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Vitrification of equine expanded blastocysts

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VITRIFICATION OF EQUINE EXPANDED BLASTOCYSTS

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ABSTRACT

The cryopreservation of equine expanded blastocysts (>300 um) has been largely unsuccessful primarily due to the low permeability to cryoprotectants and the large size of the equine embryo. Multiple experiments were performed in this study to evaluate different approaches of capsule puncture and blastocoele fluid extraction with the objective to develop a cryopreservation protocol for Day 8 equine expanded blastocysts.

In the first experiment, twenty-four Day 8 expanded blastocysts were exposed to standard equine embryo vitrification solutions following one- or two-punctures. Mean pre-treatment embryo volume was not different across treatments. A reduction of 67% of embryo volume was achieved within 5 min of embryo puncture, blastocoele fluid extraction and exposure to VS1 for both treatments. Mean embryo volume was not different for one- and two-puncture treatments during exposure to VS1, VS2, VS3, DS and culture medium. Mean embryo volume was not different during in vitro culture at 24, 48 and 72 hours. Embryo growth rate at 72 hours culture was not different for one- or two puncture treatments (75 and 50%, respectively) (P=0.21). Capsule loss was not different for one- or two-puncture treatments (25% and 50%, respectively) (P=0.21).

In the second experiment, twenty-six Day 8 expanded blastocysts were subjected to either capsule puncture, cryoprotectant injection and blastocoele fluid extraction (direct treatment) or capsule puncture and blastocoele fluid extraction (indirect treatment) prior to cryopreservation. Mean pre-vitrification embryo diameter for direct and indirect treatment groups was not different. A difference between the initial

embryo volume and the embryo volume following warming of vitrified embryos in both indirect and direct introduction treatments was detected. Following *in vitro* culture there was no difference in mean embryo volume at 24, 48 and 72 hours. Re-expansion rate and subsequent growth at 72 hours of culture was not different for one- or two puncture treatments (69.2%). However, partial or total capsule loss was different (P=0.049) across treatments, with 69% of direct treatment embryos and 30.8% of indirect treatment embryos losing the capsule. A pregnancy rate of 83.3% was obtained following transfer of vitrified expanded blastocyst subjected to the indirect introduction technique.

CHAPTER I
INTRODUCTION

It is estimated that the horse (*Equus caballus*) was domesticated approximately 5000 years ago around the Eurasian Steppes, specifically at Dereivka, Ukraine (Gordon, 1997). During the establishment and development of early human societies, horses played an important role in human activities. They were widely used for work and transportation, as well as source of meat. The horse remained beneficial to humans until the last century, at which time mechanization and the agricultural revolution diminished the horse's role. Today, the horse is used mostly for recreational activities (Gordon, 1997).

Although horses represent only a small portion of the livestock population, the economic impact of the horse industry is rather large. A study commissioned by the American Horse Council (AHC) and performed by Deloitte Consulting in 2005 stated that the American equine industry has a direct economic impact of $39 billion and an indirect impact of $102 billion.

Reproduction is considered to be a major factor in determining the productivity and profitability of most animal production systems, with reproductive rates being indicators of efficiency. Assisted reproduction technologies (ART's) are methods developed to overcome reproductive inefficiency and include technologies such as artificial insemination (AI), embryo transfer (ET), gamete and embryo cryopreservation, in vitro fertilization (IVF), intracytoplasmatic sperm injection (ICSI), and somatic cell nuclear transfer (SCNT). Embryo cryopreservation is typically utilized to preserve early-

stage embryos in most domestic livestock species, and requires controlled cooling of embryos to –196 ºC. The cryopreservation of embryos results in the cessation of active metabolic processes within the embryo. Embryos in this state can theoretically be stored for an indefinite period of time. Embryo cryopreservation has been utilized to preserve genetic variants in laboratory animals, proliferate genetically superior farm animals, and treat infertility in humans (Dobrinsky, 2002). The first successful attempt to cryopreserve mammalian embryos occurred in 1972, when it was reported that live mice were produced from frozen-thawed morulae stage embryos (Whittingham et al., 1972). Since then, advances in cryobiology, molecular biology, and farm animal embryology have resulted the successful cryopreservation of cattle and small ruminant embryos (Dobrinsky, 2002). However, in horses the cryopreservation of Day 8 blastocyst stage embryos remains a challenge. This is due to inherit special characteristics of equine embryos, such as the development of a mucin-like capsule, a large diameter, high mitotic activity, and a rapid increase in cell number during blastulation (Bruyas et al., 1993; Legrand et al., 2001; Choi et al., 2011b; Gillard Kingma et al., 2011; Stout, 2012). Therefore cryopreservation protocols should be developed to overcome these barriers.

CHAPTER II
LITERATURE REVIEW

2.1 State of assisted reproductive technologies in the Horse

In the last decade, there have been many advances in ARTs in the horse (Squires, 2009). During the 1970's and 1980's research was conducted on a whole animal basis (Freedman et al., 1979; Miller et al., 1981; Ginther and Pierson, 1984; Bergfelt and Ginther, 1985). However, over the last 15 years research has focused on reproductive processes at the cellular and molecular level (Tremoleda et al., 2003; Hinrichs et al., 2005; Galli et al., 2007; Squires, 2009; Merkl et al., 2010). Multiple techniques have been developed to address reproductive inefficiencies in the mare and stallion in an effort to increase reproductive efficiency and subsequent profitability of equine operations. Due to low success rates following cryopreservation, techniques such as semen and embryo cooling protocols have been established to allow short distance transport (Carney et al., 1991; Aurich, 2008; Vidament et al., 2012). These ARTs vary in complexity and cost, and are applied depending on specific circumstances and the degree of infertility.

2.1.1 Artificial insemination

The first reported AI in the horse occurred when Walter Heape, the father of embryo transfer, performed a semen collection from a stallion and inseminated a group of mares (Heape, 1898). Large numbers of mares were inseminated between World Wars I and II, mostly in eastern Europe and Russia (Bowen, 2011). After World War II, equine AI was extensively used to produce animals for transportation (Bowen, 2011).

During the 1950's the United States Trotting Association allowed the use of AI, and in 1963 the American Quarter Horse Association began allowing on-farm AI (Bowen, 2011). Artificial insemination has been utilized for more than 80 years, especially in Europe, the US, and China, resulting in large numbers of foals being produced (Allen, 2005; Aurich, 2012).

Artificial insemination is an ART that culminates in the deposition of fresh, cooled or frozen-thawed semen into the reproductive tract of a female thus producing a pregnancy without the physical presence of a stallion. Gordon (1997) reported multiple benefits of AI, including reduced disease transmission, more efficient use of stallions, decreased risk of injury to mares and stallions, assessment of semen quality prior to insemination, allowing the distribution of superior stallion genetics across greater distances, and increasing trade of animal genetics. In addition, higher pregnancy rates have been reported following AI compared with natural service, with Pickett and Shiner (1994) reporting an increase of 10% in the pregnancy rates of fertile mares and an increase of 30% in the pregnancy rates of subfertile mares.

Semen collection is the first step required for the AI process and multiple artificial vagina (AV) devices have been developed to facilitate this process, including the Colorado, Missouri, and Cambridge models (Allen, 2005). The AV pressure and temperature (45-50ºC) are typically stallion dependent and should be considered when preparing an AV for collection (Brinsko, 2011a). The frequency of collection can affect sperm and seminal characteristics and it is recommended that collection 3 times per

week maximizes sperm output and maintains acceptable seminal and spermatozoa characteristics (Pickett and Voss, 1975).

Extended gel-filtered semen containing antibiotics is typically utilized for AI with the non-fat dry milk-glucose extender described by Kenney et al. (1975) being the most popular. Potassium penicillin G or amikacin, or combination of these antibiotics, are most often utilized and have been shown to be the most effective (Varner et al., 1998; Brinsko, 2011b). The recommended semen dilution ratio (extender:semen) is 1:1 to 2:1 if AI will be performed within 1 hour of semen collection, however, if insemination will be performed after 1 hour, larger dilution ratios (3:1 or 4:1) should be used.

The concentration of sperm in the insemination dose is a fundamental factor that can affect pregnancy rate. Early studies showed that an insemination dose of 500 $X10^6$ progressive motile sperm (PMS) results in high pregnancy rates, but an insemination dose such as 100 X 10^6 PMS have also produced acceptable pregnancy rates (Pickett and Voss, 1975; Householder et al., 1981; Pickett and Shiner, 1994; Brinsko, 2006) . Artificial insemination programs typically utilize insemination doses ranging between 250 to 500 X 10^6 PMS, 500 X 10^6 PMS per dose considered the upper limit (Brinsko, 2011a). Recent studies have demonstrated that 300 X 10^6 PMS and 500 X 10^6 PMS result in similar pregnancy rates: 75% and 64%, respectively (Gahne et al., 1998). Likewise, in another study, it was reported that there was no difference in pregnancy rates when insemination doses of 100 and 500 X 10^6 PMS were utilized in mares that were inseminated every other day until ovulation (Demick et al., 1976). However, in

suboptimal conditions or if the fertility of stallion or mare is unknown, an insemination dose of 500 X 10^6 PMS is recommended (Pickett and Shiner, 1994).

Semen from most stallions can be stored at 4-5ºC for 24 to 72 hours and result in acceptable fertility (Allen, 2005). In a study by Douglas-Hamilton et al. (1984), a dose of 1 X 10^9 PMS was used based on the standard number of 500 X 10^6 PMS and 50% assumed sperm mortality during the cooling and storage period. Therefore, the industry standard is to store sperm at 4 ºC utilizing a number of 1 X 10^9 PMS in a dilution ratio no less than 3:1 (resulting in 25-50 X 10^6 PMS/ml), utilizing a milk-based semen extender with antibiotics (Brinsko, 2006). Samper (2011) reported that cooled semen with an initial sperm number of 1 X 10^9 PMS, exhibiting >30% PMS and good sperm morphology prior to insemination, generally results in pregnancy rates >50% when mares were inseminated within 24 hours of ovulation.

Frozen semen has been used in equine AI programs. Stallions exhibit high variability in their semen cryotolerance and post-thaw motility (Brinsko, 2006). This high variability has resulted in a practical categorization of stallions used for semen cryopreservation between “good freezers” and “bad freezers” (Allen, 2005). Good freezers exhibit post-thaw motility of 40-60% and can result in 60% to 75% pregnancy rates when a dose of >300 X 10^6 PMS is used within 24 hours of ovulation (Allen, 2005). Bad freezers are those stallions exhibiting 10-15% post-thaw motility and usually result in pregnancy rates <30% (Allen, 2005). The typical insemination dose utilizing frozen-thawed semen ranges between 200 to 800 X 10^6 PMS and results in pregnancy rates between 35% to 50% (Brinsko, 2006).

In recent years, low dose insemination has been considered as an alternative when only low spermatozoa numbers are available for insemination. Two techniques have been utilized for low dose insemination: hysteroscopic and transrectally-guided insemination (Brinsko et al., 2003). Pregnancy rates as high as 62% have been reported with an insemination dose of 5 X 10^6 PMS using both techniques. However, when the insemination dose is between 1 and 3 X 10^6 PMS, the hysteroscopic technique is preferred (Morris, 2004; Lyle and Ferrer, 2005). The low dose insemination techniques are an important tool when utilizing sex-sorted sperm or frozen-thawed semen typically resulting in higher pregnancy rates compared with the standard AI protocol (Morris, 2004; Lyle and Ferrer, 2005).

2.1.2 Embryo transfer

Embryo transfer is the transfer of a viable early stage embryo from a donor mare to a recipient mare. Allen and Rowson (1972) reported the first successful equine ET, which was performed surgically. This ART has been utilized as a clinical procedure in the equine industry since the early 1980's (Vanderwall, 2000). In the United States, the clinical application of ET has increased in the last 10-15 years due to the American Quarter Horse Association (AQHA) accepting the registration of multiple foals from a mare in a single year using ET procedures (Squires et al., 2003; Hinrichs, 2012).

Advantages of ET include multiple offspring obtained from a single mare in one year, production of progeny from young 2-year old mares, offspring obtained from older mares or mares presenting reproductive problems, offspring from competing show or

performance mares, and allowing for the use of other ARTs like embryo cryopreservation, ICSI, and NT (Vanderwall, 2000; Hartman, 2011).

Embryo transfer can be performed using a surgical or a non-surgical approach. The surgical approach involves the transfer of the embryo to one of either uterine horn. The horn is exteriorized and the embryo is deposited into the uterine lumen (Allen, 2005). Uterine horn exteriorization can be performed through mid-line laparotomy or flank laparotomy (Imel et al., 1981; Squires et al., 1982). Midline laparotomy requires general anesthesia, while flank laparotomy can be performed in a sedated standing mare (Squires et al., 1982). High pregnancy rates have been obtained using both techniques and range from 65% to 90% (Squires et al., 1999; Carnevale et al., 2000; Allen, 2005).

The non-surgical approach consists of deposition of the embryo into the uterine horn or uterine body of the recipient mare using a transcervical approach (Carnevale et al., 2000). The transcervical approach can be accomplished utilizing three different transfer instruments, depending on the preference of the technician: a disposable plastic embryo transfer pipette, a stainless steel reusable embryo transfer gun, or a standard artificial insemination pipette (Vanderwall, 2000).

Early ET studies reported lower pregnancy rates (23% to 45%) were obtained when non-surgical embryo transfer protocols were used compared with the surgical approach (68% to 72%) (Iuliano et al., 1985; Carnevale et al., 2000). However, in recent years it has been shown that non-surgical embryo transfer results in similar or higher pregnancy rates (70% to 85%) compared with surgical transfer (Meadows et al., 2000;

Jasko, 2002; Squires et al., 2003; Allen, 2005; Hinrichs and Choi, 2005; Hartman, 2011; Hinrichs, 2012). Similarly, pregnancy rates of 90% have been reported using a modified non-surgical approach utilizing a Polansky's vaginoscope and Velsellum forceps (Allen, 2005).

Non-surgical ET is typically the preferred method due to the speed of the procedure, it is more cost-efficient, and is more favorable for the recipient mare's overall welfare when compared with the surgical approach (Stout, 2006). Pregnancy rates following non-surgical transfer are highly technician-dependent, and the experience of the technician is very important for success (Squires et al., 1982; Hinrichs, 2012). It has been determined that deposition of the embryo into the uterine horn is not a necessity as the equine embryo exhibits mobility throughout the uterus, this mobility is required for maternal recognition (Hinrichs and Choi, 2005; Stout, 2006).

It has been suggested that the selection and management of recipient mares is the most critical factor in determining the success of any embryo transfer procedure (Vanderwall, 2000; Squires et al., 2003; Jacob et al., 2012). Recipient mares should be selected based on specific characteristics. Retrospective studies have determined that the characteristics most influential in the successful establishment of pregnancies are uterine tone, cervical tone, and lack of uterine edema or uterine folds (Carnevale et al., 2000). Recipient mares should have a history of normal estrous cycles and have no abnormalities in uterine or ovarian anatomy (Vanderwall, 2000). Mares ranging in age from 3 to 12 years are typically utilized as recipient females, as this reduces the probability of mares exhibiting chronic endometritis (Hartman, 2011).

Synchrony between the donor and recipient mare is another factor that should be considered. The most favorable synchrony occurs when recipient mares have ovulated 1 day before (+1) to 3 days after (-3) the donor mare. This ovulation synchrony has resulted in the highest pregnancy rates (Squires et al., 2003; Hinrichs and Choi, 2005; Stout, 2006). However, there have been reports of no difference in pregnancy rates following embryo transfer between Days +1 to -5 in commercial programs (Jacob et al., 2012). Strategies to induce ovulation synchrony between donor and recipient mares have included the use of prostaglandins ($PGF_{2\alpha}$), hCG, GnRH analogues and progestins (Squires et al., 1999; Vanderwall, 2000). Alternatives to dependency on ovulation synchrony between donors and recipients include the use of non-cyclic or ovariectomized mares subjected to progestin treatment. These progestin-treated mares have been successfully used as recipient mares (Hinrichs et al., 1987; Hinrichs and Choi, 2005).

The recovery rates of Day 7 and Day 8 embryos are higher than that of Day 6 (Jacob et al., 2012), therefore these embryos are usually preferred and are more widely used in the equine industry (Squires et al., 1999; Hinrichs, 2012; Jacob et al., 2012). Practitioners prefer Day 8 equine embryos as they are easier to identify following recovery (Hinrichs and Choi, 2005), while Day 9 and Day 10 embryos are more prone to physical damage during the recovery and transfer process (Squires et al., 1985; Hinrichs, 2012). Developmentally retarded embryos based on the day of recovery (morula stage embryos recovered on Day 8) have been associated with decreased pregnancy rates and increased embryonic loss (Carnevale et al., 2000). Embryo quality is another factor affecting embryo transfer success. It has been reported that grade 1

embryos result in higher pregnancy rates when compared with grade 2 or 3 (Carnevale et al., 2000; Squires et al., 2003). The embryo grades based in a four-point system are shown in Table 2.1.

Table 2.1 Embryo grade assignment based on a four-point system.

Grade	Comment	Description
1	Excellent	No abnormalities observed; spherical in shape; cells of uniform size, color and texture; size and development stage appropriate for age post-ovulation
2	Good	Minor imperfections, such as a few extruded blastomeres; slight irregularities in shape, size, color, or texture; limited separation between trophoblast layer and zona pellucida or capsule
3	Poor	Moderate level of imperfections, such as a larger percentage of extruded or degenerated blastomeres; partial collapse of blastocoele; or moderate shrinkage of trophoblast from zona pellucida or capsule
4	Degenerate or dead	Severe problems easily identified, such as a high percentage of extruded blastomeres, complete collapse of blastocoele, rupture of zona pellucida, or complete degeneration and embryonic death
UFO	UFO	Unfertilized oocyte

From: McCue et al., (2009).

Carnevale et al. (1987) was the first to report that equine embryos stored for 24 hours at 5 ºC produced an acceptable pregnancy rate (70%). This finding positively impacted the embryo transfer industry, allowing breeders, practitioners and veterinarians to collect embryos in different locations and ship them to centralized centers where they could be transferred to suitable recipients by experienced

technicians (Squires et al., 2003). Carney et al. (1991) reported similar pregnancy rates were obtained when equine embryos were transferred that had been stored for 24 hours at 5 ºC compared with equine embryos transferred within 1 hour of collection. The cooling of the equine embryo has usually been performed in Ham's F 10 equilibrated in an atmosphere of 5% CO_2, 5% O_2, and 90% N. Moussa et al. (2003) reported a similar number of dead cells when Ham's F 10 was compared to two commercial embryo holding media (Vigro Holding Plus and Emcare Embryo Holding Solution) following holding of equine embryos at 5 ºC for 24 hours. Similarly, pregnancy rates were not different when Ham's F 10 or Emcare embryo holding solution was utilized for embryo cooling procedures (Moussa et al., 2003).

2.1.3 Oocyte recovery

Assisted reproductive techniques such as oocyte transfer (OT), in vitro fertilization, intracytoplasmatic sperm injection, gamete intrafallopian transfer (GIFT), and nuclear transfer (NT) are all dependent on recovery of viable and developmentally competent oocytes. Oocytes can be recovered from live animals or ovaries of deceased animals from either natural causes or from slaughterhouses. However, the recovery rate of small immature equine follicles by follicular aspiration both *in vivo* and *in vitro* (20% and 30%, respectively) is lower compared with other farm animals such as the cow (45% and 63%, respectively) (Hamano and Kuwayama, 1993; Alm et al., 1997; Garcia and Salaheddine, 1998; Mari et al., 2005; Jacobson et al., 2010). The structure of the equine follicle is somewhat unique in that a thecal pad is located beneath the cumulus cell attachment (Landim-Alvarenga et al., 2008). Projection of cumulus cells to the theca

cause the cumulus cell complex to be strongly attached to the internal follicle wall, resulting in difficulty aspirating the oocyte and a low recovery rate (Landim-Alvarenga et al., 2008; Hinrichs, 2012).

Typically there are two approaches to oocyte recovery from live animals: aspiration of large follicles or aspiration of immature small follicles from the ovary (Hinrichs and Choi, 2005). For recovery of large follicles, the mare is commonly subjected to gonadotropin stimulation with a GnRH analogue such as deslorelin acetate or human chorionic gonadotropin (hCG), resulting in collection of mature and highly developmentally competent oocytes. Recovery rates using this method typically range between 65% and 80% (Carnevale and Maclellan, 2006; Jacobson et al., 2010). An advantage of this technique is that the diameter of these large follicles is ≥ 35 mm, and no special equipment is required. Aspiration can be retrieved through a cannula in the flank of the mare using a 13-ga, 20 cm needle and a syringe while the ovary is being manipulated per rectum (Hinrichs et al., 1990; Hinrichs et al., 1998). The standard transvaginal aspiration (TVA) approach utilized in cattle can also be applied for this purpose (Carnevale and Ginther, 1993).

Small oocytes can be recovered from live animals using TVA. Equipment for TVA is similar to that utilized in cattle consisting of an ultrasound scanner with a modified handle containing a convex transducer and a needle guide. Because of the strong attachment of the oocyte to the follicle wall, recovery rates are typically low and range from 10% to 30% (Blanco et al., 2009; Jacobson et al., 2010). However, recovery rates

as high as 50% have been reported when the internal surface of the aspirated follicle is repeatedly flushed (Bøgh et al., 2002).

Immature oocytes can also be obtained from the ovaries of postmortem females. Excision of ovaries, aperture of visible follicles and scrapping with a bone curette is an effective method and yields high recovery rates of cumulus-oocyte complexes (COC's) in the range of 70% (Hinrichs and Williams, 1997). The time of collection and storage conditions are variables that can affect the viability of oocytes. Controlling the temperature of the ovary is critical to achieve quality oocytes, and it has been suggested that 37°C is the optimal storage temperature for ovaries when oocytes will be recovered within two hours post-mortem and 15-20°C when collection will be performed after longer intervals (Hinrichs, 2012). Phosphate-buffered saline, normal saline with antibiotics, TCM-199, or commercial embryo holding medium are perhaps the most widely used media for storage and transport of whole equine ovaries (Carnevale and Maclellan, 2006).

2.1.4 Oocyte transfer

The surgical transfer of mature oocytes to the oviduct of a recipient mare is an ART often used as a clinical treatment for infertility. This technique can be utilized to circumvent anovulation, persistent uterine infection, pyometra and torn or scared cervices (Squires et al., 2003). Oocyte transfer has been extensively studied to provide information related to *in vivo* or *in vitro* oocyte maturation and fertilization in the mare.

The first oocyte transfer in the mare was reported by McKinnon et al. (1988) where a fertilization rate of 20% was obtained following recovery at Day 2 post-transfer followed by transfer of three fertilized oocytes which resulted in two pregnancies. One pregnancy was carried to term and the other failed at day 20. However, Carnevale and Ginther (1995) reported higher pregnancy rates after transfer of *in vivo* matured oocytes. In that study, the developmental competence of oocytes collected from young or old mares were transferred to young recipient oviducts and pregnancy rate evaluated. Pregnancy rate was higher when oocytes were transferred from young donor mares compared with oocytes from old donor mares, and were 92% and 31% respectively (Carnevale and Ginther, 1995). When using a fertile stallion and a donor mare, oocyte transfer pregnancy rates higher than 70% can be expected (Carnevale et al., 2004a). However, pregnancy rates of 9% to 18% are typical for *in vitro* matured oocytes following oocyte transfer (Hinrichs, 2012).

The pregnancy rates from sub-fertile donor mares used in commercial programs (27% to 40%) are typically lower when compared with results where fertile stallions and donor mares were used (54% to 83%) on experiments (Carnevale, 2004). Fertilization rates are usually higher when insemination occurs 15 hours prior to oocyte transfer compared with insemination 2.5 hours post-transfer (92% and 8%, respectively) (Carnevale et al., 2004a).

The successful transfer of equine oocytes into pre-ovulatory follicles (intrafollicular transfer) has been reported (Deleuze et al., 2009). In that study, the fertilization rates were 32% for intraoviductal transfer, 5.5% for intrafollicular transfer of

in vitro matured oocytes, and 12.8% for intrafollicular transfer of *in vivo* matured oocytes. Results of this study demonstrate that intrafollicular transfer of equine oocytes can be successful.

Prior to oocyte transfer, donor and recipient are synchronized with hCG on the same day to induce ovulation (Carnevale et al., 2005) and this approach requires aspiration of the recipient's pre-ovulatory follicle prior to insemination (Hinrichs and Choi, 2005). In order to avoid relying on recipient/donor synchronization, researchers have shown that non-cyclic mares can be subjected to an estrogen and progestin treatment to be successfully used as recipient mares (Hinrichs and Choi, 2005).

2.1.5 Intracytoplasmatic Sperm Injection (ICSI)

Intracytoplasmatic sperm injection is an ART that involves the direct injection of a spermatozoon into the cytoplasm of a metaphase II stage oocyte (Navarro et al., 2008). Using this approach fertilization rates of 70% to 80% and pregnancy rates close to 45% can be achieved in humans (Palermo et al., 2009). Squires et al. (1996) reported the first equine pregnancy derived from ICSI. In that study, four *in vitro* matured oocytes were injected and one oocyte cleaved. Following surgical transfer into the oviduct of a recipient mare successful pregnancy ensued. Following this success, Dell'Aquila et al. (1997) utilized ICSI to compare the developmental rates of *in vitro* fertilized oocytes and ICSI oocytes. They reported that the fertilization rate was higher for ICSI compared with IVF, which was 29.8% and 8.7% respectively. While no cleavage was reported for IVF oocytes, oocytes subjected to ICSI resulted in a 12.7% cleavage rate. Similarly, ICSI procedures utilizing fresh, cooled, frozen or lyophilized sperm (Choi et al., 2002;

Landim-Alvarenga, 2008; Choi et al., 2011a) have resulted in high fertilization rates (70% to 80%) (Choi et al., 2002; Galli et al., 2007) and blastocyst rates of 25% to 35% following *in vitro* culture (Hinrichs, 2005; Hinrichs and Choi, 2005).

There are several advantages inherit to the ICSI procedure. For example, fertilized oocytes can be cultured *in vitro* and then transferred transcervically to a recipient mare (Hinrichs, 2005). This technique, unlike oocyte transfer, does not require major surgery. Intracytoplasmic sperm injection also allows the utilization of spermatozoa from a stallion with poor semen quality or low concentrations to be utilized (Hinrichs, 2012). The disadvantages of ICSI include the use of specialized equipment requiring experience in micromanipulation, and increased oocyte and embryo handling. It appears though, that ICSI is not as efficient as oocyte transfer when competent oocytes and high quality semen are used (Hinrichs, 2005; Landim-Alvarenga et al., 2008).

2.1.6 Cloning

Cloning or nuclear transfer is the production of genetically identical individuals by non-sexual means (Landim-Alvarenga et al., 2008) and can be utilized as a tool to generate genetically identical individuals of existing or deceased animals (Galli et al., 2012). Nuclear transfer, as its name implies, involves the transfer of the nucleus from a donor cell to a recipient cell. Embryonic or somatic cells have been utilized as donor cells, and oocytes are used as recipient cells. In the early 1950's NT was first reported, where amphibian embryonic nuclei was transferred into enucleated oocytes, successfully producing the first cloned amphibian (Briggs and King, 1952). Illmensee

and Hoppe (1981) reported the first mammalian clones through NT approximately thirty years later. During this time advances in mammalian reproductive and developmental biology were achieved, allowing for successful *in vitro* culture systems for pre-implantation embryos and the development of specialized techniques for micro-manipulation of oocytes, cells, and embryos (Di Berardino, 2001). The first production of mammalian cloning was obtained after the transfer of single blastomeres derived from 8-16 cell embryos into bisected enucleated oocytes (Willadsen, 1986). In 1997, the first report of an animal to be successfully produced from sheep differentiated somatic cells became a true milestone in developmental biology research (Wilmut et al., 1997).

Shortly thereafter, the first equid cloning was reported by Woods et al. (2003), where three mules were produced using NT where 45-day-fetus' somatic cells were transferred into *in vivo* matured oocytes. These oocytes were recovered using transvaginal oocyte aspiration from gonadotropin-stimulated pre-ovulatory follicles. The same year, Galli et al. (2003) reported the production of a horse filly by adult somatic cell nuclear transfer using *in vitro* matured oocytes as recipient cells, culturing to the blastocyst stage, and transferring transcervically to recipient mares. In that study, the pregnancy was carried to term by the mare that was the donor of the somatic cell. Important differences exist in the reports of Woods et al. and Galli et al. in terms of difficulty and clinical applicability. The former used fetal cells as donor cells, which may have positively influenced the probability of live birth. Similarly, *in vivo* matured oocytes were used as recipient cells. These oocytes are highly developmentally competent in terms of nuclear reprogramming, but are limited in number and are expensive and difficult to obtain. Galli et al. (2003) used adult fibroblast as donor cells and *in vitro*

matured oocytes as recipient cells. The resultant cloned embryos were cultured to the blastocyst stage and transferred using a transcervical approach. In 2005, two cloned foals were produced from an endurance champion gelding in Italy (Lagutina et al., 2005) and two cloned foals were produced at Texas A&M University (Hinrichs et al., 2006).

Even though there are variations among protocols, the SCNT procedure consists of three basic steps: oocyte enucleation, insertion of the donor cells, and activation of the reconstructed embryos. The cloned embryos are subsequently subjected to *in vitro* culture to an optimal stage prior to transfer into a recipient female (Vajta and Gjerris, 2006). In SCNT, the zona pellucida is preserved because it is important in supporting further development of the embryo (Vajta and Gjerris, 2006). The efficiency of nuclear transfer is usually low, and reports of success range from 0.1% to 3% (Hinrichs et al., 2006; Whitworth and Prather, 2010). However, it is generally accepted that these low success rates are the result of incomplete nuclear reprogramming or the inappropriate expression of imprinted genes (Wolf et al., 2001).

Somatic cell nuclear transfer presents multiple applications to clinicians and researchers alike. These include multiplication of high value animals, rescuing endangered species to prevent extinction, multiplying high-quality farm animals, reprogramming somatic cells into undifferentiated cells, development of transgenic animals for the production of recombinant proteins, production of organs for xenotransplantation, and use in animal models for research (Bordignon, 2011).

2.2 Endocrinology and reproductive physiology of the mare: Estrous cycle

Horses are considered seasonal monovulatory breeders where breeding activity begins during increasing day length. Therefore, mares are also considered seasonal breeders, but approximately 30% ovulate during the winter (Aurich, 2011). Prior to breeding, females must attain puberty to subsequently cycle. Puberty is typically defined as the occurrence of the first estrus, and the criteria used to identify pubertal estrus are a rise in plasma progesterone, initial estrus behavior at teasing, and the first observed estrus (Eilts, 2011). Senger (2003a) has presented three definitions of puberty: the age at first estrus, the age at first ovulation, and the age at which a female can support pregnancy without deleterious effects. Fundamental factors that affect the onset of puberty are genetics, nutrition, body size, weight, fat composition, season of birth, photoperiod, and social aspects like the presence of dominant females (Gordon, 1997; Senger, 2003a; Eilts, 2011). Proper interaction of these factors appears to release a signal to the surge center of the hypothalamus causing the release of sufficient quantities of GnRH when stimulated by estradiol from the pre-ovulatory follicle to cause an LH surge from the anterior pituitary resulting in ovulation (Eilts, 2011). Puberty in fillies usually occurs between 12 and 18 months of age (Aurich, 2011), but can range between 7.8 and 37 months with a median of 15 months (Eilts, 2011). Onset of puberty varies between breeds, birth date, and management type.

Estrous cycle length is about 22 days in horses and 24 days in ponies, consisting of 14-16 days of diestrus and 5-8 days of estrus (Ginther, 1992). Length is affected by the reproductive stage of the individual, where the average length is 21.2 days for

lactating and 22.8 days for non-lactating mares (Heidler et al., 2004). Mares are monovulatory in nature but 7 to 25 % ovulate multiple oocytes.

2.2.1 Follicular phase

2.2.1.1 Development of new follicular wave

The beginning of the estrous cycle is defined as the development of a new follicular wave, starting after ovulation of a follicle from the previous cycle or after the first ovulation following vernal transition. A surge in follicle-stimulating hormone (FSH) is temporally associated with the emergence of each wave, and FSH reaches a peak when the largest follicle is approximately 13 mm in diameter (Ginther, 2000). One or two follicular waves develop during the estrous cycle (Aurich, 2011). Mares may exhibit major or minor waves throughout the estrous cycle. Major waves develop dominant follicles to a pre-ovulatory diameter of ≥ 30 mm, whereas in minor waves the largest follicle reaches only 21-27 mm in diameter with no clear dominant follicle development (Gastal et al., 1997). Major waves emerging mid-cycle are called primary waves and result in ovulation of the dominant follicle. Major waves that emerge during late estrus or early diestrus are called secondary waves and result in regression of the dominant follicle (Bergfelt and Ginther, 1993). Wave patterns during the estrous cycle can greatly differ among breeds. In quarter horses and ponies only one major wave usually develops, while in thoroughbreds it is typical to have development of two follicular waves (Ginther, 1992).

2.2.1.2 Dominant follicle deviation

Follicle selection and deviation must occur to establish a dominant follicle from the follicular wave. Deviation is the term used to describe the disassociation between dominant and subordinate follicles, and is characterized by continued growth of the largest follicle to become dominant to subordinate follicles (Ginther, 2000). Deviation is a fundamental event and prevents the development of multiple ovulatory follicles in a monoovulatory species such as the horse. By definition it occurs on the day the two largest follicles of a wave differ in growth rates (Gastal et al., 1999b). Follicular deviation occurs approximately 6.2 days after the emergence of a 6 mm follicle (Gastal et al., 1997). The follicle that is destined to become dominant typically emerges prior to subordinate follicles of that wave, and maintains a diameter 3 mm greater than other follicles of the wave until the beginning of deviation (Gastal et al., 1997). Biochemical or physiological deviation appears to precede diameter deviation (Ginther, 2000). Non-dominant follicles can develop the ability to become dominant after deviation, and this has been reported in follicle ablation studies (Gastal et al., 1999a). FSH concentration decreases after the FSH peak and continues to decline for several days after deviation (Gastal et al., 1997). This decrease in FSH concentration appears to be fundamental in the deviation process, and may be required for the establishment of a dominant follicle. The dominant follicle must acquire the capacity to suppress FSH in order to inhibit the growth of subordinate follicles (Ginther, 2000).

An increase in circulating estradiol and the production of inhibin from growing follicles appear to be involved in the decline of FSH following deviation (Donadeu and

Ginther, 2004). There is also increased production of estradiol by the largest follicle one day prior to deviation (Gastal et al., 1999b). Once the dominant follicle has been established, it has the capacity to increase estradiol synthesis by up-regulating thecal synthesis of androgens, increasing pregnenolone synthesis in the granulosa cells and preventing its metabolism to progesterone in granulosa and theca cells (Beg and Ginther, 2006).

Regulation of folliculogenesis and follicle selection involves gonadotropins, but there are also several locally produced hormones and growth factors that contribute. Intrafollicular biochemical events before deviation ensure dominance of the selected follicle. The mechanism that starts this process has not yet been elucidated, but occurs during the FSH decline and results in increased luteinizing hormone (LH) release (Ginther et al., 2003). Differences between the concentrations of intrafollicular factors at the time of gonadotropin changes are associated with diameter deviation, and this probably accounts for greater response to gonadotropins in the developing dominant follicle compared with subordinate follicles. The intrafollicular factors that have been proposed as candidates for activation of deviation include the insulin-like growth factor (IGF) system, steroids, inhibin-A/activin-A, gonadotropin receptors, angiogenic factors, and several other intrafollicular factors (Beg and Ginther, 2006). Intrafollicular growth factors present multiple paracrine/autocrine roles which include enhancing FSH action, inducing expression of LH receptors, and regulating aromatase activity.

The IGF system has received special attention because of its apparent influence on facilitating the utilization of low levels of FSH (Ginther, 2000). Differential regulation

of blood vessel formation and permeability in the theca layers of cohort follicles has been recently suggested as a new mechanism for dominant follicle selection (Mihm and Evans, 2008). The selected dominant follicle presents increased expression of angiogenic factors, such as the vascular endothelial growth factor (VEGF), in combination with increased follicle differentiation and increased blood flow (Mihm and Evans, 2008). The finding that follicular fluid concentrations of VEGF are higher in the dominant follicles than in subordinate follicles of mares one day after the beginning of deviation partially support this hypothesis (Ginther et al., 2004). Experimental reduction of VEGF signaling has resulted in follicular arrest and atresia (Mihm and Evans, 2008).

2.2.1.3 Dominant follicle maturation

It has been suggested that during the follicular phase luteinizing hormone (LH) is a gonadotropin stimulant of the selected dominant follicle. Luteinizing hormone and estradiol concentration increases two days before deviation. It has been postulated that a transition from FSH to LH-dependence is a mechanism which enables the continued development of the selected dominant follicle (Ginther et al., 2001). Luteinizing hormone is not involved in the inhibition of other follicles through FSH depression (follicle deviation), but is required for continued growth of the largest follicles after the beginning of deviation (Ginther, 2000). The role of LH during deviation has not been clarified, but indications that LH plays a role in deviation are 1) future dominant follicle develop LH receptors before diameter deviation, 2) the largest follicle does not become as large when LH concentration is suppressed and 3) a transient elevation in LH concentration occurs during deviation in mares (Ginther et al., 2003). In addition, reduction of LH

around the period of deviation results in lower circulating concentrations of estradiol and inhibin (Bergfelt et al., 2001). It has been suggested that increased LH receptor expression characterizes the dominant follicle and gives it the first developmental advantage in the cohort of growing follicles. The insulin-like growth factor system enhances FSH-induced granulosa cell differentiation, particularly LH receptor acquisition. Increased free IGF concentrations available for the dominant follicle at the beginning of deviation may be responsible for that developmental advantage (Mihm and Evans, 2008).

Established dominant follicle growth rate is approximately 3 mm/day from deviation, and this follicle reaches an average diameter of 35 mm four days before ovulation. This period is defined as the beginning of the pre-ovulatory period. Follicular growth continues for one or two days prior to ovulation until it reaches a mean diameter of approximately 40-41 mm (Ginther et al., 2008). However, this diameter is variable among mares, where pre-ovulatory follicles >55 mm have been reported (Cuervo-Arango and Newcombe, 2008). Serum LH and estradiol concentrations increase beginning five days prior to ovulation and coincide with the onset of estrus. Estradiol reaches a peak two days before the peak in LH and subsequent ovulation. This suggests that estradiol may play an important role in inducing estrus and may actively contribute to the ovulatory process (Pattison et al., 1974). Estradiol may be responsible for stimulating a positive feedback mechanism resulting in an increase in LH release from the anterior pituitary (McCue et al., 2011). The endocrine profile of LH secretion is unique in the mare compared to other domestic animals and presents a broad LH surge which peaks 1 to 2 days after ovulation. This is believed to be due to the relatively long

half-life of equine LH (Pattison et al., 1974). Luteinizing hormone concentration slowly increases until the estradiol surge (Ginther et al., 2008), and following the estradiol surge there is a rapid increase in LH which is associated with decreasing estradiol (Ginther et al., 2007c). This unique LH secretion pattern is responsible for the dominant follicle final maturation and the induction of ovulation (McCue et al., 2011). The decreased growth of the preovulatory follicle and decreased estradiol concentration two days prior to ovulation is thought to be a result of a negative effect of LH on follicular estradiol production. Reciprocally LH output variation during the surge is attributable to a negative effect of estradiol on LH throughout the surge where the extent of the negative feedback changes in function of estradiol concentration (Ginther et al., 2008).

2.2.1.4 Estrus behavior

Estrus is defined as the period when a mare develops increased interest and sexual receptiveness to the stallion. Estrus is a result of increased estradiol concentrations produced by the preovulatory follicle in the absence of progesterone (McCue et al., 2011). This hormone network is modulated by positive and negative feedback between the ovaries, pituitary, and hypothalamus. It plays a critical regulatory role in physiological and behavioral aspects of the estrous cycle (Curry et al., 2007). The onset of estrus is more gradual in mares than in other farm animals (Gordon, 1997). Estrus behavior ensures the mare is receptive to the stallion for copulation around the time of the ovulation period. A cyclical series of changes in the reproductive tract, particularly in the cervix, uterotubal junction, and oviductal isthmus, occurs in order to support deposition of the ejaculate and sperm transport to the oviduct for

fertilization. These events are endocrinologically coordinated so they occur in a proper synchronous manner (Curry et al., 2007). Estrus behavior is characterized by frequent urination, which induces the flehmen response and eventual chemosensory priming of the stallion for copulation (Marinier et al., 1988). The estrus mare approaches the stallion and frequently presents her hindquarters to him, lowering her pelvis, deviating the tail, exposing the perineal region, and presenting rhythmic eversion of the clitoris (termed clitoral winking) (Crowell-Davis, 2007). Mares in estrus exhibit sexual receptivity, primarily standing during copulation with no signs of aggressiveness towards the stallion (Crowell-Davis, 2007).

Multiple methods have been reported to induce estrus behavior. A common approach is the administration of luteolytic factors, prostaglandin, or prostaglandin analogues. This results in a decrease of progesterone concentrations through corpus luteum regression and an increase in estradiol production by the growing follicles (Curry et al., 2007). It has been reported that estrus occurs on average 3 to 4 days after prostaglandin treatment, and ovulation occurs 5 to 6 days after (Samper, 2008). However, the diameter and status of the ovulatory follicle at the time of prostaglandin treatment apparently affects the interval from treatment to onset of estrus and ovulation. This interval may range from 48 hours to 12 days (Samper, 2008). Estrus behavior can also be induced in ovariectomized or anovulatory mares with the administration of 1 mg of estradiol (Crowell-Davis, 2007), and suppressed by the administration of progesterone or progesterone analogues (Curry et al., 2007).

2.2.1.5 Ovulation

Ovulation is preceded by identifiable uterine, ovarian, and follicular characteristics that can be used for an approximate estimation of the time of ovulation. The prediction of ovulation is important in many reproductive management protocols because it allows for the efficient use of resources and human effort. In embryo transfer programs or cooled/frozen semen insemination programs it is vital to predict ovulation for the successful establishment of pregnancies. A common practice in these programs is to evaluate the mare multiple times per day or to use ovulation induction agents such as a GnRH analogue (deslorelin diacetate) or human chorionic gonadotropin. McCue et al. (2011) reported that the pre-ovulatory follicle changes from a spherical to an irregular shape just prior to ovulation, and becomes softer within 12 hours of ovulation. Just prior to ovulation, the pre-ovulatory follicle may exhibit a thickened echogenic border when observed using transrectal ultrasonography and uterine edema usually decreases. The effect of high concentrations of estradiol prior to ovulation results in the uterus and cervix becoming soft and relaxed.

The mechanics of ovulation in the mare is unique compared to other farm animals. Mares ovulate through the ovulation fossa, and all pre-ovulatory follicles will migrate to this site prior to ovulation. The preovulatory follicle consists of a fluid filled antrum encapsulated by granulosa, theca interna, and theca externa layers (Hunter, 2003). Ovulation in the mare is the result of a complex series of events under elevating concentration of LH that leads to the rupture of the pre-ovulatory follicle and the extrusion of follicular fluids, granulosa cells, and the cumulus-oocyte complex (COC)

into the infundibulum (Gastal and Gastal, 2011). Ovulation involves many cell types, sequential changes in gene expression within these cells, and the generation of an extra-cellular matrix within the expanded cumulus-oocyte complex (Richards et al., 2008). Response of the preovulatory follicle to the LH surge initiates the temporal sequence of events that affect both theca and granulosa cells, stimulating cAMP and activating selective protein kinase signaling cascades which lead to ovulation (Richards et al., 2002). Ovulation is considered an inflammatory-like response due to similarities between these processes. Follicles become highly vascularized, produce large amounts of prostaglandins, and synthesize a hyaluronan-rich matrix. These processes also occur at sites of tissue inflammation and wound healing (Richards et al., 2008).

2.2.2 Luteal phase

2.2.2.1 Corpus Luteum formation and development

The luteal phase is marked by the occurrence of ovulation, and begins with the luteinization of a previously ovulated follicle to form a functional corpus luteum. Even though the LH surge simultaneously initiates the processes of ovulation and luteinization, these events are functionally disassociated (Richards et al., 2002). In fact, it is imperative that events controlling ovulation precede those regulating the genetic program of luteinization. If not, malfunctions occur in the cycle (Richards et al., 2002). Luteinization is the transition of a preovulatory follicle into a highly vascularized corpus luteum capable of secreting large amounts of progesterone. Additionally, multiple types of ovarian cells undergo hyperplasia, hypertrophy, and migration during development of the corpus luteum (Smith et al., 1994). The luteinizing hormone preovulatory surge

begins a complex process whereby follicular cells present morphological, endocrinological, and biochemical changes (Niswender et al., 1994; Smith et al., 1994). Biochemically, LH stimulates cAMP formation and the subsequent activation of the protein kinase A (PKA) pathway. This has been suggested to serve as the initial signal for luteinization of granulosa cells (Murphy, 2000). Ovarian cells undergoing luteinization will acquire two key characteristics: the capacity to initiate progesterone production and the capacity of the cells to undergo regression or death at an appropriate time (Diaz et al., 2002). The corpus luteum is a transient endocrine organ. Its biological importance was first reported by Fraenkel (1903), who demonstrated that the removal of the CL resulted in terminated pregnancies in rabbits. Even though several compounds and molecules are produced by the CL, its primary role is considered to be progesterone production.

2.2.2.2 Luteinization

Morphological and structural changes occur once the cumulus-oocyte complex has been released from the follicle. The granulosa layer folds around the follicular antrum, which contain traces of blood and follicular fluid (Murphy, 2000). It is presumed that this infolding of the follicular wall facilitates the migration of fibroblasts, endothelial cells, and theca interna cells into the central area of the developing corpus luteum (Smith et al., 1994). These cell types have roles in corpus luteum formation and are a source of angiogenic factors (O'Shea et al., 1980; Reynolds et al., 2000). The breakdown of the basement membrane between theca interna cells and granulosa cells, which occurs during the preovulatory LH surge and ends at ovulation, will facilitate

cellular migration and tissue remodeling (O'Shea et al., 1980). Hyperemia, present in luteinization processes, facilitates access of LH into the non-vascular granulosa cell compartment (Cavender and Murdoch, 1988).

Blood supply is essential for the formation of the CL. Once it is fully developed, it becomes the most vascular tissue in the body, having the highest rates of blood flow of any tissue or organ (Wiltbank et al., 1988). Angiogenic factors play an important role in this vascularization process, where vascular endothelial growth factors (VEGF) appear to have the major effect (Reynolds et al., 2000). Expression of VEGF takes place primarily in specific perivascular cells such as arteriolar smooth muscle and capillary pericytes, which are predominantly regulated by oxygen levels (Reynolds et al., 2000). Extensive capillary branching vascularizes the CL, pulling together theca cells that were previously dispersed into a single unit or small groups. Granulosa cells develop hypertrophy causing an eightfold volume increase compared to their preovulatory size (Murphy, 2000).

In most farm animal species, numerous histological studies have shown that granulosa cells differentiate into large luteal cells and theca cells differentiate into small luteal cells (Ohleth and Bagnell, 1999). However, in the mare, luteal cells are derived exclusively from the granulosa cells (van Niekerk et al., 1975). The parenchyma of the mature CL is composed of approximately 80% large and small luteal cells, and 20% support cells, including endothelial cells, pericytes, macrophages, smooth muscle cells and fibroblasts (Niswender et al., 1994). In most farm animals, large and small luteal cells produce progesterone. Large luteal cells secrete progesterone at a higher rate (2

to 40 fold) than small luteal cells. Therefore, large luteal cells produce more than 80% of the total progesterone secreted by the CL during the midluteal phase (Niswender et al., 1994). Interestingly in the mare, progesterone receptors are found in large luteal cells but not in small luteal cells, and steroidogenic enzymes have not been detected in small luteal cells. These two findings suggest that progesterone is biosynthesized exclusively in large luteal cells in the mare (Aurich, 2011).

Progesterone production and secretion is episodic and highly regulated. As with steroid hormones, cholesterol is the precursor for the production of progesterone. The pathway of progesterone biosynthesis involves few enzymes and is considered the simplest of the steroidogenic pathways (Diaz et al., 2002). The biosynthesis of this steroid hormone is regulated by cholesterol availability and the expression of specific steroidogenic enzymes (Smith et al., 1994). Multiple cellular sources of cholesterol are available for steroidogenesis. These include cellular membranes, cholesterol ester stores, circulating lipoproteins, and de novo cholesterol biosynthesis (Diaz et al., 2002).

In mares, systemic concentrations of progesterone immediately increase after ovulation. This is in contrast to other farm animals, where a transient and less pronounced increase in progesterone occurs (Roberto da Costa et al., 2005). In the mare shortly after ovulation, circulating progesterone concentration increases rapidly from 1-2 ng/ml to concentrations of 8-16 ng/ml when the corpus luteum reaches maturity (Lofstedt, 2011). On day 8 after ovulation, peak progesterone plasma concentration is reached and will slowly decrease until the onset of luteolysis, which occurs around day 14 on average (Aurich, 2011). Because of the long estrous period

(mean 5-7 days), diestrus is shorter (14 to 16 days) than other farm animals. Diestrus behavior is characterized by the lack of sexual receptivity resulting from an increased concentration of progesterone which blocks estrus behavior (Vanderwall, 2011). The signs of diestrus are avoidance of an approaching stallion and aggressive responses with attempted mounting, including squalling, striking, and kicking (Crowell-Davis, 2007).

2.2.2.3 Luteolysis

Each estrous cycle represents an opportunity for establishing a pregnancy. During diestrus, the uterus is simultaneously prepared for luteolysis or for maintainance of the corpus luteum if a signal of maternal recognition is induced by a viable embryo (McDowell and Sharp, 2011). Luteolysis or luteal regression is essential for the initiation of a new estrous cycle. Loss of luteal function involves two processes: a decrease in progesterone production and a loss of luteal tissue (Niswender et al., 1994). After progesterone reaches maximum concentration at day 8, the CL area and progesterone concentration decrease in parallel until the onset of luteolysis (Ginther et al., 2007b).

In farm animals, including the mare, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is the luteolysin derived from the uterus (McDowell and Sharp, 2011). In contrast with ruminants, the mare lacks a well-developed countercurrent mechanism and $PGF_{2\alpha}$ is secreted into systemic circulation (Aurich, 2011). Antisteroidogenic actions of $PGF_{2\alpha}$ are mediated through activation of the protein kinase C (PKC) pathway, while luteolytic actions of $PGF_{2\alpha}$ induce increased free intracellular calcium which begins the signal for cellular apoptosis (Niswender et al., 1994). Because $PGF_{2\alpha}$ has short half-life, studies typically

measure its metabolites (PGF15-keto-13,14-dihydro- $PGF_{2\alpha}$ or II-ketotetranor PGF) and are expressed as PGFM (Goff et al., 1984). Luteolysis is initiated by a relatively small transitional pulse of PGFM (Ginther and Beg, 2011; Ginther et al., 2011). Apparently, hormones or factors (oxytocin) present at this transition increase the luteolytic response to small concentrations of PGFM (Ginther and Beg, 2012). Luteolysis starts on average 13 days after ovulation and, based on hourly blood samples, lasts for 23 hours (Ginther et al., 2011). Pulses of LH during preluteolysis and luteolysis can be detected but do not occur in synchrony with progesterone fluctuation, indicating that LH does not have a positive effect on progesterone (Ginther and Beg, 2012). Based on PGFM hourly blood sampling, concentrations of progesterone decrease linearly during PGFM pulses (Ginther et al., 2011). In other farm animals it has been suggested that luteal blood flow may be an important factor in luteal regression (Niswender et al., 1994). However, it has been shown that no acute increase or decrease in luteal blood occurs before the abrupt decrease in plasma progesterone during luteolysis (Ginther et al., 2007b). In fact, decreases in progesterone concentrations start prior to the beginning of luteal blood flow decrease (Ginther et al., 2007a). Luteolysis results in low progesterone concentration and, coupled with estradiol production from the preovulatory follicle, sets the beginning of estrus behavior (Vanderwall, 2011).

2.3 The equine embryo

2.3.1 Oocyte maturation

A developmentally competent and mature oocyte is required for fertilization to occur. Immature oocytes are arrested in the prophase of the first mitotic division until a

stimulus is provided for meiotic resumption. It has been suggested that two mechanisms control oocyte maturation: 1) an inhibitory stimulus that prevents the oocytes to resume meiosis and 2) an activating stimulus caused by gonadotropic surge that overrides the inhibitory mechanism (Grøndahl, 1998). Nuclear and cytoplasmic events are needed during the growth of the dominant follicle and periovulatory period in order to have a fully developmentally competent oocyte.

Cytoplasmic events during oocyte maturation include cortical granule migration beneath the oolema, breakdown of junctions between cumulus cells and the oolema, enlargement of perivitelline space, microfilament and microtubule reorganization, modification of kinase expression and phosphorylation, and mitochondria and lipid droplet rearrangement to a more central or semilunar domain, giving the ooplasm a polarized appearance (Grøndahl et al., 1995; Goudet, 2011). The most predominant nuclear change is the nucleolus transition from a diffuse, reticulated configuration to a dense, uniform body composed of exclusively fibrillar material. These modifications are clear indicators of intensive RNA synthesis, which gradually ceases as the oocytes develop (Fulka et al., 1998). Flattening of the spherical oocytes nucleus, undulation of the nuclear envelope (Grøndahl et al., 1995), and specific chromatin and centrosome/microtubule complex modifications are other important events that occur during maturation (Albertini, 1992; Fulka et al., 1998) . Mature, developmentally competent oocytes have progressed to metaphase II and are characterized by extrusion of the first polar body.

Once ovulation has occurred, the cumulus-oocyte complex is expelled into the infundibulum. Some evidence suggests that the final maturation of oocytes may occur in the oviduct, and is defined as pre-fertilization changes in the oocyte and zona pellucida which are required for proper fertilization and embryo development (Goudet, 2011). Multiple experiments have been conducted to determine if immature oocytes can reach developmental competence and maturity in the oviduct, but no successful fertilization has been reported (Bézard et al., 1997). Results from these experiments indicate that immature oocytes completely mature in the oviductal environment.

2.3.2 Fertilization and embryo development

Spermatozoa are present in the oviduct as little as 2 hours post insemination, reaching peak concentrations at 4 hours post insemination (Bader, 1982). Fertilization can occur within hours of insemination. However, it has also been reported that spermatozoa can remain viable for long periods in the female reproductive tract, and fertilization is still possible 6 days post insemination (Day, 1942). Sperm must be capacitated in the female reproductive tract, where it is subjected to changes in the plasma membrane and an influx of calcium ions which allow sperm to undergo the acrosomal reaction (Senger, 2003b). In the mare, fertilization takes place at the ampulla region of the oviduct and fertilization rates following natural service are as high as 90% (Betteridge, 2011).

Studies by Bezard et al. (1989) on equine fertilization and embryo development have shown that fertilization of oocytes occurs within 12 hours post-ovulation. These results agree with similar findings that spherical paternal and maternal pronuclei were

observed within 12 hours after ovulation, and by 19 hours the pronuclei usually had migrated to close apposition (Grøndahl et al., 1993). Between 24 and 34 hours after ovulation, equine embryos are at the two-cell stage (Bezard et al., 1989; Grøndahl et al., 1993), by 48 hours they are 4-6 cells, and between 72 to 96 hours they are 7-12 cells (Bezard et al., 1989).

2.3.3 Embryo oviductal transport and entrance to the uterus

Transport of the embryo through the oviduct is aided by a combination of ciliary action, muscular contractions, and fluid currents (Goudet, 2011). Studies utilizing scanning electron microscopy have determined the presence of ciliated and non-ciliated cells in the infundibulum, ampulla, and isthmus of the equine oviduct (Goudet, 2011). Ciliary motion directs the embryo toward the uterus and it has been reported that ciliary activity is more intense in the ampullar region compared with the isthmus, although both regions appear to possess similar number of ciliated cells (Ball, 1996).

A special feature of oviductal transport in the horse is the selective transport of embryos into the uterus, while unfertilized oocytes are retained at the ampulla-isthmus junction (Freeman et al., 1991). This was first described by Van Niekerk and Gemeke (1966) and Betteridge and Mitchell (1974) when they found that only viable fertilized oocytes are able to pass the ampulla-isthmus junction while spermatozoa penetrated non-fertilized oocytes were retained in this region. Studies to identify embryonic substances temporally associated with oviductal transport showed that prostaglandin E_2 (PGE_2) is produced by the embryo starting at day 5 post ovulation, the expected time of oviductal transport (Weber et al., 1991b). Other studies showed that the embryo triggers

the signal to initiate oviductal transport, but the source of this signal has not been shown (Freeman et al., 1992). Weber et al. (1991a) confirmed that PGE_2 hastens oviductal transport of equine embryos and may play a role in selective oviductal transport.

The oviductal transport time, defined as the interval from ovulation to entry of the embryo into the uterus, has been proposed to be 130 to 142 hours (Freeman et al., 1991) or 144 to 156 hours (Battut et al., 1997). Embryos enter into the uterus at the morula or early blastocyst stage (Freeman et al., 1991). It has been found that embryos at day 7 have a significantly greater mean diameter than day 6.5 (244 µm and 186 µm, respectively) (Battut et al., 1997). It appears that the equine embryo remains in the oviduct longer, resulting in a more advanced embryonic stage entering the uterus compared to other domestic animal embryos (Freeman et al., 1991; Freeman et al., 1992).

2.3.4 Embryo capsule

A special characteristic of the equine embryo is the development of an acellular glycoprotein capsule between the trophectoderm and zona pellucida. The embryos entering the uterus are at the morula or early blastocyst stage and are surrounded by a zona pellucida (Betteridge et al., 1982). The capsule appears as a uniform layer on the inner surface of the zona pellucida of equine embryos starting at day 6.5 (Betteridge et al., 1982; Flood et al., 1982; Stout et al., 2005). The zona pellucida thickness decreases during the first 24 hours as the capsule thickness increases. By day 8, capsule thickness is approximately 1 µm and the zona pellucida had been shed, and by day 11

the capsule is approximately 3 μm thick (Flood et al., 1982). By day 18 the capsule becomes thinner, and completely disappears by day 23 (Oriol et al., 1993).

The capsule is present through the second and third week of pregnancy. This period is important in embryo development and maternal recognition of pregnancy. Even though the functions of the capsule are not completely understood, it has been hypothesized to play multiple fundamental roles. Capsule glycoproteins resemble those of the mucin family, which are known to function as protective coating on surface ephithelial cells and to play important role in cell-to-cell interactions (Oriol et al., 1993). It has been suggested that the capsule is responsible for maintaining the embryo's spherical shape, preventing the trophoblast from elongating between day 10 and 16 (Allen, 2001). Because of the strong and elastic nature of the capsule, it has also been proposed that it serves as a physical protection to the embryo during a time when it is subjected to constant myometrial contractions (Leith and Ginther, 1985; Allen, 2001).

The spherical shape of the embryo, which is maintained by the capsule, is of importance during the embryo mobility phase. Embryo mobility occurs between day 7 and 17, and is required for maternal recognition of pregnancy. This process allows the embryo to distribute an antiluteolytic signal throughout the uterus, preventing the release of the luteolytic prostaglandin $F_{2\alpha}$ from the endometrium (Stout et al., 2005). This has been corroborated by experiments where surgical restriction of the equine conceptus to a small area of the uterus during the mobility phase results in luteolysis and a return to estrus (Allen, 2001).

Embryo mobility response is also thought to be facilitated by the anti-adhesive properties of the capsule glycoprotein sugar side chains, which include a high proportion of sialic acid residues. The high negative charge resulting from these residues may prevent adhesion to the endometrium (Oriol et al., 1993). Loss of sialic acid residues from capsule glycoproteins would terminate the anti-adhesion effect, which may be associated with the fixation of equine embryo on day 17. This is a unique mechanism of control of embryo mobility (Oriol et al., 1993).

The capsule glycoproteins are exclusively produced by the trophoblast cells (Albihn et al., 2003). The capsule also includes endometrial lipocalin, P19, which suggests there is a maternal contribution to capsule formation (Tremoleda et al., 2003). The capsule also contains proteins involved in the transport of materials into, and out of, the developing embryo (Betteridge and Waelchli, 2004). Because the equine conceptus implants as late as 36 days post-ovulation, the absorption of endometrial secretions through the capsule must occur during this time (Stewart et al., 1995).

It has been shown that the zona pellucida is not required for embryo survival, while the presence of the capsule is an absolute requirement for pregnancy maintenance. It was shown that bisected embryos that were denuded from the zona pellucida require the development of a capsule in the recipient uterus in order to survive following embryo transfer (McKinnon et al., 1989; Stout et al., 2005). It has been reported that the embryonic capsule exhibits low permeability to cryoprotectants (Gillard Kingma et al., 2011; Scott, 2011), and this is considered the main reason for failure to produce viable pregnancies from cryopreserved expanded blastocysts.

2.4 Cryopreservation of equine embryos

2.4.1 Slow cooling

Cryopreservation in horses was first reported by Griffin et al. (1981). In this study, 19 embryos were frozen, four of which were transferred to recipient mares. This resulted in one pregnancy that failed around day 60. The next year, Yamamoto et al. (1982) reported the birth of a live foal resulting from the transfer of a cryopreserved equine embryo.

Slade et al. (1985) reported that the plunging temperature to LN utilized in slow cooling cryopreservation protocols affects embryo quality post-thaw. In that study, thirty-two Day 6 early blastocyst and blastocyst stage embryos were utilized, and results showed that plunging embryos at -33 °C resulted in higher quality embryos compared with -38 °C after 24 hours of culture. Additionally, embryos that were frozen in straws had a higher developmental rate than embryos frozen in ampoules. Slade et al. (1985) reported that embryos frozen in straws and plunged into LN at -33°C resulted in a 53% pregnancy rate. Cryopreservation of early blastocyst stage embryos resulted in a higher pregnancy rate than expanded blastocysts (80% vs. 14% respectively).

Poitras et al. (1994) reported that morphological changes after 24 hours of *in vitro* culture of frozen-thawed Day 6.5 embryos showed that the stage of development of equine embryos is a fundamental factor in the embryonic survival following cryopreservation. Morula and early blastocyst stages were associated with higher quality and survival potential than expanded blastocyst stage embryos (Poitras et al.,

1994). Previous equine embryo cryopreservation studies showed that when early embryonic stages of development are used, such as Day 6 morulae or early blastocysts, pregnancy rates were increased (Slade et al., 1985; Poitras et al., 1994).

Different cryoprotectants have been evaluated for cryopreservation of equine embryos. Cryoprotectants vary in permeation rate and ability to protect against freezing damage. Because cellular membranes differ in their biochemical composition, the rate at which cryoprotectants move across a membrane can be affected by the molecular weight of the cryoprotectant. Low molecular weight cryoprotectants have faster permeation rates than cryoprotectants with high molecular weight. Moreover, it has been reported that different embryo developmental stages differ in their permeability to cryoprotectants (Leibo, 2008).

Glycerol is generally the cryoprotectant of choice when freezing domestic animal embryos, including horse embryos. However, other cryoprotectants have been evaluated in an effort to increase the efficiency of cryopreservation of equine embryos. In a study by Meiras et al. (1993), morulae, early blastocysts, blastocysts, and expanded blastocysts were used to evaluate the efficiency of 1,2-propanediol and glycerol as cryoprotectants. Blastocysts and expanded blastocysts frozen in glycerol resulted in no pregnancies post-transfer. In addition, it was reported that morula and early blastocyst stage embryos frozen in glycerol resulted in 40% pregnancy rate while embryos frozen in 1,2-propanediol produced no pregnancies (Meira et al., 1993). In another study by Ferreira et al. (1997), a 1,2-propanediol and glycerol mixture was compared with glycerol for cryopreservation of Day 6 or Day 7 equine embryos.

Pregnancy rates in that study were 53.3% for glycerol treatment and 0% for the 1,2-propanediol-glycerol mixture treatment. Results from both studies showed that 1,2-propanediol is not a suitable cryoprotectant for the cryopreservation of equine embryos.

In another study, Bass et al. (2004) evaluated the efficiency of methanol for the cryopreservation of Day 7-8 equine blastocysts (300-1000 µm in diameter). Embryos were randomly assigned to either a 48% methanol or a 10% glycerol treatment. Once equilibrated in cryoprotectant, embryos were frozen using a slow-cooling method and 0.25 mL plastic straws. After thawing, embryos were either transferred immediately or cultured *in vitro* for 12 hours prior to transfer. Pregnancy rates on day 16 post-ovulation for the methanol and glycerol treatments were 23% and 38%, respectively. The authors concluded that even though methanol did not exhibit any detrimental effects on equine blastocyst stage embryos, it did not provide any additional benefit when compared to glycerol based on subsequent pregnancy rates.

Ethylene glycol has become an alternative cryoprotectant for cryopreservation of equine embryos and several studies have evaluated its efficacy. Day et al. (1993) utilized Day 6 early blastocysts to evaluate the addition of ethylene glycol at two concentrations, 1.5 M and 1.37 M. One pregnancy was obtained from the 1.37 M ethylene glycol treatment and none from the 1.5 M concentration. In another study, the utilization of ethylene glycol and sucrose was evaluated for the cryopreservation of Day 6 equine embryos (Hochi et al., 1996). Treatments consisted of 10% glycerol, 10% ethylene glycol, and 10% ethylene glycol + 0.1 M sucrose. Pregnancy determined by ultrasonography on day 15 post-ovulation were 25% (2/8) for ethylene glycol and 37.5%

(3/8) for glycerol treatments. Interestingly 63.6% (7/11) of the embryos produced pregnancies after the in-straw dilution of embryos frozen using 10% ethylene glycol + 0.1 M sucrose. Results of the experiment showed pregnancy rates from frozen/thawed blastocysts could be performed, and that relatively high pregnancy rates can be obtained when utilizing ethylene glycol and sucrose in a 2-step cryoprotectant addition manner (Hochi et al., 1996).

Huhtinen and Paranko (1999) evaluated 10% glycerol in a 4-step addition manner compared with 1.5 M ethylene glycol in a 1-step protocol using embryos ranging from 140 to 190 µm in diameter. After transfer to recipient mares, pregnancy rates were 44% (4/9) for the 10% glycerol treatment and 30% (3/10) for the 1.5 M ethylene glycol treatment. Results from these experiments showed that pregnancies can be obtained when ethylene glycol is used for the cryopreservation of equine morulae and early blastocysts, but higher pregnancy rates are obtained with glycerol as a cryoprotectant. Ethylene glycol appears to be promising cryoprotectant when in-straw dilution of cryoprotectant is performed, allowing the direct transfer of embryos to recipient mares.

In addition to cryoprotectants, other compounds have been evaluated in an effort to reduce the osmotic shock and the cellular damage due to increased toxicity which embryos are exposed to during the process of slow-cooling. As the temperature decreases and water freezes, the solution within the embryo becomes hypertonic and cryoprotectants can reach high, toxic concentrations for the embryonic cells. In one study utilizing Day 6.5 embryos, the effect of the addition of 0, 20 and 100 mM of L-

glutamine to a 10% glycerol cryopreservation solution was evaluated (Lagneaux et al., 2000). Pregnancy rates, evaluated on day 12 for the 0, 20 and 100 mM of L-glutamine treatments, were 30%, 10% and 60% respectively. Moreover, 4',6-diamidino-2-phenylindole (DAPI) staining analysis showed less cellular damage for embryos frozen in solution containing 100 mM of L-glutamine than embryos frozen in a solution containing either 0 or 20 mM L-Glutamine. Multiple slow-cooling freezing protocols have typically included 50-100 mM glutamine in the cryoprotectant solutions (Huhtinen and Paranko, 1999; Duchamp et al., 2006).

Early studies in equine embryo cryopreservation utilized morulae or early blastocysts ≤300 µm in diameter. At a diameter around 300 µm, the capsule develops and is a characteristic of expanded blastocyst stage embryos. Multiple studies evaluated the permeability of expanded blastocyst stage embryos. In one such study, embryos were exposed to glycerol and ethylene glycol, and it was reported that embryos < 250 µm in diameter regained lost blastocoele volume faster than embryos that were 250-500 µm in diameter. This suggests that small embryos are more permeable to cryoprotectants than larger embryos (Pfaff et al., 1993). In that same study, it was noted that embryos >500 µm do not re-expand compared to smaller embryos, suggesting that cryoprotectants do not readily permeate the blastocyst (Pfaff et al., 1993). Similarly, Hochi et al. (1994b) reported that embryos <300 µm regained blastocoele volume when exposed to a 2-step cryoprotectant addition protocol of 5% and 10% glycerol solutions, while embryos >300 µm lost volume and did not recover.

Legrand et al. (2001) evaluated the relationship between thickness of capsule and cellular damage in embryos exposed to glycerol. Results from that experiment showed that embryos with no capsule or a thin capsule had mild cellular damage, possibly due to water outflow and glycerol penetration. In embryos with a medium thickness capsule the water outflow was not compensated by the glycerol penetration because of the thicker capsule. Therefore these embryos had a higher degree of osmotic damage. Instead, embryos with a thick capsule (0.8 µm) exhibited low osmotic damage suggesting there is no movement of glycerol or water outflow, resulting in no osmotic damage.

Estimations of equine blastocyst permeability evaluated in previous studies suggest that the capsule impedes cryoprotectant penetration into the embryo. Gillard Kingma et al. (2011) evaluated the permeability of the equine embryonic capsule to glycerol and ethylene glycol *in vitro*. Capsule pieces of Day 14 to Day 18 embryos were used, and their permeability to 1.5 M ethylene glycol, 0.74 M ethylene glycol, 0.87 M glycerol, and 0.15 M NaCl (saline) were evaluated in a dual-chambered Valia-CHien permeation apparatus. Permeation of capsule by 1.5 M ethylene glycol was significantly faster than 0.74 M ethylene glycol and 0.87 M glycerol. Both ethylene glycol and glycerol movement through the capsule were slower than saline. The authors hypothesized that ethylene glycol would be more permeable than glycerol because its volume in aqueous solution is considerably smaller ($8.86 \times 10^{-23} cm^3$/molecule) than glycerol ($12.25 \times 10^{-23} cm^3$/molecule). They concluded that the capsule acts as a filter in a size-selective manner. Results of this experiment agreed with those of Barfield et al.

(2010), who suggested that the equine capsule restricts movement of glycerol and to a lesser extent ethylene glycol into the embryo.

Scott et al. (2012) quantified the amount of glycerol permeating into Day 7 equine blastocyst stage embryos using conventional slow cooling and vitrification glycerol concentrations. Equine blastocyst stage embryos were randomly assigned to either 1.4 M glycerol or 3.4 M glycerol. Glycerol uptake percentage was not different between 1.4 M and 3.4 M glycerol for embryos >600 µm and <600 µm. However, embryos with a diameter <600 µm exhibited higher glycerol uptake than embryos >600 µm, and uptake was 3.6% ±1.5 and 0.4%±0.3, respectively. Results from this and previous studies suggested that the embryonic capsule dramatically reduces the rate at which cryoprotectants penetrate the embryo but does not completely block the movement of cryoprotectants into the embryo.

Small equine embryos (≤300 µm) appear to tolerate cryopreservation procedures better than larger embryos. Apart from capsule formation, other developmental events during blastulation could also affect the embryo's permeability to cryoprotectants. These events include variation in surface area to volume ratio, inner cell mass formation, changes in membrane aquaporin populations, blastocoele volume, rapid increase in cell number and the associated mitotic activity (Budik et al., 2008; Bruyas, 2011; Choi et al., 2011b; Gillard Kingma et al., 2011; Stout, 2012).

In order to overcome the low rate of cryoprotectant penetration through the embryonic capsule, researchers have utilized enzymatic treatments to increase permeability. In one study, Legrand et al. (2001) compared the effectiveness of trypsin

and collagenase in the elimination or increased permeability of the embryonic capsule. Concentrations for trypsin at 0.005%, 0.01%, 0.025%, 0.05%, 0.1%, 0.25%, 0.5%, 1% and 5% and concentrations of collagenase at 0.5%, 1%, 2.5% and 5% were evaluated. Day 8 equine embryos were treated with their respective enzymatic treatment for 15 minutes following by a 3-step cryoprotectant equilibration to reach a final concentration of 1.5 M glycerol. Results indicated that trypsin was more suitable than collagenase for increasing permeability of the equine capsule. It was also noted that enzymatic concentrations <0.05% were ineffective whereas concentration >1% appeared to be toxic to the embryo. They went on to report that the transfer of trypsin pre-treated frozen embryos ranging from 187 µm to 1581 µm resulted in six pregnancies on day 14 and the first live foal derived from the cryopreservation of Day 8 equine embryo.

In a second study, Legrand et al. (2002) compared the effect of enzymatic treatment and non-treated control on subsequent pregnancy rate of Day 7 and Day 8 equine embryos. The non-treated controls were frozen immediately while the enzyme treated embryos were treated with 0.2 % trypsin (w/v) for 15 minutes, and embryos were equilibrated using a 3-step protocol until a final concentration of 1.5 M glycerol was reached. The transfer of the non-treated embryos did not result in pregnancies while those treated with trypsin resulted in an 18% pregnancy rate (2/11), one Day 7 and one Day 8 embryo. These authors concluded that enzymatic treatment may be a successful alternative for the cryopreservation of large equine embryos. However, similar results have not been obtained in other studies (Maclellan et al., 2002).

Ice crystal formation that occurs during cryopreservation can affect cellular organization by lysing plasma membranes and disrupting organelles and intracellular functions. Cytochalasin-B, a microfilament inhibitor, has been shown to increase the success rate of cryopreservation of pig embryos by increasing *in vivo* survival upon thawing (Dobrinsky et al., 2000). Maclellan et al. (2002) evaluated the effect of Cytochalasin-B and trypsin pre-treatment in the post-thaw viability of frozen-thawed large equine embryos following transfer. Pregnancies were obtained from both non-pretreated embryos and cytochalasin-B pre-treated embryos, at 57% (4/7) and 42% (3/7) respectively. Trypsin and trypsin/cytochalasin-B pre-treated embryos resulted in no pregnancies. Pregnancies were obtained from cytochalasin-B pre-treated embryos, but the resultant pregnancy rates were not different compared with non-pretreated embryos.

It has been hypothesized that a reduction in the water content of expanded blastocysts would increase their cryopreservation efficiency. Barfield et al. (2009) evaluated the effect of embryo dehydration prior to standard cryopreservation procedures. Grade 1 equine blastocyst stage embryos ≥400 µm were utilized in this study. Embryos were dehydrated or served as untreated control. Dehydration was achieved through a pre-treatment with 0.6 M galactose prior to slow cooling of in 1.5 M glycerol. One pregnancy from each treatment was obtained following transfer to recipient females. Based on these results, there is no additional benefit of pre-treatment with 0.6 M galactose to induce dehydration prior to standard slow cooling cryopreservation of expanded equine blastocysts.

2.4.2 Vitrification

Two techniques are commonly used for the cryopreservation of farm animal embryos, including the horse: slow cooling and vitrification. In the slow cooling procedure, water crystallizes resulting in an osmotic gradient that draws water from the intracellular compartment until intracellular solidification occurs (Saragusty and Arav, 2011). Vitrification is the solidification of a liquid through extreme elevation in viscosity during cooling thus avoiding crystallization. Cellular fluids turn into a glass-like amorphous state where translational molecular motions are arrested and biological activity ceases (Fahy et al., 1984; Rall et al., 1987).

Rall and Fahy (1985) were the first to report the successful vitrification of a mouse embryo and since that report vitrification has become another tool utilized for the cryopreservation of embryos and oocytes in animals as well as in humans. Vitrification minimizes ice crystal formation by the utilization of high concentrations of cryoprotective agent (CPA) coupled with rapid temperature decrease (Mukaida and Oka, 2012). During the process of cryopreservation, living cells are at risk of injury by multiple factors, including toxicity of cryoprotectants, chilling damage, physical injury by extracellular ice, toxicity of concentrated electrolytes, formation and growth of intracellular ice, fracture damage, and osmotic swelling (Kasai, 1996). Ice crystal formation can lyse plasma membranes, denature critical intracellular functions and organelles, and the cytoskeleton can be de-stabilized to the point that the embryo may lose intracellular pathway communication (Dobrinsky, 1996).

Vitrification may lessen the chilling damage through rapid cooling (Kasai, 2002). However, embryos subjected to vitrification are at risk of cryoprotectant toxicity, intracellular ice formation, fracture, and osmotic damage if not performed correctly (Kasai, 1996). In order to achieve satisfactory embryo vitrification, three factors should be considered: 1) cooling/warming rate, 2) type and concentration of cryoprotectant, and 3) volume in which the embryo will be vitrified (Saragusty and Arav, 2011). High cooling and warming rates can aid in circumventing intracellular ice formation in less permeable embryos (Mukaida and Oka, 2012). Cooling and warming rates are related to the volume and the material on which the embryo is vitrified. Because smaller volumes have higher heat transfer, they facilitate higher cooling and warming rates. Saragusty and Arav (2011) reported multiple techniques that can be utilized to reduce sample volume. These can be divided in surface and tubing techniques. The surface technique includes systems such as electron microscopy grid, Cryotop, Cryoloop, nylon mesh, and cryoleaf. For the surface technique, a 0.1-1 µL drop of cryoprotectant solution allows high cooling and warming rates (aprox. 20000 ºC/min) because the devices are open and have direct contact with the liquid nitrogen or warming solution. The tubing techniques are closed systems that utilize cryoprotectant volumes >1 µL and include the 0.25 mL plastic straw, open-pulled straw, closed pulled straw, and pipette tip.

The type and concentration of cryoprotectant utilized for vitrification is important. Cryoprotectant concentration is usually 1-2 mol/L resulting in low toxicity levels for slow-freezing procedures, while for vitrification the cryoprotectant concentrations are on the order of 5-8 mol/L. The selection of a fast permeating cryoprotectant is extremely

important for vitrification (Kasai, 2002). Low molecular weight cryoprotectants are generally more permeable than high molecular weight cryoprotectants, and therefore are preferred for vitrification procedures. It has been reported that embryos may be exposed for less time to cryoprotectants with high permeation rates prior to cooling and during warming, because these cryoprotectants diffuse out quicker resulting in reduced osmotic injury (Kasai, 1996).

The proper warming of vitrified embryos is required to obtain viable embryos following any vitrification procedure. In fact, as reported by Leibo and Pool (2011), the rate at which vitrified embryos are warmed is more critical to their survival than the rate at which they were originally cooled. Removal of cryoprotectant from the embryo must occur during the warming step. If an isotonic solution is used for the warming of vitrified embryos and the removal of cryoprotectant, damage by osmotic swelling is highly probable. This is a result of water entering the embryo at a faster rate than the cryoprotectant exits (Kasai, 2002). To reduce the risk of this type of injury, a common strategy is to warm vitrified embryos in a hypertonic solution containing sucrose to counteract the inflow of excess water (Leibo, 1983; Kasai, 2002). Using this strategy it has been shown that the addition of sucrose reduces toxicity, and this may be due the reduced amount of intracellular cryoprotectant (Kasai, 1996). Other saccharides such as galactose, fructose, and glucose function similarly and are apparently non-toxic for cryopreserved cells (Kasai, 1996). In addition to small saccharides, a macromolecule (e.g. bovine serum albumin or polyvinyl pyrrolidone) has also been frequently utilized in vitrification solutions. Non-permeating agents, small saccharides, and macromolecules

all have low toxicity, and their addition can decrease the toxicity of vitrification solution by reducing the required concentration of the permeating cryoprotectant (Mukaida and Oka, 2012).

2.4.2.1 Vitrification of equine embryos

The first pregnancies resulting from the vitrification of equine embryos were reported by Hochi et al. (1994a). In this study, embryos were recovered non-surgically at day 5 to day 7 post-ovulation. Embryos were vitrified utilizing a solution of 40% ethylene glycol, 18% ficoll, and 0.3 M sucrose in phosphate-buffered saline (PBS). Three treatments were evaluated where embryos were placed directly in vitrification solution for 1-2 minutes, or embryos were exposed to 20% ethylene glycol in PBS for 10-20 minutes and then transferred to vitrification solution for 1-2 minutes. Embryo warming and cryoprotectant removal was performed in 1-step or 4-steps. *In vitro* development following 120 hours of culture was 0% for embryos exposed directly to cryoprotectant and 1-step cryoprotectant removal. Embryos exposed to cryoprotectant in a 2-step manner and warmed either in 1 or 4-steps resulted in 57% *in vitro* development each. Two pregnancies were obtained from embryos vitrified following the 2-step cryoprotectant addition and 1-step warming.

In a similar study, three treatments were evaluated according to embryo diameter: ≤200 µm, 200-300 µm, and > 300 µm. Embryos were exposed to 20% ethylene glycol in PBS for 20 minutes followed by 40% ethylene glycol, 18% ficoll, and 0.3 M sucrose for 1 minute. Re-expansion rates following 48 hours of culture were 88% for blastocysts <200 µm in diameter, 75% for blastocysts between 200-300 µm in

diameter, and 25% for blastocysts >300 µm in diameter. The re-expansion rate of embryos >300 µm was lower compared with embryos <300 µm. The authors concluded that large equine blastocyst may be damaged during the vitrification procedures (Hochi et al., 1995).

Young et al. (1997) compared 3 divergent treatments for the cryopreservation of embryos ranging between 300-700 µm. Treatments evaluated were 1-step addition of 1 M glycerol and cryopreservation by slow cooling, 2-step addition of 4 M glycerol, decrease to 2 M glycerol (step-down equilibration) and cryopreservation by slow cooling and 2-step addition of 11.9 M ethylene glycol and vitrification. The cryoprotectant removal in all treatments was performed in a 4-step manner. Following 36 hours of culture the 1-step addition of 1M glycerol resulted in 100% degenerated embryos, the step-down equilibration protocol resulted in four grade 1 embryos, one grade 2 embryo, and one grade 3 embryo. The vitrification protocol resulted in one grade 2 embryo, one grade 3 embryo and the rest of embryos were degenerated. Two pregnancies were obtained following transfer of six embryos cryopreserved by the step-down equilibration protocol.

Oberstein et al. (2001) compared the efficacy of two vitrification methods and one slow cooling method for the cryopreservation of equine embryos ≤ 300 µm in diameter. The treatments evaluated were1) 1.8 M ethylene glycol and 0.1 M sucrose placed in a 0.25 Ml straw and slow cooled at 0.5 ºC/min, 2) vitrification with open-pulled straw (OPS) in 16.5% ethylene glycol, 16.5% DMSO and 0.5 M sucrose, and 3) vitrification with cryoloop in 17.5% ethylene glycol, 17.5% DMSO, 1 M sucrose and 0.25 µM ficoll.

Following 20 hours of culture, mean grades for embryos were 2.9 for slow cooling, 3.1 for OPS, and 3.3 for cryoloop using a 4 grade scale according to McKinnon and Squires (1988). There were no differences among post-thaw embryo diameters following 20 hours of culture. It was shown that similar results can be obtained when using either of the vitrification methods compared to a slow cooling method. Similar results were reported by Moussa et al. (2005). In that study Day 6.5 to Day 6.75 equine embryos were cryopreserved with a glycerol-based slow cooling method or an ethylene glycol/DMSO-based open-pulled straw vitrification method. There was no difference in diameter, morphological grade, or percentage of degenerated embryos following 3 hours of in vitro culture across treatments (Moussa et al., 2005).

Most vitrification studies evaluated vitrification protocols utilizing multiple step removal of cryoprotectants. Most recently, the first study of successful pregnancies following direct transfer of vitrified equine embryos was reported by Eldridge-Panuska et al. (2005). In this study, equine embryos collected on Day 6, 7 and 8 post-ovulation were utilized. The treatments evaluated were exposure to 1.4 M glycerol (GLY) in PBS for 5 minutes, then to 1.4 M glycerol + 3.6 M ethylene glycol (EG) for 5 minutes, and finally moved to 3.4 M GLY + 4.6 M EG or similar procedure with a final vitrification solution of 1.4 M GLY + 6.6 EG. Embryo warming was performed in a serial dilution manner or following a 1-step manner that allows a direct embryo transfer approach. Pregnancies were only obtained with embryos ≤300 µm utilizing a final concentration of 3.4 M GLY + 4.6 M EG. Pregnancy rate at day 16 was 46% for serial dilution embryos and 62% for direct transfer embryos. Embryos >300 µm did not produce pregnancies.

The relatively high pregnancy rate obtained in the previous study has resulted in a standard vitrification protocol for equine embryos ≤300 µm that has been widely promoted in the equine industry (Carnevale et al., 2004; Carnevale, 2006). This vitrification protocol has been applied in commercial operations under field conditions. Araujo et al. (2010) reported pregnancy rates following the vitrification of Day 6 embryos obtained during the 2008 and 2009 breeding season in the northern hemisphere. Day 15 pregnancy rates were 67% in 2008 and 55% in 2009, with an embryonic loss rate until 45 days gestation at 12.5% and 9.1% for 2008 and 2009 respectively.

Hudson et al. (2006) evaluated the effect of cooling on pregnancy rates of vitrified embryos. Morulae or early blastocysts were assigned to one of the two treatments: vitrification within 1 hour of collection or storage at 5ºC for 12-19 hours prior to vitrification . Embryos were vitrified using the protocol described by Eldridge-Panuska et al. (2005). Results showed there was no difference in embryo grade, diameter, and morphology between treatments prior to vitrification. Pregnancy rates at day 16 were 75% for embryos vitrified immediately and 65% for embryos stored for 12-19 hours before vitrification. These findings showed that relatively high pregnancy rates can be obtained when cryopreserving embryos that have been cooled stored for 12-19 hours without significant loss of viability.

Alternative protocols have been evaluated in an effort to increase the viability of vitrified equine embryos. Campos-Chillòn et al. (2009) vitrified embryos at the 2 to 8-cell stage utilizing a protocol typically used for vitrification of early bovine embryos. The vitrification protocol consisted of the exposure of embryos to 1.5 M ethylene glycol for 5

minutes and moved to 7 M ethylene glycol + 0.6 M galactose for 30 seconds. While in the final vitrification solution, embryos were loaded into open-pulled straws and plunged into liquid nitrogen. Embryos were then warmed in a serial dilution manner. After transferring to recipient mares, 62% of the embryos developed into embryonic vesicles on day 16.

Because cryopreservation of large equine embryos has been challenging, other novel methods have been evaluated to increase its efficiency. Scherzer et al. (2011) evaluated a laser-assisted vitrification method for the cryopreservation of large equine embryos. This study utilized embryos ≤300 μm in diameter as a control and embryos >300 μm for the evaluated treatment. Treatment group embryos received a laser pulse at the 3 o'clock position while control group embryos received no pulse. Embryos were vitrified by placing in 7.5% ethylene glycol + 7.5% DMSO for 20 minutes, and then moved to 15% ethylene glycol + 15% DMSO. Stepwise removal of cryoprotectant was performed after warming. Following embryo transfer, the pregnancy rate at day 13 was 75% for embryos ≤300 μm and 44% for embryos >300 μm. However, just one pregnancy from the embryos >300 μm was detected at day 23 and carried to term.

In a study where the objective was to develop a method for trophoblast biopsy and posterior pre-implantation genetic diagnosis (Choi et al., 2010), it was discovered that equine embryos preserve high viability once they have been mechanically collapsed. In this regard, Choi et al. (2011b) evaluated the effect of blastocyst collapse on survival of vitrified expanded blastocysts. In one experiment, Day 7 and 8 embryos ranging between 330 to 730 μm were biopsied, and the estimated percent of

blastocoele fluid loss was recorded. Embryos were cryopreserved by either a DMSO or ethylene glycol based vitrification protocol. After warming, embryos were shipped in a portable incubator for 4-6 hours or cultured *in vitro* for 6 hours before transfer. Pregnancy rates at day 12 were 50% for the DMSO treatment and 46% for the ethylene glycol treatment. There was no difference in pregnancy rates between embryos transferred on site (44%) versus those shipped before transfer (55%). From the DMSO treatment, just two pregnancies were detected at day 25 (heartbeat stage), while all of the pregnancies were detected from the ethylene glycol treatment at day 25. It was noted that from the ethylene glycol treatment, embryos that had more than 70% of the blastocoele fluid removed resulted in 4 out of 5 pregnancies.

In another experiment of the same study, Day 7 embryos ranging between 300 to 710 µm were subjected to blastocyst fluid and cell extraction by positioning of the pipette in the center of the blastocoele. Vitrification of collapsed embryos was performed by a EG/s method, which consisted of placing embryos in 1.5 M ethylene glycol for 5 minutes then moving embryos to 7 M ethylene glycol + 0.6 M galactose. Embryos were warmed in a serial dilution manner. After warming, embryos were transferred immediately to recipient mares or were cultured for 6 hours prior to transfer. The pregnancy rate at day 13 was 13% for cultured embryos and 57% for immediately transferred embryos.

In the last experiment, Day 7 embryos ranging from 407 to 565 µm in diameter were used. Blastocoele fluid was removed by positioning the pipette in the periphery of the embryo. Embryos were vitrified and warmed by the EG/s method. After warming,

embryos were shipped for 4-6 hours before transfer. Pregnancy rates at day 13 were 86% and 71% at the heartbeat stage (day 25).

CHAPTER III
EFFECT OF EMBRYO EXPOSURE TO VITRIFICATION SOLUTIONS FOLLOWING ONE- OR TWO-PUNCTURES ON EMBRYO CRYOPROTECTANT PERMEABILITY, IN VITRO RE-EXPANSION AND CAPSULE STABILITY

3.1 Introduction

The viability of equine embryos following capsule puncture and biopsy for pre-implantation genetic diagnosis has been evaluated by Choi et al. (2010). Day 6-8 embryos (morulae, early blastocysts, blastocysts and expanded blastocysts) were subjected to puncture by a piezo drill or by a pointed ICSI micropipette. It was noted that neither capsule hole drilling nor puncture with the ICSI micropipette caused the capsule to tear. Pregnancy rates for Day 6, 7 and 8 embryos were 6/12, 10/12 and 3/4, demonstrating a high viability following capsule puncture. It was also noted that some embryos lost blastocoele fluid and collapsed, but it did not impede their ability to produce pregnancies.

Equine embryos ≥300 μm in diameter have decreased permeability of cryoprotectants compared to embryos < 300 μm (Hochi et al., 1995; Pfaff et al., 1993). The low permeability has been attributed to changes in surface to volume ratio, changes in expression of the membrane protein aquaporin (Budik et al., 2008; Gillard Kingma et al., 2011), and primarily the development of the embryonic capsule. An inverse relationship between permeability and capsule thickness has been observed (Legrand et al., 2002) and low permeability for ethylene glycol and glycerol has been measured (Gillard Kingma et al., 2011; Scott et al., 2012).

The cryoprotectant concentration of the vitrification solutions used in this study are from a vitrification protocol that has been used for successful vitrification of small equine embryos (Caracciolo di Brienza et al., 2004; Eldridge-Panuska et al., 2005; Hudson et al., 2006). Successful vitrification of ovine and buffalo embryos utilizing this protocol has also been reported (Gasparrini et al., 2001; Naitana et al., 1996). It was hypothesized that because this protocol consists of a 3-step addition of cryoprotectant, it would result in extended extracellular and intracellular embryo dehydration prior to vitrification when compared to one or two-step cryoprotectant addition protocols.

As reviewed by Leibo and Pool (2011), in early studies of embryo and gamete cryopreservation, the permeability of cryoprotectants was determined by the direct measurement of radioactive glycerol uptake. Direct permeability coefficients of radioactive glycerol uptake were compared to indirect permeability coefficients determined by the volume change of embryos or oocytes when exposed to cryoprotectants. The similar results obtained by the two techniques demonstrated the validity of the indirect volumetric method. Since then, the indirect volumetric method has usually been utilized to determine permeability coefficients due to its simplicity and low cost.

As noted by Bruyas (2011) and Stout (2012), *in vitro* approaches have been utilized in equine embryo cryopreservation research in an effort to obtain more precise and quantifiable information about the embryo damage during exposure to cryoprotectants and freezing procedures. Common *in vitro* approaches that have been utilized are embryo *in vitro* culture for re-expansion evaluation, embryo fixation for

subcellular level analysis, embryo metabolism evaluation and use of fluorescent stains to determine live/dead and apoptosis-induced cells. The objective of this experiment was to compare indirect cryoprotectant permeability, *in vitro* re-expansion, and capsule stability of equine expanded blastocysts exposed to vitrification solutions following one- or two-punctures. The mean diameter target of the study was embryos >700 µm recovered on day 8 post-ovulation.

3.2 Materials and Methods

3.2.1 Animals

All experiments were conducted at the Reproductive Biology Center, Saint Gabriel, Louisiana. Fifteen quarter horse mares were used as embryo donors and three stallions of known fertility were used as sperm donors. Mare age ranged between 5 to 16 years, and stallions were 4 to 12 years. Mare body condition score ranged from 5-7 (9 point scale). All mares were housed in Bermuda grass lots with *ad libitum* access to water and were supplemented with Bermuda grass or Ryegrass hay when necessary.

3.2.2 Estrus detection and trans-rectal ultrasonography

Detection of estrus was performed using a stallion and was followed by ovarian ultrasonography. Briefly, mares were loaded into a teasing chute and teased with a stallion. All mares exhibiting estrus were subjected to uterine and ovarian ultrasonography using a 5 MHZ linear probe (Micromaxx, Sonosite Inc., Bothell, WA). Mares exhibiting a follicle ≥ 34 mm, uterine edema and no active corpus luteum were inseminated every other day until ovulation was detected (Day 0). A dose of 2000 I.U.

human chorionic gonadotropin was utilized (i.v., Chorulon ®, Intervet Inc., Milsboro, DE, USA) when multiple embryo collection in a short time period was intended. Mares not exhibiting signs of estrus were subjected to trans-rectal ultrasonography three times per week to determine follicular growth or corpus luteum presence.

3.2.3 Semen collection and dilution

One or two stallions, depending on the number of mares to be inseminated, were collected on the day of insemination. A Colorado model artificial vagina (Animal Reproduction Systems, Chino, CA) with a disposable 22” plastic liner (Animal Reproduction Systems, Chino, CA) coupled to a semen container unit was used for semen collection. This unit was assembled prior to collection with a 118 mL sample Whirlpak bag inserted into the plastic semen container with a disposable nylon gel filter (Animal Reproduction Systems, Chino, CA). A commercial milk-based semen extender (EZ-Mixin Semen Extender, Animal Reproduction Systems, Chino, CA) was pre-warmed to 37°C for subsequent semen dilution. Prior to collection, the artificial vagina was lubricated with non-spermicidal lubricant jelly (Reproduction Provisions, LLC., Walworth, WI, USA). The stallion’s penis was evaluated for cleanliness, and when necessary the penis was rinsed abundantly with warm water. Two technicians were utilized for semen collection, one guiding the stallion and the other handling the artificial vagina. Semen was diluted with the semen extender 1:1 or 2:1 for insemination either immediately or within 2 hours, respectively. Sperm concentration was determined using an improved Neubauer hematocytometer (Hausser Scientific, Horsham, PA, USA).

3.2.4 Insemination

A standard insemination protocol was performed using a sterile equine insemination kit (Reproduction Resources, Walworth, WI, USA) containing a 50-ml syringe, glove, non-spermicidal lubricant jelly and a standard equine insemination pipette. Insemination doses contained at least 500 x 10^6 progressively motile spermatozoa. The insemination procedure consisted of a slow insertion of the sterile glove-covered hand guarding the insemination pipette through the vulva until the external os of the cervix was located with the index finger. The insemination pipette was slowly introduced through the cervix, approximately 1-2” into the uterine body, and semen was gently deposited. The pipette was removed and perineal area rinsed with water to remove any residual lube. Inseminated mares were examined daily by transrectal ultrasonography to detect ovulation.

3.2.5 Embryo recovery and holding

On Day 8 after ovulation, embryos were recovered non-surgically as described by Scott (2011). Three liters of pre-warmed (37°C) lactated ringer’s (Hospira, Lake Forest, IL, USA) supplemented with 1% bovine calf serum (Hy Clone Inc., Logan, UT) was utilized for uterine lavage. A 32 french foley catheter (Agtech Inc., Manhatten, KS) was introduced through the os of the cervix until the uterine body was reached. Once the tip of the catheter was introduced approximately 2-3 inches into the uterus, 40 to 50 ml of air was inserted into the air cuff and gently pulled to create a seal with the internal os of the cervix. Once the foley catheter was positioned, medium was infused into the uterus and extracted by gravity through a sterile y-tubing (Agtech Inc., Manhatten, KS).

Fluid recovered from the uterus passed through a large-volume filter (Miniflush Filter, Minitube, Verona, WI, USA) where embryos were retained. Typically 95% of the medium was recovered. Filter contents were searched using a stereoscopic microscope, and embryos collected were identified and moved to 35x10 mm petri dishes containing 150-200 µl drops of holding medium **(**Syngro holding medium, Bioniche Animal Health, Ontario, Canada). Embryos were rinsed four to six times in holding medium. Morphological scores from 1 to 4 were assigned to each embryo as described by Mckinnon and Squires (1988), 1 being good quality and 4 being degenerate. Embryos were held in holding medium at 37ºC approximately 15-20 minutes until treatments were applied.

3.2.6 Preparation of vitrification solutions

Solutions from the equine embryo vitrification protocol described by Eldridge-Panuska et al. (2005) were utilized in this experiment. Dulbeccos's phosphate buffered saline without calcium chloride and magnesium chloride (D-PBS) (Sigma-Aldrich, St. Louis, MO) was used as the base medium for the vitrification solutions. D-PBS was supplemented with 0.3 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 3.3 mM glucose (Sigma-Aldrich, St. Louis, MO) and 20% fetal bovine serum (v/v) (Hy Clone Inc., Logan, UT). The vitrification protocol utilized was composed of three vitrification solutions, each containing different concentrations of glycerol and ethylene glycol (Sigma-Aldrich, St. Louis, MO). Vitrification solution 1 (VS1) was composed of 1.4 M glycerol, vitrification solution 2 (VS2) was composed of 1.4 M glycerol + 3.6 M ethylene glycol and vitrification solution 3 (VS3) was composed of 3.4 M glycerol + 4.6 M

ethylene glycol. The dilution solution (DS) used for warming of embryos contained 0.5 M galactose (Sigma-Aldrich, St. Louis, MO). All solutions were prepared and stored at -20 ºC until use. Solutions were thawed the same day to perform embryo vitrification.

3.2.7 Blastocyst micromanipulation and exposure to vitrification solutions

The micromanipulation of embryos was performed using a micromanipulation tool, the Dracula pipette (Genesearch Inc., Bozeman, MT, USA). This pipette is a coaxial microinjection system that consists of a plastic holding pipette (160-300 µm i.d.) and a borosilicate injection pipette (11-17 µm o.d.) introduced from within the lumen of the holding pipette. This allows the aspiration of the fluid from the blastocoele cavity and introduction of cryoprotectant with the same pipette (Taylor et al., 2006). Micromanipulation was performed using an inverted microscope (Diaphot, Nikon Inc., Melville, NY, USA) at a 4X amplification. All micromanipulation procedures were performed at room temperature.

Vitrification solutions were thawed and maintained at room temperature before use. Following Dracula pipette assembly, the embryo was transferred to a 500-700 µL drop of VS1. While in VS1, the embryo was held by the holding pipette through negative pressure. In the one-puncture treatment, the injection pipette was slowly inserted through the embryonic capsule until the trophectoderm was penetrated. In the two-puncture treatment, injection pipette was inserted and passed completely through the embryo, to produce a double puncture from a single injection pipette insertion (Figure 3.1). The injection pipette was retracted proximal to the trophectoderm side adjacent to the holding pipette. Blastocoele fluid was slowly aspirated to reach a complete (95-99%)

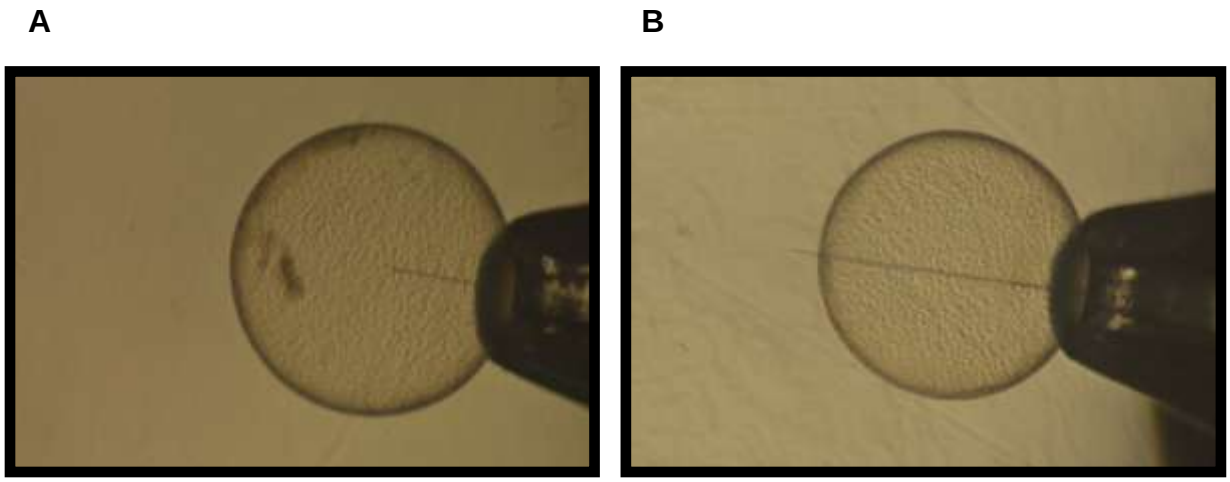

Figure 3.1: One-puncture treatment (A) and two-puncture treatment (B).

fluid reduction, and then the injection pipette was slowly removed from embryo. The entire procedure of capsule puncture and blastocoele fluid removal was completed within 3-4 minutes. The procedure of capsule puncture and blastocoele-fluid extraction is shown in Figure 3.2.

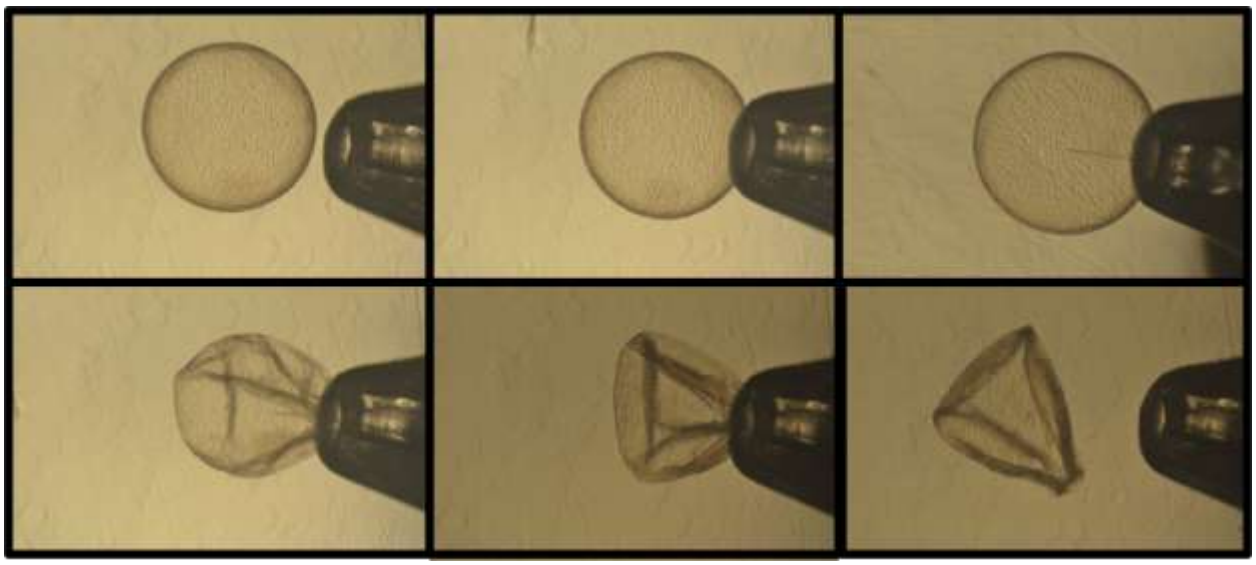

Figure 3.2: Capsule puncture and blastocoele-fluid extraction procedure.

Immediately after micromanipulation and exposure to VS1 for 5 min, embryos were sequentially exposed to VS2 for 5 min, VS3 for 45 sec, DS for 5 min and finally placed in previously equilibrated culture media.

3.2.8 Measurement of embryo diameter

To determine embryo diameter change within each solution, a digital image was captured using an EVOS microscopy system (Advance Microscopy Group, Mill Creek, WA, USA) during the final 5 seconds of exposure to a solution. The EVOS was previously calibrated with a precision ronchi ruling glass slide (Edmund optics Inc., Barrington, NJ, USA). Embryos subjected to capsule puncture and blastocoele fluid removal did not maintain a spherical shape, so two approaches were used to determine embryo diameter. The diameter of embryos with spherical shape was determined by the mean of two perpendicular measurements. To determine the diameter of embryos with an irregular shape, the mean of four perpendicular measurements was used. Embryo measurements were recorded during equilibration in VS1, VS2, VS3, DS, culture medium after 3 min of exposure and at 1, 24, 48 and 72 hours of culture. The mean embryo diameters were transformed to volume by the formula $4/3\ \pi r^3$.

3.2.9 Embryo in vitro culture

Following exposure to vitrification solutions, embryos were cultured *in vitro* for 72 hours as described by Choi et al. (2011). Embryos were cultured on Dulbecco's Modified Eagle Medium / Ham's F-12 Nutrient mixture (DMEM/F-12) supplemented with 10% fetal bovine serum and 1% of penicillin/streptomycin mixture (5000 µg/mL

penicillin and 5000 µg/mL streptomycin) (Life Technologies, Grand Island, NY, USA) in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2 at 38.2°C. Three milliliters of culture medium were placed in 35x100 petri dishes (Becton Dickinson, Franklin Lakes, NJ, USA) and covered by two mililiters of mineral oil (Sigma-Aldrich, St. Louis, MO). The medium was allowed to equilibrate for use the following day. Embryos were transferred to fresh media at 48 hours of culture. Embryo re-expansion/collapse was evaluated every 24 hours. Capsule loss was defined as partial or total loss of the embryonic capsule.

3.2.10 Experimental design

Embryos were subjected to either a one-puncture or two-puncture treatment and exposed to vitrification solutions of a standard equine embryo vitrification protocol and evaluated for re-expansion and capsule loss both without vitrification. Twenty-four Day 8 equine expanded blastocysts (Grade 1 and 2) were used in the experiment. Embryos were stratified by diameter across treatments, one-puncture (n=12) or two-punctures (n=12). Embryo volume was determined in each solution of the vitrification protocol and in culture media. Embryo re-expansion and capsule loss was assessed at 24, 48 and 72 hours of *in vitro* culture.

3.2.11 Statistical Analysis

Results of the experiment were assessed for normality using the Shapiro Wilk test. Differences between treatments in embryo volume during exposure to cryoprotectant solutions and during *in vitro* culture were determined using student

Wilcoxon-Mann-Whitney test. Re-expansion rate and capsule loss at 24, 48 and 72 hours were analyzed using a Chi-square test of independence. Level of significance was set at $P<0.05$. The Statistical Analysis System (SAS 9.3) software was used for all statistical analyses.

3.3 Results

Embryo volumes during exposure to cryoprotectants are presented in Table 3.1 and Figure 3.3. Mean embryo pre-treatment volume for one-puncture group and for two-puncture group was not different ($P=0.44$). Following exposure to VS1 for 5 minutes, mean volume for one-puncture and for two-puncture embryos was not different ($P=0.31$). An effective mean reduction of 67% of their initial embryo volume was obtained within 5 minutes of the micromanipulation procedure and exposure to VS1 for both treatments.

Following embryo exposure to VS1, embryos were exposed to VS2 for 5 minutes. Mean embryo volume for one-puncture and two-puncture embryos was not different ($P=0.34$). Mean volume for embryos exposed for 45 seconds to VS3 for one-puncture embryos and for two-puncture embryos was not different ($P=0.32$).

This experiment consisted in the exposure of embryos to the vitrification solutions, but the vitrification per se was not performed. Following exposure to VS3 embryos were exposed to DS for 5 min. Mean embryo volume for one-puncture embryos and for two-puncture embryos was not different ($P=0.41$).

Table 3.1: Effect of exposure to vitrification solutions on embryo volume following one or two punctures.

	Initial	Vitrification solution 1	Vitrification solution 2	Vitrification solution 3	Dilution solution	Culture medium
1 puncture						
Volume (mm^3)	0.686 ± 0.26[a]	0.227 ± 0.09[a]	0.184 ± 0.07[a]	0.178 ± 0.08[a]	0.270 ± 0.12[a]	0.363 ± 0.15[a]
Diameter (µm)	948 ± 114					
Rel.vol (%)	100	33.1	26.8	25.9	39.3	52.9
2 punctures						
Volume (mm^3)	0.672 ± 0.15[a]	0.224 ± 0.05[a]	0.171 ± 0.03[a]	0.176 ± 0.05[a]	0.254 ± 0.06[a]	0.319 ± 0.07[a]
Diameter (µm)	1006 ± 90					
Rel.vol (%)	100	33.3	25.4	26.2	37.8	47.5
P-value	0.44	0.31	0.34	0.32	0.41	0.44

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

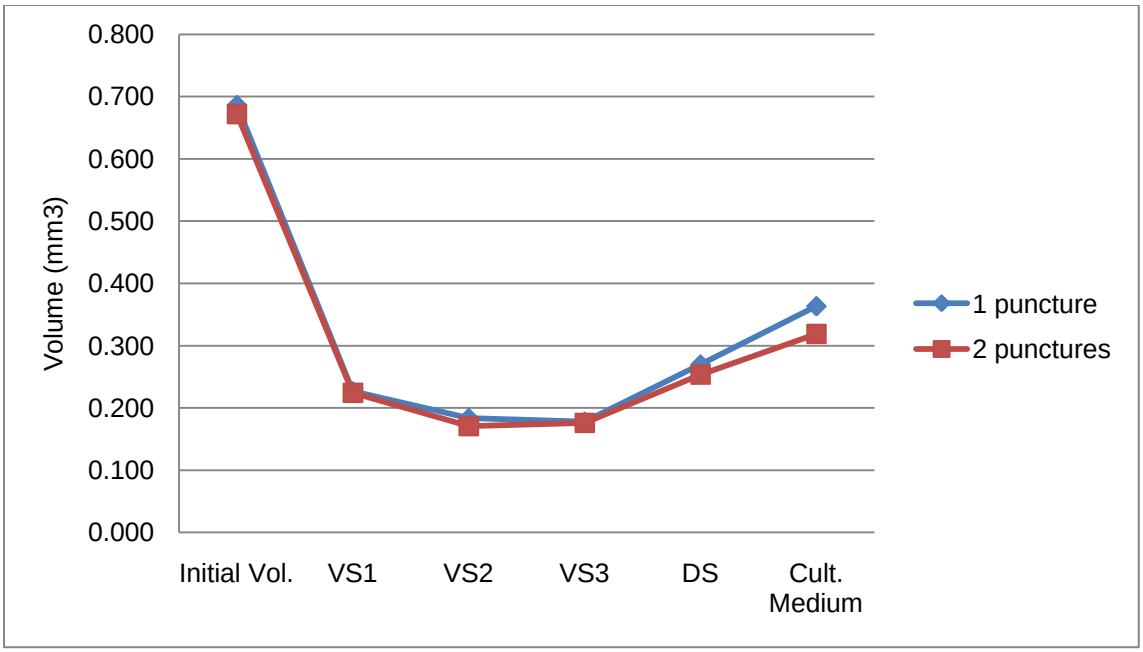

Figure 3.3: Embryo volume change following one or two punctures during embryo exposure to vitrification solutions.

Following exposure to DS, embryos were moved to equilibrated culture medium. Mean embryo volume for one-puncture and two-puncture embryos was not different (P=0.44).

Embryo volumes during *in vitro* culture are presented in Table 3.2. Mean volume following one hour of culture for one-puncture embryos and for two-puncture embryos was not different (P=0.47). Mean volume for one-puncture embryos and for two-puncture embryos was not different following 24 hours (P=0.75), 48 hours (P=0.41) and 72 hours (P=0.41) of *in vitro* culture. Embryo re-expansion and growth patterns during *in vitro* culture are shown in Figure 3.4.

Embryo re-expansion rates and capsule loss during 72 hours of embryo *in vitro* culture are presented in Table 3.3.

Table 3.2: In vitro embryo re-expansion and growth following one or two punctures and exposure to vitrification solutions.

	In vitro culture (h)			
	1	**24**	**48**	**72**
1 puncture				
Volume (mm^3)	0.309 ± 0.13[a]	0.957 ± 0.46[a]	1.546 ± 0.75[a]	2.243 ± 1.32[a]
Rel.vol (%)	45.0	140.5	225.9	333.8
2 punctures				
Volume (mm^3)	0.289 ± 0.06[a]	0.851 ± 0.33[a]	0.816 ± 0.36[a]	0.854 ± 0.44[a]
Rel.vol (%)	43.0	126.6	121.4	127.1
P-value	0.47	0.75	0.41	0.41

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

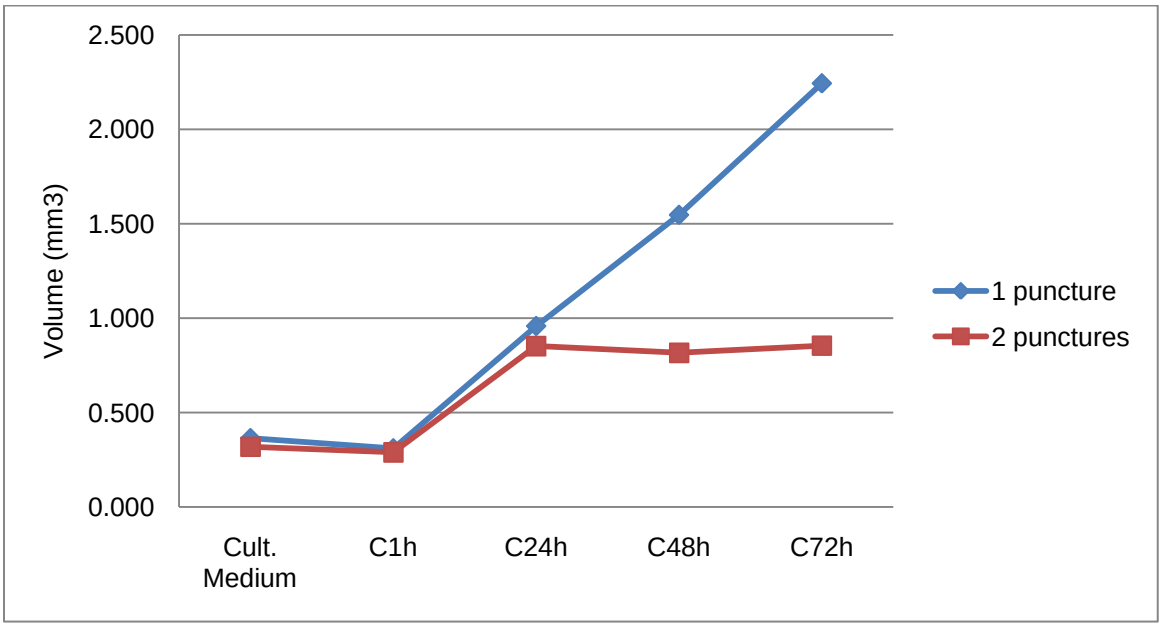

Figure 3.4: Embryo volume during *in vitro* culture following one or two punctures and embryo exposure to vitrification solutions.

Table 3.3: *In vitro* re-expansion rates and capsule stability following one or two punctures and embryo exposure to vitrification solutions.

	In vitro Culture (h)			
	24	**48**	**72**	**Capsule loss**
1 puncture	100 [a] (12/12)	83.3 [a] (10/12)	75 [a] (9/12)	25 [a] (3/12)
2 punctures	83.3 [a] (10/12)	66.6 [a] (8/12)	50 [a] (6/12)	50 [a] (6/12)
P-value	0.14	0.34	0.21	0.21
Power	0.27	0.15	0.24	0.24

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

Re-expansion rate for one-puncture embryos and for two-puncture embryos was not different during *in vitro* culture at 24 hours (P=0.14), 48 hours (P=0.34) and 72 hours (P=0.21). Similarly, capsule loss for one-puncture embryos and for two-puncture embryos was not different (P=0.21). Differences were probably not detected for re-expansion rate and capsule loss due to the relatively low embryo number utilized in the experiment, resulting in power values ranging from 0.15 to 0.27.

3.4 Discussion

In this experiment, the difference between one and two-punctures was evaluated on indirect embryo cryoprotectant permeability, re-expansion rates and capsule stability following embryo exposure to standard equine embryo vitrification solutions.

Hochi et al. (1995) exposed embryos with diameters <200 µm (mean 172.6 µm), 200-300 µm (mean 248.5 µm) and >300 µm (mean 515.4 µm) to 20% ethylene glycol in m-PBS at 25ºC for 20 min and to a vitrification solution containing 40% ethylene glycol (v/v), 18% Ficol-70 (w/v) and 0.3 M sucrose for 1 min prior to vitrification. Embryos >300 µm exhibited a 55% reduction in volume following 20 min of exposure. In contrast, embryos <200 µm and 200-300 µm reached a reduction in volume of 45% and 52% within 0.5 and 1 min, respectively.

As part of a study by Young et al. (1997), the diameter change of embryos exposed to 4.5 M ethylene glycol (~25% ethylene glycol solution) at 20-21 ºC for 15 min was evaluated. Embryos with a mean initial diameter of 409 ± 24 µm (0.0358 mm^3) exhibited a diameter of 345 ± 15 µm (0.0215 mm^3), 363 ± 30 µm (0.0250 mm^3) and 383 ± 29 µm (0.0294 mm^3) following 5, 10 and 15 min, respectively. Embryo volume was reduced to 60% of the initial embryo volume during the first 5 min and then increased to 69.8% and 82.1% of the initial embryo volume.

Contrasting results were obtained by Hochi et al. (1995) and Young et al. (1997), even though similar concentrations of ethylene glycol (20–25%) were used and embryos were exposed to solutions at similar temperatures (20-25ºC). While Hochi et al. (1995) reported a volume loss of 55% of the initial volume following 20 min of exposure to cryoprotectant solution, Young et al. (1997) reported a volume reduction of 40% following 5 min of exposure to cryoprotectants. In both reports embryos did not exhibit a major and rapid volume decrease within the first 5 min of exposure.

In our study, a reduction of 67% of the embryo volume occurred within 5 min of embryo puncture, blastocoele fluid extraction, and exposure to VS1. Rapid embryo volume reduction is beneficial due to the fact that cryoprotectant can permeate faster into embryonic cells with reduced surface area/volume ratio, and this allows for a reduced time of exposure to cryoprotectant solutions (Kader et al., 2009; Kasai, 2002). The larger the fluid-filled blastocoele, the longer the period required to allow cryoprotectant concentrations to reach required levels (Kasai, 2002). It has been shown that prolonged exposure to cryoprotectants is detrimental, and one of the major obstacles is the embryo toxicity due to the high concentrations used in vitrification protocols (Kasai, 1996).

For the one-puncture treatment mean embryo volume, as a percentage of the initial embryo volume, was reduced from 33.1% in VS1 to 25.9% in VS3. Similarly, in the two-puncture treatment, mean embryo volume was reduced from 33.3% in VS1 to 26.2% in VS3. This agrees with the observations that embryos exposed to cryoprotectant solutions lose water in response to the osmotic pressure of the extracellular cryoprotectant, resulting in a decrease in volume (Kasai, 2002; Pfaff et al., 1993). Substantial dehydration is obtained when embryos are exposed to a final concentration in the order of 30% - 50% cryoprotectant (Leibo and Pool, 2011).

In our study, mean embryo volume for both one- and two-puncture treatments increased when transferred from VS3 to DS. Similarly, mean embryo volume also increased when embryos were transferred from DS to culture medium. The increasemay be caused by water moving into the embryo. Even though dilution solution

contains 0.5 M galactose, water still moved into the embryo resulting in volume increase. Another explanation may be that the embryos were exposed to DS solution at room temperature and not at 37ºC, which is the common warming temperature in a vitrification procedure. Because we did not have a proper control to the latter, we are unsure of the exact cause of these effects.

The inclusion of non-permeating osmotic agents such as sucrose, galactose, fructose and glucose into the solution is a strategy that has been reported to prevent excess water inflow into the embryo, preventing osmotic swelling (Kasai, 2002). The non-permeating agents help to rapidly reduce the intracellular concentration of cryoprotectant, therefore reducing their toxic effects on embryonic cells (Mukaida and Oka, 2012). In our study, embryo volume increased when embryos were transferred from DS to culture medium. This effect is considered normal given that the culture medium utilized (DMEM/F-12) does not contain a saccharide hypertonic concentration, allowing the embryo to slowly recover fluid.

In our study, the mean embryo volume for one- and two-puncture treatments was not different during exposure to VS1, VS2, VS3, DS and culture media. These results show no additional benefit of two-punctures compared to one-puncture to increase cryoprotectant permeability in equine expanded blastocysts.

In our study, the mean embryo volume for one- and two-puncture treatments was not different during *in vitro* embryo re-expansion. Due the relatively low number of embryos per group (n=12), low power for statistical analysis was obtained in this study. It is hypothesized that a greater number of embryos in the experiment having similar

patterns of re-expansion rates and capsule loss would result in differences because of the 25% divergence between one puncture and two-puncture treatments at 72 hours culture. Meanwhile, results from this experiment showed that there is no additional benefit in terms of cryoprotectant permeability obtained from performing the two-puncture treatment. Additionally, the two-puncture treatment may result in an increased risk of capsule loss. Stout et al. (2005) reported that embryos subjected to capsule removal resulted in no pregnancies following embryo transfer, showing the importance of the embryonic capsule for successful establishment of pregnancy.

CHAPTER IV
EFFECT OF EMBRYO VITRIFICATION FOLLOWING INDIRECT OR DIRECT INTRODUCTION OF CRYOPROTECTANT ON IN VITRO EMBRYO RE-EXPANSION AND CAPSULE STABILITY

4.1 Introduction

Cryopreservation of equine embryos ≥ 300 µm have been difficult and usually result in pregnancy rates in the ranges of 0% to 38%, which are considered low for commercial application of the technique (Barfield et al., 2009; Bass et al., 2004; Eldridge-Panuska et al., 2005; Scherzer et al., 2011; Slade et al., 1985; Young et al., 1997). The difficulty to cryopreserve these embryos has been attributed to multiple factors: increase of blastocoele volume, increase in size and cell number, intense mitotic activity, inner cell mass formation, change in expression of membrane protein aquaporin, and development of the acellular glycoprotein capsule (Betteridge, 2007; Bruyas et al., 1993; Budik et al., 2008; Choi et al., 2011b; Gillard Kingma et al., 2011; Legrand et al., 2002; Stout, 2012).

The factors that most likely contribute to the failure of equine expanded blastocyst cryopreservation are the volume increase of the fluid-filled blastocoele and the development of the embryonic capsule. The capsule has been considered a barrier to cryoprotectant penetration because an inverse relationship between permeability and capsule thickness has been observed (Legrand et al., 2002). Equine embryos ≥300 µm in diameter have decreased permeability to cryoprotectants when compared to embryos <300 µm. This decreased cryoprotectant permeability temporally coincides with the

development of the acellular glycoprotein capsule (Hochi et al., 1995; Pfaff et al., 1993). Low permeability for both glycerol and ethylene glycol has been measured (Gillard Kingma et al., 2011; Scott et al., 2012).

In most species, one of the characteristics of the transition from the morula to the blastocyst stage is the development of the blastocoele, which is a fluid-filled cavity (Vanderzwalmen et al., 2002). At the morula stage most of the water content is intracellular, while at the blastocyst stage most of the water content is within the blastocoele cavity (Vanderzwalmen et al., 2002). In human embryo cryopreservation, the enlargement of the fluid-filled blastocoele has been correlated with reduced survival and pregnancy rates after vitrification (Cho et al., 2002; Mukaida et al., 2003; Son et al., 2003). This phenomenon has also been observed in mouse embryos (Chen et al., 2005; Vanderzwalmen et al., 1988; Zhu et al., 1993) and bovine embryos (Tachikawa et al., 1993; Van Der Zwalmen et al., 1989; Vanderzwalmen et al., 2002).

It has been suggested that the reduction in survival rates of expanded blastocysts is related to incomplete dehydration and insufficient cryoprotectant permeation into the blastocyst, resulting in ice crystal formation during the vitrification process (Cho et al., 2002; Mukaida et al., 2006; Son et al., 2003). In order to overcome this problem in human blastocyst vitrification, the removal of the blastocoele fluid has been tested and has resulted in high survival rates in the range of 87 to 100% (Hiraoka et al., 2004; Iwayama et al., 2011; Kader et al., 2009; Mukaida and Oka, 2012; Mukaida et al., 2006; Vanderzwalmen et al., 2002). Artificial collapse of human embryos is

usually performed with a glass microneedle (Vanderzwalmen et al., 2002), a 29-gauge needle (Son et al., 2003) by micropipetting (Hiraoka et al., 2004) or with a laser pulse (Mukaida et al., 2006).

Human blastocysts exhibit decreased permeability for water and cryoprotectants when compared to mouse and bovine blastocysts (Mukaida et al., 2006). In this way, human blastocysts can be considered similar to equine blastocysts in terms of decreased cryoprotectant permeability.

Puncture of equine oocytes and embryos has been proven safe and has allowed the application of multiple assisted reproductive techniques like ICSI, embryonic and SCNT, and pre-implantation genetic diagnosis (PGD) (Choi et al., 2010; Galli et al., 2003; Squires et al., 1996). Puncture of the embryonic capsule and extraction of blastocoele fluid has allowed the successful vitrification of equine expanded blastocysts with diameters between 300 µm to 650 µm (Choi et al., 2011b). The objective of this experiment was to evaluate the effect of vitrification of equine expanded blastocysts following the indirect or direct introduction of cryoprotectants on *in vitro* re-expansion and capsule stability. The mean diameter target of the study was embryos >700 µm recovered on day 8 post-ovulation. Results from experiment 1 showed that there is no additional benefit of 2 punctures in the embryo capsule for cryoprotectant permeability, and 2 punctures may result in an increased risk of capsule loss. The indirect or direct introduction of cryoprotectants was performed based on the one puncture technique.

4.2 Materials and Methods

4.2.1 Animals

All experiments were conducted at the Reproductive Biology Center, Saint Gabriel, Louisiana. Fifteen quarter horse mares were used as embryo donors and three stallions of known fertility were used as sperm donors. Mare ages ranged between 5 to 16 years and stallions ranged between 4 to 12 years. Mare body condition score ranged from 5-7 (9 point scale). All mares were housed in Bermuda grass lots with ad libitum access to water, and were supplemented with Bermuda grass or Ryegrass hay when necessary.

4.2.2 Estrus detection and trans-rectal ultrasonography

Detection of estrus was performed using a stallion and was followed by ovarian ultrasonography. Mares were briefly loaded into a teasing chute and teased with a stallion. All mares exhibiting estrus were subjected to uterine and ovarian ultrasonography using a 5 MHZ linear probe (Micromaxx, Sonosite Inc., Bothell, WA). Mares exhibiting a follicle ≥ 34 mm, uterine edema, and no active corpus luteum were inseminated every other day until ovulation was detected (Day 0). A dose of 2000 I.U. human chorionic gonadotropin was utilized (i.v., Chorulon ®, Intervet Inc., Milsboro, DE, USA) when multiple embryo collection in a short time period was intended. Mares not exhibiting signs of estrus were subjected to trans-rectal ultrasonography three times per week to determine follicular growth or corpus luteum presence.

4.2.3 Semen collection and dilution

One or two stallions, depending on the number of mares to be inseminated, were collected on the day of insemination. A Colorado model artificial vagina (Animal Reproduction Systems, Chino, CA) with a disposable 22” plastic liner (Animal Reproduction Systems, Chino, CA) coupled to the semen container unit was used for semen collection. This unit was assembled prior to collection with a 118 mL sample Whirlpak bag inserted into the plastic semen container with a disposable nylon gel filter (Animal Reproduction Systems, Chino, CA). A commercial milk-based semen extender (EZ-Mixin Semen Extender, Animal Reproduction Systems, Chino, CA) was pre-warmed to 37°C for subsequent semen dilution. Prior to collection, the artificial vagina was lubricated with non-spermicidal lubricant jelly (Reproduction Provisions, LLC., Walworth, WI, USA). The stallion’s penis was evaluated for cleanliness, and when necessary the penis was rinsed abundantly with warm water. Two technicians were utilized for semen collection, one guiding the stallion and the other handling the artificial vagina. Semen was diluted with the semen extender 1:1 or 2:1 for insemination either immediately or within 2 hours, respectively. Sperm concentration was determined using an improved Neubauer hematocytometer (Hausser Scientific, Horsham, PA, USA).

4.2.4 Insemination

A standard insemination protocol was performed using a sterile equine insemination kit (Reproduction Resources, Walworth, WI, USA) containing a 50-ml syringe, glove, non-spermicidal lubricant jelly and a standard equine insemination pipette. Insemination doses contained at least 500 x 10^6 progressively motile

spermatozoa. The insemination procedure consisted of a slow insertion through the vulva of the sterile-glove covered hand guarding the insemination pipette until the external os of the cervix was located with the index finger. The insemination pipette was slowly introduced through the cervix approximately 1-2” into the uterine body and semen was gently deposited. The pipette was removed and perineal area rinsed with water to remove any residual lube. Inseminated mares were examined daily by transrectal ultrasonography to detect ovulation.

4.2.5 Embryo recovery and holding

On day 8 after ovulation, embryos were recovered non-surgically as described by Scott (2011). Three liters of pre-warmed (37°C) lactated ringer’s (Hospira, Lake Forest, IL, USA) supplemented with 1% bovine calf serum (Hy Clone Inc., Logan, UT) was utilized for uterine lavage. A 32 french foley catheter (Agtech Inc., Manhatten, KS) was introduced through the os of the cervix until the uterine body was reached. Once the tip of the catheter was introduced approximately 2-3 inches into the uterus, 40 to 50 mL of air was inserted into the air cuff and gently pulled to create a seal with the internal os of the cervix. Once the foley catheter was positioned, medium was infused into the uterus and extracted by gravity through a sterile y-tubing (Agtech Inc., Manhatten, KS). Fluid recovered from the uterus passed through a large-volume filter (Miniflush Filter, Minitube, Verona, WI, USA) where embryos were retained. Typically 95% of the medium was recovered. Filter contents were searched using a stereoscopic microscope, and embryos collected were identified and moved to 35x10 mm petri dishes containing 150-200 µL drops of holding medium **(**Syngro holding medium,

Bioniche Animal Health, Ontario, Canada). Embryos were rinsed four to six times in holding medium. Morphological scores from 1 to 4 were assigned to each embryo as described by Mckinnon and Squires (1988), 1 being good quality and 4 being degenerate. Embryos were held in holding medium at room temperature approximately 15-20 minutes until treatments were applied.

4.2.6 Preparation of vitrification solutions

Solutions from the equine embryo vitrification protocol described by Eldridge-Panuska et al. (2005) were utilized in this experiment. Dulbecco's phosphate buffered saline without calcium chloride and magnesium chloride (D-PBS) (Sigma-Aldrich, St. Louis, MO) was used as the base medium for the vitrification solutions. D-PBS was supplemented with 0.3 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 3.3 mM glucose (Sigma-Aldrich, St. Louis, MO) and 20% fetal bovine serum (v/v) (Hy Clone Inc., Logan, UT). The vitrification protocol utilized was composed of three vitrification solutions, each containing different concentrations of glycerol and ethylene glycol (Sigma-Aldrich, St. Louis, MO). Vitrification solution 1 (VS1) was composed of 1.4 M glycerol, vitrification solution 2 (VS2) was composed of 1.4 M glycerol + 3.6 M ethylene glycol and vitrification solution 3 (VS3) was composed of 3.4 M glycerol + 4.6 M ethylene glycol. The dilution solution (DS) used for warming of embryos contained 0.5 M galactose (Sigma-Aldrich, St. Louis, MO). All solutions were prepared and stored at -20 ºC until use. Solutions were thawed the same day to perform embryo vitrification.

4.2.7 Blastocyst micromanipulation and exposure to vitrification solutions

The micromanipulation of embryos was performed using a micromanipulation tool, the Dracula pipette (Genesearch Inc., Bozeman, MT, USA). This pipette is a coaxial microinjection system that consists of a plastic holding pipette (160-300 µm i.d.) and a borosilicate injection pipette (11-17 µm o.d.) introduced from within the lumen of the holding pipette. This allows the aspiration of the fluid from the blastocoele cavity and introduction of cryoprotectant with the same pipette (Taylor et al., 2006). Micromanipulation was performed using an inverted microscope (Diaphot, Nikon Inc., Melville, NY, USA) at a 4X amplification. All micromanipulation procedures were performed at room temperature.

Vitrification solutions were thawed and maintained at room temperature before use. Following Dracula pipette assembly, the embryo was transferred to a 500-700 µL drop of VS1. While in VS1, the embryo was held by the holding pipette through negative pressure. For the direct introduction treatment, an injection pipette previously loaded with VS1 was used. Once inserted in the blastocoele, the VS1 solution was slowly injected until embryo overexpansion of approximately 10% occurred. With the injection pipette still inserted, the embryo was allowed to equilibrate for 2 min and then 95-99% of the blastocoele-fluid contents were aspirated using the injection pipette. For the indirect introduction treatment, the injection pipette was inserted into the blastocoele cavity, the contents were aspirated, and then the injection pipette was slowly removed. All micromanipulation was performed within 4 minutes, resulting in a total equilibration time in VS1 of 5 minutes.

4.2.8 Embryo vitrification and warming

Embryo vitrification was performed as described by Eldridge-Panuska et al. (2005) with modification. Embryo exposure time to vitrification solutions was VS1 for 5 min, VS2 for 5 min and VS3 for 45 sec followed by plunging into liquid nitrogen. The procedure was performed as follows: subsequent to micromanipulation, the embryo was held 5 min in VS1. The embryo was then transferred to VS2 with as little volume as possible of VS1 and held for 5 min. To prevent bubbles and minimize the volume of VS1 to contact VS2, new micropipette tips were used and were pre-loaded with 2-3 µL of VS2 prior to loading each embryo in the tip for transfer. Embryos were quickly transferred to VS3, and while in this solution the embryo was rapidly transferred to an open system vitrification device (Cryolock, Biotech Inc., Cumming, GA, USA) with the minimum volume possible. Excessive solution surrounding embryo was aspirated until a volume <1 uL was obtained. Within 45 sec, the Cryolock containing the embryo was plunged into liquid nitrogen and capped. Embryos vitrified for embryo transfer were held in LN tanks between 1 week to 1 month, depending on recipient availability.

For embryo warming, 3 mL of DS was pre-warmed in a 35x10 mm petri dish on a heated-stage (37.9ºC). The Cryolock containing the embryo was uncapped under LN to expose the embryo. While holding the base of the Cryotop, the uncapped tip of the Cryotop was moved as quickly as possible into the pre-warmed DS. The petri dish was removed from the heated-stage and observed under a stereoscopic microscope to confirm the presence of the embryo. The embryo was then maintained for 4 min in DS

until transfer to culture medium for *in vitro* culture or transfer to holding medium for subsequent transfer to a recipient mare.

4.2.9 Measurement of embryo diameter

To determine embryo diameter change within each solution, a digital image was captured using an EVOS microscopy system (Advance Microscopy Group, Mill Creek, WA, USA) during the final 5 seconds of exposure to a solution. The EVOS was previously calibrated with a precision ronchi ruling glass slide (Edmund optics Inc., Barrington, NJ, USA). Embryos subjected to capsule puncture and blastocoele fluid removal did not maintain a spherical shape, so two approaches were used to determine embryo diameter. The diameter of embryos with spherical shape was determined by the mean of two perpendicular measurements. To determine the diameter of embryos with an irregular shape, the mean of four perpendicular measurements was used. Embryo measurements were recorded following exposure to DS for 4 min, culture medium for 3 min and at 1, 24, 48 and 72 hours of culture. The mean embryo diameters were transformed to volume by the formula $4/3\ \pi r^3$.

4.2.10 Embryo in vitro culture

Following exposure to vitrification solutions, embryos were cultured *in vitro* for 72 hours as described by Choi et al. (2011). Embryos were cultured in Dulbecco's Modified Eagle Medium/Ham's F-12 Nutrient mixture (DMEM/F-12) supplemented with 10% fetal bovine serum and 1% of penicillin/streptomycin mixture (5000 µg/ml penicillin and 5000 µg/ml streptomycin) (Life Technologies, Grand Island, NY, USA) in an

atmosphere of 5% CO_2 at 38.2 °C. A volume of 950 µl culture media covered by 300 µl of mineral oil was placed in one well of a 4-well container (Thermo Scientific, Rochester, NY, USA). Medium was allowed to equilibrate for use the following day. Embryos were transferred to fresh media at 48 hours of culture. Embryo re-expansion/collapse was evaluated every 24 hours. Capsule loss was defined as partial or total loss of the embryonic capsule.

4.2.11 Embryo staining

Embryo staining was performed to determine the proportion of live/dead cells. Four embryos were stained. The first and second embryo were subjected to indirect and direct introduction treatment and vitrified. The third embryo was subjected to indirect introduction treatments and exposed for 1.5 min to VS3 to induce osmotic damage prior to vitrification. The fourth embryo was subjected to approximately 50% blastocoele fluid removal and vitrified following the indirect introduction treatment. The embryo staining protocol was performed as described by Oberstein et al. (2001) with modification. The staining solution consisted of 10 µg/mL of Propidium Iodide and 10 µg/mL of Hoescht 33342 (Sigma-Aldrich, St. Louis, MO) in a base medium of Dulbecco's phosphate buffered saline (D-PBS) without calcium chloride and magnesium chloride. Prior to staining, warmed embryos were cultured for one hour to allow the embryos to recover their spherical shape. Following culture, embryos were transferred to the pre-warmed staining solution and incubated for 15 min at 37.9 ºC, then washed through three wells of D-PBS, 10-15 sec in each well. Embryos were mounted on a slide in 8 µL of ProLong Gold antifading reagent (Life Technologies,

Grand Island, NY, USA). Fluorescence was observed under a 4X or 10X magnification (Labophot, Nikon Inc., Melville, NY, USA) using a 100-watt mercury-arc lamp (Nikon Inc., Melville, NY, USA). After excitation at the correct wave length, live cells fluoresced blue and dead cells fluoresced red. Because of the large embryo size, individual cells were not counted but a live/dead cell proportion was estimated for each treatment. All the procedures were performed in a dark room to prevent photobleaching.

4.2.12 Transfer of vitrified equine expanded blastocyst

Vitrified equine expanded blastocyst stage embryos were transferred into recipient mares to determine *in vivo* viability. Six Day 8 equine embryos were vitrified using the indirect cryoprotectant introduction technique. The initial diameter of the embryos ranged from 448 µm to 1168 µm. Recipient mares that did not show any signs of uterine infection and exhibited normal ovarian activity in the two previous cycles were pre-selected. Transfers were performed from September 5 to September 18, 2013.

On the day of transfer, mares were checked for uterine and ovarian soundness. Uterine palpation and ultrasonography was performed as gently as possible to prevent any manipulation-induced uterine prostaglandin production. The parameters considered to approve a mare as a recipient on the day of transfer were the presence of an identifiable corpus luteum, good uterine tone, and no signs of uterine edema. Ten to fiveteen minutes prior to transfer, mares received 250 mg of xylazin (i.v. Anased, Lloyd Laboratories, Shenandonah, IA, USA) and 500 mg of flunixin meglumine (i.v. Funixamine, Pfizer Inc., New York, NY, USA). The perineal and vulvar areas were cleaned as previously described for the embryo recovery procedure.

Vitrified embryos were stored in a liquid nitrogen tank until transfer. Frozen aliquots of dilution solution (DS) were thawed and 3 mL was placed in a 35x10 mm petri dish and pre-warmed. Embryo warming was performed as described earlier. The warmed embryo was removed from DS after 4 min of exposure. The warmed embryos were washed four times in holding medium drops (150 ul) prior to transfer.

Each embryo was loaded into a 0.5 mL sterile plastic straw (Minitube, Venture Ct Verona, WI, USA) as follows: one column of 180 µL of holding medium, an air bubble, an 80 µL column of holding embryo with the embryo, an air bubble, and one column with 180 µL of holding medium. A sterile equine embryo transfer (ET) pipette with a side opening for 0.5 mL straws (Minitube, Venture Ct Verona, WI, USA) was used for embryo transfer. Using scissors previously cleaned with ethanol, the bottom end of the ET pipette containing bag was cut and the straw was placed inside the ET pipette. Just before ET pipette insertion, the vulva and sorrounding area was gently sterilized with ethanol and sterile gauze. Insertion of the ET pipette into the vulva and vagina was performed as described for the artificial insemination procedure, maintaining the tip of the ET pipette within the hand to prevent contamination. Once the os of the cervix was located, the plastic cover of the ET pipette was punctured inside the vagina and the tip of the ET pipette was placed in the cervical opening. Very gently, the ET pipette was inserted into the uterine body to prevent cervical opening or damage to the endometrium. Once the ET pipette was inserted approximately 15 cm into the uterine body, the embryo was gently deposited and the ET pipette slowly removed. The tip of the pipette then was inspected under a stereoscope to confirm that the embryo had been deposited into the uterine body.

Pregnancy status was determined at day 11, 13, 17, 22 and 25 post-ovulation. Progesterone supplementation was utilized in mares presenting uterine edema with grade 1 or higher on a scale 0 to 4 (0 being no uterine edema and 4 being maximal uterine edema). Progesterone supplementation was performed by administration of 22 mg of altrenogest (p.o., Regu-mate, Intervet, Milsboro, DE, USA) daily until day 25. Pregnancies were terminated at day 25 by injecting 10 mg of dinoprost tromethamine (i.m., Lutalyse, Pharmacia & Upjohn Company, Kalamazoo, MI, USA).

4.2.13 Experimental design

Twenty-six Day 8 expanded blastocysts (Grades 1 and 2) were subjected to either capsule puncture, cryoprotectant injection and blastocoele fluid extraction (direct introduction treatment), or capsule puncture and blastocoele fluid extraction (indirect introduction treatment) prior to vitrification. Embryos were stratified by diameter into direct introduction treatment (n=13) or indirect introduction treatment (n=13). Embryo re-expansion and capsule loss was assessed at 24, 48 and 72 hours of *in vitro* culture. Embryo volume was determined in DS, culture media and at every 24 hours of *in vitro* culture. Embryo re-expansion and capsule loss was assessed at 24, 48 and 72 hours of *in vitro* culture.

4.2.14 Statistical Analysis

Results of the experiment were assessed for normality using a Shapiro Wilk test. Differences between treatments for embryo volume during exposure to DS, culture media, and *in vitro* culture were determined using Wilcoxon-Mann-Whitney test. Re-

expansion rate and capsule loss at 24, 48 and 72 hours were analyzed using Chi-square test of independence. Level of significance was set at $P<0.05$. The Statistical Analysis System (SAS 9.3) software was used for all statistical analyses.

4.3 Results

The mean embryo volumes during exposure to DS and culture media are presented in Table 4.1. Pre-treatment mean embryo volume for indirect and direct introduction treatment group was not different ($P=0.69$).

Embryo volume was not different following warming of vitrified embryos and exposure to DS during 4 min ($P=1.0$). Following exposure to equilibrated culture medium for 3 min, mean embryo volume for indirect introduction treatment and for direct introduction treatment was not different ($P=0.69$).

The effect of cryoprotectant introduction method on embryo volume change prior to vitrification and following warming was evaluated (Table 4.2). For the indirect introduction treatment, the initial embryo volume and the embryo volume following vitrification and warming were different ($P=0.29$). For the direct introduction treatment, the initial embryo volume and the embryo volume following vitrification and warming were different ($P=0.005$).

Following warming of vitrified embryos, *in vitro* re-expansion was determined during 72 hours of culture. Embryo volumes during *in vitro* culture are presented in Table 4.3. Mean volume for one-puncture embryos and for two-puncture embryos was

Table 4.1: Effect of vitrification following indirect or direct introduction of cryoprotectant on embryo volume.

	Initial	Dilution solution	Culture medium
Indirect introduction			
Volume (mm^3)	0.564 ± 0.13[a]	0.188 ± 0.04[a]	0.272 ± 0.06[a]
Diameter (µm)	912 ± 101.2		
Rel.volume (%)	100	33.3	48.2
Direct introduction			
Volume (mm^3)	0.628 ± 0.14[a]	0.176 ± 0.04[a]	0.279 ± 0.07[a]
Diameter (µm)	979.6 ± 85.6		
Rel.volume (%)	100	28.0	44.4
P-value	0.69	1.00	0.69

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

Table 4.2: Effect of cryoprotectant introduction method on embryo volume before and after vitrification.

	Mean Volume (mm^3)	
	Indirect	**Direct**
Initial	0.564 ± 0.13[a]	0.628 ± 0.14[a]
DS	0.188 ± 0.04[b]	0.176 ± 0.04[b]
P-value	0.029	0.005

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

Table 4.3: Effect of vitrification following indirect or direct introduction of cryoprotectant on *in vitro* embryo re-expansion and growth.

	In vitro culture			
	1 hour	**24 hours**	**48 hours**	**72 hours**
Volume (mm^3)				
Indirect	0.157±0.04[a]	1.406±0.45[a]	1.456±0.61[a]	2.461±1.04[a]
Rel.volume (%)	27.8	249.3	258.1	440
Direct	0.213±0.05[a]	0.935±0.29[a]	1.332±0.49[a]	2.313±0.83[a]
Rel.volume(%)	33.9	176.7	318.4	735.6
P-value	0.48	0.65	0.77	0.68

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

not different following 1 hour (P=0.48), 24 hours (P=0.65), 48 hours (P=0.77) and 72 hours (P=0.68) of culture. Embryo re-expansion and growth patterns during *in vitro* culture are shown in Figure 4.1.

Embryo re-expansion rates and capsule loss during 72 hours of embryo *in vitro* culture are presented in Table 4.4. Pictures of embryo re-expansion, growth and capsule loss are presented in Figures 4.2 and 4.3. Re-expansion rates were not different between treatments at 24, 48 and 72 hours of culture (P=1.00). Capsule loss for indirect introduction treatment embryos and for direct introduction treatment embryos was different (P=0.049).

Embryo staining results are presented in Table 4.5 and pictures are presented in Figure 4.4. Embryo staining of vitrified expanded blastocyst demonstrated a high cell

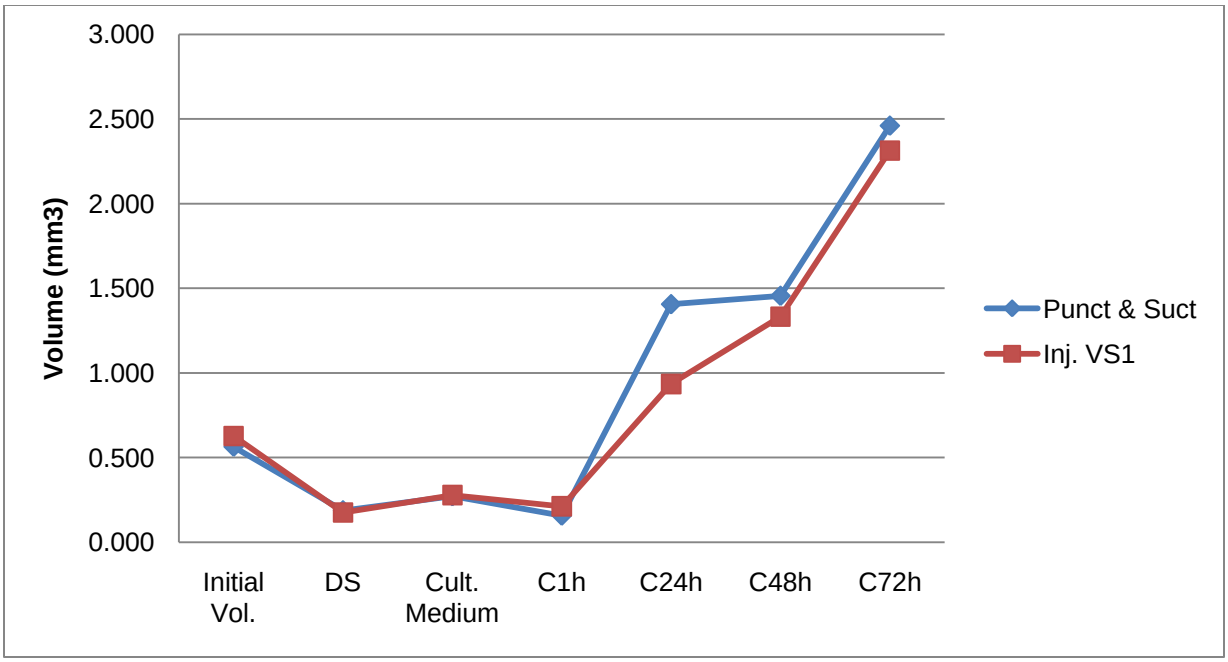

Figure 4.1: Effect of vitrification following indirect or direct introduction of cryoprotectant on embryo volume during *in vitro* culture.

Table 4.4: Effect of vitrification following indirect or direct introduction of cryoprotectant on *in vitro* embryo re-expansion rates and capsule stability.

	In vitro Culture (h)			
	24	**48**	**72**	**Capsule loss**
Indirect	100[a]	76.9[a]	69.2[a]	30.8[a]
	(13/13)	(10/13)	(9/13)	(4/13)
Direct	100[a]	76.9[a]	69.2[a]	69.2[b]
	(13/13)	(10/13)	(9/13)	(9/13)
P-value	1.00	1.00	1.00	0.0492
Power	1.00	1.00	1.00	0.41

*Different superscripts within a column denote a significant difference between parameters ($p<0.05$).

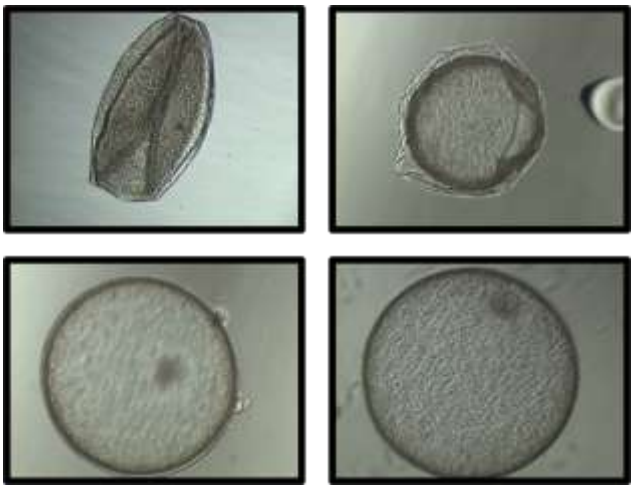

Figure 4.2: *In vitro* re-expansion and growth of a vitrified equine expanded blastocyst.

Figure 4.3: Partial capsule loss (A and B) and total capsule loss (C).

Table 4.5: Effect of embryo vitrification following indirect or direct introduction of cryoprotectant on live/dead cell proportion.

Treatment	Live cells (%)	Dead cells (%)
Indirect introduction	90	10
Direct introduction	90	10
Osmotic damage	70	30
50% fluid removal	35	65

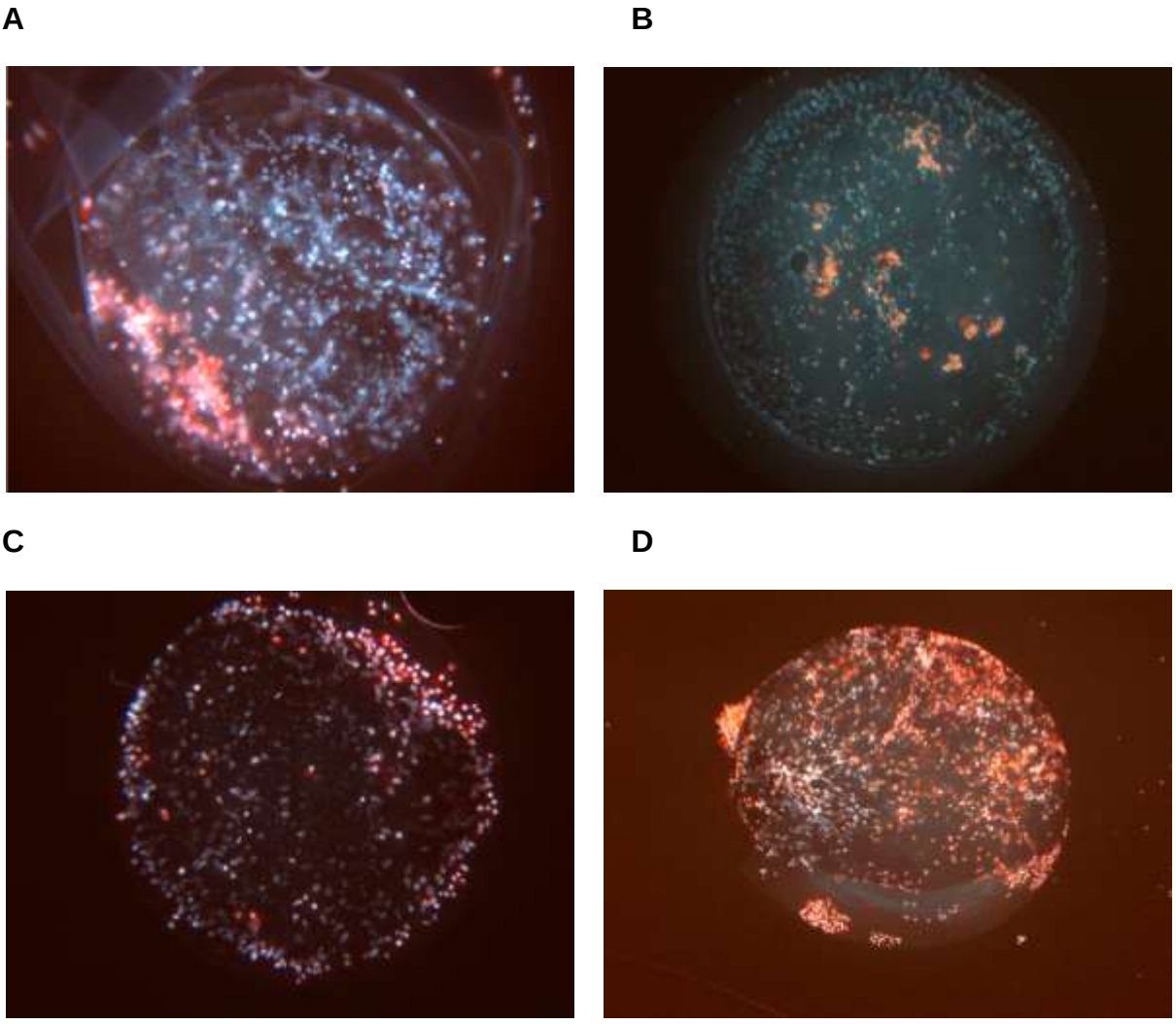

Figure 4.4: Hoescht 33342/propidium iodide embryo staining for the estimation of live/dead cell proportion. A) Indirect introduction, B) Direct introduction, C) Indirect introduction + exposure to VS3 for 1.5 min, and D) Indirect introduction + 50% blastocoele fluid removal.

survival rate (approximately 90%) for both vitrification treatments. The embryo exposed for 1.5 minutes to VS3 (osmotic damage) prior to vitrification resulted in approximately 30% cell death and embryo subjected to 50% of blastocoele fluid extraction prior to vitrification resulted in approximately 65% cell death.

Pregnancy results following transfer of vitrified expanded blastocysts are presented in Table 4.6. Following embryo transfer, two mares presented mild signs of uterine edema (grade 1) at day 11 post-ovulation and were subjected to progesterone supplementation. Pregnancy rates following transfer of Day 8 vitrified expanded blastocysts (mean= 821 µm) to Day 7 recipient mares resulted in a pregnancy rate of 83.3% (5/6). All vesicles reached the heartbeat stage (Day 25) and identifiable embryos could be observed through transrectal ultrasonography (Figure 4.5).

No anembryonic vesicles were found at day 25.The three pregnancies not subjected to progesterone supplementation were not terminated and were allowed to continue. Vitrification of expanded blastocysts with mean diameter >700 µm following the capsule puncture and blastocoele fluid extraction technique (indirect introduction treatment) was effective in producing pregnancies.

4.4 Discussion

In this experiment, a significant difference was found between the initial embryo volume and the embryo volume following warming of vitrified embryos in both indirect and direct introduction treatments. It can be concluded that embryo volume can be

Table 4.6: Pregnancy rates following transfer of vitrified equine expanded blastocysts.

				Pregnancy	
Number	**Size (µm)**	**Grade**	**P4 supplementation**	**Day 11**	**Day 25**
1	877	1	No	No	No
2	931	1	No	Yes	Yes
3	843	1	Yes	Yes	Yes
4	1168	1	Yes	Yes	Yes
5	659	1	No	Yes	Yes
6	448	1	No	Yes	Yes
Mean	821	1			
Pregnancy rate				83.3%	83.3%

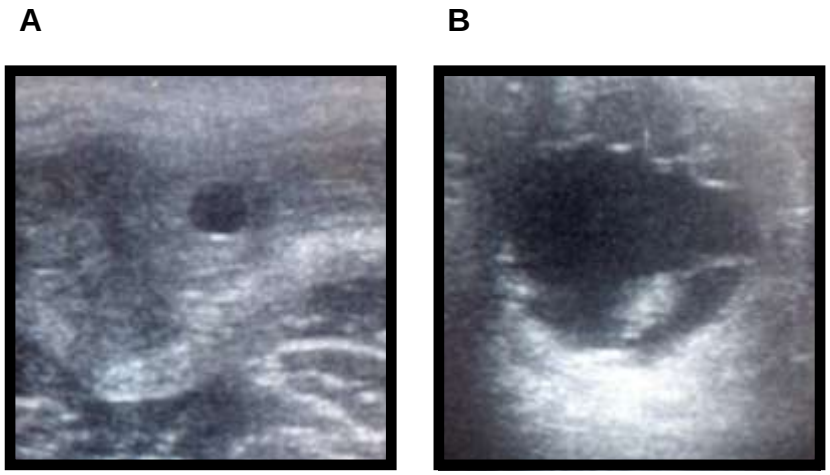

Figure 4.5: Day 11 pregnancy (A) and Day 25 pregnancy (B) from a vitrified equine expanded blastocyst.

effectively reduced by embryo puncture, blastocoele fluid extraction, and exposure to cryoprotectants. Embryo volume reduction is a desired feature to increase the embryo viability following vitrification (Vanderzwalmen et al., 2002).

Following warming and exposure to DS, embryo volume for indirect and direct introduction treatments increased after 3 min of embryo exposure to culture medium. Embryo re-expansion for indirect and direct introduction was not different during *in vitro* culture at 24, 48 and 72 hours.

Indirect and direct introduction treatments resulted in relatively high re-expansion rates following vitrification and warming. Direct introduction of cryoprotectant is not toxic for the embryo, given that indirect and direct introduction treatments exhibited the same re-expansion rates at 24, 48 and 72 hours culture. Low toxicity was expected since the cryoprotectant used for direct introduction was the first of the 3-step cryoprotectant addition protocol (1.4 M glycerol).

Poor results have been obtained during *in vitro* studies following vitrification of equine expanded blastocysts. Hochi et al. (1995) reported that only 25% of vitrified embryos (mean= 515.4 µm) developed during 48 hours of in vitro culture while the remaining 75% presented partial or total degeneration following vitrification. The vitrification protocol used in that study was the same protocol that produced a pregnancy with embryos <300 µm (Hochi et al., 1994a). Young et al. (1997) reported that from 8 vitrified embryos (mean= 435 µm) only 5 were suitable for culture after warming. At 36 hours of culture, the quality grade of cultured embryos was grade 2

(n=1), grade 3 (n=1) and grade 4 (n=3), and only a 20 µm mean increase in diameter was observed. A high proportion of embryos (3/5) showed severe signs of degeneration.

In contrast to previous studies, the methods utilized in this experiment seem to be efficient for the vitrification of expanded equine blastocysts as shown by the high *in vitro* re-expansion rates exhibited. Indirect or direct introduction of cryoprotectants resulted in 100% re-expansion rate at 24 hours culture and 69.2% at 72 hours of culture for both treatments. The direct introduction treatment resulted in significantly higher capsule loss compared with the indirect introduction treatment.

The embryonic capsule develops by approximately day 6.5, soon after the embryo arrives to the uterus coinciding with the onset of blastulation (Betteridge et al., 1982; Flood et al., 1982; Stout et al., 2005). The exact functions have not been clarified, but it is known that this is essential for embryonic viability in the uterus. The capsule plays an important role in embryo cell to cell interactions (Oriol et al., 1993). Besides mucin-like glycoproteins, the capsule contains other proteins involved in the transport of materials into, and probably out of, the developing embryo (Betteridge and Waelchli, 2004). The capsule maintains the spherical shape of the embryo and prevents the trophoblast from elongating between day 10 and 16, as occurs in bovine and swine embryos (Allen, 2001). Because of the strong and elastic nature of the capsule, is has been proposed that it aids in physical protection of the embryo during the time it is subjected to constant myometrial contractions (Allen, 2001; Leith and Ginther, 1985).

The embryo spherical shape maintained by the capsule is of special importance during the embryo mobility phase. Embryo mobility occurring between day 7 and 16 is required for maternal recognition of pregnancy, allowing the embryo to distribute an antiluteolytic signal throughout the uterus and preventing corpus luteum regression (Stout et al., 2005). The embryo mobility response is thought to also be facilitated by the anti-adhesive properties of the capsule glycoproteins, which carry a high proportion of sialic acid residues. Loss of sialic acid residues from capsule glycoproteins would terminate the anti-adhesion effect that is temporally associated with the fixation of the equine embryo on day 17, indicating that it is a unique developmentally regulated mechanism of embryo mobility control (Oriol et al., 1993). The capsule is required for pregnancy maintenance. This fact has been experimentally confirmed because half-embryos removed from the zona pellucida before transfer require the development of the capsule in order to survive (McKinnon et al., 1989). In addition, the complete capsule removal of Day 6 or 7 embryos prevents pregnancy following embryo transfer (Stout et al., 2005).

The overexpansion that direct introduction embryos were subjected to at the time of cryoprotectant introduction could have caused some degree of fracture in the embryonic capsule. Under *in vitro* conditions, embryos were unable to recover and resulted in partial or total capsule loss. Further studies are necessary to determine if the capsule damage caused by the micromanipulation procedures performed in these experiments can be recovered *in vivo* and successfully produce pregnancies. It has been observed that *in vitro* produced equine embryos do not develop a normal capsule during culture (Tremoleda et al., 2003). However, Choi et al. (2009) reported that *in vitro*

produced blastocysts transferred to recipient mares successfully develop a normal capsule, which may support the possibility that capsule fracture will recover *in vivo*.

Results of embryo staining confirm that a high proportion of viable cells are obtained following vitrification with either indirect or direct introduction of cryoprotectant. This agrees with the relatively high *in vitro* re-expansion rates following embryo vitrification obtained in this experiment. Additionally, it was shown that under the conditions of the vitrification protocol utilized in this study, the equine expanded blastocyst is more susceptible to cell death by the presence of blastocoele-fluid at vitrification rather than prolonged exposure to high concentrations of cryoprotectant (VS3) prior to vitrification.

Results of this experiment showed that similar results can be obtained from equine expanded blastocyst vitrification following either indirect or direct introduction of cryoprotectant in terms of *in vitro* re-expansion. There is no additional benefit of the direct introduction of cryoprotectant since this technique requires higher embryo manipulation skill and results in a significant increase of capsule loss *in vitro*.

Prior vitrification studies of intact equine blastocysts have not resulted in acceptable pregnancy rates. Eldridge-Panuska et al. (2005) evaluated the vitrification of expanded blastocysts (mean=609 µm) utilizing a vitrification protocol that has been used for the successful cryopreservation of equine embryos <300 µm. No pregnancies were obtained in the study. Campos-Chillon et al. (2006) utilized a 4-step cryoprotectant addition protocol using ethylene glycol as cryoprotectant for the vitrification of embryo

>300 µm. In that study, a 35% pregnancy rate was obtained where all of the resulting pregnancies were from embryos that were 300–400 µm in diameter.

Different approaches have been applied to modify the embryo characteristics and overcome the low cryoprotectant permeability of the capsule prior to cryopreservation, but have not resulted in acceptable pregnancy rates. Slow cooling cryopreservation following osmotic-induced dehydration of expanded blastocysts caused a reduction of embryo volume by 45% but has resulted in an overall pregnancy rate of 7% or 33% when only considering the embryos <500 µm (Barfield et al., 2009). Legrand et al. (2001) reported an unusual 75% pregnancy rate after enzymatic treatment and slow cooling cryopreservation of equine expanded blastocyst (187–1581 µm) but attempts to replicate these results have not been successful (Legrand et al., 2002; Maclellan et al., 2002). Pre-treatment of expanded blastocysts (300–1100 µm) with the microfilament inhibitor cytochalasin-B and slow cooling cryopreservation has resulted in similar pregnancy rates compared with control embryos (42% and 57%, respectively) (Maclellan et al., 2002). Laser-assisted vitrification has been evaluated with the objective to increase embryo permeability to cryoprotectants. A pregnancy rate of 44% was detected at day 12-14, but just one mare was still pregnant at day 23 (Scherzer et al., 2011). Laser-assisted artificial collapse on human blastocysts has been described as the simplest and most convenient technique for inducing blastocoele-fluid removal (Mukaida and Oka, 2012; Mukaida et al., 2006).This technique is frequently used in human assisted reproduction centers but it does not seem to be effective for equine expanded blastocyst cryopreservation.

In contrast to previous studies, a high pregnancy rate (83.3%) was obtained when equine expanded blastocysts ranging from 448 µm to 1168 µm (mean= 821 µm) were vitrified following the indirect introduction treatment and transferred to recipient mares. Based on this result, high live foal outcomes might be expected due to the fact that all vesicles continue to develop until the heartbeat stage (day 25) and no "anembryonic" vesicles were found in this experiment.

Choi et al. (2011b) reported the successful vitrification of equine expanded blastocysts in the range of 300 to 650 µm. The higher pregnancy rates were obtained by performing embryo puncture and aspirating >70% of the blastocoele fluid. The vitrification protocol consisted of a 2-step ethylene glycol addition and used a modified microloader tip as the vitrification device. The pregnancy rate detected at day 5 post-transfer was 86% and 71% at day 25 (heartbeat stage). The transfer of an embryo with a 780 µm diameter did not result in pregnancy.

In the report by Choi et al. (2011b), five embryos larger than 700 µm were utilized in the different experiments and one resulted in vesicle development only. There are multiple differences between the methods used in their report and the ones used in our experiment that could have positively impacted the viability of embryos larger than 650 µm following vitrification. In our experiment, the complete absorption (95-99%) of blastocoele fluid was performed. Meanwhile, Choi et al. (2011b) reported >70% of blastocoele fluid removal but did not specify further, so it is possible that aspiration of blastocoele-fluid was not complete. Incomplete aspiration, resulting in fluid within the embryo during vitrification, may result in ice crystal formation which is lethal for post-

warming embryo viability (Hiraoka et al., 2004; Vanderzwalmen et al., 2002). As shown by the embryo staining results in our study, the incomplete aspiration of blastocoele fluid resulted in a high proportion of cell death following vitrification.

In this experiment, a 3-step cryoprotectant addition protocol consisting of a glycerol and ethylene glycol mixture was used compared to the 2-step ethylene glycol addition protocol utilized by Choi et al. (2011b). It has been reported that the use of cryoprotectant mixtures in vitrification procedures is beneficial because it allows a reduced concentration of cryoprotectants to be used while maintaining the vitrification capabilities and preventing ice crystal formation (Ali and Shelton, 1993; Eldridge-Panuska et al., 2005). Additionally, the exposure of embryos to a 3-step cryoprotectant addition protocol would probably allow further embryo extracellular and intracellular dehydration prior to vitrification compared to a 2-step addition protocol. In addition to the known protective capabilities of cryoprotectants, they induce strong dehydration of embryos, therefore increasing the concentration of solutes and facilitating intracellular glass formation during cooling (Ali and Shelton, 1993; Kader et al., 2009).

Another important difference is the vitrification device used. Choi et al. (2011b) utilized a modified microloader tip. The microloader tip has to be cut at the distal end according to the diameter of the embryo to be vitrified. Additionally, embryo loading is performed by aspiration. These parameters make this system variable and inaccurate. Open system devices and microloader tips (which follow the open-pulled straw principle) can reach a cooling rate of approximately 20,000ºC/min but are extremely dependent on the volume (<1 µL) in which the embryos are vitrified (Kuwayama, 2007;

Saragusty and Arav, 2011; Vajta et al., 1998). As noted by Choi et al. (2011b), larger embryos require the microloader tip be cut to a larger diameter, resulting in increased fluid volume in the tip and thus slowing the cooling rate at which vitrification occurs. Instead, in our experiment an open system vitrification device (Cryolock) was used where a very low volume of vitrification solution (<1 µL) can be easily obtained. The use of an open system vitrification device exhibits multiple advantages. These include allowing increased cooling and warming rates by using minimal volume of solution (Kasai, 2002), preventing the formation of intracellular ice with smaller amount of intracellular cryoprotectant (Kasai, 2002; Vajta, 2000b), reduction of chilling injury (Kasai, 1996; Vajta, 2000a), shortening the time of exposure to the final cryoprotectant before cooling and after warming (Vajta, 2000a), and a reduction in extracellular induced injuries (Kasai, 1996).

The correct performance of all steps involved in embryo vitrification is important for a positive outcome. In the opinion of the authors, the critical steps for high embryo viability post-warming are the complete blastocoele fluid removal using a thin injection pipette (12-14 µm O.D.), vitrifying the embryo in the lowest volume of VS3 possible, and performing the warming procedure rapidly at the correct temperature. These steps may have accounted for the high embryo viability obtained *in vitro* and *in vivo*.

CHAPTER V
SUMMARY AND CONCLUSIONS

Cryopreservation of equine embryos ≥ 300 μm has been difficult and usually results in pregnancy rates in the ranges of 0% to 38%, which are considered low for commercial application of the technique (Barfield et al., 2009; Bass et al., 2004; Eldridge-Panuska et al., 2005; Scherzer et al., 2011; Slade et al., 1985; Young et al., 1997). The difficulty in cryopreserving these embryos has been attributed to the increase in blastocoele volume, increase in size and cell number, intense mitotic activity, inner cell mass formation, and development of the acellular glycoprotein capsule (Betteridge, 2007; Bruyas et al., 1993; Budik et al., 2008; Choi et al., 2011b; Gillard Kingma et al., 2011; Legrand et al., 2002; Stout, 2012).

Equine embryos ≥300 μm in diameter have decreased permeability of cryoprotectants compared to embryos <300 μm (Hochi et al., 1995; Pfaff et al., 1993). The low permeability has been attributed to changes in area surface to volume ratio, changes in expression of membrane protein aquaporin (Budik et al., 2008; Gillard Kingma et al., 2011), and specially the development of the embryonic capsule. An inverse relationship between permeability and capsule thickness has been observed (Legrand et al., 2002) and low permeability for ethylene glycol and glycerol has been measured (Gillard Kingma et al., 2011; Scott et al., 2012).

The indirect volumetric method determination of cryoprotectant permeability has been shown to exhibit similar results compared with cryoprotectant permeability determined by radioactive cryoprotectants (Leibo and Pool, 2011). The indirect

volumetric method has been widely utilized to determine permeability coefficients due to its simplicity and low cost. *In vitro* approaches have been utilized in equine embryo cryopreservation research in an effort to obtain more precise and quantifiable information about the different damages that embryos are prone to during exposure to cryoprotectants and freezing procedures (Bruyas, 2011; Stout, 2012). Common *in vitro* approaches that have been utilized are embryo *in vitro* culture for re-expansion evaluation, embryo fixation for subcellular level analysis, embryo metabolism evaluation, and use of fluorescent stains to determine live/dead cell proportion.

Choi et al. (2010) reported that equine embryos exhibit high viability following capsule puncture and biopsy. It was seen that loss of blastocoele fluid and collapse did not impede their ability to produce pregnancies. Choi et al. (2011b) reported the successful vitrification of equine expanded blastocysts ranging from 300 to 650 µm in diameter. In that report, five embryos larger than 700 µm were utilized in the different experiments and one resulted in vesicle development only. In this regard, multiple experiments were performed in our study to evaluate different alternatives for capsule puncture and blastocoele fluid extraction with the objective to develop a cryopreservation protocol for Day 8 equine expanded blastocysts with mean diameter of >700 µm .

The objective of the first experiment was to compare the indirect cryoprotectant permeability, *in vitro* re-expansion, and capsule stability of equine expanded blastocysts when exposed to vitrification solutions following one- or two-punctures. High embryo re-

expansion and capsule stability after two punctures would have allowed the evaluation of mechanical introduction/removal of cryoprotectant through multiple embryo punctures.

In our study we showed that a reduction of 67% in embryo volume can be obtained within 5 min of embryo puncture, blastocoele fluid extraction, and exposure to VS1 for either one- or two-puncture embryos. A rapid volume reduction is beneficial due to the fact that the cryoprotectant can permeate faster into embryonic cells with reduced surface area/volume ratio, and this allows for a reduced time of embryo exposure to cryoprotectant solutions (Kader et al., 2009; Kasai, 2002).

Mean embryo volume of one- and two-puncture treatments was not different during exposure to VS1, VS2, VS3, DS and culture media. Similarly, the mean embryo volume of one- and two-puncture treatments was not different during *in vitro* embryo re-expansion. These results show that there is no additional benefit of two-puncture compared to one-puncture to increase cryoprotectant permeability in equine expanded blastocysts. Additionally, the two-puncture treatment may result in an increased risk of capsule loss.

The objective of the second experiment was to evaluate the effect of vitrification of equine expanded blastocyst following the indirect or direct introduction of cryoprotectants on *in vitro* re-expansion and capsule stability. Based on the results of the first experiment, the indirect or direct introduction of cryoprotectants was performed following a one-puncture technique.

A significant difference was found between the initial embryo volume and the embryo volume following warming of vitrified embryos in both indirect and direct introduction treatments. It can be concluded that embryo volume can be effectively reduced by embryo puncture, blastocoele fluid extraction, and exposure to cryoprotectants. Embryo volume reduction is a desired feature to increase the embryo viability following vitrification (Vanderzwalmen et al., 2002).

In our study, vitrification of equine expanded blastocysts following indirect or direct introduction treatments resulted in similar embryo re-expansion during *in vitro* culture. Additionally, indirect or direct introduction treatment embryos resulted in 100% re-expansion rate at 24 hours culture and 69.2% at 72 hours culture for both treatments. However, the direct introduction treatment resulted in a significantly higher capsule loss compared with the indirect introduction treatment.

Results of our study showed that similar results can be obtained from equine expanded blastocyst vitrification following either indirect or direct introduction of cryoprotectant in terms of *in vitro* re-expansion. There is no additional benefit of the direct introduction of cryoprotectant and this technique requires higher embryo manipulation skill and resulted in a significant increase of capsule loss *in vitro*.

The embryonic capsule develops by approximately day 6.5, soon after the embryo arrives to the uterus, coinciding with the onset of blastulation (Betteridge et al., 1982; Flood et al., 1982; Stout et al., 2005). The exact functions have not been clarified, but it is known that it is essential for embryonic viability in the uterus and for pregnancy maintenance. This fact has been experimentally confirmed because half-embryos

removed from the zona pellucida before transfer require the development of the capsule in order to survive (McKinnon et al., 1989). In addition, the complete capsule removal of Day 6 or 7 embryos prevents pregnancy following embryo transfer (Stout et al., 2005).

Results of embryo staining confirm that a high proportion of viable cells are obtained following vitrification with either indirect or direct introduction of cryoprotectant. This agrees with the relatively high *in vitro* re-expansion rates following embryo vitrification obtained in this experiment. Additionally, it was shown that under the conditions of the vitrification protocol utilized in this study, the equine expanded blastocyst is more susceptible to cell death by the presence of blastocoele-fluid at vitrification rather than prolonged exposure to high concentrations of cryoprotectant (VS3) prior to vitrification.

A high pregnancy rate (83.3%) was obtained when equine expanded blastocysts ranging from 448 µm to 1168 µm (mean= 821 µm) were vitrified following the indirect introduction treatment and transferred to recipient mares. Based on this result, high live foal outcomes might be expected due the fact that all vesicles continue to develop until the heartbeat stage (day 25) and no “anembryonic” vesicles were found in this experiment.

Even though further studies including higher embryo numbers are required to validate the high pregnancy rates obtained, the vitrification protocol utilized in this study has the potential to become a key tool for the successful cryopreservation of equine expanded blastocysts ranging from 300 to 1100 µm in diameter.

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THE VITA

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