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# Development and permeability of equine blastocysts

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DEVELOPMENT AND PERMEABILITY OF EQUINE BLASTOCYSTS

A Thesis

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The Interdepartmental Program of  
Animal, Dairy, and Poultry Sciences

in

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by

Brittany Reshel Scott

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“Listen to advice and accept instruction, and in the end you will be wise. Many are the plans in a man’s heart, but it is the Lord’s purpose that prevails.”

- Proverbs 19:20-21

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## ABSTRACT

Equine embryo cryopreservation is unsuccessful in larger, more easily collected, day-7 embryos. It is imperative that methods to successfully cryopreserve large equine embryos or develop reliable methods to determine embryo size before collection. Therefore the objectives for this study were to quantify the amount of tritiated glycerol that would permeate various sizes of equine embryos and to determine if circulating progesterone concentration was correlated with in utero embryo size.

Mean embryo diameter ( $\pm$  SEM) across treatments (1.4M and 3.4M tritiated glycerol) was  $696.5\mu\text{m} \pm 108.6\mu\text{m}$  and  $925.9\mu\text{m} \pm 214.1\mu\text{m}$ , respectively and were not different ( $P=0.44$ ). The percent permeation for 1.4M and 3.4M glycerol were not different ( $P=0.68$ ). Embryos  $<400\mu\text{m}$  in the 1.4M glycerol treatment group had higher ( $P=0.002$ ) permeation than embryos  $>400\mu\text{m}$ ,  $8.32\% \pm 3.85\%$  and  $0.35\% \pm 0.11\%$ , respectively. Length of time, 60 or 120 minutes, did not affect amount of glycerol uptaken ( $P=0.26$ ).

Serum progesterone concentrations on day 7 post-ovulation were higher ( $P=0.009$ ) for mares who produced two viable embryos from double ovulation ( $24.17\pm 2.82\text{ng/ml}$ ) compared with mares from which a single embryo ( $14.04\pm 0.99\text{ng/ml}$ ) was collected and control mares ( $13.53\pm 1.80\text{ng/ml}$ ). No differences ( $P=0.91$ ) were detected in serum progesterone concentration on day 7 post-ovulation between mares from which a single embryo ( $14.04\pm 0.99\text{ng/ml}$ ) was collected and control mares ( $13.53\pm 1.80\text{ng/ml}$ ). Mares producing embryos  $>400\mu\text{m}$  tended to have higher ( $P=0.08$ ) circulating progesterone concentrations than mares producing embryos  $<400\mu\text{m}$ . Serum progesterone concentrations day 7 post-ovulation in mares producing embryos  $>400\mu\text{m}$  and  $<400\mu\text{m}$  were not different ( $P=0.61$  and  $P=0.68$ , respectively) than control mares. Single embryos  $<1000\mu\text{m}$  in diameter were correlated with circulating progesterone concentration day 7 post-ovulation ( $r=0.46$ ,  $P=0.006$ ). There was no significant correlation

between embryo diameter, corpus luteum diameter, and serum progesterone concentration day 7 post-ovulation.

This is the first study to quantify the amount of glycerol permeating into equine blastocysts and suggests that the capsule may be a barrier to cryoprotectant permeability. Maternal progesterone concentrations day 7 post-ovulation could be utilized in predicting embryo stage and size prior to collection for cryopreservation and in diagnosis of twin pregnancies as a result of double ovulation.



## CHAPTER I

### INTRODUCTION

Embryo transfer has made a remarkable change in the way female genetics are disbursed in all species in the livestock industry. The ability to cryopreserve embryos allows genetics to be imported and exported worldwide and to be retained prior to loss of fertility or death. Embryo cryopreservation reduces the labor and cost involved with synchronizing embryo collection to coincide with transfer to a recipient female.

Compared with embryos of other domestic species, the equine embryo has unique differences that could contribute to cryopreservation failure. Early blastocysts and blastocysts have a much shorter cell cycle (6 hours) (Colchen et al., 2000; Moussa et al., 2005) resulting in a greater number of cells and increased in diameter than embryos from other domestic species at the same developmental stage (Bruyas et al., 1993; Moussa et al., 2005). Cells of equine early blastocysts and blastocysts also contain large vesicles and large amounts of lipids stored in variably-sized droplets that make them difficult to cryopreserve (Flood et al., 1982; Bruyas et al., 1993). Equine early blastocysts also develop a capsule (acellular glycoprotein layer) between the zona pellucida and trophoblast cells after entering the uterus (Oriol et al., 1993a). This process is unique to the equine species.

Two methods, slow freezing and vitrification, allow the cryopreservation of only small equine embryos (<300 $\mu$ m) (Hochi et al., 1994; Bruyas et al., 2000b; Lascombes and Pashen, 2001). Currently, equine embryos that are collected for cryopreservation at the morula stage possess either no capsule or an incomplete capsule. These embryos can be successfully frozen in glycerol, similar to bovine embryos (Bruyas et al., 2000b).

Studies have suggested that the capsule may impede permeation of cryoprotectants to the embryo proper resulting in osmotic injury to the inner cell mass during the freezing and

thawing process resulting in inadequate concentration of cryoprotectant in the blastocoele (Slade et al., 1985a; Legrand et al., 1999a; Bruyas et al., 2000a). It has been reported that there is a negative correlation between capsule thickness and freezability of equine embryos (Legrand et al., 2000).

Monitoring serum progesterone (P4) concentrations has been a routine practice for determining phases of the estrous cycle or pregnancy in mares. Pregnancy loss occurs in 10 to 15% of mares during gestation and the majority of these losses occur during the first 40 days of pregnancy (Allen et al., 2001b). The pregnancy is most vulnerable at this stage because the primary corpus luteum is the sole source of progesterone. It has been reported that the primary corpus luteum should produce systemic blood concentrations of progesterone >4ng/ml the first 3 weeks of pregnancy (Ball et al., 1988).

Exogenous progesterone supplementation provided to ovariectomized embryo transfer and pregnant mares after luteolysis induction has been reported to save and positively stimulate mobility, fixation, orientation, and prevent early embryonic loss (Kastelic et al., 1987). In this study, it was also demonstrated that embryos could be rescued by progesterone supplementation up to 4 days after prostaglandin treatment. Results from these studies demonstrate the importance of progesterone in embryo-uterine interactions in early equine pregnancy.

Rambags (2008) describe detection of mRNA for an equine membrane associated progesterone receptor (mPR), intracellular progesterone receptor (PR), and intracellular estrogen- $\beta$  receptors (ER $\beta$ ). This suggests that maternal estrogen and progesterone stimulates the conceptus to synthesize factors that may play a role in maternal recognition and continued luteal function during early pregnancy

We propose the day 7 blastocysts are impermeable to cryoprotectant penetration into the embryo proper. Therefore this study was conducted to quantify the amount of glycerol that

permeates into the embryo of encapsulated day 7 equine blastocysts, using slow-cool (1.4M glycerol) and vitrification (3.4M glycerol) concentrations of glycerol<sup>3H</sup>. We also compared permeabilities of embryos with an incomplete or a very thin capsule (<400µm) to those possessing a fully developed, functional capsule (>400µm). Also, the use of maternal serum progesterone concentration to estimate embryo size would be beneficial in determining the proper collection time for equine embryos destined for cryopreservation and transfer. Therefore, we determined if embryo diameter was correlated with maternal serum P4 concentration on day 7 post-ovulation.

## CHAPTER II

### LITERATURE REVIEW

#### 2.1 Equine Estrous Cycle

The normal estrous cycle of the mare is approximately 21 days, and the normal length of estrus and the inter-estrous interval range from 5 to 7 days and from 14 to 16 days, respectively (Ginther, 1992). Individual variations in estrous cycles exist and can be dependent on seasonal influences, body condition, number of follicular waves, lactation, and the possibility of multiple diestrus ovulations (Robinson, 2003).

The predominant hormone during behavioral estrus is estradiol. At this time progesterone is minimal (less than 1 ng/ml), this allows mares to show signs of estrus such as winking of the clitoris, standing for a stallion, and frequent urination (Youngquist and Threfall, 2007). As the dominant follicle continues to grow, GnRH and LH are produced with the gradual decrease in FSH. These changes cause estradiol production to decrease prior to ovulation. This decline in estradiol corresponds with the end of behavioral estrus. Ovulation usually occurs 1-2 days before the end on behavioral estrus. The end of one estrous cycle and the beginning of another are marked by ovulation (day 0).

Multiple ovulations can occur in mares and there appears to be an individual predisposition for this phenomenon. Maiden and barren mares are more prone to ovulate multiple follicles than lactating mares (Henry et al., 1982), and it is postulated that double ovulations occur because of an increased number of LH receptors on two developing follicles, this in turn causes an increased sensitivity to gonadotropins (Ginther, 1992). There may also be a hereditary predisposition for double ovulations as is a common occurrence in draft mares (31%) and Thoroughbred mares (20%) (Henry et al., 1982).

Once ovulation occurs, diestrus begins and is evident by cessation of estrous behaviors. The dominant hormone during this stage of the estrous cycle is progesterone. Blood levels of progesterone increase rapidly following ovulation, leveling off around day 7 post-ovulation. GnRH pulses become infrequent and FSH pulse amplitude increases relative to LH (McKinnon and Voss, 1993). Blood concentrations of FSH reach their maximum during diestrus which encourages follicular growth.

During diestrus one or two follicular waves may occur (Ginther, 1990). If there is only one follicular wave, than it is the primary wave of folliculogenesis. The primary wave begins 9 days post-ovulation. In two wave mares, a dominant anovulatory follicle will grow and begin regression 9 days post-ovulation. At this time the primary wave will begin growth and dominant follicle will ovulate ~20 days post-ovulation. During these processes the granulosa and theca cells of the dominant follicle begin to produce inhibin in combination with small amounts of estradiol which causes FSH to decline and subordinate follicles to undergo atresia (Ginther, 1990).

The gradual increase in LH during late diestrus is a product of the regression of the CL and a decrease in the negative feedback of progesterone (Pattison, 1974). Recent research suggests that the increase in LH is held at an intermediate level by the attenuating effect of increasing but intermediate concentrations of estradiol until late diestrus (Ginther et al., 2007).

By the end of diestrus the endometrium is primed by progesterone and begins to secrete PGF<sub>2</sub> $\alpha$  (Douglas et al., 1972). At day 14 post-ovulation PGF concentrations peak and luteolysis and subsequent decline in progesterone occur. Mares return to estrus 1 to 3 days following CL regression with ovulation occurring 5 to 7 days later.

## **2.2 Equine Embryo Transportation to the Uterus**

After ovulation, the mare's oocyte is released through the ovulation fossa into the oviductal fimbria and progresses to the ampullary region of the oviduct where, if capacitated sperm are present, fertilization will occur (Boyle et al., 1987; Hunter, 2005). During the next 72 hours the newly formed zygote will reach the two cell, four to six cell, and eight to ten cell stage at 24, 48, and 72 hours post-ovulation, respectively (Bezard et al., 1989).

A unique characteristic of equine oviductal transport is the ability to identify and transport only developing viable embryos independently from unfertilized oocytes. Niekerk and Gerneke (1966) first demonstrated the differential transport of embryos and oocytes. Unfertilized oocytes (UFOs) become lodged in the ampulla and slowly degenerate (Niekerk and Gerneke, 1966). It has been reported that occasionally UFOs will be transported through the oviduct and into the uterus with a viable embryos (Freeman et al., 1992).

A series of experiments explained the reason for this unique transport involving recovery and culture in vitro of early stage embryos (Weber et al., 1991a; Freeman et al., 1992) and surgical implantation of hormone secreting minipumps onto the mesosalpinx of mares at various time after ovulation (Weber et al., 1991b; Weber et al., 1992). These experiments verified that the embryo secretes prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) at the morula stage, whereas the oocyte does not. Weber proposed that the embryo lodges at the ampullary-isthmus junction as a result of the contraction of the smooth muscle (day 4-5 post-ovulation) (Weber et al., 1995). PGE<sub>2</sub> then begins to be secreted and relaxes the smooth muscle fibers in the wall of the oviduct which allows the rapid movement and entry of the embryo into the uterus (Weber et al., 1995).

### **2.2.1 Effects of Progesterone**

Progesterone is an important hormone during oviductal development and uterine synchrony in many species. It has been reported that embryos from domestic animals can

develop in vitro without the supplementation of progesterone (Eyestone and First, 1989; White et al., 1989). In mice, progesterone is necessary for normal development and transport of embryos through the oviduct (Roblero, 1973; Roblero and Garavagno, 1979).

Administration of the antiprogestin compound RMI 12,936 has been shown to cause an arrest in embryo oviductal transport when given on day 1, 2, or 3 post-ovulation in mice (Kendle and Lee, 1980). However, this effect can be reversed if antiprogestin is administered on day 1 post-ovulation and then the female is treated with progesterone 24 to 48 hours later. These results verify that in the mouse embryo oviductal transport is mediated by progesterone and requires a continuous source of progesterone to maintain transport.

These oviductal transport requirements have also been reported in the rat. Forcelledo (1982) ovariectomized, adrenalectomized, or ovariectomized and adrenalectomized pregnant rats on days 1 to 3 post-ovulation. Embryos from the ovariectomized group exhibited no transport until progesterone implants restored serum progesterone levels to normal concentrations. The adrenalectomized group exhibited normal transport. Conclusions from this study were that secretion of progesterone from the ovaries plays a major role in regulation of oviductal embryo transport and that progesterone secretion from the adrenal glands is not essential and can not substitute for the ovarian progesterone source.

Fuentealba (1988) continued work on this hypothesis and concluded that oocyte transport in pregnant rats took 24 hours longer than nonpregnant embryo transfer recipients. Further investigation of the role of progesterone and estrogen oviductal receptors and plasma concentrations showed that the action of estrogen on the timing of oviductal egg transport in pregnant rats is controlled by endogenous progesterone (Fuentealba et al., 1988).

In cattle, ovum transport through the ampulla is rapid; however, transport of embryos through the isthmus into the uterus is delayed and results from muscular contractions (El-Banna and Hafez, 1970). It was demonstrated in this same study that embryos of super-ovulated

Hereford females reached the uterus by 72 hours post-ovulation and were at the eight cell stage. The oviducts were measured and divided into 8 equal segments with segment 1 being closest to fimbriae. In untreated single ovulating females, embryos were in segment 6 of the oviduct by 72 hours. All embryos that did not develop became lodged in the ampullary region of the oviduct and were not transported further. This study agrees with Oxenreider and Day (1965) who showed rapid oviductal transport of embryos in pigs. The rapid transport of embryos in the pig and super-stimulated cattle is attributed to large number of corpora lutea, high progesterone concentrations, and estrogenic effects.

More recently, it was proposed that early progesterone administration in the cow could alter the ratio of PGE<sub>2</sub> to PGF<sub>2α</sub> synthesis in the uterus to favor and facilitate PGE<sub>2</sub>, furthermore stimulating oviductal transport (Mann and Lamming, 2001).

Hinrichs and Watson (1991) demonstrated that administration of 250 mg of exogenous progesterone in mares on days 0, 1 and 2 post-ovulation did not hasten embryo transport to the uterus by day 5 post-ovulation. Ball (1992) later reported that 450 mg of exogenous progesterone administered to mares on days 0 to 6 post-ovulation did not affect embryonic development as determined by evaluation of morphologic evaluation, size, and number of cell nuclei of embryos recovered from the uterus at day 7 post-ovulation. However, to properly test the hypothesis embryos should be collected upon entry into the uterus (day 5 post-ovulation) to evaluate the effects of progesterone on the development and transport of oviductal embryos.

### **2.3 Uterine Equine Embryo Development**

The equine embryo enters the uterus between 144 to 156 hours (6 to 6.5 days) post-ovulation at the morula/early blastocyst stage (Allen, 2001a). After arriving in the uterus, the equine early blastocyst begins to form an acellular glycoprotein “tertiary embryo coat,” known as the blastocyst capsule (Flood et al., 1982). The capsule first appears between the



trophectoderm and the zona pellucida of the late morula/early blastocyst stage embryos (Stout et al., 2005). The blastocyst then expands rapidly and hatches from its zona pellucida and is fully enveloped in a complete capsule; at this stage (day 7 post-ovulation) the embryo contains a few thousand cells (Tremoleda et al., 2003) and is approximately 250µm (Betteridge et al., 1982).

Equine embryo morphology has been shown to be predictive of the potential for the transfer of that embryo to result in a sustained pregnancy (Carnevale et al., 2000). In this study embryos that possessed a lower grade, resulted in lower pregnancy rates than embryos of a higher grade regardless of synchrony of recipient.

During the next 7 days the conceptus continues to grow rapidly reaching 3 to 5 mm in diameter (>45,000 cells), on day 10 post-ovulation (Betteridge et al., 1982). The equine conceptus grows at a rate of 3.4 mm per day from days 11 to 16 post-ovulation (Ginther et al., 1983). This growth is a result of yolk sac fluid accumulation (Betteridge et al., 1982) and as a result the capsule increases in size and dry weight, which serves as the protective envelope around the conceptus (Oriol et al., 1993b).

The blastocyst cavity becomes lined with primitive endoderm to form a true “yolk sac” during this same period (Enders et al., 1993). The small ICM then expands and develops to form the bi-layered embryonic disc (trophectoderm and yolk-sac endoderm). Between days 10 and 12, the embryonic disc becomes macroscopically visible and around day 12 the primitive streak, precursor to embryo proper, becomes microscopically visible (Ginther, 1979). Cells then migrate through the primitive streak and differentiate to create mesoderm (gastrulation), which begins the formation of a recognizable multicellular embryo (Enders et al., 1993; Betteridge, 2000).

Hershman and Douglas (1979) demonstrated that if a pony embryo were present at day 15 of gestation, but was then removed non surgically, the next ovulation would be delayed for

several weeks. If the embryo was removed on day 14 of gestation the duration of the normal estrous cycle was not affected with luteolysis initiated on day 16 with and subsequent estrus and ovulation.

A plateau of growth occurs between day 18 to 26, after which conceptus growth continues at a rate of 1.8 mm per day until day 50 (Ginther, 1983). The endodermal wall of the yolk sac is completed from cells that are broadly distributed around the interior of the blastocyst, and seem to have other unusual characteristics (Enders et al., 1993). The fetal membranes and circulatory system develop to an extent before the first signs of a functional yolk sac (choriovitelline placenta) at day 22 (Enders et al., 1993)

At three weeks, the spherical embryo is surrounded by a single layer of trophoctoderm and the allantois is beginning to form. The highly vascularized embryo enlarges at the embryonic pole between days 21 and 40, while the yolk sack regresses to the opposite pole (Amoroso, 1952). Specialized chorionic girdle cells form around the embryonic vesicle between days 25 and 35 (Allen and Moor, 1972). These cells detach at day 38 and invade the uterine endometrium to form endocrine glands (endometrial cups) that produce equine chorionic gonadotropin (eCG) between days 40 and 130 of gestation (Allen et al., 1973; Allen, 1975).

Cole et al. (1931) reported the close relationship between the secretion of eCG and the considerable degree of secondary luteal development that occurs on the mare's ovaries between days 40 and 150 days of gestation. It has been suggested that eCG stimulates the luteal cells of both primary and secondary corpora lutea to secrete both progesterone and estrogen (Sirois et al., 1990; Daels et al., 1998).

### **2.3.1 Effects of Progesterone**

The mechanisms that prevent luteolysis and maintenance of early pregnancy are not completely understood. It is a gradual process and involves down-regulation of oxytocin

receptors in the endometrium so that releases of PGF<sub>2α</sub> from the endometrium are prevented (Sharp et al., 1997).

Dawson (1977) reported that progesterone concentrations in plasma reach a peak of 8 to 15 ng/ml between days 6 and 14 after ovulation and then decline steadily to between 4 to 6 ng/ml at days 30 to 35 of gestation. There is evidence of resurgence of the primary corpus luteum, starting on day 35 of gestation, which is believed to be in response to the release of eCG by the endometrial cups (Bergfelt et al., 1989). There is a further rise which occurs with the development of the first of the accessory corpora lutea, with concentrations of 8 to 25 ng/ml being maintained until day 150 when both primary and secondary corpora lutea begin to regress (Bergfelt et al., 1989).

Mares, more than pregnant females of any other species are administered progesterone or progestins during part or all of their pregnancy due to fear of luteal insufficiency during pregnancy. Pregnancy loss occurs in 10 to 15% of domestic species during gestation and the majority of these losses occur during the first 40 days of pregnancy in the mare (Allen, 2001b). The pregnancy is most vulnerable at this point because the primary CL is the sole source of progesterone. The primary corpus luteum should produce systemic blood concentrations of progesterone >4ng/ml the first 3 weeks of pregnancy (Youngquist and Threfall, 2007). McKinnon demonstrated that ovariectomized recipient mares can be used for embryo transfer when supplemented with a minimum dose of 0.044 mg/kg of body weight of altrenogest until endometrial cup formation (McKinnon et al., 1988), verifying that progesterone is necessary to maintain pregnancy.

Progesterone supplementation in ovariectomized and pregnant mares in which luteolysis was induced has been reported to save and positively stimulate mobility, fixation, orientation, and prevent early embryonic loss (Kastelic et al., 1987). In this study it was also demonstrated that embryos could be rescued by progesterone supplementation up to 4 days after

prostaglandin treatment. These results demonstrate the importance of progesterone in embryo-uterine interactions in early equine pregnancy and agree with previous findings (Ginther, 1985).

Papa (1998) attempted to find predictive attributes of early equine pregnancy to determine causes of early embryonic death. In this study embryonic losses occurred on day 19 (47%) and day 21 (29%) post-ovulation and agree with results report by Bergfelt et al. (1992). The mean diameter and progesterone values did not differ between pregnant and non-pregnant (early embryonic death) groups, showing that progesterone decrease is the result of and not the cause of early embryonic death. These researchers also reported that there was no correlation between corpus luteum diameter and maintenance of gestation. However, the mean plasma estrogen concentration and embryo vesicle diameter were higher in pregnant mares, which could be indicators of embryo viability.

Lane et al. (2001) demonstrated that equine morulae use equal amounts of pyruvate and glucose as energy sources; glucose consumption then increases exponentially as the embryo begins to blastulate. It has been demonstrated by Bruck (1997), that progesterone induced endometrial proteins can stimulate glucose metabolism and therefore growth of the blastocyst. These studies suggest that higher progesterone during blastulation is important to embryonic growth.

Recently, the effects of mare age and altrenogest treatment on conceptus development and secretion of hormones in early pregnancy has been investigated (Willmann et al., 2011). In this study, mares were divided into two treatments according to age (4 to 8 years and >8 years) and received either altrenogest (0.044 mg/kg once daily) or sunflower oil (control) from days 6 to 100 of pregnancy. There was no difference in sustained pregnancy rate between treatments. Altrenogest treatment had no effect on mean diameter of the embryonic vesicle between days 12 to 22 of pregnancy. There was a significant effect of mare age and altrenogest treatment on the size of the embryo proper between days 30 and 45 of pregnancy. Older mares exhibited

smaller vesicles when compared to those collected from younger mares. In the control group embryo size was negatively correlated with the age of the mares (day 30:  $r = -0.834$ ,  $P < 0.05$ ). This is a critical phase of pregnancy and corresponds with the completion of organogenesis and the beginning of placentation. This study demonstrates for the first time the positive influence of altrenogest treatment on retarded development of the embryo around the beginning of placentation in mares older than 8 years.

Administration of exogenous progesterone has been reported to increase fetal weight and crown rump length in ewes (Kleemann et al., 1994). Early evidence indicates that progesterone treatment changes cell lineage differentiation in sheep with differences in the proportion of inner cell mass and trophoblast cell number (Hartwich et al., 1995). It has also been reported that there is a proportionate increase in fetal growth when ewes were supplemented with exogenous progesterone in early pregnancy; specifically kidney, spleen, total gut, head width, and thorax circumference and a disproportionate increase in brain, heart, and tibialis anterior weight (Kleemann et al., 2001). These authors also reported that response to progesterone treatment did not occur when treated the first three days of pregnancy. Embryos responded to progesterone treatment day 3 post-ovulation when the uterus becomes progesterone-primed.

Similar studies have been conducted in the cattle and it was reported that by increasing progesterone from day 1 to 4, embryo development on day 14 was advanced (10 fold increase in conceptus length) (Garrett et al., 1988). Kerbler (1997) demonstrated a slight increase in interferon- $\tau$  production on day 18 bovine embryos collected from cows that were supplemented with progesterone on day 8 of pregnancy by induction of accessory corpus lutea. Conclusions from these studies are that it is the post ovulatory progesterone rise and not the level of progesterone secretion achieved that is most important in the control of embryo development in the cow. This is supported by a study in which progesterone was supplemented exogenously

from day 5 to 9, but not from day 12 to 16 post-ovulation which resulted in a six fold increase in the uterine concentration of interferon- $\tau$  and a fourfold increase in trophoblast length on day 16 of pregnancy (Mann et al., 2006).

It has been demonstrated in ferrets that passive immunization with a heterologous anti-progesterone monoclonal antibody arrests embryonic development during early pregnancy (Rider and Heap, 1986). The results indicate that the normal course of pregnancy is arrested as a result of antibody binding of progesterone in the circulation, causing a decrease in the amount of progesterone available to target cell receptors, and therefore blocks normal cleavage and embryonic development at an early stage.

#### **2.4 Equine Capsule Function and Effects**

The equine embryo enters the uterus between 6 to 6.5 days post-ovulation (Battut et al., 1997) at the late morula or early blastocyst stage completely enveloped in the zona pellucida (Betteridge et al., 1982). During the beginning of blastulation, an acellular glycoprotein capsule forms between the trophectoderm and zona pellucida (~6.5 days post-ovulation) (Betteridge et al., 1982). As blastulation continues and the embryo begins to rapidly expand this causes the zona pellucida to decrease in thickness, and eventually rupture to allow the blastocyst that is now completely enclosed in its capsule to hatch (Flood et al., 1982). The blastocyst capsule is formed primarily by the trophoblast cells and is composed of a mucin-like glycoprotein (Oriol et al., 1993b). Evidence of mRNA from the mucin gene MUC1 in both the trophoblast and endometrium cells suggest that the endometrium could also contribute to the formation of the capsule (Gillies et al., 1999).

Hochi (1995a) attempted to produce equine capsule material in vitro by collecting embryos day 6 post-ovulation and culturing for 5 days in various TCM-199 media. Embryos hatched from their zona pellucida and/or capsule layer at different times depending on the medium the embryos were cultured in. Hatching from capsule layers prematurely was caused

by poor capsule integrity of the cultured embryos. This study demonstrated the importance of blastocyst and uterine environment interaction during early development is instrumental in capsule development.

A more recent study (Albihn et al., 2003) utilized endometrial biopsy samples and conceptuses from six mares between days 13 to 15 after ovulation. Biopsies were prepared as 1 mm<sup>3</sup> grafts of endometrium, trophoblast and capsule material for transplantation, alone or in combination, into various sites in 88 immunodeficient mice to identify the true source of capsular production. Capsular material was produced from grafts of trophoblast material (both alone and in combination with endometrium graft on the same mouse) but not from mice with grafts of endometrium alone. These results suggest that the trophoblast may be the principal source of equine embryonic capsule formation. In addition, they demonstrate that xenogeneic grafting is a useful means of culturing endometrium and conceptus tissues outside the mare when in vitro techniques do not suffice.

The capsule is thought to provide at least three vital functions (Allen and Wilsher, 2009). First, the capsule is a pliable, elastic, strong structure providing protection from myometrial contractions and facilitating transuterine migration throughout the lumen before fixation (Betteridge, 1989). When equine blastocysts were capsule-denuded and transferred into its original uterus, capsule regeneration did not occur and embryos did not survive, showing that capsule formation is a stage specific event and necessary to maintain pregnancy (Stout et al., 2005). Second, it also appears to be instrumental in maintaining the conceptus in a spherical shape during early pregnancy, a feature that is very different from the elongation of other domestic embryos (Allen and Wilsher, 2009). Maintenance of its spherical shape and prolonged period of uterine migration are linked to suppression of PGF<sub>2α</sub> secretion by the endometrium and maternal recognition of the equine conceptus (McDowell et al., 1988). Third, the high concentration of negatively charged sialic acid residues within its constituent glycoproteins

(Oriol et al., 1993a) may function to provide an anti-adhesive effect and both regulate the embryo's intrauterine movement and assist in the accumulation and uptake of endometrial gland exocrine secretions (uterine milk) (Allen and Wilsher, 2009). These secretions contain the transport proteins uterocalin (Stewart et al., 1995) and uteroglobin (McDowell et al., 1990) that are the principal source of nutrients for the still unattached conceptus during the first 40 days of pregnancy. The capsule also acts as a "mailbox" for proteins and other molecules being exchanged between the conceptus and endometrium during early pregnancy (Crossett et al., 1998; Herrler and Beier, 2000).

It has also been suggested that the capsule plays an indirect role in fetal-maternal signaling during early pregnancy by storing conceptus produced insulin-like growth factor binding protein-3 (IGF-BP3) (Herrler and Beier, 2000), and the endometrially secreted lipocalin uterocalin (P19) (Crossett et al., 1998), both of which play direct roles in maternal-fetal dialogue.

Before fixation, the yolk-sac wall contains large amounts of GM2-activator protein (GM2AP). This protein plays a role in transport of glycolipids or phospholipids while the capsule is still intact (Quinn et al., 2006). At the time of fixation sialic acid is lost from the capsule. This loss does not occur if the mare is treated with  $\text{PGF}_{2\alpha}$  at day 14 post-ovulation (Chu et al., 1997). The loss of sialic acid in the capsule reduces the number of exposed negatively charged galactose and N-acetylgalactose residues of the major core type 1 O-glycan (Arar et al., 2007), which could be associated with changing the permeability of the capsule. It is believed that changes in charge could be involved in the mechanism(s) of conceptus attachment to the endometrium. After fixation capsule production declines until it ruptures on day 21 through enzymatic mechanisms that still remain unclear (Denker, 2000).

The capsule has been thought to be a cause of lower success of freezing large embryos (>300 $\mu\text{m}$ ) utilizing various freezing protocols. Early work by Pfaff (1993) demonstrated that smaller embryos were more permeable to the cryoprotectants glycerol and ethyl glycol by



placing embryos in each respective cryoprotectant solution and measuring volume and equilibration times. When placing embryos in hyperosmotic cryoprotectant solutions water exits the embryo (volume decreases) and cryoprotectants and water enter the embryo until equilibrated (volume increases to original size) via osmosis (Leibo and Songsasen, 2002). Pfaff (1993) reported that larger embryos (>500  $\mu\text{m}$ ) didn't equilibrate in glycerol solution after 10 minutes and continued to lose volume after 20 minutes. This effect was most likely caused by higher cryoprotectant concentration extracellularly than intracellularly, caused by cryoprotectant not entering the embryo proper. All embryos treated with ethylene glycol had higher ( $P < 0.01$ ) volumes than embryos treated with glycerol suggesting the equine blastocysts are more permeable to ethylene glycol, though there was little penetration in larger blastocysts (Pfaff, 1993).

Legrand (2000) studied the permeability of the equine capsule, using a classical freeze/thaw procedure. After thaw and culture embryos were fixed and embedded in Epon 812 and serial sectioned into semi-thin sections (1  $\mu\text{m}$ ), every fifth section was stained with 0.5% hot toluidine blue and evaluated with light microscopy. Capsule formation and cell nuclei were evaluated and categorized. Researchers found the rate of dead cells after thawing was directly proportional to the capsule thickness. Embryos exhibiting a thick capsule possessed more damaged cells as a result of the freeze-thaw process, compared with embryos with little or no capsule. A negative correlation between capsule thickness and freezability of large embryos has been demonstrated in other studies and suggests that the capsule may impede penetration of cryoprotectants into the embryo proper (Bruyas et al., 2000a; Legrand et al., 2000).

Legrand (2001) tested this hypothesis by immersing expanded blastocysts in trypsin prior to freezing to permeabilize the capsule. In this study eight embryos were transferred and resulted in six pregnancies. This study was replicated twice and resulted in reduced pregnancy rates when compared with the original study, 3/11 (Legrand et al., 2002) and 0/14 (MacLellan,

2002). It has been reported that trypsin treatment may disrupt the embryo's cytoskeleton during freezing process and also increases the adhesive properties of the capsule (Tharasanit et al., 2005).

## **2.5 Synthesis and Secretion of Equine Uterine Proteins, Receptors, and Growth Factors**

For development of embryos to continue the embryo must alter the pattern and/or amount of PGF<sub>2α</sub> secreted by the endometrium (Bazer et al., 1998). In cattle (Bazer and Thatcher, 1997), sheep (Bazer and Thatcher, 1997) and pigs (Bazer et al., 1998) the antiluteolytic signal originates from the conceptus and acts on the endometrium to suppress PGF<sub>2α</sub> production. This antiluteolytic signal varies between species. Cows and ewes secrete type-1 interferon, (interferon-τ), whereas sows secrete estrogen (Bazer and Thatcher, 1977; Bazer et al., 1998). In the mare the preimplantation period is long, which has a non-invasive epitheliochorial placenta that does not fully implant until day 40 post-ovulation (Samuel et al., 1974). The nature of embryonic signal for maternal recognition in the mare remains a mystery. The mare is unique in that the developing conceptus remains spherical and mobile until fixation at day 17 post-ovulation (Ginther, 1983).

Major secretory products that have been identified during early pregnancy include steroids, eicosanoids, proteins, and peptides (Simpson et al., 1999). The first published instance of equine conceptus steroidogenesis was in the 1970's indicating production of estrogens and androgens (Flood et al., 1979). Goff (1993) reported that 17α-hydroxyprogesterone, not estradiol, is the major steroid synthesized by the equine conceptus between 7 to 14 days post-ovulation. This study also suggested that this steroid is further metabolized to an unidentified steroid by the endometrium and it is thought that these steroids could play a role in conceptus development and prevention of luteolysis.

Recently, coexpression of two equine genes (EqIFN-δ1 and EQIFN-δ2) coding for a protein similar to interferon (INF) family which is the bovine, ovine and porcine signal for

maternal recognition (Cochet et al., 2009). EqIFN- $\delta$ 1 and EqIFN- $\delta$ 2 genes are expressed during days 16 to 22 of pregnancy (Cochet et al., 2009) which coincide with the critical time of early embryonic loss in the mare. The direct effects of progesterone and/or progesterone supplementation have yet to be studied on EqIFN- $\delta$ 1 and EqIFN- $\delta$ 2.

There are numerous progesterone-dependent proteins that have been reported to be produced by the uterus, many of which are vital to the nutrition of the equine conceptus (Betteridge, 2000). Acute loss of these proteins occurs with luteolysis (Zavy et al., 1979b). The first progesterone dependent uterine protein studied in detail is uteroglobin. Uteroglobin was first identified as a low molecular weight uterine protein secreted by rabbits and is the founder of the secretoglobin superfamily of proteins (Beier, 1968). Subsequently the gene coding for the uteroglobin gene was found in humans (Singh et al., 1988), rats (Nordlund-Möller et al., 1990), mice (Ray et al., 1993), hamsters (Sagal and Nieto, 1998b), pigs (Sagal and Nieto, 1998a), and mares (Beier-Hellwig et al., 1995).

In the mare, uteroglobin is a major endometrial progesterone-dependent secretory protein that is found in uterine secretions during the luteal phase (Beier-Hellwig et al., 1995); however, it has also been detected by northern blot analysis in the lung, uterus and prostate gland in horses (Müller-Schöttle et al., 2002). This protein has a small lipophilic binding pocket (von der Decken et al., 2005) and can bind eicosanoids such as PGF<sub>2 $\alpha$</sub>  which could influence endometrial receptivity and blastocyst implantation (Mukherjee et al., 2007). Anti-inflammatory and anti-chemotactic properties have also been suggested (Mukherjee et al., 2007). In rabbits uteroglobin has been shown to protect trophoblastic cells from the immune defense system of the mother by impairing migration of phagocytes and subsequent rejection (Vasanthakumar et al., 1988).

The best characterized equine progesterone-dependent lipocalin is P19 (uterocalin) which transports small, hydrophobic molecules such as steroids and eicosanoids through the

glycan capsule and into the yolk sac of the conceptus (Stewart et al., 2000b). Early studies determined that uterocalin is taken up by the blastocyst capsule (Stewart et al., 2000b), and transports vital vitamins and minerals to the embryo proper (Crossett et al., 1998). Uterocalin is a highly cationic protein (pI~9.4), this property explains why it readily binds to the equine capsule (Hayes et al., 2008). Uterocalin is secreted by endometrial glands in the luteal stage of the ovarian cycle and in the first 25 days of a pregnancy, and production ceases around the time of capsule deterioration (Stewart et al., 2000b; Suire et al., 2001; Kennedy, 2005). Stewart (2000b) demonstrated that uterocalin remains detectable in the endometrial glands of pregnant mares and in the trophoblast cells overlying these glands as late as day 250 of pregnancy. Uterocalin is secreted asynchronously in older mares with endometriosis and this may contribute to early embryonic death (Stewart et al., 2000a; Hoffmann et al., 2003). Amounts of uterocalin detected in uterine flushes of pregnant mares was lower with pretreatment of PGF<sub>2α</sub>, indicating the progesterone dependence of this protein (Suire et al., 2001).

Uteroferrin is another progesterone-dependent protein found in uterine secretions of the mare; however it was originally characterized and is very similar to the pig with purple coloration, acidic phosphatase activity, and a molecular mass of 35 kDa (McDowell et al., 1982). Various studies have demonstrated that the equine placental transport system for iron and its carrier protein uteroferrin is always membrane-bound in vesicles or lysosomes of maternal epithelium or columnar cells of the trophoblast (Wooding et al., 2000, 2001).

The equine conceptus produces PGE<sub>2</sub> following its passage from the oviduct to the uterus, with peak production between 11 to 15 days post-ovulation (Vanderwall et al., 1993). Watson and Sertich (1989) reported that 14 day old horse embryos in culture release PGF<sub>2α</sub>, PGE<sub>2</sub> and PGI<sub>2</sub> (measured by the stable metabolite 6-keto-PGF-1α). Embryos of other species are known to produce prostaglandins (Hyland et al, 1982; Hwang et al, 1988) and are thought to make a significant contribution to the uterine milieu in maternal recognition of pregnancy.

However, concentrations released by the embryos in the present study were very low compared with concentrations released by the endometrium. (Watson and Sertich, 1989).

Merkel (2010) used microarray analysis of endometrial biopsies from both non-pregnant and pregnant mares. This study revealed no detectable changes in gene regulation until day 12 post-ovulation where 332 genes were expressed higher and 42 at lower transcript levels. Expression of these genes was validated by real-time RT-PCR. Many of these genes were known estrogen-induced genes and genes involved in regulation of estrogen signaling. Genes known to be regulated by progesterone and PGE<sub>2</sub> were also found. It was concluded that the target genes and pathways of conceptus-derived estrogens, progesterone, and prostaglandin E<sub>2</sub> in the maternal endometrium are involved in the establishment and maintenance of pregnancy.

### **2.5.1 Effects of Progesterone**

Progesterone supplementation has been used in animals with low post-ovulatory progesterone levels during early pregnancy and in embryo transfer protocols to create a synchronous and suitable uterine environment, allowing the embryo to develop thereby preventing luteolysis. Progesterone had been called the “hormone of reproduction”, acting through its receptor and several co-factors, it orchestrates molecular, biochemical and physiological interactions in the uterus that affect embryo growth, development and viability (Spencer et al., 2004).

Rambags (2008) describes detection of mRNA for an equine membrane associated progesterone receptor (mPR), intracellular progesterone receptor (PR), and intracellular estrogen-β receptors (ERβ). These results suggest that progestins and estrogens may directly affect embryonic development in the horse. Therefore it is possible that maternal progesterone

stimulates the conceptus to synthesize factors that play a role in maternal recognition and continued luteal function during early pregnancy.

All three of the major progesterone dependent proteins (uterocalin, uteroglobin, and uteroferrin) were detected at various levels throughout pregnancy by immunohistochemistry and histological staining of biopsies in pregnant mares (Ellenberger et al., 2008). Uterocalin secretions were highest during the peak of progesterone production by the primary corpus luteum (days 16-28), then declined. Uterocalin production again increased with a sharp rise when endometrial cups were formed (day 40) and again declined when cups began to degenerate. These findings agree with previous suggestions that uterocalin is a carrier protein that provides the conceptus with essential nutrients for morphogenesis and that it is the most progesterone dependent of the three proteins. Uteroferrin did not increase until day 40 when endometrial cups formed and continued throughout gestation. Formation of endometrial cups also caused a rise in serum estrogen that is thought to stimulate uteroferrin. In ovariectomized mares it was reported that administration of progesterone increased uterine secretions of uteroferrin and that secretion was also amplified by estrogen (McDowell et al., 1987). Uteroglobin was detected in various low levels throughout gestation, suggesting that the progesterone/estrogen ratio may be very important in regulating uteroglobin secretion.

In cattle, progesterone has been reported to affect the volume of uterine secretions (Garrett et al., 1988) and the ability to produce the luteolysis inhibitor interferon- $\tau$  (Mann et al., 2006). Trophoblast protein-1, also known as interferon- $\tau$  (IFN- $\tau$ ) is a glycoprotein secreted by the conceptus of cows and sheep between days 17 to 22 of gestation (Geisert et al., 1988). IFN- $\tau$  is antiluteolytic and either inhibits or alters the pattern of endometrial release of PGF<sub>2 $\alpha$</sub>  (Helmer et al., 1989a; Helmer et al., 1989b). IFN- $\tau$  also exerts antiviral and anti-proliferative activities which assist in maintaining pregnancy (Pontzer et al., 1988; Pontzer et al., 1991).

Maternal plasma progesterone concentrations were correlated with IFN- $\tau$  production by bovine conceptuses ( $r=0.593$ ;  $P < 0.006$ ) (Kerbler et al., 1997). It was also noted that there was a tendency ( $P=0.059$ ) for an increase in synthesis of IFN- $\tau$  by conceptuses from heifers treated with hCG day 5 post-ovulation. Kerbler determined from this study that higher maternal progesterone, via human chorionic gonadotropin (hCG) treatment during early pregnancy or exogenous progesterone supplementation, provides a more suitable environment for developing bovine conceptuses.

Hicks et al. (2003) investigated the link between IFN- $\tau$  and upregulation of uterine Mx gene expression in pregnant cows, gilts, and mares. The Mx gene was first characterized in sheep in response to upregulation by IFN- $\tau$  and providing immune response to viral infection in early pregnancy (Horisberger and Gunst, 1991; Ott et al., 1998). Mx gene expression was upregulated in cows, gilts, and ewes during early pregnancy (Hicks et al., 2003). There was no difference in uterine expression of the Mx gene in pregnant versus non-pregnant mares (Hicks et al., 2003). This could be because the equine conceptus does not elongate or secrete IFN- $\tau$  during early pregnancy and fixation and maternal recognition occurs through a different mechanism (Baker et al., 1991).

Lawson and Cahill (1983) treated donor ewes with progesterone for 6 days post-ovulation and transferred day 10 embryos to progesterone treated ewes (Lawson and Cahill, 1983). Pregnancy rates at day 25 post-ovulation revealed no difference between the progesterone treated ewes receiving asynchronous embryos and ewes that had 10 day old embryos transferred 10 days post-ovulation. This suggests that the increased progesterone early in the cycle primes the uterus and can accelerate the uterine environment to facilitate transfer of older asynchronous embryos.

## **2.6 Equine Embryo Cryopreservation**

Equine embryo cryopreservation has the potential to simplify and increase the prevalence of embryo transfer in many ways. The ability to cryopreserve embryos allows recovery and transfer of embryos to be separated by both location and time, decreasing the necessity to synchronize donor and recipient animals. Embryos could be collected and frozen from sport mares, allowing production of progeny without interfering with competition schedule. The ability to cryopreserve and store equine embryos would enable producers to bank valuable genetics similar to what is done other species. International distribution of valuable bloodlines could be increased via international trade of frozen embryos in a system similar to that already exists for frozen semen and bovine embryos. Despite all of these advantages, the successful cryopreservation of equine embryos is a technique that frustrated researchers and practitioners, due to the inability to effectively cryopreserve large equine embryos.

Prior to the cryopreservation procedure, embryos must be exposed to cryoprotectants (Seidel, 1996). Cryoprotectants are classified as either intracellular (penetrating) or extracellular (non-penetrating). Intracellular cryoprotectants typically have low molecular weights allowing for cell permeation. Examples of intracellular cryoprotectants include ethylene glycol (EG), glycerol, and dimethyl sulfoxide (DMSO).

Extracellular cryoprotectants have high molecular weights and do not readily penetrate embryonic cells. Examples of extracellular cryoprotectants include sucrose, galactose, and serum albumin. Various cryoprotectants are frequently used in combination to inhibit the negative effects of high concentrations of a single cryoprotectant. It is not uncommon for cryoprotectants to be added or removed in a number of equilibration steps to prevent osmotic shock produced by the sudden osmotic changes within cells.



### **2.6.1 Conventional Slow-Cooling of Equine Embryos**

During the conventional slow freezing process, embryos are exposed to a low concentration of cryoprotectant(s), allowed to equilibrate, loaded into plastic straws, and then slowly cooled. As the cooling process progresses ice crystals begin to form in the extracellular medium causing the cryoprotectant solution within the embryo to become more concentrated and hypertonic as additional water is drawn out of the cells. The typical cooling rate for this procedure is  $-0.5$  to  $0.6^{\circ}\text{C}/\text{min}$  (Seidel, 1996). To prevent super cooling (cooling without freezing when temperatures are below  $0^{\circ}\text{C}$ ), ice crystal formation is induced (seeding) at approximately  $-6^{\circ}\text{C}$ . The seeding procedure involves dipping forceps or a cotton applicator into liquid nitrogen and then touching the straw with the frozen instrument.

Most damage to embryos during the slow cool procedure occurs during the thaw process. Rapid thawing can result in a sudden influx of water into blastomeres causing osmotic swelling. To minimize this, cryoprotectants are removed in a stepwise manner by placing embryos in decreasing concentrations of extracellular cryoprotectants. This allows the intracellular cryoprotectant to be removed from the cells during gradual rehydration.

The first foal born from a frozen equine embryo was reported in 1982 (Yamamoto et al., 1982). This study utilized glycerol as a cryoprotectant and only one foal resulted from three confirmed pregnancies following the transfer of 11 frozen-thawed early blastocysts.

Subsequent studies have established that acceptable pregnancy rates (50-60%) can be achieved if conventional slow-freezing is used when embryos are cryopreserved at an early developmental stage (morula to early blastocyst) and are  $<300\mu\text{m}$  in diameter (Czlonkowska et al., 1985; Slade et al., 1985; Skidmore et al., 1991; Squires et al., 2003).

One successful method was reported by Slade and associates (1985). In this study, glycerol was used as the cryoprotectant at a concentration of 5% and 10% v/v in a base medium of Dulbecco's phosphate-buffered saline (D-PBS) supplemented with 5% calf serum.

Embryos were exposed to 5% glycerol for 10 minutes and then transferred to a 10% glycerol solution for 20 minutes. Embryos were then loaded into plastic straws and cooled from 4°C to -6°C then seeded and held at this temperature for 15 minutes. Temperature was then reduced to -33°C at -0.5 °C/min before plunging into liquid nitrogen. Embryos were thawed first by immersing the straw in a 37°C water bath for 30 seconds. Embryos removed from plastic straws and then moved through solutions of decreasing concentrations (10%, 8.3%, 6.7%, 5.0%, 3.3% and 1.6% glycerol v/v) for 10 minutes each, to dilute internal cryoprotectant concentration before transfer. In this study 17 blastocysts were transferred, resulting in 9 pregnancies (53%). It was also noted that early blastocysts resulted in a higher pregnancy rate (8/10) compared with expanded blastocysts (1/7). Various studies have used Slade's slow-cool protocol with slight modifications. Maclellan (2002) reported pregnancy rates of 55% (embryos <250µm) by modifying time exposed to cryoprotectant and concentrations of thaw solutions.

Ethylene glycol is a smaller molecule (27.6Å) than glycerol (90.2Å) and is thought to penetrate equine embryos more readily (Pfaff, 1993; Gordiyenko et al., 2004). Bruyas (2000a) compared the effects of using 1.5M glycerol to cryopreserve day 6 equine embryos (Treatment 1) with 1.5M glycerol with the addition of sucrose during thaw media (Treatment 2), 1.5M ethylene glycol as cryoprotectant (Treatment 3), and 1.5M ethylene glycol with the addition of sucrose in thaw media (Treatment 4). After the freeze/thaw procedure embryos were cultured, fixed in glutaraldehyde and serially sectioned and evaluated with light microscopy. None of the frozen/thawed embryos treated with ethylene glycol, with or without the addition of sucrose, possessed any viable cells (Treatments 3 and 4), however those frozen in glycerol exhibited no lysed cells. Sucrose did not affect the number of lysed cells in the glycerol treatment group. The results of this study suggest that glycerol is a superior cryoprotectant when compared with ethylene glycol for early equine embryos (Huhtinen et al., 2000). In contrast, O'Donovan et al. (2000) demonstrated that incubation of large equine embryos (300-600µm) in 2M ethylene

glycol for 40 min was more effective than cryopreserving embryos with a higher molar concentration of ethylene glycol or step down equilibration.

Researchers compared the effectiveness of glycerol during slow cooling on blastocysts and expanded blastocysts and morulae and early blastocysts to 1,2-propanediol for morula and early blastocyst and fresh transferred controls embryos (Meira et al., 1993). This study also indicates that glycerol may be a superior cryoprotectant when compared with 1,2-propanediol when used for conventional slow cooling. Results also indicated that blastocyst and expanded blastocyst (day 7) embryos are unsuitable for cryopreservation via conventional slow cooling.

Researchers continued evaluating combinations of glycerol and other cryoprotectants and combined it with sucrose or 1,2-propanediol and conventionally slow cooled day 6 embryos (Ferreira et al., 1997). Pregnancy rates were 13.3% and 0%, respectively, after thawing and transfer. Non-transferred embryos from each group were also frozen-thawed and subjected to ultrastructural analysis. Both groups contained embryonic cells that were deformed and showed dilation of the intercellular and perivitelline spaces. These results indicate that these cryoprotectant combinations were not effective against damages caused during the freeze/thaw process.

Numerous cryoprotectants have also been utilized in the effort to find a suitable freezing procedure for the larger day-7 equine blastocyst. The probable cause of reduced embryo viability during freezing and thawing of large embryos is the disruption of cell organelles, in particular the cytoskeleton, as a result of intracellular ice formation (Dobrinsky, 1996). MacLellan (2002) investigated using cytochalasin-B (cyto-B) as a pretreatment for large embryos to inhibit actin polymerization by temporarily deconstructing the actin filaments. It has been previously shown to reduce cytoskeleton damage during cryopreservation of porcine embryos, thereby, possibly improving embryo viability (Dobrinsky et al., 2000). MacLellan et al. (2002) reported that equine embryos pre-treated with cyto-B had similar rates of embryonic

vesicle formation as embryos frozen with no pretreatment, indicating cyto-B is not effective for cryopreserving large embryos.

Some success in cryopreserving large equine embryos has been reported. Young (1997) utilized large equine embryos (300-680 $\mu$ m) and compared one step addition and serial dilution of 1M glycerol, step-down equilibration with 2M glycerol in the presence of galactose in media, and standard vitrification protocol with step-wise addition of 11.9M ethylene glycol. Embryos incubated in step-down equilibration with 2M glycerol with the addition of galactose in the media had higher grades and increased in post-flush diameter. These findings are consistent with previous work that demonstrated that equine blastocyst >300 $\mu$ m are more sensitive to vitrification damage than smaller blastocysts (Hochi et al., 1995b). Of the embryos exposed to 2M glycerol, five were transferred, resulting in two 26-day pregnancies. The rationale for step-down equilibration is that high extracellular concentrations of glycerol are required to produce effective intracellular concentrations in seemingly impermeable blastocysts; however, high concentrations of cryoprotectants in the thawing medium would be detrimental by causing cellular lysis (Young et al., 1997).

### **2.6.2 Vitrification of Equine Embryos**

Vitrification is defined as the conversion of liquid rapidly into glass. During the vitrification process the equine embryo is placed in high concentrations of various cryoprotectants for a short period of time, not allowing equilibration. Media can be placed in organized droplets in a tissue culture dish or in a four well plate. Generally, the droplets increase in concentration of cryoprotectant(s), with the incubation in the final droplet less than one minute (including the time in package prior to plunging in liquid nitrogen. Timing is extremely important to prevent toxic effects of the high concentration of cryoprotectants.

The first pregnancies from the vitrification of equine embryos was produced by subjecting early blastocysts to a 40% ethylene glycol, 18% Ficoll, and 0.3M sucrose solution for

2 minutes (Hochi et al., 1994). Single embryos were then loaded into 0.25-ml plastic straws and cooled in liquid nitrogen vapor for 1 minute and then immersed in liquid nitrogen. Straws were warmed by incubation in warm water (20°C) for 20 seconds and the contents were then expelled into a 0.5M sucrose in PBS. Following in vitro culture for 4 hours, five embryos were transferred non surgically into the uterine horn of recipient mares, resulting in two pregnancies.

Recently procedures previously utilized in ovine and water buffalo embryo vitrification have been modified to facilitate equine embryo vitrification. The first such equine study exposing embryos to serial dilutions of cryoprotectants during the thaw process, resulted in a 62% pregnancy rate of embryos <300 µm after direct transfer (Caracciolo di Brienza et al., 2004), while embryos >300 µm resulted in a 0% pregnancy rate after transfer. Serial dilution techniques were then modified for dilution of cryoprotectants in the straw, allowing embryos to be directly transferred after thawing into the recipient's uterus (Eldridge-Panuska et al., 2005). In these procedures the vitrification solutions were as follows: VS1 = 1.4M glycerol, VS2 = 1.4M glycerol and 3.6M ethylene glycol, VS3 = 3.4M glycerol and 4.6M ethylene glycol. The base medium for these solutions was a modified phosphate buffered saline (PBS) as previously described (Caracciolo di Brienza et al., 2004). Embryos were placed in VS1 for 5 minutes, VS2 for 5 minutes, and the total time of exposure to VS3 to plunging into liquid nitrogen <1 minute. Embryos were loaded into straws with a dilution solution (DS; 0.5M galactose, 90µl) on either side of the embryo, with each solution separated by an air pocket. To thaw, embryos were held at room temperature in air for 10 seconds before being plunged in a water bath at 37°C for 10 seconds. The straws were then "flicked" to ensure mixing of the DS solution with VS3 and transferred within 6 to 8 minutes of thawing. Results from these studies indicated that embryos >300 µm didn't produce a viable vesicle in the two-step dilution or direct transfer treatment groups. However, embryos <300 µm exhibited acceptable pregnancy rates (>50%) with both

thaw procedures and with no difference ( $P>0.05$ ) between treatments. Similar vitrification media kits are now commercially available and allow for embryo cryopreservation.

The effects of cooling embryos for an extended period of time have also been investigated (Hudson et al., 2006). Small embryos ( $<300\ \mu\text{m}$ ) were collected and prepared to be vitrified as previously described (Carnevale et al., 2004; Eldridge-Panuska et al., 2005) or collected and placed in holding media. Embryos were then cooled to  $5^{\circ}\text{C}$  and held for 12-19 hours before vitrification. After thawing all embryos were directly transferred into the uterus of recipients. Pregnancy rates at day 16 post-ovulation were not different between the two groups (15/20, 75%; and 13/20, 65% respectively). These results indicate that small equine embryos could be cooled and stored for up to 19 hours after collection to allow shipping to a facility to be cryopreserved without reduction in viability.

## CHAPTER III

### SERUM PROGESTERONE CONCENTRATION AND EMBRYO DIAMETER ON DAY 7 POST-OVULATION IN MARES

#### 3.1 Introduction

Monitoring serum progesterone (P4) concentrations has been a routine practice for determining phases of the estrous cycle or pregnancy in mares. Pregnancy loss occurs in 10 to 15% of mares during gestation and the majority of these losses occur during the first 40 days of pregnancy (Allen et al., 2001b). The pregnancy is most vulnerable at this stage because the primary CL is the sole source of progesterone. It has been reported that the primary corpus luteum should produce systemic blood concentrations of progesterone >4ng/ml the first 3 weeks of pregnancy (Dawson et al., 1977).

Papa et al. (1998) attempted to find predictive attributes of early equine pregnancy to determine possible association with early embryonic death. In this study, most embryonic loss occurred on day 19 (47%) and day 21 (29%) post-ovulation, in agreement with findings reported by Bergfelt et al. (1992). In these studies, mean embryo diameter and circulating progesterone levels did not differ between the pregnant and non-pregnant (early embryonic death) groups, suggesting that progesterone decrease is the result of and not the cause of early embryonic death in mares. It was also reported that no correlation existed between diameter of the corpus luteum and the maintenance of pregnancy in mares. However, mean plasma estrogen concentration and embryo vesicle diameter were higher in pregnant mares, and either could be an indication of embryo viability.

Exogenous progesterone supplementation provided to ovariectomized embryo transfer and pregnant mares after luteolysis induction has been reported to save and positively stimulate mobility, fixation, orientation, and prevent early embryonic loss (Kastelic et al., 1987). In this study, it was also demonstrated that embryos could be rescued by progesterone

supplementation up to 4 days after prostaglandin treatment. Results from these studies demonstrate the importance of progesterone in embryo-uterine interactions in early equine pregnancy.

Administration of exogenous progesterone has been reported to increase fetal weight and crown rump length in ewes (Kleemann et al., 1994). Early evidence indicates that progesterone treatment changes cell lineage differentiation in sheep, with differences in the proportion of inner cell mass and trophoblast cell number (Hartwich et al., 1995). Kleemann et al. (2001) reported that a proportionate increase in fetal growth occurred when ewes were supplemented with exogenous progesterone in early pregnancy; specifically affected were the kidney, spleen, total gut, head width, and thorax circumference. These authors also reported that response to progesterone treatment did not occur when treated the first three days of pregnancy, but this effect manifested itself when progesterone supplementation occurred after the embryo entered the uterus.

Similarly in cattle, increasing progesterone from day 2 to 5, produced a 10-fold increase in conceptus length at day-14 (Garrett et al., 1988). Kerbler et al. (1997) demonstrated a slight increase in interferon- $\tau$  production by day-18 bovine embryos collected from cows that were supplemented with progesterone around day 8 of pregnancy by induction of accessory corpus lutea. The conclusions from these studies are that the post ovulatory progesterone rise and not the level of progesterone secretion results from is most important in the control of embryo development in the cow. This hypothesis was supported by the finding that progesterone was supplementation from day 5-9, but not from day 12-16 post-ovulation, resulted in a six fold increase in the uterine concentration of interferon- $\tau$  and a fourfold increase in trophoblast length on day 16 of pregnancy in cows (Mann et al., 2006).

Some of the major secretory products that have been identified during early pregnancy of the mare include steroids, eicosanoids, proteins, and peptides (Simpson et al., 1999). The



first published report of equine conceptus steroidogenesis indicated embryonic production of estrogen and androgens (Flood et al., 1979). Goff et al. (1993) suggested that 17 $\alpha$ -hydroxyprogesterone, not estradiol, is the major steroid synthesized by the equine conceptus between 7 to 14 days post-ovulation. These researchers also reported that 17 $\alpha$ -hydroxyprogesterone is further metabolized into an unidentified steroid by the endometrium and it is thought that these steroids could play a role in conceptus development and embryonic-uterine cross talk.

Recently, coexpression of two equine genes (EqIFN- $\delta$ 1 and EqIFN- $\delta$ 2) coding for a protein similar to the interferon (INF) family, which is the bovine, ovine and porcine signal for maternal recognition (Cochet et al., 2009). EqIFN- $\delta$ 1 and EqIFN- $\delta$ 2 genes are produced during day 16 to 22 of pregnancy in the horse (Cochet et al., 2009), which coincides with the critical time of early embryonic loss in the mare. The direct effects of progesterone and/or progesterone supplementation have yet to be studied on EqIFN- $\delta$ 1 and EqIFN- $\delta$ 2.

Rambags (2008) described detection of mRNA for an equine membrane-associated progesterone receptor (mPR), intracellular progesterone receptor (PR), and intracellular estrogen- $\beta$  receptors (ER $\beta$ ). This suggests that maternal estrogen and progesterone stimulates the conceptus to synthesize factors that may play a role in maternal recognition and continued luteal function during early pregnancy.

There are numerous progesterone-dependent proteins, many of which are vital to the nutrition of the equine conceptus that have been reported to be produced by the uterus (Betteridge, 2000) and acute loss of these proteins occurs with luteolysis (Zavy et al., 1979b). All three of the major progesterone-dependent proteins (uterocalin, uteroglobin and uteroferrin) have been detected at various levels throughout pregnancy by immunohistochemistry and histological staining of biopsies in pregnant mares (Ellenberger et al., 2008). Uterocalin secretions were highest during the peak of progesterone production by the primary corpus

luteum (days 16-28), then declined. Production increased again with a sharp rise when endometrial cups were formed (day 40) and again declined when cups began to degenerate. These findings suggest that uterocalin may be a carrier protein that provides the conceptus with essential nutrients for morphogenesis and that it is the most progesterone-dependent of the three proteins.

It has been demonstrated that passive immunization of ferrets with a heterologous anti-progesterone monoclonal antibody arrests embryonic development during early pregnancy (Rider and Heap, 1986). This result indicates that the normal course of pregnancy is arrested as a result of antibody binding of progesterone in the circulation, causing a decrease in the amount of progesterone available to target cell receptors, blocking normal cleavage and embryonic development at an early stage.

The use of maternal serum progesterone concentration to estimate embryo size would be beneficial in determining the proper collection time for equine embryos destined for cryopreservation and transfer. Therefore, the objective of this study was to determine if embryo diameter was correlated with maternal serum P4 concentration on day 7 post-ovulation.

### **3.2 Materials and Methods**

#### Experimental Animals

Two experiments utilizing light horse mares of various breeds (n = 40) were used in this study. The mares were in good body condition (5 to 8) and ranged in age from 3 to 18 years and weighed from 440 to 590 kilograms. Two quarter horse stallions of known fertility, ages 9 and 13, were utilized in both experiments. Breeding soundness exams were conducted on both stallions prior to the 2010 breeding season to confirm fertility prior to use. All horses were housed at the LSU AgCenter Reproductive Biology Center, St. Gabriel, Louisiana where they

were allowed access to Bermuda grass and water *ad libidum*. All procedures were performed in accordance with IACUC protocols.

### Estrus Detection and Artificial Insemination

Estrus detection was performed first with a stallion followed by reproductive tract examinations via transrectal ultrasonography with a 5-MHZ linear probe (Model SSD550, Aloka, Wallingford, CT) to predict ovulation. Each donor mare was inseminated with extended fresh semen at a concentration of at least 500 million progressively motile cells when a follicle >35 mm was detected and again every other day until ovulation. On day 7 post-ovulation, a nonsurgical embryo collection was performed on inseminated mares by a single technician using a standard equine uterine flushing procedure (Imel et al., 1981) with slight modifications.

### Embryo Collection

Briefly, mares were restrained in stocks and the rectum was excavated of feces. A tail wrap was then applied. Transrectal ultrasonography was used to detect and record the corpus luteum diameter and verify the uterus was free from inflammation. The perineal area of the mare was carefully washed with warm water and clorahexadine solution, rinsed with clean warm water, and dried with a clean paper towel. A small piece of wet cotton is inserted into the vestibule to remove any additional debris.

Three liters of lactate ringer supplemented with 0.5% calf serum (Alvarenga et al., 1993) was prepared prior to collection and used as lavage media. After applying a sterile shoulder length glove with a small amount of sterile lubricating jelly on the dorsal part of hand, the arm was introduced into the vagina to identify the external os of the cervix. With the index finger, a 32 french foley catheter (Agtech Inc., Manhattan, KS) was advanced through the cervical canal into the body of the uterus, avoiding any undue dilation of the cervix. The air cuff was inflated

with 30 to 50 ml of air and pulled gently back to form a seal with the internal os of cervix. The catheter was connected with sterile Y-tubing (Agtech Inc., Manhattan, KS) to the lactate ringer and a large-volume filter (EZ-way filter, Reproduction Resources, Walworth, WI). The system was purged of air and a total of 2 to 3 L of flushing media was infused and collected via gravity flow in aliquots of 500 to 1000 ml. After flushing was completed the system was purged with additional flush media to remove any contents from tubing. Flushing media was then searched using a stereomicroscope for embryo(s). Individual embryos were identified and then washed through three to six drops of Syngro holding media (Bioniche Animal Health, Ontario, Canada) to remove any debris. Embryos were then transferred to 500 µl drop of holding media in a four-well plate. All media were pre-warmed and maintained at 37°C.

#### Serum Collection and Evaluation

Blood samples were collected via jugular venipuncture before artificial insemination (AI) (day 0) and again on day 7 post-ovulation. Blood samples were allowed to clot then centrifuged for 10 min at 2,600 x g and serum stored at -20°C until assayed. Samples were collected from mares which an embryo was collected (n=23) and noninseminated mares (n = 15) from the same research herd on the day of expected AI (day 0) and again on day 7 post-ovulation. Serum progesterone was determined using progesterone fluorometric enzyme immunoassay (FEIA) (AIA 360, Tosoh Bioscience, Inc., South San Francisco, CA). Embryo diameter was measured including the zona pellucida using Micron Software (Westover Scientific, Mill Creek, Washington).

#### Statistical Analysis

All data was assessed for normality by Shapiro Wilk test. Log-10 was used to transform and normalize all data. A one way analysis of variance (ANOVA) was used to determine

differences between progesterone concentration on day 7 post-ovulation for single, double ovulation, and control embryos. ANOVA was also used to determine differences between progesterone concentration on day 7 post-ovulation for mares from which embryos were collected. Embryos were divided into two groups, <400  $\mu\text{m}$  and >400  $\mu\text{m}$ . Embryo diameter, progesterone concentration day 7 post-ovulation, and corpus luteum diameter day 7 post-ovulation differences were analyzed by Pearson correlations. Statistics were analyzed using statistical software (SigmaStat version 3.5). Significance was set at the  $P < 0.05$  level.

### **3.3 Results**

#### Embryo Collection

A total of 40 single embryos ( $608.77\mu\text{m} \pm 63.61\mu\text{m}$ ) were collected from 23 mares in this study. A total of six pairs of embryos were collected as a result of double ovulation from three mares (Table 4.1). Control progesterone concentrations were obtained from 15 single ovulating non inseminated mares. Double ovulation control samples were obtained from two noninseminated double ovulating mares. Corpus luteum diameters on day 7 post-ovulation were recorded for 23 single embryos.

#### Experiment 1

In this experiment serum progesterone concentrations on day 7 post-ovulation were higher ( $P=0.009$ ) for mares who produced two viable embryos from double ovulation ( $24.17 \pm 2.82$  ng/ml) compared with mares from which a single embryo ( $14.04 \pm 0.99$  ng/ml) was collected and control mares ( $13.53 \pm 1.80$  ng/ml) (Figure 3.1). No differences ( $P=0.91$ ) were detected in serum progesterone concentration on day 7 post-ovulation between mares from which a single embryo ( $14.04 \pm 0.99$  ng/ml) was collected and control mares ( $13.53 \pm 1.80$  ng/ml).

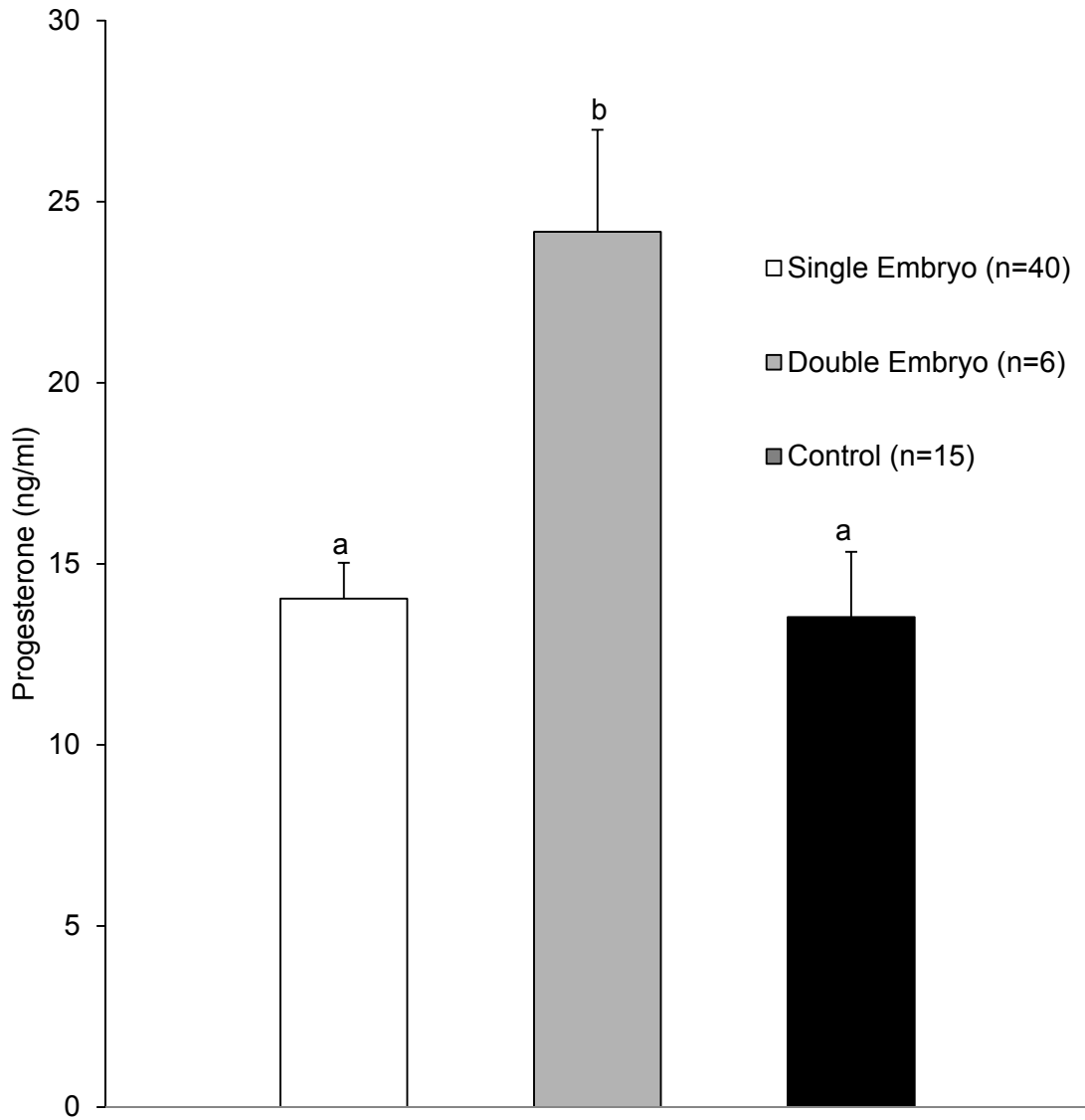


Figure 3.1 : Progesterone concentrations of single and double ovulating mares day 7 post-ovulation.

<sup>a,b</sup> Bars with different superscripts are significantly different ( $p < 0.05$ ).

Mares from which a single embryo was collected were divided into two groups, those producing embryos <400  $\mu\text{m}$  and those producing embryos >400  $\mu\text{m}$ . Embryos collected from mares that double ovulated and produced two viable embryos had higher mean serum progesterone concentration ( $24.17 \pm 2.82$  ng/ml) compared with mares producing embryos <400  $\mu\text{m}$  ( $10.78 \pm 1.14$  ng/ml), >400  $\mu\text{m}$  ( $15.61 \pm 1.28$  ng/ml), and control mares ( $13.53 \pm 1.80$  ng/ml), ( $P=0.001$ ,  $P=0.065$ ,  $P=0.012$ , respectively) (Figure 3.2). Mares producing embryos >400  $\mu\text{m}$  tended to have higher ( $P=0.08$ ) circulating progesterone concentrations when compared with mares producing embryos <400  $\mu\text{m}$ . However, serum progesterone concentrations day 7 post-ovulation in mares producing embryos >400  $\mu\text{m}$  and <400  $\mu\text{m}$  were not different ( $P=0.61$  and  $P=0.68$ , respectively) than control mares.

Diameter of single embryos were not correlated ( $r=0.15$ ,  $P=0.33$ ) with serum progesterone levels day 7 post-ovulation. However, single embryos <1000  $\mu\text{m}$  in diameter were correlated with serum progesterone concentration day 7 post-ovulation, a significant positive correlation was detected ( $r=0.46$ ,  $P=0.006$ ) (Figure 3.3). There was no correlation between embryo diameter, corpus luteum diameter, or serum progesterone concentration day 7 post-ovulation (Table 3.1).

Mares from which two viable embryos were recovered are summarized in Table 4.2. In double ovulating mares embryo diameter and serum progesterone concentration day 7 post-ovulation were correlated ( $r=0.70$ ;  $P=0.01$ ) (Figure 3.4).

### **3.4 Discussion**

Measurement of circulating blood progesterone levels has been utilized as a tool to differentiate non-pregnant from pregnant mares during early pregnancy (Palmer et. al., 1974). Normal pregnant mares typically exhibit elevated progesterone secretion from the corpus

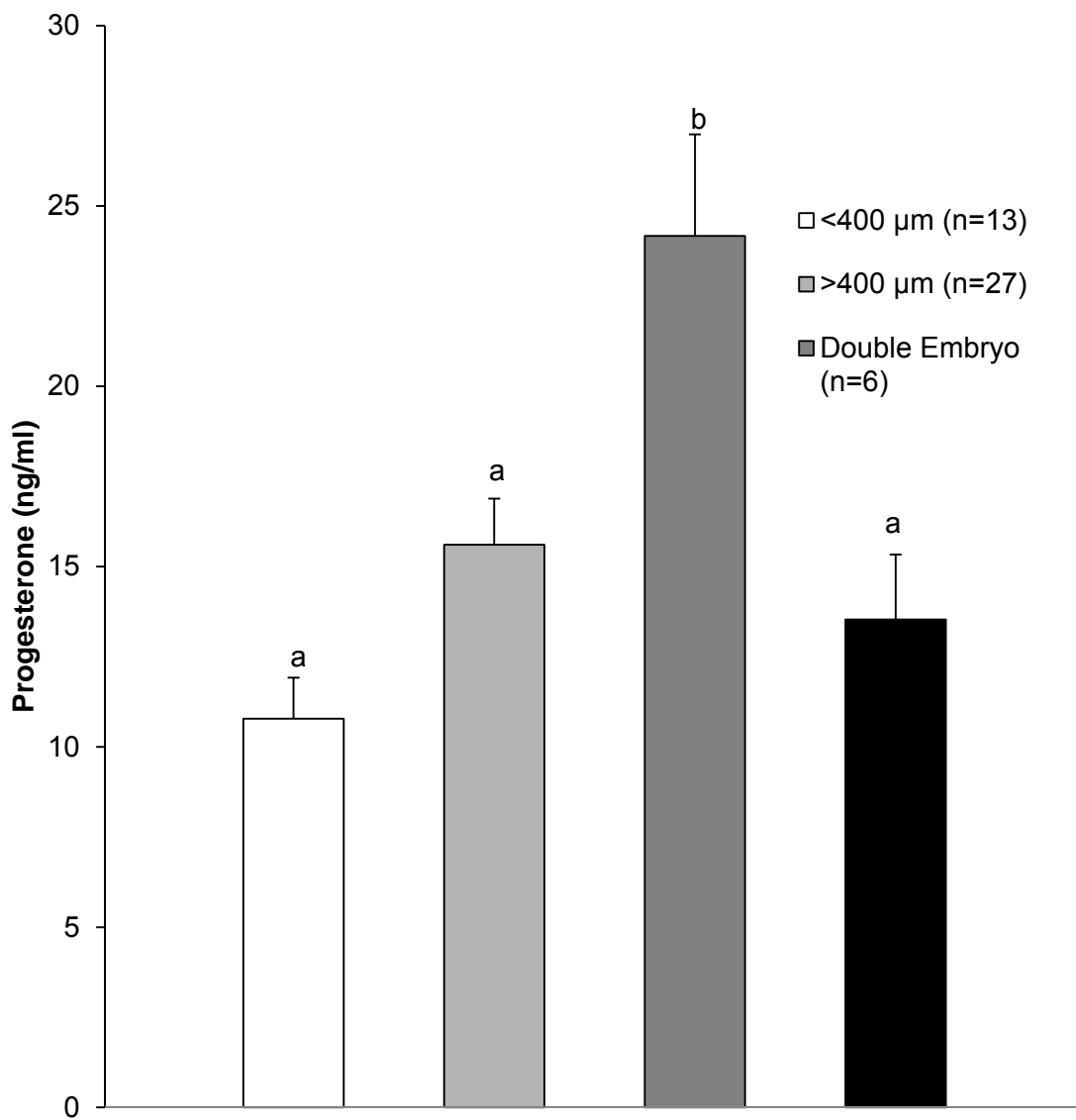


Figure 3.2 : Progesterone concentrations of single and double ovulating mares day 7 post-ovulation.

<sup>a,b</sup> Bars with different superscripts are significantly different ( $p < 0.05$ ).



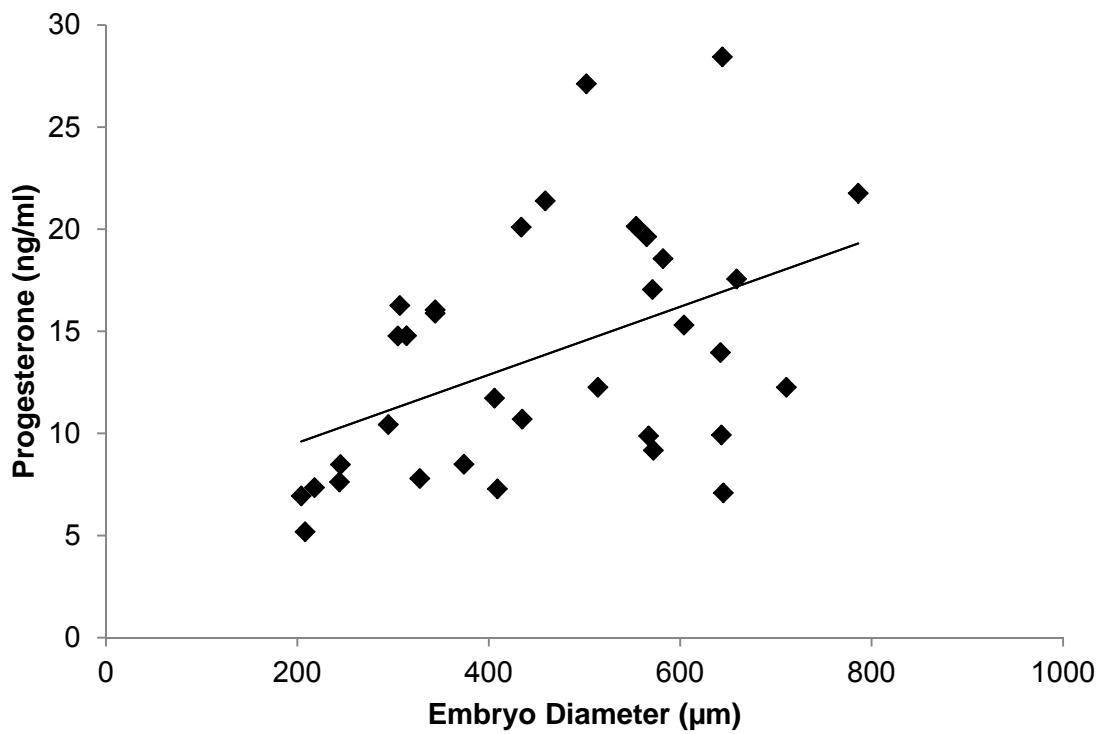


Figure 3.3: Serum progesterone concentration day 7 post-ovulation in single ovulating mares and embryos collected <1000µm in diameter. (P<0.01) (r=0.46) R<sup>2</sup> – (0.21).

Table 3.1 : Pearson correlation results for serum progesterone concentration, embryo diameter, and corpus luteum diameter day 7 post-ovulation in mares.

		P value	r	R <sup>2</sup>
Embryo diameter	CL diameter	0.19	0.28	0.08
	Progesterone concentration	0.56	0.12	0.16
CL diameter	Embryo diameter	0.19	0.28	0.08
	Progesterone concentration	0.14	0.31	0.10
Progesterone concentration	Embryo diameter	0.56	0.12	0.16
	CL diameter	0.26	0.31	0.10

Table 3.2 Embryo diameter and day 7 progesterone levels for double ovulating mares.

Mare ID	Embryo Diameter ( $\mu\text{m}$ )		Progesterone Concentration (ng/ml)
1	284	373	17.22
1	463	1029	27.58
1	672	796	28.26
1	833	1650	34.24
2	533	724	17.54
3	496	736	20.16

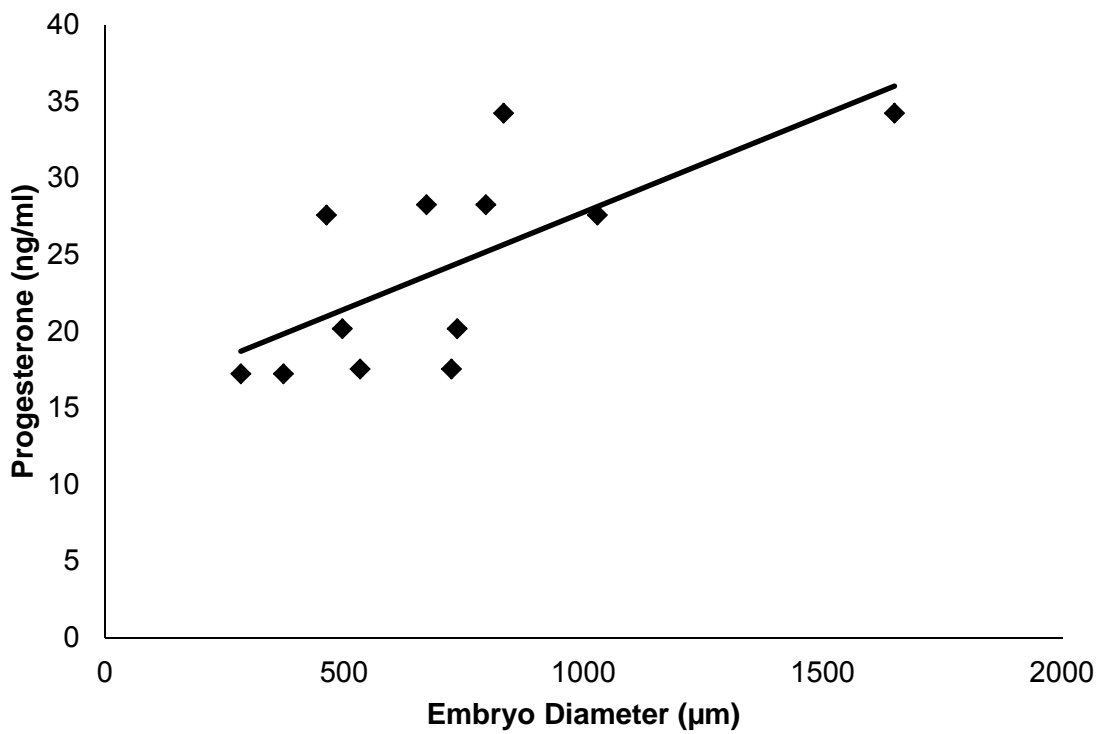


Figure 3.4 : Double ovulating mares serum progesterone concentration on day 7 post-ovulation and embryo diameter. ( $P=0.01$ ) ( $r=0.70$ ) ( $R^2=0.48$ ).

luteum beyond day 12 to 14 (maternal recognition) post-ovulation, whereas nonpregnant mares experience a sharp decline in progesterone following the release of prostaglandins from the endometrium and soon after return to estrus. The primary corpus luteum has been shown to produce systemic blood concentrations of progesterone ranging from 4 to 15ng/ml during the first 3 weeks of pregnancy (Dawson et al., 1977). In our study, mean circulating progesterone concentrations were  $13.53 \pm 1.80$  for control mares and  $14.04 \pm 0.99$  for single ovulating mares.

It has been demonstrated that circulating progesterone concentrations during diestrus are higher following double ovulations when compared to single ovulations in Thoroughbred mares (Henry et al., 1982; Urwin and Allen, 1983). In our study we demonstrated that circulating progesterone concentrations were higher in mares where two embryos were recovered compared with mares producing single embryos and control single ovulating mares. Circulating progesterone concentrations in mares producing two embryos ranged from 17 to 34 ng/ml in our study. Progesterone concentrations for single ovulating mares producing a single embryo  $>400 \mu\text{m}$  tended to be higher than single ovulating mares that produced an embryo  $<400 \mu\text{m}$ , but not different from non-inseminated control mares.

Papa et al. (1998) attempted to find predictive attributes of early equine pregnancy to determine mechanisms associated with early embryonic death. In this study the mean embryo diameter and circulating progesterone concentrations did not differ between the pregnant and non-pregnant (early embryonic death) groups. These researchers hypothesized that the decrease in progesterone is the result of and not the cause of early embryonic death. This hypothesis would explain the differences noted in our study.

Papa (1998) also reported that there was no correlation between diameter of the corpus luteum and maintenance of pregnancy. Our results are in agreement with Papa (1998) in that we also demonstrated there was no correlation between mare corpus luteum diameter,

circulating progesterone concentration, and embryo diameter during early pregnancy. However, in our study, circulating progesterone, corpus luteum diameter, and embryo diameter were recorded from 23 research mares and not an entire horse population at the station.

However, Bergfelt et al. (1998) described endocrinological and morphological relationships between the primary corpus luteum during the estrous cycle of non-pregnant mares and functional changes in circulating concentrations of progesterone related to structural changes in cross-sectional diameter and luteal echogenicity in pregnant mares until day 13. These relationships were derived from retrospective data collected over many studies, resulting in a large sample population.

It has been accepted that the initial formation of the equine blastocyst follows the pattern of most mammals which requires large amounts of energy to support the movement of fluid through the blastocyst. Lane et al. (2001) demonstrated that the equine morula will take up equal amounts of pyruvate and glucose as energy sources. Glucose consumption then increases exponentially as the embryo begins to enter the blastulation phase. It has been demonstrated by Bruck et al. (1997) that endometrial proteins influenced by circulating progesterone levels can stimulate glucose metabolism and therefore growth of the blastocyst. These studies suggest that higher progesterone during blastulation is important to embryonic growth.

Squires et al. (1985) reported that the mean diameters for embryos collected on different days after detection of ovulation were 208  $\mu\text{m}$  at 6 days, 406  $\mu\text{m}$  at 7 days, and 1132  $\mu\text{m}$  at 8 days; however in this study mares were examined once daily for ovulation, and embryos were collected at different times each day. In order to test the accuracy of predicting a true day 7 embryo we used embryos  $<1000 \mu\text{m}$  to represent this population. In our study mare circulating progesterone concentration and embryo diameter were correlated in mares producing embryos

<1000  $\mu\text{m}$  recovered on day 7 post-ovulation. Circulating progesterone concentration and embryo diameter were also correlated in mares producing twin embryos in our study. Embryo diameter has not previously been reported to be correlated with circulating progesterone concentration and could be valuable in projecting the potential diameter (and therefore stage) of embryos prior to non-surgical collection. This would alleviate the need for monitoring ovulation via transrectal ultrasonography every 12 hours, in order to collect an embryo suitable for cryopreservation (early blastocyst).

Double ovulations in equids occur in 8 to 35% of estrous cycles, the frequency depending on the breed and type of mare, with Thoroughbreds and Warmbloods having the highest rate and ponies the lowest (Henry et al., 1982). Accurate detection of such ovulations is important because twinning could result in injury and/or death to the mare and foals. Ovulation and subsequent detection of conceptuses at an early stage (preferably before day 16 of gestation) as a result of double ovulation is a very labor intensive and therefore an expensive undertaking. Currently there is a semiquantitative test (Target Equine Progesterone Assay, Biometallics, Princeton, New Jersey) that can be used to detect progesterone concentrations in whole blood, milk, or plasma. Samples are mixed with reagents and a color is produced indicating the progesterone concentration (bright blue <1ng/ml, light blue 1-5 ng/ml, and white >5 ng/ml). These tests are currently used in detecting estrus.

Positive correlations in mare circulating progesterone concentration on day 7 post-ovulation and single ovulated embryos <1000 $\mu\text{m}$  and double ovulated twin embryos recovered could be utilized to develop a more sensitive chute-side test similar to the Target Equine Progesterone Assay. This would alleviate costs and labor involved with monitoring via transrectal ultrasonography of ovulation to collect an embryo at the proper stage for cryopreservation and in detecting a twin pregnancy.

## CHAPTER IV

### USING GLYCEROL<sup>3H</sup> TO EVALUATE EQUINE BLASTOCYST CAPSULE PERMEABILITY

#### 4.1 Introduction

Commercially, equine embryos destined for cryopreservation are collected from mares on day 6, at the morula stage, due to the limited success of cryopreservation of large blastocyst stage day-7 equine embryos (Slade et al., 1985). The equine embryo enters the uterus on days 6 and 7 post-ovulation and research has shown that collection of day-7 equine embryos is more efficient than collection on day 6 (Squires et al., 1999). Developing a repeatable consistent freezing protocol for large day-7 blastocyst stage embryos would increase the overall efficiency of the equine embryo transfer procedure.

An unusual feature of early equine embryonic development is the formation of an acellular mucin-like glycoprotein layer, known as the capsule, beneath the zona pellucida on days 6 to 7 after ovulation (Flood et al., 1982; Oriol et al., 1993a). The capsule remains intact, during the embryo mobility phase, enveloping the embryo until the third week of pregnancy (Betteridge, 1989).

The equine capsule is thought to provide protection for the embryo during the mobility phase when the embryo migrates throughout the uterine lumen by myometrial contractions (Ginther, 1985). This migration is necessary for the embryo to distribute maternal recognition signal, and inhibit prostaglandin secretion from the endometrium which would result in luteolysis (Vanderwall, 2008). The capsule consists of fine circumferentially arranged fibrils and is homogenous in structure (Flood et al., 1982).

It is hypothesized that the capsule layer of the day-7 equine embryo is impermeable to cryoprotectants (CPAs), (Betteridge et al., 1982; Flood et al., 1982; Pfaff, 1993). A negative



correlation between capsule thickness and freezeability has led to the hypothesis that the capsule may impede access of CPAs into the embryo proper (Legrand et al., 1999b; Bruyas et al., 2000a). Previous work by Pfaff et al., (1993) showed that large equine embryos immersed in ethylene glycol and glycerol for various amounts of time would allow water to exit along an osmotic gradient but did not equilibrate and re-expand, suggesting that the cryoprotectants did not penetrate through the capsule.

Legrand et al. (2000) attempted to digest the capsule by immersing blastocysts in a trypsin solution prior to cryopreservation, which resulted in an unusually high pregnancy rate of 75% after thawing and transfer to recipient mares. However, other attempts to replicate these findings yielded much lower results, 27% and 0% (Legrand et al., 2002; MacLellan, 2002), respectively.

Many fish species have similar cryopreservation complications that have been described for equine embryos (Hagedorn et al., 1997). Harvey et al. (1983) quantified the amount of DMSO and glycerol that penetrated intact and dechorionated (outer chorion removed) zebra fish embryos. Intact and dechorionated zebrafish embryos were exposed to DMSO<sup>14C</sup> or glycerol<sup>3H</sup> in 1M fish ringer. Isotope uptakes were measured with a liquid scintillation counter and converted to percent permeation by volume for each embryo. Permeation of both cryoprotectants increased several fold when embryos were dechorionated, indicating that the chorion retards the free exchange of solutes. This study demonstrates that use of isotopes to measure permeation of various solutes into embryos can be utilized to identify embryonic permeability barriers. This also suggests since there are such similarities in zebrafish and equine embryonic membranes, similar permeation experiments would be informative when applied to equine embryos.

The objective of this study was to quantify the amount of glycerol penetrating the equine capsule using conventional slow cool (1.4M glycerol) and vitrification (3.4M glycerol) protocols, thereby determining if capsule impermeability to cryoprotectants is the limiting factor in cryopreservation of equine blastocysts and expanded blastocysts. The second objective was to determine if incubation in a conventional slow-cool concentration (1.4M glycerol) over an extended period of time (15-120 minutes) would increase overall uptake of cryoprotectant.

## **4.2 Materials and Methods**

### Experimental Animals

Two experiments utilizing light horse mares of various breeds (n = 14) were used in this study. The mares were in good body condition (5 to 8), and ranged in age from 3 to 18 years and ranged in weight from 444 to 590 kilograms. Two quarter horse stallions of known fertility, ages 9 and 13, were utilized in both experiments. Breeding soundness exams were conducted on both stallions prior to the 2010 breeding season to confirm fertility prior to use. All horses were housed at the LSU AgCenter Reproductive Biology Center, St. Gabriel, Louisiana where they were allowed access to Bermudagrass and water *ad libidum*. All procedures were conducted in accordance with IACUC protocols.

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Estrus detection was performed first with exposure to a stallion, followed by reproductive tract examinations via transrectal ultrasonography with a 5-MHZ linear probe (Model SSD550, Aloka, Wallingford, CT) to predict follicular development and ovulation. Each donor mare was inseminated with extended fresh semen at a concentration of at least 500 million progressively motile cells when a follicle >35 mm was detected and again every other day until ovulation. On day 7 post-ovulation, a nonsurgical embryo collection was performed on inseminated mares by

a single technician using a standard equine uterine flushing procedure (Imel et al., 1981) with slight modifications.

### Embryo Collection

Briefly, mares were restrained in stocks and the rectum was excavated of feces. A tail wrap was then applied. The perineal area of the mare was carefully washed with a warm water and weak clorahexadine solution, rinsed with clean warm water, and dried with a clean paper towel. A small piece of wet cotton was then inserted into the vestibule to remove any additional debris.

Three liters of lactated ringer's supplemented with 0.5% calf serum (Alvarenga et al., 1993) was prepared prior to collection and used as lavage media. After applying a sterile shoulder length glove with a small amount of sterile lubricating jelly on dorsal part of hand, the arm was introduced into the vagina to identify the external os of the cervix. With the index finger, the external os was dilated by massage to facilitate passing a 32 French foley catheter (Agtech Inc., Manhattan, KS) into the body of the uterus. The air cuff was inflated with 30 to 50 ml of air and pulled gently back to form a seal with the internal os of cervix. The foley catheter is connected with sterile Y-tubing (Agtech Inc., Manhattan, KS) to the lactated ringer's solution and to a large-volume filter (EZ-way filter, Reproduction Resources, Walworth, WI). The system was purged of air and a total of 2 to 3 L of flushing media was infused and collected via gravity flow in aliquots of 500 to 1000 ml. After flushing was completed the catheter was removed and the system was purged with additional flush media to remove any contents from tubing. Flushing media was then searched using a stereomicroscope for embryo(s). Individual embryos were identified and then washed through three to six drops of holding media (Syngro, Bioniche Animal Health, Ontario, Canada) to remove any debris. Embryos were then transferred to a 500  $\mu$ l drop of Syngro in a four well plate. All media was pre-warmed and maintained at 37°C.

## Preliminary Experiments

A 10ml aliquot of 1.4M glycerol was prepared in PBS with a osmolarity of 1700 mmol/kg (hyperosmotic). Holding media with a osmolarity of 287 mmol/kg was used as a hypoosmotic solution. All solution osmolarities were validated before use with vapor pressure osmometer (Vapro 5520, Wescor, Inc., Logan, Utah).

Five equine embryos were collected as previously described. After collection, embryos were washed through five drops of holding media to remove any debris. Three of these embryos were then incubated in a 1.4M glycerol for 720 minutes.

A diode laser class I 1,480 nm, infrared, solid state (Hamilton Thorne XYClone®, Hamilton Throne Biosciences, Beverly, MA) and software was used for laser ablation and/or monitoring volume changes for all embryos in this preliminary study. This system includes a 40X objective, digital video camera (Lumenera 2.0, Lumenera Corporation, Lawrenceville, GA), laser software and a laptop computer. The high transmission, visible infrared, long distance laser 40X objective was attached to a Nikon inverted microscope. Embryo volume changes were monitored by taking measurements at the 12 to 6 o'clock and 3 to 9 o'clock positions at exponential time intervals and averaged and converted to volume for each embryo. Measurements were recorded on a 10X objective and translated from pixels into micrometers. One embryo received a rent in its capsule after treatment with laser (XYClone®, Hamilton Thorne, Beverly, MA) on full power (100% power, 3000  $\mu$ s pulse).

Osmotic response of two additional embryos (early blastocyst with no capsule and expanding blastocyst with a developing capsule) were monitored in both hypertonic and hypotonic solutions. Individual embryos were exposed to 1.4M glycerol (1700 mmol/kg) for 128 minutes, and immediately transferred into Syngro holding media (287 mmol/kg) for 720 minutes.

## Experiment 1 – The Effect of Glycerol<sup>3H</sup> Concentration on Equine Capsule Permeability

### Embryo Transportation

All embryos were collected at the Reproductive Biology Center, St. Gabriel, LA as previously described and transported to the Biology Building on the main Louisiana State University campus, Baton Rouge, LA for further processing. All medium was warmed to 37°C before use and a portable incubator (Model 19180, Minitube, Verona, WI) was used to maintain a constant temperature of 37°C. Individual embryos were transferred from holding media in a four well plate to 1.5-ml microcentrifuge tubes (VWR, Batavia, IL) filled with 1.2 ml holding medium. The individual embryos were aspirated into a 0.25-ml plastic straw attached to a 1-ml syringe for transfer into the microcentrifuge tube. The end of the open end of the straw was inserted 1 to 2 cm into medium in a microcentrifuge tube and the embryo was slowly expelled. During expulsion, the microcentrifuge tubes were held in front of a light source to allow visualization of the embryo exiting the straw. After being placed in microcentrifuge tube, the embryo descended to the bottom of the tube. The tube was filled then with holding media and closed. After the top of the microcentrifuge tube was secured, a strip of parafilm was wrapped around the cap to prevent leaks. A 50-ml conical tube was used as a second container. The 50-ml tube was filled with PBS. The vial containing the embryo was then placed within the 50-ml tube and sealed. The 50-ml tube lid was then firmly wrapped with parafilm and placed in 37°C incubator for transport. All embryos were transported to the Galvez laboratory located on Louisiana States University's main campus where Experiments 1 and 2 were conducted.

### Experimental Design

Embryos (n=30) were randomly assigned to two treatments. Treatment 1 (n=15) consisted of embryos being incubated in 1.4M glycerol with a specific activity of 10 $\mu$ Ci for 15

minutes. Treatment 2 (n=15) consisted of embryos being incubated in 3.4M glycerol with a specific activity of 20 $\mu$ Ci for 15 minutes.

### Embryo Incubation in Glycerol<sup>3H</sup>

After transportation, cryovials were removed from packaging material and slowly tilted back and forth to allow embryos to float from the bottom of the tube. The contents of the tube were then poured into a tissue culture dish. The tube was thoroughly rinsed with holding media and contents were emptied into a separate tissue culture dish. Once the embryo had been located it was evaluated and is held at room temperature in holding medium until incubation in labeled glycerol.

Incubation solutions were prepared by mixing PBS and glycerol (Sigma, St. Louis, MO) to obtain a concentration of 1.4M and 3.4M glycerol. This solution was then mixed 1,2,3-<sup>3H</sup> glycerol (American Radiolabeled Chemicals Inc., St. Louis, MO) to obtain a final specific activity of 10  $\mu$ Ci/1000  $\mu$ l. Solutions were stored at -20°C until use.

Embryos were incubated in a 500- $\mu$ l drop of 1.4M or 3.4M tritiated glycerol with a specific activity of 10  $\mu$ Ci/500  $\mu$ l for 15 minutes. After incubation embryos were washed three times in 500  $\mu$ l of unlabeled glycerol identical to the treatment concentration to remove the labeled glycerol from the capsule's surface. All samples were transferred to 1.5-ml micro-centrifuge tubes (VWR, Batavia, IL) and incubated overnight in 400  $\mu$ l of nitric acid at 37°C to disintegrate embryos. After incubation, vials were vortexed for two minutes and 350  $\mu$ l of the supernatant was transferred to a scintillation vial and mixed with 5 ml scintillation fluor (Ultima Gold, Perkin Elmer, Shelton, CT). Samples were then counted on a liquid liquid scintillation Counter (TriCarb 3100TR, Perkin Elmer, Shelton, CT) with 65% counting efficiency for tritium. Results were expressed in disintegrations per minute (DPM).

The volume of each embryo was calculated and multiplied by the number of DPM in 1  $\mu$ Ci of solution. This yielded the expected maximum number of DPM for each embryo based on its volume. The actual DPM of each embryo was divided by its expected DPM to yield percent permeation for each embryo expressed in percent.

### Statistical Analysis

Results were then converted from disintegrations per minute to percent uptake of labeled glycerol by volume of the individual embryo. All data was assessed for normality by Shapiro Wilk test. Data was normalized using a LOG-10 transformation and differences were detected by Student's T-Test. Differences were detected at the ( $P < 0.05$ ) level.

### Experiment 2 - The Effect of Time on Glycerol<sup>3H</sup> Permeation in Equine Embryos

#### Embryo Incubation in Glycerol<sup>3H</sup>

Eleven equine embryos were collected and transported as previously described. After transportation cryovials were removed from the packaging material and slowly tilted back and forth so the embryo will float from the bottom of the vial. The contents of the vial were then poured into a tissue culture dish. The vial was thoroughly rinsed with holding media and contents were emptied into a separate tissue culture dish. Once the embryo had been located it was evaluated and held at room temperature in holding medium until incubation.

Incubation solutions were prepared by the addition of PBS to glycerol (Sigma, St. Louis, MO) to obtain a concentration of 1.4M and 3.4M glycerol. This solution was then mixed 1,2,3-<sup>3H</sup> glycerol (American Radiolabeled Chemicals Inc., St. Louis, MO) to obtain a final specific activity of 10  $\mu$ Ci/1000  $\mu$ l. Solutions were stored at -20°C until use.

Embryos were randomly assigned to incubation in a 500- $\mu$ l drop of 1.4M glycerol<sup>3H</sup> with a specific activity of 10  $\mu$ Ci/500  $\mu$ l for 15 minutes (n=1), 30 minutes (n=2), 60 minutes (n=5), and 120 minutes (n=3). After incubation embryos were transferred to 100-mm nylon filters (BD Falcon, Franklin Lakes, NJ) and washed using a filtration apparatus with 5 ml of unlabeled 1.4M glycerol.

All samples were then transferred to 1.5 ml-cryovials and incubated overnight in 400  $\mu$ l of nitric acid at 37°C to disintegrate embryos. After incubation, vials were vortexed for two minutes and 350  $\mu$ l of the supernatant was transferred to a scintillation vial and mixed with 5 ml scintillation fluor (Ultima Gold, Perkin Elmer, Shelton, CT). Samples were then counted on a TriCarb 3100TR liquid scintillation counter (Perkin Elmer, Shelton, CT) with 65% counting efficiency for tritium. Results were expressed in disintegrations per minute (DPM).

The volume of each embryo was calculated and multiplied by the number of DPM in 1  $\mu$ Ci/ $\mu$ l of radiolabelled glycerol solution. This yielded the expected maximum number of DPM for each embryo based on its volume. The actual DPM obtained from each embryo was then divided by its expected DPM to yield percent permeation for each embryo expressed as a percentage.

### Statistical Analysis

Results were then converted from disintegrations per minute to percent uptake of labeled glycerol by volume of the individual embryo. All data was assessed for normality by Shapiro Wilk test. Data was normalized using a LOG-10 transformation and differences were detected by Student's T-Test. Differences were detected at the (P<0.05) level.



## 4.3 Results

### Preliminary Experiments

All three embryos used in the first preliminary experiment responded to placement in the hypertonic solution with a rapid decrease to ~45% of their original volume during the first 32 minutes (Figure 4.1). After 32 minutes two expanded blastocysts with intact capsules (533 $\mu$ m and 724 $\mu$ m diameter) began to slowly increase to ~60% of their original volume between 32 and 64 minutes. Between 128 and 720 minutes the intact embryos decreased to 55% of their original volume (Figure 4.2). The compromised embryo exhibited the same rapid decrease during the first 32 minutes of incubation shrinking to 45% of its original volume. The steady decrease continued between 32 to 720 minutes of incubation and never exhibited any signs of re-expansion. After incubation for 720 minutes the compromised embryo was 30% of its original volume.

In preliminary experiment 2 the expanded blastocyst (328  $\mu$ m in diameter) response to hyperosmotic and then hypotonic solutions paralleled that of the early blastocyst (204  $\mu$ m in diameter) but at a decreased expansion level (Figure 4.3). Both embryos decreased rapidly once placed in the hypertonic glycerol solution, however the early blastocyst began to equilibrate and increase slowly in volume from 57% at four minutes to 86% at 128 minutes post-exposure. The expanded blastocyst only reexpanded from 21% at 4 minutes to 28% at 128 minutes post-exposure. After placement in the hypoosmotic both embryos reexpanded rapidly. The early blastocyst increased from 86% of its original volume at the beginning of treatment to 118% at four minutes and declined sharply to 61% by 16 minutes post-treatment. The expanded blastocyst increased from 28% of its original volume at the beginning of treatment to 71% at four minutes and declined rapidly to 33% by 16 minutes post-treatment. Both embryos increased

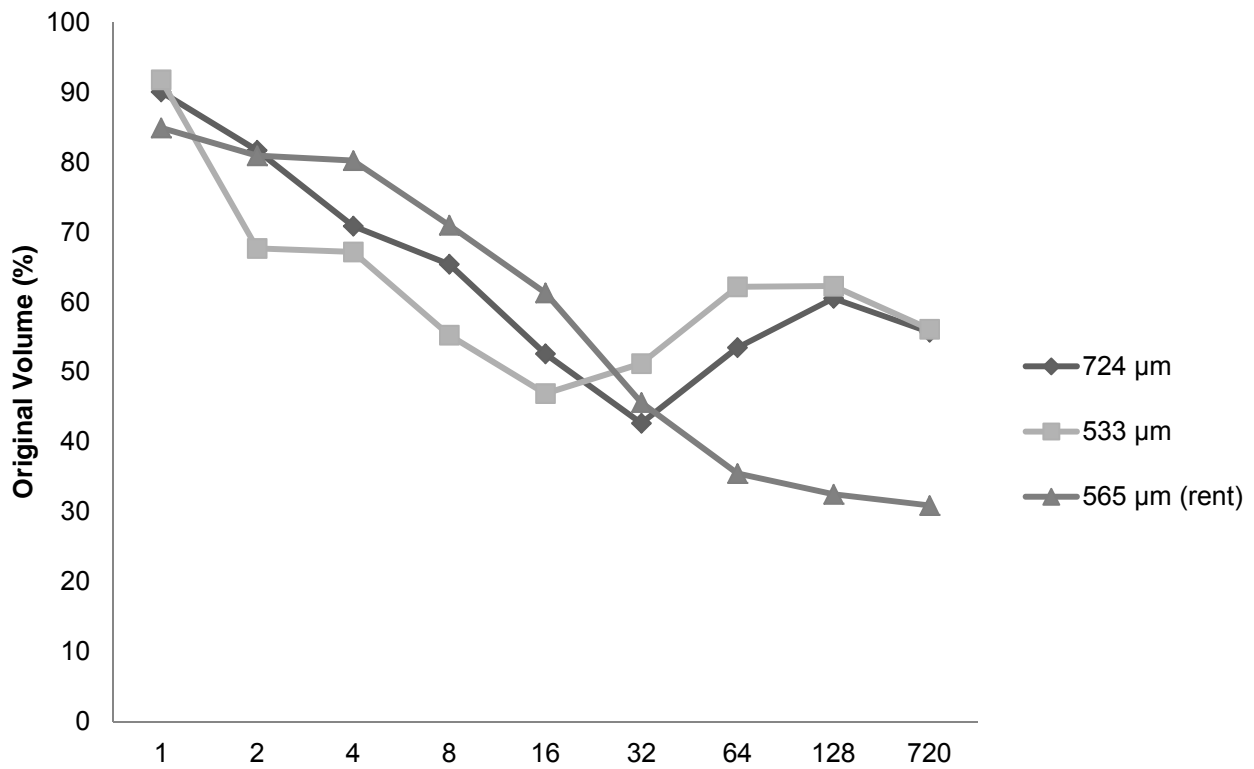


Figure 4.1 : Preliminary experiment 1 - Osmotic response of equine embryos 724  $\mu\text{m}$ , 533  $\mu\text{m}$ , and 565  $\mu\text{m}$  in diameter incubated in 1.4M glycerol solution for 720 minutes.

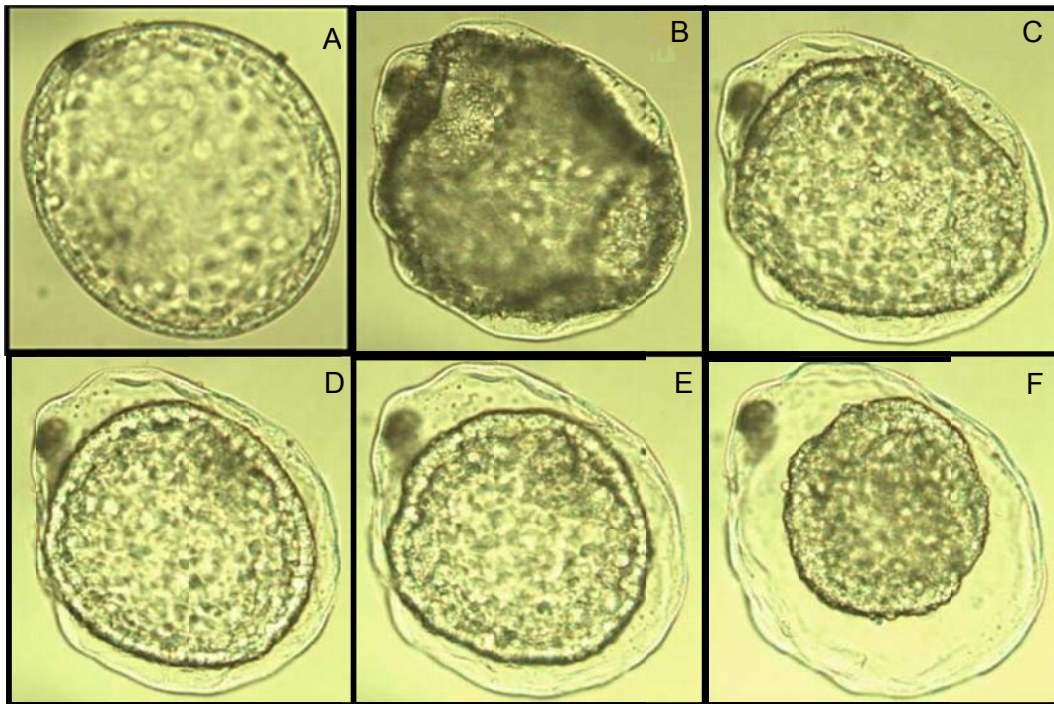


Figure 4.2 : Preliminary experiment 1 - Osmotic response of an equine embryo (533  $\mu\text{m}$ ) after exposure to 1.4M glycerol solution (1706 mmol/kg), for Panel A = pre-exposure, B = 1 minute, C = 8 minutes, D = 32 minutes, E = 128 minutes, and F = 720 minutes.

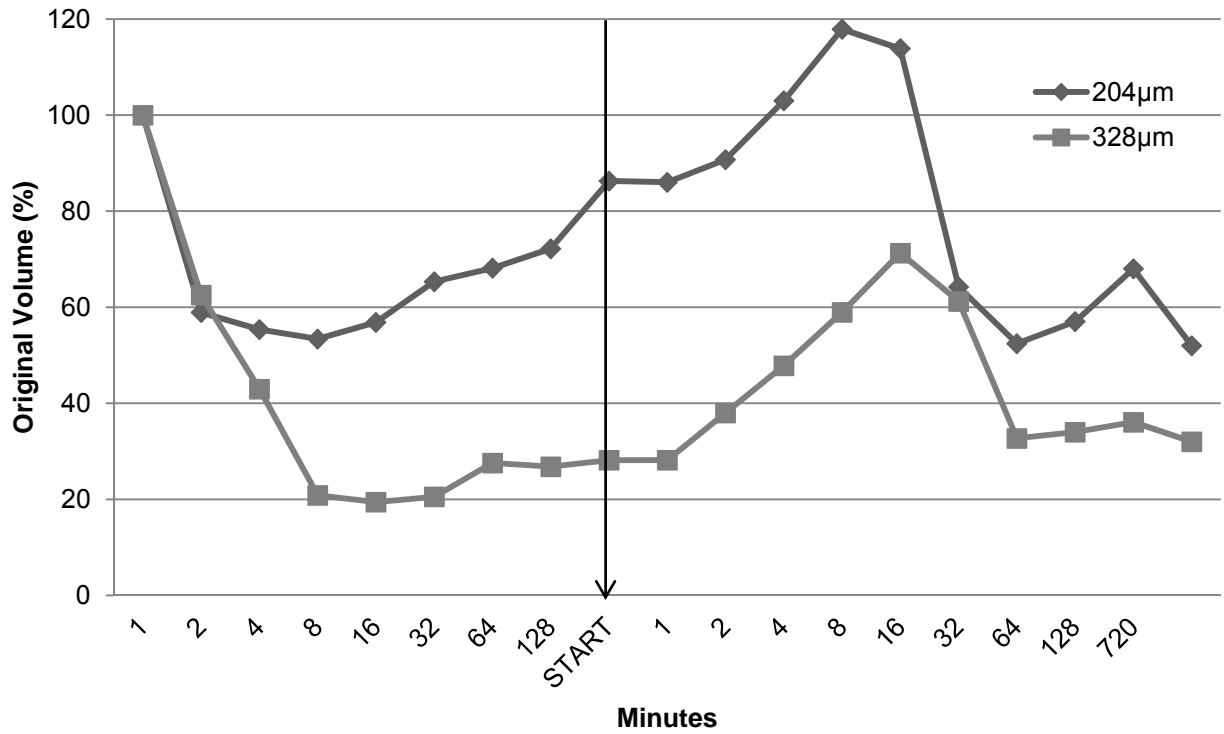


Figure 4.3 : Preliminary experiment 2 - Osmotic responses of equine embryos 204 μm and 328 μm in diameter incubated in 1.4M glycerol (right of arrow) and holding medium (left of arrow).

slightly increased from 32 minutes to 128 minutes but decreased again slightly by 720 minutes post-treatment.

### Experiment 1

In experiment 1 the mean embryo diameter ( $\pm$  standard error mean) following incubation in 1.4M glycerol<sup>3H</sup> and 3.4M glycerol<sup>3H</sup> was  $696.5 \mu\text{m} \pm 108.6 \mu\text{m}$  and  $925.9 \mu\text{m} \pm 214.1 \mu\text{m}$  respectively and were not different ( $P=0.44$ ) (Table 4.1). Percent permeation data was not normal when evaluated. Box plot analysis was then used to identify outliers in the data set (Figure 4.4). In the 3.4M group, the  $314\mu\text{m}$  embryo exhibited permeation of 79.96%. This permeation was more than two standard deviations from the mean of the group classifying it as an outlier and subsequently it removed from the data set (Table 4.1). The percent permeation between the 1.4M glycerol and 3.4M glycerol were not different ( $P=0.68$ ) (Figure 4.5).

Differences in percent permeation were then compared between embryos  $>400\mu\text{m}$  and  $<400\mu\text{m}$  in both the 1.4M and 3.4M groups. With 1.4M treatment, embryos  $<400 \mu\text{m}$  had higher ( $P=0.002$ ) permeation compared with embryos  $>400 \mu\text{m}$  ( $8.32\% \pm 3.85\%$  and  $0.35\% \pm 0.11\%$ , respectively) (Figure 4.6).

### Experiment 2

Data is presented for all samples in Table 4.2. The 60 and 120 minute incubations did not differ in diameter ( $607.2 \mu\text{m} \pm 112.2 \mu\text{m}$  and  $914.0 \mu\text{m} \pm 498.4\mu\text{m}$ , respectively). When percent permeation was compared no difference ( $P=.262$ ) was detected between 60 minute ( $0.0061\% \pm 0.0015\%$ ) and 120 minutes ( $0.0036\% \pm 0.0006\%$ ).

Table 4.1 : Experiment 1 - Equine embryo permeability when incubated in 1.4M or 3.4M concentrations of glycerol<sup>3H</sup> for 15 minutes

Treatment	Embryo Diameter	Permeation (%)	Treatment	Embryo Diameter	Permeation (%)
1.4M Glycerol <sup>3H</sup>	295	18.69	3.4M Glycerol <sup>3H</sup>	284	1.07
	307	0.44		314*	79.96*
	344	5.43		406	0.51
	373	8.70		418	2.64
	435	0.87		571	7.34
	514	0.95		582	0.25
	554	0.37		642	0.57
	567	0.02		672	0.67
	659	0.60		711	0.40
	796	0.16		786	0.49
	833	0.17		1160	0.37
	1120	0.13		1448	0.06
	1338	0.18		1650	0.79
1616	0.05	3318	0.75		
Mean±SEM	696.5 ± 108.6	2.6 ± 1.4		925.6 ± 214.1	1.2 ± 0.5

\*Sample was identified as an outlier and removed from data set

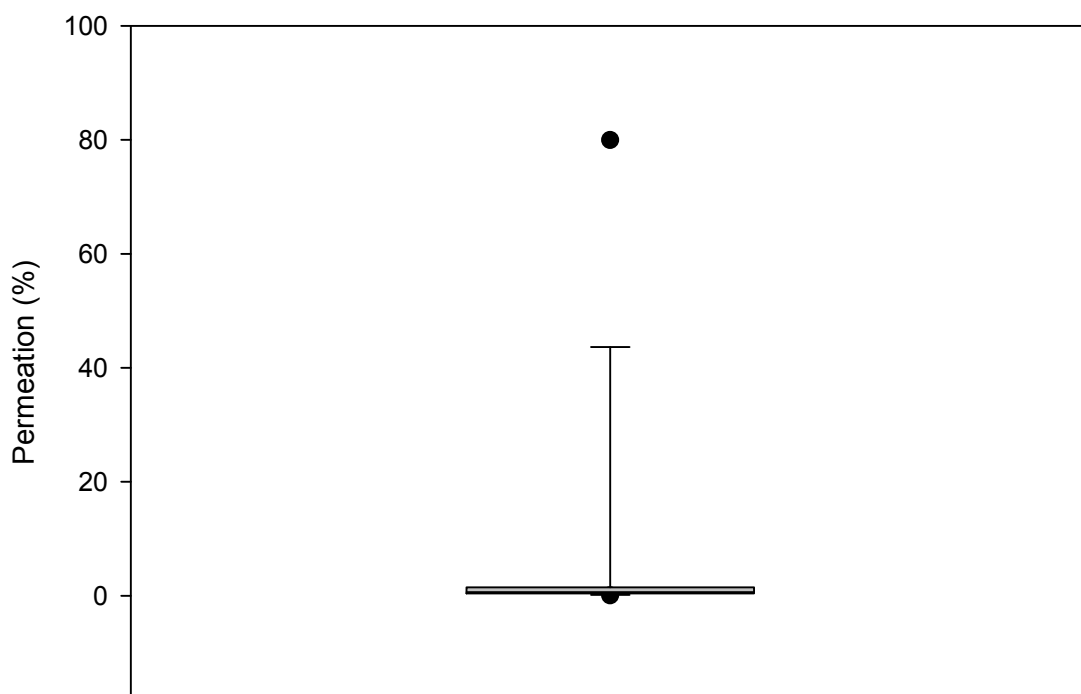


Figure 4.4 : Experiment 1 - Box plot analysis of 3.4M glycerol<sup>3H</sup>.

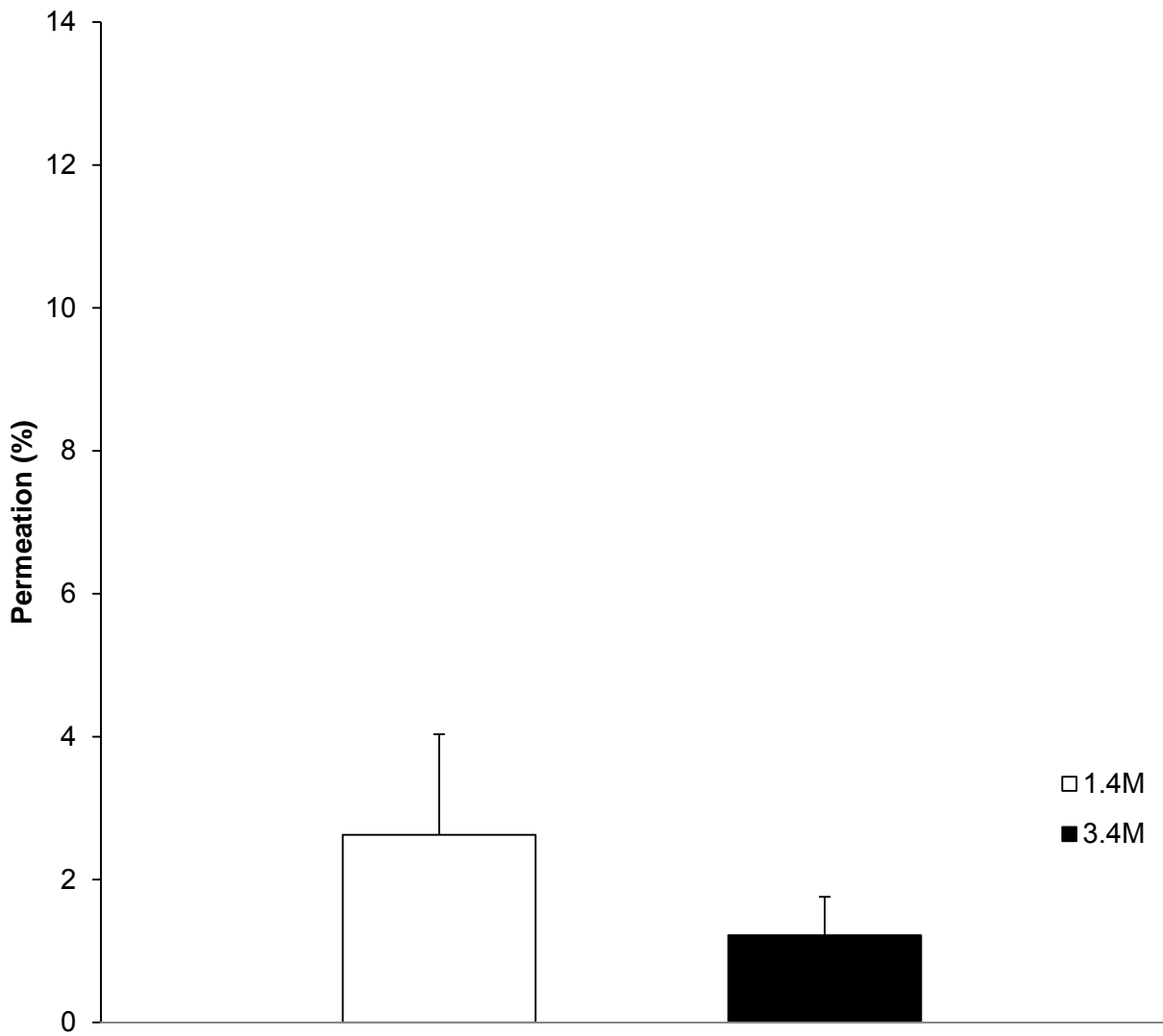


Figure 4.5 : Experiment 1 - Mean percent permeation of equine embryos incubated in glycerol<sup>3H</sup> for 15 minutes.



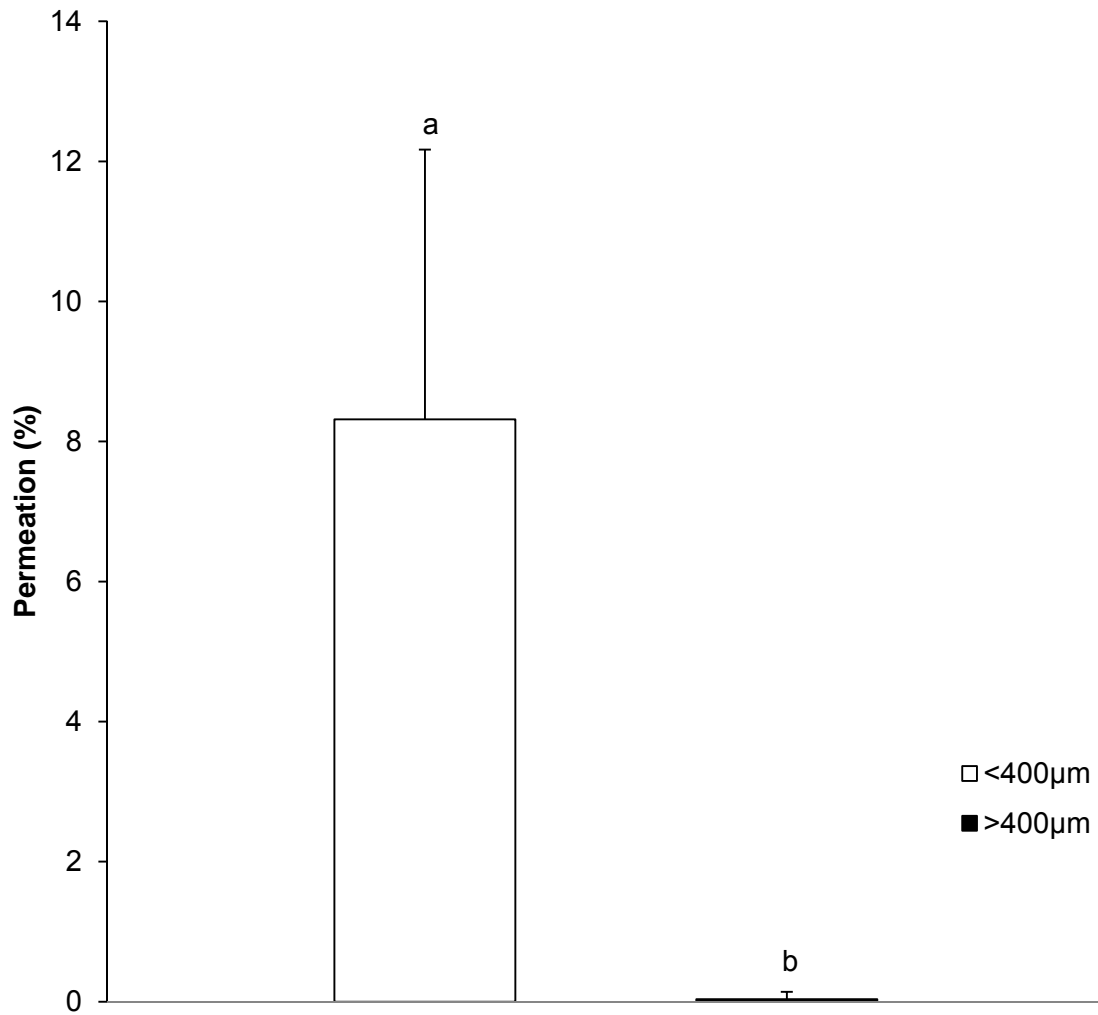


Figure 4.6 : Experiment 1 - Mean percent permeation for embryos <400µm vs. >400µm in diameter incubated in glycerol<sup>3H</sup> for 15 minutes.  
<sup>a,b</sup> Bars with different superscripts are significantly different (P=0.002)

Table 4.2 Experiment 2 - Effect of incubation time on 1.4M glycerol permeation

15 minutes <sup>1</sup>	30 minutes <sup>1</sup>	60 minutes <sup>1</sup>	120 minutes <sup>1</sup>
0.0045	0.0010	0.0006	0.0042
	0.0072	0.0092	0.0024
		0.0060	0.0040
		0.0080	
		0.0062	

<sup>1</sup>Values represented as percentage permeation.

#### 4.4 Discussion

These experiments characterized the osmotic behavior of equine embryos in response to incubation in varying osmotic solutions. Osmotic responses of bovine (Széll et al., 1989), caprine (Seidel, 1989), equine (Pfaff, 1993; Barfield et al., 2010), porcine (Men et al., 2005), and ovine (Széll et al., 1989) embryos in various osmotic solutions have also been reported. Széll et al. (1989) demonstrated that in hypertonic solutions of permeating solutes permeation and the process of osmotic equilibration begins immediately after the embryo comes into contact with the solution and is visualized as a rapid decrease followed by a gradual increase in volume. The extracellular osmotic pressure is counteracted not only by volume loss of the embryo, but also by the permeating solute. The equilibration of embryos after volume loss is the result of cryoprotectant permeation. Széll et al. (1989) concluded that the more permeable an embryo is to a cryoprotectant the less initial volume loss occurs. These findings define the processes that occurred after subjecting equine embryos to the hyperosmotic glycerol solution.

Preliminary results in our study demonstrated that equine embryos do exhibit the same rapid loss of volume when placed in hyperosmotic cryoprotectant solutions as have been reported in other domestic species. However, this initial volume was never fully recovered, even when incubated for extended periods of time. Equine embryos possess the ability to respond to hyperosmotic and hypoosmotic solutions (collapse and equilibration of the blastocoel cavity), indicating that there is some fluid movement across the capsule membrane with the embryos in our study.

Various studies have reported that ethylene glycol may be more permeable than glycerol in bovine (Széll et al., 1989), equine (Pfaff et al., 1993), and ovine (Széll et al., 1989) embryos. Pfaff et al. (1993) incubated equine embryos of various sizes (<250  $\mu\text{m}$ , 250 to 500  $\mu\text{m}$ , and >500  $\mu\text{m}$ ) in either ethylene glycol and glycerol for 20 minutes. Embryos <250 $\mu\text{m}$  and embryos

between 250-500 $\mu$ m incubated in ethylene glycol rapidly lost 50% of their original volume but regained 80% after 20 minutes of incubation. This trend is similar to that found in bovine and ovine embryos (Széll et al., 1989). Medium-sized embryos incubated in glycerol decreased to 40% of their original volume by two minutes post-incubation and volume did not change after 10 minutes. Embryos >500  $\mu$ m exhibited a steady decline in volume over the 20 minute incubation. These results are similar to the ones observed by Barfield et al., (2010) and in our study.

Barfield et al. (2010) evaluated the osmotic response of equine embryos 300 to 600 $\mu$ m (with and without capsules) when incubated in 0.75M ethylene glycol + 0.75M methanol, 1.5M methanol, or 1.5M glycerol. Upon removal of the capsule and before applying a treatment, embryos lost an average of 22% of their original volume and did not fully recover to 100% of its initial volume. Embryos incubated in 0.75M ethylene glycol + 0.75M methanol with capsules initially lost 30% of their original volume but then recovered to 92% of original volume at the end of 60 minutes. Embryos without a capsule initially lost ~15% of their volume and returned to 87% of initial volume. When embryos were incubated with intact capsules in 1.5M methanol, volume initially increased 10%, and then decreased to 95% original volume after 1 hour. Embryos without capsules remained 5% larger when incubated in 1.5M methanol. Barfield et al., (2010) incubated equine blastocysts in 1.5M glycerol. Embryos with capsules decreased to 40% of original volume within 2 minutes and did not recover any volume during incubation, while embryos without capsules decreased to 75% of their original volume gradually over 60 minutes.

These results agree with findings in our study. However, volume of all intact embryos used in our study began to regain volume after 60 minutes of incubation in 1.4M glycerol, this may be attributed a shorter incubation time in previous studies. This slight influx could be glycerol slowly permeating into the embryo causing an increase in volume. This study is the first to incubate equine embryos in cryoprotectant solutions for longer than 60 minutes. The embryo

with a compromised capsule behaved similar to those of Barfield et al. (2010) in that volume constantly declined throughout the experiment. These experiments suggest that the equine capsule may impede movement of glycerol into the embryo. Because glycerol is a larger molecule when compared to ethylene glycol (Gordiyenko et al., 2004), it may cross the capsule of large equine embryos less rapidly (Pfaff et al., 1993).

Numerous cryoprotectants have been utilized in the effort to find a suitable freezing procedure for the larger day-7 equine blastocyst. Glycerol is commonly used in equine embryo cryopreservation at different concentrations depending on freezing protocol, 1.4M is typically utilized for slow freezing (Poitras et al., 1994; Young et al., 1997) and 3.4M is typically utilized for vitrification (Eldridge-Panuska et al., 2005; Hudson et al., 2006).

Some success in cryopreserving large equine embryos has been reported. Young et al. (1997) utilized large equine embryos (300 to 680 $\mu$ m) and subjected them to step-down equilibration with 2M glycerol supplemented with galactose. In this study embryos subjected to this treatment possessed higher grades post thaw and also increased in diameter when compared to post-flush diameter, indicating growth in vitro. Young et al. (1997) also subjected large embryos to vitrification concentrations of cryoprotectant and none of the embryos were reported to be viable post-thaw. These findings are consistent with previous work that demonstrated that equine blastocyst >300 $\mu$ m are more sensitive to vitrification damage than smaller blastocysts (Hochi et al., 1995b).

In our study using large equine blastocysts, 3.4M glycerol was less permeable than 1.4M glycerol. This finding supports work reported by Hochi et al. (1995) and Young et al. (1997) and demonstrates that limited success of conventional cryopreservation and lack of success in the vitrification of large embryos may be due, in part, to the permeability of the two solutions. The probable cause of reduced embryo viability during freezing and thawing of large embryos is the

disruption of cell organelles, in particular the cytoskeleton, as a result of intracellular ice formation (Dobrinsky, 1996).

Studies have shown that acceptable pregnancy rates (50-60%) can be achieved with conventional slow-freezing of embryos at an early developmental stage (morula to early blastocyst) that are less than 300  $\mu\text{m}$  in diameter (Czlonkowska et al., 1985; Slade et al., 1985a; Skidmore et al., 1991; Squires et al., 2003). This demonstrates that embryos  $<250 \mu\text{m}$  equilibrate more readily and allowing cryoprotectant solution into protect cells during the freeze/thaw process (Pfaff et al., 1993; Barfield et al., 2010). In our study, embryos  $<400\mu\text{m}$  exhibited more permeation when subjected to 1.4M glycerol than embryos  $>400\mu\text{m}$ . These findings suggest that small equine embryos allow more cryoprotectant to permeate into the embryo and protect cells.

The equine embryo enters the uterus 6 to 6.5 days post-ovulation (Battut et al., 1997) at the late morula or early blastocyst stage completely enveloped in the zona pellucida (Betteridge et al., 1982). During the next 24 hours blastulation and the blastocyst capsule begins to form and is primarily produced by the trophoblast cells and is composed of a mucin-like glycoprotein (Oriol et al., 1993b). The capsule is completely formed by day 7 post-ovulation at the blastocyst stage (Flood et al., 1982).

In experiment 1, 1.4M samples were divided comparing samples without a developed capsule and samples with a fully developed capsule ( $<400 \mu\text{m}$  and  $>400 \mu\text{m}$  respectively). Embryos without a developed capsule ( $<400 \mu\text{m}$ ) took up more labeled glycerol solution than embryos with a developed capsule ( $>400 \mu\text{m}$ ) ( $P=0.002$ ) possibly due to a lack of, or thin capsule, and support previous studies (Pfaff et al., 1993; Legrand et al., 2002; Duchamp et al., 2006; Barfield et al., 2010). Furthermore, larger equine embryos ( $>400 \mu\text{m}$ ) allowed  $<2\%$  cryoprotectant permeation by volume indicating that embryos are composed of 98% water when

cryopreserved. This high water content increases the chance of ice crystal formation and intracellular ice damage, resulting in non-viable embryos. Our results provide quantitative data that suggest that the capsule may be the cryoprotectant permeability barrier, therefore causing limited success in the cryopreservation of large equine embryos.

Permeation of glycerol<sup>14C</sup> into fertilized and unfertilized mouse ova occurs at a constant rate and took up to 200 minutes for equilibration to complete (Jackowski et al., 1980). Our results show no differences in percent permeation between 60 and 120 minutes of incubation in 1.4M labeled glycerol. Total permeation was less than 0.05% across all treatments. Percent permeation was higher in experiment 1 were higher than those obtained from experiment 2. This may have been a result of different washing techniques used in each procedure following incubation. This washing procedure was adapted from previous work conducted in the same laboratory testing the effects and permeability of radiolabelled nicotine in zebrafish embryos (Thomas et al., 2009). Zebrafish embryos are also encased two protective layers, the chorion and yolk syncytial layer (Harvey et al., 1983; Kimmel et al., 1995). It has been demonstrated that both layers are durable and resistant to cryoprotectant permeation (Harvey et al., 1983). The physical structures of embryos processed by Thomas et al.,(2009) were easily visualized with the naked eye and unaffected during the washing procedure. However, the wash procedure in experiment 2 did not allow the visualization of equine embryos after processing. Therefore, the results indicate that the embryos may have been compromised, resulting in the loss of radiolabelled cryoprotectant. Any breach in capsule membrane would have resulted in the accelerated loss of glycerol<sup>3H</sup> due to the increased temperature during processing as demonstrated in mouse ova by Jackowski et al.,(1990).

Advancements in equine embryo cryopreservation have been restricted by the limited success of cryopreserving large day 7 blastocyst. The lack of success has been attributed to the

formation of the capsule membrane around the embryo day 7 post-ovulation (Legrand et al., 2000). Our data demonstrates that glycerol more readily permeates into embryos <400µm that do not possess a functional capsule compared with embryos >400µm with a fully formed capsule. This suggests that the capsule membrane is the cryoprotectant permeability barrier and the cause of limited success in the cryopreservation of large equine embryos.



## CHAPTER V

### CONCLUSION AND SUMMARY

Cryopreservation of embryos allows genetics to be spread across a greater population when compared to natural service and pregnancy. Collecting and cryopreserving equine embryos on day 7 post-ovulation would increase the overall efficiency of the cryopreservation procedure. Lack of success has been attributed to blastocyst capsule formation (Oriol et al., 1993b) which is completely formed by day 7 post-ovulation at the blastocyst stage (Flood et al., 1982). Studies have shown that acceptable pregnancy rates (50-60%) can be achieved if conventional slow-freezing is used, embryos to be cryopreserved are at an early developmental stage (morula to early blastocyst), and embryos are less than 300µm in diameter (Czlonkowska et al., 1985; Slade et al., 1985a; Skidmore et al., 1991; Squires et al., 2003).

Results from our preliminary study (Chapter 4) expressed in our study show that equine embryos do exhibit the same rapid loss of volume when placed in hyperosmotic cryoprotectant solutions as seen in other domestic species (Széll et al., 1989; Seidel, 1989; Men et al., 2005). Volume of all intact embryos used in our study began to regain volume after 60 minutes of incubation in 1.4M glycerol which differs from previous studies (Barfield et al., 2010). This could be attributed to a shorter of duration of incubation in previous observations. This slight influx could be glycerol slowly permeating into the embryo causing an increase in volume. This study is the first to incubate equine embryos in cryoprotectant solutions for longer than 60 minutes. These experiments suggest that the equine capsule may impede movement of glycerol into the embryo.

Numerous cryoprotectants have been utilized in the effort to find a suitable freezing procedure for the larger day 7 equine blastocyst. Glycerol is commonly used in equine embryo cryopreservation at different concentrations depending on freezing protocol, 1.4M (Poitras et al.,

1994; Young et al., 1997) and 3.4M (Eldridge-Panuska et al., 2005; Hudson et al., 2006) In our study utilizing large equine blastocysts, 3.4M glycerol was less permeable than 1.4M glycerol. This finding supports worked reported by Hochi (1995) and Young (1997) and demonstrates the lack of success in the vitrification of large embryos may be due to the poor permeability of the cryoprotectant solutions.

In our study, embryos <400  $\mu\text{m}$  exhibited higher permeation when subjected to 1.4M glycerol<sup>3H</sup> compared with embryos >400  $\mu\text{m}$  (Chapter 4, experiment 1). These results suggest that smaller equine embryos without a capsule or possess a thin capsule can allow a sufficient amount of cryoprotectant to permeate into the embryo and protect cells.

Based on our results we concluded that time had no effect on the uptake of 1.4M glycerol<sup>3H</sup> (Chapter 4, experiment 2). Results from experiment 1 were higher than those obtained from experiment 2 and may have been a result of different washing techniques used between experiments.

We showed mean circulating progesterone concentrations of  $13.53 \pm 1.80$  for control mares and  $14.04 \pm 0.99$  for single ovulating mares which are in agreement with previous work by Dawson et al. (1977). Circulating progesterone concentrations were higher in mares where two embryos were recovered compared with mares producing single embryos and control single ovulating mares. Circulating progesterone concentrations in mares producing two embryos ranged from 17 to 34 ng/ml in our study. Progesterone concentrations of single ovulation producing a single embryo >400  $\mu\text{m}$  tended to be higher than single ovulating mares that produced an embryo <400  $\mu\text{m}$ , but not different from noninseminated control mares (Chapter 3).

We also demonstrated there was no correlation between mare corpus luteum diameter, circulating progesterone concentration, and embryo diameter during early pregnancy which

agrees with results described by Papa (1998). However, in our study, circulating progesterone, corpus luteum diameter, and embryo diameter were recorded from 23 research mares and not an entire population. Mare circulating progesterone concentration and embryo diameter were correlated in mares producing embryos <1000  $\mu\text{m}$  recovered on day 7 post-ovulation, in our study. Circulating progesterone concentration and embryo diameter was also correlated in mares producing twin embryos in our study. Embryo diameter has not been correlated with circulating progesterone concentration and could be valuable in projecting the potential diameter (and therefore stage) of embryos prior to non-surgical collection. This would alleviate the need for monitoring ovulation via transrectal ultrasonography every 12 hours, in order to collect an embryo small enough for cryopreservation (early blastocyst).

Advancements in equine embryo cryopreservation have been restricted by the limited success of cryopreserving large day 7 blastocyst. The lack of success has been attributed to the formation of the capsule membrane around the embryo day 7 post-ovulation (Legrand et al., 2000). Our results provide quantitative data that suggest that the capsule may be the cryoprotectant permeability barrier, therefore causing limited success in the cryopreservation of large equine embryos. This information combined with correlations in mare circulating progesterone concentration on day 7 post-ovulation and single ovulated embryos <1000 $\mu\text{m}$  and double ovulated twin embryos recovered could be utilized to develop a more sensitive chute-side test similar to the Target Equine Progesterone Assay. This would alleviate costs and labor involved with monitoring via transrectal ultrasonography for ovulation to collect an embryo at the proper stage for cryopreservation and in detecting a twin pregnancy.

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## VITA

Brittany Reshel Scott was born April 7, 1987 in Nacogdoches, Texas. She received her elementary and high school education from Broaddus ISD in the small town of Broaddus, Texas. She was very involved in extracurricular activities during school, especially sports and agriculture and related programs. She won various awards throughout her career in agricultural mechanics and FFA leadership events, while showing broilers, steers, and horses. This involvement in agriculture is what inspired her to pursue a degree in animal science.

Following graduating high school with honors in 2005, she attended Sam Houston State University in Huntsville, Texas, for two years pursuing a bachelor's degree in animal science. Due to family illness she transferred to Stephen F. Austin State University in Nacogdoches, Texas, to complete her degree while assisting her family. In August 2008, she graduated from Stephen F. Austin Magna Cum Laude with a Bachelor of Science degree in agriculture.

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