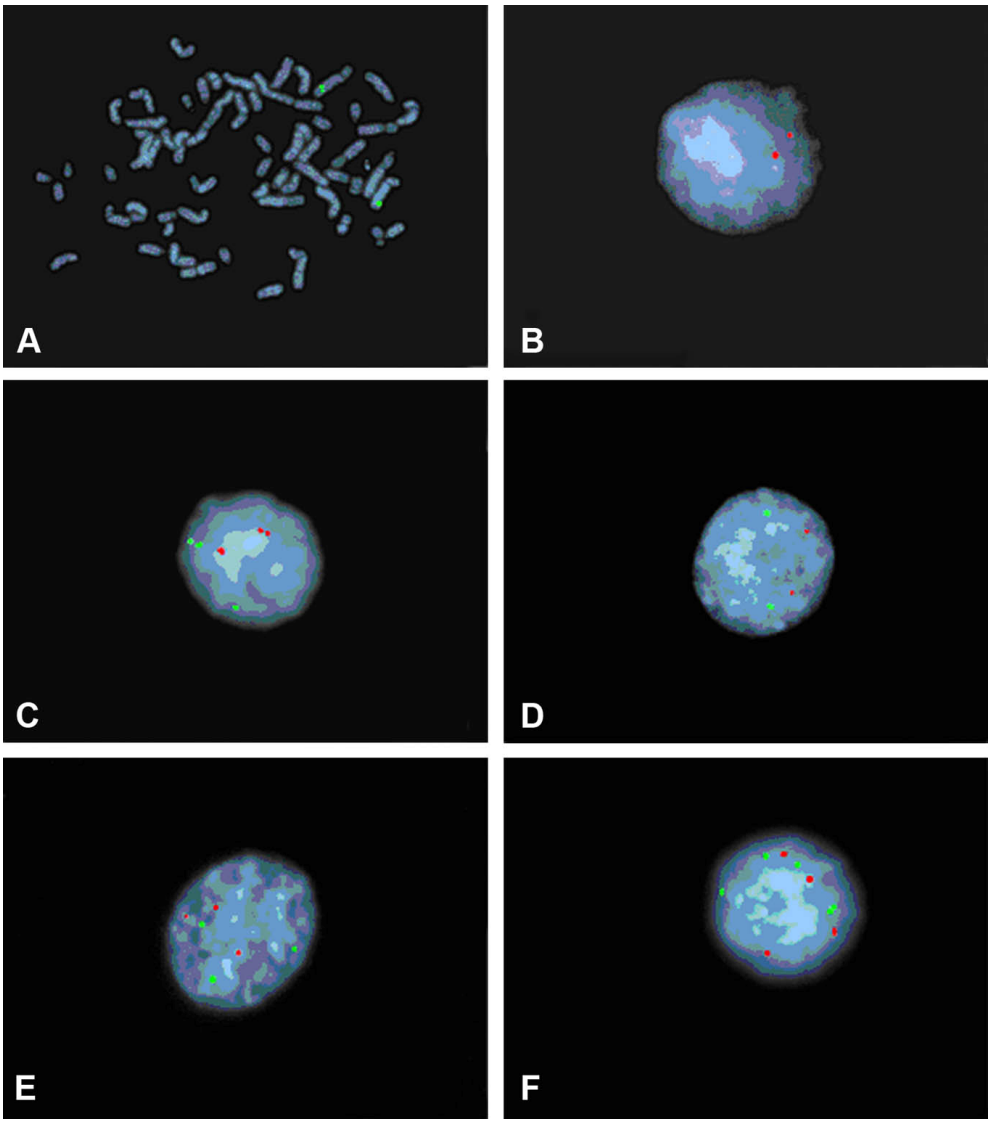

TABLE 1. Total cell numbers in equine morulae or blastocysts produced *in vivo* or *in vitro*.

Embryo production method	No. of embryos	No. of cells/embryo (mean ± SEM)	Developmental stage	No. of embryos	No. of cells/embryo (mean ± SEM)
In vivo	22	1669 ± 446.0[a]	Morula	4	161 ± 24.7[c#]
			Early blastocyst	7	575 ± 143.8[d#]
			Expanded blastocyst	11	2914 ± 716.7[e]
In vitro	20[§]	149 ± 15.8[b]	Morula	19	136 ± 9.9[c]
			Early blastocyst	1	390
			Expanded blastocyst	--	--

[a,b,c,d,e] Within a column, values with different superscripts differ significantly ($p < 0.001$; groups marked with [#] $p = 0.042$).
[§] Of the 22 *in vitro* produced embryos, 2 were excluded from this analysis because of an abnormally low cell number (i.e. 4 and 12 cells).

TABLE 2. Incidence and type of abnormal ploidy detected in equine embryos produced *in vivo* and *in vitro.*

Polyploid cells	*In vivo* produced embryos			*In vitro* produced embryos		
%	n	%	Description	n	%	Description
0	18	81.8	2N	12	60	2N
0-10	2	9.1	2N/3N/4N	5	25	2N/3N(2); 2N/4N(2); 2N/3N/4N (1)
11-20	1	4.5	2N/3N	1	5	2N/4N
21-30	0	0	--	1	5	2N/4N
61-70	1	4.5	2N/3N	0	0	--
100	0	0	--	1	5	3N
Total	22	100		20[§]	100	

[§] Of the 22 *in vitro* produced embryos, 2 were excluded from this analysis because of an abnormally low cell number (i.e. 4 and 12 cells).

TABLE 3. Number of FISH signals for chromosomes 2 and 4 (ECA2 and 4) in the nuclei of equine embryos produced *in vivo* (n=4) or *in vitro* (n=7).

Embryo production method	Total no. of nuclei	Number of FISH signals for ECA2-ECA4																	Assumed embryonic ploidy (following the classification of Viuff *et al.* 1999)
		n/a*	1-1	1-0	0-1	2-2	2-1	2-0	1-2	0-2	3-3	3-2	3-1	3-0	2-3	1-3	0-3	4-4	4-3	
In vivo	674	122	3			527	4		3	2	8				2			3		2N/3N/4N
	753	166				169	3	5	3	3	391	3	3	2	4		1			2N/3N
	192	27				147	2		3	2	4		1					6		2N/3N/4N
	222	24				194	2	1	1											2N
In vitro	164	48		1		109		2	1	3										2N
	144	42		1		81	2	3	1	2								11	1	2N/4N
	136	20	1	1	2	107	2	2	1											2N
	160	32	1	2	1	117		2		1								4		2N/4N
	196	2	1			187	3		3											2N
	101	16									80	2	1	1			1			3N
	57	26				29		1	1											2N
Total no. of nuclei (%)	2799	525 (97.4)	6	5	3	1667 (96.3)	18	16	17	13	483 (95.8)	5	4	4	6	0	2	24 (96.0)	1	

* This category includes overlapping, damaged or otherwise unreadable nuclei as well as intact, non-overlapping nuclei in which no signal could be detected (0-0).

DISCUSSION

Chromosomal aberrations are widely assumed to be a significant cause of early embryonic death in the mare. To date, however, there has been no concrete proof (cytogenetic or otherwise) of their existence in either obviously abnormal or apparently normal conceptuses. This report describes the validation and use of interphase FISH for analysing the chromosomal constitution of equine embryonic cells and, despite the use of a simplified scoring system that only recorded increases in the number of copies of chromosomes 2 or 4 as abnormal, it was possible to demonstrate that equine embryos developed either *in vivo* or in *vitro* commonly contain chromosomally abnormal cells. Moreover, although only a relatively small number of embryos were examined, the rates of chromosomal abnormality (40% of *in vitro* and 18% of *in vivo* embryos) were similar to those reported for bovine embryos when a similar system for identifying 'abnormalities of ploidy' was employed (72% and 25%: Viuff et al., 1999). As will be discussed later, the use of probes for more chromosome pairs, or of techniques to verify cases of suspected chromosome loss, would have led to a higher number of embryos recorded as containing a greater percentage of chromosomally abnormal cells of a more diverse nature. By contrast, previous studies using conventional karyotypic analysis had not detected any abnormalities in either failing or apparently normal horse conceptuses (Blue 1981; Haynes and Reisner 1982; Romagnano et al. 1987). However, karyotypic analysis using conventional cytogenetic staining methods can only be performed on the small proportion of nuclei in metaphase, and indeed only on those metaphases with well spread-out, non-overlapping chromosomes; a chromosome paint library of the type needed to analyse poor quality metaphases, and as used for example in human cancer studies, has only recently been developed for the horse (Yang et al., 2004). Interphase FISH is, therefore, a particularly useful tool because it dramatically increases the number of analyzable nuclei per embryo, while rendering cell culture to increase the number of metaphases superfluous. This enables the detection of numerical chromosomal abnormalities even when it affects only a small proportion of cells.

In the present report, *in vivo* and IVP equine embryos were examined for differences in development and the incidence of numerical chromosomal aberrations. As reported previously (Tremoleda et al. 2003a), IVP embryos comprised significantly fewer cells than their *in vivo* counterparts (149 ± 16 versus 1669 ± 446; $p<0.001$). However, all but one of the IVP embryos were morulae, whereas the majority of day 6.5-7.5 *in vivo* embryos examined were blastocysts. When total cell numbers in morulae were compared, they did not differ significantly between *in vivo* and IVP embryos. This suggests that IVP equine, like bovine (Dieleman et al. 2002), embryos develop more slowly than their *in vivo* counterparts. Although more IVP (8/20) than *in vivo* embryos (4/22) contained cells with more than 2 copies of ECA 2 and/or 4, this difference was not statistically significant at the 5% level ($p=0.118$) presumably, at least in part, because of the low number of embryos analysed. A higher rate of chromosomal abnormalities in IVP embryos might be expected to result from deficiencies in the chromosome segregation machinery developing during *in vitro* maturation of the oocytes (Ocana-Quero et al. 1999) and/or during the the periods of post-fertilisation culture (2 days in SOF, 5 days in a ewe's oviduct). In this respect, it is possible that the effect of IVP in the current study was minimised by the choice of the sheep oviduct as the primary culture system; Lonergan *et al.* (2004) reported that the sheep oviduct system resulted in a lower incidence of mixoploidy in bovine blastocysts than a

SOF based system. Although freezing and thawing has also been suggested to increase the likelihood of chromosomal abnormalities (e.g. in human embryos: Salumets et al. 2004), the fact that the IVP but not *in vivo* embryos were cryopreserved is unlikely to have influenced the incidence of chromosomal abnormalities in the current study because the IVP embryos were lysed, spread and fixed immediately after thawing i.e. they were not subjected to a period of post-thaw culture when anomalies of chromosome segregation could have arisen. On the other hand, it could be argued that the tendency to higher levels of chromosomal abnormality in IVP embryos might be an artefact of the earlier developmental stage of these embryos compared to those developed *in vivo*. In man (Bielanska et al., 2002b) and cattle (Viuff et al., 2002), the detected incidence of chromosomal abnormality decreases at later developmental stages, probably because embryos with severe chromosomal abnormalities are non-viable and have already degenerated.

The great majority (98.1%) of embryonic cells analyzed in the current study were scored as diploid (2N). And although a number of cells were recorded as triploid (3N) and tetraploid (4N), all but one of the chromosomally abnormal embryos were classified as mixoploid, i.e. they contained diploid as well as apparently triploid and/or tetraploid cells. The one exception was an IVP embryo scored as entirely triploid. In this respect, while Viuff *et al.* (1999) did not encounter any entirely polyploid cattle embryos, triploidy is one of the most frequent chromosomal abnormalities detected in failed human pregnancies, accounting for >10% of spontaneous abortions (Jacobs et al. 1982; Kaufman 1991; Jacobs and Hassold 1995; Golubovsky 2003). In man, the majority of triploids (> 65%) appear to be paternal in origin (diandric) and to arise from dispermic fertilisation. In horses, spontaneous polyspermy has not been reported, and available evidence suggests that triploidy is more likely to be maternal in origin, where maternal (digynic) triploidy originates primarily from errors in meiosis and consequent failure to extrude chromosomes in a polar body (Zaragoza et al. 2000). In this respect, 2.7% of *in vitro* matured oocytes were reported to be diploid rather than haploid (King et al. 1990; Lechniak et al. 2002) and 12% of equine zygotes that developed abnormally after fertilisation by ICSI had formed 2 female pronuclei as a result of failure to extrude the second polar body (Tremoleda et al. 2003b). Although only 1 entirely triploid embryo was identified in the current study, a further 11 embryos were scored as mosaics of diploid, triploid and/or tetraploid cells. The most plausible mechanism for the development of tetraploid cells is failure of cytokinesis some time after the first zygotic division, leading to 2 rounds of DNA replication without an intervening chromosome division (endoreduplication), thereby giving rise to a 2N/4N mixoploid conceptus (Wilson et al. 1988; Edwards et al. 1994; Alonso et al. 2002). It is, however, much more difficult to explain the possible aetiology of diploid/triploid mixoploids, even though these are reported to be common among cattle embryos produced *in vivo*, *in vitro* (Viuff et al., 1999) or by nuclear transfer (Li et al. 2004a, 2004b), and are also found among D5 IVP human embryos (Daphnis et al. 2005). One theoretical possibility is the "postzygotic diploidisation" of a triploid embryo, where a proportion of cells revert to diploidy by excluding a haploid genome from the metaphase plate during mitosis (Golubovsky, 2003); there is, however, no physical proof that such postzygotic diploidisation actually occurs. Other proposed mechanisms by which 2N/3N mixoploids could arise include: (1) dispermy, where the second sperm fuses not to the other pronuclei but to a diploid embryonic nucleus after initial cleavage; (2) fusion of the second polar

body to an embryonic cell after the first cleavage division; and (3) chimeric union of a diploid and a triploid embryo (Donnai et al. 1988). However, all of these mechanisms are unlikely and would, therefore, be expected to account only for very occasional 2N/3N mixoploids, which would be expected to contain a large proportion of triploid cells. These mechanisms are, therefore, unlikely to account for triploidy in all 6 of the 2N/3N and 2N/3N/4N mosaic embryos found in the current study that contained a low proportion of triploid cells. A theoretically more plausible explanation for these chromosomal "mixoploids" is post-zygotic mitotic non-disjunction of either, or both, chromosomes 2 and 4 leading to trisomy (or double trisomy) and establishment of a trisomic cell line within an otherwise diploid embryo (trisomic mosaicism: Hsu et al. 1997; Kalousek 2000). In this respect, the analytical criteria used in the current and comparable reports (Viuff et al. 1999, 2002) could have led to incorrect classification of monosomic nuclei as either diploid (2+1) or unscorable (1+1, double monosomic) and of trisomic (3+2) or double trisomic (3+3) nuclei as triploid. On the other hand, for the 11 embryos for which the number of FISH signals were recorded in detail, the vast majority of cells (95.6%) showed an equal number of copies for chromosomes 2 and 4, supporting their classification as diploid, triploid or tetraploid. In the remaining 4.4% of cells, 'missing' FISH signals could have represented true absences of the chromosomes in question and therefore monosomies, trisomies, nullisomies or haploidies. However, since false negatives are also likely (e.g. due to overlapping signals or failed hybridisation), it is impossible to be certain that these findings truly represented sporadic instances of chromosome loss or gain. Daphnis *et al.* (2005) recently verified the occurrence of individual cell chromosome loss or gain in human embryos by using two sets of FISH probes per chromosome examined, but simultaneously demonstrated that a single FISH probe yields artefacts in approximately 5% of nuclei analysed. In the absence of a means of verification (i.e. more than one FISH probe per chromosome), it seems prudent to ascribe incidents of 'chromosome loss' in the current study to experimental artefact, even though this necessarily involves an underestimation of the incidence of chromosomal abnormality and, in some instances, a misdiagnosis of their nature. Similarly, confirmation that all cells recorded as, for example, triploid were indeed triploid and not trisomic would require FISH examination of more chromosome pairs, comparative genomic hybridisation (CGH: Wells and Levy 2003) or, if sufficient metaphase plates could be generated, conventional cytogenetics or chromosome paints.

Although morphologically normal equine conceptuses were shown to contain chromosomally abnormal cells, it is not clear to what degree this would affect the viability of the embryo/conceptus. In this respect, the significance of the chromosomally abnormal cells is likely to depend on their relative abundance and distribution between the embryo proper and the trophoblast. For example, in bovine (Viuff et al. 2002) and porcine (Long and Williams 1982) mixoploid embryos most polyploid cells are located in the trophoblast and relatively few are found in the embryonic disc or inner cell mass. Furthermore, Hare *et al.* (1980) reported that tetraploidy in up to 25% of the trophoblast cells can be compatible with the 'normal' progression of pregnancy in cattle. On the other hand, triploidy and tetraploidy are very rare in live-born children but common in human spontaneous abortions (Hassold et al. 1980; Eiben et al. 1990), suggesting that higher rates of polyploidy, or polyploidy within the fetus itself, greatly impairs conceptus/fetal viability. This hypothesis is supported by Sandalinas *et al.* 's (2001) report that polyploidy of more than 38% of the cells of an early embryo greatly reduced the likelihood of development, and it is therefore

likely that the 2 embryos in the current study with more than 65% presumptive triploid cells were non-viable, while it is quite possible that the less severely affected embryos could have given rise to a viable fetus/foal, particularly if the abnormal cells were concentrated in the trophectoderm. Although there is no evidence that preferential allocation of aneuploid cells to the trophectoderm occurs in human blastocysts, differential distribution of aneuploid cells between the fetus and placenta has been observed in some aneuploid-diploid mosaic fetuses such that the pregnancies were able to progress to term and give rise to a chromosomally normal infant (Kalousek 1994, 2000). Irrespective of whether the chromosomal abnormalities observed in the current study were true polyploidies, or in fact other types of aneuploidy, the current findings are compatible with chromosomal abnormalities accounting for a proportion of unexplained early embryonic losses in the mare and also possibly contributing to the low blastulation and pregnancy rates recorded for equine IVP embryos (Squires et al. 2003).

In summary, this study used interphase FISH to demonstrate that chromosomal abnormalities occur in morphologically normal horse embryos developed *in vivo* or *in vitro*. However, the use of just 2 chromosome specific probes to study chromosomal abnormalities in a species with a diploid total of 64 chromosomes, and the use of analytical criteria (Viuff et al. 1999, 2002) that ignore or, in some cases, may incorrectly classify certain categories of aneuploidy, imposes clear limitations. Most significantly, because only 2 chromosome pairs were studied and the absence of any signals was regarded as a false negative, it is likely that the observed incidence of aneuploid cells and/or embryos are considerable underestimates of the true number. On the other hand, while classifying all instances of extra FISH signals as an increase in ploidy is also a simplification inherent to a system using only 2 chromosome probes, the fact that more than 95% of 2274 cells examined fully had equal numbers of ECA 2 and 4 suggests that, in this case, it was a reasonable working hypothesis. More accurate classification of the nature of the numerical chromosome abnormalities commonly present in equine embryos could be achieved by validating probes for many more (preferably all 32) chromosome pairs, and using combinations in multi-colour FISH experiments (Baart et al. 2004). And whereas the use of CGH or chromosome paints would allow more precise categorisation of aneuploidies together with detection of other types of chromosome abnormality (e.g. translocations), the former is laborious when large numbers of cells are involved and the latter is only applicable to metaphase cells. Nevertheless, a combination of all 3 techniques would enable more extensive investigation of the incidence and nature of chromosomal aberrations in horse embryos, and their significance as a cause of pregnancy loss.

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Chapter 5

Expression of progesterone and oestrogen receptors by early intrauterine equine conceptuses

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ABSTRACT

Progestagens and oestrogens play essential roles in the maintenance of pregnancy in eutherian mammals and are thought to exert their effects on the developing conceptus indirectly, via the endometrium. In some species, early embryos have themselves been shown to express steroid receptors, thereby suggesting that reproductive steroids may also influence embryonic development directly. The aim of this study was to determine whether early intrauterine equine conceptuses express either the classical intracellular progesterone (PR) and oestrogen receptors (ERα and ERβ) or the more recently characterised membrane-bound progesterone receptors (PGRMC1 and mPR). Horse conceptuses recovered on days 7, 10 and 14 after ovulation (n=8 at each stage) were examined for steroid receptor mRNA expression using quantitative rtPCR. Where commercial antibodies were available (PR, ERβ), receptor localisation was examined immunohistochemically in day 10, 12, 14, 15 and 16 conceptuses (n=2 at each stage). mRNA for PR, PGRMC1 and mPR was detected at all stages examined, but while PGRMC1 and mPR expression increased during the day 7-14 period, PR expression decreased. ERα mRNA was not detected at any stage examined, whereas ERβ was detected in all day 14, some day 10 and no day 7 conceptuses. Immunoreactive ERβ receptors were localised to the trophectoderm of day 14-16 conceptuses; PR were not detected immunohistochemically in conceptus tissue. In summary, this study demonstrates that equine conceptuses express mRNA and, in the case of ERβ, protein for steroid hormone receptors during the period encompassing rapid conceptus growth, differentiation and maternal pregnancy recognition.

INTRODUCTION

Progesterone and oestrogens play important roles in conceptus development and, until fairly recently, it was widely accepted that their effects were indirect and mediated almost exclusively by modulation of endometrial receptivity (Wu et al. 1971; Baulieu 1989; Clarke and Sutherland 1990; Singh et al. 1996). In addition, the steroid hormones were thought to bind exclusively to intracellular receptors and to exert their biological effects via a relatively slow 'genomic pathway' involving the migration of the steroid-receptor complex to the nucleus, where it can modulate gene transcription and subsequent translation of mRNA into protein (Jensen et al. 1968; Beato 1989; Tuohimaa et al. 1996). There is, however, increasing evidence that the reproductive steroids may also directly influence pre-implantation conceptus development. For example, both progesterone (PR) and oestrogen receptor (ER) mRNA and protein have been detected in mature cumulus oocyte complexes and embryonic cells from several mammalian species (Hou and Gorski 1993; Hou et al. 1996; Ying et al. 2000; Kowalski et al. 2002; Hong et al. 2004; Hasegawa et al. 2005).

It is also increasingly clear that steroid hormones can exert their biological effects via messenger systems other than the classical genomic pathway. Indeed, steroid hormones sometimes induce their effects rapidly, whereas *de novo* protein synthesis would be expected to take hours to days (Losel *et al.* 2003), and do so even in cells lacking a (functional) nucleus and, therefore, incapable of gene expression (Losel and Wehling 2003). For example, aldosterone can induce cardiovascular effects within 5 minutes (Klein and Henk 1963) and alter sodium ion exchange in nucleus-free cells such as erythrocytes (Spach and Streeten 1964). Similarly, progesterone can induce the acrosome reaction in mammalian sperm within seconds of binding to a specific cell membrane receptor (Meizel and Turner 1991; Roldan et al. 1994; Sabeur et al. 1996; Cheng et al. 1998a; Cheng et al. 1998b), even though the sperm's nuclear DNA is transcriptionally inactive (Gilbert 1997). In fact, it has been proposed that steroids can exert non-genomic effects in two ways: (i) non-specifically (i.e. in the absence of a specific receptor) by altering cell membrane fluidity, or (ii) via ligand-specific steroid receptors including modified classical nuclear receptors and non-classical membrane-associated receptors (Falkenstein *et al.* 2000). In this latter respect, a membrane-bound progesterone receptor initially isolated and cloned from hepatic and smooth muscle tissue (Falkenstein et al. 1996; Meyer et al. 1996; Selmin et al. 1996; Falkenstein et al. 1998), has subsequently been detected in various other cell types, including sperm (Losel et al. 2004; Losel et al. 2005). This receptor has been variously termed mPR in pigs (Falkenstein et al. 1999), 25-Dx in rats (Selmin *et al.* 1996) and Hrp 6.6 in man (Gerdes *et al.* 1998), but has recently been reclassified as PGRMC1 (progesterone membrane receptor component 1: Losel et al. 2004). A second, unrelated membrane bound progesterone receptor (mPR) has since been discovered and cloned in trout oocytes (Zhu *et al.* 2003b), and homologues have subsequently been detected in a range of human, murine and porcine tissues (Zhu *et al.* 2003a). The existence of plasma membrane-associated oestrogen receptors has also been proposed (Nadal et al. 2000; Levin 2002; Toran-Allerand et al. 2002), but has not yet been confirmed.

Presumably to enhance their roles in the maintenance of pregnancy, reproductive steroids, in particular oestrogens, are produced in considerable quantities by pre-implantation

conceptuses of a wide range of species including the horse (Zavy et al. 1979; Marsan et al. 1987; Walters et al. 2001), donkey (Heap *et al.* 1991), pig (Perry et al. 1973; Gadsby et al. 1980), cow (Eley *et al.* 1983), camel (Skidmore *et al.* 1994), rabbit (Hoversland *et al.* 1982), hamster (Sholl *et al.* 1983), rat (Dickmann and Dey 1974; Dey and Dickmann 1974) and man (Edgar *et al.* 1993). While it is therefore clear that reproductive steroid production is a common feature of mammalian embryo development and an important component of fetal-maternal communication, the presence of receptors in the conceptus raises the additional possibility that steroid production helps to modulate conceptus development in an autocrine or paracrine fashion.
The aim of the current study was to determine whether equine conceptuses express classical and/or non-classical progesterone or oestrogen receptors which might allow the reproductive steroids to directly influence conceptus development. To this end, reverse transcriptase PCR (rtPCR) was used to examine whether pre-implantation conceptuses express mRNA for the intracellular progesterone (PR) and oestrogen (ERα and ERβ) receptors or the membrane-bound progesterone receptors (PGRMC1 and mPR). If mRNA expression was detected, quantitative rtPCR (q-rtPCR) would be used to determine whether gene expression altered during the early intra-uterine period. Where antibodies were available for the genes expressed, immunohistochemistry (IHC) would then be used to determine whether translation took place, and in which cell layers the receptor protein was localised.

MATERIALS AND METHODS

Collection and preparation of conceptuses

Thirty-four conceptuses were recovered from a group of 13 Warmblood mares, inseminated at numerous oestrous cycles. During early oestrus, a mare's reproductive tract was examined three times a week by *per rectum* palpation and ultrasonography. Once the dominant ovarian follicle(s) exceeded 35 mm in diameter, the mare was inseminated with 300-500 million motile sperm from one of two fertile stallions. Conceptuses were recovered on days 7 (n=8), 10 (n=10), 12 (n=2), 14 (n=10), 15 (n=2) or 16 (n=2) after ovulation by non-surgical uterine lavage using either Dulbecco's phosphate buffered saline (DPBS: day 7 conceptuses) supplemented with 0.5% (v:v) fetal calf serum (FCS; Sigma-Aldrich-Chemicals BV, Zwijndrecht, The Netherlands) or lactated Ringer's solution (day 10-16 conceptuses). After recovery, the conceptuses were washed repeatedly in large volumes of fresh DPBS or lactated Ringer's solution to remove FCS and any contaminating maternal cells. Next, the yolk sac fluid and capsule were removed from day 10-14 conceptuses, and the conceptus membranes were snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction. Day 7 embryos were snap-frozen immediately after recovery and washing.
Conceptuses for IHC were, after washing, carefully punctured before being examined using a stereomicroscope. The capsule was removed and the embryonic disc (ED), together with a generous margin of surrounding bilaminar trophoblast, was excised using a pair of micro-surgical scissors. The excised tissue was fixed by overnight incubation in 4% formaldehyde

before being dehydrated by immersion for 30 min in 70% ethanol. The tissue was then stained for 2 min with eosin, so that it could be identified during embedding, and dehydrated further by immersion for 3 x 15 min in 96% and 100% ethanol solutions and 2 x 15 min in xylene, before being embedded in paraffin. Once the paraffin had solidified, the eosin stained tissue was identified, excised with a scalpel and incorporated into a regular paraffin block. The paraffin blocks were subsequently orientated in the microtome such that the ED could be sectioned transversely from posterior to anterior. Five μm thick sections were then cut and adhered onto successively numbered coated glass slides (Superfrost® Microscope Slides, Menzel-Gläser, Braunschweig, Germany) by overnight incubation at 55˚C. Every tenth slide was stained with Haematoxylin and Eosin (HE) for orientation purposes, and the remaining slides were stored at room temperature until IHC.

PCR primer design

The primers used for rtPCR are listed in Table 1. The primer pairs for ERα, ERβ, PR and a reference gene (β-actin) were designed using the equine coding sequences. Since the equine sequence was not available for mPR and PGMRC1, the primers were designed using areas of sequence conserved well across other mammalian species; by preference, primers of a pair were located on separate gene exons. All primer pairs were validated using equine cDNA from conceptus, liver and endometrial samples.

TABLE 1. Primers used for rtPCR to examine expression of mRNA for reproductive steroid hormone receptors by day 7-14 equine conceptuses.

Gene	Genbank accession number	Primer sequence (5'→3')	T_a	Product size (bp)
ERα	AF124093	s: TCCATGATCAGGTCCACCTTCT as: GGTGTCTGTCATCTTGTCCA	55˚C	341
ERβ	AJ439894	s: TCAGCCTGTTCGACCAAGTG as: CCTTGAAGTCGTTGCCAGGA	60˚C	194
PR	AF007798	s: GTCAGTGGACAGATGCTGTA as: CGCCTTGATGAGCTCTCTAA	55˚C	255
mPR	AY424286	s: GCCAAGTATCGTTACCGGAG as: AAGAGGATCTGGAGCGTGTG	55˚C	173
PGMRC1	NM_213911	s: TCAACGGCAAGGTGTTCGAC as: GGCTCTTCCTCATCTGAGTA	58˚C	280
β-actin	AF035774	s: CCAACCGCGAGAAGATGACC as: ACCGGAGTCCATCACGATGC	60˚C	128

T_a = annealing temperature; s = sense; as = anti-sense.

RNA extraction and cDNA synthesis

RNA isolation and on-column DNAse digestion was performed using the Invisorb® Spin Cell RNA mini kit (Invitek GmbH, Berlin, Germany) combined with the RNAse-free DNAse set (Qiagen, Valencia, CA, USA). First, conceptuses were lysed in 700 µl lysis buffer. Next, either 350, 50 or 10 µl of the lysate (for day 7, 10 and 14 conceptuses, respectively) made up to a final volume of 350 µl with lysis buffer, was applied onto a DNA-binding spin filter. After incubation for 2 min at room temperature and centrifugation for 2 min at 11,000g, the binding filter containing the DNA was discarded, and the remaining lysate was diluted (1:1) with 70% ethanol and applied directly onto a RNA-binding filter. Next, the column was washed with 300 µl of wash buffer before RNAse-free DNAse was applied to the RNA-binding filter to remove any remaining DNA during a 15 min incubation at room temperature. After three further washes with wash buffer, the RNA was eluted from the RNA-binding filter using 33 µl RNAse-free water.
Reverse transcription was performed in a total volume of 20 µl, made up of 10 µl sample RNA, 4 µl of 5xRT buffer (Invitrogen, Breda, The Netherlands), 8 units RNAsin (Promega, Leiden, The Netherlands), 150 units Superscript II reverse transcriptase (Invitrogen), 0.036 units of random primers (Invitrogen), 10 mM DTT (Invitrogen) and 0.5 mM of each dNTP (Promega). After denaturation of the RNA for 5 min at 70°C, the mixture was incubated for 1 h at 42°C, and 5 min at 80°C to inactivate the reverse transcriptase, before being stored at –20°C. Minus RT blanks were prepared similarly, but in the absence of reverse transcriptase. RNA extraction and cDNA synthesis were then checked by rtPCR with primers for β-actin; samples that failed to yield cDNA, or those for which the minus RT blank was positive, were excluded from further analysis.

Experiment 1: mRNA expression

Expression of mRNA for ERα, ERβ, PR, PGRMC1, mPR and β-actin by day 7-14 conceptuses was analysed by rtPCR using the primers described in Table 1 and the following thermal cycling profile: 94°C for 15 min followed by 40 cycles of 15 sec at 94°C, 30 sec at the primer specific annealing temperature (see Table 1) and 45 sec at 72°C; final extension was performed by incubation at 72°C for 10 min. After completion of the PCR reaction, 10 µl of product was resolved on a 1% agarose gel containing 0.4µg/ml ethidium bromide to visualize any PCR products. A 100 basepair (bp) ladder was included as a reference for fragment size, and a standard sequencing procedure was used to verify the identity of the PCR products.
If mRNA expression for a given receptor was detected, quantitative PCR (q-rtPCR) was performed using a real-time PCR detection system (MyiQ Single-color Real-Time PCR Detection System; Bio-Rad Laboratories, Veenendaal, The Netherlands) on cDNA from day 7, 10 and 14 conceptuses (n=8 at each stage). For each target gene, a 96-well plate was loaded with triplicate samples of cDNA from the 24 conceptuses, a negative control (double distilled water) and 7 standards. The standard curve was constructed from 10-fold serial dilutions of known amounts of target gene PCR product. The 25µl q-rtPCR reaction mixture contained 1 µl cDNA, 0.5mM of each primer (Isogen Bioscience BV, Maarssen,

The Netherlands) and 12.5 µl of IQ™ Sybr® Green Supermix (Bio-Rad Laboratories). Initial DNA denaturation at 95˚C for 5 min was followed by 40 cycles of 15 sec at 95˚C, 30 sec at the primer specific annealing temperature (see Table 1) and 45 sec at 72˚C. The purity of the amplified product was checked by examining the melting curves. Standard curves were produced by plotting the log of the starting amount of product against the threshold cycle for detection, and gene expression was then calculated as the relative expression level (REL), i.e. the ratio of the quantity of a target gene to that of β-actin in the same sample.

Experiment 2: Immunohistochemistry

Commercial antibodies were available for only two of the receptors of interest, ERβ and PR. Immunohistochemical staining of day 10, 12, 14, 15 and 16 conceptus membranes (n=2 at each stage) was performed for these two receptors using the ABC-peroxidase procedure (DAKO, Hamburg, Germany). After deparaffinising and re-hydrating the 5-µm thick sections in xylene (2 x 5 min), followed by 100% ethanol, 96% ethanol, 70% ethanol and water (2 x 3 min each), antigen retrieval was performed by microwaving (1000 Watts) in preheated citrate buffer (pH = 6) for 30 min. The sections were then left to stand for 20 min to cool down to room temperature and rinsed for 5 min in water. Next, endogenous peroxidase activity was blocked by immersing the sections in 1% H_2O_2 in methanol for 30 min. After washing in phosphate buffered saline (PBS: 3 x 5min), either with (PR) or without (ERβ) 0.1% Tween-20, the sections were exposed to normal goat serum (1:10 dilution in PBS) for 10 min to block non-specific binding, before being incubated for 16 h at 4°C with primary antibody for ERβ (1:10 dilution of Monoclonal Mouse Anti-Human Estrogen Receptor β1; Dakocytomation, Glostrup, Denmark) or PR (1:80 dilution of Monoclonal Mouse Anti-Human Progesterone Receptor; Immunotech Inc., Marseille, France). After washing for 3 x 5 min in PBS, with (PR) or without (ERβ) 0.1% Tween-20, sections were exposed to the secondary antibody-peroxidase conjugate (Envision Goat-Anti-Mouse-Peroxidase: Dako, Hamburg, Germany) for 30 min at room temperature. Three further 5 min washes in PBS were followed by induction of the colour reaction by incubation for 10 min with freshly prepared 3,3'-diaminobenzidine tetrahydrochloride (DAB). The slides were then washed twice for 5 min in running tap water before the nuclei were counterstained for 45 sec with Mayer's haematoxylin. After a final 10 min rinse in running tap water, the sections were sealed under a cover slip using Eukitt™ Mounting Medium (Electron Microscopy Systems, Hatfield, PA, USA) and examined using an Olympus BX41 microscope (Olympus Nederland BV, Rotterdam, the Netherlands) at a magnification of 400x.

Statistical analyses

Statistical analysis was performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, IL, USA). To obtain continuous, normally distributed data sets, RELs were subjected to natural logarithmic transformation; the normality of distribution was then confirmed by plotting the quantiles of the residuals against the expected normal residuals (qq plot). The

effect of conceptus age on the REL for a given gene was analysed using a one-way-ANOVA, followed by a post-hoc Bonferroni-test to determine the site of differences in gene expression. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Experiment 1

ERα mRNA expression was not detected in any of the day 7-14 conceptuses examined. For the remaining steroid receptors (PR, PGRMC1, mPR and ERβ) mRNA was detected and q-rtPCR was performed. Although mRNA for PR was detected at all stages of development examined, relative expression was lower on day 14 than on days 7 or 10 (Fig. 1A). mRNA for the membrane-bound progesterone receptors (PGRMC1 and mPR) was also detected in all conceptuses examined (Fig 1 B-C) but, in these cases, the relative level of expression increased significantly between days 7 and 10. ERβ mRNA was not detected in day 7 conceptuses, but was detected in three of the eight day 10 and in all eight day 14 conceptuses (Fig. 1D).

Experiment 2

The ERβ antibody was validated by staining endometrium from an oestrous mare (Fig 2A); the nuclei of the glandular epithelial cells stained positively, and there was no evidence of non-specific binding in the negative control section (i.e. in the absence of primary antibody: Fig 2B). In day 14-16 conceptuses, ERβ-staining was present in the extra-embryonic trophoblast cells of both the trilaminar and bilaminar omphalopleure (Figs 2C-H); immunoreactive ERβ receptor was not detected in day 10 or 12 conceptuses.
The PR-antibody was validated on endometrium from a dioestrous mare. Again, the nuclei of the glandular epithelial cells showed positive staining, while omission of the primary antibody confirmed the absence of non-specific binding. Nevertheless, no immunoreactive PR was detected in any of the day 10-16 conceptus membranes examined (results not shown).

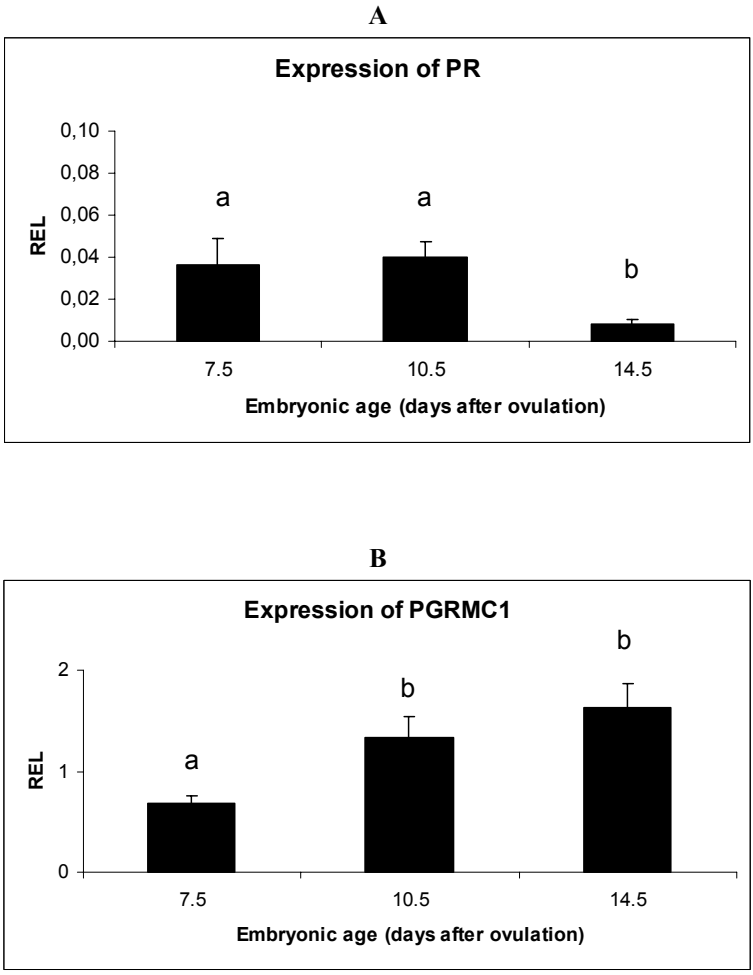
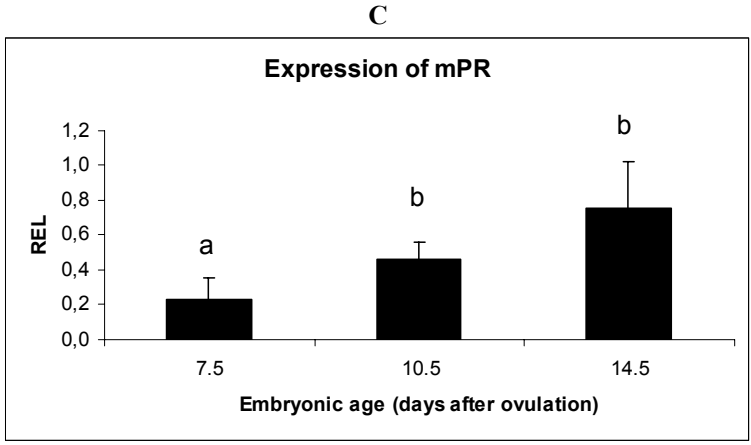
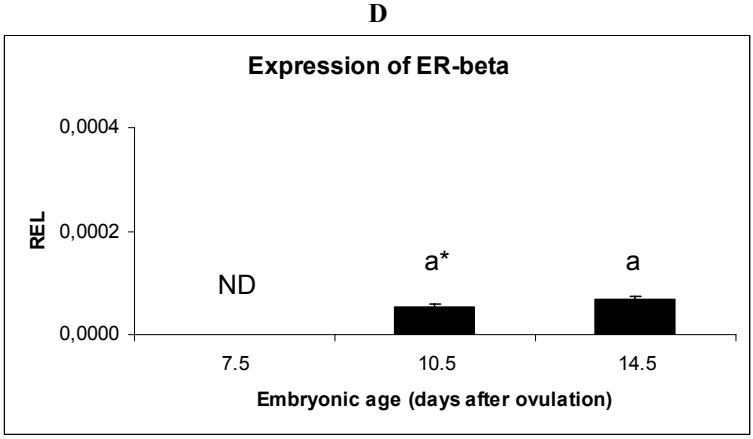


FIG. 1. Expression of mRNA for PR (Fig 1-A), PGRMC1 (Fig 1-B), mPR (Fig 1-C) and ERβ (Fig 1-D), in day 7, 10 and 14 equine conceptuses. In each case, gene expression is expressed as the relative expression level (REL), i.e. the ratio to the expression of a reference gene (β-actin). Unless otherwise indicated, each bar represents the mean (± sem) REL for 8 conceptuses.
Columns with different letters differ significntly ($p < 0.05$).
'ND' = non-detectable. * n=3.





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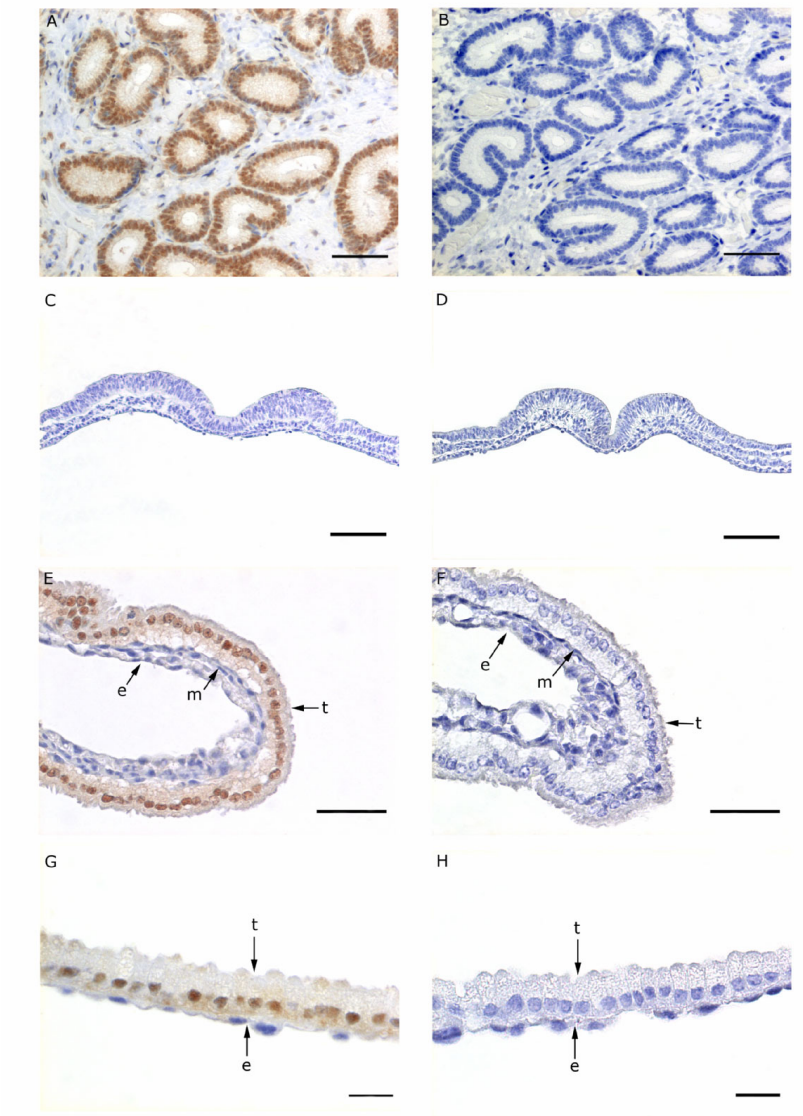

FIG. 2. Micrographs to illustrate the immunolocalisation of ERβ protein in equine endometrium and conceptus membranes.
(**A,B)** Endometrial tissue from an oestrous mare (positive control); ERβ receptors were localised to the endometrial gland cell nuclei (**A**), while no staining was observed in the absence of primary ERβ antibody (**B**).
(**C,E,G)** Images of a day 16 equine conceptus to show that ERβ antibodies do not stain the embryonic disc (**C**), extraembryonic mesoderm (m) or endoderm (e) (**E,G**). However, ERβ staining was observed in both the trilaminar (**E**) and bilaminar (**G**) extraembryonic trophectoderm (t).
(**D,F,H**) Negative controls for C, E and G, respectively.

DISCUSSION

This study aimed to determine whether equine conceptuses express receptors for progesterone and oestrogen during early intrauterine development. In the case of the genomic progesterone receptor (PR), mRNA levels were low and decreased between days 10 and 14 after ovulation, while receptor protein was not detected at any of the developmental stages studied. A similar conceptus-age related fall in PR mRNA levels has been reported in the pig, in which PR mRNA and protein were present in 1-4 cell embryos but undetectable in 5-cell or day 10-12 stages (Yelich *et al.* 1997; Ying *et al.* 2000). By contrast, PR mRNA was detectable only after blastulation in murine embryos (Hou and Gorski 1993). Although the decrease in PR mRNA with increasing equine conceptus age could be explained by expression in the pre-ovulatory oocyte and not in the developing embryo/conceptus, this would assume a time course for oocyte mRNA degradation greatly exceeding the norm (2-cell stage: Hamatani et al. 2004). Alternatively, it is possible that PR was transcribed, and possibly translated, during intra-oviductal development (up to day 6-7: Battut et al. 1997) but that expression ceased at around the time of uterine entry and blastulation (day 7: Betteridge et al. 1982). In either case, since the diminishing quantities of PR mRNA were apparently not translated into receptor during days 10-16 of gestation, progesterone must operate via an alternative route if it is to influence early intrauterine conceptus development. In this respect, mRNA for the membrane-bound progesterone receptors, PGRMC1 and mPR, was detected at levels 10-100 times higher than that for PR. In addition, since the RELs increased with developmental stage, this clearly represented active PGRMC1 and mPR expression. Previously, the presence of PGRMC1 mRNA has been described only in recently cleaved bovine embryos 28-40 hours after IVF (Dode *et al.* 2006); there are no previous reports of later embryonic or conceptus mPR expression. Although we were not able to procure antibodies to perform IHC for these membrane-bound progesterone receptors, the high and increasing mRNA expression levels make it conceivable that both receptor proteins are expressed in at least day 10 and 14 conceptuses.
With respect to the nuclear oestrogen receptors, we were unable to detect mRNA for ERα at any stage of development examined. ERβ mRNA was detected, but the level of expression was modest and evident in only 3 (of 8) day 10 and the day 14 conceptuses. On the other hand, ERβ was the only receptor for which we were able to prove translation of mRNA into protein, where the latter was localised to the trophoblast cells of day 14-16 conceptuses. This suggests that ERβ gene expression begins on or around day 10 of gestation in equine conceptuses, with translation to protein following shortly after (in any case, by day 14). Interestingly, ERβ expression in day 14-16 horse conceptuses was thus localised to the same cells, the extraembryonic trophectoderm, that express the enzymes for oestrogen production (Walters *et al.* 2000). In addition, the expression pattern for the nuclear oestrogen receptors was similar to that described previously in porcine and murine conceptuses. For example, ERα mRNA was not detectable in day 11-14 porcine conceptuses, whereas ERβ mRNA expression increased during this period and immunoreactive protein could be localised to the trophoblast cells from day 12 (Kowalski *et al.* 2002). In mice, maternally derived ER mRNA is present in the oocyte, and while levels decrease to undetectable during the first days after fertilisation, they climb again after

activation of the embryonic genome (Wu *et al.* 1992; Hou and Gorski 1993; Hou *et al.* 1996).

Horse conceptuses therefore express mRNA for reproductive steroid receptors during a period when the appropriate ligands are abundantly present in the uterine environment, and when a number of critical developmental events take place. These events include hatching from the zona pellucida, capsule formation and blastulation (Betteridge 2000), followed by rapid expansion (from 300 μm on day 7 to 20mm on day 14: Betteridge 2000), a dramatic increase in cell number (from 1,500 on day 7 to >40,000 on day 10: Tremoleda et al. 2003) and critical cell differentiation events including gastrulation (Enders *et al.* 1993) and subsequent neurulation, angiogenesis and organogenesis (Betteridge 2000). In addition, between days 8-10 (Stout et al. 1999) and days 14-16 (Hershman and Douglas 1979) the conceptus must signal its presence to its dam to ensure the prolongation of the primary corpus luteum in a process known as maternal recognition of pregnancy.

If progesterone does have direct actions on conceptus development, the current study suggests that this is most likely to be mediated via the membrane-associated, non-genomic receptors PGRMC1 and mPR. And while little is known about the second messenger pathways triggered via PGRMC1 (but see Falkenstein et al. 1999), mPR is a G-protein coupled receptor binding of progesterone to which leads to an inhibition of adenylyl cyclase and decrease in intracellular cAMP which activates cyclin B and indirectly promotes MAP Kinase signalling (Maller 2003). In this respect, cyclin B is a known regulator of mitosis (Pines 2006) and the MAPK pathways are involved in several embryonic processes including mesodermal differentiation, angiogenesis and cardiovascular development (Kuida and Boucher 2004).

In the case of oestrogens, their ability to potently modulate fetal development *in utero* has been demonstrated tragically by the teratogenic effects of the synthetic oestrogen, diethylstilboestrol (DES), in the offspring of women and animals treated during early pregnancy (for review see Marselos and Tomatis 1992a; Marselos and Tomatis 1992b). More recent studies have indicated that oestrogens can directly influence developmental processes such as embryonic neural stem cell proliferation and differentiation (Brannvall *et al.* 2002), and embryonic brain gene expression (Beyer *et al.* 2003), although it is not clear how these oestrogenic effects are mediated.

To our knowledge, this is the first study to report the expression of mPR by mammalian conceptuses, or the expression of progesterone or oestrogen receptors by equine conceptuses. These findings raise the possibility that reproductive steroids may exert their effects on pre-implantation equine conceptus development directly, and not only via the endometrium.

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Chapter 6

Effects of exogenous insulin on luteolysis and reproductive cyclicity in the mare

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ABSTRACT

Insulin is a pancreatic hormone that classically regulates carbohydrate and fat metabolism, but also appears to play a role in various reproductive processes. A preliminary study suggested insulin production by day 10-18 equine conceptuses. The aim of the present study was to examine the hypothesis that insulin is the conceptus signal responsible for maternal recognition of pregnancy (MRP) in the mare, or otherwise influences reproductive cyclicity during the MRP period. Six Warmblood mares were treated daily during days 7–17 after ovulation of two successive oestrous cycles with either (short and intermediate acting) insulin or control saline. Mares were assigned randomly to treatment, and crossed over during the subsequent cycle. Time of ovulation and corpus luteum surface area were determined by serial transrectal ultrasonographic examination of the mares' ovaries, and daily jugular vein blood samples were analysed for progesterone and luteinising hormone (LH) concentrations. On day 14 of dioestrus, the luteolytic drive was examined by measuring systemic 15-ketodihydroprostaglandin $F_{2\alpha}$ (PG-metabolite) release in response to oxytocin challenge. In addition, yolk sac fluid recovered from 32 day 10-14 equine conceptuses was analysed for insulin concentrations. Insulin administration did not affect luteal size, dioestrus length, the interovulatory interval, or circulating LH concentrations. Insulin administration also failed to suppress oxytocin-induced $PGF_{2\alpha}$ release, and tended to depress systemic progesterone concentrations. Finally, insulin could not be detected in the yolk sac fluid of day 10-14 equine conceptuses by radio-immunoassay. It is concluded that insulin administered daily during days 7-17 of dioestrus has little or no effect on reproductive cyclicity in the mare, and is unlikely to be the MRP signal.

INTRODUCTION

Insulin is a 6 kDa polypeptide hormone secreted by the pancreatic islets of Langerhans, and made up of a 2.5 kDa α-chain and a 3.5 kDa β-chain. Classically, insulin is the primary regulator of carbohydrate and fat metabolism, and its role in these activities is mediated by insulin and insulin growth factor 1 (IGF-1) receptors and glucose transporters (GLUTs) in liver, adipose and muscle tissue (Poretsky and Kalin 1987; Kaye 1997). It has become increasingly clear that insulin also plays important roles in many reproductive processes, at both the ovarian and uterine levels. In women suffering from insulin-dependent diabetes mellitus, for example, the low insulin levels appear to predispose to ovarian hypofunction characterised by low circulating oestrogen and progesterone concentrations and associated primary amenorrhea, late menarche, anovulation, low pregnancy rate, and early menopause; symptoms that can largely be overcome by exogenous insulin administration (for review see Poretsky and Kalin 1987). In farm animal species, both insulin administration and dietary-induced differences in endogenous circulating insulin concentrations affect numerous reproductive parameters including follicle growth, ovulation rate, activation of oocyte maturation (germinal vesicle breakdown), luteal function and secretion of reproductive steroids and LH (Diamond et al. 1989a; Clarke et al. 1990; Cox 1997; Downing et al. 1999; Daniel et al. 2000; Mao et al. 2001; Armstrong et al. 2002; Gong et al. 2002; Mihm and Bleach 2003; Hunter et al. 2004; Schams and Berisha 2004; Bhushan et al. 2005). Conversely, reproductive steroid hormones, in particular progesterone, modulate sensitivity to insulin, as illustrated by increased insulin resistance in women (Buchanan and Xiang 2005) and mares (Barnes et al. 1979; Fowden et al. 1980; Fowden et al. 1984) during mid and late pregnancy, in women during the luteal phase of the menstrual cycle (Diamond et al. 1989b), and in both men and women treated with progesterone (Kalkhoff et al. 1970; Kahn et al. 2003).

Insulin also appears to play at least a facilitative role in early embryonic development. In this respect, insulin has been reported to enter the uterine lumen in primates and mice (Heyner et al. 1989; Chi et al. 2000). Since it has also been shown to bind to embryonic insulin (Heyner et al. 1989) and/or IGF-1 (Schultz et al. 1992) receptors and be internalized (Heyner et al. 1989), maternal insulin can presumably affect the embryo directly. Indeed, insulin supplementation *in vitro* enhances embryonic uptake of glucose, amino acids and proteins, stimulates RNA and protein synthesis (Harvey and Kaye 1988; Lewis et al. 1992; Dunglison and Kaye 1993), prevents apoptosis (Herrler et al. 1998; Augustin et al. 2003), increases embryonic cell number and promotes blastocyst formation (Harvey and Kaye 1992; Matsui et al. 1995; Herrler et al. 1998; Augustin et al. 2003). *In vivo*, insulin supplementation has been reported to improve embryonic development and pregnancy rates in diabetic animals (Diamond et al. 1989a; Moley et al. 1991; De Hertogh et al. 1992).

Recently, Stout et al. (2004) proposed insulin as a candidate maternal recognition of pregnancy (MRP) signal in the mare. The horse is one of the few large domestic animal species in which the MRP signal, i.e. the conceptus factor responsible for prolonging the lifespan of the primary corpus luteum (CL), has yet to be identified. It is currently accepted that conceptus signalling for MRP in the mare must be completed before days 14-16 after ovulation (Hershman and Douglas 1979; Sharp et al. 1989b) and depends principally on an

anti-luteolytic (absolute reduction in uterine $PGF_{2\alpha}$ secretion: Stout and Allen 2002) rather than a luteoprotective or a luteotropic action (Watson and Sertich 1989). Previous studies have, for various reasons, ruled out other postulated equine MRP signals such as interferons (Sharp et al. 1989a; McDowell et al. 1990; Baker et al. 1991), oestrogens (Woodley et al. 1979; Goff et al. 1993; Vanderwall et al. 1994) and PGE_2 (Vanderwall et al. 1994; Ababneh et al. 2000). On the other hand, experiments involving fractionation of conceptus products have suggested that the equine MRP signal has a molecular weight between 3 and 10 kDa (Sharp et al. 1989b; Ababneh et al. 2000). In a preliminary study, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and n-terminal amino acid sequencing identified the β-chain (3.5 kDa) of insulin (6 kDA) in culture medium used to incubate day 10-18 equine conceptuses (Stout, Stewart and Allen, unpublished results). The aim of the current study was to determine whether insulin administration throughout the MRP period would suppress luteolysis or otherwise affect reproductive cyclicity in mares. Insulin was administered to cycling mares on days 7-17 after ovulation and the effects on cycle length, corpus luteum size, circulating progesterone and LH concentrations, and the ability of the endometrium to release $PGF_{2\alpha}$ in response to oxytocin challenge were examined. In addition, insulin concentrations in yolk sac fluid recovered from day 10, 12 and 14 equine conceptuses were examined by radioimmunoassay (RIA).

MATERIALS AND METHODS

Animals and insulin treatment

The study was performed using six normally cycling Dutch Warmblood mares ranging from 4-10 years of age, weighing 555-720 kg and in moderate-good body condition; experimental procedures were approved by Utrecht University's Ethics Committee. Throughout the experimental period, the mares were stabled individually and provided with haylage and water *ad libitum*; they were not exercised. Mares were assigned randomly to treatment with either insulin or 0.9% sodium chloride solution (control); during the subsequent oestrous cycle, mares were crossed over to the alternative treatment, and thus served as their own controls. The day of ovulation (day 0) was determined by daily transrectal ultrasonographic examination of a mare's ovaries. Insulin or saline administration was performed during days 7 to 17 after ovulation, the period when an equine conceptus would be mobile within the uterine lumen, beginning with uterine entry on day 6-7 (Battut et al. 1997) and ending with fixation on day 16-17 (Ginther 1983), and when MRP must be accomplished (Sharp et al. 1989b; Stout et al. 1999). Treatment took the form of 0.01 IU/kg BW intravenous short acting insulin (Actrapid; Novo Nordisk A/S, Bagsværd, Denmark) and 0.2 IU/kg BW intramuscular intermediate-acting insulin (Caninsulin; Intervet International BV, Boxmeer, the Netherlands), or equivalent volumes of 0.9% sodium chloride solution, administered daily at 9 am. The combination of the two types of insulin was chosen to produce a (near) continuous hyperinsulinaemia throughout the treatment period. Although the pharmacokinetics of insulin preparations have not been reported in the horse, the short acting intravenous Actrapid would be anticipated to induce a

rapid increase in plasma insulin concentrations of short duration (4-6 hours in cats; Martin and Rand 2001), while the intermediate acting 'lente' insulin, Caninsulin, would be absorbed more slowly and reach a later peak concentration (4 h and 11 h after injection in dogs; Graham et al. 1997: 4 h post-injection in cats; Martin and Rand 2001) that would be maintained for much longer (14-24 h in dogs; Graham et al. 1997: 12 h in cats; Martin and Rand 2001).
As a part of a concurrent study on the effects of reproductive cycle stage on peripheral insulin sensitivity, mares were subjected to a euglycemic-hyperinsulinemic clamp test (Rijnen and van der Kolk 2003) at 9 am once during oestrus (1-2 days before ovulation) and once during dioestrus (day 15 after ovulation) of each cycle. The day before the clamp test the insulin-treated mares received only the short-acting insulin, and all mares were fasted overnight. On the day of the clamp, the mares received only the short acting insulin used for the test itself (Rijnen and van der Kolk 2003).
Throughout the insulin administration period, clinical hypoglycaemia was avoided by restricting exercise and feeding extra concentrates at 7 am and 3 pm. Jugular vein blood samples were recovered at random intervals to verify that blood glucose levels remained within the physiological range (3.9 – 5.6 mmol/L).

Cycle length and corpus luteum size

To determine when they returned to behavioural oestrus, mares were teased on alternate days with a vigorous stallion, beginning on day 12 after ovulation. During oestrus, mares' reproductive tracts were examined daily by transrectal palpation and ultrasonography to determine when ovulation occurred; cycle length was calculated as the number of days between the first ovulation of two successive oestrous periods. Starting on day 4 after ovulation, the maximum cross-sectional area of each corpus luteum (CL) was measured ultrasonographically by a single operator every other day until CL regression.

Progesterone and LH concentrations

Throughout the study, jugular vein blood samples were collected daily into lithium heparinised vacutainer tubes (Vacuette®; Greiner Bio-one B.V., Alphen a/d Rijn, the Netherlands) and immediately centrifuged at 1000 G for 10 min. The plasma was stored at −20 °C until hormone concentrations were assayed. Plasma concentrations of progesterone were measured using a solid-phase I^{125} RIA (Coat-A-Count® TKPG; Diagnostic Products Corporation, Los Angeles, CA, USA) developed for cattle (Dieleman and Bevers 1987) and previously validated for horses (Pycock et al. 1995). The sensitivity of the test was 0.05 ng/ml, and intra- and interassay coefficients of variation were 11 and 14%, respectively. Plasma LH concentrations were determined using a heterologous RIA developed for cattle (Dieleman et al. 1983) and previously validated for the horse (Parlevliet et al. 2001). The LH assay had a sensitivity of 0.2 ng/ml, and intra- and interassay coefficients of variation were 2.3 and 10.5%, respectively.

Oxytocin challenge

On day 14 after ovulation, the luteolytic pathway was tested by measuring the $PGF_{2\alpha}$ release after oxytocin challenge (Starbuck et al. 1998). Jugular vein blood samples were collected into lithium heparinised vacutainer tubes at 15 min intervals, from 30 min before until 60 min after intravenous administration of 20 IU oxytocin (Oxytocin-S; Intervet International BV, Boxmeer, the Netherlands). Immediately after collection, blood samples were centrifuged at 1000 G for 10 minutes and the resulting plasma was immediately frozen at -20˚C. Subsequently, concentrations of 15-ketodihydroprostaglandin $F_{2\alpha}$ (PG-metabolite), the major circulating metabolite of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), was measured using a RIA developed for cattle (Kindahl et al. 1976) and previously validated for the horse (Kindahl et al. 1982). The sensitivity of the assay was 75 pmol/L, and the intra- and interassay coefficients of variation were 8.5 and 14%, respectively.

Yolk sac fluid insulin concentrations

Thirty-two conceptuses were recovered from Dutch Warmblood mares 10 (n=20), 12 (n=6) and 14 (n=6) days after ovulation detected by daily ultrasonographic examination of the ovaries. During the preceding oestrus, the mares had been inseminated every second day with at least $300x10^6$ progressively motile sperm from a fertile stallion. Conceptuses were collected by non-surgical uterine lavage using Ringer's solution introduced and recovered via a standard cuffed uterine flushing catheter with an internal diameter of 8 mm (Bivona, Gary, Indiana, USA; day 10 embryos) or a 24 mm endotracheal tube, in a modification of the technique described by Stout and Allen (2002). Immediately after recovery, conceptuses were washed 3-5 times with sterile Ringer's solution, which was removed before a syringe and 28 gauge needle were used to puncture the capsule and trophoblast and aspirate the yolk sac fluid. Yolk sac fluid samples were stored at -20ºC until analysed for insulin concentrations using a RIA (Coat-A-Count® Insulin TKIN2 836; Diagnostic Products Corporation, Los Angeles, USA) previously validated for insulin in equine plasma (van der Kolk et al. 1995). The assay had a sensitivity of 1.2 µU/ml and intra- and interassay coefficients of variation of 5 and 7%, respectively.

Statistical analysis

Differences in oestrous cycle length and dioestrus length (measured both as the time at which circulating progesterone concentrations fell below 1 ng/ml and when mares returned to behavioural oestrus) were analysed using a generalized linear model (R Foundation for Statistical Computing, Vienna, Austria, 2005; http://www.R-project.org) assuming a poisson distribution with random mare effects; treatment (insulin versus control), treatment order (control–insulin versus insulin–control) and their interaction were treated as fixed factors. Other statistical analyses were performed using SPSS 12.0.1 for Windows (SPSS Inc., Chicago, Illinois, USA). Analysis of variance (ANOVA) was used to determine whether insulin treatment affected corpus luteum surface area or concentrations of PG-metabolite, LH and progesterone; in all cases, hormone concentrations were subjected to

natural logarithmic transformation prior to analysis. Again, the analyses assumed random mare effects and considered treatment, treatment order and their interactions as fixed factors. For each parameter, normality of distribution was verified by examining plots of actual versus estimated residuals (qq-plot). Differences were considered statistically significant if $P<0.05$.

RESULTS

Cycle length, corpus luteum size, progesterone and LH concentrations

Daily insulin administration during days 7-17 did not delay the return to behavioural oestrus (mean ± s.d.: 17.3 ± 2.3 versus 16.8 ± 1.0 days in treated and control cycles, respectively), nor did it affect oestrous cycle length (21.7 ± 1.8 versus 21.7 ± 2.4 days) or total CL cross-sectional area (Fig. 1).

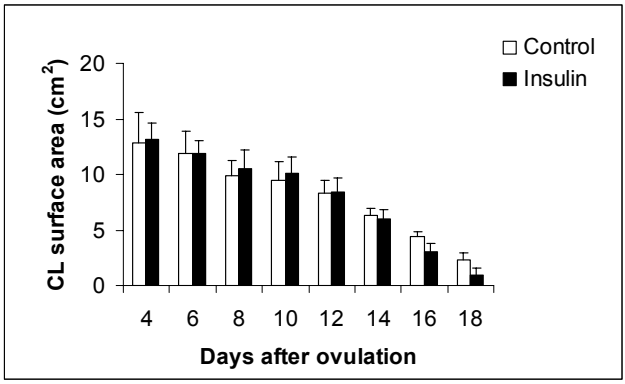

FIG. 1. Mean (± s.e.m.) total corpus luteum cross-sectional surface area (cm^2) in mares treated daily with insulin or saline (control) during days 7-17 after ovulation.

However, plasma progesterone concentrations tended to be lower in insulin-treated than in untreated cycles ($P=0.07$) during the period of insulin treatment, largely because of an apparently earlier onset but more gradual luteolysis in treated cycles (5 days to reach 1ng/ml progesterone versus 3 days for control cycles: Fig. 2).

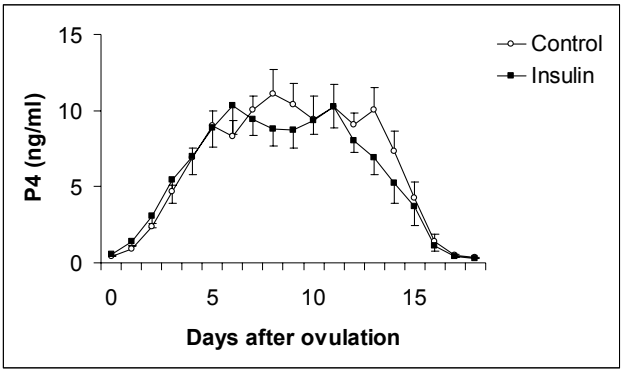

FIG. 2. Mean (± s.e.m.) plasma progesterone (P4) concentrations (ng/ml) in mares treated daily with insulin or saline (control) during days 7-17 after ovulation. During the treatment period, progesterone concentrations tended to be lower in insulin-treated mares (P=0.07)

However, the average interval from ovulation to completion of physiological luteolysis (i.e. when plasma progesterone concentrations dropped below 1.0 ng/ml) did not differ between control and treated cycles (16.5 ± 0.8 days and 16.2 ± 1.0 days, respectively). While there was also no effect of insulin administration on mean systemic LH concentrations (Fig. 3; P=0.44), treatment order did have a statistically significant effect (higher LH in mares treated with insulin in the first cycle: P=0.02). Subsequent analysis of individual LH profiles suggested that the treatment order effect was due to a single mare with unusually high LH concentrations at all time points; removal of this mare from the analysis ablated the treatment order effect (P=0.74).

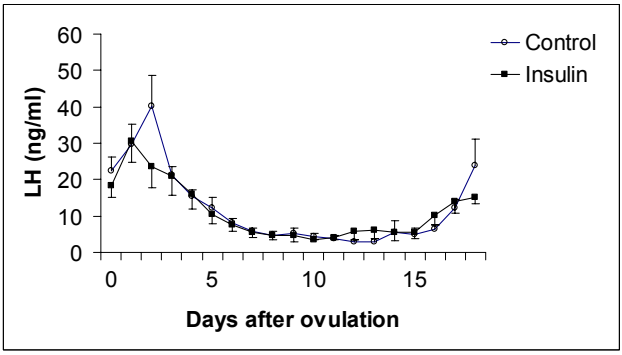

FIG. 3. Mean (± s.e.m.) plasma luteinising hormone (LH) concentrations (ng/ml) in mares treated daily with insulin or saline (control) during days 7-17 after ovulation.

Oxytocin challenge

In both control and insulin-treated cycles, the intravenous administration of 20 IU oxytocin (t=0) resulted in a rapid rise in PG-metabolite concentrations that peaked within 15 minutes (>1000 pmol/L) and returned to baseline over the following 45 minutes (Fig. 4). Neither peak nor total (area under the curve) $PGF_{2\alpha}$ release differed significantly between insulin-treated and control cycles.

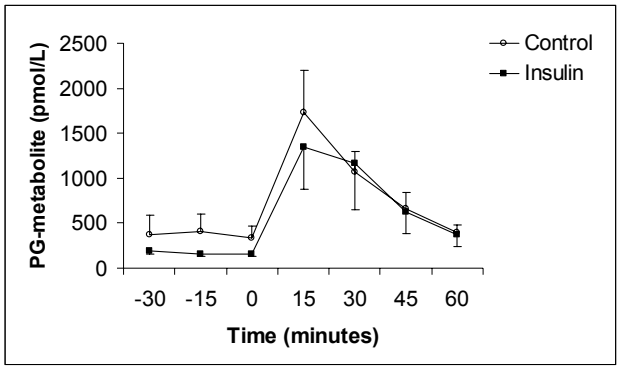

FIG. 4. Mean (± s.e.m.) plasma 15-ketodihydroprostaglandin $F_{2\alpha}$ (PG-metabolite) concentrations (pmol/L) before and after intravenous administration of 20 IU oxytocin on day 14 after ovulation in mares treated daily with insulin or saline (control) during days 7-17 after ovulation.

Yolk sac fluid insulin concentrations

To ensure sufficient volumes for assay, yolk sac fluid samples from day 10 conceptuses were pooled in 4 groups of 5; samples from day 12 (n=6) and day 14 (n=6) conceptuses were analysed individually. Measurable quantities of insulin were not detected in yolk sac fluid, even though insulin was readily detected in blood plasma samples from horses in another experiment analysed in the same assay, i.e. the assay was capable of detecting equine insulin.

DISCUSSION

The results of this study suggest that insulin is very unlikely to be the conceptus derived MRP factor in the mare. Firstly, insulin could not be detected by RIA in the yolk sac fluid of day 10-14 equine conceptuses, whereas one would expect the MRP signal to be produced in large amounts if it is to reliably ensure suppression of luteolysis. Other hormones produced in large quantities by horse conceptuses, such as oestrogens (Zavy et al. 1979; Marsan et al. 1987; Walters et al. 2001), are present in high concentrations in yolk sac fluid (Flood et al. 1979; Raeside et al. 2004). The second reason that insulin is unlikely to be the

equine MRP signal is the fact that administering insulin to cycling mares during days 7 to 17 after ovulation, in a manner aimed at inducing a continuous elevation in plasma insulin concentrations, did not delay or prevent luteolysis or suppress the endometrium's ability to release $PGF_{2\alpha}$ (measured as PG-metabolite) in response to oxytocin challenge. In pregnant mares during the MRP period, the $PGF_{2\alpha}$ response to oxytocin challenge is almost completely abolished (Goff et al. 1987; Starbuck et al. 1998). The failure of chronic insulin administration to prolong CL lifespan was manifested as an inability to delay any of: the return to behavioural oestrus, the rate of CL regression (as measured ultrasonographically), or the fall in systemic progesterone concentrations.

The failure to detect insulin in yolk sac fluid recovered from day 10-14 equine conceptuses need not be surprising. Although insulin production by the yolk sac of late gestation rat fetuses has been described (Muglia and Locker 1984), conceptus insulin was reported to be undetectable at earlier stages of pregnancy in species including the rat, mouse, man, sheep and cow (for reviews see Kane et al. 1997; Kaye 1997). On the other hand, the rationale for the current study was the presumptive detection using SDS PAGE and n-terminal amino acid sequencing of the β-chain of insulin in culture medium conditioned by day 10-18 equine conceptuses (Stout, Stewart and Allen; unpublished data). Although the reason for the discrepancy is not clear, it is unlikely to relate to assay sensitivity since amino acid sequencing requires a relatively large amount of protein, whereas the RIA used can detect concentrations as low as 1.2 μU/ml. It is more likely that the preliminary study either detected contaminating insulin of maternal origin (maternal insulin has previously been detected in the reproductive tract of female mammals: Heyner et al. 1989; Chi et al. 2000) or, more probably, that the molecule detected by n-terminal amino acid sequencing was in fact another member of the IGF family. IGF-1 in particular is structurally similar to the insulin β-chain (Zapf et al. 1984; Poretsky and Kalin 1987). Moreover, IGF-1 is produced by both the equine conceptus and endometrium, and is present in both the yolk sac fluid and uterine flushings recovered from mares during early pregnancy (Walters et al. 2001).

Insulin did not have the anticipated antiluteolytic or luteoprotective effects. On the contrary, circulating progesterone concentrations tended to be lower in insulin-treated cycles. Specifically, circulating progesterone levels appeared to start falling earlier in insulin-treated cycles, although the return to endocrinological oestrus (progesterone <1ng/ml) was not accelerated. The absence of any marked effect of chronic insulin administration on CL function mirrors Sessions et al.'s (2004) finding that manipulating endogenous insulin concentrations by intravenous lipid administration did not affect circulating progesterone levels in mares. By contrast, *in vitro*, insulin enhances progesterone production by both ruminant follicular and luteal granulosa cells (Spicer et al. 1993; Wathes et al. 1995; Bhushan et al. 2005) and porcine luteal tissue (Mao et al. 2001). Even in these latter species, however, the *in vivo* effects of insulin on progesterone production are difficult to decipher since they critically depend on the nutritional and reproductive status of the animals involved (Matamoros et al. 1990; Matamoros et al. 1991; Cox 1997; Almeida et al. 2001).

The failure of insulin to affect systemic LH concentrations in the current study agreed with previous studies in which neither a single treatment with 0.1 or 0.2 IU insulin/kg body weight (Deichsel et al. 2005) nor increased endogenous insulin concentrations as a result of

intravenous lipid infusion (Sessions et al. 2004) affected LH concentrations in cycling mares. In ewes, insulin treatment has been associated with both increased (Daniel et al. 2000) and decreased LH concentrations (Clarke et al. 1990), with the direction of the effect apparently dependent on blood glucose levels (Clarke et al. 1990; Arias et al. 1992) and dietary circumstances (Daniel et al. 2000).

In conclusion, the results of this study do not support the hypothesis that insulin is the 3-10 kDa conceptus signal responsible for maternal recognition of pregnancy in the mare. Furthermore, and in contrast to some other species, chronic systemic insulin administration appears to have no obvious effect on reproductive hormone secretion and cyclicity in mares in reasonably good body condition.

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Chapter 7

Summarising discussion

INTRODUCTION

During the last 30 years, equine reproductive management has been revolutionised by the introduction and/or widespread exploitation of novel diagnostic, therapeutic or assisted reproductive techniques such as ultrasonography, artificial insemination, embryo transfer (ET), cryopreservation of semen and embryos, and the *in vitro* production of embryos via intra-cytoplasmic sperm injection (ICSI) or cloning (Loomis 2001; Squires et al. 2003; Allen 2005; Stout 2006). As a result, equine reproductive performance has improved significantly (Whitwell 1980; Giles et al. 1993; Hong et al. 1993; Morris and Allen 2002; Smith et al. 2003), genetic improvement has accelerated, the genetic material of valuable stallions and mares has become available to breeders all over the world, and many important causes of subfertility have become amenable to treatment (Troedsson 1997; Hurtgen 2006).

Despite these improvements in overall reproductive success, early pregnancy loss remains a poorly understood and significant cause of economic loss to the equine breeding industry (Morris and Allen 2002; Allen et al. 2007). Known predispositions to reduced embryonic survival include advancing **maternal age** (Morris and Allen 2002; Allen et al. 2007) and ***in vitro* embryo production** (IVP: Hinrichs 2005; Galli et al. 2007). Intriguingly, the majority of equine pregnancy losses occur within the first 5 weeks of pregnancy, a period characterised by intense **fetal-maternal interaction** (Allen 2001), including the maternal recognition of pregnancy (MRP), the process by which a conceptus signals its presence to its dam to prolong the lifespan of the primary corpus luteum (Short 1969; Allen 2001). This thesis examined several factors thought to influence embryonic quality and survival during early gestation in the mare, and the results of the various studies will now be summarised and discussed with respect to the principal predisposing factors to early pregnancy loss and reduced fertility.

MATERNAL AGE

On average, mare fertility begins to decline beyond the age of 14-15 years. In this respect, aged mares have been reported to suffer a higher rate of embryo loss during both the first week of gestation (Ball et al. 1986; Ball et al. 1989), such that the day 7 embryo recovery rate in commercial ET programs is significantly lower for aged than for younger mares (Vogelsang and Vogelsang 1989), and thereafter, resulting in both a lower per cycle pregnancy rate and a higher incidence of day 15-35 pregnancy loss (Morris and Allen 2002; Allen et al. 2007). The underlying causes of the age-related increase in embryonic death are likely to include impairment of the oviductal and/or uterine environments, and factors intrinsic to the oocyte and embryo. Indeed, Carnevale and Ginther's (1995) finding that transferring oocytes to the oviducts of young recipients results in lower pregnancy rates if the oocytes are recovered from aged as compared to young mares, suggests that the deleterious effects of mare age on fertility are more likely due to intrinsic defects of the oocyte (and resulting embryo) than a deficient oviductal and/or uterine environment. A

similar conclusion has been reached for women because donation of oocytes from younger women overcomes the majority of age-related fertility problems (Templeton et al. 1996; Sauer 1997).

Mitochondrial quality and quantity in equine oocytes

A reduction in the quality or quantity of oocyte mitochondria has been proposed to contribute to the age-related decline in female fertility (Nagley and Wei 1998; Tilly 2001). Mitochondria are maternally-inherited cell organelles (Hutchison et al. 1974; Giles et al. 1980; Kaneda et al. 1995; Sutovsky et al. 1999) that generate energy, in the form of ATP, via the OXPHOS pathway. Concurrently, however, mitochondrial activity liberates reactive oxygen species (ROS) with the capacity to damage cellular lipids, proteins and DNA (Lee and Wei 2001). Over time, ROS-induced damage may accumulate and thereby contribute to an overall decline in oocyte quality. Indeed, De Bruijn *et al.* (2004) have reported significantly higher levels of damage in the mitochondrial membranes of resting phase oocytes in the ovaries of aged compared to younger women. These authors therefore postulated that, when an oocyte reactivates during maturation within a pre-ovulatory follicle and its energy demands increase, the damaged mitochondria may release increased quantities of ROS and thereby inflict further damage. In this respect, mitochondrial DNA (mtDNA) is thought to be particularly susceptible to ROS inflicted damage because of its close proximity to the ROS production site (mitochondrial membranes) and because, in contrast to nuclear DNA, it lacks protective histones and DNA repair mechanisms (Wallace et al. 1987). Moreover, mtDNA mutations are known to accumulate with age in slowly or non-dividing, post-mitotic tissues with high energy demands, such as brain and muscle (Cortopassi and Arnheim 1990; Shigenaga et al. 1994; Jazin et al. 1996; Nagley and Wei 1998). Since oocytes are also essentially (temporarily) non-dividing post-mitotic cells, it is tempting to speculate that a similar mechanism may underlie declining oocyte quality in aged females. However, previous studies have produced equivocal results, with no clear evidence of an age-related increase in the incidence of oocyte mtDNA mutations and deletions (Chen et al. 1995; Keefe et al. 1995; Muller-Hocker et al. 1996; Blok et al. 1997; Brenner et al. 1998; Barritt et al. 1999; Barritt et al. 2000) or an age-related decrease in oocyte mtDNA number (Steuerwald et al. 2000; Reynier et al. 2001; Barritt et al. 2002). On the other hand, most of the early studies examined relatively small numbers of oocytes, and were biased because the oocytes available were those considered unsuitable for IVF following recovery from women attending fertility clinics. In chapter 2, we circumvented many of these limitations by using an equine model. Moreover, using quantitative PCR we were able to demonstrate that mtDNA numbers decreased during maturation *in vitro* in oocytes from aged but not young mares. Transmission electron microscopy (TEM) confirmed that mitochondria in oocytes from aged, but not young, mares swelled and lost their internal architecture during maturation *in vitro*. On the other hand, because we did not find any evidence of age-related mtDNA mutations, we were forced to conclude that if accumulated ROS-induced damage to the mitochondrial membranes of oocytes from aged females is the trigger to mitochondrial swelling and destruction following GVBD, then the mtDNA is not the principle site of damage. While an oocyte could become atretic and/or

die as a result of extensive mitochondrial damage, it is possible that many survive but suffer a decrease in fertilisability or developmental competence. Indeed, oocytes with a reduced mtDNA number (Reynier et al. 2001; El Shourbagy et al. 2006), damaged mitochondria (Thouas et al. 2004; Thouas et al. 2005) or subnormal energy production capacity (Van Blerkom et al. 1995) are compromised, as witnessed by reduced embryo development rates and an increased incidence of pre-blastulation embryonic death. We conclude that increased susceptibility to mitochondrial damage is an important contributor to decreased developmental competence in oocytes from aged females. This no doubt in part explains why transfer of cytoplasm from the oocytes of young women has been reported to improve the developmental competence of oocytes from aged women (Cohen *et al.* 1998; Perez *et al.* 2000).

Mitochondrial quantity in early equine embryos

Mitochondrial number increases dramatically during oogenesis from tens in a primordial germ cell to hundreds of thousands (Jansen and de Boer 1998) or millions (chapter 2) in an antral follicle. However, shortly before fertilisation mitochondrial replication arrests and, at least in murine embryos, does not restart until the developing conceptus has embarked upon gastrulation (Piko and Taylor 1987; Ebert et al. 1988; Larsson et al. 1998; Thundathil et al. 2005). As a consequence, embryo survival depends on the fertilised oocyte from which that embryo was derived containing sufficient functional mitochondria to support development up to gastrulation, despite the subdivision of those mitochondria over a rapidly increasing number of blastomeres. A decrease in oocyte mitochondrial quantity and quality, as described in chapter 2, could thus hamper subsequent embryo development. This prompted us to examine the influences of maternal age on the quality of equine blastocysts in terms of their mtDNA content (chapter 3). Having confirmed histologically that gastrulation in the horse begins between 11.5 and 12.5 days after ovulation (Enders et al. 1993), we discovered that mitochondrial replication in equine embryos starts much earlier than in the mouse, at around the time of blastulation (day 7). However, on day 7.5 both IVP embryos and embryos from aged mares had mtDNA copy numbers more comparable to those in oocytes than those in 'normal' embryos form young mares. The relatively low mtDNA numbers could conceivably result from either a low initial mtDNA number (chapter 2) or a delay in the onset of mitochondrial replication and/or gene expression in general. Indeed, Hamatani *et al.* (2004b) have described a maternal age-related delay or decrease in mRNA expression for genes involved in transcription, cell cycle regulation, preimplantation development and mitochondrial function in mouse oocytes that presumably underlies delayed embryo development in aged rodents (Sopelak and Butcher 1982; Ishikawa and Yamauchi 2003; Hamatani et al. 2004a; Trejo et al. 2005). Similarly, we noted a tendency for day 7.5 embryos produced *in vitro* or recovered from aged mares to be developmentally retarded in comparison to embryos from young mares (chapter 3). And while a general retardation in development may explain the reduced embryonic mitochondrial content soon after the restart of mitochondrial replication, both would predispose to early pregnancy loss and probably contribute to the age-related decrease in developmental competence.

Chromosomal abnormalities

A potential consequence of oocyte mitochondrial dysfunction is aberrant **meiotic** spindle function, abnormal chromosome disjunction and an increased incidence of embryonic chromosomal abnormalities (Eichenlaub-Ritter et al. 2004). Indeed, experimentally induced mitochondrial damage has been shown to impair germinal vesicle break down, meiotic spindle formation and chromosome segregation in mouse embryos (Takeuchi et al. 2005). It is therefore tempting to speculate that the age-related decline in mitochondrial function underlies the increased incidence of meiotic spindle aberrations (Eichenlaub-Ritter 1989; Battaglia et al. 1996; Liu and Keefe 2002) and chromosomal abnormalities in both oocytes (Pellestor et al. 2003; Pellestor et al. 2005) and blastocysts (Munne et al. 1995) from aged females. A reduction in the number of mitochondria per embryonic cell may also predispose to aberrant chromosome segregation during **mitosis** (Eichenlaub-Ritter et al. 2004) resulting in 'mosaic' embryos containing normal diploid cell lines alongside aneuploid or polyploid lines; these latter embryos are also known as 'mixoploids'. Mixoploid embryos have been described in a number of mammalian species, including man (Munne et al. 1994; Bielanska et al. 2002), rabbit (Fechheimer and Beatty 1974), pig (McFeely 1967; Long and Williams 1982), sheep (Murray et al. 1986) and cow (Hare et al. 1980; Viuff et al. 1999). On the other hand, previous studies to examine the chromosomal constitution of equine gametes and embryos revealed only low incidences of chromosomal abnormalities in spermatogonia (Scott and Long 1980) and oocytes (King et al. 1990; Lechniak et al. 2002), and none in conceptuses (Blue 1981; Haynes and Reisner 1982; Romagnano et al. 1987). However, those studies employed conventional karyotypic analysis which can only be performed on the small proportion of embryonic cells that are in, or can be driven into, metaphase and which have clearly spread-out, non-overlapping chromosomes. For this reason, chromosomally abnormal cells are easily missed, especially if they are in relatively low abundance. In chapter 4, we validated and used fluorescent in situ hybridisation (FISH) probes for equine chromosomes 2 and 4. The use of FISH probes dramatically increases the proportion of nuclei per embryo analysable for chromosome number because non-dividing (interphase) cells can also be examined. Despite using only 2 randomly selected chromosomes, we discovered that nearly all embryos contain apparently aneuploid cells. On the other hand, relatively few *in vivo* generated embryos (4/22) were indisputably mixoploid; although in one, 65% of the cells were polyploid. To our knowledge, this is the first demonstration that equine embryos can contain chromosomally abnormal cells. Unfortunately, we were not able to examine sufficient embryos to determine whether increasing mare age is associated with a higher incidence of embryonic chromosomal abnormalities. In women, the links between advanced maternal age, an increased incidence of embryonic chromosomal abnormalities and a higher incidence of spontaneous abortion have been made (Hook et al. 1983; Menken et al. 1986; Nybo Andersen et al. 2000; Heffner 2004). Indeed, approximately half of all spontaneously aborted human fetuses is chromosomally abnormal, and the incidence of certain chromosomal abnormalities shows a clear maternal age effect (Hassold et al. 1980; Eiben et al. 1990). Studies to examine the incidence and type of chromosomal abnormalities in failing equine pregnancies have yet to be performed, and neither is it known if particular

chromosomes are more prone to age-related non-disjunction in the mare. Nevertheless, now that it is clear that chromosomal abnormalities occur in equine embryos, it would be worthwhile to examine the extent to which they contribute to pregnancy loss, how and when the abnormalities arise, and whether particular chromosomes are predisposed to non-disjunction.

IN VITRO EMBRYO PRODUCTION

A second factor associated with decreased embryonic developmental competence in the horse is *in vitro* production. Although foals have been produced by conventional *in vitro* fertilisation (Palmer et al. 1991; Bezard 1992) and somatic cell nuclear transfer (Galli et al. 2003; Woods et al. 2003), this section will focus on ICSI because it is the most common method of producing equine embryos *in vitro*. Techniques for *in vitro* oocyte maturation and zygote culture have improved appreciably over the last 10 years, such that a high percentage of oocytes can now be matured successfully *in vitro* (Choi et al. 2002; Tremoleda et al. 2003) and cleave following ICSI (Choi et al. 2002; Choi et al. 2004a). On the other hand, only a small proportion of ICSI zygotes successfully develop to the blastocyst stage during subsequent *in vitro* culture (Choi et al. 2004b; Choi et al. 2006). In addition, transcervical transfer of ICSI embryos to the uterus of recipient mares results in a lower likelihood of pregnancy (Hinrichs et al. 2005; Galli et al. 2007) than transfer of *in vivo* embryos (Squires et al. 2003) and, even then, ICSI pregnancies are more prone to early embryonic loss (Hinrichs et al. 2005; Galli et al. 2007). That *in vitro* maturation of oocytes from aged mares leads to a significant reduction in mitochondrial quality and quantity (chapter 2) and that day 7 ICSI embryos are developmentally retarded with a mtDNA content comparable to that of an oocyte rather than a 'normal' *in vivo* embryo (chapter 3) may well contribute to the apparent reduction in developmental competence. Moreover, when compared to *in vivo* embryos, ICSI embryos are more likely to contain chromosomally abnormal cells (8/20 versus 4/22; chapter 4), which form a greater proportion of the total, significantly lower cell population. While, as in women (Templeton et al. 1996), maternal age will probably continue to affect the outcome of equine IVP, it is equally evident that present oocyte and embryo culture systems are suboptimal and predispose to degenerative changes that reduce developmental competence (chapters 2, 3 and 4). Identifying the precise types and causes of degeneration will be an important part of improving culture techniques to optimize both blastocyst and foal production.

FETAL-MATERNAL INTERACTION

An adequate and properly timed communication between a conceptus and its dam is essential for pregnancy maintenance. This concept is illustrated clearly by the fact that, in an ET programme, the recipient mare's oestrous cycle must be synchronized with the developmental stage of the transferred embryo (Squires et al. 1982; McCue and Troedsson 2003; Stout 2003). Otherwise the conceptus may fail to adequately signal its presence to its

dam to ensure the prolongation of the primary CL, or it may be exposed to an asynchronous endometrial environment with 'out of phase' levels of growth factors, which may result in embryonic death or growth retardation (Barnes 2000). In chapters 3 and 4, we described how both day 7 ICSI embryos and *in vivo* embryos from aged mares are developmentally retarded. If severe, this developmental retardation may in itself be sufficient to cause a mismatch between endometrial and embryonic developmental stage and lead to early pregnancy loss; the 'small for dates' early equine conceptus is known to be at heightened risk of embryonic death (Chevalier and Palmer 1982).

Molecules likely to play an important role in fetal-maternal (mis)communication in the mare include the reproductive steroids, progesterone and oestrogens, and the, as yet uncharacterized, maternal recognition of pregnancy signal.

Reproductive steroids

Sufficient maternal circulating progesterone is a prerequisite for pregnancy maintenance in eutherian mammals, including the horse (Daels et al. 1991). Moreover, the dogma has long been that the stimulatory effects of progesterone, and indeed oestrogens, on conceptus development are indirect, and mediated primarily via the endometrium (Wu et al. 1971; Baulieu 1989; Clarke and Sutherland 1990; Singh et al. 1996). Until recently, it was also believed that steroid hormones acted exclusively by binding to intracellular receptors and that their biological effects were mediated via a relatively slow 'genomic pathway' that relied upon the modulation of gene transcription and translation into functional proteins (Jensen *et al.* 1968; Beato 1989; Tuohimaa *et al.* 1996). There is, however, increasing evidence that the reproductive steroids may also directly influence pre-implantation conceptus development. In particular, both progesterone (PR) and oestrogen receptor (ER) mRNA and protein have been detected in embryonic cells from several mammalian species (Hou and Gorski 1993; Hou *et al.* 1996; Ying *et al.* 2000; Kowalski *et al.* 2002; Hong *et al.* 2004; Hasegawa *et al.* 2005). Other recent studies have indicated that steroid hormones do not exclusively act via nuclear receptors. Indeed, two distinct membrane-bound progesterone receptors (PGRMC1, Losel et al. 2004; mPR, Zhu et al. 2003a; Zhu et al. 2003b) have been described which trigger rapid effects via non-genomic pathways, and the existence of comparable plasma membrane-associated oestrogen receptors has also been proposed (Nadal *et al.* 2000; Levin 2002; Toran-Allerand *et al.* 2002). In chapter 5, we used quantitative rtPCR to show that day 7-14 equine conceptuses express genes for both the classical nuclear (PR and ERβ, but not ERα) and the novel non-genomic (PGRMC1 and mPR) steroid receptors. Horse conceptuses therefore express mRNA for reproductive steroid receptors during a period of gestation when the appropriate ligands, progesterone and oestrogens, are present in the uterine lumen in abundance (Zavy et al. 1984; Marsan et al. 1987; Walters et al. 2001). This raises the possibility that the reproductive steroids may exert at least some of their effects on pre-implantation equine conceptus development directly, and that the conceptus may be able to respond both rapidly (via non-genomic pathways) or over a longer period (via genomic pathways). However, it is not yet clear

what, if any, direct effects the reproductive steroids have on conceptus development or how critical these pathways are to conceptus development and survival.

Maternal recognition of pregnancy

The single most important aspect of fetal-maternal communication during the first three weeks of pregnancy is the production and timely release of the signal for maternal recognition of pregnancy, i.e. the conceptus factor responsible for prolonging the lifespan of the primary corpus luteum (CL) beyond its normal 14-16 days. The horse is, however, the only commercially important large domestic animal species for which the MRP signal has yet to be identified. Previous studies have ruled our several postulated equine MRP signals such as interferons (Sharp et al. 1989a; McDowell et al. 1990; Baker et al. 1991), oestrogens (Woodley et al. 1979; Goff et al. 1993; Vanderwall et al. 1994) and PGE_2 (Vanderwall et al. 1994; Ababneh et al. 2000). And currently it is believed that conceptus signalling for MRP starts before day 10 after ovulation (Goff et al. 1987; Stout et al. 1999), must be completed by days 14-16 (Hershman and Douglas 1979; Sharp et al. 1989b), and that the MRP signal has a molecular weight of between 3 and 10 kDa (Sharp et al. 1989b; Ababneh et al. 2000). In chapter 6, we tested the hypothesis advanced by Stout *et al*. (2004) that conceptus insulin (a 6 kDa protein) might be the equine MRP signal. However, we were unable to detect insulin in the yolk sac fluid of day 10-14 conceptuses by radioimmunoassay, and since administering insulin to cycling mares throughout the MRP period (days 7-17 after ovulation) neither delayed or abolished luteolysis nor suppressed the endometrium's ability to release $PGF_{2\alpha}$, it is extremely unlikely that conceptus insulin is the 3-10 kDa conceptus signal responsible for MRP in the mare. Indeed, in contrast to other mammalian species (Clarke et al. 1990; Spicer et al. 1993; Wathes et al. 1995; Daniel et al. 2000; Mao et al. 2001; Bhushan et al. 2005), chronic parenteral insulin administration appeared to have no significant effects on reproductive hormone secretion in mares.

CONCLUSIONS

The principal conclusions of this thesis are as follows:

- The mitochondria of oocytes from aged (≥12 years old) mares exhibit a heightened susceptibility to damage, e.g. during maturation *in vitro* (chapter 2).
- Gastrulation in the horse conceptus begins between days 11.5 and 12.5 after ovulation (chapter 3).
- Mitochondrial replication in equine embryos restarts somewhere around blastulation (day 7), much earlier than the gastrulation stage onset described previously for rodent embryos (chapter 3).
- Day 7.5 embryos recovered from young mares have significantly more mtDNA copies than oocytes, whereas day 7.5 IVP embryos and embryos from aged mares do not; the latter 2 classes of embryos are also developmentally retarded (chapter 3).

- Chromosomally abnormal cells are a common finding in early equine embryos produced either *in vivo* or *in vitro* (chapter 4).
- In comparison to 'normal' *in vivo* embryos, ICSI embryos are developmentally retarded and contain fewer cells which are more likely to be chromosomally abnormal (chapter 4).
- During the second week of development, equine embryos express genes for both nuclear (PR and ERβ) and membrane-bound (PGRMC1 and mPR) reproductive steroid receptors. This suggests that the reproductive steroids may directly influence equine embryonic development (chapter 5).
- Insulin is almost certainly not the equine MRP signal (chapter 6).
- Chronic parenteral insulin administration has no significant effects on reproductive hormone secretion or cyclicity in the mare (chapter 6).

FUTURE PERSPECTIVES

Characterisation of ROS induced oocyte damage

In chapters 2 and 3 of this thesis, the effects of maternal age on the mitochondrial content of oocytes and embryos were examined. Advanced maternal age was associated with susceptibility to damage, swelling and loss of mitochondria during oocyte maturation *in vitro*. Although we postulated that this damage was inflicted by ROS, this hypothesis was not tested. To determine whether ROS are an important contributor to age-related oocyte mitochondrial degeneration, future studies could examine the timing and localisation of intracellular oxidative damage in maturing oocytes and early embryos from young and aged females, e.g. using probes for ROS (Squirrell *et al.* 1999; Diaz *et al.* 2003), lipid peroxidation (Drummen *et al.* 2004; Brouwers *et al.* 2005) and oxidised proteins (Suh *et al.* 2004; Judge *et al.* 2005). Once the occurrence, underlying causes and consequences of ROS-inflicted damage have been better characterised, it may be possible to design treatments to reduce or prevent oxidative damage and thereby improve the developmental competence of embryos derived from aged females or produced *in vitro*. Treatments that might be worth testing include antioxidants (Guerin *et al.* 2001) or low oxygen tensions during *in vitro* culture (Jansen and Burton 2004), or more invasive approaches like the transfer of cytoplasm from the oocytes of young females to the oocytes of the older females of interest (Cohen *et al.* 1998; Perez *et al.* 2000).

Further characterisation of chromosomal abnormalities

In chapter 4, we used just 2 chromosome specific FISH probes to study chromosomal abnormalities in horse embryos. Although this enabled the detection of polyploidy and/or aneuploidy for the two chromosomes examined, because the horse has 32 chromosome pairs it is likely that the observed incidence of aneuploid cells and/or embryos considerably underestimates the true number. In human pre-implantation genetic diagnosis (PGD), probes for up to 10 of the 23 chromosome pairs are employed using multi-colour FISH

(Baart et al. 2004). Moreover, the labelled chromosomes are not selected randomly but rather because aneuploidy thereof has been linked to spontaneous abortion and/or shown to increase with maternal age (Hassold et al. 1980; Eiben et al. 1990). In short, a panel of FISH probes for the chromosomes most likely to suffer non-disjunction is used to screen single blastomeres from human (*in vitro* produced) embryos for chromosomal abnormalities (Baart et al. 2004). To develop a comparable approach for equine reproductive medicine, FISH probes would need to be constructed and validated for each equine chromosome (Milenkovic et al. 2002) and studies performed to determine which are most prone to non-disjunction in equine conceptuses produced *in vitro* or *in vivo* and, in particular, in abnormally developing pregnancies. This approach should enable identification of the chromosomes most susceptible to non-disjunction in elderly mares and/or following IVP, and whether chromosomal aberrations are a common accompaniment to failed embryonic development / impending pregnancy loss. Such a screening panel for chromosomal abnormalities could, for example, be used to compare different methods of IVP or to screen and select embryos (PGD) before ET (especially if it can be combined with superovulation) in order to eliminate abnormal embryos and increase pregnancy rates for (aged) mares that had suffered from repeated pregnancy losses, in an approach comparable to that successfully practiced in human medicine (Sermon et al. 2004; Maroulis and Koutlaki 2006).

Novel techniques for gene expression studies

In chapters 5 and 6, we examined the expression of specific 'target' genes (reproductive steroid receptors and insulin, respectively) that we suspected to play a role in conceptus development. Although this is a valid approach, it is time-consuming and therefore limited to a relatively small number of known genes. To investigate the expression profiles of larger numbers of genes, or to identify additional genes likely to be involved in poorly understood processes, like MRP in the mare, future studies could usefully apply more powerful, high through-put techniques, such as micro-arrays (Seligson 2005), to identify those genes up- or down-regulated in the appropriate tissue at the appropriate time. Because equine micro-arrays have not yet been developed it may, in the short term, be most productive to perform pilot studies using micro-arrays developed for closely related species, like the pig (Affymetrix: Tsai et al. 2006; Tuggle et al. 2007); previous cross-mammalian species gene expression studies (porcine tissue on human micro-arrays) have proven to be informative (Ji et al. 2004). On the other hand, now that the first draft of the horse genome has been made available (Spencer and Davis 2007), it may not be long before equine specific micro-arrays are also available. Once a suitable array has been identified, cDNA from equine embryos of different developmental stages could be analysed for genes up-regulated during a specific period of development (e.g. the MRP period); a comparable approach has already been initiated to identify genes involved in early porcine embryonic development (Lee et al. 2005). Following the production of a list of 'candidate genes', quantitative rtPCR and immunohistochemistry could be used to verify the micro-array results and examine whether, and in which cells, the mRNA is translated into protein. Finally, the role of the candidate gene would need to be examined or proven using

functional studies, which might range from simply observing the effect of a given protein on a defined developmental event or signalling pathway, to inhibiting expression of a target gene either by gene silencing via RNA interference (RNAi: Janitz et al. 2006; Martin and Caplen 2007) or using a specific inhibitor of the signalling molecule/pathway in question. Certainly, the functional studies will be critical in defining the roles, significance and mechanisms of action of any genes up-regulated during early embryonic development. Nevertheless, using a step-wise approach, it should be possible to identify how, and to what extent, maternal age or *in vitro* culture influence gene expression in oocytes and embryos, to clarify the roles of the reproductive steroids in fetal-maternal interaction and, last but not least, to identify the MRP signal(s) in the horse. Identifying the genes involved and determining how they act should help in the design and testing of improved *in vitro* oocyte, zygote and embryo culture systems and in the development of diagnostic tests to predict, or treatments to prevent, pregnancy failure, particularly where the latter is threatened as a result of failing fetal-maternal communication or moderately impaired embryo developmental competence.

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Samenvatting

INLEIDING

Gedurende de laatste 30 jaar heeft de fertiliteitbegeleiding bij het paard stormachtige ontwikkelingen doorgemaakt door de introductie en/of het op grote schaal inzetten van nieuwe diagnostische en therapeutische voortplantingstechnieken zoals: de echografie, de kunstmatige inseminatie, de embryotransplantatie (ET), het succesvol invriezen, opslaan en transporteren van sperma en embryo's en de *in vitro* productie (IVP) van embryo's door middel van intra-cytoplasmatische sperma-injectie (ICSI) of door middel van kerntransplantatie ('klonen'). Hierdoor is de efficiëntie van de commerciële paardenfokkerij significant toegenomen, is de genetische vooruitgang binnen de paardenpopulatie versneld, is het genetische materiaal van waardevolle hengsten en merries verkrijgbaar geworden voor fokkers over de gehele wereld terwijl vele vormen van subfertiliteit bij de merrie inmiddels goed gediagnosticeerd en behandeld kunnen worden.

Ondanks deze vooruitgang binnen de veterinaire voortplantingsgeneeskunde is vroeg-embryonale sterfte (VES) nog altijd een belangrijke maar slecht begrepen oorzaak van grote economische verliezen binnen de paardenfokkerij. Er zijn meerdere predisponerende factoren voor VES bij de merrie bekend zoals een hoge **maternale leeftijd** en de ***in vitro* productie van embryo's**. Het merendeel van de VES treedt op gedurende de eerste 5 weken van de dracht. Gedurende deze periode vindt er een intensieve interactie plaats tussen de merrie en de conceptus (**foetale-maternale interactie**). De conceptus geeft een signaal af in de baarmoeder waardoor het moederdier de dracht 'herkent' zodat uiteindelijk de levensduur van het primaire gele lichaam, en dus de productie van progesteron, wordt verlengd. Dit proces wordt ook wel de 'maternale drachtherkenning' (MDH) genoemd. Dit proefschrift beschrijft een aantal onderzoeken gericht op factoren die van invloed kunnen zijn op de kwaliteit en de vitaliteit van paardenembryo's tijdens de eerste weken van de dracht. Hieronder zullen de resultaten van deze onderzoeken besproken worden aan de hand van de drie belangrijkste predisponerende factoren voor VES bij het paard: hoge maternale leeftijd, IVP en foetale-maternale (mis)communicatie.

MATERNALE LEEFTIJD

De vruchtbaarheid van de merrie begint vanaf een leeftijd van ongeveer 14-15 jaar duidelijk af te nemen. Zo treedt embryonale sterfte tijdens de eerste 2 weken van de dracht beduidend vaker op bij oudere merries dan bij jongere merries. In de praktijk uit zich dat bij oude merries door een lagere embryo-opbrengst in commerciële ET programma's en door een lager drachtpercentage per cyclus. Tevens hebben oudere merries een grotere kans op het verlies van een vrucht tussen de 15^{e} en de 35^{e} dag van de dracht. Deze leeftijdsgerelateerde toename in VES bij de merrie wordt waarschijnlijk veroorzaakt door een verminderde kwaliteit van de eileiders en de baarmoeder, enerzijds, en door intrinsieke factoren die leiden tot een verminderde ontwikkelingscompetentie van de eicel en/of het vroege embryo, anderzijds. Echter, wanneer eicellen van jonge en oude merries overgebracht worden naar de eileider van een jonge merrie wordt, na inseminatie van de

ontvangster en bevruchting van de eicellen, meer VES waargenomen wanneer de eicellen afkomstig waren van oudere merries dan wanneer ze gedoneerd waren door een jonge merrie. Dit suggereert dat de leeftijdsafhankelijke afname in de vrouwelijke fertiliteit eerder door intrinsieke factoren van de eicel (en het daaruit ontstane embryo) dan door een afwijkend milieu binnen de eileider en/of de baarmoeder veroorzaakt wordt; een vergelijkbaar fenomeen is bekend van humane eiceldonatieprogramma's.

De kwaliteit en hoeveelheid van mitochondriën in paardeneicellen

Een verminderde kwaliteit en/of hoeveelheid van mitochondriën in de eicel speelt waarschijnlijk een belangrijke rol in de leeftijdsafhankelijke afname van de fertiliteit van vrouwelijke zoogdieren. Mitochondriën zijn celorganellen die exclusief via de moederlijke lijn worden doorgegeven aan de nakomelingen. Zij zorgen voor de cellulaire energievoorziening in de vorm van ATP dat gevormd wordt via de oxidatieve fosforylering (OXPHOS). Echter, tegelijkertijd met de productie van ATP komen er ook reactieve zuurstofdeeltjes ('reactive oxygen species': ROS) vrij. Deze ROS zijn schadelijk voor intracellulaire lipiden en eiwitten en het DNA; in theorie zou deze schade in de loop der tijd kunnen accumuleren binnen de eicel en zo bijdragen aan de totale afname van de kwaliteit van die eicel. Inderdaad hebben morfologische studies van ongematureerde eicellen van oudere vrouwen aangetoond dat deze significant meer schade aan de mitochondriale membranen vertonen dan eicellen van jonge vrouwen. Wanneer deze beschadigde eicellen op een later tijdstip aan de laatste fase van de eicelrijping beginnen, zal de behoefte aan energie (ATP) stijgen en zullen er nog meer ROS geproduceerd worden die nog meer schade aan de mitochondriën kunnen toebrengen en zo kunnen leiden tot een negatieve spiraal. Met name het mitochondriale DNA (mtDNA) is erg gevoelig voor deze door ROS veroorzaakte schade omdat het mtDNA zich vlak bij de plaats bevindt waar de ROS gevormd worden. Daarnaast heeft mtDNA, in tegenstelling tot nucleair DNA, geen beschermende histonen en geen DNA-reparatiemechanismen waardoor er gemakkelijk mutaties in het mtDNA kunnen ontstaan. Van deze mtDNA mutaties is het bekend dat ze met name voorkomen in langzaam of niet-delende post-mitotische weefsels met een hoge energiebehoefte, zoals de hersenen en de dwarsgestreepte spieren, en dat hun incidentie inderdaad toeneemt met de leeftijd. Aangezien eicellen in feite ook (tijdelijk) niet-delende post-mitotische cellen zijn is het verleidelijk om aan te nemen dat een vergelijkbare stapeling van mtDNA mutaties ook optreedt in eicellen en zo bijdraagt aan de leeftijdsgerelateerde subfertiliteit van vrouwelijke zoogdieren. Echter, de vele studies die deze hypothese hebben onderzocht hebben tegenstrijdige resultaten opgeleverd. Het probleem van het merendeel van deze studies is dat ze een duidelijke bias vertonen: slechts een klein aantal eicellen kon worden onderzocht en dan alleen eicellen die ongeschikt werden geacht voor *in vitro* fertilisatie (IVF) en die bovendien afkomstig waren van vrouwen die in een fertiliteitkliniek werden behandeld. In hoofdstuk 2 van dit proefschrift hebben wij veel van deze methodologische problemen kunnen omzeilen door het paard te gebruiken als diermodel voor de mens. We hebben door middel van kwantitatieve PCR aangetoond dat in de eicellen van oudere merries het aantal mtDNA-kopieën afnam tijdens *in vitro* maturatie (IVM) terwijl een dergelijke afname niet werd waargenomen bij eicellen

die afkomstig waren van jonge merries. Tevens hebben we door middel van transmissie elektronenmicroscopie (TEM) aangetoond dat de mitochondriën in eicellen van oudere merries tijdens IVM opzwollen en daarbij hun interne structuur verloren terwijl een dergelijk fenomeen niet optrad tijdens IVM van eicellen van jonge merries. In tegenstelling tot de verwachting werden er in deze eicellen geen leeftijdsgerelateerde mtDNA mutaties aangetroffen. Blijkbaar zijn de mitochondriale membranen, en niet het mtDNA, de belangrijkste locatie van de door ROS veroorzaakte schade. Als gevolg van deze schade kunnen eicellen atretisch worden en ten gronde gaan. Echter, het is ook mogelijk dat de aangetaste eicellen weliswaar levensvatbaar blijven maar dat deze minder goed te bevruchten zijn of dat deze nog wel te bevruchten zijn maar na de bevruchting een abnormale embryonale ontwikkeling doormaken. Experimenten met eicellen en embryo's van verschillende zoogdiersoorten hebben inderdaad aangetoond dat een verminderd aantal mtDNA-kopieën, beschadigde mitochondriën of een verminderde energieproductie leiden tot een abnormale ontwikkeling of zelfs het afsterven van het embryo. Concluderend kan gezegd worden dat met het toenemen van de leeftijd de mitochondriën van eicellen meer beschadigd zijn en dat de accumulatie van deze schade waarschijnlijk een grote rol speelt bij de leeftijdsafhankelijke afname van de eicelkwaliteit en dus van de fertiliteit van oudere vrouwelijke individuen. Dit verklaart waarschijnlijk ook waarom het overbrengen van cytoplasma van eicellen van jonge vrouwen naar die van oudere vrouwen leidt tot een verbeterde ontwikkelingscompetentie van die oude eicellen.

De kwaliteit en hoeveelheid van mitochondriën in paardenembryo's

Tijdens de oögenese neemt het aantal mitochondriën sterk toe: primordiale eicellen hebben slechts enkele tientallen mitochondriën terwijl eicellen in de laatste maturatiestadia honderduizenden tot miljoenen (hoofdstuk 2) mitochondriën bevatten. In muizeneicellen stopt de toename in het aantal mitochondriën vlak voordat de bevruchting plaatsvindt. Pas nadat het embryo het gastrulastadium bereikt heeft, begint de vermenigvuldiging van mitochondriën weer. Dit houdt in dat een embryo gedurende de periode van bevruchting tot aan de gastrulatie voor zijn energievoorziening volledig afhankelijk is van het aantal mitochondriën dat tijdens de bevruchting in de eicel aanwezig is. Een afname in de kwaliteit en de hoeveelheid van deze mitochondriën, zoals beschreven in hoofdstuk 2, heeft daarom waarschijnlijk een negatieve invloed op de vroeg-embryonale otwikkeling. Dit was de reden om de invloed van de maternale leeftijd en IVP op het aantal mitochondriën in blastocysten te bestuderen (hoofdstuk 3). Hoewel aangetoond werd dat paardenembryo's pas rond de 12^{e} dag na ovulatie het gastrulastadium bereiken, begon de mitochondriale vermenigvuldiging in paardenembryo's in een eerder ontwikkelingsstadium dan bij muizenembryo's, namelijk rond de 7^{e} dag na de ovulatie wanneer het paardenembryo zich tot blastocyst ontwikkeld heeft. Echter, IVP embryo's en embryo's afkomstig van oudere merries hadden relatief veel lagere aantallen mitochondriën dan vergelijkbare embryo's die afkomstig waren van jonge merries. Deze relatief lage aantallen mitochondriën kunnen het gevolg zijn van een (te) laag aantal mitochondriën in de pre-ovulatoire eicel voorafgaand aan de bevruchting (zoals beschreven in hoofdstuk 2) maar ze kunnen ook veroorzaakt worden door het vertraagd op gang komen van de embryonale mitochondriale

vermenigvuldiging doordat het embryonale genoom te laat tot expressie wordt gebracht. Inderdaad is bij knaagdiereicellen aangetoond dat een hoge maternale leeftijd geassocieerd is met een afname van de expressie van genen die een rol spelen in de transcriptie van andere genen, de regulatie van de celcyclus, de vroeg-embryonale ontwikkeling en de functie van mitochondriën; deze afname in genexpressie ligt waarschijnlijk ten grondslag aan de vertraagde ontwikkeling van knaagdierembryo's afkomstig uit oudere moederdieren. In dit proefschrift vonden wij een vergelijkbare leeftijdsafhankelijke vertraging van de ontwikkeling van paardenembryo's (hoofdstuk 3). Afgezien van het feit dat het vooralsnog niet duidelijk is of de vertraagde embryonale ontwikkeling veroorzaakt wordt door het relatief lage aantal mitochondriën (of juist andersom), is het zeer waarschijnlijk dat beide fenomenen predisponeren voor VES en zo bijdragen aan de leeftijdsgerelateerde afname van de ontwikkelingscompetentie van paardenembryo's.

Chromosomale afwijkingen

Een mogelijk gevolg van een disfunctie van eicelmitochondriën is een verminderd functioneren van de spoelfiguur tijdens de meiose, een abnormale segregatie van de chromosomen en dus een verhoogde incidentie van chromosomale afwijkingen. Inderdaad is het zo dat experimenteel veroorzaakte schade aan eicelmitochondriën bij de muis leidt tot afwijkingen tijdens de 'germinal vesicle break down' (GVBD), de vorming van de meiotische spoelfiguur en de segregatie van de chromosomen. Het ligt daarom voor de hand om aan te nemen dat een leeftijdsafhankelijke afname in de functie van de eicelmitochondriën (zoals beschreven in hoofdstuk 2) ten grondslag ligt aan de verhoogde incidentie van afwijkende spoelfiguren en chromosomale afwijkingen in eicellen en blastocysten van oudere vrouwelijke individuen. Echter, dit fenomeen hoeft niet beperkt te blijven tot meiotische celdelingen. Een afname in het aantal mitochondriën per blastomeer kan ook leiden tot een afwijkende segregatie van chromosomen tijdens de mitotische celdelingen. In dat geval kunnen er zogenaamde 'mozaïekembryo's' ontstaan, oftewel embryo's die naast een normale diploïde cellijn ook aneuploïde of polyploïde cellijnen bezitten. Het laatste type mozaïekembryo's staat ook wel als 'mixoploïde' embryo's bekend; dergelijke embryo's zijn aangetoond bij de mens, het konijn, het varken, het schaap en de koe. Uit onderzoek blijkt dat bij het paard slechts een klein deel van de eicellen en spermacellen chromosomaal afwijkend is terwijl er tot op de dag van vandaag nog nooit chromosomale afwijkingen gevonden zijn in paardenembryo's. Eén van de oorzaken hiervoor ligt waarschijnlijk in het feit dat deze studies gebruik maakten van conventionele karyotypering. Bij deze techniek kunnen slechts een zeer beperkt aantal cellen onderzocht worden, namelijk alleen die cellen die zich na een kweek *in vitro* in de metafase van de celcyclus bevinden en waarvan de chromosomen goed verspreid liggen en elkaar niet of nauwelijks overlappen. Hierdoor kunnen chromosomale afwijkingen gemakkelijk over het hoofd gezien worden, zeker wanneer deze slechts in een beperkt deel van alle embryonale cellen voorkomen. Daarom hebben we voor onze studie (hoofdstuk 4) gebruik gemaakt van twee chromosoomspecifieke DNA probes die door middel van 'fluorescent in situ hybridisation' (FISH) gelabeld waren. Aangezien deze FISH probes ook gebruikt kunnen worden op cellen die zich in de interfase van de celcylus bevinden, kon het aantal

onderzochte cellen drastisch verhoogd worden. Hoewel er slechts van 2 chromosoomspecifieke probes gebruik is gemaakt, bleek dat chromosomaal afwijkende cellen regelmatig voorkomen in paardenembryo's. Van de *in vivo* geproduceerde embryo's (embryos die gewonnen werden uit merries door middel van een baarmoederspoeling) werden 4 van de 22 embryo's geclassificeerd als mixoploïde; van deze 4 embryo's bestond er één voor meer dan 65% uit polyploïde cellen. Voor zover wij weten is dit de eerste keer dat chromosomale afwijkingen zijn aangetoond bij paardenembryo's. Helaas was het door het lage aantal onderzochte embryo's niet mogelijk om uitsluitsel te geven over de vraag of een hoge leeftijd van de merrie gepaard gaat met een toename in het aantal chromosomaal afwijkende embryo's. Een dergelijke correlatie tussen maternale leeftijd enerzijds en een verhoogde incidentie van zowel het optreden chromosomale afwijkingen als het voorkomen van een miskraam anderzijds is bekend uit de humane geneeskunde: ongeveer de helft van de spontaan geaborteerde humane vruchten heeft chromosomale afwijkingen terwijl de incidentie van bepaalde chromosomale afwijkingen een duidelijke positieve correlatie vertoont met een hoge maternale leeftijd. Bij het paard moet nog onderzocht worden of een dergelijke verhoogde incidentie van chromosomale afwijkingen bij geaborteerde vruchten voorkomt en of bepaalde chromosomenparen gevoeliger zijn voor een leeftijdsafhankelijke non-disjunctie dan andere chromosomenparen. Hoe dan ook is het nu duidelijk dat chromosomale afwijkingen voorkomen bij paardenembryo's; toekomstige studies moeten uitmaken in hoeverre zij bijdragen aan de verhoogde incidentie van VES bij oudere merries.

DE *IN VITRO* PRODUCTIE VAN PAARDENEMBRYO'S

Een tweede factor die geassocieerd wordt met een verminderde ontwikkelingscompetentie van paardenembryo's is de IVP van embryo's. Hoewel er in het recente verleden enkele IVF en gekloonde veulens geboren zijn, worden de meeste IVP embryo's geproduceerd door middel van ICSI. Gedurende de laatste 10 jaar zijn de *in vitro* kweektechnieken dermate verbeterd dat paardeneicellen zeer goed *in vitro* gematureerd kunnen worden terwijl het merendeel van de zygoten direct na de ICSI begint aan de eerste klievingsdelingen. Echter, slechts een klein percentage van deze ICSI-zygoten ontwikkelt zich verder tot het blastocyststadium. Daarnaast is het zo dat ICSI-embryos, nadat ze overgezet zijn naar de baarmoeder van een ontvangstermerrie, tot lagere drachtpercentages en tot een hogere incidentie van VES leiden in vergelijking tot 'normale' *in vivo* embryo's. Mogelijke oorzaken voor deze verminderde ontwikkelingscompetentie van ICSI-embryo's zijn o.a. de significante afname in de kwaliteit en de hoeveelheid van mitochondriën in eicellen van oudere merries na IVM (hoofdstuk 2) en het feit dat ICSI-embryo's achterlopen in hun ontwikkeling en minder mitochondriën bevatten in vergelijking tot *in vivo* embryo's die afkomstig zijn van jonge merries (hoofdstuk 3). Daarnaast lijken chromosomale afwijkingen vaker voor te komen bij ICSI-embryo's dan bij *in vivo* embryo's (8/20 versus 4/22; hoofdstuk 4). Tot slot, hoewel de maternale leeftijd van de donormerrie waarschijnlijk ook een belangrijke rol speelt bij de uiteindelijke resultaten van de IVP van paardenembryo's, is het waarschijnlijk zo dat de huidige *in vitro*

kweektechnieken zelf nog niet helemaal geoptimaliseerd zijn voor paardeneicellen en embryo's. Om in de toekomst de productie van blastocysten (en veulens) door middel van ICSI te verbeteren, zullen eerst het type en de exacte oorzaken van de verhoogde degeneratie van eicellen en embryo's tijdens de *in vitro* kweek geïdentificeerd moeten worden.

FOETALE-MATERNALE INTERACTIE

Een adequate en goed getimede communicatie tussen de vrucht en het moederdier is essentieel voor de voortgang van de dracht. Dit concept wordt het beste geïllustreerd door het feit dat in commerciële ET programma's het stadium van de cyclus van de ontvangstermerrie aangepast moet zijn aan het ontwikkelingsstadium van het over te zetten embryo. Wanneer niet aan deze voorwaarde wordt voldaan, kan het embryo zijn aanwezigheid in de baarmoeder niet op het juiste moment aan de ontvangster kenbaar maken en/of komt het embryo op het verkeerde moment in contact met de verkeerde groeifactoren. Hierdoor kan het embryo ernstig gehinderd worden in zijn ontwikkeling en uiteindelijk zelfs afsterven. In hoofdstuk 3 en 4 hebben we beschreven dat zowel ICSI-embryo's als embryo's die afkomstig zijn van oudere merries, onderontwikkeld zijn. Een dergelijke vertraging in de embryonale ontwikkeling kan, zeker wanneer deze achterstand groot is, op zichzelf al leiden tot een mismatch tussen het ontwikkelingsstadium van het endometrium, enerzijds, en dat van het embryo, anderzijds, en uiteindelijk tot het verlies van de dracht. Uit de dagelijkse veterinaire praktijk is het inderdaad bekend dat (te) kleine ('small for dates') vruchten een verhoogde kans hebben op afsterven gedurende de eerste weken van de dracht.

Moleculen die waarschijnlijk een belangrijke rol spelen in deze foetale-maternale (mis)communicatie bij het paard zijn progesteron, oestrogenen en het nog onbekende signaal dat zorgt voor de MDH.

Progesteron en oestrogenen

Voldoende hoge progesteronspiegels zijn een essentiële voorwaarde om bij zoogdieren, inclusief het paard, de dracht in stand te houden. Tot voor kort ging men ervan uit dat progesteron en oestrogenen de ontwikkeling van de vrucht alleen indirect (via het endometrium) kunnen beïnvloeden en dat zij hun effect bewerkstellingen via een genomisch werkingsmechanisme waarbij de binding van het steroïdhormoon aan intracellulaire receptoren via transcriptie- en translatiestappen leidt tot de expressie van bepaalde eiwitten. Er komen echter steeds meer aanwijzingen dat progesteron en oestrogenen hun effect ook direct op pre-implantatie embryo's kunnen uitoefenen aangezien er in de embryo's van verschillende zoogdieren progesteron- (PR) en oestrogeenreceptoren (ER) zijn aangetoond. Daarnaast hebben recente studies aangetoond dat beide steroïden niet alleen aan de klassieke intracellulaire receptoren kunnen binden. Inmiddels zijn er twee membraangebonden progesteronreceptoren (PGRMC1 en mPR)

geïdentificeerd die na binding door progesteron zeer snel tot een effect kunnen leiden zonder dat daar transcriptie- en translatiestappen voor nodig zijn (non-genomisch werkingsmechanisme). Daarnaast bestaan er waarschijnlijk ook membraangebonden oestrogeenreceptoren. In hoofdstuk 5 hebben we door middel van kwantitatieve rtPCR aangetoond dat 7-14 dagen oude paardenembryo's zowel de klassieke intracellulaire steroidreceptoren (ERβ en PR) als de nieuwe membraangebonden receptoren (mPR en PGRMC1) tot expressie brengen. Paardenembryo's brengen dus receptoren voor oestrogenen en progesteron tot expressie op een tijdstip van de dracht waarop beide type steroïden in grote hoeveelheden aanwezig zijn in de directe omgeving van het embryo. Dit suggereert dat oestrogenen en progesteron hun effecten niet alleen via het endometrium maar ook direct op het embryo zelf uit kunnen oefenen en dat de conceptus zowel heel snel en kortdurend (non-genomisch werkingsmechanisme) of juist langzaam en langdurig (genomisch werkingsmechanisme) op deze hormonale prikkels kan reageren. De exacte aard van deze effecten en hun belang voor de ontwikkeling en vitaliteit van de vrucht moeten nog verder onderzocht worden.

Maternale drachtherkenning (MDH)

Het allerbelangrijkste aspect van de interacties tussen de vrucht en het moederdier is de tijdige productie en afgifte van het signaal dat leidt tot MDH, oftewel het signaal geproduceerd door het embryo dat er voor zorgt dat het primaire corpus luteum tot ver na de 14^{e}-16^{e} dag na ovulatie progesteron blijft produceren. Het paard is het enige landbouwhuisdier waarvan de identiteit van dat MDH-signaal nog steeds niet bekend is. Eerdere studies hebben een aantal mogelijke kandidaten uitgesloten zoals oestrogenen, interferonen en PGE_2. Er zijn echter wel enkele eigenschappen van het MDH-signaal van het paard bekend. Momenteel gaat men er van uit dat de MDH bij het paard begint voor de 10^{e} dag na ovulatie, dat de MDH voltooid moet zijn rond de 14^{e}-16^{e} dag van de dracht, en dat de MDH-signaalcomponent een grootte heeft die varieert tussen de 3 en 10 kDa. In hoofdstuk 6 hebben we de hypothese onderzocht dat insuline (een eiwit van 6 kDa) het MDH-signaal van het paard zou kunnen zijn. Echter, insuline kon niet aangetoond worden in de dooierzakvloeistof van 10-14 dagen oude paardenembryo's. Daarnaast kon de parenterale toediening van insuline gedurende de MDH periode (van 7 tot 17 dagen na de eisprong) het optreden van de luteolyse niet voorkómen noch kon het de afgifte van $PGF2_{\alpha}$ door het endometrium onderdrukken Bovendien had de toediening van insuline aan de merries geen invloed op hun cyclus noch op de plasmaspiegels van progesteron en LH. Daarom is het zeer onwaarschijnlijk dat insuline de 3-10 kDa grote signaalcomponent is verantwoordelijk voor MDH bij het paard.

CONCLUSIES

De belangrijkste conclusies van dit proefschrift zijn:

- De mitochondriën van eicellen van oude (≥ 12 jaar) merries hebben een verhoogde kans op schade tijdens IVM (hoofdstuk 2).
- Bij paardenembryo's begint de gastrulatie rond de 12^{e} dag na ovulatie (hoofdstuk 3).
- De vermenigvuldiging van mitochondriën in paardenembryo's start in de periode dat het embryo het blastocyststadium bereikt (hoofdstuk 3); dit is veel eerder dan beschreven is voor muizenembryo's (gastrulastadium).
- Zeven dagen oude embryo's afkomstig van jonge merries hebben significant meer mtDNA-kopieën dan eicellen terwijl een dergelijke toename niet optreedt bij 7 dagen oude IVP embryo's of embryo's die afkomstig zijn van oudere merries; daarbij zijn de laatste twee groepen embryo's vertraagd in hun ontwikkeling (hoofdstuk 3).
- Chromosomaal afwijkende cellen komen regelmatig voor bij zowel *in vivo* als *in vitro* geproduceerde paardenembryo's (hoofdstuk 4).
- In vergelijking tot 'normale' *in vivo* geproduceerde paardenembryo's zijn IVP embryo's vertraagd in hun ontwikkeling en bezitten ze minder cellen met een grotere incidentie van chromosomale afwijkingen (hoofdstuk 4).
- Tijdens de tweede week van hun ontwikkeling brengen paardenembryo's genen voor zowel intracellulaire (PR en ERβ) als membraangebonden (PGRMC1 en mPR) steroïdreceptoren tot expressie (hoofdstuk 5); dit suggereert dat progesteron en oestrogenen de embryonale ontwikkeling direct, zonder tussenkomst van het endometrium, kunnen beïnvloeden.
- Insuline is hoogstwaarschijnlijk niet het MDH-signaal bij het paard (hoofdstuk 6).
- Langdurige parenterale toediening van insuline aan merries heeft geen significant effect op de cyclus van deze merries noch op de secretie van progesteron en LH (hoofdstuk 6).

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Curriculum Vitae

Björn Rambags was born on the 5th of April 1973 in Venray, the Netherlands. In 1991, he passed his final exams at "Gymnasium Bernrode" in Heeswijk-Dinther, and started his studies in veterinary medicine at Utrecht University. After finishing his Master in Veterinary Medicine in Utrecht, he passed the examination for a Norwegian veterinary license (*'midlertidig vetrinærlisens'*) at the Norwegian College of Veterinary Medicine in Oslo, and subsequently worked as a locum veterinarian in a mixed veterinary practice on the isles of Tustna and Smøla just off the west coast of Norway. He subsequently returned to Utrecht University for his final clinical rotations in large animal medicine and herd health management, and performed an elective rotation in equine surgery at the College of Veterinary Medicine in Knoxville, Tennessee. After his graduation as Doctor of Veterinary Medicine from Utrecht University in 1999, Björn started a combined residency and PhD track in equine reproduction at Utrecht University and spent a period as a resident veterinarian at Alabar Bloodstock, a stud farm for pacers in Echuca, Australia. In 2006, he was registered as a Specialist in Equine Reproduction by the Royal Netherlands Veterinary Association and one year later he completed his PhD studies into "Embryonic quality and survival in the horse; maternal and intrinsic aspects". At present, he is working as technical service manager at Boehringer Ingelheim Pharmaceuticals, and pursuing a MBA degree at TiasNimbas Business School.