Optimizing equine assisted reproductive technologies

Optimaliseren van moderne voortplantingstechnieken bij het paard

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

Chapter 1

General introduction

The more widespread application of assisted reproductive technologies (ART) in modern horse breeding started with a rapid increase in the popularity of artificial insemination (AI) in the late 20th century. A marked switch from natural mating to AI with fresh or chilled-transported stallion semen in sport horses in Western Europe took place during the 1980-90s. AI with frozen-thawed semen initially accounted for only a modest proportion of inseminations because of a combination of variable, often poor, results and the need for more intensive mare management to achieve reasonable success rates. However, primarily as a result of improvements in cryopreservation protocols and extenders, frozen semen AI has become a routine procedure in the 21st century. The shift from natural cover to AI in sport horse breeding is well illustrated by the changes that have taken place in the breeding management of Warmblood mares in the Netherlands. In the late 1970s, less than 5% of mares were inseminated artificially; this percentage increased progressively over the following 20 years to over 90% in the early 90s; in the early 21th century the use of frozen semen varied between 2.5% in the Hanoverian horse to 22% in the French sport horse (Aurich and Aurich, 2006).

AI has several advantages over natural mating as a means of impregnating mares, among which is more rapid genetic improvement by increasing the availability and use of genetically 'superior' stallions. When using standard insemination doses of 500 x 10^6 motile or 300 x10^6 morphologically normal motile sperm, more than 5-10 mares can be inseminated with semen from a single ejaculate, thereby significantly increasing the breeding potential of a stallion. If 'low-dose' insemination is applied, by depositing the semen at the tip of the uterine horn, close to the utero tubal junction, the number of sperm required can be reduced further by a factor of 10, such that even more mares can be inseminated. An additional advantage of AI is that it is considerably easier and more animal friendly to transport semen rather than live horses, thereby also increasing the availability of a stallion's genetic material both within a country and beyond national borders. This is even more so for cryopreserved (frozen) semen which, following appropriate screening for pathogens, can be easily transported transcontinental over great distances with few time constraints, and can therefore be used to import or export genetic material and promote genetic diversity within previously restricted populations. Clearly, not needing to transport live horses for breeding purposes can significantly improve animal welfare by removing the stress and possible health risks of long distance transport. Another advantage of the routine use of AI, is more secure control of transmissible diseases. Clearly, since a breeding stallion, used in an AI program does not need to have direct genital contact with any mares, the risk of him contracting or spreading venereal transmitted bacteria such as *Taylorella Equigenitalis* (the contagious equine metritis organism: CEMO; Schulman *et al.,* 2013) or

viruses such as equine arteritis virus (EAV). EAV is removed, as long as the stallion has been tested negative at the onset of the AI program. In addition, maintaining the stallion in a quarantine facility with other animals (stallions and teaser mare) of similar health status and removing the need to interact with mated mares greatly reduces the risk of transmitting other more general infectious agents spread primarily by direct animal-to-animal contact such as *Streptococcus equi equi* (the bacterium causing 'strangles') or equine herpes virus 1 (Samper and Tibary, 2006). A last important advantage of AI over natural mating, is the opportunity to routinely evaluate the quality of the stallions' semen. Semen can be evaluated after every collection, by microscopy and other laboratory techniques (Varner, 2008), thereby enabling monitoring and the development of strategies to cope in case of a quantitative or qualitative changes or fluctuations in semen production or subfertility.

While artificial insemination became a routine procedure in horse breeding during the late 1980s, embryo recovery and transfer (ET) was still essentially unknown to most breeders at that time. Just as AI can be used to increase male selection intensity within a breeding program, ET can be used to increase the contribution of individual high genetic merit mares. Although techniques for equine ET were first described in the mid-1970s (Stout, 2008), both the stud books and breeders were initially reluctant to, respectively, allow or implement the technique due partly to conservatism but also ignorance of the potential benefits and risks. The use of ET in the field began to increase gradually during the nineties, in particular in high-goal polo ponies in Argentina (Stout, 2008). Embryos were flushed out of the uterus of selected donor mares during the off-season for polo and transferred non-surgically to the uterus of synchronised recipient mares. In this way, ET made it possible to obtain multiple foals per year from high-goal polo mares that that were still active in competition. This was significant development because the best polo ponies are 10-15 year old mares, and the physiological horse breeding season overlaps significantly with the polo season. These days, once the polo season has finished, many of the best mares are transferred to ET stations where repeated cycles of AI and embryo flushing are performed. In total more than 20,000 embryo flushes are performed annually in South America, producing more than 15,000 foals, and accounting for more than 80% of all of the polo ponies foals born in South America each year (IETS annual reports 2013, 2014). In the USA and Europe, uptake of was slower, in large part due to the refusal of some of the larger breed registries to accept foals born by ET or to limit numbers to one per donor mare per year such that it was only attractive for mares that were incapable of carrying a foal to term. The absence of any commercially available hormones to induce superovulation and the poor success of cryopreserving horse embryos were other factors that hindered the early growth

of ET in practice. Nowadays, most of the larger sport and pleasure horse registries, with the notable exception of the Thoroughbred studbooks, accept ET foals and there is much wider understanding among horse breeders of the potential advantages of the technique as a means of getting multiple foals from valuable mares, or indeed any foals at all from aged mares unable to carry a foal to term as a result of endometrial degeneration or other uterine or cervical abnormalities. In 2013, approximately 25,000 equine embryo transfers were reported worldwide to the International Equine Embryo Transfer Society (IETS), however there are probably at least as many transfers that are not reported to the IETS, while even these numbers are small when compared to those for *in vivo* produced cattle embryos (> 500,000; Hinrichs, 2013).

In some species (e.g. man, cow), rather than recovering embryos from the uterus of a donor female, embryos are produced by *in vitro* fertilization (IVF) of oocytes recovered directly from ovarian follicles. In the horse, conventional IVF i.e. placing spermatozoa and oocytes together in a petri dish to facilitate fertilisation, was a technique initially investigated in the nineties, despite the birth of two foals (Palmer *et al.,* 1991; Bézard *et al.,* 1992), conventional IVF proved to be extremely unsuccessful for equine gametes (Leemans *et al.,* 2016). The major impediment to conventional equine IVF appears to be an inability to adequately trigger capacitation of equine sperm cells *in vitro*, such that although the sperm bind to the zona pellucida of the oocyte, they do not complete the acrosome reaction and fail to penetrate into the perivitelline space. Intracytoplasmic sperm injection (ICSI) was developed as a technique to overcome 'male factor infertility' and has been adopted to tackle the hurdle of inadequate sperm cell activation in equine IVF. The first pregnancies / foals produced using ICSI were born in the late 90s (Squires *et al.,* 1996; Cochran *et al.,* 1998; McKinnon *et al.,* 1998). Additional theoretical advantages of using ICSI to fertilize oocytes, include possibility of using sperm with poor motility or other abnormalities that may reduce their ability to penetrate an oocyte, or that have been sex-sorted (Galli *et al.,* 2013) prior to injection into the oocyte and subsequent initiation of embryonic development.

A major constraint for the wider application of ICSI was relatively low yield of oocytes obtained via ultrasound guided aspiration from ovarian follicles in the living donor (ovum pick-up: OPU). In women and cattle, oocytes are generally recovered from follicles stimulated to mature using gonadotrophins with high FSH activity. Unfortunately, the mare is uniquely resistant to the stimulatory effects of exogenous FSH-like preparations (Tharasanit *et al*., 2006). Indeed, to date only equine FSH (pituitary derived or recombinant) has been shown to have sufficient biological activity at the equine FSH receptor to reliably stimulate development of

multiple mature follicles (G Meyers-Brown *et al.*, 2013); however, neither pituitary-derived equine FSH (due to biosecurity concerns) nor the new recombinant equine FSH are currently available commercially. To overcome the inability to stimulate multiple pre-ovulatory (mature) follicles in the mare, it is common to puncture all immature follicles and subsequently mature the oocytes collected *in vitro*; while this, historically, was hindered by a low oocyte recovery percentage (<30%: Hinrichs, 2012), recent improvements to the technique have seen improvements to 50-60% oocyte recovery per aspirated follicle, equating to a mean of 8-14 oocytes per OPU session (Galli *et al.,* 2014; Choi *et al.,* 2016).

A final ART available for creating horse offspring is somatic cell nuclear transfer (SCNT); i.e. reproductive cloning. The first cloned equid reported was a mule (Woods *et al.,* 2003), followed shortly by the first cloned horse (Galli *et al.*, 2003). Reproductive cloning is now carried out on a fairly large scale by commercial companies in the USA / Canada (Viagen) and South America. In general, the donor cells are skin cells recovered by biopsy, while oocytes are collected from the ovaries of slaughtered mares (Lagutina *et al.,* 2005; Galli *et al.,* 2008; Choi *et al.,* 2015). The oocytes are matured i*n vitro* and then enucleated, the nucleus of the donor cell (skin) is than either injected into the enucleated oocyte or the cell is fused with the zona-free oocyte using an electrical charge. Further development is stimulated using chemical triggers such as 6-dimethylaminopurine and cycloheximide. At present, SCNT is still an inefficient process with only around 5-30% of reconstructed embryos developing to the blastocyst stage (Choi *et al.,* 2015; Olivera *et al.,* 2016); the inefficiency is exacerbated by relatively low pregnancy rates after transfer to recipient mares, and high early embryonic loss rates (Galli *et al.,* 2008; Choi *et al.,* 2015; Olivera *et al.,* 2016). In addition, acceptance of SCNT by the general public is low, and the ethical questions regarding, in particular the relatively high rates of fetal or neonatal abnormalities in some farm animal species (although notable not the horse: Hinrichs, 2012) has led to SCNT being forbidden by the law in the Netherlands. On the other hand, in 2012 the Fédération Equestre Internationale announced that it would allow cloned horses to compete in international events, and a number of the larger stud books accept cloned animals as registered breeding stallions. These are the first concrete signs from the sport horse industry that it will consider SCNT as being of potential value for horse breeding (Galli *et al*., 2013). Although the major ARTs are now commercially available, superovulation, embryo cryopreservation and IVF (by ICSI) are not yet as successful as in other species. Improved understanding of the requirements of embryo culture media, and of how to predict or optimize oocyte and semen quality should help improve the results, and will in turn yield valuable information about the normality of embryonic, fetal and postnatal development of *in vitro*

produced embryos. This general introduction reviews some challenges that equine ART will have to face.

Normal fetal development

Mare reproductive success is influenced by a number of factors including the mare's age and reproductive status (maiden, barren or foaling), the intensity of reproductive management and type of mating system (natural cover; fresh, chilled or frozen semen insemination). In naturally mated, Thoroughbred mares, the pregnancy rate at day (D) 15 after ovulation has been reported in various studies as approximately 60%, although foaling rates are subsequently reduced by a 7-13% pregnancy loss rate before D42 (Allen *et al.,* 2007; Bosh *et al.,* 2009). In sport horse breeding (including polo, show jumping and dressage horses), pregnancy rates are more variable because a greater range of mating systems are used. In addition, the importance of mare age is heightened because mares that have exceptional sporting qualities tend to start their reproductive carriers at a later age having been used primarily for sport during their most fertile years. As for women, age has a significant impact on mare fertility, with mares older than 14 years old suffering from a reduced pregnancy rate; for example, Carnevale (2008) reported pregnancy and foaling rates of 31% and 48%, respectively, in older (≥ 14 yr) compared to 57% pregnancy rate and 82% foaling rate in 2-11 year old mares (Carnevale 2008). A study from Marinone and others showed also reduction of fertility ~15% in polo mares older than 12 yr (Marinone *et al.,* 2015). The origin of mare age-related reduced fertility is multifactorial but encompasses factors such as reduced oocyte quality, an increased incidence of persistent post-breeding endometritis and reduced endometrium quality (i.e. endometrosis), all of which lead to increased incidences of both early embryonic death and later fetal losses (Stout, 2006; Allen *et al.,* 2007).

Embryo based ARTs are often used in attempt to overcome mare age – related sub-fertility, because they reduce the importance of uterine quality to pregnancy survival (Stout, 2006; Hinrichs, 2012; Galli *et al.,* 2014) and offer the possibility of increasing the number of chances of producing a successful pregnancy (i.e. by flushing multiple embryos or producing multiple embryos in vitro). While commercial equine OPU-ICSI is only at a very early stage of development, it is clear that embryos produced by ICSI currently display reduced developmental competence after transfer, compared to embryos recovered by flushing the uterus of young donor mares. This may in part be because OPU-ICSI is often used to overcome pre-existing sub-fertility in either mare or stallion, but it is also likely that current culture conditions during IVM, ICSI and IVP are suboptimal and result in embryos of lower quality,

as evidenced for example by lower cells per embryo at the morula or blastula stage, and higher incidences of apoptotic cells (Tremoleda *et al.,* 2003; Rubio-Pomar *et al.,* 2005; Gad *et al*., 2012; Galli *et al.,* 2013), and a retarded stage of development compared to *in vivo* produced embryos (Tremoleda *et al.*, 2003; Rambags *et al.*, 2005). Adequate synchrony between the early embryo and the uterus into which it is transferred is also critical to embryo development. For example, ET to sub-optimally synchronised recipient mares (i.e. that ovulated more than 1 day before or more than 3 days after the donor mare) has been reported to predispose to early embryonic loss (Carnevale *et al.*, 2000). Moreover, while pregnancies can be established at a reasonable rate in recipient mares that ovulated as much as 5 days after the donor mare, such a degree of embryo-uterine asynchrony markedly delays embryonic development (Wilsher *et al.,* 2012). It is also increasingly clear that peri-conception period is critical with regard to the erasure and re-establishment of epigenetic markings; any perturbations during this period can result in modifications in genomic reprogramming, thereby permanently altering gene expression potential with possible repercussions for the subsequent development of pregnancy, and the health and viability of the neonate and resulting adult (Ventura-Junca *et al*., 2015). On some occasions, the epigenetic alterations may only become apparent during adulthood (e.g. reduced incidence of type II diabetes, heart and vascular disease in people) or they may influence the size and viability of the full term foetus (Gad *et al.,* 2012). For example, in man IVF neonates are often premature with 40-60% greater risk of being born with a below average birth weight (Vulliemoz and Kurinczuk, 2012). By contrast, calves born in the early days of cattle IVF were often delivered after an extended gestation and were oversized, a phenomenon known as the 'large offspring syndrome'. It subsequently transpired that the addition of serum or bovine serum albumin (BSA) to the embryo culture medium altered the expression of genes involved in placental development resulting in embryos containing more cells and developing a larger placenta than their in vivo counterparts. Changing culture system to a serum-free system based on synthetic oviductal fluid (SOF) medium greatly reduced these unwanted side-effects (Galli *et al.,* 2001; Galli and Lazzari, 2008). Identification of deviations in fetal growth from normal curves can be made during mid to late gestation using ultrasonography; this can be useful in cases where multi-fetal pregnancy is suspected, where maternal age or disease is thought to have compromised fetal development, or to monitor development after ARTs.

To understand how and whether ARTs influence embryonic and fetal development, it is therefore important to understand normal fetal development; in farm animals and horses, this also needs to take account for breed or type, since there are marked differences between breeds in fetal growth rates. Previous studies of fetal development in horses have concentrated on mid-

sized breeds (Thoroughbreds, Quarter horses and Standardbreds; Bucca *et al.,* 2005; Renaudin *et al.,* 2000), whereas as little is published about normal fetal development in breeds in which ART is more common, such as Warmbloods.

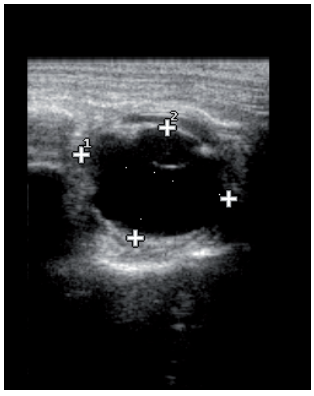

Figure 1. Ultrasonographic image of a fetal eye at 8 months of gestation (length and width displayed between crosses).

ART in mares

Mares selected as ET/IVF donors by breeders generally come from a proven bloodline, have proven their 'phenotypic superiority' by success in competition and/or by producing offspring with superior athletic or other phenotypic qualities. ARTs such as ET (using fresh or cryopreserved embryo's) and IVF (via OPU and ICSI) make it possible to increase the number of offspring per year from these valuable donor mares. In addition, where focus is particularly on genetic merit the techniques can be used to reduce the generation interval by producing embryos from mares as young as 18 months old, and enhance genetic progress both in this way or by increasing the selection pressure for only the best (genetic merit) mares.

However, the success of ARTs in equine breeding is often suboptimal. Factors such as mare age, breed, illness and stallion subfertility can result in disappointing embryo recovery, pregnancy of live foal rates (Galli *et al.,* 2013; 2014). For example, Warmblood mares selected as ET donors on the basis of a successful sports career or successfully competing offspring are by definition 'older' (>12yrs) mares. Just as in women, increasing mare age is associated with decreased fertility primarily as a result of an increased risk of early pregnancy loss (Ball *et al*., 1989; Madill, 2002; Carnevale, 2008; Scoggin, 2015). Other indications for the application of ART are young mares currently in sport, or mares that have become sub-fertile due to for example repeated failure of normal ovulation, oviductal pathology, chronic endometritis,

uterine luminal adhesions, cervical damage etc. ART can also offer a solution for some cases of stallion infertility, since gradient centrifugation can be used to select the best quality sperm (Morrel *et al.,* 2009).

However, since sperm selection is likely to result in a significant reduction in total sperm number this technology needs to be accompanied with methods to minimize sperm use such as deep intrauterine insemination or, preferably, ICSI. OPU and ICSI also make it possible to create offspring from mares that die: the ovaries need to be removed immediately after death and the oocytes harvested, followed by *in vitro* maturation, ICSI and embryo culture. Hinrichs and her team produced foals from six of 16 mares from which oocytes were recovered post mortem (Hinrichs, 2012).

Although equine ARTs occasionally yield disappointing results / success rates, it is not always clear whether this is inherent on the ART itself, or an indication that the process is not yet optimal, or whether it may reflect inherent subfertility of the donor mare and/or stallion used. Factors that could contribute to poor results include impaired oocyte and embryo developmental competence (e.g. due to advanced maternal age), DNA damage to sperm cells, and suboptimal culture conditions. In human embryos, it has been demonstrated that at day 3 of development 50% of embryos contain at least one cell that deviates from the 46-chromosome complement, i.e. aneuploidy (McCoy *et al.,* 2015) where aneuploidy can either arise during meiosis (but would affect all cells; e.g. trisomy 21 in human embryos) or during later mitotic divisions and therefore affect one or more subsets of cells. Advancing maternal age is known to lead to an increased rate of aneuploidy, due primarily to a heightened incidence of chromosome segregation errors during meiosis (McCoy *et al.,* 2015). In this respect, mitochondrial dysfunction is an important contributor to impaired oocyte maturation resulting from advancing maternal age, in particular affecting nuclear spindle activity and chromosome segregation (Bentov *et al.,* 2011). In horses, IVP resulted in an increase in embryos showing cells with abnormal ploidy (40% compared to 18% of *in vivo* embryos) (Rambags *et al.,* 2005). In man, spermatozoal DNA quality is also affected by donor age. Advancing paternal age is related to an increase in DNA fragmentation, gene mutations and chromosomal abnormalities within the spermatozoa. This leads to an increase in achondroplasia, a higher incidence of spontaneous abortions, and increased frequencies of autosomal dominant diseases (Wyrobek *et al.,* 2006). Identifying specific factors that hamper IVF procedures might enable adjusting these procedures and thereby help to improve results.

An embryo transfer program

For *in vivo* embryo production, the donor mare is monitored daily to detect the time of ovulation. Depending on the wish of the breeder, she is inseminated with fresh, chilled or frozen semen at the appropriate time. After insemination, evaluation for post-breeding endometritis is performed and when necessary treated (Coutinho da Silva, 2008; Stout, 2006). A standard ET program includes collection of an embryo from a donor mare 6.5 to 9 days after ovulation by non-surgical lavage of the uterus with Ringer's solution or a commercial embryo flushing solution, with the embryo (fresh or chilled) being transferred non-surgically to a suitable recipient mare within 1 (fresh) - 24h (chilled) after collection (Stout, 2006). Embryo recovery rate is variable and influenced by intrinsic fertility of the mare and stallion. Use of ET with 'older mares' (>14 yr.) as donor can lead to disappointing results: embryo recovery rate is lower (30-50%), initial pregnancy rate is lower and the pregnancy loss rate after an initial positive pregnancy scan is higher than those for younger donors (>70%; Stout, 2006). The factors involved in the reduced developmental competence of embryos from old donor mares are intrinsic to the oocyte / embryo as proven by studies in which oocytes from old mares were transferred to the oviduct of younger mares; pregnancy rates were significantly lower than when oocytes from young mares were transferred (Carnevale 2008). Unfortunately, little is known about what changes in the oocytes of old mares, or indeed, older women to compromise their developmental competence. For example, little is known about the metabolism of the equine oocyte or embryo, or the regulation of mitochondrial number or function. In species like cattle and mice, it is demonstrated that oocytes of older females contain lower mitochondrial content compared to younger females resulting in a lower fertility rate. Oocytes from 'older' pigs also contain lower mitochondrial copy number resulting in reduced blastocyst formation. Oocytes of 'aged' mice, contain genes with an altered expression involved in mitochondrial function and regulation of oxidative stress (May-Panloup *et al.,* 2016). Studies to examine how factors such as maternal ageing or *in vitro* maturation affect the developmental competence of an oocyte or early embryo would help determine whether the processes can be ameliorated and/or the better quality oocytes or embryos could be identified and preferentially selected for IVM, fertilization and/or transfer into a recipient.

Recipient mares are also a critical component of an equine ET program. A recipient mare is typically young (3-10 yrs of age), has a sound reproductive tract, a similar size as the donor mare, and has previously carried a foal to term. When an embryo is transferred to a 'too small' recipient mare, the foal will be restricted in fetal development and glucose metabolism will be altered in post-natal development. An embryo transferred into a 'too large' recipient

mare create a relatively large and too heavy foal, with weak tendons/muscles and changes in insulin sensitivity (Peugnet *et al.,* 2014). The oestrous cycle of the recipient mare needs to be reasonably well synchronized with that of the donor mare; in this respect ovulation of the recipient mare should occur within a time frame of 1 day prior to the ovulation day of the donor mare to 4-5 days thereafter (Jacob *et al*., 2012; Wilsher *et al*., 2010). The embryo transfer will be performed nonsurgical, via the cervix, after proper preparation (rectal / ultrasonographic evaluation, cleaning vulva / perineum and when necessary sedation) of the recipient mare. Surgical embryo transfer does not enhance the result of an experienced veterinarian, therefor nonsurgical transfer is preferred (Carnevale *et al.,* 2000). When ET is performed on a small scale, the availability of suitable synchronized recipient mares may be difficult to guarantee. This can be overcome by the shipment of the embryo at 4-5ºC within 24h after collection to another facility where a synchronized recipient is available; early studies demonstrated that storing horse embryos for up to 24h at 4ºC has little or no effect on subsequent pregnancy and foaling rates (Carney *et al.,* 1991; Carnevale *et al.,* 2000), and chilled transport is now a routine part of many equine ET programmes. A less routinely applied technique for reducing the pressure on oestrus cycle synchronisation is the long term storage of embryos by cryopreservation. The slow development and implementation of equine embryo cryopreservation can be largely explained by a small number of interconnected impediments, these include; 1) the absence of commercially-available products for reliably stimulating superovulation in horses (Squires and McCue, 2007; Meyers-Brown *et al.,* 2011); 2) very poor pregnancy rates following cryopreservation of embryos >300 μm in diameter (0-38% pregnancy; Choi *et al.,* 2011) difficulty in recovering embryos at early developmental stages amenable to cryopreservation (Robinson *et al.,* 2000); and 4) inter-embryo variation in susceptibility to cryodamage. However, acceptable success rates (> 55% pregnancy) have been reported for both slow-frozen (Lascombes *et al*., 2000) and vitrified small embryos (<300 μm: Eldridge-Panuska *et al.,* 2005), and there is renewed interest in cryopreservation, not only in the context of standard ET programmes, but also because it would facilitate pre-implantation genetic testing and allow wider access to techniques for producing embryos *in vitro* (Choi *et al.,* 2015b).

***In vitro* embryo production**

During IVF, mature (metaphase II stage of meiosis) oocytes are required for fertilization. One approach to achieve this goal is to harvest the oocyte from a (pre)ovulatory follicle, roughly 24-43 hours after injection of hCG to induce follicle maturation, by

transvaginal ultrasound-guided follicle aspiration (OPU). Although the oocyte recovery rate from pre-ovulatory follicles is high (51-86%: Carnevale 2016), the oocyte yield per cycle is low because most mares produce only one preovulatory follicle per cycle. Superovulation in horses is not applied routinely because there is no commercially available FSH product that is capable of reliably stimulating the development of multiple follicles (Squires and McCue, 2007). This is not to say that stimulation is not possible, but it is only possible with equine FSH (Tharasanit *et al*, 2006), and the number of pre-ovulatory follicles that develop is variable and, in general not very high with an average of 3-4 (McCue *et al.,* 2007). The protocols described for stimulating multiple follicle development in mares involve once or twice daily injection of pituitary derived equine FSH (no longer commercially available) or recombinant equine FSH for 3-5 days, starting at around the time that the dominant follicle would normally start to deviate from subordinate follicles (22.5 mm in diameter; Ginther *et al.*, 2004) which is generally somewhere around seven days after ovulation. On the second day of treatment, a PGF2α analogue is administered to induce luteolysis and, once the largest follicles reach 30-35mm in diameter, hCG or recombinant equine LH are administered to induce final follicle maturation and ovulation (McCue *et al*., 2007). However, when this follicle stimulation protocol was used in combination with OPU, a disappointing oocyte recovery rate was obtained (15%: Blanco *et al.,* 2009).

At present therefore, OPU in the mares is focussed on aspiration of the more numerous smaller follicles, followed by *in vitro* maturation of the collected oocytes. In the past, oocyte recovery rates were disappointing (18-35%) and it subsequently transpired that the equine oocyte is unusally firmly attached to the follicle wall by a wide attachment of the cumulus hillock to the mural granulosa (Hinrichs, 2013; see Fig. 2).

Improving the OPU procedure by flushing and scraping 8-10 times the inside of the immature follicle using a large (12 gauge) double-lumen needle has resulted in a significant improvement in oocyte recovery rate to 50-65% of all punctured follicles (>5-10mm in diameter; Galli *et al.,* 2014; Hinrichs 2013). After collection of the oocytes, the oocytes are either shipped to the laboratory in H-SOF medium at room temperature (23° C) or directly transferred into maturation medium. After 24-30h maturation, 50-80% of oocytes have reached metaphase II stage showing extrusion of the first polar body and these oocytes are selected for fertilization (Carnevale, 2016).

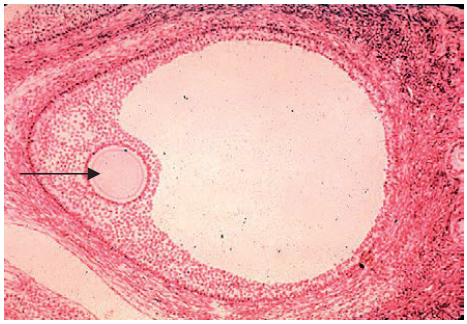

Figure 2. Equine oocyte (arrow) in follicle (microscopic section; Gluck Equine Research Centre, Lexington, KY, USA) demonstrating the broad attachment to the mural granulosa layer.

As mentioned above, horse sperm cells cannot currently be induced to capacitate and fertilize equine oocytes *in vitro* in commercially viable percentages, with documented fertilization rates ranging between 0 and 33%, and exposure of the equine spermatozoa to calcium ionophore appearing to be the only method capable of stimulating some degree of oocyte penetration by sperm (Dell'Aquila *et al.,* 1996; Hinrichs *et al.,* 2012). While McPartlin *et al.* (2009) reported very high fertilization rates (40-80%) using a combination of bicarbonate to trigger the tyrosine phosphorylation aspect of capacitation and procaine to stimulate the hyperactivated motility necessary for the capacitated sperm to penetrate the ZP. However, none of the putative embryos produced by this technique developed beyond the 8 cell stage, and Leemans *et al*. (2016) recently demonstrated that a large part of the developing embryos were actually the result of parthenogenetic activation of cytokinesis accompanied by aberrant condensation and division of the nuclear DNA, explaining why the embryos were not viable.

The failure to develop a successful protocol for conventional IVF has led to ICSI being adopted as the method of choice for IVF in horses. This technique was initially developed as a means of treating male factor infertility in man, and subsequently adopted as an alternative to conventional IVF. However, ICSI requires expensive equipment, expertise in micromanipulation, and involves increased oocyte and embryo handling. Nevertheless, OPU-ICSI has the advantages of reduced animal handling compared to conventional ET, since minimal donor mare management prior to the procedure is required, there is no immediate need for available recipient mares (since the embryos can be cryopreserved), more oocytes can be fertilised and poor quality semen (low sperm numbers or even immotile sperm) can be used (Hinrichs, 2013).

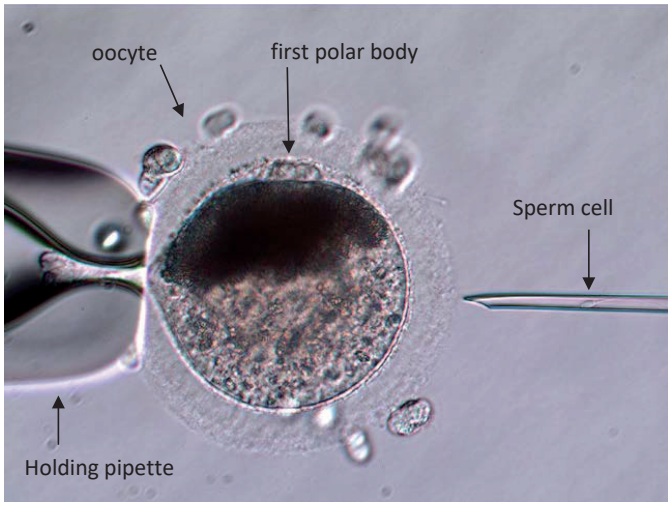

Figure 3. ICSI of an equine oocyte

Fertilization rates, as diagnosed by the presence of two polar bodies and two pronuclei, have increased from 5 to 40% in the early days of equine ICSI, to a current mean of around 70-90% (Lazzari *et al.,* 2010; Rader *et al.,* 2016). Transferring the presumptive zygotes directly to the oviduct of a recipient mare (Hinrichs, 2005) or to the oviduct of progesterone-treated ewes (Lazzari *et al*., 2010) as alternatives to *in vitro* embryo culture resulted in relatively high (50%) blastocyst production rates, but have the disadvantage of requiring surgery. Nowadays, the presumptive zygotes are cultured until the (early) blastocyst stage followed by transcervical, nonsurgical transfer into a suitable recipient mare. However, only 15-25% of injected oocytes will reach this stage primarily because culture conditions still need to be optimized (Alm *et al.,* 2008; Hinrichs 2013; Jacobson *et al.,* 2010).

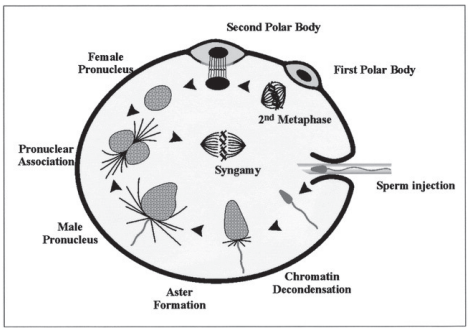

Figure 4. A schematic representation of the changes in microtubule and chromatin organization during zygote formation following fertilization by ICSI (Tremoleda *et al.,* 2003).

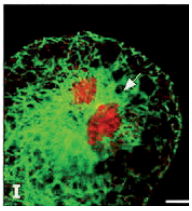

Figure 5. Laser-scanning confocal image of a fertilized horse oocyte. Two pronuclei (in red) are present (Tremoleda *et al.,* 2003).

Factors affecting developmental competence of the oocyte and embryo

Chromosomal and cytoplasmic abnormalities

In several species, including man, cattle and horse the incidence of aneuploidy in developing embryos increases with advancing maternal age. Mechanisms responsible for these increased incidences of numerical chromosome abnormalities include embryonic aberrations arising via the transmission of pre-existing parental chromosome abnormalities to the gamete, e.g. balanced translocations, or be generated via failure of normal segregation of bivalents or sister chromatids during gametogenesis, at fertilisation or during early embryonic development (King 1990). Even if they do not completely stop embryo development, it will alter transcriptional potential (McCoy *et al.,* 2015). In man, genetic screening of IVF embryos has revealed that most chromosome abnormalities are whole-chromosome abnormalities (including polyploidy), with the majority mitotic origin and not related to abnormalities of meiosis in oocytes from mothers of advancing age. In the study by McCoy and others, pre-implantation genetic screening patients with previous IVF failure had elevated rates of mitotic errors, while patients with recurrent pregnancy loss had elevated rates of meiotic errors, controlling for maternal age (McCoy *et al.,* 2015). In horses chromosomal abnormalities have been proposed as an important contributor to early embryonic death. Although the topic has been little studied, numerical chromosome abnormalities certainly arise in equine embryos (Rambags *et al*., 2015) and IVP is thought to increase. Advancing maternal age also affects cytoplasmic morphology as a result of relatively low expression of spindle assembly checkpoint proteins, resulting in a high incidence of spindle aberrations, which is considered another significant cause of chromosomal abnormalities (McCoy *et al*., 2015).

Oocyte mitochondrial quantity and function are affected by maternal age and IVP

A possible contributor to the maternal age related decrease in oocyte quality is a decline in mitochondrial quantity and function. Mitochondria are the structures within the (embryonic) cell that generate energy via oxidative phosphorylation. In addition to energy production, they are also involved in other metabolic processes such as steroid hormone production, β-oxidation, calcium homeostasis and processes associated with cell deterioration, including the production of reactive oxygen species (ROS) and apoptosis.

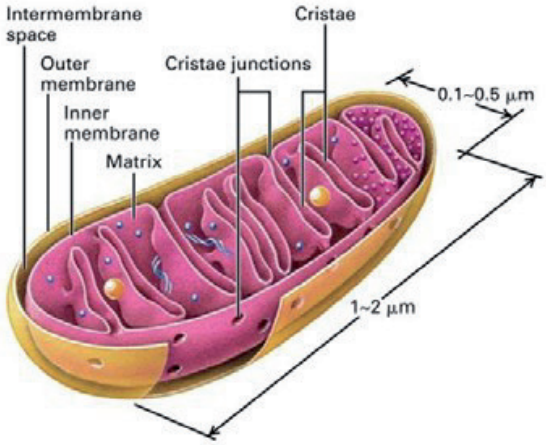

Figure 6. Cross section of a mitochondrion.

A species-specific minimum number of mitochondria in a mature oocyte is required for successful early embryonic development. Mouse oocytes are reported to require at least 50,000 - 200,000 mtDNA copies (Cao *et al.,* 2007; Cree *et al.,* 2008; Wai *et al.,* 2008; 2010), whereas bovine, porcine and human oocytes are thought to need between 100,000 and 700,000 mtDNA copies if they are to retain developmental competence (Steuerwald *et al.,* 2000; Reynier *et al.,* 2001; May-Panloup *et al.,* 2005; Almeida-Santos *et al.,* 2006; Spikings *et al.,* 2007; Zeng *et al.,* 2007). During early embryonic development, mtDNA replication is transiently arrested (St. John *et al.,* 2010) and the mtDNA copy number therefore either remains constant (e.g. mouse and rat; Thundathil *et al.,* 2005; Kameyama *et al.,* 2007; Wai *et al.,* 2010) or decreases over time (e.g. cow and pig; May-Panloup *et al.,* 2005; Spikings *et al.,* 2007). Mitochondrial replication is not reinitiated until the blastocyst stage in the cow and pig (May-Panloup *et al.,* 2005; Spikings *et al.,* 2007) or until gastrulation in the mouse (Facucho-Oliveira *et al.,* 2007). This temporary arrest in mitochondrial replication, despite a near exponential increase in the number of cells, underlines why the number of functional mitochondria present in a mature

oocyte is of critical importance for subsequent developmental competence (May-Panloup *et al.,* 2007).

An increase in the incidence of mitochondrial DNA (mtDNA) aberrations such as point mutations and deletions, and increased production and release of reactive oxygen species from damaged mitochondria, will further compromise mitochondrial function and initiate apoptosis of embryonic cells. Advancing maternal age and IVP both negatively influence mitochondrial function and the incidence of apoptosis (Seidler and Moley, 2015). Another effect of advancing maternal age and IVP is a reduction in mtDNA copy number in oocytes and embryos. For example, maturation of oocytes from young (21-89 months) bovine donors resulted in a mean increase in mtDNA copy number and ATP content, whereas maturation of oocytes from 'aged' (>89 months) bovine donors led to a decrease in both parameters (Iwata *et al.,* 2011). A similar effect was observed in equine oocytes after *in vitro* maturation (IVM), with oocytes retrieved from older mares (≥12yr) suffering a significant decrease in mtDNA copy number that was not seen in oocytes from younger mares (<12yr) (Rambags *et al.,* 2014).

Although increasing maternal age led to a reduction in mtDNA copy number in oocytes after *in vitro* maturation, it is not known which factors are responsible for this reduction. In this respect, it is logical that expression of genes involved in mitochondrial replication, such as mitochondrial transcription factor *(TFAM)* (Facucho-Oliveira *et al.,* 2007), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single stranded DNA binding protein (*SSB*) (St. John *et al.,* 2010) could play a role by failure to restart mtDNA replication at the appropiate time or protect existing mtDNA copies; it is also possible that dysfunction of genes involved in energy production and oxygen free radical scavenging could both result from and predispose to further mitochondrial damage and loss of function. Glutathione peroxidase (*GPX*) and ATP synthase (*ATP-synth_F6*) are enzymes involved in balancing the generation and decomposition of ROS (Ufer and Wang, 2011) to help prevent ROS levels reaching a point likely to induce oxidative damage. For example, in oocytes collected from women >35 years of age an increased expression of *ATP-synth_F6* has been reported (Wang *et al.,* 2009). By adjusting the culture media used for oocyte maturation, it might be possible to improve fertilization rates and number if offspring produced from the oocytes of 'aged' mares despite a reduction in mitochondrial number and function.

Cryopreservation of equine embryos

Cryopreservation of embryos is a useful solution for temporary unavailability or inadequate availability of synchronized recipient mares. It also allows transfer of embryos at a

desired time of year and enables shipment of embryos intercontinentally. Unfortunately, until recently only very early stage equine embryos, namely day 6-7 morulae or early blastocysts less than 300 µm in diameter resulted in acceptable pregnancy rates after freezing and thawing (Stout, 2012). For embryos larger than 300 µm (expanded blastocysts), pregnancy rates were under 25%. The reasons for the poor freezability of large equine embryos has been proposed to be related to formation of the acellular blastocyst capsule, which was hypothesized to hinder access of cryoprotectant to the embryonic cells (Bruyas *et al.,* 2000). The capsule is an acellular glycoprotein structure composed of mucin-like glycoproteins that develops around the embryo soon after its entry into the uterus at day 6.5 after ovulation (Flood *et al*., 1982) and increases in dry-weight until approximately day 18 (Oriol *et al*., 1993). From around day 18, the capsule begins to attenuate before losing continuity at around days 21-23 and disappearing completely by about day 28-30 (Stout *et al.*, 2005).

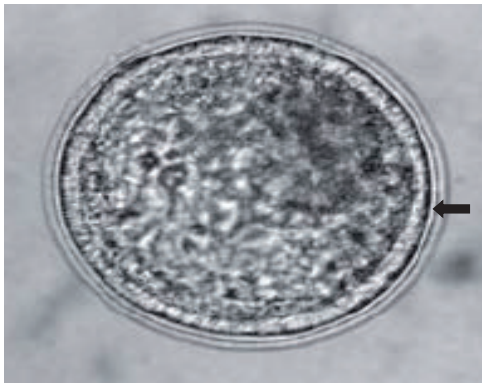

Figure 7. Equine early blastocyst surrounded by zona pellucida and capsule (arrow).

The poor permeability of the capsule has been proposed to hinder cryoprotectant accessand increase the risk of catastrophic intracellular ice crystal formation within embryonic cells during cryopreservation, as evidenced by improved embryo survival rates after thinning of the capsule by trypsin treatment (Legrand *et al*., 2002), and amelioration of damage to the embryonic cytoskeleton (Tharasanit *et al.*, 2005). It has also been proposed that the large volume of fluid within the blastocoele of larger (> 300 µm) equine embryos (Choi *et al.,* 2011) and the much reduced surface area to volume ratio (Tharanasit *et al.,* 2005) were greater impediments to successful CPA entrance and cryopreservation studies demonstrating high survival rates of large equine blastocysts after puncture to remove fluid appears to confirm the importance of minimal blastocoele volume in improving cryopreservability (Choi *et al.*, 2011).

Another reason for the historical relatively low uptake of cryopreservation of equine embryos is that controlled rate freezing was relatively labour intensive and required an expensive programmable freezing machine. The advent of vitrification as a successful technique for cryopreserving oocytes and embryos (Vatja, 1998) has changed this paradigm, since the process is quick and involves minimal equipment. While good results have been reported after vitrification and warming of equine embryos (Eldridge Panuska *et al*., 2005), the high concentrations of cryoprotectants used mean that timings of exposure to the various equilibration and vitrification media are critical and has meant that in field results have been variable.

Scope of the thesis

This thesis aims to increase the understanding of the effects of maternal age and ART on the oocyte and early embryonic developmental competence. In Chapter 2, the mid-late gestation conceptus was monitored ultrasonographical (rectal and abdominal) in Dutch Warmblood mares. This study yielded growth curves for various fetal parameters as references for normal intra-uterine development. The curves should be helpful in identifying deviations from normal fetal growth that could arise in mares with poor endometrial quality, suffering from placental pathology or other disease but also for monitoring development of pregnancies derived from ART, for either intra-uterine growth retardation or excessive fetal growth as a result of epigenetic perturbance. In Chapters 3 and 4, mitochondrial quantity in and function of the oocyte and early embryo were investigated to assess their potential contribution to reduced developmental competence of *in vitro* produced embryos and embryos recovered from older mares. In Chapter 5, the type and amount of damage within embryonic cells resulting during cryopreservation were studied. These results enable an easy way of monitoring freezing damage and thus validate potential improvements in freezing techniques. The described techniques are quicker and less extensive and expensive than waiting for the outcome of a large number of embryo transfers. Finally the results of these studies are summarized and discussed in Chapter 6 and new perspectives for further research are indicated.

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Chapter 2

Maternal age and parity influence ultrasonographic measurements of fetal growth in DutchWarmblood mares

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ABSTRACT

Ultrasonographic examination of the equine fetus in mid-late gestation is usually performed only if there are concerns about fetal or maternal health. Even then it is difficult to determine whether development is 'normal' for gestational age because the reference values include considerable error margins. This study examined maternal factors that influence fetal growth with the aim of producing more precise late gestation fetal growth curves for Dutch Warmblood horses. Fetal development was monitored at 2-week intervals from day 100 of gestation until term in 32 mares ranging from 4 to 18 years in age; seven of the mares were primiparous. Transrectal and/or transabdominal ultrasonographic measurement of the fetal eye orbit, cranium, aorta, heart rate and of the combined thickness of uterus and placenta (CTUP) were performed using a portable ultrasound machine equipped with 6 MHz linear and 3.5 MHz curved array probes.

During days 100–250 of gestation, the CTUP was thicker in primiparous than multiparous mares ($p < 0.05$). After day 220 the maximum cross-sectional area, but not diameter, of both the eye orbit and cranium were also greater in primiparous than multiparous mares ($p < 0.05$). Fetal aorta diameter was not influenced by parity but was affected by maternal age, being smaller in mares $\geq$15 years of age than younger animals ($p < 0.05$). Only biparietal cross-sectional surface area and aorta diameter increased linearly throughout late gestation. However, even allowing for the effects of parity and maternal age, the late gestational variation in fetal size is such that serial measurements may be required to definitively identify abnormal development.

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1. Introduction

Since its first description for the early detection of pregnancy in mares (Chevalier and Palmer, 1982), real time (B-mode) ultrasonography has become established as invaluable for detecting pregnancy, demonstrating pregnancy loss (Ball et al., 1986; McKinnon et al., 1987; Squires et al., 1988; Chevalier-Clément, 1989; Sertrich, 1997; Madill, 2002) and identifying multiple conceptus vesicles early enough to allow reduction to a singleton by manual crushing (Roberts, 1982; McKinnon et al., 1987; Squires et al., 1988; Macpherson and Reimer, 2000). Generally, 2–4 scans are performed during the first 45 days of gestation, the period when pregnancy loss is most common (Morris and Allen, 2002). Currently, it is popular to perform an additional fetal examination between days 60 and 70 to determine gender (Curran and Ginther, 1989; Sertrich, 1997; Mari et al., 2002). Otherwise, ultrasonographic examination of the fetus after day 45 is uncommon unless there are indications that all may not be well (e.g. the mare exhibits behavioural oestrus, vaginal discharge, premature mammary development, lactation or prolonged gestation). This contrasts markedly with the approach in human medicine, where routine fetal ultrasonography is performed much later in development (11–14 weeks and 20–22 weeks; Vankayalapati and Hollis, 2004) and aims predominantly to determine whether the fetus is developing normally; this includes examination for abnormal organ development, markers that correlate with developmental abnormalities (e.g. nuchal translucency for Down's syndrome: Snijders et al., 1999; Vankayalapati and Hollis, 2004) and evidence of intrauterine growth retardation (IUGR). Because IUGR is associated with a higher risk of fetal mortality and increased morbidity for a range of conditions, if a human fetus falls below the 10th percentile for estimated fetal weight at a given gestational age, fetal growth is monitored by serial measurement of parameters such as biparietal diameter, head circumference, abdominal circumference and femur length (Baschat and Hecher, 2004; Lerner, 2004).

It could be argued that there is little rationale for monitoring equine fetal development in mid-late gestation, since abnormalities are uncommon and the options for intervention are limited. In addition, until relatively recently, while most veterinarians had an ultrasound probe suitable for transrectal examination, few had equipment suitable for examining an equine fetus transabdominally. In this respect, from approximately 3 months of gestation, the only structures that can be reliably imaged transrectally are the uterine wall and placenta in the region of the cervix (Renaudin et al., 1997, 1999, 2000), and the fetal eye (vitreous body) and head if the fetus is in anterior presentation (Pantaleon et al., 2003; Bucca et al., 2005; Turner et al., 2006). On the other hand, examination of the caudal uteroplacental segment is clinically relevant because ascending placentitis is a common cause of abortion beyond day 100 of gestation (Hong et al., 1993; Madill, 2002; Smith et al., 2003), that may be amenable to treatment if detected early (for review see Macpherson, 2005). Moreover, ultrasonographically detected thickening, oedema and/or separation of the placenta may be useful indicators of placentitis or impending abortion (Renaudin et al., 1997, 1999; Kelleman et al., 2002; Morris et al., 2007). Assessing fetal development during late gestation may also be a valuable tool for identifying foals at increased risk of perinatal disease (Pantaleon et al., 2003; Bucca et al., 2005). In this respect, the equine fetus can be examined transabdominally throughout the latter two thirds of gestation with the help of a low frequency transducer (2–3.5 MHz: Pipers and Adams-Brendemuehl, 1984; Reef, 1998; Renaudin et al., 2000). Indeed, previous studies have provided useful data on fetal growth in Thoroughbreds, Quarter horses, Standardbreds and Shetland ponies during months 4–11 of gestation via measurements of the eye orbits, cranium, abdomen, thoracic width, intercostal spaces and aorta (see Reef et al., 1995; Reef, 1998; Renaudin et al., 2000; Pantaleon et al., 2003; Bucca et al., 2005; Turner et al., 2006). However, a plateau in the growth of many fetal parts and considerable variation in fetal size during the last 1–2 months of gestation limit the utility of many ultrasonographic parameters for assessing normality of fetal development, or for estimating gestational age if it is not known. Another problem is that fetal development in the Dutch Warmblood mare has never been studied ultrasonographically; therefore no fetal growth curves are available for this breed. While the documented variability in fetal size may in part be due to a combination of the relatively low number of animals examined, long inter-examination intervals (Renaudin et al., 1997, 1999, 2000; Pantaleon et al., 2003; Bucca et al., 2005; Turner et al., 2006) or the absence of serial examinations of individual fetuses (Pipers and Adams-Brendemuehl, 1984; Adams-Brendemuehl and Pipers, 1987;

Reef, 1998), it may also reflect considerable differences in fetal growth between mares of different breeds, age, size and parity, since these factors significantly influence foal birth weight (Allen et al., 2002; Wilsher and Allen, 2002; Forhead et al., 2004; Ousey et al., 2004; Turner et al., 2006). The aim of the current study was to monitor mid-late gestation fetal growth longitudinally in a cohort of Dutch Warmblood mares, to identify maternal factors that influence fetal growth and parameters other than aorta diameter (Pipers and Adams-Brendemuehl, 1984) that increase linearly in size during late gestation, and finally to produce fetal growth curves with smaller error margins. Measurements would be performed with a simple, portable ultrasound machine of a type affordable for most practices.

2. Materials and methods

2.1. Animals

This study was performed using 32 pregnant Dutch Warmblood mares ranging in age from 4 to 18 years (8.5 ± 0.7 years; median ± S.E.M.) and weighing between 616 and 707 kg (661.3 ± 14.6 kg; mean ± S.E.M.) at the beginning of the study. Seven of the mares were primiparous, i.e. carrying their first foal. The mares were maintained in three groups in loose yards during autumn and winter and at grass during the spring and summer. During the autumn-winter housed period, the mares were fed hay and concentrates. Mares were inseminated with chilled, transported semen from 15 commercial stallions between May 16th and August 30th, and the day of ovulation was recorded during ultrasonographic examinations of the ovaries performed every second day (i.e. the true ovulation date could have been up to 2 days previously). Every two weeks from day 100 of gestation ultrasonographic examinations were performed and the mares were weighed by placing them on a weight scale (W9000: Welvaarts BV, Den Dungen, The Netherlands). The foals were weighed with the same system by placing them in a special box on the weight scale, immediately after birth.

Fetal parameters were included in statistical analyses only if the pregnancies met the following criteria:

a. Healthy foal delivered at full-term (≥320 days of gestation)
b. Puerperium: the fetal membranes were expelled within 3 h after foaling without manual assistance or oxytocin administration
c. The fetal membranes were intact and had a normal appearance when examined after expulsion:
   i. smooth, whitish allantoic surface containing prominent vessels,
   ii. reddish velvet-like chorionic surface,
   iii. bluish-white, translucent amnion
   iv. no extensive twisting of umbilical cord (Schlafer, 2004)
d. Normal fetal parameters after foaling, as detailed by Koterba (1990)

During the course of the study, two mares aborted for reasons unrelated to the study (one as a result of mycotic placentitis and one due to umbilical cord torsion) and their measurements were therefore excluded from statistical analysis.

2.2. Ultrasonographic examinations

Transrectal and transabdominal ultrasonographic examination of the fetus and placenta was carried out at 2-week intervals from day 100 of gestation until parturition. Ultrasonography was performed using a standard ultrasound machine (Parus 240: Pie Medical, Maastricht, The Netherlands) equipped with a 6 MHz linear array transducer for transrectal examination and a 3.5 MHz curvilinear transducer for transabdominal examination. On the day prior to each transabdominal examination, the ventral abdominal mid-line was clipped free of hair from the mammary glands to the xiphoid sternum. Immediately prior to fetal examination, the ventral abdomen was washed with warm water, and ultrasound-coupling gel was applied liberally. The parameters measured were:

2.2.1. Combined thickness of uterus and placenta (CTUP)

The CTUP was measured transrectally close to the cervical–placental junction where a branch of the uterine artery could be seen coursing between uterus and bladder, as described by Renaudin et al. (1997), by using electronic callipers of the ultrasound machine and displayed in millimeters (mm). Both ventral and dorsal CTUP were measured four times, and the means of the replicates were recorded.

2.2.2. Fetal eye orbit dimensions

Fetal eye orbits were imaged transrectally, except for in a limited number of cases when the fetus was in posterior presentation relatively late in gestation and the orbits could only be imaged transabdominally. All measurements were made at the maximum orbital cross-section, using the lens capsule and inner margins of the vitreous body as landmarks. The length and width of the orbit were measured in perpendicular directions, as described by Renaudin et al. (2000) (see Fig. 1) by the use of electronic callipers of the ultrasound machine, and the mean of these two figures was recorded as "diameter" and displayed in mm. In addition, because diameter is not the best predictor of the growth of a spherical structure (Lofstedt and Ireland, 2000), the circumference (in mm) and surface area of the orbital cross-section (in mm^2) were measured by tracing around the inner margins of the vitreous body with the electronic callipers of the ultrasound machine and applying software installed in the ultrasound machine. Each measurement was duplicated and, if there was a significant discrepancy, the measurements were repeated; recorded figures were the mean of all replicates.

2.2.3. Fetal biparietal dimensions

The diameter of the fetal cranium (the biparietal diameter) was measured as described by Renaudin et al. (2000), i.e. where the maximum biparietal diameter was ovoid, and with the ultrasound calipers placed on the outer margin of the cranium nearest the transducer, and the inner margin of the cranium farthest from the transducer (see Fig. 2). The diameter measurement was performed twice and displayed in mm. The circumference (in mm) and cross-sectional surface area (in mm^2) were measured by tracing around the outer margins of the cranium with the electronic callipers of the ultrasound machine and applying software installed in the ultrasound machine. Biparietal dimensions were mea-

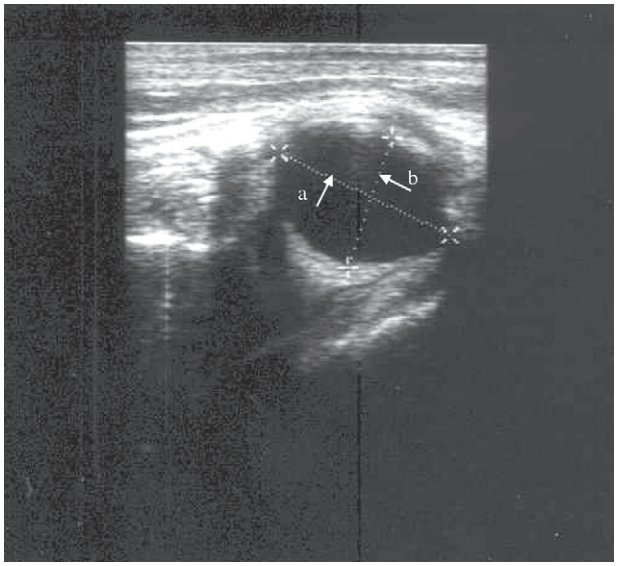

Fig. 1. Transrectal ultrasonographic picture of the fetal eye at 326 days of gestation. a = length of fetal eye orbit; b = width of fetal eye orbit.

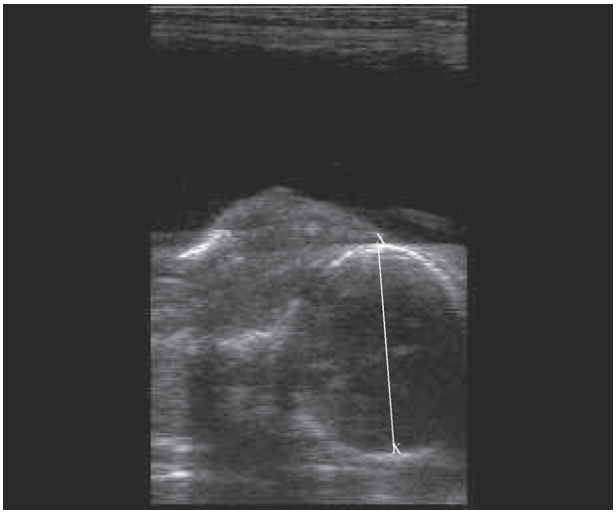

Fig. 2. Transrectal ultrasonographic picture of a measurement of fetal biparietal diameter. X marks the placement of the callipers.

sured transrectally except when the fetus was in posterior presentation or when biparietal diameter exceeded the maximum that could be measured with the 6 MHz probe; in these cases, measurement was performed transabdominally.

2.2.4. Fetal heart rate

After locating the fetal heart transabdominally using B-mode ultrasound, heart movements were recorded using M-mode. Fetal heart rate was calculated over two full cardiac cycles using software built-in to the ultrasound machine. Fetal heart rate measurement was repeated twice during the 20–30 min. examination.

2.2.5. Fetal aorta diameter

The aorta was located in a longitudinal section of the fetal thorax and distinguished from the trachea and vena cava by its pulsatility. As described by Renaudin et al. (2000), aorta diameter was measured close to its exit from the heart, during systole, by using the electronic callipers of the ultrasound machine and displayed in mm; the cine-loop function of the ultrasound machine was used to ensure that the maximum/systolic diameter was selected. Measurement of aorta diameter was repeated three times, and the mean was recorded.

2.3. Statistical analysis

Statistical analysis was performed using "The R Project for Statistical Computing" software (R foundation for statistical computing, Vienna, Austria 2005). Fetal growth curves were constructed using a linear mixed-effects model (Pinheiro and Bates, 2000) that included a random mare effect and an autocorrelation structure of order 1, for the following parameters: dorsal and ventral CTUP, eye orbit diameter, eye orbit cross-sectional surface area, biparietal diameter, biparietal cross-sectional surface area, aorta diameter and heart rate. The starting model included the effects of gestation stage, parity, mare's age and parity-gestation stage interaction. Akaike's Information Criterion was used to determine which of the parameters should be included in the final model; Akaike's Information Criterion is an index that helps to determine which of a number of alternative statistical models best fits the data by including or excluding competing factors. The index takes into account both the statistical 'goodness of fit' and the number of parameters necessary to achieve this degree of fit. The lowest value

indicates the best model, i.e. the one including the fewest parameters that still provides an adequate fit to the data with a normal distribution (Everitt, 1998). The preliminary models were based on calculations that included gestation length, (gestation length)^2, parity, mare's age and parity: gestation length. Factors that significantly influenced fetal size parameters ($p < 0.05$) were included in the final model. A 'random mare effect' was included to account for the fact that any measurement within a mare is necessarily related to both the preceding and subsequent measurements. The final model was used to calculate the profile ("average mare") and 95% confidence intervals.

Where a given maternal factor (e.g. age or parity; $p < 0.05$) significantly affected a fetal size parameter, separate curves were created for the two subsets of mares. With respect to parity and age, mares were classified as primiparous or multiparous and <15 or ≥15 years, respectively. Factors affecting uteroplacental thickness (CTUP) were identified similarly, after subjecting CTUP values to a natural logarithmic transformation to normalise their distribution. Finally, mathematical formulae and corresponding graphs were produced to demonstrate model fetal growth.

Descriptive parameters, such as gestation length, mare's weight and foal's weight were obtained by using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL), displayed as the mean ± S.E.M. and differences were considered significant when $p < 0.05$.

3. Results

Thirty of the mares foaled between May and September following a gestation of 339.4 ± 1.5 days (range 329–356 days); the remaining two mares aborted at 216 and 236 days, respectively. Since development in the mares that aborted could not be assumed to be normal, 399 sets of ultrasonographic measurements of fetal and placental dimensions from 30 mares were eventually used to create the fetal growth curves. Seven of the foaling mares were primiparous and 23 multiparous; 26 mares were younger than 15 years of age and four were 15 years of age or older. Mean mare body weight increased from 669.5 ± 11.5 kg (range 628–707 kg) at day 100–120 of gestation to 734.8 ± 16.0 kg (range 652–867 kg) at parturition (D330–350). There was no correlation between mare's body weight and fetal size parameters measured during this study. Foal birth weight ranged from 51 to 76 kg and did not differ significantly between primiparous and multiparous mares (58.4 ± 4.2 kg versus 62.3 ± 1.4 kg, respectively).

The following final model growth curves were created for the various fetal parameters (for abbreviations see below):

Dorsal CTUP (mm): $\ln(\text{CTUPd}) = 1.83 - 0.007x + 0.000025x^2$ (Fig. 3)
Ventral CTUP (mm): $\ln(\text{CTUPv}) = 2.007 - 0.012x + 0.000038x^2 - 0.37p + 0.0013x/p$ (Fig. 4)
Fetal eye orbit diameter (mm) $= -5.62 + 0.21x - 0.0003x^2$ (orbit diameter = (orbit length + orbit width)/2) (Fig. 5)
Fetal eye orbit cross-sectional area (mm^2) $= -5.20 + 0.065x - 0.00008x^2 + 0.798p - 0.0041x/p$ (Fig. 6)
Fetal biparietal diameter (mm) $= -26.64 + 0.597x - 0.0008x^2$ (Fig. 7)
Fetal biparietal cross-sectional surface area (mm^2) $= -21.25 + 0.22x + 5.68p - 0.03x/p$ (Fig. 8)
Fetal aorta diameter (mm) $= -3.67 + 0.077x - 0.088z$ (Fig. 9)
Fetal heart rate (beats per min) $= 167.97 - 0.249x$ (Fig. 10)
x = current gestational length
p = parity: 0 = primiparous and 1 = multiparous
z = age group: 0 = ≥15 years and 1 = <15 years

The CTUP just cranial to the cervix was consistently thicker on the dorsal than on the caudo-ventral aspect, and neither measurement increased markedly until day 200 of gestation (Figs. 3 and 4). Between approximately day 250 of gestation and term, utero-placental thickness increased rapidly, as indicated by linear increases in the log of both the dorsal and ventral CTUP measurements. Between days 100 and 250 the ventral CTUP was significantly thicker in primiparous than multiparous mares ($p = 0.006$: Fig. 4).

Fetal eye orbit diameter increased linearly until approximately day 240 of gestation. Thereafter, the rate of increase slowed to a plateau from around day 300 (Fig. 5). Fetal eye orbit cross-sectional area showed a similar trend (Fig. 6), and was significantly influenced by parity ($p = 0.03$), being smaller in primiparous than multiparous mares until approximately day 200 of gestation, and greater in primiparous mares thereafter (Fig. 6).

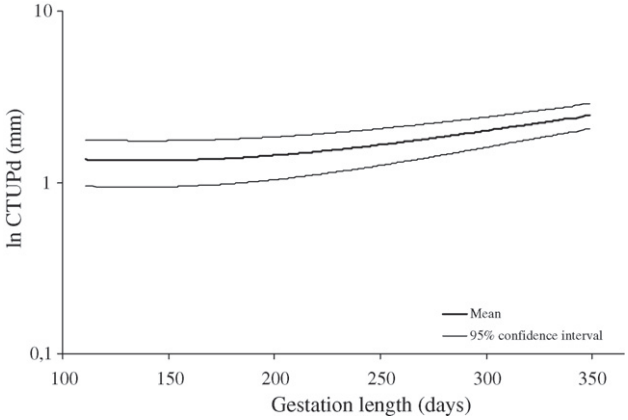

Fig. 3. Mean and 95% confidence limits for the combined thickness of uterus and placenta measured dorso-cranially from the cervical pole (dorsal CTUP) in Dutch Warmblood mares from day 100 of gestation to term. The predicted mean growth curve is described by the formula: ln (CTUP) (mm) = 1.83–0.007x + 0.000025x^2; where x = current gestational length in days.

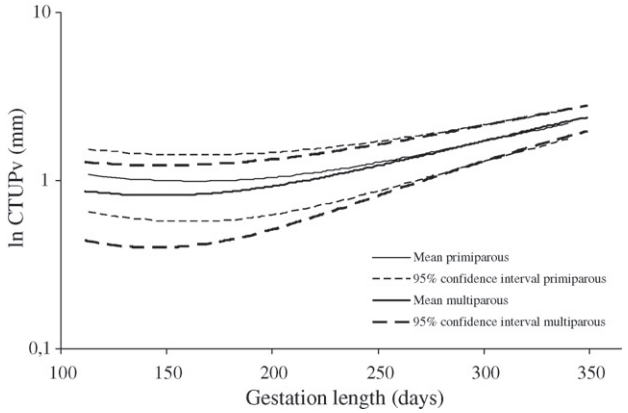

Fig. 4. The combined thickness of uterus and placenta measured ventrally from the cervical pole (ventral CTUP) in primiparous and multiparous Dutch Warmblood mares from day 100 of gestation to term (mean, 5th and 95th percentiles). The predicted mean growth curves are described by the formula: ln (CTUP) (mm) = $2.007 - 0.012x + 0.000038 \times 2 - 0.37p + 0.0013x/p$, where x = current gestational length in days, and p = parity (0 = primiparous; 1 = multiparous).

Fetal biparietal diameter also increased linearly during mid-gestation before plateauing during the last 3 months (Fig. 7). However, the fetal biparietal cross-sectional surface area increased linearly throughout gestation and was influenced by parity ($p = 0.02$), being larger in the fetuses of primiparous compared to multiparous mares after day 200 of gestation (Fig. 8).

Fetal aorta diameter increased linearly throughout the period of gestation examined and was influenced by mare age ($p = 0.02$); the fetal aorta in mares ≥15 years of age was smaller than in younger mares (Fig. 9). Fetal heart rate decreased linearly with advancing gestational age, from 144.7 ± 3.7 beats per min (bpm) at day 100 to 77.1 ± 3.8 bpm at term, but was not affected by either maternal age or parity (Fig. 10).

4. Discussion

This study demonstrates that the following parameters for examining equine placental and fetal growth are affected by maternal age or parity: CTUPv (D100-D250 of gestation) fetal eye cross-sectional

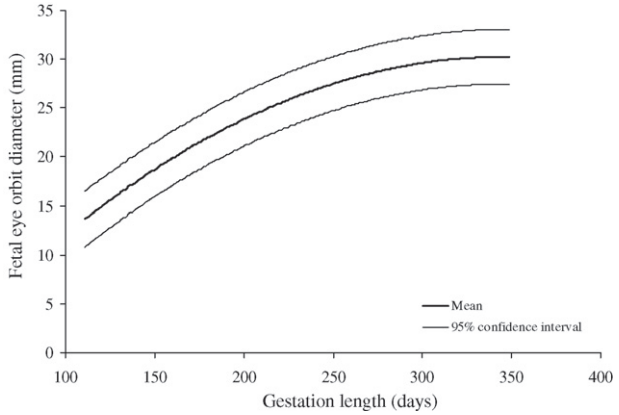

Fig. 5. Fetal eye orbit diameter [(length + width)/2] measured in Dutch Warmblood mares from day 100 of gestation to term (mean, 5th and 95th percentiles). The predicted mean growth curve is described by the formula: Fetal eye orbit diameter (mm) = $-5.62 + 0.21x - 0.0003x^2$ where x = current gestational length in days, and fetal eye orbit diameter (mm) = (length + width)/2.

surface area and fetal biparietal cross-sectional surface area by parity and fetal aorta diameter by maternal age. However, it was also clear that even when the effects of parity and maternal age were accounted for, there was still considerable variation in fetal size during late gestation due to a low number of mares per group (7 primiparous mares and 4 mares ≥15 years of age). This means that while the curves created are useful for monitoring the development of a single fetus of known gestational age, they provide only a rough indication when attempting to estimate fetal age or predict foaling date during late gestation. Technically, it was useful that the fetus could be imaged transabdominally throughout gestation with inexpensive, portable ultrasound equipment. However, the penetration of the 3.5 MHz probe did become limiting during very late gestation (>D300) with regard to measuring fetal biparietal diameter, fetal biparietal cross-sectional surface area and fetal aorta diameter, particularly in mares with substantial ventral oedema. In this period (D301–349) 79 examinations were performed of which 18 (aorta diameter) and 55 (fetal biparietal cross-sectional surface area) data could

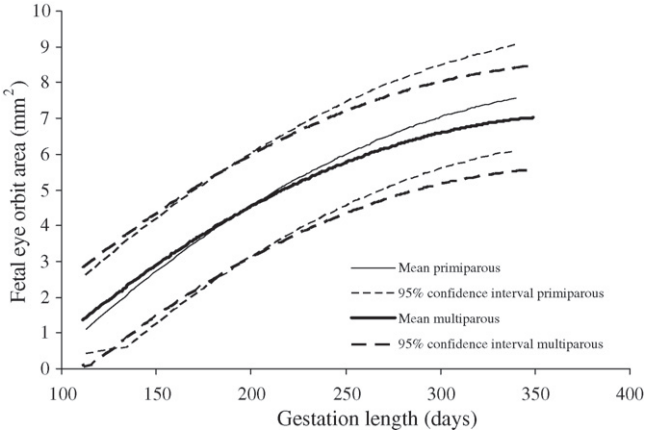

Fig. 6. Cross-sectional surface area of the fetal eye orbit in primiparous and multiparous Dutch Warmblood mares from day 100 of gestation to term (mean, 5th and 95th percentiles). The predicted mean growth curves are described by the formula: Fetal eye orbit cross-sectional area (mm^2) = $-5.20 + 0.065x - 0.00008x^2 + 0.798p - 0.0041x/p$ where x = current gestational length in days, and p = parity (0 = maiden; 1 = multiparous).

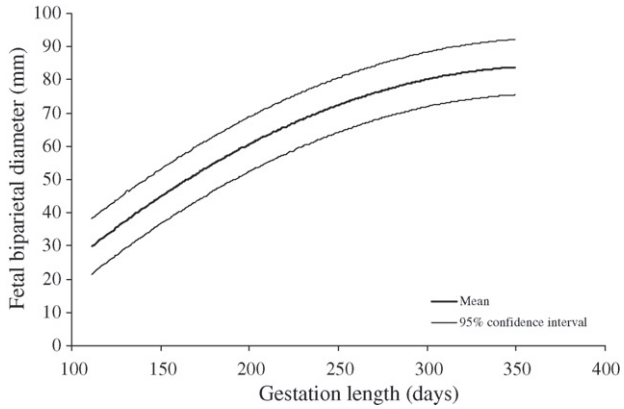

Fig. 7. Fetal biparietal diameter in Dutch Warmblood mares during day 100 of gestation to term (mean, 5th and 95th percentiles). The predicted mean growth curve is described by the formula: Fetal biparietal diameter (mm) = $-26.64 + 0.597x - 0.0008x^2$, where x = current gestational length in days.

not be obtained due to the limitations of the 3.5 MHz probe. To assess more accurate measurements a lower frequency probe would be suitable.

Wilsher and Allen (2006) showed that mare's body weight, at least when it changes suddenly, influences fetal development. In the current study, we did not find any correlation between mare's body weight and fetal size or growth. The major difference between the two studies is that the sudden, mid-gestation, illness-induced drop in maternal body weight in Wilsher and Allen's study could reasonably be expected to have resulted in compromised placental nutrient supply. In our study, it is possible that even though body weight differences between mares were appreciable (616–707 kg at D100 and 638–867 kg at term) they were insufficient to outweigh 'genetic' contributions to fetal size; in this respect, mares were all of the same 'type' (warmbloods), numbers were relatively small (30) and we did not include stallion size as a variable.

The gestation-related changes in the CTUP largely mirrored those reported previously by Renaudin et al. (1997) and Bucca et al. (2005); i.e. that CTUP does not noticeably increase until 5 months of gestation, with rapid thickening beginning at around 8 months. This increase in placental thickness

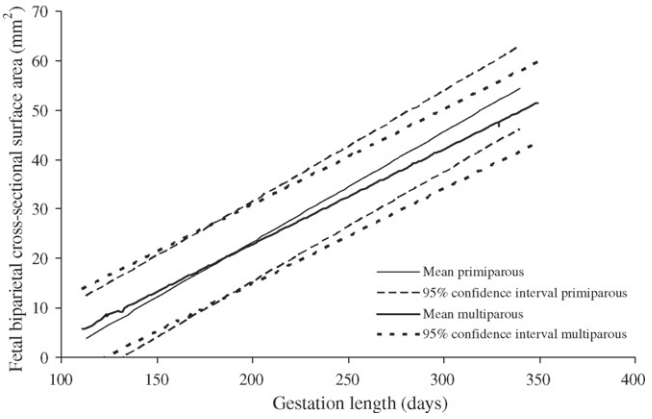

Fig. 8. Cross-sectional surface area of the fetal cranium in primiparous and multiparous Dutch Warmblood mares during day 100 of gestation to term (mean, 5th and 95th percentiles). The predicted mean growth curves are described by the formula: Fetal biparietal cross-sectional surface area (mm^2) = $-21.25 + 0.22x + 5.68p - 0.03x/p$; where x = current gestational length in days, and p = parity (0 = primiparous; 1 = multiparous).

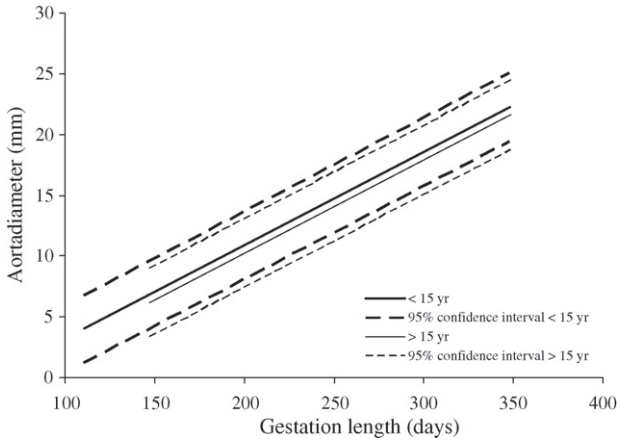

Fig. 9. Fetal aorta diameter in Dutch Warmblood mares less than 15 years of age and greater than or equal to 15 years of age (mean, 5th and 95th percentiles). The predicted mean growth curves are described by the formula: Fetal aorta diameter (mm) = −3.67 + 0.077*x* − 0.088*z*; where *x* = current gestational length in days, and *z* = age group (≥15 years = 0: < 15 years = 1).

has also been shown using scanning electron microscopy examination at different stages of gestation (Macdonald et al., 2000). Renaudin et al. (1997) proposed that the caudo-ventral CTUP should be used preferentially when assessing placental normality because it was the most repeatable measurement of utero-placental thickness. It was, therefore, interesting that in the current study the ventral CTUP was thinner in multiparous than primiparous mares during days 100–250 of gestation. However, there was little difference between the two groups in late gestation, because the ventral CTUP in multiparous mares thickened more rapidly than in primiparous mares from approximately day 200. The greater mid-gestation CTUP in primiparous mares was surprising given that overall placental size and weight is greater in multiparous than primiparous mares (Wilsher and Allen, 2002) and since Abd-Elnaeim et al. (2006) reported longer microplacentomes at 179 days of gestation albeit in a single multiparous mare. The relative thickness of the placenta in primiparous mares during mid gestation could relate to the greater depth of chorion reported for this category of mares (Wilsher and Allen, 2002; Abd-Elnaeim et al., 2006), or may reflect differing mechanical forces; the more pendulous abdomen in a multiparous mare may result in a deeper lying 3–8 month fetus that 'stretches' the uterus and placenta, an effect that may become less marked later in pregnancy.

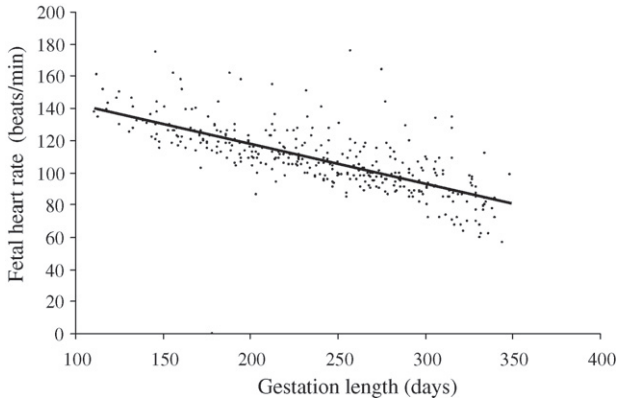

Fig. 10. Fetal heart rate in Dutch Warmblood mares from day 100 of gestation to term (raw data). The effect of gestational age is described by the formula: Fetal heart rate (beats per min) = 167.97 − 0.249*x*; where *x* = current gestational length in days.

CTUP measurements are considered useful indicators of placentitis or placental abnormality. For example, transabdominal measurement of a CTUP exceeding 2 cm in the gravid horn has been proposed to suggest a generalised placentitis (Reef, 1998), because it is more than 2 standard deviations thicker than the mean (12.6 ± 3.3 mm: Adams-Brendemuehl and Pipers, 1987). However, transabdominal examination of uteroplacental thickness requires a high quality ultrasound image, and is affected by the precise uterine location at which the measurement is performed and whether the fetus is in contact with the placenta at the time of measurement (Bucca et al., 2005). Transrectal assessment of the CTUP is easier, more repeatable and has the advantage that most placentitides begin in the region of the cervical pole. Using a branch of the uterine artery as an anatomical landmark, Renaudin et al. (1997, 1999) established a 95% upper confidence limit for the CTUP ventral to the cervical pole in the last month of gestation of 12 mm; higher values were proposed to indicate abnormalities. In the current study, two multiparous mares showed marked thickening and oedema of the intrapelvic uteroplacenta late in gestation (>D300; CTUPv 14–16 mm) without further clinical signs or post-partum evidence of placental pathology. In fact, other studies have also reported incidences of ultrasonographically detected placental thickening without pathology (Renaudin et al., 1997; Bucca et al., 2005). This suggests that while an elevated CTUP may suggest pathology, confirmation of placentitis will rely on the presence of other clinical signs (e.g. placental detachment, vaginal discharge, premature mammary development). Conversely, not all mares suffering from an ascending placentitis show ultrasonographically detectable placental thickening. Indeed, when LeBlanc et al. (2004) initiated placentitis by inoculating bacteria via the cervix, only 60% of the mares exhibited a CTUP greater than 12 mm prior to abortion. Nevertheless, the 12 mm calculated by Renaudin et al. (1997) as the 95th percentile above which placental pathology should be considered in lighthorse mares during the last month of gestation, also appears to apply to Dutch Warmblood mares; our study indicated a 95th percentile of 12.1 mm (8.03 ± 1.28 mm) in primiparous and 12.6 mm (8.31 ± 1.23 mm) in multiparous mares in the last month of gestation. Bucca et al. (2005) reported a mean and standard deviation of 15.8 and 5.6 mm, respectively, for standardbred mares.

The current study also revealed effects on fetal size of both mare age (aorta diameter) and parity (fetal eye orbit and biparietal cross-sectional surface area), even though neither age nor parity significantly influenced foal birth weight. Given that primiparous mares generally produce smaller and lighter foals than multiparous mares (Wilsher and Allen, 2002; Turner et al., 2006), it was surprising that fetal eye orbit and biparietal cross-sectional surface areas were larger in the primiparous mares ($p < 0.05$), i.e. the effect of parity was opposite to that anticipated. This finding also contrasts with a study of fetal growth in small ponies in which Turner et al. (2006) did not find any significant effects of parity on fetal eye-orbit length or width. Although the reason for the converse finding of larger fetal orbit and cranium in primiparous mares is not known and is based on a few mares, it does illustrate that not all ultrasonographic measurements of fetal size relate directly to foal birth weight. The fetal parameter reported to best predict foal birth weight is aorta diameter (Adams-Brendemuehl and Pipers, 1987; Reef et al., 1995). In the current study, aorta diameter was not affected by parity, but was affected by mare age; fetal aorta diameter in mares 15 years of age or older was lower than in younger mares ($p = 0.02$). In theory, this age-related difference in aorta diameter could represent a mild IUGR caused by poor placental development due to age-related endometrial degeneration (Bracher et al., 1996; Wilsher and Allen, 2002). However, the differences in fetal aorta diameter were not reflected by significant differences in foal birth weight (58.5 ± 4.0 kg (mares <15 years of age) versus 62.1 ± 1.7 kg (mares ≥15 years of age) or gestation length (347 ± 5.1 days in mares ≥15 years of age and 338.4 ± 7.8 days in mares <15 years of age). Whether these differences in fetal aorta diameter were caused by a possible age-related endometrial degeneration was not investigated by uterine biopsies of the mares (prior or after pregnancy).

Fetal heart rate is primarily dependent on fetal activity. However, persistent bradycardia or tachycardia is generally an indication of fetal stress, where bradycardia is often a result of CNS depression due to hypoxia (Baschat, 2004; Bucca et al., 2005). Previous studies have largely concentrated on the normal fetal heart rate and rhythm during very late gestation (e.g. Adams-Brendemuehl and Pipers, 1987; Reef et al., 1995). In this respect, the description in the current study of mean fetal heart rates that decline from 144.6 ± 3.7 bpm at day 100 of gestation to 77.1 ± 3.8 bpm at term (Fig. 10), together

with similar values reported by Bucca et al. (2005), provide a useful database for identifying heart rate abnormalities earlier in gestation.

In order to meaningfully monitor fetal development, or estimate fetal age during late gestation, parameters are required that show a steep linear increase during the period of interest. Previous studies have suggested that aorta diameter is the most useful indicator of fetal size in late gestation (Renaudin et al., 2000). Renaudin et al. (2000) also noted linear increases in biparietal diameter and estimated eye volume throughout gestation. In the current study, using more data points, biparietal diameter did not increase linearly throughout gestation but levelled off some 1–2 months before term (Fig. 7). However, the biparietal cross-sectional surface area did continue to increase linearly throughout pregnancy. Unfortunately, even where fetal size parameters increased linearly late in gestation there was considerable between-fetus variation. In essence, this means that a single measurement is insufficient to either estimate gestational age or, except in extreme cases, diagnose IUGR and identifies a foal likely to need extra post-partum care. For this reason, if there are doubts about fetal development it may be sensible to measure a number of parameters (e.g. aorta diameter, biparietal cross-sectional surface area) serially and determine whether the rates of growth follow the expected trends. A similar approach is used in human perinatology where it is accepted that, although every fetus follows its own growth curve, growth-restricted and macrosomic fetuses can be identified because their growth rates eventually diverge from predictions. In man, the best predictors of IUGR are abdominal circumference (Lerner, 2004), and fetal weight estimated on the basis of abdominal circumference, biparietal diameter, head circumference, and femur length (Gallivan et al., 1993; Mongelli and Biswas, 2001; Lerner, 2004; Vankayalapati and Hollis, 2004). Unfortunately, two of these parameters (abdominal circumference and femur length) cannot be measured during late gestation in horses. Further studies are therefore required to determine which combination of parameters best identifies abnormal equine fetal growth. In the meantime, combining as many measurements that show a linear increase (i.e. aorta diameter, biparietal cross-sectional surface area and, after 9 months, ventral CTUP) as possible, preferably at more than one examination, may allow more accurate monitoring of fetal development, as described for cattle (Kähn, 1994).

In conclusion, the current study demonstrated that aorta diameter, biparietal cross-sectional surface area and ventral CTUP increase linearly during late gestation. While these parameters should be of use for monitoring fetal growth and development, they are influenced by maternal age or parity, and vary considerably between individuals, probably due to a relatively low number of mares used in this study. Serial or combined measurements are therefore likely to be more informative than single examinations. In addition, while ultrasonographic examination of the CTUP is a useful part of investigating suspected placental pathology, ultrasonographically detected thickening in the absence of other clinical signs need not always be pathological.

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Chapter 3

Maternal age and *in vitro* culture affect mitochondrial number and function in equine oocytes and embryos

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Maternal age and *in vitro* culture affect mitochondrial number and function in equine oocytes and embryos

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Abstract. Advanced maternal age and *in vitro* embryo production (IVP) predispose to pregnancy loss in horses. We investigated whether mare age and IVP were associated with alterations in mitochondrial (mt) DNA copy number or function that could compromise oocyte and embryo development. Effects of mare age (<12 vs ≥12 years) on mtDNA copy number, ATP content and expression of genes involved in mitochondrial replication (mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*)), energy production (ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*)) and oxygen free radical scavenging (glutathione peroxidase 3 (*GPX3*)) were investigated in oocytes before and after *in vitro* maturation (IVM), and in early embryos. Expression of *TFAM*, *mtPOLB* and *ATP-synth-F6* declined after IVM ($P < 0.05$). However, maternal age did not affect oocyte ATP content or expression of genes involved in mitochondrial replication or function. Day 7 embryos from mares ≥12 years had fewer mtDNA copies ($P = 0.01$) and lower mtDNA : total DNA ratios ($P < 0.01$) than embryos from younger mares, indicating an effect not simply due to lower cell number. Day 8 IVP embryos had similar mtDNA copy numbers to Day 7 *in vivo* embryos, but higher *mtPOLB* ($P = 0.013$) and a tendency to reduced *GPX3* expression ($P = 0.09$). The lower mtDNA number in embryos from older mares may compromise development, but could be an effect rather than cause of developmental retardation. The general down-regulation of genes involved in mitochondrial replication and function after IVM may compromise resulting embryos.

Additional keywords: ATP, gene expression, mitochondrial quantity, mitochondrial replication.

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Introduction

Mares selected for breeding based on their performance in sports other than flat-racing (e.g. show jumping, dressage, polo) are often of advanced age (8–16 years old) at the onset of their breeding career. Unfortunately, fertility also begins to decline from around 12–14 years of age, as indicated by lower pregnancy rates on Day 15 after ovulation and an increased incidence of pregnancy loss, particularly in the Day 15–42 period (Morel *et al.* 2005; Allen *et al.* 2007). Embryo transfer (ET) is attractive because it enables sport and breeding to be combined and it can also help overcome one of the age-related contributors to reduced fertility, namely chronic endometrial

fibrotic degeneration (Carnevale and Ginther 1992). However, endometrial degeneration is probably not the primary contributor to the age-related decline in mare fertility. Embryos flushed from the oviducts of mares ≥20 years of age have been reported to undergo delayed cleavage and to contain fewer cells than those from younger mares (Carnevale and Ginther 1995), analogous to the situation in older compared with younger women (Baird *et al.* 2005). Combined with studies demonstrating that embryos from mares >18 years show lower survival rates than embryos from mares <6 years after transfer to young recipients (Ball *et al.* 1989), this indicates that the loss of developmental potential is intrinsic to the ovulated oocyte or arises during very early (intra-oviductal) embryo development. Further studies have clearly demonstrated that the principal contributor to the age-related decline in mare fertility is reduced oocyte quality, because oocytes from mares ≥15 years often exhibit morphological abnormalities associated with functional deficiencies that, in turn, contribute to a decrease in developmental competence (Carnevale and Ginther 1992; Carnevale 2008; Altermatt *et al.* 2012).

Another way of producing offspring from mares with impaired fertility, or without interrupting their sporting career, is *in vitro* embryo production (IVP). Despite the relatively low per oocyte success rates (18%–36% of recovered oocytes develop into a transferable blastocyst; Hinrichs *et al.* 2005; Galli *et al.* 2007), during the past 5 years IVP has become a commercially viable means of breeding horses. The increasing popularity of IVP despite the relatively high costs is explained by the number of transferable blastocysts per ovum pick-up (OPU) session (0.6–0.8; Galli *et al.* 2014), which compares favourably with embryo flushing in the field. Moreover, OPU–IVP is applicable to mares incapable of producing embryos in the normal manner (e.g. due to severe damage to the uterus or oviducts or because they repeatedly fail to ovulate normally; Galli *et al.* 2007) and/or when stallion semen availability or fertility is limiting (Lazzari *et al.* 2002).

In women, the principal symptom of the age-related reduction in oocyte quality is an increased risk of numerical chromosome abnormalities (aneuploidy) as a result of aberrant chromosome segregation (Franasiak *et al.* 2014). One of the proposed underlying predispositions to aneuploidy and compromised developmental competence of oocytes and embryos from older women is mitochondrial dysfunction. Compromised mitochondrial function appears to stem from defective mitochondrial replication (leading to a fall in mitochondrial numbers), an increase in the incidence of mitochondrial (mt) DNA aberrations, such as point mutations and deletions, and increased production and release of reactive oxygen species (ROS); Takeuchi *et al.* 2005; Carnevale 2008; Fragouli *et al.* 2011). The number of mitochondria in an oocyte can be estimated by analysis of mtDNA copy number because oocytes contain only one to two mtDNA copies per mitochondrion (Pikó and Matsumoto 1976; Pikó and Taylor 1987; Jansen 2000; Santos *et al.* 2006*b*; Jiao *et al.* 2007; Chiaratti and Meirelles 2010). For successful early embryonic development, a species-specific minimum number of mitochondria appears to be required; for example, mouse oocytes are thought to require at least 50 000–200 000 mtDNA copies (Cao *et al.* 2007; Cree *et al.* 2008; Wai *et al.* 2008, 2010), whereas bovine, porcine and human oocytes have been reported to require between 100 000 and 700 000 mtDNA copies if they are to retain developmental competence (Steuerwald *et al.* 2000; Reynier *et al.* 2001; May-Panloup *et al.* 2005*a*; Almeida-Santos *et al.* 2006*b*; Spikings *et al.* 2007; Zeng *et al.* 2007). However, during early embryonic development, mtDNA replication is transiently arrested (St. John *et al.* 2010) and therefore the mtDNA copy number either remains constant (e.g. mouse and rat; Thundathil *et al.* 2005; Kameyama *et al.* 2007; Wai *et al.* 2010) or decreases over time (e.g. cow and pig; May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007). Mitochondrial replication is not reinitiated until the blastocyst stage in the cow and pig (May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007) or until gastrulation in the mouse (Facucho-Oliveira *et al.* 2007). This temporary arrest in mitochondrial replication, despite a near exponential increase in the number of cells, underlines why the number of functional mitochondria present in a mature oocyte is of critical importance for subsequent developmental competence (May-Panloup *et al.* 2007).

The combination of advancing maternal age and IVP has been reported to negatively influence mtDNA copy number in oocytes and embryos. For example, maturation of oocytes from young (21–89 months) bovine donors resulted in a mean increase in mitochondrial copy number and ATP content, whereas maturation of oocytes from 'aged' (>89 months) bovine donors led to a decrease in both parameters (Iwata *et al.* 2011). Similarly *in vitro* maturation (IVM) of oocytes retrieved from older mares (≥12 years) led to a significant decrease in mitochondrial copy number that was not seen in younger mares (<12 years; Rambags *et al.* 2014).

Advancing maternal age and IVP have both been reported to induce alterations in mitochondrial function that can result in mitochondrial damage (Wilding *et al.* 2001; Wang *et al.* 2009). This manifests as a decrease in mtDNA copy number, a reduction in mitochondrial gene expression and an increased production of ROS within embryonic cells (Giritharan *et al.* 2007, 2010). Changes in mtDNA quantity can also result in alterations in the expression of genes involved in various aspects of the mitochondrial replication process, such as mitochondrial transcription factor (*TFAM*; Facucho-Oliveira *et al.* 2007), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*; St. John *et al.* 2010). Expression of these genes is normally upregulated when mtDNA replication is reactivated (Pikó and Taylor 1987; May-Panloup *et al.* 2005*b*; Thundathil *et al.* 2005; Spikings *et al.* 2007). Glutathione peroxidase (GPX) and ATP synthase (ATP synthase-coupling factor 6, mitochondrial-like (ATP-synth_F6)) are enzymes involved in balancing the generation and decomposition of ROS (Ufer and Wang 2011) to help prevent ROS levels reaching a point likely to induce oxidative damage. Increased expression of *ATP-synth_F6* has been described in oocytes collected from women >35 years, and in embryonic cells during IVP (Wang *et al.* 2009).

The aim of the present study was to determine whether advancing maternal age and IVP induce changes at the level of mitochondrial number and function in horse oocytes that may underlie reduced developmental competence. To this end we examined the effects of maternal age and IVP on mtDNA copy

number and the expression of genes involved in mitochondrial replication (*TFAM*, *mtPOLB*, *SSB*). Mitochondrial function was evaluated by measuring total oocyte ATP content and the expression of genes involved in energy production (*ATP-synth_F6*) and oxygen free radical scavenging (*GPX3*).

Materials and methods

Collection of cumulus–oocyte complexes

Ovaries were collected from 101 mares immediately after they had been killed and were divided into two groups based on donor mare age, namely younger (<12 years) or older (≥12 years), which was determined on the basis of the date of birth recorded in the mare's passport. The ovaries were transported to the laboratory at 30°C in a thermos flask and processed within 4 h postmortem. Cumulus–oocyte complexes (COCs) were collected as described previously (Tharasanit *et al.* 2006). Only oocytes with a complete multilayered cumulus investment (Hinrichs *et al.* 1993) were selected and maintained in HEPES-buffered M199 (GIBCO BRL Life Technologies; Bleiswijk, The Netherlands) supplemented with 0.014% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands). For each mare age group, the COCs recovered were divided randomly into two groups. In the first group (not-IVM), oocytes were immediately denuded by vortexing for 4 min in calcium- and magnesium-free Earle's balanced salt solution (EBSS; GIBCO BRL Life Technologies) containing 0.25% (v/v) trypsin-EDTA (GIBCO BRL Life Technologies); removal of all cumulus cells was confirmed by microscopic examination. The denuded oocytes were washed twice in phosphate-buffered saline (PBS; Sigma-Aldrich Chemicals) containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals) and placed individually in 10 µL lysis buffer (RLT + β-mercaptoethanol; Qiagen, Venlo, The Netherlands) in a 0.5-mL Eppendorf tube, snap-frozen and stored at −80°C until further processing (ATP measurement or DNA and RNA extraction). For the second group (IVM), COCs were incubated in groups of four to five for 30 h in 500 µL maturation medium consisting of M199 (GIBCO BRL Life Technologies) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; GIBCO BRL Life Technologies), 0.01 units mL^{-1} porcine FSH and 0.01 units mL^{-1} LH (Sigma-Aldrich Chemicals) in four-well plates (Nunc, Roskilde, Denmark) at 38.7°C in a humidified atmosphere of 5% CO_2 in air. After incubation, only the fully expanded COCs were transferred to EBSS containing 0.25% (v/v) trypsin-EDTA (GIBCO BRL Life Technologies) and denuded by vortexing for 4 min. The completely denuded oocytes were further processed as for Group 1 and stored at −80°C until ATP quantification or DNA and RNA extraction.

Embryo collection

Ten Dutch warmblood mares (age 4–22 years) were used to recover embryos on Day 7 after ovulation. All animal procedures were approved by Utrecht University's Institutional Animal Care and Use Committee (DEC 2007.III.02.036). During oestrus, the ovaries and uteri of the mares were examined daily by transrectal palpation and ultrasonography using a MyLab Five ultrasound machine (Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5-MHz linear array transducer. Once the dominant follicle exceeded 35 mm in diameter, ovulation was induced with intravenous injection of 1500 IU hCG (Chorulon; Intervet, Boxmeer, The Netherlands) and the mare was inseminated with >500 million motile spermatozoa from a single fertile stallion. Embryos were collected by non-surgical uterine lavage using three flushes with 1 L prewarmed (37°C) lactated Ringer's solution (LRS; Baxter, Lessines, Belgium) supplemented with 0.5% FCS (Greiner Bio-One, Alphen aan den Rijn, The Netherlands). Eight embryos each were recovered from younger (<12 years; average age 6.87 years, range 4–11 years) and older (>16 years; average age 19.25 years, range 17–22 years) mares. Embryos were washed 10 times with LRS to remove maternal cells and residual FCS before assessment under a dissecting microscope (SZ60; Olympus, Zoeterwoude, The Netherlands). Embryos were classified according to developmental stage (morula, early blastocyst or expanded blastocyst) and quality (1–4, good to degenerate), as described previously (Tremoleda *et al.* 2003), and their diameter was measured using a calibrated eyepiece micrometer. The embryos were then transferred with 10 µL LRS into 0.5-mL Eppendorf tubes, snap frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction.

Eight *in vitro* embryos were produced as described by Galli *et al.* (2001), with minor modifications. Briefly, COCs recovered from the ovaries of mares of mixed breed and unknown age that had been killed were subjected to IVM by incubating for 22–24 h in Dulbecco's modified Eagle's medium (DMEM)–F12 containing 10% serum replacement (SR; GIBCO BRL Life Technologies), ITS supplement (1.0 mg mL^{-1} insulin, 0.55 mg mL^{-1} transferrin, 0.5 µg mL^{-1} sodium selenite; Sigma-Aldrich Chemicals), 1 mM sodium pyruvate, 50 ng mL^{-1} epidermal growth factor (Sigma-Aldrich Chemicals), and 0.1 IU LH and FSH (Pergovet, Serono, Italy). Next, oocytes were denuded and returned to maturation medium for a further 2–4 h (i.e. up to 26 h maximum IVM). Thirty-two oocytes with an extruded first polar body were then fertilised by intracytoplasmic sperm injection (ICSI) with spermatozoa selected by Redigrad (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation. Sperm injection was performed using a Piezo-driven unit (PrimeTech, Tsuchiura-shi, Japan) fixed on a micromanipulator equipped with a 37°C heated stage. Oocytes were held with the polar body oriented at the 6- or 12-o'clock position using a holding pipette with an inner diameter of 50 µm and outer diameter of 150 µm and injected with a blunt-ended needle with an inner diameter of 5 µm. Only motile spermatozoa were selected for ICSI and immobilised by two or three Piezo pulses before injection. The resulting presumptive zygotes were cultured in synthetic oviductal fluid (SOF) supplemented with minimum essential medium (MEM) essential and non-essential amino acids, glutamine and BSA and examined for cleavage on Day 2 after injection; those showing development to the 2-cell stage or more (26/32; 81.25%) were cultured *in vitro* for an additional 6 days, with the culture medium refreshed on Days 4 and 6 (Galli *et al.* 2007). On Day 8 of development, eight of the 12 resulting embryos (12/32; 37.5%) were washed twice in PBS containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals) and transferred individually to DNase–RNase-free tubes

in RLT buffer (Qiagen, Milan, Italy), snap-frozen in liquid nitrogen and stored at −80°C.

RNA and gDNA extraction and cDNA synthesis

Total RNA and genomic (g) DNA were extracted from groups of pooled oocytes (nine per group) and from individual embryos in 350 or 600 µL RLT buffer, respectively, using an AllPrep DNA/RNA/Protein Mini Kit and subjected to on-column DNase I digestion using an RNase-Free DNase Set (both from Qiagen), as described previously (Paris *et al.* 2011). DNA was eluted in 50 µL elution buffer (Qiagen). RNA was eluted in 35 µL RNase-free water (Qiagen) and then transcribed into cDNA. For conventional polymerase chain reaction (PCR), the total reaction volume was 25 µL, containing 1 µL cDNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.5 µM forward primer, 0.5 µM reverse primer and 0.625 U HotStarTaq DNA polymerase (all Qiagen, except dNTPs). The PCR cycle conditions consisted of 15 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at the primer-specific annealing temperature (Table 1) and 1 min at 72°C, with a final extension for 10 min at 72°C. Products were visualised on 1% agarose gels. If suitable for PCR amplification and free of gDNA contamination, both +RT (with RT) and –RT (without RT) cDNA samples were diluted 1 : 9 and frozen at −20°C until further quantitative reverse transcription–polymerase chain reaction (qRT-PCR) analysis.

DNA quantification

Total cell number was estimated by quantifying total gDNA with a Quant-iT PicoGreen dsDNA assay kit (Molecular Probes, Invitrogen, Eugene, OR, USA). A standard curve ranging from 25 ng mL^{-1} to 2.5 pg mL^{-1} was created via a 1 : 9 dilution series. Briefly, 50 µL Pico Green reagent was mixed with 9.95 mL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and 40 µL was added to 20 µL sample (standard curve or embryo extract) per well in a FLUOTRAC 600 96-well microplate (Greiner Bio-One, Frickenhausen, Germany). During preparation and plate setup, the materials were protected from light. Fluorescence was measured using a multimode detector (Beckman Coulter DTX 880; Beckman, Woerden, The Netherlands) and the Fluorescein Top Read software program for Anthos Multimode detectors (Anthos Microsystem, Krefeld, Germany).

qRT-PCR

Primers for the *TFAM*, *mtPOLB*, *SSB*, *ATP-synth_F6* and *GPX3* genes were optimised and tested for specificity using an iQ5 RT-qPCR Detection System with iQ5 Optical System Software v2.0 (Table 1; BioRad, Hercules, CA, USA); equine kidney served as the positive control tissue.

One hundred and sixty-five oocytes were recovered from younger mares (<12 years) and 170 were recovered from older mares (≥12 years). Seventy-seven oocytes from younger mares and 92 oocytes from older mares were analysed directly after harvesting from follicles, whereas 88 from younger mares and 78 from older mares were subjected to IVM before analysis. The same oocytes and embryos were used to study mtDNA copy number and to examine the relative expression of genes associated with mitochondrial replication or function.

Quantitative real-time PCR was performed separately for oocytes and embryos, but using the same equipment, settings

Table 1. Primers used for gene amplification in quantitative reverse transcription–polymerase chain reaction
T_A, annealing temperature; F, forward; R, reverse

Symbol	Gene name	GenBank Accession no.	Primer sequence (5′–3′)	Product size (bp)[C]	T_A (°C)
PGK1	Phoshoglycerate kinase 1	XM_001502668	F: 5′-CAAGAAGTATGCTGAGGCTG-3′ R: 5′-AGGACTTTACCTTCCAGGAG-3′	260	57.0
SRP14	Signal recognition particle 14kDa	XM_001503583	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	100	55.0
RPL4	60S ribosomal protein L4-like	XM_001497094	F: 5′-CATCCCTGGAATTACTCTGC-3′ R: 5′-CGGCTAAGGTCTGTATTGAG-3′	203	61.5
TFAM	Transcription factor A, mitochondrial	NM_001034016	F: 5′-GGCAGGTATACAAGGAAGAG-3′ R: 5′-GTTATAAGCTGAGCGAGGTC-3′	170	56.5
mtPOLB	DNA polymerase subunit gamma-2, mitochondrial	NM_015810	F: 5′-CCGAGTAAGGAACAGCTAGT-3′ R: 5′-ACTCCAATCTGAGCAAGACC-3′	155	57.5
SSB	Mitochondrial single stranded DNA binding protein	XM_003364867	F: 5′-CATGAGACAGGTGGAAGGAA-3′ R: 5′-GATATGCCACATCTCTGAGG-3′	167	57.1
ATP-synt_F6	ATP synthase-coupling factor 6, mitochondrial-like	XM_001499999	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	148	59.5
GPX3	Glutathione peroxidase 3 (plasma)	XM_001917631	F: 5′-ACTCACCCTCAAGTATGTCC-3′ R: 5′-CCAGTATGTCCATCTTGACG-3′	289	58.9
mtDNA	Mitochondrial DNA	NC_001640.1	F: 5′-CATGATGAAACTTCGGCTCC-3′ R: 5′-TGAGTGACGGATGAGAAGGCAG-3′	118	67.7

and software described by Paris *et al.* (2011). The total reaction volume was 25 μL per well, and included 1× iQ5 SYBR Green Supermix (BioRad), 0.5 μM forward primer and 0.5 μM reverse primer (Ocimum Biosolutions, IJsselstein, The Netherlands). Each well included 10 μL standard or sample, and the following were included in the final plates: (1) standard curve: 100 fg–6.4 ag fivefold dilution series ($n = 7$); (2) 1 : 9 diluted sample cDNA (pooled oocytes $n = 24$; embryos $n = 24$); (3) 1 : 9 diluted –RT sample (pooled oocytes $n = 24$; embryos $n = 24$); and (4) DNase and RNase-free water (Invitrogen) as a no-template control.

All samples except the –RT samples were run as duplicates. Both frozen–validated and freshly prepared standards were included on each plate. Because of space limitations, –RT samples for the embryos were run on a separate plate in a subsequent run on the same day but using identical standards. The PCR cycle conditions consisted of 4.5 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at optimal annealing temperature (T_A; Table 1) and 30 s at 72°C, during which fluorescence was acquired, followed by a melting curve protocol consisting of 1 min at 95°C, 1 min at optimal T_A, then 10 s at optimal T_A increasing to 95°C by 0.5° per cycle during which a second round of fluorescence was acquired. Baseline and threshold (~100 relative fluorescence units (RFU)) were manually adjusted and samples with non-uniform or failed amplification, primer dimers or with amplified products in the corresponding –RT sample were excluded from further analysis for all genes (only 12 samples in total from all the reactions for gene expression and mtDNA quantification). Raw gene expression data from non-matured pooled oocytes and embryos obtained from younger mares was set at 1, and the results of the other groups were expressed relative to these values. Relative gene expression was calculated using iQ5 Optical Software v2.0 (BioRad, Hercules, CA, USA). The relative expression of all genes of interest was then normalised using three stable reference genes (phosphoglycerate kinase 1 (*PGK1*), ribosomal protein L4 (*RPL4*) and signal recognition particle 14kDa (*SRP14*)) previously identified to be suitable for early equine embryos (Paris *et al.* 2011).

For mtDNA quantification, DNA was used from pooled groups of oocytes and individual embryos. The same protocol was used as for cDNA quantification, and all samples were amplified on one plate using the same plate setup as above. The PCR product length for mtDNA is 118 bp, which is equivalent to 1.21×10^{-4} fg and represents 1 mtDNA copy. Therefore, mtDNA copy number was calculated by dividing the starting quantity (i.e. absolute quantity in fg measured during the amplification process) by 1.21×10^{-4}.

Analysis of ATP quantity

The ATP content was determined using an ATP-dependent luciferin–luciferase bioluminescence assay (ATP Bioluminescent Somatic Cell Assay Kit; Sigma-Aldrich) as described by Van Blerkom *et al.* (1995). After removal of any adherent cumulus cells, zona-intact oocytes were frozen at −80°C in 200 μL ultrapure water. They were subsequently thawed immediately before the automated addition of the luciferin–luciferase mixture. A standard curve containing 11 ATP concentrations ranging from 10 fmol to 10 pmol was generated for each analysis. Oocytes from the different experimental groups were analysed simultaneously on a single 96-well plate (all samples on one plate). Measurements were repeated five times within 10 min and the mean values of these five measurements were used for statistical analysis.

Statistical analysis

Data were analysed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). For gene expression, the effects of maternal age, IVM (oocytes) and IVP (embryos) were analysed using one-way ANOVA, followed by a post-hoc Bonferroni test to determine the source of any differences. Data for mtDNA (mtDNA copy number and mtDNA : DNA ratio) and for ATP quantity were $\log_{10}$ transformed before analysis to achieve equivalence of variance. After transformation, data were analysed using univariate analysis. Differences were considered significant at two-tailed $P < 0.05$.

Results

mtDNA copy number

The eight Day 7 embryos recovered from younger and older mares were all Grade 1–2 and ranged in developmental stage from the morula to the expanded blastocyst stage, and in size from 150 to 800 μm in diameter (younger mares: two early blastocysts, six expanded blastocysts; older mares, two morulae, three early blastocysts, three expanded blastocysts). The eight Day 8 IVP embryos were all early blastocysts. The gDNA quantity (an indicator of cell number) did not differ significantly among embryos retrieved from younger mares, older mares or those produced *in vitro* (Fig. 1). In contrast, there was considerable variation in mtDNA copy number among groups. Indeed, both mtDNA copy number per embryo and the ratio of mtDNA : total DNA (i.e. corrected for approximate cell number) were significantly ($P < 0.05$) lower in embryos recovered from older (>16 years) mares ($0.26 \times 10^6 \pm 0.09 \times 10^6$ and

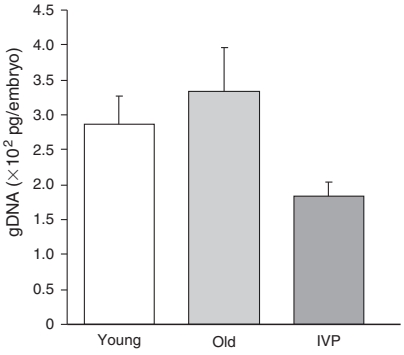

Fig. 1. Total DNA quantity (pg per embryo) in Day 7 *in vivo* equine embryos recovered from younger mares (<12 years) and older mares (>16 years), as well as in *in vitro*-produced (IVP) embryos recovered from mares of unknown ages. Values are the mean ± s.e.m. No significant differences were observed between the different groups. gDNA, genomic DNA.

$1.42 \times 10^{-4} \pm 0.45 \times 10^{-4}$, respectively) than in those from younger mares ($32.91 \times 10^{6} \pm 21.54 \times 10^{6}$ and $2.84 \times 10^{-2} \pm 1.89 \times 10^{-2}$, respectively) or produced *in vitro* ($7.64 \times 10^{6} \pm 3.13 \times 10^{6}$ and $0.95 \times 10^{-2} \pm 0.51 \times 10^{-2}$, respectively; Fig. 2).

Expression of genes involved in mitochondrial replication or function

All –RT cDNA samples were free of gDNA contamination. The PCR products for each primer pair were of the expected size when examined by agarose gel electrophoresis (Fig. 3) and DNA sequencing confirmed that the products were specific to the target genes (data not shown). Evaluation of the stability of gene expression for *PGK1*, *RPL4* and *SRP14* in the pooled oocyte groups, using geNorm v3.5 (Biogazelle NV, Zwijnaarde, Belgium), confirmed that they were suitable for use as reference genes (gene expression stability measure (M) values of 0.94, 1.40 and 0.96, respectively).

Most oocytes or embryos contained detectable quantities of mRNA for the genes of interest. The notable exception was *GPX3*, for which expression was below the detection limit in 30 of the 48 pooled oocyte samples. However, there was no group effect or bias in that 6–8 of the 12 samples were below the detection limit in all four groups examined. For *mtPOLB*, mRNA expression was undetectable in one IVP embryo and one embryo from an older mare, such that there was also no group or treatment bias. For the purposes of statistical analysis, samples with gene expression values under the detection limit were assigned a value of 0.

Normalised relative gene expression for *TFAM*, *mtPOLB* and *ATP-synth_F6* was lower in oocytes after IVM than in oocytes that had not been cultured ($P < 0.05$; Fig. 4*b*), although the differences were not statistically significant for *TFAM* and *ATP-synth_F6* in young mare oocytes. Relative expression of the five target genes in embryos was unaffected by maternal age. However, IVP embryos showed higher expression of *mtPOLB* than *in vivo* embryos ($P = 0.013$), whereas their expression of *GPX3* tended ($P = 0.09$) to be lower than in *in vivo* embryos from young mares (Fig. 5*e*).

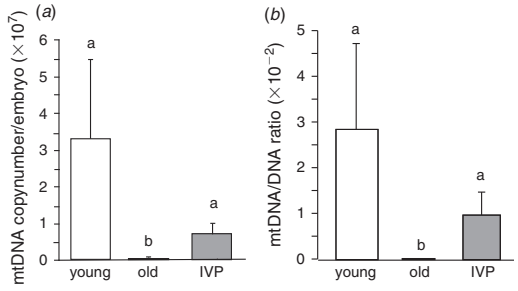

Fig. 2. (*a*) Mitochondrial (mt) DNA copy number per embryo and (*b*) mtDNA quantity in relation to total DNA quantity (mtDNA : DNA ratio) in Day 7 *in vivo* equine embryos recovered from younger mares (<12 years) and older mares (>16 years), as well as in *in vitro*-produced (IVP) embryos recovered from mares of unknown ages. Values are the mean ± s.e.m. Columns with different letters differ significantly ($P < 0.05$).

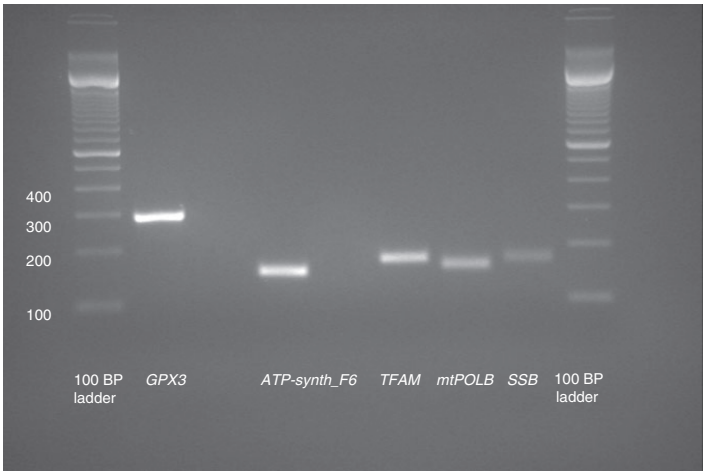

Fig. 3. Amplified products of glutathione peroxidase 3 (*GPX3*), ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*) separated on a 2% agarose gel in parallel with a 100-bp DNA ladder and visualised by ethidium bromide staining. Products were of the expected size (*GPX3*, 289 bp; *ATP-synth_F6*, 148 bp; *TFAM*, 170 bp; *mtPOLB*, 155 bp; *SSB*, 167 bp).

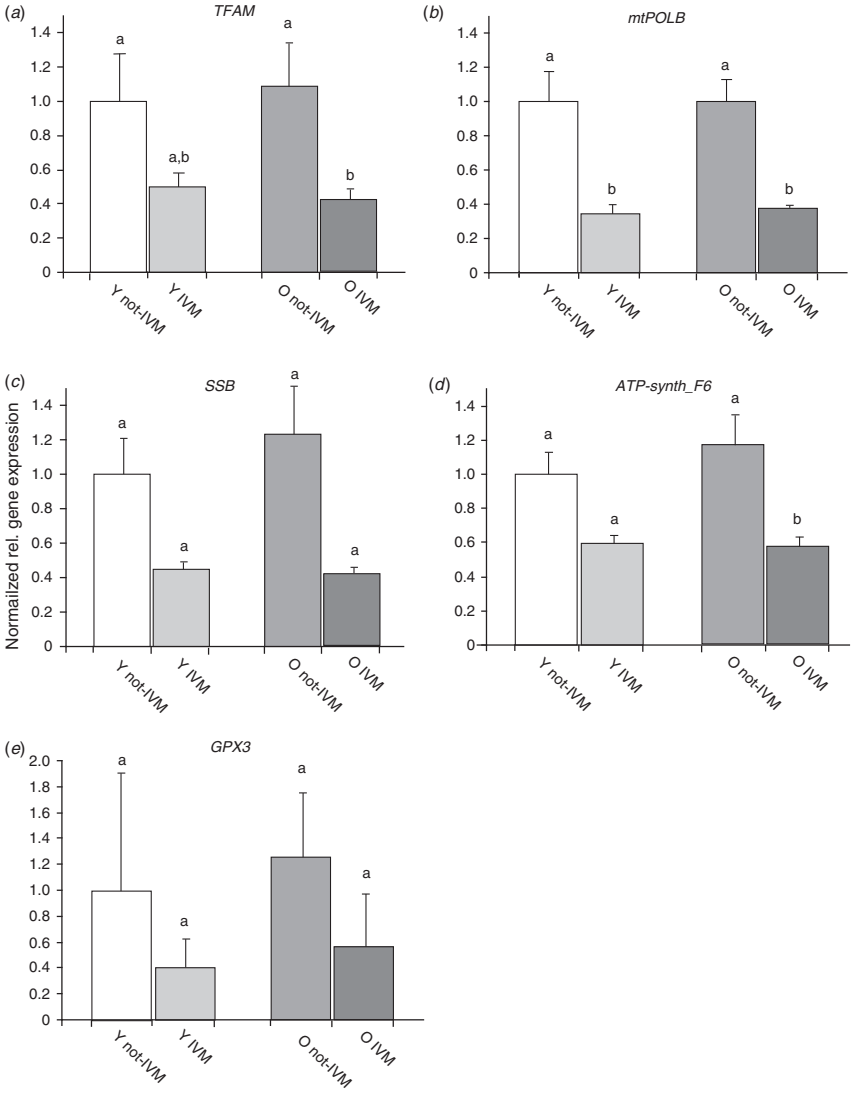

Fig. 4. Normalised relative gene expression (mean ± s.e.m.) for genes involved in mitochondrial replication, namely mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*), and function, namely glutathione peroxidase 3 (*GPX3*) and ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), in pooled equine oocytes obtained from younger mares (<12 years) and older mares (≥12 years) that were either matured *in vitro* (IVM) or not matured (not-IVM). The expression of most genes was detectable in all oocyte pools in each treatment group with the exception of *GPX3*, for which only a few oocyte pools per group yielded a product. Columns with different letters differ significantly ($P < 0.05$).

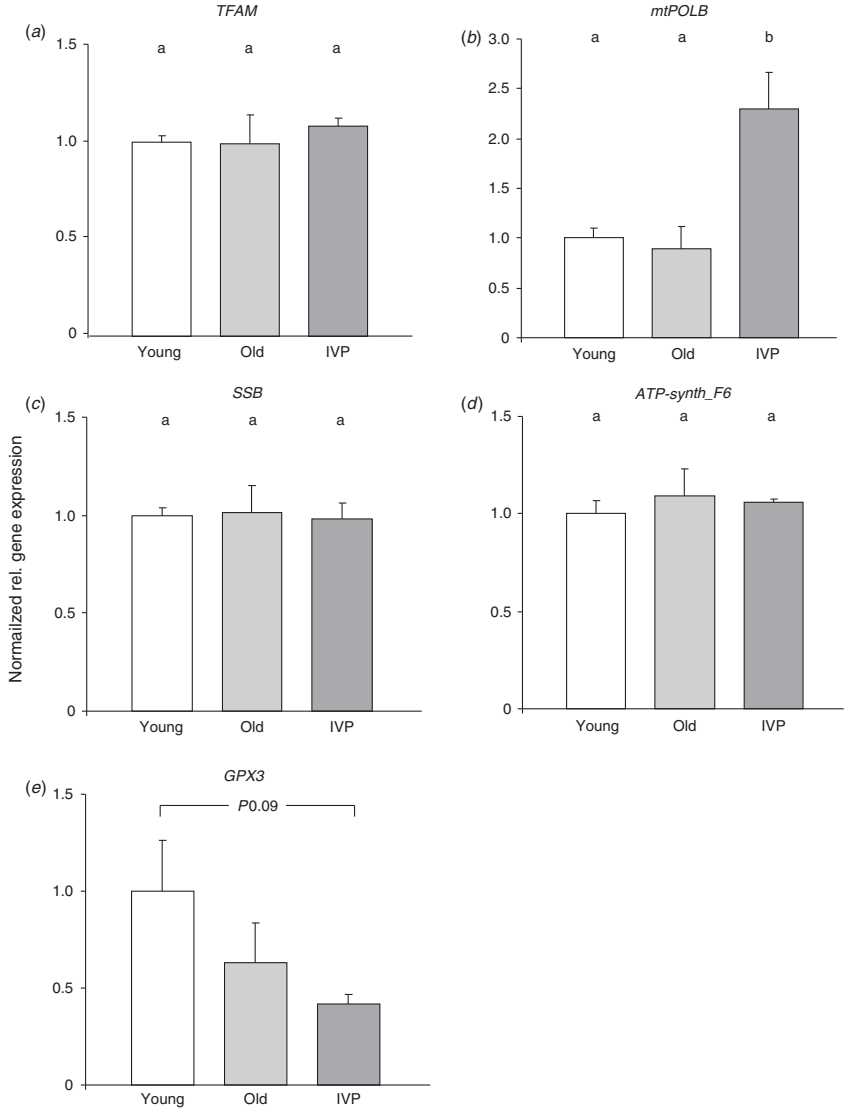

Fig. 5. Normalised relative gene expression (mean ± s.e.m.) for genes involved in mitochondrial replication, namely mitochondrial transcription factor (*TFAM*), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single-stranded DNA-binding protein (*SSB*), and function, namely glutathione peroxidase 3 (*GPX3*) and ATP synthase-coupling factor 6, mitochondrial-like (*ATP-synth_F6*), in day 7 equine embryos obtained from younger mares (<12 years) and older mares (>16 years) and in day 8 *in vitro*-produced (IVP) embryos from mares of unknown ages. Columns with different letters differ significantly ($P < 0.05$).

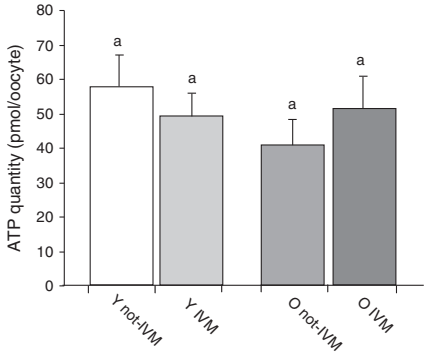

Fig. 6. ATP quantity (pmol per oocyte) in individual equine oocytes obtained from younger mares (<12 years) and older mares (≥12 years) that were either matured *in vitro* (IVM) or not matured (not-IVM). Values are the mean ± s.e.m. No significant differences were observed between the different groups.

ATP quantity

The effect of maternal age on metabolic capacity of oocytes during IVM was evaluated by measuring ATP quantity in oocytes before and after IVM. The mean ATP quantity before IVM was 40.5 ± 7.3 pmol per oocyte in oocytes from older mares and 57.6 ± 9.1 pmol per oocyte in those from younger mares. ATP quantities did not change significantly after oocyte maturation and did not differ significantly between the two mare age groups (Fig. 6).

Discussion

Equine oocytes contain an average of more than one million mtDNA copies (Rambags *et al.* 2014) and, presumably, a similar number of mitochondria. During early development, the mitochondria in a blastomere are distributed sequentially over the newly formed daughter cells, leading to falling numbers of mitochondria per cell until replication is restarted (i.e. at the time of gastrulation in the mouse (Facucho-Oliveira *et al.* 2007) and at blastocyst formation in cattle and pigs (May-Panloup *et al.* 2005*b*; Spikings *et al.* 2007)).

In the present study, embryos collected from older mares on Day 7 after ovulation contained significantly fewer mtDNA copies than those from younger mares. The mtDNA : gDNA ratio in older mares was also lower, indicating that there were also fewer mitochondria per cell. IVP embryos showed an intermediate mtDNA copy number that could reflect, at least in part, their generation from oocytes recovered from mares of unknown, but presumably mixed, age. The reduced mtDNA copy number per embryo (and cell) in aged mares could arise in one of at least three different ways: (1) a lower number of mitochondria at the onset of development, as suggested by a reduced mtDNA content in oocytes from mares ≥12 years after IVM (Rambags *et al.* 2014); (2) a delay in the onset of mitochondrial replication or a reduced replication rate; or (3) a higher rate of mitochondrial deterioration induced, for example, by oxidative stress. In this respect, various genes involved in mitochondrial replication and mtDNA maintenance, namely *TFAM*, *mtPOLB* and *SSB* (Facucho-Oliveira *et al.* 2007; St. John *et al.* 2010), were not differently expressed between embryos from younger versus older mares in the present study. The relative gene expression of *GPX3* (a key factor in neutralising the effects of ROS; Eichenlaub-Ritter *et al.* 2011) was also not significantly affected by maternal age.

The relative mRNA abundance of *TFAM* and *mtPOLB*, both of which are enzymes involved in mitochondrial replication, decreased after IVM of equine oocytes. A similar general trend was observed for all target genes examined and may explain, in part, why mtDNA copy numbers have been reported not to increase during IVM of equine oocytes (Rambags *et al.* 2014). A decrease in relative gene expression for *TFAM* and *mtPOLB* after oocyte maturation has previously been reported for porcine and murine oocytes (Spikings *et al.* 2007; Mahrous *et al.* 2012) and related to the arrest in mitochondrial replication that is observed during early embryonic development. However, the absolute reduction in mtDNA copy number in oocytes of mares ≥12 years after IVM observed by Rambags *et al.* (2014) was thought to be a factor of mitochondrial deterioration, as indicated by electron microscopic evidence of mitochondrial swelling and disintegration.

Expression of genes associated with mitochondrial replication or function differed little between embryos of differing origins (older mares, younger mares, IVP). The only verifiable difference was an increase in *mtPOLB* expression in IVP compared with *in vivo* embryos. *mtPOLB* is primarily involved in mtDNA maintenance, by mediating replication, recombination and repair (St. John *et al.* 2010). This increase may therefore indicate a response to a suboptimal environment (*in vitro* culture) aimed at maintaining sufficient mtDNA and mitochondria to safeguard embryonic development. Under suboptimal conditions, ROS production is likely to be higher, resulting in an increased risk of mtDNA damage; an increase in *mtPOLB* expression could therefore be a reaction to mitigate mtDNA damage (Takahashi 2012; Giritharan *et al.* 2007).

Expression of *GPX3* is associated with the protection of mitochondria against their own potentially toxic by-products, namely ROS, which *GPX3* can neutralise (Guérin *et al.* 2001; Eichenlaub-Ritter *et al.* 2011). Reduced *GPX3* expression can result from elevated production of ROS and is associated with an increased risk of damage to mitochondrial and nuclear DNA, which can lead to developmental retardation and arrest (Takahashi 2012). Although there were no significant differences in *GPX3* expression in oocytes recovered from younger versus older mares, and no significant effect of *in vitro* oocyte maturation on *GPX3* expression, relative gene expression for *GPX3* was approximately halved after IVM. It is therefore possible that there was a fall in ROS-scavenging capacity within oocytes during IVM that failed to reach statistical significance because of the low number of oocyte pools examined. Alternatively, the apparent decrease in gene expression may be a more general phenomenon, given that the abundance of all other target mRNAs roughly halved during IVM. It is also possible that ROS-scavenging activity is not a function of the oocyte alone, because an effect of IVM on *GPX3* expression has been recorded in the surrounding cumulus cells (Luciano *et al.* 2006).

Future studies examining ROS-scavenging activity during oocyte maturation should therefore encompass both the oocyte and its cumulus investment. The trend to reduced *GPX3* gene expression in IVP equine embryos may have similar implications for the prevention of ROS accumulation, and could therefore contribute to the elevated levels of apoptosis and DNA damage seen in equine IVP compared with *in vivo* embryos (Tremoleda *et al.* 2003; Pomar *et al.* 2005).

Relative expression of *ATP-synth_F6* mRNA was reduced in oocytes from older mares after IVM. However, this was not reflected by a reduction in the ATP content of aged mare oocytes following IVM. *ATP-synth_F6* is involved in ATP production via the electron transport system; however, oocyte maturation, both *in vivo* and *in vitro*, takes place in a hypoxic environment. This may promote ATP production via glycolysis rather than via oxidative phosphorylation and the electron transport system (Leese *et al.* 2008; Ramalho-Santos *et al.* 2009). During blastocyst formation, embryos are thought to become more dependent on oxygen consumption for the production of energy from mitochondria (Houghton and Leese 2004) and reduced *ATP-synth_F6* expression at this stage may therefore be detrimental. In the present study, we did not find any evidence of a difference in *ATP-synth_F6* expression between Day 7 embryos produced *in vitro* or recovered from mares <12 or >16 years.

In conclusion, Day 7 equine embryos recovered from older mares have lower mitochondrial DNA copy numbers than Day 7 embryos recovered from younger mares; this seems to be independent of any change in the rate of development because the mtDNA : gDNA ratio (i.e. corrected for cell number) is also reduced. This reduction in the number of mtDNA copies per cell may push the cells in some developing embryos below the threshold number required for normal development and thereby contribute to compromised cell survival and, as a result, reduced embryo developmental competence. This rationale has been used previously to propose treatments to improve the developmental capacity of oocytes from aged females undergoing fertilisation by ICSI, namely using the transfer of cytoplasm containing 'high-quality mitochondria' from a young oocyte (for a review, see Chappel 2013).

Two genes were found to be differentially expressed in IVP compared with *in vivo* embryos, namely *mtPOLGB* and *GPX3*. This suggests that some of the detrimental effects of present *in vitro* culture procedures on developmental competence (as evidenced by higher rates of early pregnancy loss than after fertilisation *in vivo*; Carnevale *et al.* 2005; Alexopoulos *et al.* 2008; Giritharan *et al.* 2010) may be mediated via effects on mitochondrial replication, repair or ability to resist the effects of ROS. In addition, IVM led to a generalised reduction in the expression of genes involved in mitochondrial replication and function, irrespective of maternal age. However, because these comparisons have not yet been performed on oocytes matured *in vivo*, it is not possible to determine whether this is an effect of IVM or inherent to the oocyte maturation process.

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Chapter 4

Mitochondrial DNA replication is initiated at blastocyst formation in equine embryos

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In submission

Mitochondrial DNA replication is initiated at blastocyst formation in equine embryos

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Abstract

Intracytoplasmic sperm injection (ICSI) is the technique of choice for equine *in vitro* fertilization (IVF) and, in a research setting, 18-36% of injected oocytes develop to blastocysts. However, blastocyst development in clinical programs is lower, presumably due to a combination of variable oocyte quality (e.g. from old mares), suboptimal culture conditions and marginal fertility of some stallions. Furthermore, mitochondrial constitution appears to be critical to developmental competence, and both maternal ageing and *in vitro* embryo production (IVEP) negatively influence mitochondrial number and function in murine and bovine embryos. This study examined the onset of mitochondrial DNA (mtDNA) replication in equine embryos and investigated whether IVEP affects the timing of this important event, or the expression of genes required for mtDNA replication (*TFAM*, *mtPOLB* and *SSB*). We also investigated whether developmental arrest was associated with low mtDNA copy number. MtDNA copy number increased ($P < 0.01$) between the early and expanded blastocyst stages both *in vivo* and *in vitro*, while the mtDNA/total DNA ratio was higher in IVEP embryos ($P = 0.041$). Mitochondrial replication was preceded by an increase in *TFAM* but, unexpectedly, not *mtPOLB* or *SSB* expression. There was no association between embryonic arrest and lower mtDNA copy numbers.

Additional keywords: embryonic development, horse, oocyte, *in vitro* fertilization, gene expression

Introduction

Conventional *in vitro* fertilization (IVF) with equine gametes is poorly successful, and the primary reason for this failure appears to be inadequate activation of equine spermatozoa to penetrate the zona pellucida *ex vivo* (Hinrichs, 2012). As for human male factor infertility, this deficit can be overcome by injecting the spermatozoon directly into the oocyte's cytoplasm (i.e. intracytoplasmic sperm injection: ICSI). To date, however, only a handful of laboratories have been able to achieve acceptable blastocyst production (18-36% of injected oocytes) after *in vitro* oocyte maturation, ICSI and *in vitro* embryo culture (Galli *et al*., 2007; Hinrichs, 2012). A combination of factors including variable oocyte quality, suboptimal culture conditions and questionable fertility of donor stallions or semen, appears to compromise the developmental potential of zygotes and embryos in a commercial setting, and contribute to sub-optimal blastocyst production (Galli *et al.*, 2007; Hinrichs, 2013). In this respect, several processes are disturbed in the follicles, and the oocytes they contain, in aged mares (Carnevale, 2008), and during oocyte maturation and the cleavage stages of early embryo development *in vitro*. These deficits can in turn influence developmental kinetics, energy and glucose metabolism, and predispose to a higher incidence of embryonic cell apoptosis, and to an altered epigenetic constitution (Badr *et al.,* 2007). One critical contributor to normal early embryo development is mitochondrial number and activity (Bentov *et al.,* 2011). Impaired mitochondrial function is thought to be a key factor in the reduced developmental competence of oocytes from older women, and can be successfully overcome by the transfer of cytoplasm containing 'healthy' mitochondria from younger donors (Barritt *et al.,* 2001).

During oocyte development, the mitochondrial DNA (mtDNA) copy number increases from tens of copies to hundreds of thousands (McConnell and Petrie, 2004; Shoubridge and Wai, 2007). By the time of germinal vesicle breakdown and the onset of oocyte maturation,

the oocyte contains a large but stable quantity of mitochondria and copies of mitochondrial DNA. A species-specific minimum number of mitochondria is thought to be required to permit normal post-fertilization development of the early embryo (Shoubridge and Wai, 2007; Wai *et al*., 2010; Fragouli *et al.,* 2015). This is primarily because mitochondrial DNA replication is transiently arrested between fertilization and the onset of cell lineage segregation (St. John *et al.,* 2010). Indeed, the mtDNA copy number remains constant in mouse and rat embryos until gastrulation (Thundathil *et al.,* 2005; Facucho-Oliveira *et al.,* 2007; Kameyama *et al.,* 2007; Wai *et al.,* 2010), and even decreases over time in cattle, pig and human embryos up to the time of blastocyst formation (May-Panloup *et al.,* 2007; Spikings *et al.,* 2007; Hashimoto *et al.,* 2017). Coincident with blastocyst formation in the cow and pig (May-Panloup *et al.,* 2007; Spikings *et al.,* 2007), mitochondrial DNA replication is reinitiated in trophectoderm, but not in ICM, cells (Hashimoto *et al.,* 2017). In general, the ratio of mtDNA to mitochondria is cell-type specific and, since the ratio in oocytes and early embryos is 1-2 mtDNA copies per mitochondrion (Shoubridge and Wai, 2007), mtDNA copy number is a useful indicator of mitochondrial number. The developmentally programmed arrest in mtDNA replication at the early cell cleavage stages, despite a near exponential increase in the number of cells, underlines why the number of functional mitochondria present in a mature oocyte is of critical importance for subsequent developmental competence of the embryo (May-Panloup *et al.,* 2007).

In vitro embryo production (IVEP) predisposes oocytes and early embryos to alterations in mitochondrial function leading to mitochondrial damage (Wilding *et al.,* 2001; Wang *et al.,* 2009) and resulting in a decrease in mtDNA copy number, a reduction in mitochondrial gene expression, and an increased production of reactive oxygen species (ROS) within embryonic cells (Giritharen *et al.,* 2007; 2010). Reduced mtDNA quantity can also result in reduced expression of genes, such as mitochondrial transcription factor *(TFAM)*

(Facucho-Oliveira *et al.,* 2007), mtDNA polymerase γ subunit B (*mtPOLB*) and mitochondrial single stranded DNA binding protein (*SSB*: St. John *et al.,* 2010), involved in the various steps of the mitochondrial replication process. Expression of these genes is normally up-regulated shortly before mtDNA replication is reactivated (Piko and Taylor, 1987; Thundathil *et al.,* 2005; May-Panloup *et al.,* 2007; Spikings *et al.,* 2007).

We previously reported that equine IVEP embryos contain lower numbers of mitochondria than their *in vivo* counterparts (Hendriks *et al.*, 2015). However, IVEP embryos develop more slowly and have lower cell numbers at the same time point than *in vivo* embryos (Tremoleda *et al.,* 2003), such that mtDNA quantity per embryonic cell did not differ significantly between *in vivo* and IVEP embryos. At present therefore, it is not clear whether the reduced mtDNA copy number in IVEP embryos is primarily a factor of retarded development, whether it reflects a reduction in the starting number of mtDNA copies, or whether it results from alterations in the timing or efficiency of mtDNA replication.

The current study was performed to determine the time of onset of mitochondrial DNA replication in equine embryos, and to examine whether this and the expression of genes required for mtDNA replication (*TFAM*, *mtPOLB* and *SSB)* were affected by IVEP.

Materials and methods

Collection of cumulus oocyte complexes

Ovaries from 11 mares (age < 12 years) were recovered immediately after slaughter, transported to the laboratory at 30°C in a thermos flask and processed within 4 hours. Cumulus oocyte complexes (COCs) were recovered as described previously (Tharasanit *et al.,* 2005). Only oocytes with a complete multi-layered cumulus investment (Hinrichs *et al*., 1993) were selected and maintained in HEPES buffered M199 (Gibco BRL Life Technologies, Paisley, Strathclyde, UK) supplemented with 0.014% (w/v) BSA (Sigma-

Aldrich Chemicals B.V., Zwijndrecht, The Netherlands). The oocytes were immediately denuded by vortexing for 4 min in calcium and magnesium free Earle's Balanced Salt Solution (EBSS; Gibco BRL Life Technologies) containing 0.25% (v/v) trypsin-EDTA (Gibco BRL Life Technologies). The denuded oocytes were washed twice in phosphate buffered saline (PBS; Sigma-Aldrich Chemicals B.V.) containing 0.1% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals B.V.) and placed individually in 10 µl lysis buffer (RLT + β-mercaptoethanol; Qiagen, Venlo, The Netherlands) in a 0.5 ml eppendorf tube, snap-frozen and stored at -80°C until further processing (DNA/RNA extraction).

Collection of embryos

Embryos were recovered from ten Dutch Warmblood mares (aged 4 - 12 years) on day 7 after ovulation. All animal procedures were approved by Utrecht University's Institutional Animal Care and Use Committee (DEC 2007.III.02.036). During oestrus, the reproductive organs of the mares were examined daily by transrectal palpation and ultrasonography using a MyLab Five ultrasound machine (Esaote Pie Medical, Maastricht, The Netherlands) equipped with a 7.5 MHz linear array probe. Once the dominant follicle exceeded 35 mm in diameter, ovulation was induced by the intravenous injection of 1500 IU hCG (Chorulon®, Intervet, Boxmeer, The Netherlands) and the mare was inseminated with >500 million motile sperm from a single fertile stallion. Thereafter, mares were examined daily until ovulation was detected by the evacuation of the pre-ovulatory follicle. Seven days after ovulation, embryos (n = 21) were collected by non-surgical uterine lavage using 3 x 1 L pre-warmed (37°C) Lactated Ringer's solution (LRS: Baxter, Lessines, Belgium) supplemented with 0.5% fetal calf serum (FCS: Greiner Bio-One, Alphen aan den Rijn, The Netherlands). After recovery, embryos were washed 10 times with LRS to remove maternal cells and residual FCS prior to assessment using a dissecting microscope (SZ60: Olympus, Zoeterwoude, The Netherlands).

The embryos were classified by developmental stage (morula, early blastocyst or expanded blastocyst) and quality (1-4: good to degenerate) as described previously (Tremoleda *et al.,* 2003), and their diameter was measured using a calibrated eye-piece micrometer. Only grade 1 - 2 embryos were used further, and these were transferred with 10 µl LRS into 0.5ml eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until DNA/RNA extraction.

In vitro embryos were produced as described by Galli *et al.* (2001), with minor modifications. Briefly, COCs recovered from the ovaries of slaughtered mares of mixed breed and unknown age were matured *in vitro* (IVM) by incubating for 22-24h in DMEM-F12 containing 10% serum replacement (SR: Gibco BRL Life Technologies), ITS supplement (1.0 mg/ml insulin, 0.55 mg/ml transferrin, 0.5 µg/ml sodium selenite: Sigma-Aldrich Chemicals B.V.), 1 mM sodium pyruvate, 50 ng/ml EGF (Sigma-Aldrich Chemicals B.V.), and 0.1 IU LH and FSH (Pergovet, Serono, Italy). Next, oocytes were denuded and returned to maturation medium for a further 2 - 4h (i.e. up to 26 - 28 h total duration of IVM). Oocytes with an extruded first polar body were then fertilised by intracytoplasmic sperm injection (ICSI) with sperm selected by Redigrad (Amersham Biosciences AB, Uppsala, Sweden) density gradient centrifugation. The resulting presumptive zygotes were cultured in modified synthetic oviductal fluid (mSOF; Tervit *et al.,* 1972) supplemented with MEM essential and non-essential amino acids, glutamine and BSA, and examined for cleavage at day 2 post injection; those showing development to the 2-cell stage or further were cultured *in vitro* for an additional 6 days, refreshing the medium at days 4 and 6 (Galli *et al*., 2007). At day 7 or 8, culture was stopped when embryos reached the morula, early blastocyst or expanded blastocyst stage.

IVEP embryos (n = 32) that appeared to be developing normally were also harvested at various time-points during culture: 48 h (2 days), 96 h (4 days), 144 h (6 days) and 192 h (8

days) after ICSI (8 embryos per group). In addition, 15 embryos showing arrested development were collected at the same time points; not cleaved (n=5) on day 1, blocked at 2-cell stage (n=5) on day 2, blocked at 8-cell stage (n=2) on day 3 and blocked at >16-cell stage (n=3) on day 4 after ICSI. All embryos were washed twice in phosphate buffered saline (PBS) containing 0.1% polyvinyl alcohol (PVA; Sigma-Aldrich Chemicals B.V.), and transferred individually to DNase-RNase free tubes in RLT buffer (Qiagen), snap-frozen in liquid nitrogen and stored at -80°C.

DNA and RNA extraction, and cDNA synthesis

Total RNA and DNA (tDNA) were extracted from individual oocytes and individual embryos in 350 or 600 µl RLT buffer, respectively, using an AllPrep DNA/RNA/Protein Mini Kit and subjected to on-column DNase I digestion using an RNase-Free DNase Set (both Qiagen) as described by Paris *et al.* (2011). tDNA was eluted in 50 µl elution buffer (Qiagen) and RNA was eluted in 35 µl RNase-free water (Qiagen). The RNA from individual oocytes or embryos was then transcribed into cDNA. For conventional PCR, the total reaction volume was 25 µl containing 1 µl cDNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs (Promega, Madison, WI, USA), 0.5 µM forward primer, 0.5 µM reverse primer and 0.625 U HotStarTaq DNA polymerase (all Qiagen). PCR cycle conditions consisted of 15 min at 95°C followed by 35 cycles of 30 sec at 94°C, 30 sec at the primer-specific annealing temperature (Hendriks *et al.,* 2015) and 1 min at 72°C; with final extension for 10 min at 72°C. Products were visualized on 1% agarose gels. If suitable for PCR amplification, and free of gDNA contamination, both +RT and –RT cDNA samples were diluted 10-fold and frozen at -20°C prior to qRT-PCR.

DNA quantification

Cell number was estimated by quantifying total DNA (tDNA) using a Quant-iT™ PicoGreen® dsDNA assay kit (Molecular Probes, Invitrogen, Eugene, USA). A standard curve ranging from 25 ng/ml to 2.5 pg/ml was created via a 1 in 10 dilution series. Fifty µl Pico Green reagent was mixed with 9.95 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and 40 µl per well was added to 20 µl sample (standard curve or embryo extract) in a FLUOTRAC™ 600 ninety-six well microplate (Greiner Bio-One GmbH, Frickenhausen, Germany). During preparation and plate setup, the materials were protected from light. Fluorescence was measured using a multimode detector (Beckman Coulter DTX 880, Beckman Inc., Woerden, The Netherlands) and the 'Fluorescein Top Read' software program for Anthos Multimode detectors (Anthos Microsystem GmbH, Krefeld, Germany).

Quantitative RT-PCR

Primers for the genes *TFAM, mtPOLB* and *SSB* were optimized and tested for specificity using an iQ5 RT-PCR Detection System with iQ5 Optical System Software v2.0 (BioRad, Hercules, CA, USA; Table 1); equine kidney was used as the positive control tissue.

Quantitative Real-Time PCR (qRT-PCR) was performed separately for oocytes and embryos, but using the same equipment, settings and software as described by Paris *et al.* (2011). The total reaction volume was 25 µl per well, and included 1x iQ5 SYBR Green Supermix (BioRad), 0.5 µM forward primer and 0.5 µM reverse primer (Ocimum Biosolutions, IJsselstein, The Netherlands). Each well included 10 µl of standard or sample, and the following were included in the final plates: (1) Standard curve: 100 fg – 6.4 ag 5-fold dilution series (n=7: fresh or frozen); (2) Positive control tissue (n=1: kidney); (3) 10-fold diluted sample cDNA (*in vivo*: n = 5 morulae, n = 8 early blastocysts, n = 8 expanded blastocysts; IVP: n = 8 morulae, n = 9 early blastocysts, n = 9 expanded blastocysts) or 10-fold diluted sample cDNA (normal: n = 8 oocytes, n = 8 cleaved embryos 2 days after ICSI, n

= 8 embryos 4 days after ICSI, n = 8 embryos 6 days after ICSI, n = 8 embryos 8 days after ICSI; arrested: n = 5 uncleaved embryos, n = 5 embryos blocked at 2-cell stage, n = 2 embryos blocked at 8-cell stage, n = 3 blocked at >16-cell stage); (4) 10-fold diluted –RT sample; (5) DNase/RNase-free water (Invitrogen) as no-template control.

All samples except for the –RT samples were run as duplicates. Both validated frozen and freshly-prepared standards were included on each plate. –RT samples for the embryos were run on a separate plate, in a subsequent run on the same day using identical standards. PCR cycle conditions consisted of 4.5 min at 95°C followed by 40 cycles of 15 sec at 95°C, 30 sec at optimal annealing temperature (T_A; Table 1) and 30 sec at 72°C, during which the fluorescence was acquired; followed by a melt-curve protocol consisting of 1 min at 95°C, 1 min at optimal T_A, then 10 sec at optimal T_A increasing to 95°C by 0.5°C per cycle during which a second round of fluorescence was acquired. Baseline and threshold (~100RFU) were manually adjusted and samples with non-uniform/failed amplification, primer dimers, or with amplified products in the corresponding –RT sample, were excluded from further analysis for all genes (only 3 samples in total of all the reactions for gene expression and mtDNA quantification). Raw gene expression data from single extracted oocytes were set at 1, and the results of the other groups were expressed relative to these values. Relative gene expression was calculated using the iQ5 Optical Software v2.0.

For mtDNA quantification, DNA was used from individual oocytes and individual embryos. The same protocol was used as for cDNA quantification, and all samples were amplified on one plate using the same plate setup as above. Further calculations were based on the fact that the PCR product length for mtDNA is 118 base pairs; such that 1 mtDNA copy weighs 1.21×10^{-4} fg. Therefore, mtDNA copy number could be calculated by dividing the starting quantity (i.e. absolute quantity in fg measured during the amplification process) by 1.21×10^{-4}.

Statistical analysis

Data were analysed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). For gene expression, the effects of IVEP, stage of development, and effect of developmental arrest were analysed using one-way ANOVAs, followed by a post-hoc Bonferroni-test. Data for mtDNA (mtDNA copy number, tDNA quantity and mtDNA/tDNA ratio) were $\log_{10}$ transformed prior to analysis to achieve equivalence of variance. After transformation, the data was analysed using univariate analysis. For gene expression comparison, oocyte values were set as one. The dataset was $\log_{10}$ transformed prior to analysis, followed by one-way ANOVA and post-hoc Bonferroni-testing. Differences were considered statistically significant if $P < 0.05$. Data are displayed as mean ± S.E.M.

In a number of instances, mRNA was below the detection limit in extracts from individual oocytes or IVEP embryos. For statistical analysis this data was subjected to 'left-censoring'; i.e. observations below the detection limit were replaced by a value just below the detection limit, rather than being recorded as 0, and were treated as left censored (Klein and Moeschberger, 2003). Akaike information criterion (AIC) was used to select the appropriate model for statistical analysis. According to AIC, this approach gave the best fit for the following distributions: Weibull, normal, log-normal, logistic, log-logistic, extreme value, Raleigh and the t-distribution.

Results

Mitochondrial quantity in normal in vivo and in vitro produced embryos

MtDNA copy number increased significantly between the early and expanded blastocyst stages in both *in vivo* and IVEP embryos ($P < 0.01$; Fig. 1A). The tDNA quantity (an indicator of cell number) did not differ significantly between the morula and early

blastocyst stages; however, there was a marked increase in tDNA in *in vivo* but not *in vitro* expanded blastocysts ($P < 0.01$; Fig. 1B). When tDNA was used to correct for cell number, the mtDNA/tDNA ratio increased significantly between the morula and early blastocyst stage in both the *in vivo* and the IVEP embryos ($P = 0.03$; Fig. 1C). Despite the wide variation in the mtDNA/tDNA ratio, especially among the IVEP embryos (range 7.7×10^{-6} - 1.4×10^{-4} in the expanded IVEP blastocysts; 1.6×10^{-5} - 1.4×10^{-4} in *in vivo* expanded blastocysts), there was a significant overall difference between embryo production methods; the mtDNA/tDNA ratio was higher in IVEP than *in vivo* embryos ($P = 0.041$).

To further examine changes in mtDNA quantity during early embryo development *in vitro*, mtDNA copy number comparisons were performed for immature oocytes and developing zygotes/embryos 2, 4, 6 and 8 days after ICSI. An increase in mtDNA copy number was seen from $0.3 \pm 0.1 \times 10^6$ in oocytes to $28.8 \pm 16.3 \times 10^6$ in normally developing embryos on day 8 of culture (Fig. 2A). None of the embryos on day 2 after ICSI contained more than 1×10^6 mtDNA copies. By contrast on day 4 after ICSI six embryos contained more than 1×10^6 mtDNA copies, and on day 8 after ICSI four had more than 10×10^6 mtDNA copies. The mean tDNA quantity appeared to increase in embryos on day 8 after ICSI (Fig. 2B), as did the mtDNA/tDNA ratio (from $0.03 \pm 0.01 \times 10^{-3}$ in oocytes to $1.84 \pm 1.44 \times 10^{-3}$ in embryos on 8 day after ICSI); however, there was a high degree of inter-embryo variation (range from 0.11×10^{-4} to 0.12×10^{-1} in embryos 8 days after ICSI; Fig. 2C) such that a significant effect of embryo developmental stage was not detected ($P = 0.266$).

Expression of genes involved in mitochondrial replication

Expression of two potential (reference) genes for normalizing gene expression between oocytes and embryos, *PGK1* (phosphoglycerate kinase 1) and *RPL4* (ribosomal protein L4), was evaluated in oocytes and in the *in vivo* and IVEP embryos described above.

All –RT cDNA samples were free of gDNA contamination. Evaluation of the stability of gene expression for *PGK1* and *RPL4* (stably expressed in *in vivo* embryos: Paris *et al.,* 2011), across the single oocytes and IVEP embryos using geNorm v3.5 indicated that both *RPL4* and *PGK1* were suitable for use as reference genes (M values 1.44; 1.35). Since the combination of *RPL4* and *PGK1* did not lower the M value and some results for *PGK1* were close to the limit of detection, *RPL4* alone was used for normalization of gene expression values.

The normalised relative gene expression for *TFAM* increased significantly between fertilization and day 4 after ICSI, with a 16 fold increase compared to oocytes, but subsequently tended to decrease from day 4 to day 8 (Fig. 3A). *mtPOLB* expression was high in oocytes and IVEP embryos during the first 2 days of culture, but decreased to baseline levels thereafter (Fig. 3B). *SSB* expression had increased significantly by day 2 after ICSI, but thereafter decreased to levels similar to those seen in oocytes (Fig. 3C).

mtDNA quantity and gene expression in arrested in vitro produced embryos

To study a possible contribution of mitochondrial quantity and replication to failure of *in vitro* development in arrested embryos: uncleaved zygotes (n=5) and embryos blocked at the 2-cell (n=5), 8-cell (n=2) and ≥16-cell (n=3) stages were evaluated. Due to the low numbers of arrested embryos collected, the groups 'uncleaved' and 'blocked at 2-cell stage' were combined for statistical analysis, as were the groups 'blocked at 8 cell stage' and 'blocked at ≥16-cell stage'.

Surprisingly, mitochondrial copy number in zygotes that failed to cleave or arrested at the 2-cell stage tended to be higher ($2.4 \pm 0.6 \times 10^6$), rather than the expected lower, compared to oocytes ($0.3 \pm 0.1 \times 10^6$) and developing embryos at day 2 after ICSI ($0.45 \pm 0.1 \times 10^6$; Fig. 2A). Similarly, mtDNA/tDNA ratio in uncleaved zygotes and embryos blocked at

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the 2-cell stage was not lower than in oocytes or apparently normal embryos 2 days after ICSI (Fig. 2C).

Gene expression data from the groups 'uncleaved' and 'blocked at 2-cell stage' were combined for statistical analysis as were data from the groups 'blocked at 8-cell stage' and blocked at ≥16-cell stage', because the amplification products of some of these samples were undetectable. Overall, IVEP embryos that arrested during development showed, or tended to show, higher normalized relative gene expression than normal IVP embryos for *mtPOLB* (P = 0.045) and *SSB* (P = 0.051) respectively (Fig. 3B and C).

Discussion

This study demonstrated a relatively stable mtDNA copy number in early equine embryos up to the time of blastocyst formation and expansion, at which time both the mtDNA copy number and the mtDNA/tDNA ratio increased significantly in both *in vivo* and IVEP embryos. This indicates a similar time of onset of mtDNA replication, and/or the time at which mtDNA replication outpaces any underlying mtDNA degradation, to that reported previously in porcine and bovine embryos. In the latter species, an initial increase in mtDNA copy number at the time of blastocyst formation was followed by a near exponential increase during expansion and hatching (Facucho-Oliviera *et al*., 2007; May-Panloup *et al*., 2007; Spikings *et al.,* 2007). In the current study, mtDNA copy number was similar in *in vivo* and IVEP embryos at the morula and early blastocyst stages, and it was only following blastocyst expansion that *in vivo* embryos diverged, with a much more rapid increase in both absolute mtDNA copy number and in tDNA quantity (i.e. cell number). This suggests that the lower mtDNA copy number in IVEP, compared to *in vivo,* blastocysts on day 7-8 after fertilization is primarily a function of the more rapid increase in cell number (predominantly trophectoderm cells) that the latter undergo at the time of blastocyst expansion (Tremoleda *et*

al., 2003; Rambags *et al.,* 2005). That is, the difference does not seem to be due to an initial deficit in mtDNA copy number, e.g. as a result of increased mtDNA degradation (Rambags *et al*., 2014) during *in vitro* oocyte maturation or reduced replication because of lower expression of components of the mtDNA replication machinery (Hendriks *et al*., 2015). However, this finding may in part depend on the status of the oocytes used for IVEP (e.g. age of the mare from which the oocyte was recovered), since Rambags *et al.* (2014) reported a drop in mtDNA copy number during *in vitro* oocyte maturation in oocytes from old (>12 years) but not young mares.

We monitored mtDNA copy number throughout early equine embryo development *in vitro*, and found the mean values to be relatively stable up to the time of blastocyst formation, despite considerable variability between individual oocytes and embryos, as reported in other species (May-Panloup *et al.,* 2007; Hashimoto et al., 2017). The major increase in mtDNA copy number was observed between days 6 and 8 after ICSI, coincident with the onset of blastocyst formation and formation of the trophectoderm cell lineage. We also attempted to examine the effect of cell number by measuring total DNA (tDNA) levels; however, the enormous number of mtDNA copies in equine oocytes (mean exceeds 10^6; Rambags *et al*., 2014) and early embryos means that mtDNA accounts for a large proportion of the total DNA (May-Panloup *et al.,* 2007), and in part explains why tDNA quantity in IVP embryos did not increase significantly until relatively late (6-8 days after ICSI) even though cell number should double on a daily basis (Grondahl and Hyttel, 1996).

One unexpected observation during the current study was that failure of oocytes to cleave and embark on embryo development following fertilization by ICSI was not associated with a reduced mtDNA copy number, as had previously been reported for human (Reynier *et al*., 2001) and porcine oocytes (El Shourbagy *et al.,* 2006). The reasons for this discrepancy are not clear but may relate to reports that, while mtDNA copy number is relatively constant

between oocyte maturation and blastocyst formation, this stability masks a single mtDNA replication event that takes place in the period between fertilization and the first cleavage division (McConnel and Petrie, 2004). McConnel and Petrie (2004) further suggested that environmental conditions that disturb the normal regulation of this replication event, which they mimicked by including homocysteine in the medium, could result in an increase in mtDNA copy number (i.e. synthesis outpacing degradation) during this window of replication. The biological function of this early zygotic mtDNA replication event has been proposed to relate to an additional opportunity to remove paternal and/or defective mtDNA copies, but also presents an opportunity for adverse environmental conditions to untowardly affect zygote mtDNA copy number. While speculative, this mechanism combined with sub-optimal *in vitro* conditions for supporting immediate post-fertilization equine embryo development could explain the unexpectedly high mtDNA copy numbers (and mtDNA:tDNA ratio) observed in equine zygotes that either failed to cleave or arrested at the 2-cell stage. The potential for an early mtDNA replication event is supported by the presence of abundant mRNA transcripts for *TFAM, mtPOLB* and *SSB*, important components of the mtDNA replication machinery, in oocytes and 2-cell stage embryos.

In several species including the mouse, cow and pig, the onset of mitochondrial replication in the early embryo is preceded by up-regulation of genes for components of the mitochondrial replication machinery, such as *TFAM*, mt*POLB* and *SSB* (Wang *et al.,* 2009; St John *et al.,* 2010; Cagnone *et al.,* 2016), at some point between embryonic genome activation and compaction of the developing morula. In the current study, *TFAM* increased at day 4 of *in vitro* embryo culture, corresponding to the expected time of equine embryonic genome activation (Grondahl and Hyttel, 1996), and preceding the increase in mtDNA copy number. Thereafter, *TFAM* expression tended to decrease in a fashion similar to that described in porcine embryos (Spikings *et al*., 2007). What was less expected was the relatively high

mtPOLB and *SSB* expression present in equine oocytes and zygotes 2 days after ICSI, including those that failed to develop further (Fig. 3). As discussed above, these presumably represent maternal transcripts either remaining from the intense period of mtDNA replication and stabilisation during oocyte growth and maturation, or involved in the mtDNA replication event proposed to take place between fertilization and the first cell cleavage division (McConnel and Petrie, 2004). The subsequent drop in *mtPOLB* expression 6 days after ICSI could be explained by the global degradation of maternal mRNA transcripts that occurs at the time of embryonic genome activation (St. John *et al.,* 2010). However, the absence of a more obvious upregulation of *mtPOLB* and *SSB* transcription at the time of onset of mtDNA replication, as described in mouse, pig, cattle and sheep embryos (Bowles *et al.*, 2007; May-Panloup *et al.,* 2007) was unexpected. This may in part reflect a masking effect of the very high transcript numbers persisting from the early post-fertilization mtDNA replication event.

In conclusion, mitochondrial replication commences in equine *in vivo* and IVEP embryos just prior to blastocyst expansion. This is preceded by an increase in *TFAM*, but not *mtPOLB* or *SSB* expression. Further research should focus in greater detail on the period in which mitochondrial replication begins (between D6 and D8) *in vivo* and *in vitro* and on other aspects of the establishment of the mitochondrial replication machinery. Differences between mitochondrial DNA regulation in inner cell mass cells versus trophectoderm cells could help establish in which cells mtDNA replication primarily occurs, and could be used as a marker to help improve IVEP conditions in the future.

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Figure legends

Figure 1. Mitochondrial DNA copy number (A), total DNA quantity (fg/embryo; B), and ratio of mitochondrial DNA to total DNA quantity (mtDNA/tDNA ratio; C) in *in vivo* (i.e. flushed) horse embryos (morula (M) n=5; early blastocyst (EA) n=8; expanded blastocyst (EX) n=8) and *in vitro* produced embryos (morula n=8; early blastocyst n=9; expanded blastocyst n=9). Values are mean ± SEM.

Within production method values marked with different letters differ significantly ($P < 0.05$).

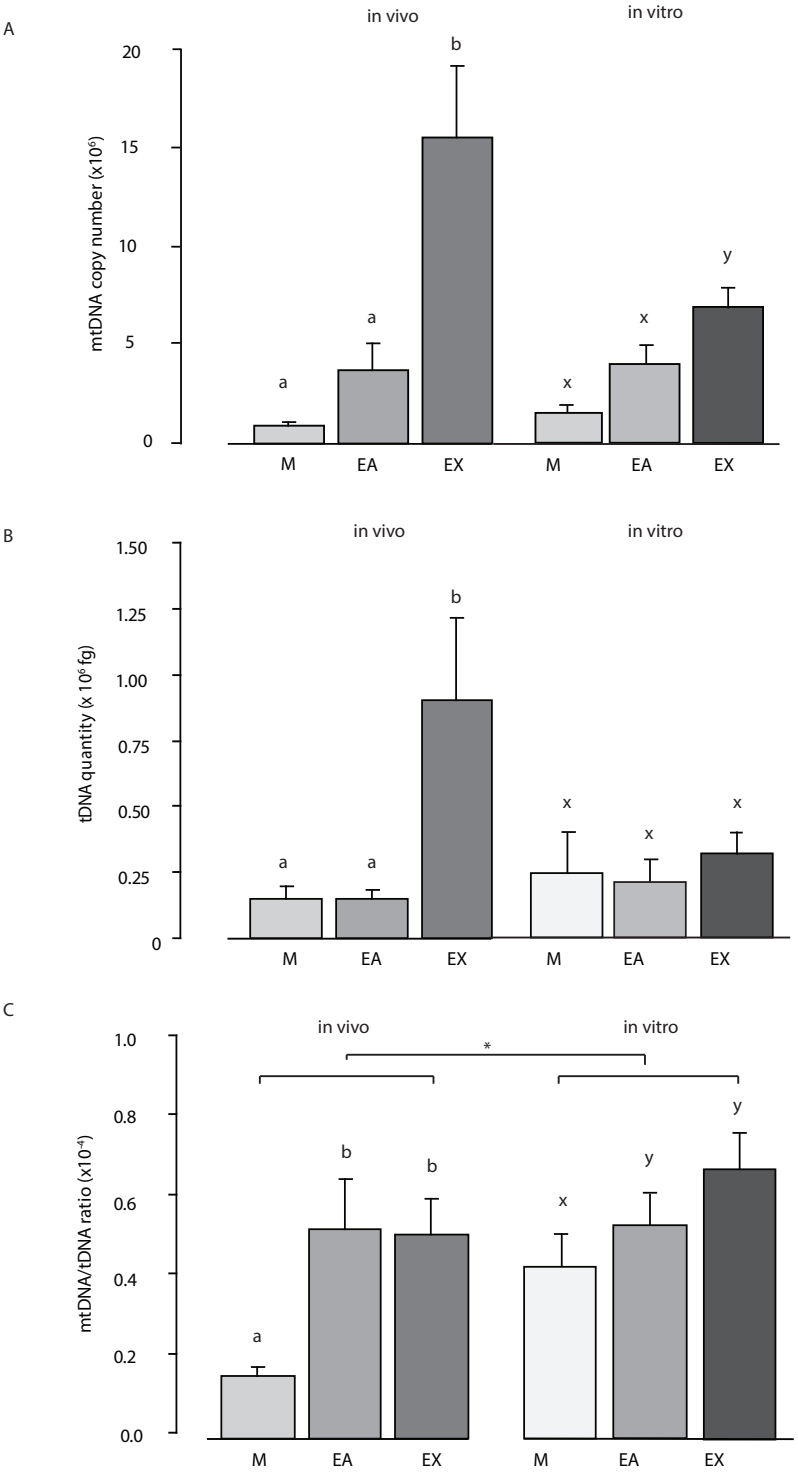
Between production method values marked with * differ significantly ($P < 0.05$).

Figure 2. Mitochondrial DNA copy number (A), total DNA quantity (fg/oocyte or fg/embryo; B), and ratio of mitochondrial DNA to total DNA quantity (mtDNA/tDNA ratio; C) per oocyte (n=8) and for normally developing *in vitro* produced horse embryos (2, 4, 6 and 8 days after ICSI; n=8 per group left panel) and in arrested *in vitro* produced embryos (uncleaved (nc) n=5; blocked at 2 cell stage (b2) n=5; blocked at 8 cell stage (b8) n=2; blocked at >16 cell stage (b16) n=3; right panel). Values are mean ± SEM.

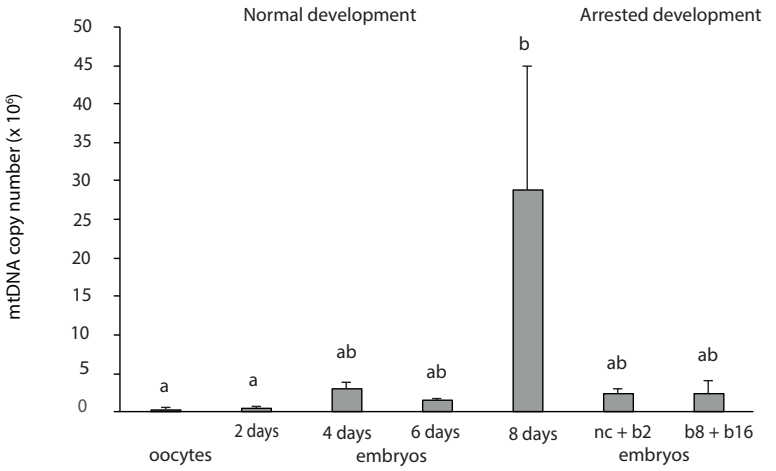
Values marked with different letters differ significantly ($P < 0.05$).

Figure 3. Normalized relative gene expression (mean ± SEM) for genes involved in mitochondrial replication (*TFAM* (A); *mtPOLB* (B); *SSB* (C)) in equine oocytes (n=8) and normally developing *in vitro* produced horse embryos (2, 4, 6 and 8 days after ICSI (n=8 each); left panel) and in arrested *in vitro* produced embryos (uncleaved (nc) n=5 + blocked at 2 cell stage (b2) n=5; blocked at 8 cell stage (b8) n=2 + blocked at >16 cell stage (b16) n=3; right panel). Values marked with different letters differ significantly within the group 'normal

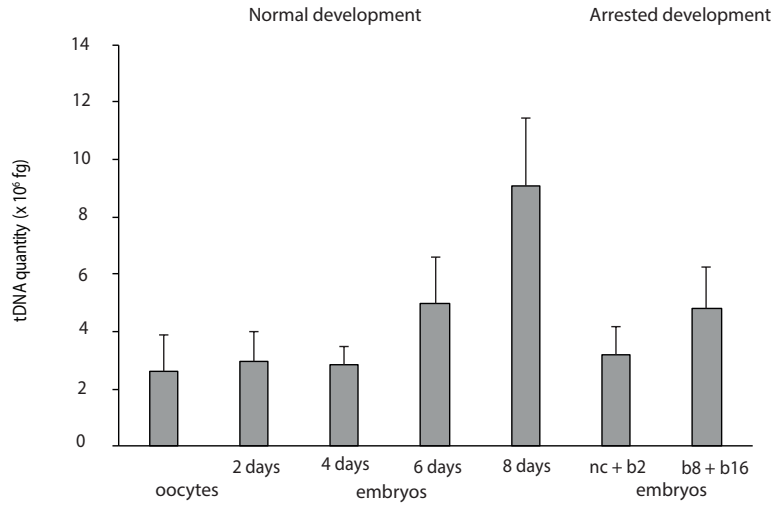
development' ($P < 0.05$). The groups marked with * differ (SSB $P = 0.051$) significantly (mtPOLB $P = 0.045$).


A
in vivo
in vitro
mtDNA copy number (x10⁶)
20
15
10
5
0
a
a
b
x
x
y
M
EA
EX
M
EA
EX
B
in vivo
in vitro
tDNA quantity (x 10⁶ fg)
1.50
1.25
1.00
0.75
0.50
0.25
0
a
a
b
x
x
x
M
EA
EX
M
EA
EX
C
in vivo
in vitro
*
mtDNA/tDNA ratio (x10⁻⁴)
1.0
0.8
0.6
0.4
0.2
0.0
a
b
b
x
y
y
M
EA
EX
M
EA
EX

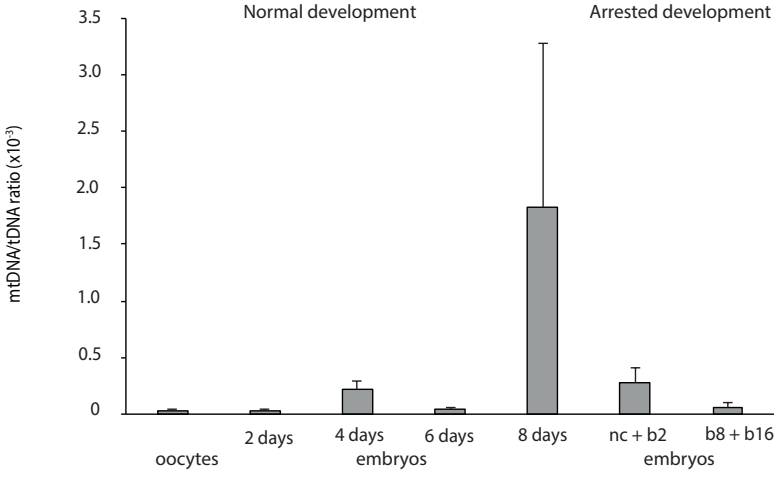
A


Normal development
Arrested development
mtDNA copy number (x 10⁶)
50
45
40
35
30
25
20
15
10
5
0
a
a
ab
ab
b
ab
ab
oocytes
2 days
4 days
6 days
8 days
nc + b2
b8 + b16
embryos
embryos

B


Normal development
Arrested development
tDNA quantity (x 10⁶ fg)
14
12
10
8
6
4
2
0
oocytes
2 days
4 days
6 days
8 days
nc + b2
b8 + b16
embryos
embryos

C


Normal development
Arrested development
mtDNA/tDNA ratio (x10⁻³)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
oocytes
2 days
4 days
6 days
8 days
nc + b2
b8 + b16
embryos
embryos

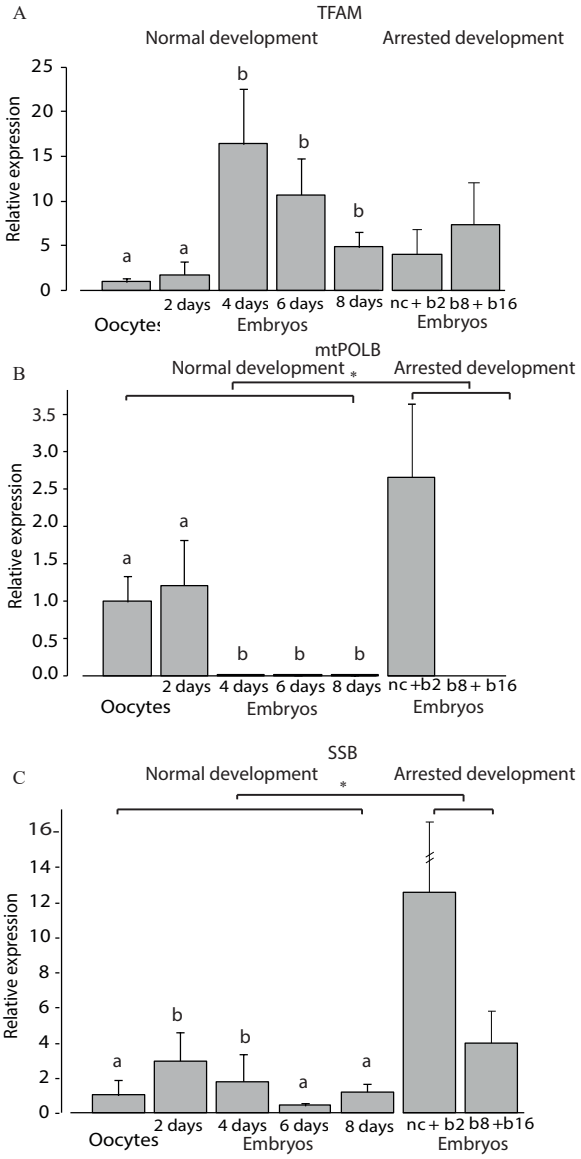

A
TFAM
Normal development
Arrested development
Relative expression
25
20
15
10
5
0
a
a
b
b
b
2 days
4 days
6 days
8 days
nc + b2
b8 + b16
Oocytes
Embryos
Embryos
B
mtPOLB
Normal development
*
Arrested development
Relative expression
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
b
b
b
2 days
4 days
6 days
8 days
nc +b2
b8 + b16
Oocytes
Embryos
Embryos
C
SSB
Normal development
*
Arrested development
Relative expression
16
14
12
10
8
6
4
2
0
a
b
b
a
a
2 days
4 days
6 days
8 days
nc + b2
b8 +b16
Oocytes
Embryos
Embryos

Table 1. Primer details used for gene amplification in quantitative RT-PCR

Symbol	Gene name	GenBank accession number	Primer sequence (5' – 3')	Product size (bp)*	T_A (°C)
PGK1	phoshoglycerate kinase 1	XM_001502668	f 5'-CAAGAAGTATGCTGAGGCTG-3' r 5'-AGGACTTTACCTTCCAGGAG-3'	260	57.0
RPL4	60S ribosomal protein L4-like	XM_001497094	f 5'-CATCCCTGGAATTACTCTGC-3' r 5'-CGGCTAAGGTCTGTATTGAG-3'	203	61.5
TFAM	transcription factor A, mitochondrial	NM_001034016	f 5'-GGCAGGTATACAAGGAAGAG-3' r 5'-GTTATAAGCTGAGCGAGGTC-3'	170	56.5
mtPOLB	DNA polymerase subunit gamma-2, mitochondrial	NM_015810	f 5'-CCGAGTAAGGAACAGCTAGT-3' r 5'-ACTCCAATCTGAGCAAGACC-3'	155	57.5
SSB	mitochondrial single stranded DNA binding protein	XM_003364867	f 5'-CATGAGACAGGTGGAAGGAA-3' r 5'-GATATGCCACATCTCTGAGG-3'	167	57.1
mtDNA	mitochondrial DNA	NC_001640.1	f 5'-CATGATGAAACTTCGGCTCC-3' r 5'-TGAGTGACGGATGAGAAGGCAG-3'	118	67.7

T_A = annealing temperature; f = forward; r = reverse
† used to check quality and purity of cDNA only
¥ touchdown protocol decreasing from 67 to 57 °C (1 °C per cycle over the first 10 cycles)
* numbers in parentheses represent gDNA amplicons

Chapter 5

Cellular damage suffered by equine embryos after exposure to cryoprotectants or cryopreservation by slow-freezing or vitrification

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Cellular damage suffered by equine embryos after exposure to cryoprotectants or cryopreservation by slow-freezing or vitrification

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Summary

Reasons for performing study: Equine embryos are cryopreserved by slow-freezing or vitrification. While small embryos (<300 μm) survive cryopreservation reasonably well, larger embryos do not. It is not clear if slow-freezing or vitrification is less damaging to horse embryos.
Objectives: To compare the type and extent of cellular damage suffered by small and large embryos during cryopreservation by slow-freezing vs. vitrification.
Study design: Sixty-three Day 6.5–7 embryos were subdivided by size and assigned to one of 5 treatments: control, exposure to slow-freezing or vitrification cryoprotectants (CPs), and cryopreservation by either technique.
Methods: After thawing/CP removal, embryos were stained with fluorescent stains for various parameters of cellular integrity, and assessed by multiphoton microscopy.
Results: Exposing large embryos to vitrification CPs resulted in more dead cells (6.8 ± 1.3%: 95% confidence interval [CI], 3.1–10.4%) than exposure to slow-freezing media (0.3 ± 0.1%; 95% CI 0.0–0.6%: P = 0.001). Cryopreservation by either technique induced cell death and cytoskeleton disruption. Vitrification of small embryos resulted in a higher proportion of cells with fragmented or condensed (apoptotic) nuclei (P = 0.002) than slow-freezing (6.7 ± 1.5%, 95% CI 3.0–10.4% vs. 5.0 ± 2.1%, 95% CI 4.0–14.0%). Slow-freezing resulted in a higher incidence of disintegrated embryos (P = 0.01) than vitrification. Mitochondrial activity was low in control embryos, and was not differentially affected by cryopreservation technique, whereas vitrification changed mitochondrial distribution from a homogenous crystalline pattern in control embryos to a heterogeneous granulated distribution in vitrified embryos (P = 0.05).
Conclusions: Cryopreservation caused more cellular damage to large embryos than smaller ones. While vitrification is more practical, it is not advisable for large embryos due to a higher incidence of dead cells. The choice is less obvious for small embryos, as vitrification led to occasionally very high percentages of dead or damaged cells, but a lower incidence of embryo disintegration. Modifications that reduce the level of cellular damage induced by vitrification are required before it can be considered the method of choice for cryopreserving equine embryos.

Keywords: horse; cryopreservation; embryo; vitrification; slow-freezing

Introduction

Equine embryos are not routinely cryopreserved, in part because of the absence of commercially available superovulation protocols [1,2], such that few embryos are available per mare. In addition, pregnancy rates can be disappointing [1–3], and the success depends critically on embryo size and technical experience [4,5]. Pregnancy rates of 45–62% have been obtained for embryos <220–300 μm in diameter [6,7], compared with <20% for larger embryos [5]. Collecting embryos <220 μm is, however, labour intensive and, even when mares are examined for ovulation every 6 h and embryos recovered 156 ± 6 h after ovulation, 14% of those embryos exceed the 220 μm threshold [6]. Moreover, the embryo recovery rate is significantly lower on Day 6 after ovulation, than on Day 7 or 8 [8]. Reasons proposed for poor cryopreservability of large equine embryos (>300 μm) include impaired access of cryoprotectants due to the presence of the blastocyst capsule [9,10], and increased embryo volume [11]. In this latter respect, promising results have recently been reported for large equine blastocysts vitrified following piezo drill-assisted blastocoele collapse [11].

The 2 major cryopreservation techniques are slow-freezing and vitrification [2,3]. Slow-freezing employs relatively low concentrations of cryoprotectants (CPs), but is time consuming and requires a (relatively expensive) programmable freezing machine. Vitrification is rapid and requires only simple materials; however, embryos are exposed to high concentrations of CPs, particularly during the last incubation before immersion in liquid nitrogen [12]. While vitrification avoids ice crystal formation, the high concentrations of CPs can cause cellular damage [13]. Since the pregnancy rates for slow-frozen and vitrified human [14,15], bovine [12] and equine embryos [7,16–18] appear to be similar, vitrification has become the preferred technique because it is rapid [19].

Detecting between freezing-technique differences is difficult because the proof of success, i.e. pregnancy, is an all-or-nothing parameter; unrealistically large numbers of embryos are therefore required to prove definitively that one technique is 'better' than another [3]. Moreover, stereomicroscopic assessment of embryo morphology, performed prior to embryo transfer, appears to be a misleading indicator of embryo viability [20,21]. Alternative analyses that generate discriminative information about embryo quality include post thaw growth *in vitro* [22], metabolic capacity [23] and ultrastructural integrity [24–26]. In this respect, exposure to CPs (e.g. glycerol) has been reported to alter embryo metabolic capacity [23], damage mitochondria and induce nuclear pycnosis [24,25,27,28]. While the effects of exposure to glycerol are reversible, freezing and thawing induces cell death and irreversible disruption of the cytoskeleton [26], cytoplasm (vacuolisation) and cell organelles [20]. The extent of cellular disruption or metabolic compromise may therefore be a useful aid for identifying the least damaging, and for improving, freezing technique(s). Promising candidate markers for reduced viability include mitochondrial damage (morphology [25]; metabolic capacity [23]), and cell membrane and actin cytoskeleton integrity [26]; these parameters are probably interdependent given that cytoskeleton disruption in pronuclear embryos also affects mitochondrial distribution and thereby compromises adenosine triphosphate (ATP) generating capacity [29–31], while ATP is required to repair cytoskeletal damage [25].

This study assessed the amount and types of damage caused by slow-freezing or vitrification at the levels of cellular integrity and mitochondrial activity (an index of metabolic capacity) in equine embryos

(≤300 μm and >300 μm) to determine which was least damaging, and to establish baseline data for future attempts to improve the efficiency of equine embryo cryopreservation.

Materials and methods

Animals

Nine Dutch Warmblood mares, aged between 6 and 15 years, were used as embryo donors. During early oestrus, the reproductive tract of the mares was examined every other day by transrectal palpation and ultrasonography. Once the dominant follicle reached 30 mm in diameter, mares were examined daily until that follicle exceeded 35 mm, at which point the mares were treated with 3000 iu human chorionic gonadotropin (Chorulon)[a] i.v., to induce ovulation, and inseminated with at least 300 × 10^6 morphologically normal, motile sperm from a single fertile stallion. Twice daily examination continued until ovulation was detected.

Embryo recovery and handling

Embryos were collected 156–168 h after ovulation by transcervical lavage of the uterus with 3 × 1 l Dulbecco's phosphate buffered saline (PBS)[b], supplemented with 0.5% v/v fetal calf serum[b]. Embryos were examined using a stereoscopic dissecting microscope[c], and the external diameter (including coverings) and quality score (1–4) [32] were recorded. Next, the embryos were washed 4 times in Syngro holding medium[d] and transferred to 3 ml of holding medium for transportation to the laboratory (approximately 10 min) in an insulated container at room temperature. Embryos graded with a quality score of 1 or 2 were divided into size classes (≤300 or >300 μm) and assigned to one of the following treatment groups (embryos from individual donor mares were evenly distributed over groups):

1) control (≤300 μm, n = 5; >300 μm, n = 7);
2) exposure to slow-freezing CP (≤300 μm, n = 5; >300 μm, n = 5) without freezing;
3) exposure to vitrification CP (≤300 μm, n = 5; >300 μm, n = 6) without cooling;
4) cryopreservation by slow-freezing (≤300 μm, n = 7; >300 μm, n = 8); stored in liquid nitrogen for at least 7 days prior to analysis;
5) cryopreservation by vitrification (≤300 μm, n = 8; >300 μm, n = 7); stored in liquid nitrogen for at least 7 days prior to analysis.

Slow-freezing

Slow-freezing was performed as described by Czlonkowska *et al*. [4]. Briefly, the embryos were equilibrated in 4 steps by incubation at room temperature (22–24°C) for 10 min in each of 0.34 mol/l, 0.67 mol/l, 1.03 mol/l and 1.37 mol/l (10% v:v) glycerol (BioWhittaker)[e] in PBS (pH 7.2–7.4). Subsequently, the embryo was loaded into a 0.25 ml polyvinyl chloride straw (PVC)[f] in a droplet of 1.37 mol/l glycerol in PBS sandwiched between 2 air bubbles. The straw was sealed with a plastic plug and placed vertically in a programmable controlled-rate freezing machine (Kryo 10 Series II)[g]. Initial cooling from ambient temperature down to -6°C was at 1°C/min. The straw was then held for 5 min at -6°C and 'seeded'. After seeding, the straw was cooled at 0.3°C/min to -33°C, and maintained at -33°C for 10 min before submersion into liquid nitrogen.

Thawing slow-frozen embryos

After removal from the liquid nitrogen, the straw was held in the air at room temperature for 10 s before being placed into a 35°C water bath for 1 min. Next, the embryos were expelled into PBS containing 1.37 mol/l glycerol and 0.25 mol/l sucrose[b]. The cryoprotectant was then washed out in 3 steps by incubating the embryo for 10 min each in PBS containing 0.25 mol/l sucrose and decreasing concentrations of glycerol (1.03 mol/l, 0.67 mol/l, 0.34 mol/l). Prior to staining, the embryos were transferred to PBS.

Vitrification

Embryos were vitrified as described by Eldridge-Panuska *et al*. [7]. In brief, embryos were exposed to the following vitrification solutions at room temperature: 5 min in a 200 μl drop of 1.4 mol/l glycerol in PBS, 5 min in a 200 μl drop of 1.4 mol/l glycerol and 3.6 mol/l ethylene glycol[b] in PBS, and finally transferred to a 30 μl drop of 3.4 mol/l glycerol and 4.6 mol/l ethylene glycol in PBS. Within 1 min of transfer to the final vitrification solution, the embryo was loaded into the centre of a 0.25 ml PVC straw, separated on either side by air columns of approximately 10 mm from 90 μl columns of 0.5 mol/l galactose[b] in PBS and the straw was sealed. After a total of 60 s exposure to the high CP concentrations, the straw was placed for 1 min in a plastic goblet (10 × 120 mm)[f] held in liquid nitrogen, before being plunged directly into liquid nitrogen.

Warming vitrified embryos

After removal from the liquid nitrogen, the straw was held at room temperature for 10 s before being submerged for 10 s in a 20°C water bath. Thereafter, the straw was shaken to mix CP and galactose solution, after which the contents of the straw were expelled into a Petri dish and gently stirred to mix the vitrification and dilution solutions. Embryos were incubated for 5 min in a 200 μl drop of 0.25 mol/l galactose in PBS before transfer to PBS.

Exposure to cryoprotectants without cryopreservation

Embryos exposed to the CPs without cryopreservation underwent the same treatments (equilibration and washing) at room temperature as described above, but without cooling or freezing.

Staining of DNA, mitochondria and microfilaments

To label the nuclei of dead cells, embryos were incubated for 15 min at 37°C in PBS containing 0.1 mg/l 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI)[h]. The embryos were then examined using a conventional fluorescence microscope[c] equipped with a digital camera, to count the dead (DAPI-stained) cells.

Next, the embryos were washed in PBS supplemented with 0.1% polyvinylpyrrolidone (PBS-PVP)[b] and exposed to 1 μmol/l Mitotracker Red CMHX-Ros[h] at 37°C for 30 min to label active mitochondria. This was followed by washing 3 times with PBS-PVP at 37°C and exposure to 1 μmol/l Mitotracker Deep Red[h] at 37°C for 30 min, to label all mitochondria. The embryos were subsequently washed 3 times in PBS-PVP at 37°C, before being fixed overnight in 4% paraformaldehyde. Fixed embryos were transferred to PBS-PVP and stored at 4°C before further staining.

To stain all the cell nuclei, and thereby allow determination of total cell number and identify cells with evidence of nuclear fragmentation or condensation (i.e. late stage apoptosis), embryos were again stained for 15 min with 0.1 mg/l DAPI.

Finally, the fixed embryos were permeabilised by incubation in 0.1% Triton X-100[b] for 10 min at room temperature to allow staining of the actin cytoskeleton by incubation for 30 min in 0.165 μmol/l Alexa Fluor 488 phalloidin[h] [26].

Microscopic analysis

Embryos were placed individually into 1 μl droplets of antifade medium (Vectashield)[i] in a fluorodish[j], covered with oil[k] and stored in the dark at 4°C until analysis. The embryos were analysed using a Bio-rad Radiance 2100MP multiphoton system[l] mounted on a Nikon TE300 inverted microscope[m] and equipped with 488 nm Argon-ion, 543 nm Helium-Neon and 568 nm Argon-Krypton lasers. Identical settings were used for all embryos, and imaging was performed in slices and stacks to create a 3D image of the entire embryo.

Cytoskeleton structure was graded as described previously [26]; a *grade 1* cytoskeleton showed uniform, intense actin staining along the cell borders; a *grade 2* cytoskeleton had less distinct cell borders (less sharp) with patchy distribution, including moderate 'clumping', of the actin; a *grade 3* cytoskeleton was characterised by absence of actin staining over large areas, with most of the visible actin in large clumps (Fig 1).

The proportion of active mitochondria was calculated by comparing the distribution of Mitotracker Red CMHX-Ros staining (active mitochondria) with that of Mitotracker Deep Red (all mitochondria). Mitochondrial activity was classified into 4 categories (0–25%, 26–50%, 51–75%, 76–100% active mitochondria: Fig 2).

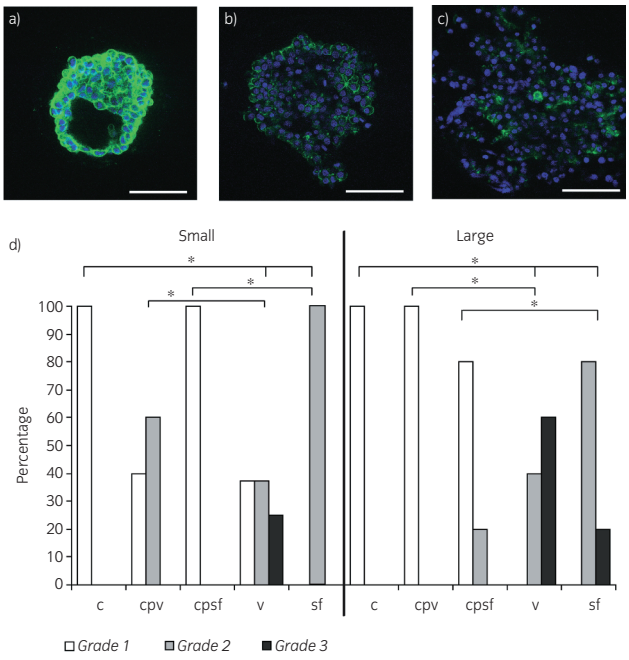

Fig 1: Effect of vitrification or slow-freezing on actin cytoskeleton quality in small (≤300 μm) and large (>300 μm) Day 6.5–7 equine embryos. (a–c) Multiphoton laser scanning micrographs (a) *grade 1*, (b) *grade 2*, (c) *grade 3* actin cytoskeletons. The cytoskeleton is labelled with phalloidin (green) and the nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride (blue). Scale bars represent 100 μm. (d) Cytoskeleton quality scores in different treatment groups, displayed as the percentage of embryos within a treatment group that fell into the different quality categories: control (c); exposure to vitrification cryoprotectant (cpv); exposure to slow-freezing cryoprotectant (cpsf); cryopreserved by vitrification (v) or slow-freezing (sf). *Indicates groups that differ significantly (P<0.05).

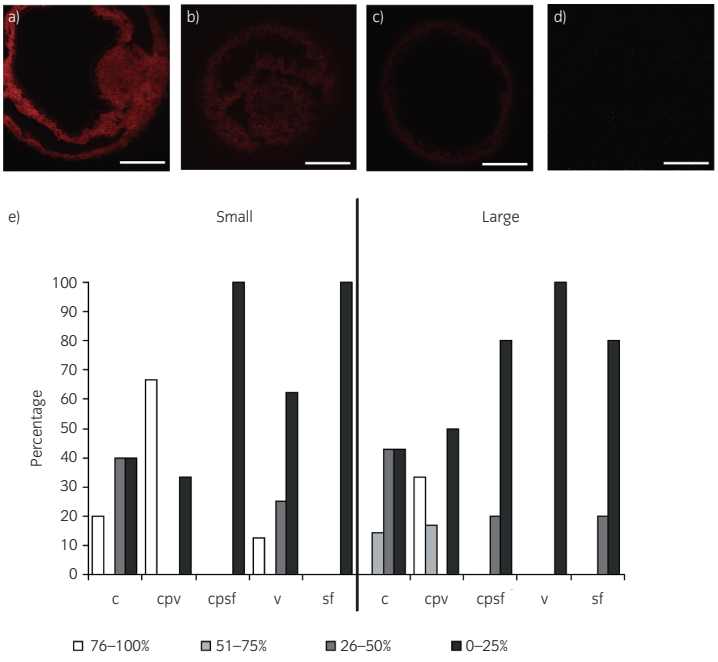

Fig 2: Effect of vitrification or slow-freezing on mitochondrial activity in small (≤300 μm) or large (>300 μm) equine embryos. (a–c) Multiphoton laser scanning micrographs; (a) 76–100%, (b) 51–75%, (c) 26–50%, (d) 0–25% of all mitochondria showing evidence of activity, i.e. Mitotracker Red CMHX-Ros (red) staining. Scale bars represent 100 μm. (e) Proportion of mitochondria showing evidence of activity in the various treatment groups, displayed as the percentage of embryos within a treatment group that fell into the different quality categories: control (c); exposure to vitrification cryoprotectant (cpv); exposure to slow-freezing cryoprotectant (cpsf); cryopreserved by vitrification (v) or slow-freezing (sf).

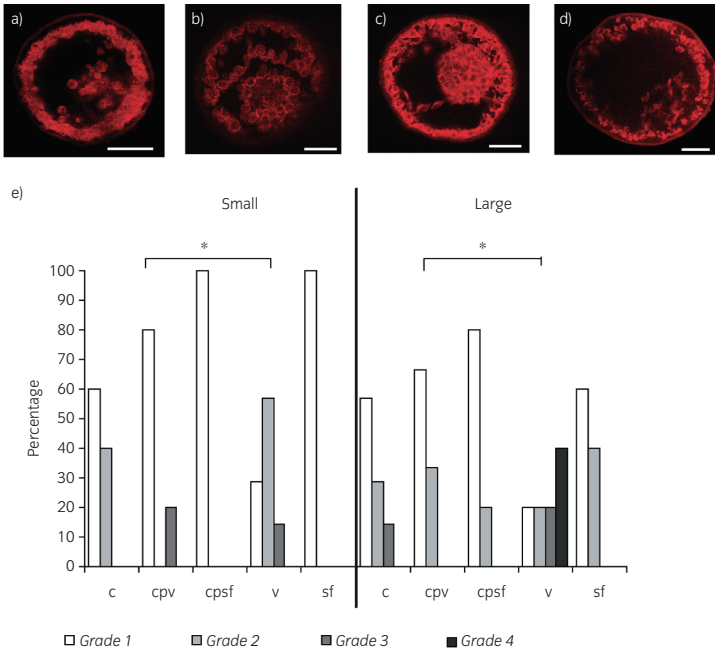

Fig 3: Effect of vitrification or slow-freezing on mitochondrial distribution in small (≤300 μm) or large (>300 μm) equine embryos. (a–d) Multiphoton laser scanning micrographs; (a) *grade 1*, (b) *grade 2*, (c) *grade 3*, (d) *grade 4* mitochondrial distribution patterns, as visualised by Mitotracker Deep Red (red) staining. Scale bars represent 100 μm. (e) Changes in mitochondrial distribution patterns in the different treatment groups, displayed as the percentages of embryos within a treatment group that fell into the different quality categories: control (c); exposure to vitrification cryoprotectant (cpv); exposure to slow-freezing cryoprotectant (cpsf); cryopreserved by vitrification (v) or slow-freezing (sf). *Indicates groups that differ significantly (P<0.05).

The distribution of the mitochondria within embryonic cells was also evaluated and graded as described previously [33]. A *grade 1* mitochondrial distribution referred to homogenous, crystalline distribution of the organelles; *grade 2* indicated homogenous, granulated (more punctate) distribution; *grade 3* referred to heterogeneous, crystalline distribution; and *grade 4* to heterogeneous, granulated distribution (Fig 3).

The 3D images obtained using the multiphoton microscope system were analysed using FIJI software, for which an additional mathematical procedure (Supplementary item 1) was designed to count the total number of nuclei in each embryo automatically.

To count the number of fragmented and condensed nuclei, labelled embryos were additionally examined using a conventional fluorescence microscope equipped with a digital camera.

Data analysis

Statistical analyses were performed using SPSS Statistics 22.ⁿ Data for total cell number, percentages of dead cells and cells with fragmented or condensed nuclei were subjected to logarithmic transformation to fulfil the assumptions of normality of the statistical tests applied.

For total number of cells, percentage of dead cells and percentage of cells with fragmented or condensed nuclei, ANOVA was performed using size, type of cryoprotectant, freezing technique and their interaction as factors. *Post hoc* Bonferroni tests were applied to evaluate further the source of any differences (Supplementary items 2–4).

To compare mitochondrial activity and mitochondrial distribution between treatment groups, ordinal logistic regression was performed to exclude interaction (using embryo size, treatment group and the interaction between these 2 factors as independent categorical variables), followed by univariable analyses of variance using *post hoc* Bonferroni correction for evaluation of statistical differences between treatment groups (Supplementary items 5, 6). Cytoskeleton quality was evaluated by binary logistic regression using the categories *grade 1* and *grade 2*, *3* and *4* combined (because few embryos were *graded 3* and none as *4* for cytoskeleton quality; Supplementary item 7).

Because of the low embryo numbers, the statistical analysis for the prevalence of embryo disintegration was performed using Fisher's exact test to exclude interaction, followed by ANOVA with a *post hoc* Bonferroni correction for evaluation of statistical differences between treatment groups (Supplementary item 8). Differences were considered statistically significant when P<0.05.

Results

Seventy Day 6.5–7 embryos were collected. Seven of those embryos were of poor morphological quality (*grade 3* or *4*) and not used further.

Initial statistical analysis indicated no interaction between total number of cells and percentage of damaged nuclei; therefore, further analyses evaluated differences between the treatment groups: control, exposure to slow-freezing CPs without freezing, exposure to vitrification CPs without cooling, slow-freezing and vitrification. For the percentage of dead cells, there was an interaction with total cell number (P = 0.005) and an ANOVA was run for the 10 different treatment groups (i.e. the 5 groups above, divided over the 2 size categories: ≤300 μm or >300 μm).

Eight embryos disintegrated during the thawing or warming process and could not, therefore, be used for detailed examination of cell integrity. While the proportion of embryos that disintegrated did not differ significantly between slow-freezing (6/15; 40%) and vitrification (2/14; 14%), or between small (3/15; 20%) and large embryos (5/15; 33%), small embryos (P = 0.013) were more likely to disintegrate after slow-freezing than after vitrification. The remaining 55 *grade 1* or *2* embryos were analysed in detail, although one slide bearing 3 large control embryos was damaged during analysis such that the total cell numbers could not be calculated.

Exposure of large embryos to vitrification CPs resulted in higher rates of cell death than exposure to slow-freezing CPs (P = 0.001; Table 1). Both freezing techniques were damaging to large embryos; there was no significant difference between vitrification or slow-freezing (Table 1).

Cryopreservation by vitrification also damaged small embryos, as indicated by an increase in the percentage of cells with a fragmented or condensed nucleus compared with control embryos (P = 0.002: Table 1).

TABLE 1: Effect of cryopreservation technique on percentages of cells recorded as dead or exhibiting nuclear fragmentation or condensation in small (≤300 μm) or large (>300 μm) Day 7 equine embryos (mean ± s.e.m.)

	Small					Large				
	c	cpv	cpsf	v	sf	c	cpv	cpsf	v	sf
n	5	5	5	8	4 *(3ʳ)*	4 *(3ᵇ)*	6	5	5 *(2ʳ)*	5 *(3ʳ)*
Diameter (μm)	254 ± 27^{A}	175 ± 14^{A}	225 ± 22^{A}	197 ± 19^{A}	225 ± 15^{A}	475 ± 74^{B}	521 ± 72^{B}	420 ± 37^{B}	438 ± 61^{B}	559 ± 46^{B}
Total no. of cells	1294 ± 416^{A}	409 ± 68^{A}	521 ± 145^{A}	548 ± 210^{A}	735 ± 154^{A}	3092 ± 617^{B}	2581 ± 1153^{B}	2498 ± 521^{B}	2033 ± 665^{B}	4992 ± 1976^{B}
Dead cells (%)	0.1 ± 0.1^{A}	1.2 ± 0.3^{A}	1.5 ± 0.9^{A}	19.9 ± 13.5^{A}	3.3 ± 0.6^{A}	0.1 ± 0.0^{A}	6.8 ± 1.3^{A}	0.3 ± 0.1^{A,c}	53.3 ± 27.0^{B,a}	26.3 ± 18.5^{B,a}
Fragmented/ condensed nucleus (%)	2.4 ± 1.3^{A}	3.4 ± 0.7^{A}	2.2 ± 0.7^{A}	6.7 ± 1.5^{B}	5.0 ± 2.1^{A}	4.3 ± 1.3^{A}	7.6 ± 2.0^{A}	9.2 ± 3.2^{A}	4.8 ± 0.8^{A}	5.8 ± 1.3^{A}

c = control; cpv = exposure to vitrification cryoprotectant; cpsf = exposure to slow-freezing cryoprotectant; v = vitrification; sf = slow-freezing. Italics indicate disintegrated embryos *(ʳ)* or those lost due to a broken slide *(ᵇ)* and therefore not included in the calculations. Within a row, values with different superscripts (capital or small letters) differ significantly (P<0.05).

Exposure to CPs without freezing had no effect on cytoskeleton quality, whereas cryopreservation by either technique of both small and large embryos led to a reduction in mean cytoskeleton quality (P<0.001 for vitrification, P<0.001 for slow freezing; Fig 1d). However, there was no statistically significant difference between cryopreservation techniques.

Mitochondrial activity was surprisingly variable in control embryos (Fig 2e), with around 40% of untreated embryos showing low percentages (0–25%) of actively respiring mitochondria. However, neither exposure to CPs nor cryopreservation of embryos markedly affected apparent mitochondrial activity within the cells of either small or large embryos (Fig 2e).

Freezing large embryos by vitrification led to a significant change (P = 0.048) in mitochondrial distribution pattern, namely an increase in heterogeneous granulated distribution (Fig 3e).

Discussion

In the current study, the percentages of dead cells in intact embryos were relatively low. The means of 3.3% and 26.3% dead cells in small and large embryos, respectively, after slow-freezing compared favourably to the 8.5% and 19% reported by Tharasanit *et al*. [26]. Similarly, the 3.3% and 19.9% dead cells in small embryos after slow-freezing and vitrification compared favourably with the 42% and 46% reported by Moussa *et al*. [17] and the 26% and 49% documented by Oberstein *et al*. [18]. These differences presumably stem, at least in part, from differences in the way in which the percentage of dead cells was calculated. In this respect, Moussa *et al*. [17] counted dead cells, but estimated total cell number on the basis of embryo diameter, whereas Oberstein *et al*. [18] estimated the percentage of dead cells on the basis of the relative areas of staining for propidium iodide and Hoechst 33342. By contrast, Tharasanit *et al*. [26] provided precise figures by counting individual cells; while undoubtedly accurate, cell counting is laborious and would be difficult in the large embryos examined in the current study, which contained many thousands of cells. To overcome this problem, we developed a 'macro' for the Image J software to automatically count the nuclei in stacks of confocal laser scanning images spanning the entire depth of an embryo (i.e. a virtual 3D image), this allowed rapid but accurate estimation of the cell number.

An unexpected finding of the current study was the absence of significant differences in cytoskeleton disruption between vitrification and slow-freezing. This is at odds with Dobrinsky's [34] suggestion that intracellular ice crystal formation is the primary cause of cytoskeleton disruption during embryo cryopreservation, since vitrification should have circumvented ice crystal formation. While this difference may, in part, be due to the small number of embryos examined, we did confirm that slow-freezing caused cytoskeleton disruption [26], especially in larger equine embryos. The unexpected element was therefore the finding that vitrification was also detrimental to cytoskeleton integrity. Moreover, it was vitrification *per* se, rather than exposure to the CPs that seemed to cause the majority of the damage observed.

The low percentage of active mitochondria (<25%) in many control embryos was, in retrospect, not surprising given that 'healthy' embryos are reported to have a 'quieter metabolism' than arrested or damaged embryos, because they do not, unlike the latter, have to generate energy to repair damage [25–27]. As a result of elevated mitochondrial activity, compromised embryos are subject to higher levels of metabolic stress and an elevated risk of reactive oxygen species production, which can in turn result in apoptosis-like DNA damage (including fragmentation and condensation), and ultimately cell death [35]. The hypothesis that embryos damaged by exposure to CPs and/or cryopreservation would display elevated levels of active mitochondria was not confirmed; in this respect, the high degree of inter-embryo variability and low numbers of embryos meant that it was difficult to discern between-treatment differences.

As expected, large embryos were more severely affected by cryopreservation using either technique than small embryos. Reasons for the high incidence of cell death may include inadequate penetration of CPs into the cells of larger blastocysts or increased embryo volume *per se* [10,11]. Recently, blastocoele collapse induced by aspirating fluid prior to vitrification, via a hole drilled through the capsule and trophoblast with a piezo drill [11] or laser [36], has been reported to improve the freezability of large blastocysts, suggesting that increased volume is the primary impediment; of course, both are specialised interventions requiring expensive equipment. It will therefore be interesting to determine both whether blastocoele collapse can be reliably induced by simpler methods that can be performed in practice, and the extent to which blastocoele collapse ameliorates cell death and damage inflicted during vitrification and whether this protection also applies to slow-freezing, the technique that was less damaging to large embryos in the current study.

In conclusion, equine embryos >300 μm in size are considerably more susceptible than small embryos to freezing-induced cellular damage; this is particularly true for vitrification which, unless the utility of blastocoele collapse can be verified, should be avoided in larger horse embryos. Although vitrification has an inherent advantage in terms of practicality, and tended to result in a lower incidence of disintegration among small embryos, it carries a higher risk of cell death and damage, as evidenced by an increased incidence of late-stage cell apoptosis, and occasional high levels of cell death never observed in slow-frozen (small) embryos.

Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

All animal procedures were approved by the local ethical committee (DEC 007.III.02.036) of Utrecht University.

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Authorship

All authors made significant contributions to the study design, data analysis and interpretation and preparation of the manuscript. All authors have approved the manuscript submitted for publication.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary item 1: Mathematical procedure to count cell nuclei.

Supplementary item 2: SPSS analysis; descriptive statistics for cell number, percentage of dead cells and percentage of damaged nuclei.
Supplementary item 3: SPSS analysis log cell number.
Supplementary item 4: SPSS analysis log percentage of dead cells and log percentage of damaged nuclei.
Supplementary item 5: SPSS analysis mitochondrial activity.
Supplementary item 6: SPSS analysis mitochondrial distribution.
Supplementary item 7: SPSS analysis cytoskeleton quality.
Supplementary item 8: SPSS analysis disintegrated embryos.

Chapter 6

Summarizing discussion

Introduction

Assisted reproductive technologies (ARTs) such as embryo transfer (ET), embryo cryopreservation and *in vitro* embryo production (IVEP) via intracytoplasmic sperm injection (ICSI) are in increasing demand in the horse breeding industry. In general, the high economic value of some individual mares and stallions justifies the efforts and costs required to generate offspring using these techniques. Moreover, success rates in terms of ongoing pregnancies or birth of live foals after applying most of these ARTs are now high enough to vindicate their use in clinical practice; nevertheless, there is still room for improvement. Inadvertent and undesirable effects described when using ARTs include epigenetic alterations, which result in (often permanent) changes in gene expression (switching off or switching on of genes) without any changes in DNA composition. Exposure to fluids and contact with plastic-ware during oocyte maturation, IVEP, embryo transport/storage and cryopreservation (e.g. exposure of plastics to culture medium causes leakage of polymers that can induce epigenetic alterations in mouse embryos) have all been reported to induce changes in the epigenome that influence the transcriptional potential of the nuclear DNA (for review see Ventura-Junca *et al.,* 2015; Pinborg *et al.,* 2016). For example, in man and cattle, complications such as low birth weight (man: Dumoulin *et al.,* 2010) and the large offspring syndrome (cattle: Badr *et al*., 2007) are, respectively, the result of epigenetic changes triggered by culture medium components during IVEP. In horses, epigenetic changes resulting from ARTs have not yet been described or studied.

Other aspects of ARTs that are disappointing or require optimizing for the horse breeding industry include the low embryo recovery rate after uterine lavage in older mares (Marinone *et al.,* 2015), lower pregnancy rates after transfer of oocytes recovered from older mares to the oviduct of (young) recipients (Carnevale *et al.,* 2005), poor success of cryopreserving expanded equine blastocysts (Choi *et al.,* 2011), and a relatively low blastocyst rate after using ICSI for IVEP with patient derived material, as compared to in a research setting (Hinrichs, 2010, 2012; Galli *et al.,* 2007, 2014). In addition, a higher incidence of early embryonic loss can be expected when recovering embryos from older mares or after oocyte recovery and IVEP. This thesis investigated factors involved in ART procedures, to better understand the underlying causes of the intrinsic difficulties, and to encourage the creation of solutions.

Summary of results

Chapter 2 *Fetal development and ultrasonography*

In vitro embryo production compromises early embryonic development and quality, presumably because culture conditions inadequately mimic the *in vivo* environment. In general, after an equivalent number of days post-fertilization (usually 7-8) IVEP horse blastocysts are smaller (~140 µm vs. ~375 µm) than *in vivo* blastocysts, have a lower cell number (85-150 cells vs. ~1200-2000 cells), a higher percentage of apoptotic cells (Rubio-Pomar *et al.,* 2005), and a less well defined microfilament cytoskeleton (Tremoleda *et al.,* 2003). Advancing maternal age is another factor known to reduce reproductive efficiency. Recent surveys in Thoroughbred horses reported higher pregnancy rates at day (D) 15 of gestation in mares younger than 14 years (63-72%) compared to mares 14 years and older (48-61%) (Allen *et al.,* 2007; Bosh *et al.,* 2009; Scoggin, 2015). This was compounded by a higher incidence of early embryonic death in the older mares (10-23% versus 4-10%) such that D40-42 pregnancy rates diverged even more markedly (58-63% in mares <14 years old versus 37-52% in mares > 14 years old) (Allen *et al.,* 2007; Bosh *et al.,* 2009). It was also reported that the embryonic vesicles in aged mares are smaller at D11 than in younger mares (Scoggin, 2015), and this retarded embryonic/fetal development in aged mares continues throughout gestation, ultimately resulting in foals of lower birth weight and with other problems (Wilsher and Allen, 2003). In some species, IVEP has been shown to predispose to intrauterine growth retardation (IUGR), for example during human fetal development (Leese, 2012). In both man and horse, ultrasonography can help in the monitoring of fetal development.

Ultrasonography is a valuable tool for evaluating and monitoring fetal growth, and can be used to diagnose abnormal fetal development and intrauterine growth retardation. In man, the growth curves of growth-restricted and macrosomic (large for gestational age) fetuses diverge significantly from expected growth curves (Lerner, 2004). Similarly in cattle, divergent fetal development following IVEP in serum enriched medium that resulted in the 'large offspring syndrome' (Knijn *et al*., 2005; Badr *et al.,* 2007; Galli *et al.,* 2012), could be detected by serial ultrasonographic evaluation. However, while studies in cattle have gone on to identify changes in gene expression during IVEP that predispose to the large offspring syndrome (Heras *et al.,* 2016), the influence of IVEP on fetal development in horses has not been examined. Advancing mare age results in a smaller, slower growing fetus; however, the reason for restricted fetal development in older mares may be based on an age-related reduction in endometrium quality (Allen *et al*., 2007) rather than on intrinsic deficits of the oocyte and resulting embryo. In order to monitor development and determine whether factors such as IVEP

and maternal age compromise fetal development, and whether the latter can be remedied by transferring the embryo into a younger recipient mare (i.e. dissect the effects of oocyte quality and endometrium quality), fetal growth curves for normal intra-uterine development in mares of the appropriate breed or type are required as a reference. Since initial equine fetal growth curves concentrated on Thoroughbred and Standardbred horses (Pantaleon *et al.,* 2003; Bucca *et al.,* 2005), which are significantly smaller than the Warmblood horses typically used for show jumping and dressage, Chapter 2 monitored fetal growth in Dutch Warmblood mares longitudinally to generate fetal growth curves as a baseline for future monitoring.

Ultrasonography allows visualization and measurement of some fetal body parts and, rather than concentrating on measurements performed at a single examination, is most useful when applied in the form of serial examinations to monitor intra-uterine fetal growth and detect divergence from an expected growth curve. While it is unclear whether IVEP leads to fetal oversize (such as after culturing ruminant embryos in serum containing medium) or intra-uterine growth retardation (IUGR: as described for human IVF programmes) in horses, factors such as advanced maternal age, severe maternal illness and placentitis are known to predispose to IUGR (Baschat and Hecher, 2004; Morris *et al.,* 2007; Klewitz *et al.,* 2015). Moreover, the severity of IUGR may be a useful indicator for the ultimate outcome in terms of whether the foal will be carried to term and, if it is, its likely health and susceptibility to disease.

A significant result from our study was that, of the parameters considered, only aorta diameter and biparietal (i.e. the fetal cranium) cross-sectional surface area increased linearly during late gestation; these should therefore be considered the parameters of choice for evaluating equine fetal size and growth. Another interesting result from our study was that aorta diameter and biparietal cross-sectional surface area were significantly influenced by maternal age and parity, respectively. At a given stage of gestation (time after ovulation), aorta diameter of the fetus in mares aged >15 years was smaller than that in mares ≤ 15 years, suggesting retarded fetal growth that presumably explaining the mean lower birth weight of foals from older mares (Wilsher and Allen, 2003). The biparietal cross-sectional surface area in primiparous mares was, surprisingly, larger than that in pluriparous mares. The opposite had been expected given that primiparous mares generally produce foals with a lower birth weight than in subsequent pregnancies (Wilsher and Allen, 2003). Most importantly, the study provided the necessary curves with which fetal growth during late gestation can be compared.

To accurately evaluate fetal growth and recognize deviations from normal development, it is essential to perform serial measurements since this will give a more accurate indication of not only whether fetal measurements are within normal limits (two standard deviations from

the mean) but whether growth is parallel to, or deviates increasingly, from the expected growth curve. To date, only very limited information about equine fetal development after IVEP or somatic cell nuclear transfer is available and this concentrated on pregnancy loss, the combined thickness of uterus and placenta (which increases during late pregnancy), live foal birth rate and post-natal development of 'cloned' embryos (Galli and Lazzari, 2008; Johnson *et al.*, 2010). Our study should facilitate detection of deviations from normal fetal growth and development in cases where there are reasons to suspect that pregnancy may be at risk of compromise, this would support decisions on whether specific steps need to be taken to support pregnancy, or to prepare to assist parturition and provide neonatal care.

Chapter 3 and 4 *IVP and mitochondria*

A downside of current equine IVEP protocols is compromised early embryonic development: cleavage rates after intracytoplasmic sperm injection (ICSI) of *in vitro* matured oocytes average around 70% (Galli *et al.,* 2007) compared to the 90-95% reported after *in vivo* oocyte maturation (Ball *et al.,* 1989); on the other hand, ovum pick up can yield around 6-7 mature oocytes (Jacobsen *et al.,* 2010; Galli *et al.,* 2014) for attempted fertilization compared to only 1 or 2 for an *in vivo* cycle. Development of the cleaved embryo to the blastocyst stage is however much lower *in vitro* at 5-35% (Jacobsen *et al*., 2010) than the 66-73% reported *in vivo* (Ball *et al.,* 1989; Marinone *et al*., 2015). One factor that might affect the success of fertilisation *in vitro* and subsequent early embryonic development, is inadequate mitochondrial capacity in the oocyte, as a result of insufficient mitochondrial numbers or suboptimal function of the available mitochondria (Eichenlaub-Ritter *et al.,* 2011). Mitochondria are critical to cellular metabolic processes and are one of the first organelles to fail during cell deterioration and programmed cell death (apoptosis). As a result of their role in ATP production, mitochondria are the site of reactive oxygen species production while, because of the absence of normal DNA repair mechanisms within the mitochondria, mitochondrial DNA (mtDNA) is more prone to ROS-induced damage that can accumulate over time than genomic DNA. Advanced maternal age and the conditions experienced during IVM, ICSI and embryo culture could augment this mtDNA damage. In this respect, IVEP-induced changes in mitochondrial quantity and function have been demonstrated in mouse, cattle and pig oocytes (El Shourbagy *et al.,* 2006; May-Panloup *et al.,* 2007; Iwata *et al.,* 2011; Lee *et al*., 2014). In horses, *in vitro* maturation of oocytes from old mares led to a reduction in mitochondrial number with swelling and loss of cristae indicative of the loss of mitochondrial function (Rambags *et al.,* 2014).

To determine whether IVEP affected mitochondrial number, function or mtDNA replication capacity in horse oocytes and early embryos, we investigated the influence of advancing maternal age and IVEP on mitochondrial quantity and function in equine oocytes and embryos (Chapters 3 and 4). In Chapter 3, the effects of maternal age and IVEP on mitochondrial quantity were investigated. It transpired that D7 embryos recovered from older mares (> 16 years) have fewer mtDNA copies than D7 embryos from younger mares (<12 years). By contrast, there was no obvious effect of IVEP, in that D8 IVEP embryos had similar mtDNA copy numbers to D7 *in vivo* embryos, while the stages cover the morula to blastocyst transition period.

To examine possible effects on mitochondrial transcription and replication, we examined expression of genes that regulate these processes, including mitochondrial transcription factor (*TFAM*) (Facucho-Oliveira *et al.,* 2007; Kasashima *et al.,* 2014), mtDNA polymerase γ subunit B (*mtPOLGB*) and mitochondrial single stranded DNA binding protein (*SSB*) (St John *et al.,* 2010). *TFAM* is involved in the initiation of mitochondrial transcription and also in mtDNA packaging, and plays a critical role in the regulation of mitochondrial copy number (Kasashima *et al.,* 2014). In fact, the actions of *TFAM* appear to be dose dependent in that low levels of *TFAM* are required for mtDNA replication to take place, whereas high levels are associated with repressed transcription. Nevertheless, because homozygous *TFAM* knockout mice suffer embryonic death as a result of post-gastrulation mtDNA depletion (Larsson *et al*.,1998), it is clear that *TFAM* is critical for dynamically regulating mtDNA replication. *mtPOLGB* similarly helps to maintain mtDNA copy number by stimulating replication, whereas *SSB* promotes Twinkle activity, where Twinkle is required to unwind the mtDNA to allow replication (St John *et al.,* 2010). All of these genes are up-regulated in the embryonic period during which mtDNA replication is reactivated (Piko and Taylor, 1987; May-Panloup *et al.,* 2007; Thundathil *et al.,* 2005; Spikings *et al.,* 2007).

In D8 IVEP horse embryos, *mtPOLGB* and *GPX3* (involved in balancing the generation and decomposition of reactive oxygen species (ROS) expression deviated from that observed in *in vivo* embryos. *mtPOLGB* expression in IVEP embryos was double that in D7 *in vivo* embryos (presumably to support mtDNA maintenance) whereas *GPX3* expression was halved. This halving of *GPX3* expression might lead to reduced protection against ROS induced damage, while the overexpression of *mtPOLGB* may be part of an attempt to maintain mitochondrial number in the face of increasing rates of damage. Alternatively, a reduced ability

to neutralize ROS might lead to damage to mitochondria and other cellular organelles and molecules, which would compromise embryonic quality and developmental competence.

Since both IVEP and advanced maternal age have been proposed to compromise the onset of mtDNA replication, we also wanted to determine when mtDNA replication is reinitiated after fertilization. In cattle and pig embryos, the onset of mitochondrial DNA replication was shown to occur at the early blastocyst stage in *in vivo* embryos (El Shourbagy *et al.,* 2006; May-Panloup *et al*., 2007; Spikings *et al*., 2007). We investigated the timing of onset of mtDNA replication in both *in vivo* and IVEP horse embryos (Chapter 4). The first significant post-fertilization increase in mtDNA copy number took place between the early and expanded blastocyst stages, in both *in vivo* and *in vitro* embryos. The onset of mitochondrial replication was preceded by an increase in *TFAM* expression in *in vitro* embryos at 96h of culture, 4 days prior to blastocyst formation, although this was not accompanied by increases in *mtPOLGB and SSB*. By contrast, *mtPOLGB* and *SSB* expression were high in oocytes and in embryos after 48h of culture. The reasons for the relatively large numbers of *mtPOLGB* and *SSB* transcripts in oocytes and 2-cell stage embryos is unclear, but may relate to a round of mtDNA replication postulated to take place in the period between fertilization and the first cleavage division (McConnel and Petrie, 2004) and which has been proposed to be linked to, and help compensate for, the removal of paternal and defective mtDNA copies.

Chapter 5 *Cryopreservation and mitochondria*

Cryopreserving embryos can greatly improve the efficiency of equine ART by removing the need to have synchronized recipient mares available every time a fresh *in vivo* or *in vitro* produced embryo might become available. The major impediment to wide scale embryo cryopreservation has been suboptimal results, coupled to the high value and relative scarcity of horse embryos, which discourages the performance of clinical trials with commercial embryos. While IVEP embryos and small *in vivo* embryos can be frozen and thawed with only a moderate reduction in pregnancy rates (Choi and Hinrichs, 2016), large flushed blastocysts (>300 μm) fair much less well. Recently blastocoel aspiration was described as a means to reduce the volume of expanded blastocysts (Choi *et al.,* 2011) and has improved the likelihood of pregnancy after vitrification and warming, but levels of success are still significantly lower than for smaller embryos (Choi and Hinrichs, 2016). Clearly, cryopreserving embryos causes damage that reduces their viability; to improve cryopreservation techniques in a targeted fashion, it would be useful to know what type of damage results during the cryopreservation process. In this respect, freezing/thawing of equine embryos is known to cause pyknosis

(shrinking of the nucleus leading to cell death) while exposure to the cryoprotectant glycerol has been reported to damage the mitochondria (Bryas *et al.,* 2000; Dalcin *et al.,* 2013); mitochondrial dysfunction was also proposed as a possible contributor to the failure of frozen-thawed embryos to completely regenerate their actin cytoskeleton during post-thaw culture (Tharasanit *et al*., 2005). In chapter 5, the effect of different cryopreservation methods on embryos of different sizes was examined, with read-out parameters including mitochondrial activity, nuclear fragmentation indicative of apoptosis and cytoskeleton integrity.

Exposure to cryoprotectants without freezing/cooling did not affect mitochondrial activity, nor did it result in nuclear fragmentation or cytoskeleton disruption. Overall, small embryos cryopreserved by controlled-rate freezing suffered less damage than large controlled-rate frozen or vitrified embryos. Although the proportion of mitochondria showing evidence of activity on the basis of Mitotracker red CMHX-Ros (a marker for mitochondrial membrane potential) was not a useful indicator of embryo quality or damage, vitrification of large embryos resulted in a change in mitochondrial distribution within the cells, from a homogenous to a heterogeneous pattern, that has previously been proposed to be associated with an alteration in function. In control embryos, mitochondrial activity was surprisingly variable with about 40% of untreated embryos displaying very low mitochondrial activity. That 'healthy' embryos have a 'quieter metabolism' than damaged embryos, because they do not have to generate energy to repair damage (Leese, 2012; Takahashi, 2012), may in large part explain why it was difficult to monitor mitochondrial damage in terms of a reduction in activity.

ART and aging

Breeding with older mares is common; many breeders first want evidence of a mare's sporting capacities before deciding on whether she is suitable for a breeding career. In some cases, however, a show jumping or eventing mare will be bred to produce a foal at 3 or 4 years of age, before being 'broken' and embarking on a competitive career. The advantage is that at the time that the mare begins to compete at a higher level, her first offspring will also have entered training and it may be apparent that she is a promising transmitter of genetic potential. The difficulty with breeding an older mare is the age-related decline in fertility. Surveys of commercial Thoroughbred mares have demonstrated that reproductive performance decreases from approximately 9 years of age onwards (Allen *et al.,* 2007; Bosh *et al.,* 2009; Scoggin, 2015). Age-related factors known to influence fertility include reduced endometrium quality, blocked oviducts and reduced oocyte quality (Carnevale *et al.,* 2005; Snider *et al*., 2011). ART can be used to bypass some of the issues of advanced age e.g. using a young recipient mare

with better endometrial quality; however reduced oocyte quality in older mares remains a potential problem. Age-related reduced oocyte quality is sometimes grossly visible by morphological changes, for example an increase in the number and size of cytoplasmic vacuoles, but will also be associated with a higher incidence of invisible DNA modifications and aberrations (Carnevale, 2008), that explain the significant reduction in pregnancy and foaling rates after transfer of oocytes from mares aged >23 years into the oviduct of young recipients compared to transfer of oocytes from mares < 15 years old (Carnevale, 2004).

Another structure within the oocyte known to be affected by maternal age is the mitochondrion. As described for oocytes from older women (Shoubridge and Wai, 2007), it is possible that oocytes from older mares contain more mitochondrial DNA mutations , that may result in compromised mitochondrial function which, in turn, could lead to increased ROS production. Increased ROS production from damaged or defective mitochondria (Carnevale, 2008) has been proposed to be one of the underlying causes of the increased incidence of apoptosis seen in IVEP horse embryos (Tremoleda *et al*., 2003; Rubio Pomar *et al.,* 2005) and may predispose to retarded embryo development and reduced developmental competence, as will be discussed in a later section.

ART, aging and ultrasonography

Ultrasonographic evaluation of the mare's reproductive tract is a standard procedure in equine breeding management. For embryo transfer, it is important to know the number of follicles ovulated in the donor mare, and the time of ovulation of both donor and recipient mares to enable adequate synchronization of ovarian cycles (Stout, 2006; Wilsher *et al.,* 2012; Jacob *et al.*, 2012). In addition, ultrasonography enables the detection of abnormalities of the reproductive tract that may compromise fertility of either donor or recipient, like the presence of intrauterine fluid or air. During ovum pick-up (OPU), ultrasonography is critical for visualizing the follicles for correct needle placement to allow aspiration and flushing of the follicular contents (Galli *et al.,* 2001; Hinrichs *et al.,* 2012; Rader *et al*., 2016) . In addition, ultrasonography can be used to evaluate development of the embryonic vesicle, embryo proper and fetus, and also for fetal sexing (Aurich and Schneider, 2014; Resende *et al.,* 2014) between either days 60 and 70 of gestation or between approximately days 90 and 120, a procedure that has become routine in many breeding facilities over the last decade. Regular examination of pregnant mares facilitates the recognition of abnormalities during fetal development that may require further monitoring or intervention (Bucca *et al.,* 2005; Renaudin *et al.,* 2000). Chapter 2 revealed that serial measurements of fetal aorta diameter and biparietal cross-sectional surface

area are the most reliable ways of monitoring late fetal development, because they are the only parameters that increase linearly rather than reaching a plateau.

In theory, any disturbance of, or deviation from the normal, maternal environment during early (in particular, preimplantation) development can lead to permanent changes at the level of the epigenome that either affect subsequent pregnancy development or post-natal health and susceptibility to disease (Sinclair and Singh, 2007). The developing embryo responds to the prevailing environment by irreversibly altering its functional genome by methylation or histone modifications that permanently change gene expression capacity (Stout and Troedsson, 2015). Beckwith-Wiedemann syndrome in children and the large offspring syndrome in calves (Ren *et al.,* 2015) both result from changes to the epigenome that arise in response to IVF conditions, and both can also be detected by ultrasonographic monitoring of fetal growth and development. Since IVEP in horses is still in its infancy, little is known about deviations in fetal growth and development; fortunately, to date the majority of IVEP foals appear to be grossly normal. On the other hand, a recent report documented a high rate of abnormalities in placental development in pregnancies produced by somatic cell nuclear transfer (Pozor *et al.,* 2016), emphasizing the importance of monitoring fetal and placental development following ART procedures. Since ART is popular among Warmblood breeders, parameters for normal fetal and placental growth (Chapter 2) will be useful references for monitoring the consequences of ART procedures in the field.

ART and mitochondria

As described above in the section "ART and aging", mitochondrial DNA copy number and mitochondrial number and activity can all be affected by either ART, increasing maternal age or a combination of the two. Moreover, suboptimal mtDNA copy number is associated with poor embryo viability. How mtDNA copy number and mitochondrial function in equine oocytes and embryos are affected by ART and aging in horses, and how this affects pregnancy and embryonic loss rates, are only just beginning to be unravelled. The following paragraphs give some additional discussion and suggest possible avenues to overcome deficiencies.

Mitochondrial function

Mitochondria are the cellular organelles responsible for various metabolic processes, notably oxidative phosphorylation (OXPHOS) for the aerobic generation of ATP; however, mitochondria are also responsible for potentially harmful processes such as the production of reactive oxygen species (ROS) and the regulation of apoptosis (via the intrinsic pathway)

(Chappel, 2013). Because of the huge energetic demands of oocyte maturation and early embryo development, reduced mitochondrial or metabolic capacity of the oocyte has a marked negative effect on oocyte developmental competence (Van Blerkom *et al.,* 1995; Takeuchi *et al.,* 2005). Both advanced maternal age and IVEP can increase the risk of mitochondrial dysfunction (Fragouli *et al.,* 2015; Meldrum *et al.*, 2016), which will in turn result in increased ROS production, accompanied by a higher incidence of apoptosis (Pomar *et al.,* 2005) and of mitophagy/autophagy (Ramalho-Santos and Amaral, 2013). In turn, this will reduce the bioenergetic capacity of the oocyte or embryo and predispose to meiotic spindle disorders, chromosomal mis-segregation, and increased incidences of aneuploidy and pregnancy loss (Bentov *et al.,* 2011; Van Blerkom, 2011). It is likely that this explains why *in vitro* embryo culture leads to an increased rate of chromosomal abnormalities, illustrated by studies of *in vitro* produced embryo's (horse: Rambags *et al*., 2005; farm animals: Viuff *et al*., 2002).

Mitochondrial quantity

Oocyte mitochondrial quantity is significantly affected by IVEP and maternal aging, and a drop in mtDNA copy number below a threshold level is thought to have a deleterious effect on subsequent embryonic development (Ge *et al*., 2012; Chappel, 2013). In this respect, suboptimal conditions during oocyte maturation and embryonic development can lead to the activation of pathways that trigger or permit apoptosis and cell death (O'Neill *et al*., 2012). Moreover, an increase in ROS production can lead to mitochondrial damage and since damaged mitochondria are less able to regulate ROS production, a vicious circle can result that ends in mitochondrial deterioration and could explain the drop in the number of mtDNA and mitochondria per oocyte seen in old mare oocytes subjected to IVM (Rambags *et al.,* 2014) and, indeed, in the oocytes of older women (Bentov *et al.,* 2011).

However, the optimal mtDNA copy number per embryonic cell and the effects of reduced mtDNA copy numbers on embryo development need to be studied in more detail, because preliminary observations are contradictory. Fragouli (2015) reported that human IVF blastocysts that were unable to implant contained significantly higher numbers of mtDNA per cell compared to those that were capable of initiating a clinical pregnancy. By contrast, other authors have suggested that diminished mtDNA copy numbers lead to compromised ATP generation that retards or compromises embryonic development leading to suboptimal implantation rates and a higher incidence of early embryonic death and intra-uterine growth retardation (for review see Chappel, 2013). In human, pig and mouse embryos a minimum of 100,000 mtDNA copies is thought to be required to ensure successful fertilization and

embryonic development (Piko and Taylor, 1987; El Shourbagy *et al.,* 2006; Reynier *et al.,* 2001; Santos *et al.,* 2006). In our studies, D7 equine embryos recovered from older mares (>12 years) had lower mtDNA copy numbers than those from younger mares, which may in part explain why embryos from older mares are often developmentally retarded and are less likely to result in an on-going pregnancy (Chapter 3). Nevertheless, it is still not entirely clear whether the problem arises due to a lower number of mitochondria/mtDNA in the oocyte before or after maturation (Rambags *et al*., 2014), or whether the lower mtDNA copy number is primarily a factor of retarded development and a delayed onset of mtDNA replication. Moreover, in human embryos, it has recently been suggested that an optimal, rather than a minimum, mtDNA quantity is required because cells biopsied from embryos recovered from older women (38-42 years) and aneuploid embryos (irrespective of donor age) had higher mtDNA copy numbers per blastomere, despite being of reduced viability (Fragouli *et al.,* 2015). In principle, this fits the 'quiet embryo' hypothesis that a good quality embryo is less metabolically active than a poor quality embryo, because the latter has to work hard to repair defects and damage (Leese, 2012). Given the diametrically opposed reasons for considering both too few and too many mtDNA copies or active mitochondria to be a sign of poor embryo quality, further studies should focus on establishing whether there really is an optimal mtDNA number in oocytes and developing embryos, and whether mtDNA copy number of individual cells recovered by biopsy can be used to predict the likelihood of pregnancy after transfer.

Mitochondrial replication

IVEP can significantly compromise aspects of oocyte maturation, fertilization and subsequent early embryo development, simply because the *in vitro* environment inadequately mimics that of the oviduct. Effects of culture include alterations at all levels of processing genetic information (i.e. transcription, translation and post-translational regulation), energy metabolism, and cytoskeleton organization (Ren *et al.,* 2015). While the mitochondrion is unique in possessing its own genome, which appears to be less susceptible to epigenetic modification but more susceptible to mutation and damage, the proteins involved in mitochondrial replication, such as *TFAM*, *mtPOLGB* and *SSB,* are generally encoded in the nucleus, and their initial up-regulation appears to be associated with the loss of pluripotent status in trophectoderm cells during the first major cell lineage segregation event (Piko and Taylor, 1987; May-Panloup *et al.,* 2007; Thundathil *et al.,* 2005; Spikings *et al.,* 2007). Although it is not yet clear whether the genes involved in mtDNA replication are subject to epigenetic regulation, there are some early indications that this could be the case (see St John

et al., 2010 for review). If it turns out that the genes involved in mtDNA replication are subject to epigenetic regulation either by methylation or by micro RNAs, it would provide extra possible mechanisms by which *in vitro* culture or maternal ageing could influence oocyte and embryo mitochondrial number and function. In this respect, inhibition of *mtPOLB* and *SSB* has been shown to result in 'embryonic lethality' by impaired IVEP embryo development, defects of mtDNA replication and impaired mitochondrial biogenesis (Ren *et al.,* 2015). Although human IVF clinics have already begun to advertise mtDNA copy number screening as an indicator of embryo quality, there is clearly much to learn about the importance of mtDNA and mitochondrial number, and how the genes responsible for mtDNA replication, packaging and repair are themselves regulated during oocyte maturation and early embryo development.

Mitochondrial distribution

During oocyte maturation, fertilization, and early embryonic development, mitochondria in a range of species (including the mouse and pig; Sun *et al.,* 2001) have been shown to undergo characteristic changes in distribution, i.e. from around the germinal vesicle to a more peripheral location followed by an accumulation around the meiotic spindle and, following fertilization, the mitotic spindle. These changes in mitochondrial distribution have been proposed to allow partitioning of ATP generating potential to the areas of the cell where it is most needed (Van Blerkom, 2011) e.g. the microtubular spindle during chromosome segregation. Indeed, disturbances in the localization of mitochondria within the cell appear to predispose to changes in cytoskeleton organization that have, in turn, been proposed to be causal related to the reduced developmental capacity of, for example, vitrified sheep embryos (Dalcin *et al.,* 2013). Mitochondria appear to use the cytoskeleton and, in particular, the microtubules for their translocations while simultaneously providing the energy to maintain the embryonic cytoskeleton, and to fuel the reorganization of actin filaments and microtubules that are, respectively, essential for normal embryo cleavage and chromosome segregation (Ge *et al.,* 2012).

Actin filaments and microtubules are an essential but fragile dynamic network, required for normal function of mammalian oocytes and embryos. However, both IVEP (Tremoleda *et al.,* 2003) and cooling or cryopreservation (Tharasanit *et al.,* 2005) of horse embryos have been shown to alter or disrupt normal cytoskeleton architecture; cooling/freezing induced damage can however largely recover during post-warming or post-thawing culture. Nevertheless, disturbed cytoskeletal function can result in mitochondria being unable to supply energy at the places where it is required, leading to retarded or aberrant development (Dalcin *et al.*, 2013).

It is important that the cytoskeletal structure of embryonic cells recovers after cryopreservation and thawing; that this recovery is often compromised after thawing equine embryos (Tharasanit *et al.,* 2005) may in part be due to the changes in mitochondrial distribution that were present after vitrification of horse embryos (Chapter 5). Monitoring the effects of cryopreservation on cytoskeletal architecture and mitochondrial distribution may therefore be a more immediate and sensitive way to assess the suitability of changes in cryopreservation procedures than transferring embryos.

Cytoplasmic donation as therapy?

One approach to improve IVF outcome for women that have suffered repeated embryonic developmental failure, is cytoplasmic transfer (Cohen *et al*., 1997). This involves the transfer of cytoplasm from women with 'healthy' oocytes; although the transferred cytoplasm will contain numerous mRNAs and other molecules, it will also include large numbers of mitochondria (Babayev and Seli, 2015). One potential risk of this technique is the introduction of third party mitochondria leading to mitochondrial heteroplasmy i.e. the presence of more than one mitochondrial DNA variant. This is unusual in that paternal (sperm derived) mtDNA is generally labelled with ubiquitin and thereby targeted for destruction at the time of embryonic genome activation (see St John *et al*., 2010 for review), ensuring that the mtDNA of the developing embryo is homoplasmic. Following cytoplasmic transfer, a variable proportion of embryos will remain heteroplasmic with respect to their mtDNA, and while these can develop into live offspring they are at increased risk of physiological abnormalities, such as systemic and pulmonary hypertension and increased body fat mass (Acton *et al*., 2007), which most likely arise because genetic differences between the two populations of mtDNA disrupt both strict control of mitochondrial function and communication between the nuclear and mitochondrial genomes, thereby predisposing to mtDNA type diseases.

On the other hand, there is reasonable evidence that mitochondria from developmentally competent oocytes will improve the outcome for incompetent oocytes, and harvesting them from a genetically identical source may have value as a treatment for improving the outcome of IVF without the risk of mtDNA heteroplasmy. One way to achieve this would be to pool mitochondria from several oocytes of a single donor, and introduce them into oocytes selected for fertilization (see St John *et al*., 2010 for review). Another suggested source of suitably 'matched' mitochondria are the polar bodies of a patient's own oocytes, even though there are fewer mitochondria than in the oocyte itself (Babayev and Seli, 2015). Another option would be to retrieve mitochondria from oocytes of maternal-line relations such as a daughter or sister;

since mitochondria are maternally inherited, the risk of heteroplasmy should be low. Nevertheless, it would be prudent to evaluate the mitochondrial genome for quality and to preferentially select a donor without any mtDNA deletions or mutations (Schatten *et al.,* 2014; Babayev and Seli, 2015). Finally, while mitochondria from somatic cells are detrimental to oocyte development (Takeda *et al.*, 2005), and therefore not a suitable source of donor mitochondria, one notable exception is the mitochondria from granulosa cells which improve preimplantation development of oocytes with poor mtDNA content (Hua *et al*., 2007). This suggests that the mitochondria in granulosa cells differ in maturation status and energy requirements to those in other somatic cells and more closely resemble those in oocytes. Since granulosa cells will become available during oocyte recovery for IVF, they may become an attractive potential source of mitochondria for transplanting to improve oocyte competence. While the pros and cons of oocyte mitochondrial supplementation has been addressed in mouse studies and human clinical trials, it has not been examined as a treatment for improving fertility in aged mares. The feasibility is likely to be limited by costs unless, for example, granulosa cells prove to be a suitable and cost effective source of suitable mitochondria.

ART and apoptosis

The frequency, location and significance of apoptotic cells in mammalian embryos is unclear, in part because apoptosis has been proposed to play a useful function in ridding a developing embryo of abnormal cells (e.g. aneuploid cells) or even for controlling cell number (for review see Fabian *et al*., 2005). However, while apoptosis may play a vital role in some aspects of post-implantation development, the presence of too many apoptotic cells can reflect problems with embryo culture conditions (Levy*,* 2001). In this respect, equine IVEP embryos have been reported to display a much higher proportion of apoptotic cells than their *in vivo* counterparts (Tremoleda *et al*., 2003; Pomar *et al*., 2005), which typically have no or only very occasional (<0.1%) apoptotic cells. In general, apoptosis does not feature before embryonic genome activation and is unusual before compaction prior to blastocyst formation (for review see Fabian *et al*., 2005). However, apoptosis may play important roles in the blastocyst e.g. to regulate cell number, primarily in the inner cell mass as demonstrated in bovine embryos (Leidenfrost *et al.,* 2011). The apoptotic cascade can be initiated by oxidative stress, DNA damage or growth factor deprivation, which all release cytochrome C from mitochondria to further enhance the apoptotic cascade via activating caspase-9 (Boeddeker and Hess, 2015). Indeed, excellent quality human IVEP blastocysts, are reported to show relatively high rates of apoptosis and it has been argued that the process might play an important role in normal

development, e.g. by removal of defective cells and regulating cell number or type. In this respect, it is striking that a higher incidence of programmed cell death is seen in inner cell mass (ICM) than in trophectoderm cells of well-developed hatching human and bovine blastocysts (Hardy, 1999; Leidenfrost *et al.,* 2011), presumably because (genetic) integrity of ICM cells is more important than those of trophectoderm cells, since the former could well contribute to the cells of the embryo/fetus whereas the latter are restricted to placental tissues. In the early blastocyst, ICM cells are also able to regenerate trophectoderm cells, which is impossible in the later stage of blastocyst. ICM cells in later blastocyst stages could therefore be preferably eliminated by apoptosis (Hardy, 1999).

To determine whether apoptosis in equine embryos is more a feature of trophectoderm or ICM cells, we performed a pilot study to compare the incidence and location of apoptotic cells between IVP and *in vivo* embryos. To this end, we stained equine embryos using DAPI for total cell number, used terminal deoxynucleotidyl transferase-dUTP nick end labeling (TUNEL) to detect DNA damage consistent with apoptosis (Tremoleda *et al.,* 2003), and CDX2 to distinguish trophectoderm cells from inner cell mass cells (Kuijk *et al.,* 2012; Choi *et al.,* 2015). The embryos were evaluated by confocal laser-scanning microscopy, and total cell number and cells stained with CDX2 were counted using a customized software application. As in previous studies, *in vivo* produced equine embryos (i.e. flushed from the uterus of inseminated mares) showed nearly no or very few apoptotic cells (Fig. 1), while CDX2 staining did enable differentiation of trophectoderm from ICM cells (Fig. 1 and 2). In contrast to reports for other species, apoptosis in the equine IVP embryos was observed primarily in trophectoderm cells (Fig. 2). With respect to embryo viability, apoptosis in trophectoderm cells is considered of marginal importance because this contribute exclusively to future placental tissue and therefore embryo viability should not be impaired. Our preliminary data suggest that combined staining protocols to demonstrate different cell lineages (e.g. CDX2 for trophectoderm and GATA6 for primitive endoderm; Choi *et al.,* 2015) combined with markers for apoptosis (e.g. TUNEL or active caspase-3, which plays a central role in apoptosis; Wydooghe *et al.,* 2011) or other indicators of cell damage or compromise might help in attempts to optimize culture conditions for equine IVEP and also to determine if and when apoptosis plays an important role in the normal development of equine embryos.

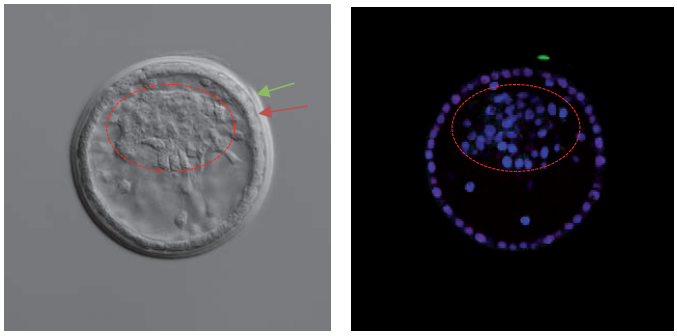

Figure 1. *In vivo* equine expanded blastocyst – zona pellucida (green arrow), capsule (red arrow), ICM (within red dotted line); Nuclei (stained blue using DAPI); apoptotic cells (labelled green using TUNEL); trophectoderm cells (stained red using CDX2)

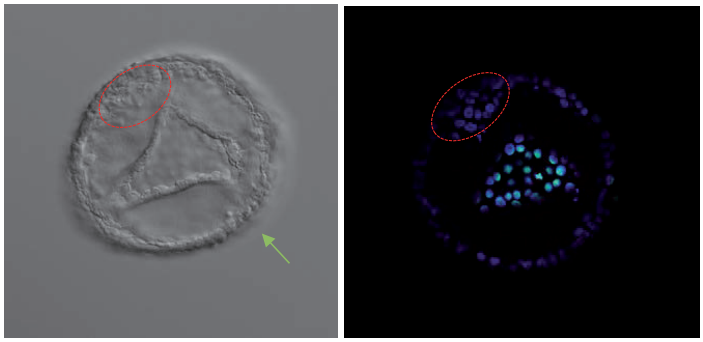

Figure 2. IVP equine expanded blastocyst - zona pellucida (green arrow), ICM (within red dotted line); Nuclei (blue; DAPI); apoptosis (green; TUNEL); trophectoderm cells (red; CDX2)

ART and genomics

ART and epigenetics

ICSI is a promising technique for obtaining offspring from sub-fertile mares and/or stallions, but care should be exercised before widespread application. *In vitro* embryo production by ICSI may disrupt biological processes during fertilization and early (preimplantation) development: most importantly, the pre-implantation embryo appears to be particularly susceptible to epigenetic modification leading to permanent changes in gene expression that could predispose to aberrant *in utero* development and/or increased morbidity to certain diseases during post-natal life. The major conserved systems for epigenetic regulation are covalent modifications of the DNA (e.g. methylation), which typically represses gene

transcription (Pinborg *et al.,* 2016; Ghosh *et al.,* 2017), and post-transcriptional modifications of histone proteins (Figure 3), where the histones acts as spools around which the DNA is wound and thereby play a role in regulation of gene expression: modifications can be associated with either activation or repression of gene transcription (Pinborg *et al.,* 2016).

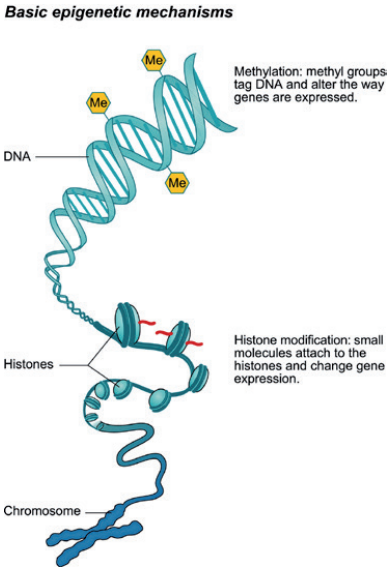

Figure 3. Basic epigenetic mechanism (Pinborg *et al.,* 2016)

Epigenetic modifications affect transcription of DNA without altering DNA sequence. During normal development, epigenetic marks reinforce cell-fate decisions, and establish barriers against reversal to more primitive (less differentiated) cellular states. However, to ensure that a newly produced embryo can make a 'fresh' start, after fertilization and during primordial germ-cell specification, epigenetic marks are largely erased in a process known as epigenetic reprogramming to return the cells to a pluripotent state (Canovas and Ros, 2016). Epigenetic reprogramming is increasingly considered a critical control point in fetal adjustment to predicted conditions in postnatal life (Sinclair and Singh, 2007).

One group of genes that appear to be particularly susceptible to disturbance as a result of an altered environment during very early development are those subject to 'genetic imprinting', in which mono-allelic expression results from the preprogrammed inactivation of one allele or other, in a parent-of-origin (maternal or paternal) determined fashion (for review see Pinborg *et al.,* 2016). The imprint is erased at the primordial germ cell stage, after the cells have migrated to the genital ridge, when their genome undergoes erasure of epigenetic marks. This enables subsequent, post-fertilization, epigenetic reprogramming and parent-of-origin

controlled activity of specific genes (for review see Pinborg *et al*., 2016). Imprinted genetic regions are protected from demethylation-methylation during gametogenesis, and remethylation is completed at the time of implantation. Aberrances in epigenetic programming, such as hyper- or hypomethylation of DNA within a specific region can result in disturbances of gene activity and imprinting disorders such as the large offspring syndrome in cattle, and Angelman syndrome or Beckwith-Wiedemann syndrome in IVF children (Ren *et al.,* 2015).

During very early embryonic development, epigenetic marks appear to be particularly vulnerable to changes in the external environment (Ghosh *et al.,* 2017). Transient environmental disturbances can produce permanent changes in epigenetic marks that have life-long phenotypic consequences. A classic example is the periconception exposure (first 10 weeks *in utero*) to famine during the 'Dutch Hunger Winter' which was associated with reduced birth weight associated with hypomethylation of the IGF2 DMR 6, and which decades later presented as adult onset diseases characterized by insulin resistance in not only the children conceived during the hunger winter but also second and third generation offspring (Heijmans *et al.,* 2008; Pinborg *et al.,* 2016).

It is highly likely that epigenetic alterations occur during equine *in vitro* embryo culture due, for example, to suboptimal culture media, light, oxygen levels and contact with plastic-ware (Ventura Junca *et al*., 2015). However, since IVP has only relatively recently been applied to equine breeding, no information is yet available about how and to what extent IVEP affects the epigenome of the equine embryo and whether this has consequences for the health, morbidity, fertility or athletic prowess of resulting foals. A follow up of equine offspring produced via IVEP should be performed to examine whether their health, susceptibility to specific diseases (e.g. those associated with insulin resistance) and sporting capacities are different to those of naturally conceived peers.

ART and genetic testing

A recent development in equine reproduction, is pre-implantation genetic testing for heritable mono-allelic diseases such as hereditary equine regional dermal asthenia (HERDA), glycogen branching enzyme deficiency, hyperkalaemic periodic paralysis (HYPP) and polysaccharide storage myopathy in American Quarter Horses, severe combined immunodeficiency and cerebellar abiotrophy in Arabian Horses, and hydrocephalus and dwarfism in Friesian horses. All of these tests can be performed on a small number of embryonic cells recovered by biopsy of day 7-8 blastocysts, with the embryo then cryopreserved to allow transfer only if the testing indicates freedom from the undesirable genetic mutation (Choi *et al.,*

2015). In this way, genetic testing can help to select "healthy" embryos for transfer. At present, however, recovering a biopsy from a horse embryo is only possible in a specialized clinic. Although only a few cells are required for the genetic tests, recovering a biopsy from an *in vivo*-produced embryo and then producing a viable pregnancy can be challenging, because the equine embryo only arrives in the uterus late on D6 after ovulation, when it is at the late morula to early blastocyst stage of development (Battut *et al.,* 1997). After arrival in the uterus, it rapidly expands and develops an acellular capsule which appears to be essential for intra-uterine survival (Stout *et al.,* 2005). While microblade dissection has been trialled as a technique to recover embryonic cells (Choi *et al*., 2015), there was a high incidence of capsule damage and loss, with the expectation that the embryo would not subsequently survive transfer into the uterus. Moreover, freezing of large expanded equine embryos is less successful than freezing of smaller embryos (see Stout, 2012 for review). Using a Piezo drill to biopsy equine blastocysts appears to be a solution to this problem, because the blastocoele fluid can be removed to reduce embryonic volume, while the hole made in the capsule is small and allows the latter to maintain its structure, both of which seem to improve the ability of biopsied-vitrified embryos to survive after vitrification-warming and transfer, respectively (Hinrichs 2012; Choi *et al.,* 2015). Producing embryos by IVEP may, however, allow further improvements since IVEP embryos freeze well as a result of their very small size (Galli *et al.,* 2007), and they are not at risk of losing their capsule since they have yet to form a confluent capsule, presumably because they have yet to be exposed to the uterine environment (Tremoleda *et al*,. 2003). In short, genetic screening is already a promising technique for breeds with known heritable monogenic diseases, allowing screening of early embryos and thereby preventing the birth of offspring carrying debilitating genetic mutations, or more simply to facilitate selection of offspring with the desired sex (Herrera *et al.,* 2014) or coat colour.

ART – future prospects

In other livestock species, notable dairy cattle (Aksu *et al.,* 2012) genetic profiling of embryos has been carried a step further to encompass genome wide association studies (GWAS) to identify embryos displaying single nucleotide polymorphisms (SNPs) associated with desirable phenotypic criteria, e.g. performance traits, for transfer. Of course, the ability to identify mutations associated with desirable or undesirable phenotypic traits opens the door for future potential application of gene editing techniques. Techniques like zinc-finger nucleases (ZFN's), transcription activator-like effector nucleases (TALENs) and clustered regulatory interspaced short palindromic repeats (CRISPRs) are leading methods. The first two techniques

can be customized to recognize almost any sequence, but require complex engineering. The last group of nucleases (CRIPSRs) are found naturally in bacteria where they provide immunity against invading foreign DNA by using RNA-guided DNA cleavage. CRISPR-associated endonuclease (Cas9) can create a cut in the selected gene. This technique is easy to design, relatively cheap and effective (for review see Lotti *et al*., 2017), and can be used to introduce or delete specific genetic sequences or mutations, and also to erase epigenetic marks. At present, however, the efficiency of gene editing at the early zygote stage is relatively low (~5-6% of the embryos were correctly spliced; Vassena *et al.,* 2016) and the edited embryos are often mosaic (Liang *et al.,* 2015; Travis, 2015). Recently, authorization was given in the UK for CRISPR-Cas9 to be tested on human embryos, with the aim of developing treatments for preventing heritable diseases like Huntington's disease and achondroplasia (Ma and Liu, 2015); moreover, the first reports of gene editing of human embryos have emerged from China. Assuming that the efficiency of the editing process can be significantly improved, it is likely that CRISPR-Cas9 could be applied to zygotes produced by IVEP as an alternative way to eliminate mutations associated with heritable genetic diseases; in the longer term, and only after extensive studies of possible 'off target' effects and an extensive debate about the ethics and desirability of genome editing for performance traits, it is possible that CRISPR-Cas9 of IVEP embryos could be used to produce horses with genetic modifications aimed at improving sporting performance.

As indicated earlier, various aspects of the ART/IVEP process have the potential to influence the epigenetic signature of the embryo, these include culture media components, exposure to light and altered oxygen tensions (Pinborg *et al*., 2016; Ghosh *et al.,* 2017). To minimize the risks of epigenetic perturbations arising during *in vitro* culture of oocytes or embryos, it would help to develop conditions that better mimic the *in vivo* environment, one possibility is to culture embryos in an '*in vivo*-like' in vitro system, such as the 'oviduct-on-a-chip' system described recently in which an oviduct epithelial cell culture system was developed that allows cells to maintain or re-establish their typical *in vivo* polarized, differentiated state and to support early embryonic development (Ferraz *et al.,* 2017). Even closer to the *in vivo* situation, is the use of an 'intra-uterine incubator' such as the AneVivo device recently described for the culture of human IVF embryos (www.anecora.com/the-anenvivo-device, 2016). The AneVivo device is a small capsule (1 cm long and 1 mm wide) with pores large enough to allow exchange of secretions but large enough to prevent the entry of inflammatory cells; mature oocytes and sperm cells or fertilized oocytes are placed inside

the device which is then introduced into the uterus of a woman for a pre-determined period (24 hours) to allow initial development of the embryos. After the period of intra-uterine incubation, healthy embryos are selected for transplantation after 2-4 days of further *in vitro* culture. In this way, the embryo culture environment should be closer to the natural environment, thereby improving the chance of healthy embryos developing while minimizing the risk of epigenetic perturbation. In the horse, *in vivo* incubator approaches or the development of organs-on-a-chip may be interesting novel avenues to explore in attempts both to develop a system in which conventional *in vitro* fertilization can be supported, and in which to improve the blastocyst production rate and both the developmental competence and epigenetic integrity of IVEP horse embryos. In cattle, an oviduct-on-a-chip has been shown to allow *in vitro* fertilization without any requirement for the factors normally required to stimulate sperm capacitation *in vitro* (Ferraz *et al.,* 2017); since the inability to adequately capacitate stallion sperm appears to be the primary impediment to successful conventional IVF in horses (McPartlin *et al.,* 2009), an *in vivo* like approach may both provide a solution to the problem and a method to better study the process of stallion sperm capacitation. Similarly, since transfer to the oviduct of either a mare (Hinrichs *et al*., 2002) or a progesterone treated sheep (Lazzari *et al.,* 2010) improves both the rate of blastocyst development and the quality of the blastocysts (Tremoleda *et al.,* 2003), an *in vivo* incubator or an oviduct-on-a-chip might improve the percentage of zygoytes developing to the blastocyst stage, and the quality of those blastocysts.

While there are clear potential advantages of equine ARTs as a solution to some forms of sub-fertility, to facilitate genetic improvement and/or to help eliminate monogenic diseases, there are also a number of limitations that affect the costs of the procedure. One of these, is the ability to successfully cryopreserve horse embryos. Since there is a restricted window of acceptable synchrony in which a recipient mare can be used (Stout, 2006), recipient mares can become a limiting factor for conventional embryo transfer at the height of the breeding season. Cryopreservation has clear potential benefits in improving the efficiency with which recipient mares can be used (Stout, 2012), since an embryo can simply be thawed at the time that a suitable recipient reaches the ideal time for transfer. However, the relative scarcity and high costs of producing embryos in the absence of commercially available products for reliably inducing superovulation, combined with the poor success of cryopreserving expanded blastocysts, have discouraged wider uptake of embryo cryopreservation in the field. The recent description of techniques to puncture and aspirate the blastocoele fluid from larger expanded blastocysts (up to 600µm; Choi *et al.*, 2011) is a promising advance, assuming that the technique can be simplified for simpler equipment than a micromanipulator equipped with a piezo drill.

As described in this thesis several assisted reproductive techniques are available in equine reproduction, but all are associated with (dis)advantages. If a breeder would is considering applying one of the ART techniques, he/she should be well informed of the potential downsides as well as the benefits, before making a decision on whether to continue. Focussing further studies to improve the practicality, and reduce the potential for epigenetic disturbance, of techniques like cryopreservation and ICSI would enhance genetic progression and could improve the efficiency of assisted reproduction programmes.

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Samenvatting

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Inleiding

Binnen de paardenfokkerij is er steeds meer interesse in de toepassing van moderne voortplantingstechnieken (MVT) als embryo transplantatie (ET), invriezen van embryo's en *in vitro* embryo productie (IVEP) met behulp van intracytoplasmatische sperma injectie (ICSI). De grote economische waarde van sommige merries en hengsten verantwoorden de moeite en de hoge kosten die met deze technieken gepaard gaan. Daarnaast zijn de drachtpercentages en "levend geboren veulen" percentages goed genoeg om deze technieken toe te passen, maar er is zeker nog ruimte voor verbetering. Ongewenste effecten als gevolg van MVT kunnen o.a. veranderingen in genexpressie zijn, zonder dat er wijzigingen optreden in DNA samenstelling. De blootstelling aan vloeistoffen en contact met plastics tijdens de eicelrijping, IVEP, embryo transport/opslag, en invriezen kunnen tot deze veranderingen leiden. Voorbeelden hiervan zijn bij de mens een laag geboorte gewicht en bij het rund het 'grote kalf syndroom': bij het paard is niet bekend of MVT dergelijke epigenetische veranderingen veroorzaken.

Andere punten die bij MVT verbeterd kunnen worden zijn de resultaten bij oudere merries na het spoelen voor en transplanteren van een embryo, resultaat na het invriezen van geëxpandeerde blastocysten en het blastocyste percentage na toepassen van ICSI bij patiënten in de praktijk. Tevens kan men er rekening mee houden dat er meer vroeg embryonale sterfte optreedt bij embryo's verkregen van oudere merries of na eicel winning (ovum pick up; OPU) en IVEP. Dit proefschrift onderzocht factoren betrokken bij MVT om beter te kunnen begrijpen wat de onderliggende oorzaken van de intrinsieke problemen zijn om op grond daarvan tot oplossingen te komen.

Samenvatting van de resultaten

Hoofdstuk 2 *Foetale ontwikkeling en echografie*

In vitro embryoproductie vertraagt de vroeg embryonale ontwikkeling en vermindert de embryo kwaliteit, zeer waarschijnlijk omdat de kweekomstandigheden niet voldoende de *in vivo* omstandigheden nabootsen. In het algemeen zijn IVEP paarden blastocysten kleiner ten opzichte van *in vivo* blastocysten op eenzelfde aantal dagen na bevruchting, hebben minder cellen, meer dode cellen en zijn dus van mindere kwaliteit.

Een andere factor die de vruchtbaarheid negatief beïnvloedt is een toegenomen maternale leeftijd. Uit verschillende onderzoeken is gebleken dat het drachtpercentage op dag (D) 15 na bevruchting in merries < 14 jaar hoger is dan bij merries van 14 jaar en ouder. De vruchtblaas op D11 is kleiner bij oudere merries ten opzichte van die bij jonge merries en dit achterblijven in embryonale/foetale ontwikkeling bij oudere merries is de gehele drachtperiode zichtbaar. Dit leidt vervolgens tot een hoger percentage vroeg embryonale sterfte bij oudere merries maar ook tot veulens met een lager geboorte gewicht.

Door de toepassing van echografisch onderzoek is de foetale ontwikkeling te beoordelen en te monitoren, het kan helpen afwijkende foetale groei vast te stellen. Bij de mens verschillen de groeicurven van achterblijvende vruchten en te snel groeiende foetussen significant van de verwachte groeicurven. Bij het rund wordt een afwijkende foetale ontwikkeling na IVEP in serum verrijkt medium gezien (‘grote kalf syndroom’), die echografisch vast te stellen is. Het toenemen van de maternale leeftijd resulteert in kleinere, traag groeiende vruchten. De oorzaak van het achterblijven van de foetale ontwikkeling bij oudere merries kan liggen in een leeftijd gerelateerde verslechtering van de endometriumkwaliteit en niet *per se* in de intrinsieke defecten van de eicel en het embryo. Om de ontwikkeling te kunnen monitoren en te kunnen vaststellen of IVEP en/of maternale leeftijd foetale ontwikkeling vertragen is het noodzakelijk dat er foetale groeicurven beschikbaar zijn van paarden van verschillende rassen. Tot redelijk kort geleden waren er alleen foetale groeicurven van Volbloeden en Dravers, dus bij rassen die duidelijk kleiner zijn dan de Warmbloed paarden die worden ingezet voor het springen en de dressuur. In Hoofdstuk 2 wordt de foetale groei bij het KWPN paard beschreven; dit kan worden gebruikt als referentie voor toekomstige monitoring.

Door herhaaldelijk bepaalde foetale lichaamsdelen te meten, kan intra-uteriene groei gemonitord worden en afwijkende groei worden vastgesteld. Er zijn bij paarden nog geen extremen in groei waargenomen maar het is wel bekend dat toegenomen maternale leeftijd, ernstige ziekte bij de merrie en placentitis predisponeren voor intra-uteriene groei achterstand. De ernst van deze groei achterstand kan een belangrijke indicator zijn voor het voorspellen of een dracht wordt uitgedragen en of deze vrucht dan gezond is bij geboorte.

Een belangrijke uitkomst van onze studie was dat de aorta diameter en het maximale oppervlak van een dwarsdoorsnede van de schedel de enige twee parameters waren die lineair toenemen tijdens de dracht. Dit zijn daarmee de gewenste parameters om foetale grootte en groei bij paarden te evalueren. Verder werd duidelijk dat aorta diameter en het maximale oppervlak van een schedeldwarsdoorsnede significant beïnvloed worden door maternale leeftijd en pariteit. De aorta diameter van de vrucht bij merries > 15 jaar was kleiner dan bij merries

≤15 jaar, mogelijk indicatief voor het achterblijven van foetale groei en lager geboorte gewicht bij veulens van oudere merries. Het oppervlak van de schedeldwarsdoorsnede van de vrucht in primipare merries was verrassend groter dan in pluripare merries; dit was in tegenstelling tot het verwachte omdat primipare merries lichtere veulens bij geboorte geven.

De ontwikkelde groeicurves bij het KWPN paard kunnen helpen vast te stellen of foetale ontwikkeling afwijkt van de normale ontwikkeling bij patiënten waarbij afwijkingen worden verwacht. Tevens kan het helpen bij de beslissing of de dracht ondersteund moet worden met bijvoorbeeld medicatie en of er voorbereidingen getroffen moeten worden om de geboorte te begeleiden en neonatale zorg te verlenen.

Hoofdstuk 3 en 4 *IVP en mitochondriën*

Een nadeel van de huidige IVEP protocollen bij het paard is de tegenvallende vroeg embryonale ontwikkeling. Het winnen van eicellen via ovum pick-up (OPU) en de start van de embryonale ontwikkeling verlopen nog voorspoedig, echter het opkweken tot een te transplanteren embryo verloopt zeer wisselend (5-35%). Een mogelijke oorzaak voor het matige resultaat is het suboptimaal functioneren van de mitochondriën of de aanwezigheid van onvoldoende mitochondriën. Mitochondriën zijn belangrijke onderdelen in het cellulaire metabole proces en zijn een van de eerste celorganellen die beschadigd raken tijdens cel afbraak en geprogrammeerde celdood (apoptosis). Mitochondriën zijn betrokken bij de energie productie waarbij tevens vrije zuurstof radicalen gevormd worden. Mitochondriaal DNA (mtDNA) heeft geen DNA herstel mechanisme. Hierdoor is mtDNA gevoeliger dan nucleair DNA voor schade veroorzaakt door vrije zuurstof radicalen welke schade in de loop van de tijd toeneemt. Toename van de leeftijd van de eiceldonor en suboptimale kweek omstandigheden tijdens IVM, ICSI en embryo vorming kunnen de mtDNA schade verergeren. Bij andere diersoorten als runderen, muizen en varkens is aangetoond dat IVEP schade aan mitochondriën veroorzaakt en ook bij paarden is gezien dat tijdens *in vitro* rijping van eicellen de mitochondriën beschadigd raken.

Om vast te stellen of IVEP de mitochondriale hoeveelheid en het functioneren en de deling van eicellen/embryo's van paarden beïnvloedt, hebben wij de invloed van veroudering en IVEP op mitochondriale hoeveelheid en functioneren in eicellen en embryo's van paarden onderzocht (Hoofdstukken 3 en 4). In hoofdstuk 3 zijn de effecten van merrie leeftijd en IVEP op mitochondriale hoeveelheid onderzocht. Het bleek dat D7 embryo's van oudere merries (>16 jr) minder mtDNA kopieën bevatten dan D7 embryo's van jongere merries (<12 jr). Een duidelijk effect van IVEP kon niet worden vastgesteld; D8 IVEP embryo's hadden een gelijk

aantal mtDNA kopieën als D7 *in vivo* embryo's, waarbij de periode van morula tot blastocyste vorming werd onderzocht.

De genen *TFAM*, *mtPOLGB* en *SSB* zijn betrokken bij de processen rondom mitochondriale vermenigvuldiging en de werking ervan lijkt concentratie afhankelijk te zijn: bijvoorbeeld een geringe hoeveelheid *TFAM* is nodig voor mtDNA vermenigvuldiging, terwijl grote hoeveelheden juist gerelateerd zijn aan het afremmen van de vermenigvuldiging. In D8 IVEP paardenembryo's is aangetoond dat *mtPOLGB* en *GPX3* (betrokken bij het vormen en afbreken van vrije zuurstof radicalen) expressie verschilde van de *mtPOLGB* en *GPX3* expressie in *in vivo* embryo's. *MtPOLGB* expressie in IVEP embryo's was verdubbeld ten opzichte van die in D7 *in vivo* embryo's (waarschijnlijk om mtDNA kopieën in stand te houden) terwijl *GPX3* expressie was gehalveerd. De halvering van *GPX3* expressie zou kunnen leiden tot een verminderde bescherming tegen door vrije zuurstof radicalen veroorzaakte schade, terwijl de hoge expressie van *mtPOLGB* mogelijk zou kunnen helpen de hoeveelheid mitochondriën te waarborgen. Daarnaast zou een verminderde capaciteit voor het wegvangen van vrije zuurstof radicalen kunnen leiden tot schade aan de mitochondriën, andere cel organellen en moleculen. Dit zou vervolgens kunnen leiden tot verminderde embryo kwaliteit en een verminderde potentie tot verdere ontwikkeling.

Omdat IVEP en veroudering beide als factoren worden genoemd die de mtDNA vermenigvuldiging afremmen hebben we onderzocht wanneer mtDNA vermenigvuldiging start na bevruchting. In runder en varkens embryo's start de mtDNA vermenigvuldiging in het vroege blastocyste stadium van *in vivo* embryo's. Wij hebben het moment van mtDNA vermenigvuldiging in *in vivo* en IVEP embryo's onderzocht (Hoofdstuk 4). De eerste duidelijke toename van mtDNA kopieën na bevruchting vindt plaats tussen het vroege en geëxpandeerde blastocyste stadium, in *in vivo* en *in vitro* embryo's. De start van deze vermenigvuldiging werd vooraf gegaan door een toename van *TFAM* expressie in *in vitro* embryo's 96 uur na kweek, 4 dagen voordat de blastocyste gevormd wordt. Dit werd niet vooraf gegaan door een toename in *mtPOLGB* en *SSB* expressie. Echter, *mtPOLGB* en *SSB* expressie was hoog in eicellen en embryo's 48 uur na kweek. De reden voor deze relatief hoge *mtPOLGB* en *SSB* transcripten in eicellen en 2-cellige embryo's is onduidelijk, maar zou gelinkt kunnen worden aan een mtDNA vermenigvuldigingsronde die mogelijk plaatsvindt tussen bevruchting en de eerste celdeling waarbij mogelijk paternaal mtDNA en beschadigd mtDNA worden verwijderd.

Hoofdstuk 5 *Invriezen en mitochondriën*

Door het invriezen van paarden embryo's kan de efficiëntie van MVT enorm verbeterd worden, omdat het niet meer noodzakelijk is om draagmerries te synchroniseren voor het moment dat er een *in vivo* of *in vitro* verkregen embryo beschikbaar komt voor transplantatie. Een belangrijke belemmering om embryo's grootschalig in te vriezen zijn de tegenvallende resultaten. Gerelateerd aan de hoge waarde en relatieve schaarste van paardenembryo's wordt het uitvoeren van onderzoek met commerciële embryo's bemoeilijkt. In Hoofdstuk 5 is het effect van verschillende invriesmethoden bij embryo's van verschillende grootte onderzocht, waarbij duidelijk zichtbare parameters zoals mitochondriale activiteit, kern fragmentatie als indicatie van apoptosis en het intact zijn van het cytoskelet zijn beoordeeld.

Blootstelling aan cryoprotectanten - zonder invriezen/koelen - had geen negatief effect op mitochondriale activiteit, kern fragmentatie of kwaliteit van het cytoskelet. In zijn algemeenheid hadden kleine embryo's die waren ingevroren via een gecontroleerd invriesprogramma minder schade dan de grotere embryo's of gevitrificeerde embryo's. Er was grote variatie in mitochondriale activiteit zoals gemeten met een fluorescerende marker; gezonde embryo's vertoonden relatief zeer lage activiteit, waarschijnlijk omdat ze geen energie hoeven te leveren voor herstel. Embryo's van slechte kwaliteit kunnen zowel een verhoogde als een verlaagde activiteit vertonen. Dit verklaart waarom mitochondriale activiteit een weinig geschikte parameter is gebleken voor beoordeling van de embryo kwaliteit.

MVT, veroudering en echografie

Het fokken met oudere merries is een gebruikelijk fenomeen, omdat de meeste fokkers willen weten of de merrie goede sport kwaliteiten heeft, alvorens ze wordt ingezet in de fokkerij. Dit heeft als nadeel dat de vruchtbaarheid bij de (oudere) merrie afneemt, onder andere als gevolg van verminderde baarmoederslijmvlies kwaliteit, verstopte eileiders en verminderde eicel kwaliteit. MVT kunnen worden gebruikt om enkele van deze problemen te omzeilen, zoals bijvoorbeeld het gebruik van een jonge merrie met een goede baarmoederslijmvlies kwaliteit als draagmoeder bij ET, echter de verminderde eicel kwaliteit bij de oudere merrie blijft een probleem. De verminderde eicel en embryo kwaliteit bij de oudere merrie maar ook bij IVEP vindt o.a. zijn oorsprong in verminderde kwantiteit van mitochondriën, en verminderde kwaliteit doordat er meer mtDNA mutaties aanwezig zijn door de suboptimale kweekomstandigheden.

Echografie is een techniek die wordt toegepast voor het winnen van eicellen bij donormerries (OPU) en de begeleiding van donoren en draagmerries bij ET procedures. Daarnaast maakt echografie het mogelijk om geslachtsbepaling bij de ongeboren vrucht uit te

voeren, iets waar de laatste jaren in de paardenpraktijk meer om gevraagd wordt. Doordat er nog weinig bekend is over de gevolgen van IVEP bij paard kan echografie ook hulp bieden om de dracht te monitoren en tijdig afwijkingen te constateren. De groeicurves in Hoofdstuk 2 zullen hierbij helpen.

MVT en nieuwe ontwikkelingen

Een mogelijke manier om de resultaten van IVEP bij het paard te verbeteren is het toevoegen van mitochondriën aan de eicel wanneer de mitochondriale hoeveelheid of kwaliteit van deze eicel onvoldoende is. In de humane praktijk lijkt deze techniek al veelbelovend; echter het heeft wel het risico van introduceren van "derde partij" mitochondriën die een verhoogd risico op fysiologische afwijkingen zou kunnen geven. Door mitochondriën te gebruiken uit cellen van een genetisch identieke bron (bijvoorbeeld uit eicellen van de moeder of zuster) kan dit probleem geminimaliseerd worden. Haalbaarheid van deze techniek bij merries zal met name door kosten bepaald worden.

Een recente ontwikkeling in de paarden voortplanting is het genetisch testen van embryo's voor de implantatie fase op erfelijke mono-allel afwijkingen, zoals 'hereditary equine regional dermal asthenia' (HERDA), 'hyperkalemic periodic paralysis' (HYPP) en 'polysaccharide storage myopathy' (PSSM) bij Quarter Horses, 'severe combined immunodeficiency' (SCID) en cerebellaire abiotrofie (CA) bij Arabieren en waterhoofd en dwerg bij Friezen. Al deze testen kunnen worden uitgevoerd op een klein aantal embryonale cellen verkregen via een biopt bij D7-8 blastocysten, waarna het embryo wordt ingevroren in afwachting van het resultaat. Dit maakt het mogelijk om alleen transplantatie uit te voeren van embryo's die vrij zijn van deze aandoeningen. Tot op heden is het biopteren van een paardenembryo alleen mogelijk in gespecialiseerde klinieken, waarbij met behulp van een Piëzo drill embryonale cellen verkregen kunnen worden, het kapsel intact blijft en vervolgens het embryo met redelijk succes kan worden ingevroren in afwachting van de testresultaten.

De laatste jaren wordt genetische profilering van embryo's toegepast, bijv. bij rundvee om te selecteren op hogere melkgift alvorens te transplanteren. Andere technieken zijn meer vooruitstrevend zoals CRISPR-Cas9, die het mogelijk maken om specifieke mutaties te benutten die kunnen leiden tot verbeteren van een kenmerk of verwijderen van een overdraagbare aandoening. Dit lijkt een veelbelovende toepassing die bij het paard ingezet zou kunnen worden voor selectie op gen profielen die gekoppeld zijn aan sport prestaties.

Een recente noviteit is de enkele centimeters grote AneVivo kweekkamer die door een klein aantal humane IVF klinieken in het Verenigde Koninkrijk ingezet wordt om embryo's in

een meer fysiologische omgeving te laten ontwikkelen. Gezien de suboptimale verloop van IVEP voor paarden embryo's zou een vergelijkbare systeem een mogelijkheid voor paarden IVEP kunnen worden. Het is een "incubator" waarin gerijpte eicellen en spermacellen worden samengebracht. Deze incubator, - met een semidoorlaatbare wand - wordt vervolgens in de baarmoeder geplaatst zodat bevruchting en de eerste ontwikkeling van het embryo in een intra-uteriene omgeving plaatsvindt. Na het terugwinnen van de "incubator" worden geproduceerde gezonde embryo's vervolgens – al of niet na een korte *in vitro* periode - naar een (draag) moeder getransplanteerd voor verdere ontwikkeling.

Zoals beschreven in dit proefschrift zijn er verschillende MVT bij paarden beschikbaar, maar vele hebben zowel voor- als nadelen. Wanneer een fokker één van deze technieken wil gaan toepassen, is het belangrijk dat hij/zij goed geïnformeerd wordt over de voor- en nadelen voordat een beslissing wordt genomen de techniek toe te passen. Door het toekomstige onderzoek te focussen op praktische toepasbaarheid en het verminderen van epigenetische verstoringen, kunnen invriezen en ICSI het mogelijk maken sneller genetische vooruitgang te boeken en de efficiëntie van de MVT verbeteren.

Dankwoord

Dankwoord

Het project “Promotie-onderzoek” ben ik ruim 10 jaar geleden gestart. Mijn steun, toeverlaat en echtgenoot Dave Hendriks heeft mij in alle opzichten daarbij gefaciliteerd. Het behalen van de titel van Doctor werd vandaag bewaarheid. Nooit hebben wij twijfel over het halen van de eindstreep uitgesproken, ondanks dat er op gezette momenten daartoe misschien wel aanleiding was. Gelukkig stond Dave er niet alleen voor in al die jaren. Mijn moeder Betty Verdonk, mijn zus Ciska Onstein en Jan Otten hebben hem en mij met alles wat mogelijk was, gesteund. Het is heel spijtig dat mijn vader het resultaat van een ieders inspanning niet heeft kunnen meemaken door zijn voortijdig overlijden. Dit geldt ook voor mijn tante Rita Verdonk die kort geleden helaas is overleden. Ook zij had het vertrouwen dat ik dit project tot een goed einde zou brengen.

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Professor emeritus Ben Colenbrander verdient eveneens speciale aandacht in het onderzoekstraject. Hij heeft me gestimuleerd om de draad van het promotietraject weer op te pakken nadat mijn aandacht in beslag was genomen door het opstarten van een eigen praktijk, mijn zwangerschap en de geboorte van onze zoon Duco. Het promotie-onderzoek was door deze belangrijke veranderingen in mijn leven op een laag pitje komen te staan en ik had er gaandeweg ook minder vertrouwen in gekregen dat e.e.a. tot een goede afronding zou kunnen komen. Hij was degene die mij stimuleerde en wilde gaan begeleiden in het vervolgtraject onder de voorwaarde dat Tom het goed zou vinden. ‘Het was alleen nog maar schrijfwerk..’, zo sprak hij vol enthousiasme. Veel dank ben ik hem derhalve verschuldigd voor zijn initiatief en het uitgesproken vertrouwen in mijn vakmansschap. Hij leerde me het gestructureerd schrijven van de artikelen en het kritisch analyseren van de inhoud ervan.

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Dank u allen, thanks to all of you

Curriculum vitae

Curriculum vitae

Wilhelmina Karina Hendriks-Onstein was born on the 1st of September 1974 in Velp, The Netherlands. In 1993, she passed het final exams at "Het Rhedens Lyceum" in Rozendaal, and started Medical Biology at Utrecht University. After passing her propedeuse, she started Veterinary Medicine at Utrecht University in 1994. After finishing her Master in Veterinary Medicine in 2001, she started working as veterinarian at the Division of Equine Reproduction, Department of Equine Health, Faculty of Veterinary Medicine, Utrecht University. Subsequently she worked as a teacher at Groenhorst College, Barneveld for half a year and returned to Utrecht University to become resident in Equine Reproduction. She passed her ECAR exam in 2008 and became Diplomate ECAR Equine Reproduction. In 2007, she started her PhD in Equine Reproduction. In 2012, she left Utrecht University as employee and started her own business Hendriks EQ Repro Consultancy in collaboration with De Graafschap Dierenartsen. In 2016 she became partner of Seldsum EQ Hendriks, a business in application of modern equine reproductive techniques. In 2018 she finished her thesis "Optimizing Equine assisted reproductive technologies".