EFFECTS OF EXERCISE OR OOCYTE HEAT SHOCK ON EMBRYO DEVELOPMENT AND GENE EXPRESSION IN THE HORSE

A Dissertation

by

CHRISTOPHER JOHN MORTENSEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2007

Major Subject: Animal Science

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ABSTRACT

Effects of Exercise or Oocyte Heat Shock on Embryo Development and Gene Expression in the Horse. (May 2007)

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Horse owners commonly maintain their broodmares in training and competition during the breeding season. The effect this has on mare reproductive efficiency has received limited attention. Heat stress has shown to be detrimental to oocyte competence in other species and heat shock protein 70 has been shown to be an important gene in regulating cellular response to heat. Mares were exercised in a hot humid environment to determine the effects on reproductive efficiency. Embryos were collected at d 7 after ovulation from exercised and control mares. Oocyte developmental competence was measured after oocytes were subjected to a one time heat shock, 42 ºC for 2 or 4 h, at the onset or near completion of in vitro maturation. Embryos from both previous experiments were examined for HSP70 gene expression by real time RT-PCR. Exercised mares ovulated significantly smaller follicles, 39.8 vs. 41.5 mm diameter, and ovulated later after being given PGF2α, 8.5 vs. 9.2 d. Twenty-two embryos (22/35) were recovered from control mares, recovery rate of 63%. Significantly fewer embryos were recovered in exercised mares (11/32), recovery rate of 34%. A lower proportion of grade 1 embryos were recovered from exercised versus control mares (4/11 vs.16/22,

respectively). No effect was observed on oocyte nuclear maturation or embryonic development after ICSI when oocytes were exposed to heat shock at the onset of IVM. A heat shock of 42 ºC for 2 or 4 h on oocytes during late IVM resulted, however, in a significantly lower rate of nuclear maturation, and a significant decrease in advanced embryo development (morulae plus blastocysts). Heat shock protein 70 gene expression was shown to be related to quality score of in vivo-recovered embryos, with lower quality embryos recording a significantly higher relative expression. Heat shock of late stage IVM oocytes for 4 h resulted in significantly higher blastocyst HSP70 expression. Results of this study indicate that exercise in a hot humid environment is detrimental to mare reproductive efficiency, late-stage maturing oocytes are sensitive to heat, and HSP70 expression in equine embryos is related to embryo quality score and oocyte quality.

DEDICATION

To the Horse

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A wise man once said "Whoever ceases to be a student, has never been a student" (George Iles). My philosophy in pursuing my advanced degrees is to dedicate my life to the pursuit of knowledge to better this world for horses and other animals. This road has proven to be tough at times but has been rewarding just the same. No one man accomplishes extraordinary things alone. Thank you to all who have helped me along the way.

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CHAPTER I

INTRODUCTION

Efficiency of reproduction is of vital importance to the equine industry. Many complex processes are involved in reproduction and interruption during any of these steps can lead to failure of the mare to conceive or maintain pregnancy. Many owners today maintain their mares in competition and/or training throughout the breeding season. These owners utilize the reproductive technology of embryo transfer to allow their mares to remain in training while being bred and may unwittingly be lessening their chances of recovering an embryo. If an embryo is recovered, it may be of poorer quality and/or retarded in its growth, thus reducing their chances for a foal.

When compared to other species of livestock, horses are often seen as inefficient in conception and maintenance of pregnancy. This in reality is a misconception. Due to the demands we have placed on the horse, particularly the annual January 1st birth date for most breed registries, producers try to breed their horses early in the calendar year, outside of their natural breeding season. Other factors that may induce stress such as transport and/or heat have the capacity to suppress conception rates.

In 2002, the American Quarter Horse Association (AQHA), the largest horse breed registry in the United States, altered their rules and regulations to allow multiple registrations of foals per mare per year. Previously, only one foal per year could be

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This dissertation follows the style of The Journal of Animal Science.

registered from a given mare and, if desired, the owner could utilize a surrogate mare (recipient) to carry this foal to term. With these changes in regulations, the industry saw an increased use of embryo transfer and increased interest in reproductive efficiency of the mare.

It is common for young, valuable mares to remain in training throughout the breeding season, with the owner utilizing embryo transfer to prevent interruption of the mare's training and/or competition schedule (Squires et al., 2003). Exercise or training can possibly alter the reproductive cycle of mares and/or lessen their chance to conceive a viable embryo. One of the byproducts of exercise is a substantial increase of heat in the horse (Hodgson et al., 1994). Exercise for as little as 30 minutes can lead to a body temperature increase of 2.5 ºC (McCutcheon and Geor, 2000). Exercise also stimulates a stress hormonal response, and an increase in corticosteroids has been shown to suppress important reproductive hormones such as gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH; Kalantaridou, et al., 2004).

Sertich (1989) reported a depressed rate of 40.5 % in embryos recovered (n = 111) in performance mares over a 4 yr period. All of the mares studied were maintained in training and competition during the breeding season. This is in contrast to reported embryo recovery rates of a slightly higher 50% (Squires et al., 2003) and up to an optimal 75% rate (Vogelsang et al., 1985). The lower recovery reported by Sertich (1989) may be due to individual mare or stallion fertility, or to recovery technique, as no control mares were utilized in that report. However it is possible a slightly lower embryo recovery rate is found in performance mares. Supporting this, among the

veterinary community, it is often perceived that mares subjected to work have inferior fertility rates; mares that remain in competition during the breeding season produce fewer embryos and/or fail to conceive (K. Hinrichs, Texas A&M University, College Station, TX, personal communication 2005).

Thermal stress and its detrimental effects on female mammalian fertility have been well documented in other species. Hansen et al. (2001) states that it is likely that reproductive function will be compromised in a cow whose body temperature increases approximately 1º C or more for a period of more than 4 hours, indicating that temperatures above homeothermy can disrupt maternal reproductive function.

The reproductive processes in the male and female mammal are very sensitive to disruption by hyperthermia with the most pronounced consequences being reduced quantity and quality of sperm production in males and decreased oocyte and early embryonic developmental competence in females (Hansen et al., 2001). Thermal stress and its detrimental effects in mammalian fertility were first documented by Dr. Lester C. Ulberg in 1958 at North Carolina State University (Ulberg, 1958). Since Dr. Ulberg's early scientific investigation into heat stress, there has been substantial research into its possible detrimental effects in many species, however little work has been done in the horse.

The aim of this research was to investigate the effects of exercise and heat stress on mare fertility, equine oocyte developmental competence, and heat shock expression by the early developing equine embryo. Heat stress was induced in vivo through exercise in a hot and humid environment and the effects of this treatment were measured by

evaluating embryo recovery rate and embryo quality score. To investigate heat effects on oocyte competence, early- and late-stage maturing oocytes were subjected to a one-time heat shock, and their competence measured by their progression to metaphase II and to the blastocyst stage after fertilization by intracytoplasmic sperm injection (ICSI). Heat shock protein 70 (HSP70) expression in d 7 in vivo- and in vitro-derived embryos was measured utilizing real time RT-PCR to gauge the embryo's ability to respond to cellular stress. Therefore the objectives of this research were as follows:

1. To investigate the effects of exercise in the mare on embryo recovery rates and quality grade;
2. To investigate equine oocyte meiotic and developmental competence after a one- time heat shock during in vitro maturation;
3. To investigate HSP70 expression in d 7 equine embryos exposed to exercise stress in vivo or heat shock in vitro, through the use of real time RT-PCR.

The results of this research can provide valuable information to horse owners on how to better manage their mares during the breeding season. If exercise and heat stress proves to be detrimental to mare fertility, owners may want to closely manage their breeding mares to limit their training/competition schedule, or limit the exposure of their mares to other stressors such as heat and/or transport during times of high environmental heat and humidity.

CHAPTER II

REVIEW OF LITERATURE

Historical Perspective

Horses were first thought to be domesticated in the Near East between 4500 and 2500 B.C. (Evans, 2001). Through the centuries, equine reproduction has progressed from a hobby to serious scientific investigation. In 1332 A.D., legend has it; an Arab chieftain who coveted a rival's stallion had a spy steal some of the stallion's semen from a recently mated mare and used the semen to artificially inseminate one of his own mares (Allen, 2005). Reproductive technologies, such as artificial insemination, did not emerge as a documented science in the horse until 1898 with Sir Walter Heape's report of artificial insemination of the mare (Heape, 1898; Allen, 2005). Since this first documented report, equine reproduction has been a focus of researchers worldwide.

The earliest report of heat stress and its negative impact on female reproduction was documented in the ewe by Ulberg (1958). Early researchers of the era stated that reproductive efficiency is lowered during periods of high environmental temperature and this lower efficiency may result from the abnormality of ova, a lowering of the fertilization rate, or an increase in early embryonic mortality (Alliston et al., 1961). This began a flurry of research to investigate the detrimental effects of high ambient temperature and humidity on female reproductive efficiency.

Poor reproductive performance of domestic farm animals in hot, humid environments was a generally accepted premise at the time (Dunlap and Vincent, 1971 Ulberg and Sheean, 1973). Investigation involved species such as cattle (Dunlap and Vincent, 1971; Gwazdauskas et al., 1973; Ingraham et al., 1974), pigs (Warnick et al., 1965; Tompkins et al., 1967; Omtvedt et al., 1971), sheep (Alliston et al., 1961; Dutt, 1963; Thwaites, 1969), rabbits (Ulberg and Burfening, 1967) and mice (Burfening et al., 1970). These early studies all have shown detrimental effects of hyperthermia with harmful effects on embryo development and depressed fertility rates. The conclusions drawn by these scientists were that periods of transitory infertility exist in females subjected to high air temperature (Ulberg and Sheean, 1973).

Ewes subjected to high air temperature at the time of mating have a reduced reproductive rate because of an increased embryonic death rate (Ulberg and Burfening, 1967). Alliston et al. (1961) reported ewes maintained at 33.3 ºC had an increased rectal temperature of 39.4 ºC in comparison to ewes maintained at 18.3 ºC with reported rectal temperatures of 38.7 ºC. Both Alliston et al. (1961) and Dutt (1963) reported that ewes maintained under hot environmental conditions had reduced fertility rates. In addition, Alliston et al. (1961) reported higher percentages (86 vs. 13%) of abnormal embryos recovered from ewes maintained at 35 ºC.

In cattle, Dunlap and Vincent (1971) reported that short-term (72 h) exposure to a continuous heat stress (32.2º C) following breeding increased average rectal temperatures to 40 ºC and had a substantial detrimental effect on conception rates. In swine, Warnick et al. (1965) reported a trend toward decreased number of live embryos

in sows maintained at 32.2 ºC for 25 d post breeding. Omtvedt et al. (1971) agreed with these findings in that gilts subjected to heat stress (37.8 ºC for 17h daily) either d 0 to 8 post-breeding or d 8 to 16 post-breeding tended to have lower conception rates and fewer viable embryos at d 30 post-breeding.

The stage at which thermal stress disrupts the reproductive process has been debated among many published reports. Early workers felt that there was a direct effect of heat stress on the sperm or the developing embryo, while other studies suggested that heat stress altered the hormonal or biochemical status of the animal (Dunlap and Vincent, 1971). It was also possible that heat stress affected all aspects of the reproductive axis, leading to suppressed fertility. Investigation continued to explore all possibilities.

Ulberg and Burfening (1967) stated that in rabbits and sheep, the stress of a slight increase in temperature for a short period of time, acting on either the spermatozoa before fertilization or on the ovum immediately after fertilization, causes the resulting embryo to die some time later in its development. They reported that exposure of 1-cell fertilized rabbit eggs to elevated temperatures in vitro reduced survival, but the harmful effects were reduced with further embryonic development suggesting that the embryos became more heat resistant as they developed. Rabbit embryos cultured in vitro through the first cell division at 40 ºC had a significantly lower survival rate than those cultured at 38 ºC when both were placed in a normal unstressed surrogate rabbit (Ulberg and Burfening, 1967). These results are in agreement with Dutt (1963) who proposed that in

the sheep the negative effects of thermal stress are diminished when the embryo is exposed later in development.

The careful examination of thermal stress and its detrimental effect on mammalian fertility began through the diligent work of these scholars. Research has continued in this area in most livestock species, with one exception: the horse. Heat stress or exercised induced-stress in the horse is an area of research that needs to be examined.

Physiological Effects of Exercise on Thermal Load in the Horse

Exercise can be very demanding on the mare and can be even more stressful in a hot and humid environment, leading to hyperthermia (overheating). One of the main physiological byproducts in exercising horses is heat. Conversion of stored energy into mechanical energy during exercise is relatively inefficient in horses, with approximately 80% of the energy being given off as heat (Hodgson et al., 1994). Under most exercise conditions, heat production is in excess of that required for homeothermy, resulting in a need for activation of thermoregulatory mechanisms if body temperature is to be maintained within a few degrees of that at rest (Hodgson et al., 1994).

The combined effects of exercise and heat stress place the cardiovascular and thermoregulatory systems under severe strain (Williams et al., 2002). When compared to humans, the horse has a low surface area:body mass ratio (1:40 man vs. 1:100 horse) and consequentially enormous demands are placed on the thermoregulatory system of the horse during exercise (Hodgson et al., 1994). McCutcheon and Geor (2000) stated that

thermoregulatory mechanisms in the exercising horse are stressed more than those in humans due to the use of a larger proportion of body mass during locomotion (greater rate of heat production per unit body mass) and a higher metabolic rate at any given workload. They further stated that due to the horse's larger surface area:body mass ratio, horses suffer from a lower mass-specific area for heat dissipation (McCutcheon and Geor, 2000). The 4 basic mechanisms that aid horses in thermoregulation are: 1) radiation, transfer of heat from the body to surrounding surfaces; 2) convection, movement of air around the horse; 3) conduction, heat transfer away from working muscles to the skin surface and 4) evaporation from the skin and respiratory tract (Hodgson et al., 1994).

Exercising horses in hot, humid environments decreases the ability of the horse to properly thermoregulate. Failure to dissipate heat during exercise can result in a potentially dangerous increase in body temperature (Kohn et al., 1999) and the horse can become hyperthermic. When air temperature is high, the skin-to-air thermal gradient is decreased and the ability to dissipate heat is reduced. Alternatively, if the relative humidity is high, rate of evaporation and, consequentially, heat loss, is diminished (Kohn et al., 1999). If exercise is continued when the mechanisms for heat dissipation are unable to match those of production, muscle and core temperatures may approach critical levels (Hodgson et al., 1994). These substantial elevations in body temperature may have deleterious effects on central nervous system function, muscle metabolism, exercise capacity (Hodgson et al., 1994) and if horses respond as other species (Hansen et al., 2001) reduced reproductive function.

The average resting normal body temperature of the horse ranges from 37.5 to 38.6 ºC (Evans, 2001). McCutcheon and Geor (2000) reported that exercise training for 30 min at 32-34 ºC (hot) and at 50% (moderate) relative humidity caused a mean increase in rectal temperatures of 2.5 ºC in exercising horses. Even in cool conditions, the high rate of metabolic heat production combined with physical constraints on convective, conductive, and evaporative heat loss result in core (rectal) temperature of 2.5 ºC after <30 min of exercise (McCutcheon and Geor, 2000). These results may be indicative of the thermal load experienced by horses even under cool environmental conditions. Exercised horses may experience significant thermal increases in body temperature despite environmental conditions.

While the study of McCutcheon and Geor (2000) reported that thermal increases in the exercising horses did not differ under cool vs. hot environmental conditions, Kohn et al. (1999) reported that exercising horses in a hot (30.6 ºC) and humid (85%) environment produced a pulmonary arterial temperature of 41.5 ºC in an average of only 21.1 min. However, in a cooler (20.2 ºC) environment with moderate humidity (53.6%) and after exercise of 46 min, the average pulmonary arterial temperature was only 40.2 ºC (Kohn et al., 1999). The differences between temperature increases in the two studies can be possibly explained by the differing exercise intensities utilized by the researchers. Furthermore, Kohn et al. (1999) reported rectal temperatures did not differ significantly from pulmonary arterial temperatures. The horses exercised in hot and humid conditions had an average rectal temperature of 41 ºC and those exercised in cooler and moderately

humid conditions averaged 40 ºC. While pulmonary arterial temperatures are slightly higher, rectal temperature seems to correlate to that of the circulation.

Geor et al. (1995) reported that the added thermal load of high ambient temperature and relative humidity impaired the regulation of body temperature in horses during both exercise and recovery. When exercised under hot (32-34 ºC) and humid (80-85%) conditions, horses attained a pulmonary arterial temperature of 41ºC in an average of 16.5 minutes versus 37 minutes under cool (20 ºC) and dry (45-55%) conditions (Geor et al., 1995). This differs slightly from the results of Kohn et al. (1999) but reflects the fact that the horses were exercised under slightly higher temperatures (32-34 ºC versus 30.6 ºC). At the conclusion of exercise, under hot and humid conditions the horses had an average muscle (middle gluteal) temperature of 42.6 ºC and under the cool and dry conditions the muscle temperatures measured an average of 42 ºC. Geor et al. (1995) further noted that impairment of thermoregulation was more profound under hot and humid conditions and was reflected by an increase in the rate of heat storage during exercise and a delay in dissipation of metabolic heat during recovery. Kohn et al. (1999) observed in horses at the conclusion of exercise in hot and humid conditions, temperatures would spike during the recovery period before they declined. This can be explained as a "lag" period, in that muscle and core temperature increased before the body's thermoregulatory mechanisms activated and excess heat was then diverted into the bloodstream (Hodgson et al., 1994). As the mechanisms for heat dissipation are often complex, time is needed for their activation and during this "lag", excess heat is stored in

body tissues which is reflected in the increases seen in muscle, blood and core temperatures (Hodgson et al., 1994).

Exercise undoubtedly leads to an increase thermal load in the horse that can last past the exercise period. Geor et al. (1995) reported that after 30 min of exercise in cool and dry conditions, rectal temperatures did not return to rest after 60 min post exercise and under these conditions horses can still maintain body temperatures exceeding 40 ºC. How this accumulated heat within the body affects mare fertility is currently unknown.

Physiological Effects of Exercise and Heat-Stress on Female Hormonal Circulation and Estrus Expression

The effects of exercise on stress hormones and female reproductive function have been well documented in other species. The hypothalamic-pituitary-adrenal (HPA) axis under stress exerts an inhibitory effect on the female reproductive system (Kalantaridou et al., 2004). In humans, the HPA hormones corticotropin-releasing hormone (CRH) and ß-Endorphin have been shown to exert an inhibitory effect on gonadotropin-releasing hormone (GnRH; Kalantaridou et al., 2004). Cortisol, as an HPA hormone, has been shown to inhibit both GnRH and luteinizing hormone (LH) secretion (Kalantaridou et al., 2004). Both GnRH and LH are important hormones in the regulation of the female mammalian reproductive cycle (McKinnon and Voss, 2005). This can possibly explain the disruption of reproductive function observed in cattle (Hansen et al., 2001; Rensis and Scaramuzzi, 2003) swine (Wettemann and Bazer, 1985) and humans (Kalantaridou, et al., 2004).

Plasma adrenaline, noradrenaline, ß-Endorphin and cortisol have all been shown to be involved in the response to exercise and thermoregulation in the horse (Williams et al., 2002). Williams et al. (2002) reported that ß-Endorphin concentrations in horses increased by 40 min of exercise under hot (30 ºC) and humid (80% humidity) conditions but did not increase until 60 min of exercise under cool (20 ºC) and dry (40% humidity) conditions. Cortisol concentrations were significantly different from baseline after 60 min of exercise under hot and humid conditions (Williams et al., 2002). Cortisol, ß-endorphin and CRH have not been correlated with effects on mare fertility. However, the exercise-induced stress response may elicit an inhibitory effect on GnRH and LH in the horse, as documented in other species.

Heat-stressed cows have shown an increased corticosteroid concentration and it has been suggested that this alters the concentrations of circulating reproductive hormones by its inhibition of GnRH and LH secretion (Gilad et al., 1993). Gilad et al. (1993) reported that heat stress inhibited the secretion of gonadotropins to a greater degree in cows with low plasma concentrations of estradiol compared to those with higher concentrations. Heat-stressed cows may have responded with increased levels of cortisol, ß-endorphin or CRH, that suppressed GnRH and consequentially estradiol. The mechanisms by which heat stress alters the concentrations of circulating reproductive hormones in cattle are not known (Rensis and Scaramuzzi, 2003), but corticosteroid increase may partially explain altered reproductive hormone function.

Estradiol concentrations are decreased in the heat stressed cow, however there is controversy with regards to progesterone (Rensis and Scaramuzzi, 2003). Progesterone

has been shown to be increased, decreased and unchanged in various published reports as reviewed by Rensis and Scaramuzzi (2003). Rensis and Scaramuzzi (2003) hypothesize that progesterone concentrations could be influenced by either the type of heat stress observed (acute or chronic) or by the cows' dry matter intake.

In swine, it is not known whether the increase in plasma progesterone and decrease in estradiol, which occurs when gilts are exposed to elevated ambient temperature during the first 8 d after estrus, are of sufficient magnitude to alter conception rate or embryonic survival (Wettemann and Bazer, 1985). They did observe the concentration of estradiol during d 12-14 of pregnancy were lower in gilts exposed to 32 ºC than in gilts exposed to 21 ºC and concluded that these lower concentrations could possibly interrupt pregnancy recognition. The authors hypothesized that an alteration in the time of the estradiol increase in heat-stressed gilts may be related to increased embryonic losses.

Hansen et al. (2001) stated that heat stress decreased follicular growth and follicular fluid concentrations of estradiol-17ß of the first wave of dominant follicles in cattle. The plasma levels of LH and estradiol were deceased in heat-stressed cows and this was one of the main factors contributing to low fertility during the hot months of the year (Rensis and Scaramuzzi, 2003). Concentrations of plasma FSH were higher during the preovulatory period in summer (Gilad et al., 1993) and there is general agreement among researchers that FSH secretion in summer is increased due to decreased inhibin secretion from small follicles (Rensis and Scaramuzzi, 2003). Plasma inhibin concentrations in summer were lower in heat-stressed cows. These lower concentrations

were surmised to be reflecting reduced folliculogenesis since a significant proportion of plasma inhibin comes from small and medium size follicles (Rensis and Scaramuzzi, 2003).

With reduced circulating reproductive hormones disrupted by exercise and heat stress, the estrous cycle could be adversely affected resulting in reduced reproductive efficiency and estrous behavior. The mechanism by which heat stress reduces expression of estrus may be hormonal in part, since heat stress reduces circulating estradiol-17ß concentrations and can possibly increase adenocorticotropin secretion, which itself can block estradiol-induced estrous behavior (Hansen et al., 2001). Ealy et al. (1994) observed that heat stress reduced the intensity and duration of estrus in cows. Hansen et al. (2001) stated that even with lower circulating estradiol, it was also likely that estrous expression was reduced by the physical lethargy experienced by heat-stressed cows.

If exercise and heat stress cause corticosteroid elevations that suppress GnRH and LH secretion, with increasing FSH due to decreased inhibin concentrations, it is logical to conclude that folliculogenesis could be affected. Wolfenson et al. (1995) stated that follicular dynamics are altered and follicular dominance is depressed by heat stress. Heat stress in cattle changed folliculogenesis, as shown by increases in the number of large follicles and an associated decrease in number of medium-sized follicles. Furthermore, heat stress caused a decline in plasma concentration of estrogen, earlier emergence of the preovulatory follicle, and lack of decline in the number of medium-sized follicles during the follicular phase (Wolfenson et al., 1995). Hansen et al. (2001) stated heat stress seemed to suppress the ability of the first-wave dominant follicle to

exert dominance since heat stress beginning at d 1 of the estrous cycle caused an increase in number of follicles > 10mm in diameter and earlier emergence of the dominant follicle of the second follicular wave. This is supported by Wolfenson et al. (1995), who reported that heat stress induced a marked increase in the number of large follicles during wave 1, resulting in a 50% increase in the number of large follicles during the second half of wave 1. Furthermore, heat stress seemed to depress not only dominance of the first dominant follicle but also that of the second-wave dominant follicle (Wolfenson et al., 1995). In cattle during summer months, environmental heat has been shown to reduce the degree of dominance of the dominant follicle, as more medium-size subordinate follicles survive (Wolfenson et al., 1995; Wilson et al., 1998; Roth et al., 2000; Rensis and Scaramuzzi, 2003).

The consequences of heat stress in cows delaying follicle selection is that it lengthens the follicular wave and thus has potentially adverse effects on the quality of oocytes and follicular steroidogenesis (Badinga et al., 1993; Roth et al., 2000; Rensis and Scaramuzzi, 2003). Disruption in patterns of folliculogenesis could lead to ovulation of aged oocytes with lower potential for fertilization (Hansen et al., 2001).

Effect of Heat Stress on Oocyte Quality

The bovine preovulatory oocyte appears to be highly susceptible to heat stress (Lenz et al., 1983; Ealy et al., 1993, Hansen et al., 2001; Roth and Hansen, 2005). Similar observations have been reported in sheep (Dutt, 1963) and pigs (Omtvedt et al., 1971). During the immediate preovulatory stage, the oocyte is maturing through meiosis

under the influence of hormones and growth factors. Additionally, the follicle is undergoing angiogenesis, which increases the vascular supply to the growing follicle (McKinnon and Voss, 2005). Oocyte maturation involves a series of nuclear and cytoplasmic maturational events that results in acquisition of the capacity for fertilization and subsequent development (Roth and Hansen, 2005).

The negative effect of heat stress on fertility might be the result of a direct effect of high ovarian temperature on oocyte quality (Rensis and Scaramuzzi, 2003). Broussard et al. (1996) reported that oocytes obtained during hot and humid summer months were compromised and that this contributed to the increased rate of failed fertilization and/or early embryonic mortality in cattle. Under exercise or environmental heat-stress in cattle, increased body temperature can disrupt meiosis (Roth and Hansen, 2005) and negatively affect oocyte developmental competence (Putney et al., 1988a; Rocha et al., 1998).

Rutledge et al. (1999) reported seasonal effects on embryo yield in cattle when using abattoir-derived oocytes; blastocyst production was reduced in mid- to late summer when compared to stable high yields of blastocysts in the winter and fall months. Putney et al. (1988b) reported that dairy heifers subjected to heat stress during the later stages of oocyte maturation yielded a decreased number of normal embryos as compared with non-stressed heifers. Rocha et al. (1998) noted no differences detected in quality of oocytes between hot and cool seasons from *Bos indicus* cattle while there was a marked decline in oocyte quality in *Bos taurus* cattle during the hot versus cool seasons (oocytes were collected ex vivo via follicular aspiration). The capacity of the

heat-tolerant Brahman cows to produce embryos of high quality during the hot season may be due to their known ability to maintain normal body temperature, even during periods of high environmental temperature (Rocha et al., 1998; Paula-Lopes et al., 2003).

In the immediate pre-ovulatory period (24 h before ovulation, as the oocyte undergoes meiosis) the oocyte appears sensitive to damage. Exposure of superovulated cows to heat stress for 10h beginning at the onset of estrus (which correlates to the onset of resumption of meiosis in cattle) had no effect on fertilization rate but reduced the proportion of normal embryos recovered on d 7 after estrus (Putney et al., 1988b). In addition, Broussard et al. (1996) reported oocytes that were deemed "normal" based on oocyte morphology during hot summer months had significantly lower cleavage and developmental rates to the morula stage in vitro than oocytes collected during cooler periods.

Heat shock during oocyte maturation can impair resumption of meiosis and thereby attenuate the ability of the oocyte to be fertilized (Roth and Hansen, 2005). Roth and Hansen (2005) reported that when bovine oocytes were given a heat shock at 40 or 41 ºC during the first 12 h of in vitro maturation the majority of heat-shocked oocytes did not fully mature. They observed while most of the oocytes held at 38.5 ºC matured to the expected metaphase II stage of meiosis, the majority of heat-shocked oocytes were blocked at the first metaphase, first anaphase or first telophase stages (Roth and Hansen, 2005). The authors further noted that not only did heat shock block the

normal progression of oocyte maturation but that a higher proportion of these oocytes were apoptotic.

Ju et al. (2005) reported that when a one-time heat shock was applied to bovine oocytes for 1, 2 or 4 h at 42 ºC at the end of in vitro maturation, there was a trend toward decreasing oocyte developmental competence (those oocytes that when fertilized developed to the blastocyst stage in vitro). Control oocytes maintained at 38.5 ºC had a 44% blastocyst formation rate; whereas 1 h treatment at 42 ºC yielded a 38% blastocyst rate and those treated for 2 h yielded a 36% blastocyst rate (Ju et al., 2005). A significant finding of this research was that those oocytes cultured at 42 ºC for 4 hours reported a significant decrease with a 27% blastocyst formation rate. Furthermore, when studying spindle configuration of metaphase II oocytes, the authors observed that heat shock changed the spindle configuration, caused chromatin to aggregate within the oocyte and thus reduced subsequent development of the oocytes following in vitro fertilization. The changes in spindle morphology and size indicated that heat shock affected polymerization or depolymerization of meiotic spindle microtubules (Ju et al., 2005).

It is possible that oocyte maturation in the mare can be disrupted by heat stress as demonstrated in other species. In the exercising or heat-stressed mare, increased temperatures in the vascular supply to the preovulatory follicle may possibly disrupt oocyte maturation. While oocytes appear to be sensitive to disruption by heat, the early developing embryo is thought to be the stage most susceptible to thermal temperature change as it has only a limited ability to respond to stress (Al-Katani and Hansen, 2002). While oocytes that resume meiosis mature from only prophase I to metaphase II, cells of

early equine embryos continually divide and may have more opportunities in the cell cycle to be susceptible to thermal stress.

Heat Stress and Early Embryonic Development

Heat stress has been associated with poor conception rates in many species. This may not only be due to poor oocyte quality but also to disruption of the process of fertilization and/or early embryonic development. Increased average daily heat and humidity above 21ºC on a day prior to breeding was associated with a significant linear decrease in conception rates in dairy cattle (Ingraham et al., 1974). Ingraham et al. (1974) reported conception rates for cows serviced with an average heat index under 18.9 ºC was 67%, compared to cows serviced on d averaging above 24.4 ºC was 21%. Furthermore, Lenz et al. (1983) stated that the production of hyaluronic acid by the cumuli, occurrence of the acrosome reaction in sperm cells, and fertilization are all temperature-dependent processes and under elevated temperatures may be disrupted, causing reproductive failure.

Poor reproductive performance associated with thermal stress may be due to high temperature acting directly on the developing embryo (Gwazdauskas et al., 1973) with early embryonic development possibly compromised by heat stress (Putney et al, 1988b; Rivera and Hansen, 2001). During the early cleavage stages of embryonic development, the embryonic genome is largely suppressed (Al-Katani and Hansen, 2002) and the developing embryo is dependent upon the proteins and RNA stored by the oocyte for survival and further development. The equine genome is not thought to be active until

the 4-8 cell stage (Brinsko et al., 1995) and early equine embryonic development is comparable to other species.

Temperatures that have little effect on survival of most cells can be lethal to pre-implantation embryos (Rivera et al., 2003). Putney et al. (1988b) stated that the early bovine embryo is extremely sensitive to maternal heat stress prior to d 7 of embryonic development, which may severely compromise its viability and may lead to an increased rate of early embryonic mortality. The detrimental effect of heat stress on early embryonic development may be multi-factorial, with effects seen on the intrauterine environment and/or direct effects on the conceptus.

The intrauterine environment is compromised in heat-stressed cows with a decrease in blood flow to the uterus and increased uterine temperature (Roman-Ponce et al., 1977; Rensis and Scaramuzzi, 2003). These changes can inhibit embryonic development, increase early embryonic loss and reduce the proportion of successful inseminations (Roman-Ponce et al., 1977; Rensis and Scaramuzzi 2003). In contrast, Wettemann and Bazer (1985) reported that heat stress does not appear to alter uterine blood flow in gilts, although fertility is reduced.

Elevated uterine temperature may result in an increase in the metabolic rate of the bovine conceptus and thus, alter nutrient uptake and growth (Biggers et al., 1987). Increased conceptus metabolic rate, as well as decreases in nutrient secretion by the uterus, may result in retarded development (Biggers et al., 1987). In agreement with this concept, Putney et al. (1988a) stated that high environmental temperature may severely alter bovine conceptus metabolic activity in vivo and lead to reduced growth rates and

failure of the conceptus to produce biochemical signals in adequate amounts for pregnancy recognition and maintenance of corpus luteum (CL) function. Furthermore, in agreement with Biggers et al. (1987), high oviductal and uterine temperatures may have directly altered the metabolic activity of stressed embryos, resulting in developmental retardation and a reduced rate of embryonic growth (Putney et al., 1988a).

The rate of embryonic development appeared to be slowed considerably in heat-stressed heifers (Putney et al., 1989). Ryan et al. (1992) reported in cattle that chronic elevation of temperature above the normal culture temperature (40 vs. 38.6 ºC) in vitro resulted in a higher incidence of embryo death that was evident shortly after embryo hatching. While Biggers et al. (1987) reported pregnancy rates and d 17 conceptus wet weights were significantly lower in heat-stressed groups and these embryos were less than half the weight of embryos from non-heat stressed cows.

Experimental application of heat stress from d 1 to 7 after estrus reduced developmental stage attained and morphological characteristics of embryos flushed from the reproductive tract of superovulated cows (Putney et al., 1989). Putney et al. (1989) reported that when heifers were exposed to a daily heat stress (30 ºC 16 h and 42 ºC 8 h) the superovulatory response and embryo recovery efficiency were not different from control. However, the researchers reported that the embryo recovery quality was significantly lower in the thermally stressed heifers versus control (Putney et al., 1989). Additionally, they classified the embryos recovered as being at an earlier developmental (retarded) stage with the mean number of cells significantly lower in heat stressed cattle than controls, and that the proportion of embryos classified abnormal, retarded or as

unfertilized ova was significantly higher (Putney et al., 1989). A higher incidence of abnormal embryos from heat-stressed heifers included morphological abnormalities such as the presence of blastomeres extruded from the cellular mass, or degenerating blastomeres of irregular shape and with a dark granular appearance (Putney et al., 1989).

During early embryonic cleavage, the majority of the embryo cell cycle is spent in the synthesis (S) and mitotic (M) phases (Rivera et al., 2003).The S and M phases are disrupted by elevated temperatures; exposure of cells to heat shock during the S phase results in spontaneous premature chromosome condensation and micronuclear formation (Swanson et al., 1995), while heat shock during the M phase results in disassembly of the mitotic spindle, failure of cytokinesis and polyploidy (Coss et al., 1982; Vidair et al., 1993).

Rivera et al. (2003) reported that disruption of development caused by exposure of two-cell bovine embryos to a physiologically relevant heat shock (41 ºC at 6 h) involves alterations to the cytoskeleton and mitochondria. Elevated temperatures in vitro caused alterations in the nucleus, cytoplasm, mitochondria, and other organelles of early embryonic cells (Rivera et al., 2003). Some of the changes, particularly to the cytoskeleton and mitochondria, occur at temperatures characteristic of cows experiencing hyperthermia-induced infertility (Rivera et al., 2003).

Upon heat shock, it has been shown that the organelles of 2-cell bovine embryos moved centrally, leaving the periphery of the blastomere devoid of organelles which led the cellular mitochondria to be swollen (Rivera et al., 2003). Rivera et al. (2003) hypothesized that the loss of mitochondria at the two-cell stage could adversely affect

later energy metabolism unless the loss is compensated for by increased mitochondrial biogenesis. When mitochondria are damaged they can no longer maintain a proton gradient, aerobic ATP synthesis is arrested, a progressive unfolding of the inner mitochondrial cristae occurs, and several apoptosis initiator components such as cytochrome c and apoptosis-inducing factor are released (Ichas and Mazat, 1998). In addition, heat shock damages the nucleus, induces protein aggregation and induces functional and ultrastructural changes in membranes (Rivera et al., 2003). The translocation of organelles toward the center of the blastomere suggests that heat shock affected the cytoskeleton because this system is involved in active transport of organelles and other components of the cell using both microtubule and actin filament system of transport (Rivera et al., 2003). The three components of the cytoskeleton (i.e. actin filaments, microtubules, and intermediate filaments) work together to enhance structural integrity, impart cell shape, provide cell motility, and position mRNA and protein (Capco, 1993). The cellular cytoskeleton plays an important role in cell signaling (Huang et al., 1999; Rivera et al, 2003), mitosis and cytokinesis (Lodish et al., 2000; Rivera et al, 2003). These results reported in bovine embryos are in agreement with the report of Coss and Linnemans (1996) in which heat shock induced morphological alterations to the cytoskeleton in hamster embryos and hindered further development.

Embryos become more resistant to heat stress as pregnancy progresses (Ealy et al., 1994; Hansen et al., 2001). Ealy et al. (1993) observed that embryos were sensitive to deleterious effects of maternal heat stress at d 1 of pregnancy but became more resistant to heat stress effects by d 3. Rivera et al. (2003) found that hypersensitivity of

the pre-implantation embryo to elevated temperatures is a transient phenomenon; effects of heat shock on the development of cultured bovine embryos were reduced as embryos advanced in development past the 2 to 4-cell stage. Ealy et al. (1995) suggested that increased embryonic heat tolerance in d 3 and older bovine embryos may be due to the ability of the embryos to produce heat-shock proteins after activation of the embryonic genome at the 8-to 16-cell stage.

Heat Shock Protein 70 Genes

There are several proposed mechanisms that aid in helping the early embryo in surviving cellular insults, such as heat. One of the primary mechanisms hypothesized is that individual blastomeres depend on stored (constitutive) heat shock proteins or have the ability to transcribe and translate new (heat inducible) proteins in response to cellular stress. Heat shock proteins are so called due to their discovery in limiting cellular oxidative damage when cells were treated with heat (Freeman et al., 1990). These classes of proteins are known as "molecular chaperones" and can be defined as a protein that prevents improper interactions between potentially complementary surfaces and disrupts any improper liaisons that may occur (Ellis and Hemmingsen, 1989). The proposed function is to assist in self-assembly of proteins by inhibiting alternative assembly pathways that produce nonfunctional structures (Kampinga, 2006).

Heat shock proteins are members of a multi-gene family whose genes are expressed under a variety of physiological conditions (Lindquist and Craig, 1988). Four members of the smaller heat shock protein 70 family have been identified and have been

commonly called HSP70, HSC70, HSP72, P72 and GRP78 (Linquist and Craig, 1988). Members of this family are classified according to molecular weight and cytosolic members include the heat-inducible HSP70 and the constitutively expressed heat shock cognate 70 (HSC70), both play an important role in maintaining cellular function during heat shock by acting as molecular chaperones to stabilize or refold proteins damaged by heat and by blocking apoptosis (Morimoto et al., 1996; Mosser et al., 1997; Al-Katani and Hansen, 2002; Chen et al., 2006). In addition, the members of this family are nuclear genes and their proteins have been localized to various cellular compartments, including the cytosol, mitochondria, chloroplast, and endoplasmic reticulum (Chen et al., 2006).

Heat shock proteins, and more specifically HSP70, are one of the most highly conserved and widely studied stress proteins and are abundant in a variety of mammalian cells (Lindquist and Craig, 1988; Chen et al., 2006) The structure of HSP70 consists of a conserved N-terminal 44-kDa ATPase domain, a less well-conserved central 18-kDa peptide-binding domain and a C-terminal 10-kDa variable domain of unknown function (Kampinga, 2006). Heat-inducible HSP70 and related heat shock proteins bind ATP with high affinity (Zylicz et al., 1983; Welch and Feramisco, 1985; Chappell et al., 1986). ATP binding allows substrate binding, followed by ATP hydrolysis that locks the substrate to the binding domain (Kampinga, 2006).

During a number of fundamental cellular processes, including protein synthesis, transport and function, the interactive protein surfaces are transiently exposed to the intracellular environment (Kampinga, 2006). This is an unavoidable internal form of

stress that enhances the probability of formation of intracellular aggregates that can lead to eventual cell death (Kampinga, 2006). Heat shock proteins act to protect individual proteins during these processes by binding with them, and as described above, act as a molecular chaperone that prevents protein aggregation and maintains the proteins in a competent state for either refolding or degradation (Kampinga, 2006). The heat shock response can be induced when cells are treated with arsenite, amino acid analogs or oxidative stress (Kampinga, 2006). Ellis and van der Vies (1991) stated that the heat shock protein response (i.e., generation of increased amounts of heat shock proteins in response to a stimulus), can be viewed as an amplification of these proteins' basic chaperone function and that furthermore, heat shock seems to be a valid study model in mammalian cells to observe HSP gene function.

Heat shock proteins are important in protecting developing embryonic cells from the effects of heat or other cellular stress (Chandolia et al., 1999). The most predominant of the heat shock proteins, the mammalian 70-kDa heat-shock protein has been detected in mouse (Bensaude et al., 1983; Wittig et al, 1983; Thompson et al., 1995), rat (Mirkes, 1987), rabbit (Heikkila et al., 1986), porcine (Bernardini et al., 2003) and bovine (Putney et al., 1988a; Edwards and Hansen, 1997; Chandolia et al., 1999; Matwee et al., 2001) embryos under thermal stress. However, the expression of this protein has not been demonstrated in equine embryos.

As stated earlier, the limited ability of early pre-implantation embryos to respond to heat stress suggests that this is due to the embryonic genome being largely suppressed during the early cleavage stages (Al-Katanani and Hansen, 2002). Only one study

(Brinsko et al., 1995) is available on the time of onset of transcription in the equine embryo. While in this study, genomic activity did not begin until the 4 or 8-cell stage, studies have shown that bovine embryos at the 2-cell stage can undergo transcription and synthesis of the heat-inducible form of HSP70 after thermal stress (Edwards and Hansen, 1997; Chandolia et al., 1999). Investigation in the mouse has shown that HSP70 can be expressed as early as the single cell (Christians et al., 1995) and 2-cell stage (Loimas et al., 1998). In addition, Christians et al. (1995) stated that during the onset of zygotic genome activity, marked expression of the HSP70 gene indicates it appears to be the first major inducible heat shock protein.

Activation of the murine zygotic genome has been described as a succession of two phases of transcriptional activity (Christians et al., 1995). The first phase is described as "minor" at the single or 2-cell stage (this may include the ability to transcribe heat shock proteins), with "major" activation at the blastocyst stage marked by significant increased polypeptide synthesis (Vernet et al., 1992; Schultz, 1993; Christians et al., 1995). Ealy et al. (1993) suggested that increased embryonic heat tolerance in d 3 (~8 cell) and older bovine embryos may be due to the ability of the embryos to produce heat-shock proteins after activation of the embryonic genome at the 8 to 16-cell stage. While HSP70 expression has not been demonstrated in early equine embryos, due to the highly conserved nature of heat shock proteins (Lindquist and Craig, 1988; Matwee et al., 2001; Kampinga, 2006) and their demonstration to be expressed in early developing embryos in other species, it may be possible that equine embryos may respond similarly in embryonic gene expression.

Heat shock protein 70 has not been previously associated with maternal gene imprinting. However, assisted reproductive technologies (ART) in oocytes have been shown to have an effect on embryonic gene expression through these imprints (Ertzeid and Strong, 2001; Gardner and Lane, 2005; Sato et al., 2007). Hiura et al. (2006) stated that the majority of imprinted genes are thought to be epigenetically modified during the oocyte growth period. While, Lucifero et al. (2002) surmised that maternal imprints are established later in oogenesis. How heat affects oocytes and consequentially HSP70 gene expression in early embryos is not clear. However, it may alter gene expression through maternal gene imprinting and these effects can possibly be measured at the blastocyst stage of development.

CHAPTER III

MATERIALS AND METHODS

Exercise-Induced Heat Stress

Sixteen mares, two Arabian Horses, two Thoroughbreds and twelve Quarter Horses, belonging to the Texas A&M University Department of Animal Science, were utilized for this study. The mares ranged from 2 to 16 yr of age and prior to this experiment were determined to be reproductively and physically sound. Mares were maintained on pasture throughout the duration of the study, fed coastal bermuda grass hay and a daily 13% crude protein commercial concentrate. Horses used in this study were maintained under the approval of the Texas A&M University Institutional Agriculture Animal Care and Use Committee using guidelines set forth by the Federation of Animal Science Societies (1999).

This portion of the research was conducted from May to September during the years of 2004 and 2005 at Texas A&M University, College Station, TX. These were determined to be the warmest months of the year with high relative humidity for College Station, TX (NWS, 2006). Mares were randomly assigned to either an exercise group or a non-exercise (control) group. Mares initially assigned to the exercise group were given a minimum 2 wk training period before breeding. Mares were evaluated, inseminated, and subjected to uterine flush for embryo recovery for 2 consecutive cycles, then moved to the opposite group, given a cycle without reproductive manipulation and then

inseminated and flushed during 2 more consecutive cycles. Mares transferring from the control into the exercise group began exercise on the day after the second control embryo recovery. The cycle free of reproductive manipulation provided time for mares in this category to be exercised for a minimum of 2 wks (time from prostaglandin administration to ovulation, and a natural diestrus period) before being inseminated. Additionally this allowed mares that had completed the exercise regimen to go through one cycle without exercise to reduce any deleterious effects exercise may have had on reproductive function of the upcoming estrous cycle.

Throughout the duration of the study, mares were teased in groups or individually with a proven teaser stallion to determine each mare's stage of estrus. Mares displaying estrus behavior were evaluated by transrectal ultrasonography (Medison SonoVet 600®, Universal Medical Systems, Inc., Bedford Hills, NY, USA) daily to further assess ovarian follicular activity. Once a follicle greater than 35 mm diameter was detected in an estrous mare, the mare was artificially inseminated with a minimum of 500 million motile sperm from 1 of 2 proven, fertile Quarter Horse stallions. This insemination dose was diluted to 20 ml with a commercial semen extender (EZ-Mixin-CST ®, CST-ARS, Chino, CA, USA). Once mares were inseminated, transrectal ultrasonography was used daily to detect ovulation of the dominant follicle. Mares were inseminated every other d until ovulation was detected.

Mares in the exercise group were worked at a long trot and canter in a mechanical exercise pen (free exerciser) for 30 min between 1300 and 1500 h daily. Rectal temperatures were recorded before the onset of exercise, every 10 min throughout

the exercise period, at the conclusion of exercise and at 30 min post-exercise. On two separate occasions over the two years, each individual mare's temperatures were taken beyond the 30 min to determine when the mares returned to resting body temperature.

Non-surgical embryo collection by uterine flush was performed on d 7 after ovulation. Mares' uteri were infused 4 times with 500 ml flush media (Emcare Complete ®, ICP-Bio, New Zealand) per infusion utilizing a silicone 34 French catheter (Next Generation®, Exodus, York, PA, USA) and the effluent was collected through Y-junction tubing (VCI®, Har-Vet, Spring Valley, WI, USA) into a 75 um filter (Emcon ®, Exodus, York, PA, USA). Mares were administered prostaglandin (Lutalyse®, Pfizer, New York, NY, USA), 10 mg i.m., at the conclusion of the flush to cause luteolysis. The contents of the filter were rinsed into collection dishes, which were searched using a stereomicroscope at 20x for presence of an embryo. Embryos were evaluated based on a 1 (excellent) through 4 (non-viable) scale as previously described (Vanderwall, 1996) by an observer blind to the treatment.

Embryos were vitrified as described by Moussa et al. (2005) for future HSP70 expression analysis. Individual embryos were washed 4 times in DPBS (Emflush® Har-Vet) with 1% fetal calf serum. Embryos were exposed to 7.5% dimethyl-sulfoxide (DMSO) (Sigma Chemical Co., St. Louis, MO) plus 7.5% ethylene glycol (EG) (Sigma) for 3 min and in 18% DMSO plus 18% EG plus 0.4M sucrose for 1 min. Embryos were loaded into ¼ cc French mini-straws and plunged directly into liquid nitrogen.

All statistical analysis was performed using SPSS software (SPSS 10.1, Chicago, IL, USA). Days between administration of PGF2α and the subsequent ovulation, and

follicle size at the day prior to detected ovulation were analyzed by paired sample T-Test. Only those mares for which values were available in both exercise and non-exercise groups were included in these calculations. Embryo recovery rate and proportions of embryos with the different quality scores was evaluated using Fishers exact test. Differences were considered significant when $P < 0.05$.

Effect of One-Time Heat Shock on Equine Oocytes

This portion of research was conducted from January to October 2006 at Texas A&M University, College Station, within Dr. Katrin Hinrichs' research laboratory. Oocytes were collected and matured as described by Choi et al. (2006a). To measure oocyte competence under thermal stress, 2 separate experiments were utilized with oocytes subjected to a 2 or 4 h one-time heat shock of 42 ºC either at the beginning (Experiment 1) or near the end (Experiment 2) of the 24 hour in vitro maturation (IVM) period. To determine any deleterious effects of the heat shock on the oocytes, those that matured to metaphase II with the extrusion of the first polar body underwent intracytoplasmic sperm injection (ICSI) (Choi et al., 2003). All ICSI procedures were conducted by Dr. Young-Ho Choi. After ICSI, oocytes that developed to the blastocyst stage of development in vitro at d 7 or d 8 were considered developmentally competent.

Oocyte Collection. Ovaries were obtained from two abattoirs and transported to the laboratory at Texas A&M University, College Station, Texas. Ovaries were maintained at room temperature (26-34 ºC) during the 3-4 h transport time. Once the

ovaries arrived, adnexa were trimmed and ovaries were cleaned with sterilized gauze. Visible follicles were opened with a scalpel blade and the granulosa layer of each follicle was scraped using a 0.5 cm bone curette. The contents of the curette were washed into individual Petri dishes with 25 mM Hepes-buffered TCM199 with Hanks' salts (Gibco Life Technologies Inc., Grand Island, NY, USA) plus 25 μg/ml gentamicin (Gibco).

Individual Petri dishes were searched using a dissection microscope at 10 to 20x magnification for the presence of an oocyte. Classification of oocytes was conducted as previously described by Hinrichs and Williams (1997). Oocyte-cumulus complexes were classified as compact (Cp), expanded (Ex), or degenerating depending on the expansion of both mural granulosa and cumulus cells. Those oocytes with both a compact cumulus and compact mural granulosa were classified as compact. Oocytes with any sign of slight to full expansion of either the cumulus or the mural granulosa led to the classification of expanded. Only Ex oocytes were used in this study.

Oocyte In Vitro Maturation and a One Time Heat Shock. Oocytes classified as Ex were washed once in holding medium and placed in the same medium in 1.1 ml autosampler borosilicate glass vials (National Scientific Co., Duluth, GA, USA) as described by Choi et al. (2006a). The holding medium was the mixture of 40% TCM199 with Earle's' salts, 40% TCM199 with Hanks' salts, and 20% fetal bovine serum (FBS, Gibco). Each vial had 27-50 oocytes. The vial was sealed with a lid and parafilm to prevent leakage, wrapped in aluminum foil to limit light exposure and laid down on its side at 22-27 °C for 13-16 h. For in vitro maturation, oocytes were washed twice in

maturation medium consisting of TCM199 with Earle's salts (Gibco), 5 mU/ml FSH (Sioux Biochemical Inc., Sioux Center, IA, USA), 10% FBS and 25 µg/ml gentamicin.

In Experiment 1, oocytes were subjected to heat shock at the onset of in vitro maturation. In Experiment 2, oocytes were subjected to heat shock after 20 h (4 h treatment) or 22 h (2 h treatment) in vitro maturation. Five treatment groups were used in each experiment. The zero control consisted of standard in vitro oocyte maturation at 38.2 ºC for 24 h in a gassed atmosphere of 5% CO_2 in air as described by Choi et al. (2003). Zero control oocytes were cultured in droplets of maturation medium at a ratio of 10 µl medium per oocyte under light white mineral oil (Sigma). To subject the oocytes to the heat treatment, oocytes stored overnight as described above were placed in previously equilibrated maturation medium in a 1.1 ml autosampler borosilicate glass vial. The vial was sealed with a lid and covered in parafilm to prevent leakage, wrapped in aluminum foil to limit light exposure, and submerged in a programmable water bath (Lindberg/Blue, Ashville, NC, USA) at 42 ºC, for 2 or 4 h. To control for possible effects of handling, two further control groups were similarly packaged and were submerged for 2 or 4 h in a water bath (Thermo Electron Corp., Waltham, MA, USA) set at 38.2 ºC. After treatment in the water baths, oocytes were removed from the vials, placed in droplets of maturation medium under oil, and incubated (adjusted to 22 h and 20 h to make up for the time in the water bath), as the zero control oocytes. After maturation culture oocytes were denuded by pipetting in 0.05% hyaluronidase and those with a polar body were subjected to ICSI. Oocytes without a polar body were fixed in buffered formol saline, mounted on a slide with a 6.5 µl of 9:1 glycerol:PBS containing

2.5 µg/ml Hoechst 33258, and examined using fluorescence microscopy to determine the chromatin configuration.

ICSI Procedures and In Vitro Culture. The ICSI procedures were conducted as previously described by Choi et al. (2003) using a Piezo drill (Prime Tech LTD., Ibaraki, Japan). Sperm cells for ICSI were obtained from previously frozen equine semen. Semen straws were thawed at 37 ºC for 30 s, and 200 µl of the semen was placed on the bottom of a 5 ml tube containing 1 ml of Sp-CZB for swim-up. The semen was incubated for 20-min at 38.2 ºC in 5% CO_2 in air. Approximately 0.6 ml of medium was collected, transferred into a 1.7 ml polypropylene tube and then centrifuged at 327 g for 3 min. The sperm pellet was resuspended, washed once with the medium, and then the supernatant was removed leaving the sperm cells for ICSI.

All ICSI manipulations were performed at room temperature. The outside diameter of the pipette used for ICSI procedures was 7 to 8 µm, while an additional pipette of 120 to 140 µm was used to hold the oocytes. Prior to sperm injection, 1 µl of sperm suspension was placed in 3 µl of Sp-CZB (Choi et al., 2003) containing 10% polyvinylpyrrolidone (Sigma) under mineral oil. Once an individual sperm cell was selected, each spermatozoon was immobilized by applying a few pulses with the Piezo drill. Sperm injection was carried out in a separate 50 µl drop of CZB-M containing 10% FBS under oil. Once injected, oocytes were held in CZB-H containing 10% FBS at 38.2 °C in 5% CO_2 in air until all manipulations were completed.

After ICSI, injected oocytes were cultured in DMEM/F-12 (Sigma) with 10% FBS under mineral oil at a ratio of 1 µl medium per oocyte at 38.2 °C in 5% CO_2, 5% O_2, and 90% N_2 (Hinrichs et al., 2005). Two to five oocytes were cultured per droplet and maximum volume of each droplet was 5 µl. Medium was completely replaced at 3 and 5 d of culture. Any uncleaved or retarded embryos were removed at the time of each medium change. Presumptive embryos were cultured for a total of 8 d and development to blastocyst was determined on morphological examination at d 7 and 8 under a dissection microscope at 63x. Embryos not determined to be blastocysts were fixed and stained with Hoechst 33258 as described above for oocytes, to examine the number and status of nuclei. Embryos with $\geq$64 nuclei, and with an outer layer of apparent differentiating trophoblast cells were considered to be blastocysts; and those with $\geq$32 nuclei but lacking apparent organization were classified as morulae. Those embryos morphologically deemed to be blastocysts were frozen in liquid nitrogen for future HSP70 gene expression analysis.

All statistical analysis was utilized using SPSS software (SPSS 10.1, Chicago, IL, USA). Differences between treatment groups were analyzed by Fishers exact test. Differences were considered significant when $P < 0.05$.

Heat Shock Protein 70 Gene Expression Detected via Real Time RT-PCR

This portion of research was conducted from June to August 2006 at Texas A&M University, College Station, within Dr. Nancy Ing's research laboratory. The embryos used were derived from the 2 previous experiments, in vivo embryos were from the exercised induced heat stress study and the in vitro blastocysts resulting from the oocyte heat shock study. Both in vivo and in vitro embryos were classified, vitrified and stored separately in liquid nitrogen as previously described. Prior to each real time RT-PCR experiment, isolated frozen embryos were identified and thawed in DPBS (Emflush®, Har-Vet). The target gene for this research was heat shock protein 70 (HSP70).

To isolate RNA, each embryo was individually washed 4 times in RNase free PBS and put into an 0.65 ml microcentrifuge tube (Sorrenson Bioscience, Inc., Salt Lake City, UT, USA) with 50 µl of Tripure Reagent (Roche Applied Science, Indianapolis, IN, USA) and 10 µg glycogen (Sigma). To induce zona pellucida rupture and ensure cell lysis, each tube was submerged in liquid nitrogen for a period of 5 min 4 times. Upon completion, tubes were vortexed (VWR Scientific, West Chester, PA, USA) at room temperature (RT) and 10 µl of chloroform (Sigma) was added and vortexed which allowed RNA isolation and extraction. Tubes were centrifuged for 5 min at 4 ºC and 12,000 g (Eppendorf 5417R®, Eppendorf, Westbury, NY, USA). At completion of the centrifugation, the contents of each tube had separated into a lower phenol-chloroform phase, an interphase and a clear aqueous upper phase that contained isolated RNA. The upper phase was removed and combined with 25 µl of isopropanol (Sigma), vortexed and incubated at room temperature for 10 min which allowed RNA precipitation.

Following precipitation, each tube was centrifuged for 10 min at 4 ºC and 12,000 g. The supernatant was carefully discarded and the remaining pellet containing RNA was washed with 50 µl of 75% ethanol (Sigma). Tubes were spun for 5 min and the pellet was air dried. Pellets were then resuspended in 10 µl DEPC water and warmed in a 70 ºC heating block (VWR) for 15 min with vortexing every 2 to 3 minutes.

To purify RNA, a DNase reaction was utilized to cleave any double stranded DNA remaining in the precipitate, thus removing any possible contamination for the real time RT-PCR reaction. Added to a separate tube were 1 µl first strand buffer (Invitrogen, Carlsbad, CA, USA), 0.5 µl RQ1 DNase (Promega, Madison, WI, USA) and 9 µl of the RNA solution. The mixture was incubated for 30 min in a 37 ºC heating block (VWR) and then for 10 min in the 70 ºC heating block to inactivate the Dnase. At the conclusion, RNA concentration was determined utilizing 1 µl in a Nanodrop spectrophotometer (Nanodrop DN-1000®, Nanodrop Technologies, Wilimington, DE, USA).

To synthesize first strand cDNA for the real time RT-PCR reaction, Superscript II (Invitrogen) enzyme was utilized with an RNA concentration of 500 ng. Added to a nuclease-free microcentrifuge tube was the following: 1 µl of random octomer (Roche), 1 µl 10 mM dNTP (Roche), 500 ng mRNA (volume determined by RNA concentration in mixture), and brought to a total volume of 12 µl with PCR quality water (Sigma). The mixture was heated in the 70 ºC heating block for 5 min then slowly cooled on ice. Contents were briefly centrifuged and added were 4 µl 5xFirst Strand Buffer (Invitrogen), 2 µl of 0.1 M DTT (Invitrogen) and 1 µl RNasein (Promega). Contents

were mixed gently and held at room temperature for 2 min. One microliter of Superscript II was added and mixed by pipetting up and down gently. The tube was then incubated in a 42 ºC water bath (VWR) for 50 minutes and it was then incubated in the 70 ºC heating block for 15 minutes to inactivate RT enzyme. Contents were stored in a -20 º C freezer until real time RT-PCR analysis.

Quantification of mRNA was performed using an ABI 7900HT Fast Real-Time PCR System® (Applied Biosystems, Foster City, CA, USA). SYBR®Green (Applied Biosystems) was utilized as the double-strand DNA-specific fluor for HSP70 cDNA analysis. This sensitive fluor binds to double-stranded DNA and upon excitation emits light. When utilizing SYBR®Green, as the PCR products accumulate the fluorescence increases. Normalization of RNA concentration was performed using 18S ribosomal RNA as the housekeeping gene and TAQMAN® (Applied Biosystems) served as the double-strand DNA-specific fluor reagent. This fluor differed from SYBR®Green in that TAQMAN® depends on a specialized probe for fluorescence. TAQMAN® probes depend on the 5'-nuclease activities of the DNA polymerase used for PCR and are specialized due to a fluorescent reporter dye (FAM, 6-carboxy-fluorescein) attached to the 5' end and a quencher moiety (TAMRA, 6-carboxy-tetramethyl-rhodamine) coupled to the 3' end. In the unhybridized state the proximity of the of the reporter dye and quencher moiety prevent the detection of a fluorescent signal from the probe. However, during PCR and during replication when polymerase replicates a template, the 5'-nuclease activity cleaves the probe, decoupling the reporter and quencher, and in response fluorescence increases in proportion to the amount of probe cleaved.

Specific amplification primers for the real time RT-PCR reaction (Equus caballus heat shock protein 70, Genbank accession no. AF397192 (Roinick et al., 2001, unpublished data): forward primer, 5'-AGGTGCAGGTGAGCTACAAGG-3'; reverse primer: 5'-GGTCAGCACCATGGACGA-3'; were designed using Primer Express software (Applied Biosystems). Primers and probe for 18S expression were supplied by Dr. Nancy Ing and sequences checked to ensure compatibility; Equine caballus 18S, Genbank ascension no. AJ311673 (Janke et al., 2002): forward primer 5'-ATTCTTGGCAAATGCTTTCG-3'; reverse primer: 5'-GCGCCGCTAGAGGTGAAAT-3'; and 18S probe 5'-(FAM)ACCGGCGCAAGACGGACCAG(TAMRA)-3')).

For real time RT-PCR, an optic quality 96-well plate and sealing lid was used with each sample being run in triplicate. Into each individual well for HSP70 mRNA expression the following was added: 6.25 µl SYBR®Green, 1.25 µl of 3 µM forward HSP70 primer, 1.25 µl of 3 µM reverse HSP70 primer, 1.75 µl PCR quality water and 2 µl of reverse transcribed cDNA for a total volume of 12.5 µl. For 18S rRNA expression the following was added: 6.25 µl TAQMAN®, 1.25 µl of 3 µM forward 18S primer, 1.25 µl of 3 µM reverse 18S primer, .125 µl 18S probe, 3.125 µl PCR quality water and 1 µl of cDNA mixture for a total volume of 13 µl. The thermal cycler initiated the polymerase chain reaction with an initial 10 min at 95 ºC to melt all the products within the 96-well plate, and then ran at 95 ºC for 15 s followed by 60 ºC for 1 min for 40 cycles.

Positive control of HSP70 gene expression was conducted utilizing the HSP70 target sequence cloned into a pCMV-SPORT6 vector (Open Biosystems, Huntsville, AL, USA). As negative control, wells were prepared as described above in which cDNA was omitted during the real time RT-PCR reaction. To ensure no signal from genomic DNA contamination or possible amplification during the real time RT-PCR reaction, samples were prepared and analyzed without reverse transcriptase.

Quantitative analysis was conducted using the $2^{-\Delta\Delta C_T}$ method as described by Livak and Schmittgen (2001). This method was used to calculate relative changes in gene expression determined from the real time RT-PCR experiments. At completion of the experiments, data were analyzed by threshold cycle (C_T), the relative cycle number at which the fluorescence of the reaction crossed the set threshold, and normalization of the real time RT-PCR data. The C_T value was used to indicate at which cycle amplification of the target gene rose above the background and recorded by the ABI 7900HT Fast Real-Time PCR System®. Normalization analysis required an internal control gene (18S) and a choice of a specific sample data results (C_T) as a calibrator. For the embryos derived from the oocyte heat shock study, blastocysts from the zero control treatment served as the source of calibration. Quality grade 1 embryos recovered from non-exercised mares served as those experimental results calibrator. Each individual samples triplicate C_T results and then each samples result from replicate real time RT-PCRs were averaged prior to the $2^{-\Delta\Delta C_T}$ analysis. Additionally, during analysis each individual sample was treated separately and results between similar treatment (oocyte heat shock study) and embryo quality (exercise study) were averaged after $2^{-\Delta\Delta C_T}$ calculation (Livak

and Schmittgen, 2001). Prior to analyzing the relative expression utilizing $2^{-\Delta\Delta C}{}_{T,}$ first ΔC_T (average HSP70 C_T – average 18S C_T) and then $\Delta\Delta C_T$ (average ΔC_T – average $\Delta C_{T,\ calibrator}$) was calculated. The resulting value is termed "relative expression" and represents fold change in gene expression from control.

All statistical analysis was performed using SPSS software (SPSS 10.1, Chicago, IL, USA). Differences in HSP70 and 18S C_T values, relative expression between in vitro derived embryos from heat shocked oocyte treatments and differences for embryos from exercised vs. non-exercised mares and quality grade was analyzed by ANOVA with Tukey's HSD for multiple comparisons. Differences were considered significant when $P < 0.05$.

CHAPTER IV

RESULTS

Environmental Temperature and Relative Humidity

The daily temperature, humidity and precipitation data for the time periods studied is listed in Appendix A1-A10. Average daily high temperatures for the months of the experiment in 2004 ranged from 22.8 to 36.7 ºC with relative humidity ranges from 26 to 100%. Average daily high temperature for the months of the experiment in 2005 ranged from 27.8 to 40.6 ºC, with relative humidity ranges from 22 to 100%. Temperatures typically would reach their highest point in late afternoon and relative humidity would be at its lowest, while temperatures typically reached their lowest points in the early morning with relative humidity at its highest. Mares in this study were exercised in the early afternoon when daily temperatures were beginning to peak and relative humidity was decreasing.

Mares' Response to Exercise

The monthly average environmental temperatures and relative humidity for the exercise periods (1300 to 1500 h daily) for the months studied are depicted in Tables 1 and 2, respectively. The lowest temperature reported for any exercise period was 22.8 ºC with the highest at 36.4 ºC. Reported relative humidity ranged from a low of 27% with a high of 94%. During the research period, 60 d averaged $\leq$30.0 ºC whereas 205 d

averaged >30 ºC, Appendix A11. Relative humidity was <50% for 120 d and >50% for 145 d, Appendix A12. Temperature and humidity were inversely correlated with one another.

Table 1. Minimum, maximum and mean values of ambient temperatures (ºC) during the exercise period (1300 to 1500 h) for the months studied

Month	Min Temperature (ºC)	Max Temperature (ºC)	Mean Temperature (ºC)	S.D.
May-04	24.80	34.15	29.59	2.15
Jun-04	22.80	33.60	29.57	2.81
Jul-04	24.00	33.60	31.75	1.86
Aug-04	28.60	35.25	31.93	1.88
Sep-04	24.70	35.55	31.20	2.18
May-05	23.60	33.90	29.12	2.81
Jun-05	27.25	34.15	32.63	1.38
Jul-05	26.95	36.40	33.11	2.19
Aug-05	24.00	36.15	32.77	2.36
Sep-05	29.45	35.40	33.38	1.41

Table 2. Minimum, maximum and mean values of ambient relative humidity (%) during the exercise period (1300 to 1500 h) for the months studied

Month	Min Humidity (%)	Max Humidity (%)	Mean Humidity (%)	S.D.
May-04	57	76	64.06	5.18
Jun-04	55	94	71.90	9.81
Jul-04	36	69	49.58	6.26
Aug-04	46	68	56.19	7.05
Sep-04	27	77	47.90	11.69
May-05	40	84	52.94	11.86
Jun-05	35	60	45.73	7.29
Jul-05	31	80	50.41	11.43
Aug-05	31	94	51.61	11.67
Sep-05	28	57	40.37	8.67

The average rectal temperatures of the mares during exercise and after the 30 min cool-down period are depicted in Table 3. Rectal temperature at the beginning of exercise was an average of 38 ºC. The mares' rectal temperatures steadily increased under exercise to an average of 38.8 ºC at 10 min, 39.4 ºC at 20 min and 39.9 ºC at end of the exercise period. The mean increase from the beginning of exercise to completion was 1.97 ºC. At 30 min post-exercise, the average rectal temperature was 39.7 ºC. Mares varied on their return to resting body temperature, with a range of 45 min to 120 min post-exercise.

Table 3. Average mare rectal temperatures (ºC) during the exercise regimen and 30 minutes post-exercise

Time Period	Min Temperature (ºC)	Max Temperature (ºC)	Mean Temperature (ºC)	S.D.
Initial	36.70	38.44	37.96	0.52
10 minutes	38.22	40.11	38.84	0.79
20 minutes	38.44	41.06	39.42	1.12
End	39.00	41.28	39.93	1.09
Post 30 min	38.33	41.28	39.72	1.20

Mare Reproductive Performance

The interval from PGF2α administration on d 7 after ovulation (the d of uterine flush) to the subsequent ovulation was significantly ($P < 0.05$) longer in mares when in the exercise group than when in the control group (Table 4). When exercised, mares

ovulated on average d 9.27 (± .294) days after prostaglandin administration, versus d 8.47 (± .337) when not exercised.

Table 4. Mean days to ovulation during the interval after PGF2α administration in non-exercised vs. exercised mares

Treatment	n	Average day to Ovulation (d)	SEM
Exercise	15	9.27 [a]	0.294
Non-exercise	15	8.47 [b]	0.337

[a, b] Within columns, values with different superscript differ significantly ($P < 0.05$) by paired T-test (one-tailed)

Follicle diameter the d prior to detected ovulation was significantly greater when mares were in the control (non-exercise) group (41.5 ± 0.5 mm) (Table 5) than when they were in the exercise group (39.8 ± 0.5 mm).

Table 5. Mean follicle diameter the day prior to detected ovulation in non-exercised vs. exercised mares

Treatment	n	Average Follicle Diameter at Ovulation (mm)	SEM
Exercise	28	39.8[a]	0.544
Non-exercise	28	41.5[b]	0.500

[a, b] Within columns, values with different superscript differ significantly ($P < 0.05$) by paired T-test (two-tailed)

Embryo Collection and Evaluation

Sixty-seven uterine flushes for embryo collection were performed over the 2 yr. Results are shown in Table 6. The embryo recovery rate for the control, non-exercise group was 22/35 (63%). A significantly lower recovery rate was obtained with the exercise group of mares (11/32, 34%; $P < 0.05$). The proportion of embryos in the different grades is presented in Table 6. The proportion of embryos considered to be grade 1 tended strongly ($P = 0.051$) to be lower in exercised mares, 4/11 (36%) than in non-exercised mares, 16/22 (73%).

Table 6. Embryo recovery rate and embryo quality score in embryos from non-exercised vs. exercised mares

	Non-exercise	Exercise
Flushes performed	35	32
Embryos recovered	22 (63%)[a]	11 (34%)[b]
Embryos classified as Grade		
1	16/22 (73%)[*]	4/11 (36%)[*]
2	1/22 (4%)	3/11 (27%)
3	2/22 (9%)	0/11 (0%)
4	3/22 (14%)	4/11 (36%)

[a,b] Within a row, values without a common superscript differ significantly ($P < 0.05$)
[*] Depicts a strong trend ($P = 0.051$)

To try and establish a correlation between mare temperature and embryo recovery rate and quality score, the highest daily achieved temperatures for the 14 d prior to an embryo collection were compared. Exercised mares that yielded an embryo at

collection had a mean of temperature of 39.95 ± 0.15 (SEM). This was not significantly different ($P > 0.05$) from mares that did not yield embryos (mean rectal temperatures of 40.05 ± 0.10). Furthermore, these temperatures under exercise were not significantly associated with quality score of embryos recovered, with embryos of quality grade 1 recovered from mares with a mean temperature of 39.87 ± 0.20 and of those that yielded quality grade 4 embryos of a mean high rectal temperature of 39.88 ± 0.30.

Effect of a One Time Heat Shock on Equine Oocyte Competence

A total of 510 ovaries were processed with 2681 follicles scraped, an average of 5.26 follicles per ovary. Of the oocytes recovered, 484 were classified as Cp, 1067 classified as Ex, and 91 were degenerating or classified as other. For this project, 1067 Ex oocytes were used.

Experiment One. A total of 531 Ex oocytes were used for this experiment. The number of oocytes used and their status after maturation culture is presented in Table 7. After maturation, 20 oocytes were broken during removal of cumulus cells. Thus 511 oocytes were evaluated for presence of a polar body. Of oocytes without polar bodies, 9 (2%) were found to be in metaphase I on fixation and staining. There were no significant differences ($P < 0.05$) in maturation rate among groups (56 to 60% MII).

Table 7. Effects of exposure of equine oocytes at the onset of IVM to heat shock (42 °C) on oocyte maturation

Treatment	# Oocytes n	Oocytes in MI n	Oocytes in MII n (%)
Control	105	1	61 (58)
38.2 ºC (2 h)	100	1	60 (60)
38.2 ºC (4 h)	101	4	57 (56)
42.0 ºC (2 h)	103	2	59 (57)
42.0 ºC (4 h)	102	1	60 (59)

Of 297 oocytes with polar bodies, 2 were lysed after ICSI. Thus 295 were cultured. Cleavage and blastocyst development rates for the 5 treatments are given in Table 8. Cleavage rates were not significantly different among groups (75 to 90%).

Table 8. Effects of exposure of equine oocytes at the onset of IVM to heat shock (42 ºC) on embryonic development following fertilization via ICSI

Treatment	# Oocytes n	Oocytes cleaved n (%)	Morulae n	Total blastocysts n (%)
Control	60	45 (75)	5	16 (27)
38.2 ºC (2 h)	59	51 (86)	7	10 (17)
38.2 ºC (4 h)	57	46 (81)	7	13 (23)
42.0 ºC (2 h)	59	53 (90)	4	14 (24)
42.0 ºC (4 h)	60	49 (82)	4	13 (22)

There was no significant difference in advanced development (morula and blastocyst) between 2 and 4 h controls (38.2 ºC) or 2 and 4 h treatments (42 ºC), therefore time groups were combined for analysis and are presented in Table 9. Advanced embryo development was not significantly different between groups, being 37/116, 32% and 35/119, 29% for control and treated oocytes, respectively.

Table 9. Effects of exposure of equine oocytes at the onset of IVM to heat shock (42 ºC) on subsequent advanced (morulae and blastocysts) embryonic development following fertilization via ICSI

Treatment	# Oocytes (n)	Oocytes cleaved n (%)	Total morulae and blastocysts n (%)
Control	60	45 (75)	21 (35)
38.2 ºC (2 and 4 h)	116	97 (84)	37 (32)
42 ºC (2 and 4 h)	119	102 (86)	35 (29)

Experiment Two. A total of 536 Ex oocytes were used for Experiment 2. The distribution of the oocytes among groups is given in Table 10. Of the oocytes used, 17 oocytes were broken during the removal of cumulus cells. Thus 519 oocytes were evaluated for presence of a polar body. Of oocytes without polar bodies, 6 (1%) were found to be in metaphase I and 1 (0.2%) in metaphase II on fixation and staining. The rate of maturation to metaphase II was significantly ($P < 0.05$) lower in oocytes subjected to 42 ºC for 2 or 4 h (40 and 44%, respectively) when compared to their corresponding control water bath, 38.2 ºC for 2 or 4 h (68 and 59%, respectively).

Table 10. Effects of exposure of equine oocytes near completion of IVM to heat shock (42 ºC) on subsequent oocyte maturation

Treatment	# Oocytes (n)	Oocytes in MI (n)	Oocytes in MII n (%)
Control	103	0	54 (52)[a,b]
38.2 ºC (2 h)	103	0	70 (68)[a]
38.2 ºC (4 h)	102	0	60 (59)[a]
42.0 ºC (2 h)	105	2	43 (41)[b]
42.0 ºC (4 h)	106	4	47 (44)[b]

[a-c] Within a column, values without a common superscript letter differ ($P < 0.05$)

Cleavage and blastocyst development rates for Experiment 2 are presented in Table 11. Of 273 oocytes with polar bodies, 3 were lysed after ICSI. Thus 270 oocytes were cultured. There were no differences in cleavage rates among the groups (77 to 85%).

Table 11. Effects of exposure of equine oocytes near completion of IVM to heat shock (42 ºC) on subsequent embryonic development following fertilization via ICSI

Treatment	# Oocytes (n)	Oocytes cleaved n (%)	Morulae n	Total blastocysts n (%)
Control	53	41 (77)	5	13 (25)
38.2 ºC (2 h)	68	54 (79)	5	19 (28)
38.2 ºC (4 h)	60	51 (85)	6	13 (22)
42.0 ºC (2 h)	42	34 (81)	3	7 (17)
42.0 ºC (4 h)	47	38 (81)	2	6 (13)

There was no significant difference in advanced development (morula and blastocyst) between 2 and 4 h controls (38.2 ºC) or 2 and 4 h treatments (42 ºC),

therefore time groups were combined for analysis like those in experiment 1 and are presented in Table 12. Advanced embryo development was significantly lower (20 vs. 34%; $P < 0.05$) in oocytes that were heat-shocked (42 ºC 2 and 4 h) when compared to controls (38.2 ºC 2 and 4 h).

Table 12. Effects of exposure of equine oocytes near completion of IVM to heat shock (42 ºC) on subsequent advanced (morulae and blastocysts) embryonic development following fertilization via ICSI

Treatment	# Oocytes (n)	Oocytes cleaved n (%)	Total morulae and blastocysts n (%)
Control	53	41 (77)	18 (34)[a]
38.2 ºC (2 and 4 h)	128	105 (82)	43 (34)[a]
42 ºC (2 and 4 h)	89	72 (81)	18 (20)[b]

[a,b] Within a column, values without a common superscript letter differ ($P < 0.05$)

Heat Shock Protein 70 Gene Expression Detected via Real Time RT-PCR

Pilot test runs were conducted to confirm the capability of detecting HSP70 mRNA and 18S rRNA expression in individual equine blastocysts. Both in vivo and in vitro derived equine embryos demonstrated the ability to express both HSP70 mRNA and 18S rRNA confirmed by real time RT-PCR using the primers and procedures as previously described. A total of 21 in vivo embryos were individually processed, with non-exercised mare derived embryos representing the following quality grades; 7 grade 1, 1 grade 2, 1 grade 3 and 1 grade 4; and from exercised mares, 4 grade 1, 2 grade 2, and 4 grade 4. Data from Grade 3 and 4 embryos were pooled together in the non-

exercised mare group due to the low availability of lower quality embryos for analysis. From the in vitro derived embryos that were from the heat shock experiment near the end of IVM, a total of 19 embryos were individually processed, with 3 embryos representing the zero control (normal IVM procedures), and 4 embryos each from the 38.2 ºC 2 h and 4 h and 42 ºC 2 h and 4 h water bath treatment groups.

From both experimental groups, individual embryos yielded greater than 60 ng/µl of RNA, determined by the Nanodrop spectrophotometer, which allowed individual samples to continue with reverse transcription and real time RT-PCR analysis. The concentration of total RNA isolated from individual embryos and then data pooled according to treatment and quality score ranged from as low 145.5 ± 38.2 (SEM) from quality grade 4 embryos recovered from exercised mares to as high as 507.5 ± 267.5 in embryo quality grade 2 embryos also from exercised mares (Table 13). There were no significant differences between any of these groups.

Table 13. Mean values of total RNA concentration determined by a Nanodrop spectrophotometer isolated from individual embryos recovered from non-exercised (control) and exercised mares (treatment)

Treatment	n	Embryo Quality Grade	Total RNA (ng)	SEM
Non Exercised Mares	7	1	2070.5	453.4
	1	2	1653.7	-
	2	≥ 3	2177.5	1007.5
Exercise Mares	6	1	2773.7	889.2
	2	2	5075.0	2675.0
	3	≥ 3	1454.6	382.1

Total RNA isolated from individual blastocysts produced in vitro is depicted in Table 14. The concentrations ranged from as low as 249.9 ± 146.1 to as high as 1023.5

± 590.9. There were no significant differences between groups. However, when comparing in vitro versus in vivo embryos overall total isolated RNA, in vitro embryos (6994.5 ± 1461.5) recorded higher concentrations versus in vivo recovered embryos (2460.7 ± 405.2).

Table 14. Mean values of total RNA concentration determined by a Nanodrop spectrophotometer isolated from individual embryos grown in vitro from oocytes that were treated near the end of IVM

Treatment	n	Total RNA (ng)	SEM
0 control	3	10234.9	5909.2
38.2 ºC (2h)	4	4207.4	2103.7
38.2 ºC (4h)	4	6759.8	3902.8
42 ºC (2h)	4	2983.6	1491.8
42 ºC (4h)	4	2499.4	1249.7

The relative gene expression of HSP70 mRNA of d 7 equine embryos derived by non-surgical collection from non-exercised and exercised mares are depicted in Table 15. Significant differences ($P < 0.001$) were observed between the quality grades of the embryos in HSP70 mRNA relative to 18S rRNA gene expression. Although exercised mare grade 1 embryos had a slight 2 fold increase in expression versus the non-exercised mare grade 1 embryos, there were no statistical differences. However, as quality grade decreased in both treatment groups, HSP70 relative to 18S rRNA gene expression increased. Quality grade 2 embryos reported 52 (non-exercised) to an almost 800 (exercised) fold increase in relative expression that was significantly ($P < 0.05$) higher than the quality grade 1 embryos. As the quality of embryos further decreased, HSP70 relative gene expression increased significantly due to decreasing 18S rRNA C_T values.

As embryo quality decreased, the C_T value for 18S rRNA significantly ($P < 0.05$) increased and the C_T values for HSP70 remained relatively unchanged. Quality grade 3 or 4 embryos in the non-exercised mare treatment group had an over 1000 fold increase in normalized expression that was significantly ($P < 0.05$) higher than both quality grade 1 and 2 groups. The quality grade 4 embryos from the exercised mares reported the highest significant ($P < 0.01$) relative expression over any other group examined when compared to the non-exercise quality grade 1 embryos, with an almost 18,000 fold increase in relative HSP70 to 18S gene expression. The determining factor in higher relative expression was due to the ratio of lower expression values (C_T) of 18S rRNA when compared to the relatively unchanged values (C_T) in HSP70 mRNA. Additionally, due to the low availability of lower quality embryos, SEM tended to be higher in these groups, however statistical significance was still achieved in the lower quality grade 4 embryos.

Table 15. Mean values of reported threshold cycles (C_T) for the housekeeping gene (18S) and target gene (HSP70) with normalized gene expression in embryos recovered from non-exercised (control) and exercised mares (treatment)

Treatment	n	Embryo Quality Grade	HSP70 (C_T)	SEM	18S (C_T)	SEM	Normalized Expression	SEM
Non Exercised Mares	7	1	31.6	0.505	15.6	0.435	1.0^{a}	0.53
	1	2	33.5	-	23.2	1.790	$52.0^{a,c}$	-
	2	≥ 3	31.7	1.589	25.7	0.625	1099.3^{b}	399.9
Exercise Mares	6	1	30.9	0.658	16.0	0.356	2.6^{a}	0.79
	2	2	32.5	2.310	24.0	2.950	$788.1^{b,c}$	660.07
	3	≥ 3	31.6	0.512	29.3	1.062	17927.9^{d}	7571.72

[a-d] Within columns, values with different superscript differ significantly ($P < 0.05$)

The relative gene expression of HSP70 mRNA of equine blastocysts derived from the in vitro heat shock near the end of IVM experiments are depicted in Table 16. There were significant ($P < 0.01$) differences between HSP70 relative to 18S rRNA gene expression between the oocyte treatment groups. The relative gene expression of HSP70 trended to be higher in the blastocysts that were derived from the two 4 h water bath treatments. The 38.2 ºC 4 h water bath oocyte treatment reported an over 6 fold increase while the 42 ºC 4 h derived blastocysts reported an almost 10 fold increase in HSP70 mRNA relative to 18S rRNA gene expression. However, the only statistically significant ($P < 0.05$) difference were from the embryos derived from the oocytes that were heat shocked for 4 h at 42 ºC. This group recorded higher relative HSP70 gene expression than the zero control and both 2 h water bath treatments. While there were no statistical differences between the C_T values in 18S expression in any of the in vitro derived embryos, 18S responded significantly ($P <0.001$) later in the real time RT-PCR than any of the in vivo derived quality grade 1 embryos from the earlier experiment.

Table 16. Mean values of reported threshold cycles (C_T) for the housekeeping gene (18S) and target gene (HSP70) with normalized gene expression in embryos grown in vitro from oocytes that were treated near the end of IVM

Treatment	n	HSP70 (C_T)	SEM	18S (C_T)	SEM	Normalized Expression	SEM
0 control	3	33.5	0.167	22.4	1.360	1.00 [a]	0.12
38.2 ºC (2h)	4	34.1	0.411	22.0	1.350	1.02 [a]	0.59
38.2 ºC (4h)	4	34.0	0.814	23.3	1.980	6.31 [a,b]	5.44
42 ºC (2h)	4	33.3	0.838	21.8	0.925	1.64 [a]	0.66
42 ºC (4h)	4	30.7	0.474	22.9	0.569	9.92 [b]	6.08

[a,b] Within columns, values with different superscript differ significantly ($P < 0.05$)

The difference between in vivo versus in vitro derived embryos was also examined. The control ΔC_T for the $2^{-\Delta\Delta C}{}_T$ method was set as the non-exercised mare quality grade 1 embryos, the optimal environment and grade for equine embryos. Embryos grown in vitro under optimal conditions (0 control) when compared to the in vivo embryo had a 31 fold increase in HSP70 expression. Determining this difference utilizing the $2^{-\Delta\Delta C}{}_T$ method was a 1.9 increase in HSP70 and a 6.8 increase in 18S C_T values observed in the processed in vitro embryos.

CHAPTER V

DISCUSSION

Environmental Temperature and Relative Humidity

The temperatures and relative humidity reported are in the normal ranges for College Station, TX for the time periods studied. The exceptions were May and June 2004 with higher than normal precipitation recorded. Of the 265 d observed, 205 d recorded temperatures of over 30 ºC. However, temperature typically would only drop under 30 ºC during periods of precipitation. The lowest mean temperatures during the exercise period were observed in May and June 2004, with ranges of 22.8 to 33.6 ºC. During these two months, 19.88 and 29.85 cm of precipitation were recorded respectively, contributing to the lowered temperatures. The highest mean temperatures were from July through September 2005 with mean monthly temperatures during the exercise period ranging from 32.7 to 33.3 ºC.

Relative humidity during the exercise period could be classified as moderate. While the highest relative humidity recorded was 94%, this was not typical during exercise. Humidity would only reach this high of a level after a period of precipitation and would correspond with a drop in temperature. Relative humidity hovered around 50% for the majority of d studied. The two months May and June 2004, humidity averaged 64% and 72%, respectively. This higher percent humidity compared to the

other months observed was due to the higher than average precipitation recorded during those months.

Mares' Response to Exercise

The exercise regimen prescribed during this study was not considered intense. Mares would long trot or canter for 10 min, followed by a brief rest to allow recording of rectal temperatures. Mares then would continue for another 10 min with a brief pause again allowing for temperatures to be recorded, and then 1 final 10 min exercise period. At the conclusion of exercise, temperatures were recorded and mares were walked for at least 30 min to cool down. Mares that did not demonstrate a decline in body temperature following the 30 min cool-down period continued to be walked and were not allowed to return to the band of mares until they had began to decline in temperature.

The rectal temperature increases observed in this study are reflective of what has been reported in the equine. The rectal temperatures after 30 min exercise reported here had a mean of 39.72 ºC. These means are slightly lower than Geor et al. (1995) who reported after 30 min of exercise, horses in hot (32-34 ºC) and humid (80-85%) or dry (45-55%) conditions, recorded average rectal temperatures of 40.5 ºC. In another report, Kohn et al. (1999) observed that horses exercised for 30 min in hot (30.6 ºC) and humid (84%) conditions achieved mean rectal temperatures of 41 ºC and even those horses exercised in cool (20.2 ºC) and dry (54%) conditions achieved a mean rectal temperature of 40 ºC. Meanwhile, McCutcheon and Geor (2000) subsequently reported mean rectal temperature increases of 2.5 ºC in just 18 to 25 min of exercise and indicated they

thought that the intensity of exercise influenced the temperature increase seen when compared to other reports. Agreeing with this concept, the lower rectal temperatures recorded in this study may also be a reflection of the less intense exercise regimen that was employed.

The slight decrease of rectal temperatures 30 min post-exercise of only 0.21ºC is probably indicative of the high environmental temperatures and moderate humidity. Evaporative cooling is the primary means of heat loss during exercise and under adverse ambient conditions, high temperature and humidity, severely limits the horses ability to thermoregulate and can induce heat-stress (Hodgson et al., 1994). In addition, convection, the heat dispersal mechanism by air movement around the horse, is severely limited under temperatures that approach the body temperature of the horse.

A similar phenomenon was observed in this study compared to others in that, of the temperature observations made 30 min following exercise (n = 77), 16 showed mean increases of 0.16 ºC in rectal temperature. Hodgson et al. (1994) conferred that a period of "lag" exists during exercise, in that muscle and core temperature increased before the bodies thermoregulatory mechanisms are activated and excess heat then can be diverted into the bloodstream. Therefore, the excess heat stored within the body during exercise takes time to dissipate and can explain the increases seen here. Similar results have been published and discussed. Kohn et al. (1999) reported significant increases in rectal temperatures of horses exercised under hot and humid conditions and proposed that horses develop large endogenous heat loads or are unable to dissipate heat during exercise under such conditions. Additionally, Geor et al. (1995) stated that the added

thermal load of high ambient temperature and relative humidity impaired the regulation of body temperature, both during exercise and recovery. The mares in this study after exercise appeared to be challenged to thermoregulate, first due to the almost 2 ºC increase in rectal temperatures at the conclusion of exercise and secondly by 30 min post exercise, mares rectal temperatures had either slightly decreased (- 0.21 ºC) or slightly increased (+ 0.16 ºC).

Mare Reproductive Performance

Much has been discussed in other species about heat stress and its detrimental impact on the estrous cycle. The results of this study are in agreement that heat stress, associated with exercising mares in a hot and humid environment, during the breeding season may alter the dynamics of the mares' estrous cycle. The mares under exercise ovulated significantly smaller follicles than they did when not under exercise and this may be an indication of impaired folliculogenesis. Furthermore, mares under exercise had significant delays in time to ovulation after luteolysis with PGF2α on d 7 of diestrus, indicating again that the exercise protocol employed in this study affected timing of ovulation with possible impairment of folliculogenesis.

The ovulation of smaller follicles seen in these mares is similar to what has been observed in cattle. Wilson et al. (1989) reported that heat stressed heifers had smaller dominant second wave follicles. Consequentially, the heifers in their study had a longer estrous cycle (Hansen et al., 2001).

Wolfenson et al. (1995) and Rensis and Scaramuzzi (2003) reported that heat stress in cows delays follicle selection and lengthens the follicular wave, thus interrupting and lengthening the estrous cycle. Mares on the exercise regimen ovulated on average at d 9.3 after administration of PGF2α on d 7, versus non-exercised mares which ovulated on average at d 8.5.

How these two abnormalities in the estrous cycle affect mare fertility is not clear, however, Hansen et al. (2001) stated that disruption in patterns of folliculogenesis, leading to a lengthened period of dominance for the largest follicle, leads to poor fertility. Mihm et al. (1999) reported that in cattle with lengthened follicular dominance (> 9 d), oocytes ovulated had reduced competence. These trends observed in cattle could possibly be associated with the poorer fertility seen in the mares in this study based on the decreased embryo recovery rate. Further investigation into the effects of heat stress and exercise on follicular dynamics in the mare are warranted based on these results.

Embryo Recovery Rate and Quality Score

The most striking data from this research is the disparity in embryo recovery rate in exercised vs. non-exercised horses. Mares that were exercised produced significantly fewer embryos (34% recovery rate) than did non-exercised (63%) mares. Additionally, a strong trend was observed in the proportion of grade 1 embryos, this being lower in exercised than in non-exercised mares. Therefore, under the conditions of this study, it appears that exercising mares for 30 min per d in a hot and moderately humid

environment significantly reduces embryo recovery rate and may lower the chances of recovering a grade 1 embryo in the mare.

Hansen et al. (2001) stated that reproductive efficiency in the cow would be compromised if body temperature exceeded 1 ºC for a period of more than 4 h. The mares in this study began to exceed this temperature threshold set for cattle after only 20 min of exercise. The mares' mean rectal temperatures increased almost 2 ºC from resting levels in this study by the completion of exercise. Although the longest duration of elevated body temperature in the horses was deemed to be no longer than 2 h post exercise, the temperature increase was twice that of the ceiling proposed for cattle. Furthermore, the mares in this study experienced daily temperature increases resulting from exercise rather than a one-time heat shock, which may have increased the effects of the heat on embryo recovery.

Putney et al. (1988b) reported a close correlation between the rectal temperature of cows (from 39 to 41 or 42 °C) and the percentage of abnormal embryos recovered. However, there appeared to be no association between temperature and embryo recovery in the mares in the current study. This may be due to the low numbers (n = 32) of collections or to the possibility that the intensity was enough to disrupt reproductive efficiency in all mares studied. Duration of thermal increases in the mares past 30 min was not consistently recorded; therefore no correlation could be established of elevated temperature duration and embryo recovery. However future work in differing exercise intensities and duration of thermal increases in the mare would be beneficial.

Rectal temperature seems to be indicative of circulation temperature (Kohn et al., 1999). However, muscle temperature and core body temperature have been reported to be higher than that of pulmonary arterial temperature. Geor et al. (1995) reported when pulmonary arterial temperatures were 41 ºC; the middle gluteal muscle temperature was 42.6 ºC, a 1.6 ºC difference. This may indicate that rectal temperature does not provide an accurate representation of temperature within the reproductive tract. Under the conditions of this study it is possible temperatures within the tract may have been higher or lower, than were the reported rectal temperatures, or elevated for a longer duration.

Within the design of this experiment, the mechanism of action of the exercise on the mares' fertility cannot be determined. The effect of exercise and heat stress on the preovulatory oocytes in this study cannot be discounted. Mares under exercise in this study ovulated significantly smaller follicles, showing an effect on follicle physiology that may have been associated with altered oocyte competence. Oocytes may not have been fully mature at ovulation, or could have been aged, as suggested in other species. Exercised mares had a delay in time to ovulation after PGF2α administration this being 9.3 d compared with 8.5 d for non-exercised mares. The longer time to ovulation may support the theory that exercise disrupted normal follicle physiology and may have resulted in ovulation of aged oocytes, thus lessening their competence for further development.

The disparity in embryo recovery rate and the higher proportion of poorer quality embryos found in exercised versus non-exercised mares could possibly be explained by the effect of exercise on the early developing equine conceptus. This is supported by

Putney et al. (1989) who reported that cows whose rectal temperatures increased to 2 to 2.5 ºC in response to daily heat treatments (42 ºC for 8 h) from d 1 to 7 post breeding had a significant decrease in embryos recovered and a larger proportion of embryos recovered had significantly more morphological defects. While the heat stress experienced by the cows in that report was for a longer duration than that by the mares in this study, the similarities are compelling. Additionally, the higher incidence of abnormal embryos recovered from heat-stressed cows included morphological imperfections such as extruded blastomeres and degenerative blastomeres of irregular shape with a dark granular appearance (Putney et al., 1989). These abnormalities are similar to those found in the lower quality grad embryos recovered in the mares in this study. As noted in the introduction, Rivera et al. (2003) reported that heat-shock of 2-cell bovine embryos resulted in cytoskeleton disruption and mitochondria swelling. Furthermore, these disruptions under thermal stress in the current study may have led to compromised early development evidenced by the lower embryo recovery rate and embryo quality seen in exercised mares.

Other studies have shown reduced embryo quantity and quality in response to heat stress. Ryan et al. (1992) reported that incidence of bovine embryonic death significantly increased in vitro under a chronic 40 ºC and Rivera et al. (2003) reported a significant reduction in development of 2-cell bovine embryos to the blastocyst stage after exposure to 41 ºC for 6 h. The changes under thermal stress are two-fold for embryos; the embryo's metabolism appears to increase and cytological changes within disrupt development, which may eventually lead to embryonic death. While the embryos

recovered in this study were not transferred into recipient mares for further development, it would be worth investigating the effect exercise has on post-transfer development of those embryos that are recovered.

While heat stress is inferred to be the mode of reduction in quantity and quality of embryos recovered in this study, the effects of exercise directly on reproduction efficiency in the mare cannot be discounted. Specifically, the effect exercise has on the hormonal stress response and any consequential reproductive effects in the mare was not evaluated. Further research is needed to separate the effects of heat and exercise on reproductive efficiency in the mare.

Effect of a One Time Heat Shock on Equine Oocyte Competence

A one-time heat shock near the end of in vitro maturation had a detrimental effect on oocyte meiotic and developmental competence. From the results obtained in Experiment 1, it appears oocytes are not measurably affected by heat (as applied in this study) when it is administered at the onset of in vitro maturation (IVM). This is a time period where the oocyte in vivo would still be within a growing follicle and would be in the germinal vesicle (GV) stage, paused at prophase I. However, the results from Experiment 2 in which oocytes were treated near the end of IVM produced much different results. A significant decrease in maturation rates was found in the heat treatment groups, indicating that the heat-shock applied affected the oocytes' ability to proceed to MII. The significantly lower oocyte advanced developmental competence

(morulae and blastocysts) of the treated groups, indicates that heat shock during late IVM may have compromised oocyte developmental competence.

The explanations for this resistance to heat shock at the GV stage may be related to the fact that in Ex horse oocytes (those from atretic follicles), the chromatin within the GV is in a condensed formation. Only expanded (Ex) oocytes were used in this study. The majority of oocytes recovered by follicular scraping from abattoir obtained ovaries are classified as Ex (Choi et al., 2004; Choi et al., 2006a, b) due to the large number of atretic follicles per ovary. Expanded oocytes originate from atretic follicles that allow pre-maturational changes (Hinrichs and Williams, 1997) that aid in allowing those oocytes to have greater meiotic competence versus compact (Cp) oocytes (Hinrichs and Schmidt, 2000). The chromatin condensation seen in Ex oocytes mimics that of oocytes in preovulatory follicles over 20 mm diameter (Hinrichs and Schmidt, 2000). Furthermore, Hinrichs et al. (1993) reported that no change in chromatin configuration in Ex oocytes during the first 8 h of IVM indicating that there appeared to be a pause before oocytes resumed meiosis. The maintenance of the condensed configuration during the onset and early stages of IVM equine oocytes could conceivably allow the oocyte to withstand the stresses placed upon it.

In contrast to the findings in Experiment 1, results in the bovine (Edwards and Hansen, 1997; Payton et al., 2004; Roth and Hansen, 2005) have shown disruption in oocyte competence in GV-stage oocytes due to heat. Chromatin is diffuse in GV-stage bovine oocytes. Payton et al. (2004) reported that a heat shock of 41 ºC for 6 h at the onset of oocyte IVM slowed the developmental ability of the fertilized oocytes to

proceed to the blastocyst stage of development. While a reduction in oocyte maturation rates has not been reported in heat-shocked bovine GV-stage oocytes, a heat shock of 41 ºC for greater than 12 h did affect the number of embryos developing in vitro to the blastocyst stage (Edwards and Hansen, 1997; Payton et al., 2004; Roth and Hansen, 2004). A longer-duration heat shock was not considered for the current study, due to relevance of such research to the industry. Horses have the ability to thermoregulate proficiently (Hodgson et al., 1994) and under the exercise portion of this research (exercise for 30 minutes), horses did not sustain physiological elevated temperatures for more than 2 h. However, if horses in the industry were exercised for a longer duration or under more intense conditions, they might possibly sustain elevated temperatures for more than 4 h. Since there were no noticeable differences in maturation, cleavage or blastocyst rates in any treatment group, it appears from the results of Experiment 1 that equine GV-stage oocytes can tolerate a heat shock of 42 ºC for up to 4 h.

As the oocyte matures in vitro and meiosis resumes, the oocyte progresses from prophase I to metaphase II with the extrusion of the first polar body, and then pauses at metaphase II prior to ovulation. Hinrichs et al. (1993) reported that the majority of IVM oocytes do not proceed from the GV-stage until 8 h after the initiation of maturation. By 16 h IVM, the preponderance of oocytes has resumed meiosis and by 24 h are in metaphase II, the second phase of meiosis (Hinrichs et al., 1993). During this period, the chromatin or its superstructure (microtubules of the spindle) may be more susceptible to heat. It was during these later stages of oocyte development that a one-time heat shock was applied in Experiment 2.

In Experiment 2 it appeared that the heat shock had a definite effect on oocyte competence. Significantly fewer oocytes were found to be in MII in the heat shocked groups (42 ºC) than the corresponding control groups (38.2 ºC). Additionally, fewer heat shocked oocytes developed to the morula or blastocyst stage, indicating a trend for decreased competence. Of all the groups studied, the 42 ºC 4 h treatment group recorded the lowest blastocyst rate (13%). However, no significant difference was found in blastocyst rate alone in either of the 42 ºC treatment groups, most likely due to the low number of oocytes involved for IVM and ICSI procedures. This is caused by the low availability of equine ovaries, by the decreased rate of maturation to MII of the treated groups, and by the limited ability of fertilized MII equine oocytes to develop to the blastocyst stage after ICSI, with a maximum expected blastocyst development rate of 27% in this study. Therefore, time groups were combined and overall advanced embryonic development (morulae and blastocysts) was analyzed between combined treatment and combined control groups. Through this analysis, heat shock produced a significant reduction in those oocytes that proceeded to a more advanced stage of development after fertilization. Therefore, the results of Experiment 2 indicated that heat shock of 42 ºC for 2 or 4 hours in later stages of IVM can be detrimental to equine oocyte competence when measured not only by those oocytes that mature in vitro, but by the capability of those that do mature to develop after ICSI to either a morula or blastocyst in vitro.

The results of Experiment 2 are similar to what has been observed in other species. While studies examining longer duration of heat shock on bovine oocytes (41 ºC

at 12 h) have reported significant decreases in oocyte meiotic competence (Rivera and Hansen, 2001; Roth and Hansen, 2005), studies examining shorter durations are limited. Ju et al. (2005) reported a significant decrease in the proportion of bovine oocytes developing to blastocysts in vitro after a heat shock of 42 ºC for 4 h at the end of 20 h of standard IVM culture conditions. Of the oocytes (n = 95) subjected to the heat shock, 27% developed to the blastocyst stage compared to the 44% of the control group (n = 101) (Ju et al., 2005). In contrast to the results of this study in equine oocytes, Ju et al. (2005) reported that a 2-h heat shock at 42 ºC did not significantly decrease blastocyst development rates (36 vs. 44%) in bovine oocytes. However, the authors did note that a decreasing trend was evident in the blastocyst development rate and furthermore, that both the 2 and 4-h treatments had significantly decreased trophectoderm cell numbers. Trophectoderm is important in formation of the extraembryonic tissues of the placenta (Rossant, 1995). The mechanism by which heat shock of an oocyte affects future embryonic trophectoderm development is currently unknown; however, this decrease could lead to early embryonic death and could possibly explain pregnancy decreases seen in heat stressed cattle as previously discussed.

Ju and Tseng (2004) reported that in porcine oocytes, a heat shock of 41.5 ºC for 1 to 4 h resulted in chromosomal changes. The chromosomal changes observed were not typical of normal developing oocytes in that the heat-shocked fully mature oocytes, chromosomes did not fully decondense into pronuclei but rather into separate segregated structures, which resulted in lower oocyte developmental competence (Ju and Tseng, 2004). The authors hypothesized that the elevated temperatures may have depolymerized

the meiotic spindle and partially activated the oocytes leading to this segregation (Ju and Tseng, 2004). In another report, Ju et al. (2005) found that short term heat-shocked bovine oocytes (42 ºC 2 or 4 h) underwent a change in spindle morphology and size and these authors felt this indicated that heat shock affects polymerization or depolymerization of meiotic spindle microtubules. This previous research may offer evidence to as why the late IVM oocytes in this study appeared to be compromised, evidenced by lower developmental competence.

In summary, heat shock at 42 ºC for 2 or 4 h near the end of IVM reduced equine oocyte competence when measured by those oocytes that mature in vitro and those that, once fertilized via ICSI, developed to either a morula or blastocyst. This differs from oocytes shocked before IVM, in that these equine oocytes appear to be resistant to a one-time heat shock. These findings are similar in what has been reported in other species, except that equine oocytes seem to be more resistant at the GV-stage of development.

Heat Shock Protein 70 Gene Expression Detected via Real Time RT-PCR

Heat shock protein 70 (HSP70) gene expression of in vivo derived d 7 or in vitro grown equine blastocysts has not been previously reported in the equine. Based on the results of this study, measurement of both HSP70 mRNA and 18S rRNA gene expression in either d 7 in vivo embryos or in vitro derived equine blastocysts is possible. The results of the in vivo derived d 7 embryos indicated that HSP70 mRNA ratio when related to 18S rRNA gene expression increased with decreasing embryo quality. Interestingly, as the embryo quality decreased, measurement levels (C_T) of 18S

rRNA also decreased. However, this phenomenon was not observed in the HSP70 mRNA C_T values. The data collected on the in vitro blastocysts indicated that a one-time heat shock during late IVM on equine oocytes has an effect as measured by increased HSP70 gene expression at the blastocyst stage of development. When comparing in vitro derived blastocysts versus the most optimal embryo conditions (quality grade 1 from non-exercised mares), in vitro embryos had lower 18S expression and higher relative expression of HSP70. Furthermore, this is the first known study to not only examine HSP70 in early developing equine embryos, but that examination of equine gene expression in a single d 7 in vivo embryo or in vitro grown blastocyst is possible utilizing real time RT-PCR technology.

Research in other species has shown early embryos retain the ability to transcribe HSP70 in response to stress (heat) that can be measured employing polymerase chain reaction technology (Chandolia et al., 1999; Matwee et al., 2001; Fiorenza et al., 2004; Hartshorn et al., 2005). More specifically, real time RT-PCR is an emerging technology that is increasingly being utilized in understanding genomic expression in the early mammalian embryo. Other scientists have demonstrated that not only can single-cell murine oocytes or multi-cellular embryos be utilized for RT-PCR (Loimas et al., 1998), but measurement of HSP70 mRNA gene expression in a single cell from a murine embryo is possible (Fiorenza et al., 2004; Hartshorn et al., 2005).

The observations measuring HSP70 mRNA and 18S rRNA gene expression from in vivo and in vitro equine blastocysts indicated that both are measurable genes during early equine embryonic development. This conclusion is based on the results that

showed in all the in vivo (n = 21) and in vitro (n = 19) equine embryos processed, each sample prepared as described by this study, recorded HSP70 mRNA and 18S rRNA gene expression when measured by real time RT-PCR. These results are supported by Christians et al. (1995) who stated that HSP70 is the first major inducible heat shock gene as evidenced by higher levels of transcription in early murine embryos after heat treatment. Chandolia et al. (1999) found similar results in 2-cell bovine embryos after heat treatment. The authors of both studies concluded that HSP70 is most likely important for protecting embryonic cells from adverse effects of heat shock or other cellular stresses. Furthermore, Robert et al. (2002) confirmed the ability of detecting 18S rRNA gene expression via real time PCR in bovine d 7 embryos.

The mares in this study that underwent exercise in a hot and humid environment conceivably suffered from higher core body temperatures that may have had an effect on the developing embryo within the mare's oviductal or uterine environment. Therefore, it is plausible that the early developing equine embryo would respond to this detrimental environment by higher expression of HSP70. However, in this study, there was no statistical difference in HSP70 expression between quality grade 1 embryos from non-exercised mares or those who suffered from higher body temperatures due to exercise.

An interesting observation of the in vivo derived embryos real time RT-PCR results is that as embryo quality decreased, 18S rRNA C_T values significantly increased, indicating that 18S rRNA concentration in the samples were diminished. In addition, there were no differences in HSP70 C_T values despite decreased embryonic quality. The results of decreased 18S rRNA expression appears to be in agreement with Smith et al.

(2007) who reported significant reductions in gene expression profiles in d 7 quality grade 3 bovine embryos. The authors reported a significant decrease (50%) in GATA6 gene expression and a 40% decrease in ß-actin expression measured via real time RT-PCR when compared to quality grade 1 embryos. The gene GATA6 is a developmentally important gene while ß-actin is a common housekeeping gene (Smith et al., 2007), similar to 18S. Furthermore, they observed that grade 3 embryos contained significantly (65%) fewer cells than the grade 1 embryos (Smith et al., 2007), and this trend is similar to lower quality equine embryos (Vanderwall, 1996). Although neither HSP70 nor 18S were one of the genes examined in the previous mentioned study, the authors demonstrated that with reduced embryo quality, expression of certain genes are reduced. This could help explain the reduction of 18s rRNA expression seen in this study.

While 18S decreased with lower embryo quality, HSP70 mRNA gene expression levels (C_T) appeared to be similar across all quality grades of in vivo recovered embryos. However, the embryos that had cellular insults evident by their darkened cellular appearance, extruded blastomeres and/or other abnormalities, leading to their lower quality grade, demonstrated substantially higher HSP70 mRNA when related to 18S rRNA gene expression. This led to a significantly higher ratio of HSP70 compared to 18S expression in quality grade 4 embryos recovered from both exercised and non-exercised mares. It is possible that these embryos experienced HSP70 mRNA expression increases to try and salvage damaged proteins and block further programmed cell death

(apoptosis). However, these results are not clear due to the benchmark genes (18S) decreased expression observed in these lower quality embryos.

Research has shown that HSP70 plays an important role in maintaining cellular function during cellular stress by acting as molecular chaperones to stabilize or refold proteins damaged by heat and by blocking apoptosis (Morimoto et al., 1996; Mosser et al., 1997; Al-Katani and Hansen, 2002; Chen et al., 2006). Apoptosis is a cellular process required for not only cell selection and for further embryonic development but it also is a reaction to eliminate cells under stress or ones that are malfunctioning (Hernandez et al., 2004). This is accomplished by the activation of caspases that cleave key cellular substrates, which result in an orderly dismantling of the affected cell. In addition, components of the cellular translation machinery are targets of caspase-mediated cleavage during apoptosis that leads to inhibition of protein synthesis (Holcik and Sonenberg, 2005). During cellular apoptosis, 18S and 28S rRNA (constituents of the 80S ribosomal complex) are cleaved and it is postulated that this breakdown leads to protein synthesis inhibition (Degen et al., 2000; Bushel et al., 2004; Rudra and Warner, 2004). Additionally, HSP70 levels elevate and are involved in apoptosis obstruction by inhibiting JNK activation and by its ability to interfere with the formation of the apoptosome, one of the cell death execution caspase complexes that is induced after the leakage of cytochrome-c form the mitochondria (Kampinga, 2006). Therefore, lower quality scored embryo characteristically have more apoptotic cells that led to increased cleavage of 18S rRNA and this can help further explain why lower expression levels of 18S rRNA were observed here. Additionally, this bolsters the possibility that the

embryonic cells in lower quality scored embryos responded to increased cellular stress and apoptosis with markedly increased HSP70 gene expression. However, due to the results of 18S lowered expression, no quantifiable conclusions on HSP70 expression can be drawn from these data.

Although the heat shock response in almost all cell types is well documented, the impact heat shock on mature oocytes and their subsequent development are poorly understood (Curci et al., 1987; Ju et al., 2005). Fiorenza et al. (2004) did report however, that when murine dictyate oocytes were subjected to a heat shock (43 ºC 30 min), increased HSP70 expression was observed. The authors further reported that when oocytes are isolated from their follicle a heat shock factor 1 (HSF1) migrates to the nucleus in response to cellular stress and that this migration was only coupled with HSP70 transcription when oocytes were heat shocked. This stress response was also seen in the murine 1-cell embryo (Fiorenza et al. 2004). While no equine oocytes were analyzed for HSP70 gene expression in this study, equine oocytes were heat shocked during late IVM, fertilized and cultured in vitro to measure developmental competence and then processed for gene analysis.

The results of the in vitro derived blastocysts resulting from a one-time heat shock on equine oocytes showed that the 42 ºC 4 h heat treatment had a lasting effect on HSP70 mRNA relative to 18S rRNA, indicated by an almost 10 fold significant increase in relative gene expression. It was observed that in vitro derived blastocysts 18S rRNA levels did not fluctuate like those observed in the in vivo recovered embryos, indicating the uniform quality of these blastocysts. This observed increase can be explained by the

earlier reported activity (30.7 C_T) of HSP70 in the real time RT-PCR versus all the other observed groups (33.7 C_T). Interestingly, the 2 h treatment at 42 ºC did not seem to have the same lasting effect on HSP70 relative gene expression like the longer 4 h treatment, evidenced by only a 1.6 fold increase. While surprisingly it appears that removing oocytes during IVM for 4 h and held in a control (38.2 ºC) water bath also had an effect that increased HSP70 gene expression relative to 18S rRNA. It is interesting to note that while no differences were noted in oocyte maturation rate or subsequent blastocyst development in this group in the previous oocyte study, it was observed that they recorded the second highest relative expression with a 6 fold increase, though not statistically significant. While oocyte competence was not comprised evidenced by acceptable blastocyst development rates, by examining HSP70 mRNA gene expression relative to 18S rRNA, the removal may have been more stressful than at first thought. This may indicate another use of real time RT-PCR based technology by aiding researchers in examining different applications or laboratory techniques and their positive or negative effects on gene expression. Based on these results, it appears that only a one time heat shock of 42 ºC for 4 h during the late stages of IVM significantly increased HSP70 relative expression and these effects can be measured in d 7 in vitro cultured blastocysts. How the in vitro equine embryos maintained elevated levels of HSP70 expression from the oocyte, through fertilization, up to the blastocyst stage in vitro is unclear. However, it is possible that the maternal genes within the oocyte were affected due to heat and this could possibly explain the results seen here.

It has been shown that maternal gene expression is affected by assisted reproductive technologies (ART) due to the effects on oocytes. Research on mice and hamsters has shown that superovulation results in aberrant embryo development in culture and subsequently compromised fetal development following transfer (Ertzeid and Strong, 2001; Gardner and Lane, 2005). Shi and Haaf (2002) demonstrated that the methylation patterns of 2-cell murine embryos collected from females having undergone superovulation were disturbed at twice the frequency of that for embryos from naturally ovulated oocytes. In agreement, Sato et al. (2007) stated that superovulation changed methylation patterns in mouse oocytes and concluded that different stages of oogenesis and folliculogenesis may have specific time sensitivities to their environment. The majority of imprinted genes are thought to be epigenetically modified during the oocyte growth period (Hiura et al., 2006) and the time period most sensitive to disruption appears to be later in oogenesis, during the diplotene stage, which these maternal imprints are established (Lucifero et al., 2002). While these oocytes were heat shocked near the late final stages of oogenesis, it is possible that a heat shock during this period may leave a lasting effect on the maternal genes in the developing equine embryos genome by stimulating hyper-expressive transcription of HSP70 as observed in this study.

When examining any possible differences in gene expression between in vivo versus in vitro derived equine blastocysts, it is evident that artificial conditions influence embryonic gene expression. The first evidence of this was observed in the data from total RNA isolated from individual embryos. Those embryos grown and maintained in

artificial conditions had higher concentrations of total RNA than the embryos recovered from the live mare. Why embryos grown and maintained in an artificial environment have higher concentrations of total RNA is unclear. However, further evidence of altered gene expression was observed. In vitro derived embryos had similar 18S C_T values (22.3) between groups, however when compared to quality grade 1 in vivo embryos 18S C_T values (15.6), the rRNA expression was significantly diminished. Furthermore, HSP70 relative expression between the in vitro embryos derived from the optimal oocyte maturation environment (0 controls) was compared to the optimal quality grade 1 embryos recovered from non-exercised mares. In vitro embryos had a 31 fold increase in relative HSP70 mRNA to 18S rRNA gene expression. The differences observed are due to that artificially grown blastocysts had lower expression of 18S rRNA thus increasing the ratio comparing the two genes, rather than possible increases in HSP70 expression. However, these results do suggest that a correlation exists in the conditions of the in vitro growth and development environment, by possibly adding more stress upon early developing equine embryos thus altering gene expression. Rycke et al. (2002) stated that through the use of RT-PCR for mRNA phenotyping in bovine preimplantation embryos that the expression pattern of several genes important for development differs between embryos cultured in vitro and embryos obtained in vivo. Furthermore, research into other species has shown embryos grown and maintained under in vitro conditions experience stresses that alter gene expression that would not have occurred had development occurred in vivo (Fiorenza et al., 2004; Gardner and Lane, 2005; Oliveira

et al., 2005). Therefore, it does appear that in the horse, embryonic gene expression is significantly altered under an artificial versus a natural environment.

This research has demonstrated that as in vivo embryo quality decreased, 18S rRNA expression also decreased while HSP70 mRNA gene expression appeared similar across all quality grades. These results validate the widely held view that HSP70 is active and expressed in the early developing embryo. The in vitro blastocysts analysis showed that a one-time heat shock of 42 ºC for 4 h on equine oocytes stimulate an effect on gene expression, evidenced by the higher relative HSP70 expression in the d 7 blastocyst. Additionally, handling oocytes outside of normal IVM procedures may be more stressful to the gamete, and RT-PCR may be a beneficial research tool to analyze laboratory techniques that may alter normal gene expression. Finally, this research is in agreement that artificial culture conditions (in vitro) alter gene expression when compared to more natural in vivo conditions. These results have opened the door for future investigation into the early developing equine embryo with the use of ART and their effects on gene expression, specifically HSP70 expression. Furthermore, this research has shown that the use of real time RT-PCR in the early developing equine embryo is a valid study technique to better understand early embryonic gene expression.

CHAPTER VI

SUMMARY

The results of this research indicate that exercising mares during the breeding season in a hot and moderately humid environment can reduce their reproductive efficiency. The mechanism of this reduction is suspected to be an increase in thermal load on the mare thereby stressing her thermoregulation abilities. However, the additional strain on the stress hormonal response on the hypothalamic-pituitary-axis cannot be discounted, and further research is needed to separate the effects of heat and exercise on mare reproductive function. The exercise regimen followed in this study was associated with a reduction in the diameter of the dominant follicle at ovulation and also delayed the mares' ovulation after administration of PGF2α in diestrus. Furthermore, the exercised mares produced fewer d 7 embryos and had embryos of lesser quality, than those mares not exercised. The results seen here are in agreement with what has been observed in other species.

Germinal-vesicle stage oocytes appeared to be able to withstand a one-time heat shock of 42 ºC for up to 4 h. However, later stage IVM oocytes appeared to be susceptible to the same heat shock regimen. Both a heat shock of 2 or 4 h at 42 ºC was associated with lower rates of oocytes at MII. Advanced embryonic development was significantly lower in the combined heat shock treatments. Therefore, from results of the in vitro experiments in this study, the late-stage preovulatory oocytes are susceptible to

heat shock. These results could possibly explain in part the reduced embryo recovery rate seen in the live mares. While GV stage equine oocytes appeared to be more resistant to heat shock that are oocytes of other species, the results of late-stage oocyte heat shock are in agreement with research in other species, and warrant further investigation into oogenesis and the possible stressors leading to diminished oocyte competence.

Real Time RT-PCR is an innovative technology that is opening new avenues into research. The use of this technology to investigate equine early embryonic development is still in its infancy. The results of this study have shown that single d 7 in vivo and single in vitro-produced equine blastocysts can be analyzed using these procedures. Heat shock protein 70 has received much attention in early embryonic gene analysis. It appears to be a major gene in early genomic activity and, based on this study, this holds true for the equine. The examination of HSP70 gene expression from embryos derived from exercised mares indicates that as embryo quality diminished, 18s rRNA expression decreased and relative to this, HSP70 expression increased. Embryos produced from heat-shocked oocytes during late stage IVM and then subsequently grown in vitro expressed higher levels of HSP70. Total RNA isolated from individual embryos appeared to be higher in artificially produced embryos when compared to embryos recovered from the live animal. Furthermore, in vitro derived embryos when compared to in vivo quality grade 1 embryos expressed lower levels of 18S rRNA and had a higher relative HSP70 expression and this is associated with their unnatural environment. This is in agreement with other studies in that the artificial environment of IVM changes the embryo's natural gene expression during early development

This research has shown that exercise in a hot and humid environment and the possible consequential thermal load experienced by the mare can be detrimental to her fertility during the breeding season. The in vitro portion of this research has indicated that heat can be detrimental to equine oocytes and has proven again to be a valid study model to be applied to the live animal. Investigation into early embryonic genomic function can continue to aid our understanding of how environmental conditions affect early development and cellular function.

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APPENDIX

Table A.1. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for May 2004

May-04	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
18	31.11	27.22	23.33	93	74	55	10	2.03
19	31.67	27.22	22.22	93	72	50	9	0
20	31.67	27.22	22.78	93	72	50	11	0
21	31.11	26.67	21.67	100	79	57	12	0
22	31.67	27.22	22.78	93	69	44	14	0
23	31.67	26.67	21.67	93	73	53	11	0
24	32.22	27.22	21.67	93	70	47	12	0
25	32.78	28.33	23.33	93	72	50	12	0
26	32.22	27.22	22.22	93	72	50	11	0
27	33.33	28.33	22.78	93	70	47	13	0
28	33.33	28.89	23.89	94	77	59	10	0
29	32.78	28.89	24.44	94	77	59	14	0
30	31.67	29.44	26.67	88	80	72	11	0
31	35.56	29.44	22.78	100	76	51	6	0

Table A.2. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for June 2004

Jun-04	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	35.56	28.33	20.56	93	73	53	9	1.07
2	34.44	28.33	22.22	85	71	56	10	0
3	31.67	25.56	19.44	93	74	55	8	1.27
4	33.33	27.78	22.22	93	73	52	6	1.83
5	30.56	26.11	21.67	84	71	57	8	0.13
6	32.78	28.33	23.33	100	76	52	11	0
7	31.11	26.67	21.67	100	85	70	10	2.18
8	26.11	23.89	21.67	100	96	91	10	6.12
9	31.11	27.22	22.78	100	82	63	11	0.69
10	32.22	28.89	25.00	94	75	56	13	0.08
11	31.67	27.78	23.89	94	79	63	11	0.81
12	32.78	28.89	25.00	91	74	56	11	0
13	33.33	27.78	22.22	100	78	56	9	0
14	31.11	26.67	21.67	100	82	63	4	0
15	26.11	23.33	20.00	100	90	79	4	7.44
16	28.89	24.44	20.00	100	87	74	5	0
17	32.22	27.78	22.78	100	86	72	4	0
18	33.89	28.89	23.89	100	78	56	6	0
19	33.33	28.89	23.89	100	73	46	4	0
20	33.89	28.89	23.89	94	73	52	5	0
21	33.89	29.44	24.44	100	78	56	8	0
22	28.89	25.56	22.22	100	85	69	6	0.97
23	30.56	27.22	23.89	100	85	70	6	0
24	26.67	25.00	23.33	100	96	91	5	0.86
25	30.56	26.67	22.78	100	84	68	5	0.56
26	29.44	26.11	22.22	100	83	65	8	0.03
27	29.44	25.56	21.11	100	90	80	6	4.57
28	31.67	27.22	22.22	100	79	57	7	0.51
29	28.89	25.56	22.22	100	87	74	6	0.30
30	27.78	25.00	22.22	100	87	74	6	0.43

Table A.3. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for July 2004

Jul-04	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	32.22	27.78	22.78	100	78	56	7	0.03
2	32.22	28.33	24.44	100	78	56	9	0
3	32.78	28.89	24.44	94	75	56	8	0
4	32.78	28.33	23.89	94	72	49	9	0
5	33.33	28.89	23.89	100	76	52	9	0
6	33.89	28.89	23.89	100	76	52	7	0
7	33.33	28.89	23.89	100	76	52	8	0
8	33.89	28.89	23.89	100	76	52	9	0
9	30.00	26.67	22.78	94	82	69	6	0.99
10	32.78	27.22	21.11	93	73	52	5	0
11	32.22	27.78	22.78	93	72	50	5	0.20
12	32.22	27.22	21.67	100	74	47	5	0
13	33.33	27.78	21.67	93	67	41	3	0
14	34.44	28.89	22.78	93	70	46	3	0
15	35.00	29.44	23.33	87	67	46	3	0
16	34.44	29.44	24.44	94	72	49	4	0
17	35.00	29.44	23.89	94	70	46	7	0
18	33.33	29.44	25.56	85	61	36	9	0
19	33.89	27.78	21.11	84	63	41	4	0
20	33.89	28.89	23.33	94	68	41	6	0
21	34.44	28.33	22.22	93	70	46	5	0.00
22	33.89	28.89	23.33	100	75	49	7	0
23	35.00	29.44	23.33	100	73	46	5	0
24	35.56	30.00	23.89	94	68	41	3	0
25	35.56	29.44	23.33	94	73	52	6	4.42
26	30.56	25.56	20.00	90	71	51	9	0
27	32.22	26.67	21.11	97	74	50	7	0
28	33.33	28.33	22.78	87	72	56	9	0
29	33.89	28.33	22.78	94	72	49	6	0.28
30	34.44	28.89	23.33	100	76	52	5	0
31	35.00	30.00	24.44	88	72	56	6	0

Table A.4. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for August 2004

Aug-04	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	33.89	30.00	25.56	94	75	56	4	0.18
2	35.56	30.00	24.44	94	71	48	3	0
3	36.11	30.56	25.00	94	69	43	3	0
4	36.67	30.56	24.44	94	68	42	4	0
5	36.67	30.56	23.89	94	68	42	5	0
6	32.78	29.44	25.56	85	69	52	8	0
7	32.22	27.22	22.22	84	61	37	7	0
8	32.78	27.22	21.11	84	61	38	5	0
9	35.00	29.44	23.33	82	59	36	6	0
10	32.78	28.33	23.33	88	72	56	4	2.69
11	35.00	28.33	21.67	94	68	41	6	0.05
12	30.00	25.00	19.44	90	61	32	11	0
13	30.56	23.89	17.22	87	63	39	5	0
14	30.00	25.00	20.00	78	59	40	5	0
15	30.56	25.00	18.89	78	56	34	5	0
16	32.22	25.56	18.33	81	58	34	4	0
17	32.78	26.11	19.44	81	59	36	4	0
18	33.89	27.22	20.00	90	69	47	7	0
19	33.33	27.78	22.22	100	78	56	12	0.36
20	36.11	28.89	21.67	94	68	42	8	0.15
21	32.78	27.22	21.11	100	80	59	7	1.52
22	33.89	28.33	22.78	94	73	52	7	0.94
23	32.22	28.33	23.89	97	81	64	8	0.56
24	35.00	30.00	25.00	94	70	46	10	0
25	35.00	30.00	25.00	97	73	49	10	0
26	35.56	31.11	26.11	97	71	45	12	0
27	34.44	30.00	25.00	94	73	52	10	0
28	32.78	28.33	23.33	100	78	56	6	0.18
29	32.78	27.22	21.67	93	74	55	4	0
30	33.89	27.22	20.56	90	63	36	6	0
31	31.67	26.67	21.67	84	58	31	8	0

Table A.5. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for September 2004

Sep-04	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	30.56	24.44	18.33	81	61	40	6	0
2	30.00	26.11	21.67	84	66	48	3	0
3	28.33	25.00	21.11	94	82	69	5	0
4	32.22	27.22	22.22	100	76	52	4	0.33
5	33.89	28.33	22.22	100	76	52	5	0
6	33.33	28.33	23.33	100	78	55	8	0
7	27.22	23.33	19.44	93	74	54	10	0.10
8	31.67	26.67	21.67	61	47	32	8	0
9	32.78	25.00	17.22	72	53	34	6	0
10	33.89	27.22	20.56	73	55	36	6	0
11	33.89	28.89	23.33	84	64	44	4	0
12	34.44	28.33	22.22	87	62	36	5	0
13	34.44	28.33	22.22	88	69	49	6	0
14	32.22	28.33	23.89	100	80	59	7	0.18
15	34.44	29.44	23.89	100	73	46	5	0
16	36.11	29.44	22.22	93	65	36	3	0
17	37.22	30.00	22.22	87	61	35	4	0
18	35.56	29.44	23.33	93	66	39	5	0
19	35.56	29.44	22.78	93	60	26	6	0
20	33.33	26.67	20.00	74	55	36	6	0
21	32.78	27.22	21.11	93	68	43	7	0
22	33.33	27.78	22.22	90	66	41	8	0
23	34.44	27.22	20.00	93	65	36	8	0
24	30.56	25.56	20.56	97	76	55	10	0
25	31.11	26.67	22.22	94	75	55	10	0.05
26	32.78	26.67	20.00	93	66	38	8	0
27	31.67	25.56	18.89	87	57	27	5	0
28	33.33	27.22	20.56	73	50	26	6	0
29	32.22	25.56	18.89	73	50	27	5	0
30	32.78	25.56	18.33	73	57	40	7	0

Table A.6. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for May 2005

May-05	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	23.89	16.11	8.33	86	59	31	5	0
2	25.56	18.89	11.67	77	52	27	7	0
3	23.89	18.89	13.89	64	53	41	9	0
4	23.89	18.89	13.89	86	66	46	9	0.05
5	27.22	19.44	11.67	77	57	36	5	0
6	28.89	22.22	15.56	84	59	34	8	0
7	28.33	22.22	15.56	93	70	47	13	0
8	21.67	18.89	15.56	100	90	79	11	3.73
9	28.33	21.11	13.89	100	82	63	6	0.05
10	30.56	25.56	20.56	97	77	57	9	0
11	30.56	25.56	20.56	97	73	48	13	0
12	29.44	25.00	20.56	97	74	51	12	0
13	29.44	24.44	19.44	100	72	44	10	0
14	29.44	24.44	19.44	93	71	48	6	0
15	29.44	24.44	18.89	73	54	34	9	0
16	24.44	21.11	17.78	87	69	50	5	0
17	30.00	22.78	15.56	97	70	43	8	0
18	31.11	25.56	19.44	100	73	45	9	0
19	30.56	25.56	20.00	97	73	48	6	0
20	32.22	27.22	22.22	93	70	47	5	0
21	35.56	28.33	21.11	90	62	34	3	0
22	35.56	28.89	21.67	84	59	34	3	0
23	33.33	27.78	21.67	87	63	38	8	0
24	32.78	27.22	21.11	100	71	41	7	0
25	34.44	27.78	20.56	97	69	41	6	0
26	27.78	24.44	20.56	87	74	60	7	0.20
27	31.67	25.56	18.89	87	62	37	5	0
28	31.67	27.22	22.22	93	68	43	8	0.46
29	30.00	25.00	20.00	100	79	57	6	1.78
30	29.44	25.00	20.56	97	78	58	5	0.03
31	31.11	26.11	21.11	97	72	46	5	0

Table A.7. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for June 2005

Jun-05	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	29.44	24.44	18.89	93	72	51	6	0.9398
2	32.78	26.11	19.44	90	69	47	6	0
3	32.22	27.22	21.67	93	73	52	11	0
4	33.33	28.89	24.44	88	69	49	13	0
5	33.33	28.89	23.89	88	65	41	9	0
6	34.44	29.44	23.89	94	71	47	7	0
7	34.44	29.44	24.44	94	70	46	11	0.1524
8	33.89	30.00	25.56	91	70	49	12	0
9	34.44	29.44	24.44	94	69	44	12	0
10	34.44	28.89	22.78	94	68	41	11	0
11	34.44	28.89	22.78	94	68	41	6	0
12	34.44	28.89	23.33	94	67	39	9	0
13	33.33	28.33	23.33	94	73	52	8	0
14	35.56	29.44	23.33	94	69	43	5	0
15	36.67	30.00	23.33	87	63	38	4	0
16	36.11	30.00	23.33	87	62	36	6	0
17	35.00	29.44	23.33	94	68	41	7	0
18	35.56	30.00	23.89	94	67	39	7	0
19	35.56	30.56	25.00	79	58	36	7	0
20	34.44	28.33	22.22	73	51	28	7	0
21	34.44	27.78	20.56	73	52	30	4	0
22	35.00	28.89	22.22	79	53	26	3	0
23	35.00	29.44	23.33	87	60	33	6	0
24	35.56	28.89	22.22	79	56	33	6	0
25	35.00	28.33	21.67	84	59	34	5	0.0508
26	35.56	29.44	22.78	87	59	30	6	0
27	35.56	28.89	22.22	87	60	33	6	0
28	36.11	29.44	22.78	87	61	35	5	0
29	36.67	30.56	23.89	87	60	33	6	0
30	37.22	30.56	23.89	88	63	38	6	0

Table A.8. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for July 2005

Jul-05	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	37.78	31.67	25.56	93	72	51	6	0
2	37.78	31.11	23.89	90	69	47	6	0
3	37.78	31.11	23.89	93	73	52	11	0
4	38.33	32.22	25.56	88	69	49	13	0
5	36.67	30.56	23.89	88	65	41	9	0
6	38.89	32.22	25.00	94	71	47	7	0
7	38.89	30.00	21.11	94	70	46	11	0
8	33.89	27.78	21.67	91	70	49	12	0.13
9	35.00	29.44	23.33	94	69	44	12	0
10	36.11	30.00	23.33	94	68	41	11	0
11	36.11	30.56	24.44	94	68	41	6	0
12	36.11	30.00	23.89	94	67	39	9	0
13	36.11	30.56	24.44	94	73	52	8	0
14	35.56	28.89	22.22	94	69	43	5	0
15	31.67	27.22	22.22	87	63	38	4	1.93
16	30.00	26.67	23.33	87	62	36	6	0.15
17	30.00	27.22	23.89	94	68	41	7	0.91
18	33.33	28.89	24.44	94	67	39	7	0.381
19	34.44	29.44	23.89	79	58	36	7	0
20	33.89	30.00	25.56	73	51	28	7	0
21	33.33	28.89	24.44	73	52	30	4	0
22	35.56	30.00	24.44	79	53	26	3	0
23	33.89	28.89	23.89	87	60	33	6	0.051
24	34.44	29.44	23.89	79	56	33	6	0
25	35.00	29.44	23.89	84	59	34	5	0
26	33.33	28.33	23.33	87	59	30	6	0
27	34.44	28.33	22.22	87	60	33	6	0
28	35.00	28.89	22.22	87	61	35	5	0
29	34.44	29.44	23.89	87	60	33	6	0
30	35.56	29.44	22.78	88	63	38	6	0
31	36.11	28.33	20.00	97	72	46	5	0

Table A.9. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for August 2005

Aug-05	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	35.56	30.00	23.89	79	63	46	5	0.102
2	35.56	29.44	23.33	87	63	39	3	1.42
3	34.44	29.44	23.89	94	70	46	6	0.025
4	34.44	28.89	23.33	87	68	49	6	0
5	34.44	28.33	22.22	87	67	46	5	0
6	35.56	28.89	21.67	93	67	41	3	0.025
7	35.00	29.44	23.89	87	63	39	4	0
8	33.89	28.89	23.33	94	72	49	6	0.41
9	30.00	26.11	21.67	90	76	61	6	0
10	28.33	25.56	22.22	94	84	74	6	4.88
11	33.89	28.33	22.78	94	70	46	7	0
12	35.00	29.44	23.89	94	69	43	7	0
13	34.44	29.44	23.89	94	70	46	9	0
14	31.67	28.33	25.00	91	79	67	7	0
15	33.89	28.89	23.33	94	73	52	6	0.79
16	33.33	28.33	23.33	100	76	52	5	0
17	33.89	28.89	23.33	94	72	49	6	0
18	35.00	30.00	24.44	94	70	46	7	0
19	33.89	29.44	24.44	94	75	56	7	0
20	35.00	30.00	24.44	94	67	39	6	0
21	36.11	30.00	23.89	94	65	35	3	0
22	36.11	30.56	24.44	94	67	40	4	0
23	36.11	30.56	24.44	94	68	42	5	0
24	36.11	30.56	24.44	88	64	40	5	0
25	35.00	30.00	25.00	91	70	49	4	0
26	36.11	30.56	25.00	88	65	42	3	0
27	37.22	31.67	25.56	91	65	38	6	0
28	35.00	29.44	23.33	87	62	36	8	0
29	35.56	30.56	25.00	79	59	38	9	0
30	36.67	29.44	22.22	93	62	30	4	0
31	37.22	30.00	22.78	87	57	26	3	0

Table A.10. Mean temperatures (ºC), relative humidity (%), wind (kph) and precipitation (cm) for September 2005

Sep-05	Temperature (ºC)			Humidity (%)			Wind (kph)	Precipitation (cm)
	high	avg	low	high	avg	low	avg	sum
1	37.22	30.00	22.78	87	56	24	5	0
2	35.56	30.00	23.89	82	59	36	6	0
3	35.00	29.44	23.33	87	62	36	5	0
4	36.11	30.00	23.33	82	54	25	7	0
5	36.11	28.89	21.11	61	42	22	6	0
6	35.00	28.33	21.11	73	52	31	6	0
7	33.89	27.22	20.56	79	54	29	7	0
8	35.00	27.22	19.44	76	52	28	5	0
9	35.00	28.33	21.67	84	56	28	4	0
10	32.22	27.22	22.22	91	75	59	7	0
11	33.33	28.89	23.89	94	69	44	9	0
12	33.33	28.89	23.89	87	67	47	8	0
13	35.00	29.44	23.33	94	69	44	8	0
14	36.67	30.56	23.89	94	66	38	10	0
15	37.22	31.11	24.44	94	66	38	10	0
16	35.56	30.56	25.56	91	66	41	7	0
17	37.22	31.11	24.44	94	63	31	6	0
18	36.67	31.11	25.00	91	63	35	8	0
19	36.67	30.00	23.33	94	65	35	7	0
20	36.67	29.44	22.22	93	60	27	2	0
21	38.89	31.11	22.78	74	50	26	3	0
22	38.89	30.56	22.22	74	49	24	5	0
23	36.67	30.56	23.89	64	46	28	15	0
24	34.44	28.89	22.78	76	57	38	18	0
25	40.00	30.56	21.11	84	55	25	5	0
26	40.56	32.78	24.44	94	58	22	7	0
27	39.44	31.11	22.78	87	59	31	6	0
28	39.44	32.22	24.44	100	65	29	5	0
29	28.89	25.56	22.22	93	74	54	11	0
30	32.22	26.67	20.56	78	63	47	7	0

Table A.11. Mean temperatures and relative humidity for the exercise period (1300-1500 h) for the months studied during 2004

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
May	18	24.80	71
	19	25.45	65
	20	29.45	61
	21	28.90	61
	22	30.00	68
	23	29.70	57
	24	30.00	63
	25	30.55	59
	26	30.00	61
	27	31.10	61
	28	30.30	59
	29	31.40	68
	30	29.15	68
	31	34.15	76

Table A.11 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
June	1	33.60	63
	2	33.05	64
	3	30.00	65
	4	31.95	63
	5	27.50	64
	6	31.40	64
	7	28.83	78
	8	23.45	94
	9	30.00	73
	10	30.85	66
	11	30.85	71
	12	32.25	65
	13	31.70	67
	14	30.00	73
	15	22.80	85
	16	28.15	81
	17	30.85	79
	18	31.95	67
	19	32.75	60
	20	32.25	63
	21	32.80	67
	22	27.15	77
	23	29.15	78
	24	25.55	94
	25	29.00	76
	26	28.45	74
	27	26.75	85
	28	27.45	68
	29	26.10	81
	30	30.00	65

Table A.11 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
July	1	31.65	56
	2	30.55	56
	3	31.40	56
	4	31.95	49
	5	32.25	52
	6	31.95	52
	7	32.20	52
	8	31.95	52
	9	24.00	69
	10	30.60	52
	11	31.70	50
	12	31.40	47
	13	31.70	41
	14	32.20	46
	15	33.60	46
	16	33.35	49
	17	33.05	46
	18	33.35	36
	19	33.05	41
	20	32.75	41
	21	32.20	46
	22	33.05	49
	23	32.30	46
	24	31.95	41
	25	32.45	52
	26	33.60	51
	27	31.10	50
	28	27.70	56
	29	30.00	49
	30	31.90	52
	31	32.25	56

Table A.11 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
August	1	30.85	54
	2	34.15	54
	3	34.70	63
	4	34.45	61
	5	35.25	60
	6	30.00	62
	7	30.25	64
	8	31.40	64
	9	32.80	59
	10	31.40	55
	11	33.35	63
	12	28.85	59
	13	28.86	66
	14	28.85	67
	15	29.15	68
	16	30.00	49
	17	31.50	48
	18	31.40	46
	19	32.25	50
	20	34.15	49
	21	30.85	50
	22	32.50	68
	23	31.70	48
	24	30.35	50
	25	33.85	50
	26	33.85	60
	27	33.87	55
	28	34.15	49
	29	32.80	54
	30	31.40	47
	31	32.55	50

Table A.11 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
September	1	29.20	42
	2	29.40	49
	3	26.35	77
	4	30.55	60
	5	31.65	59
	6	31.95	62
	7	24.70	60
	8	30.00	37
	9	30.55	36
	10	30.55	41
	11	32.75	57
	12	31.50	38
	13	33.65	63
	14	31.40	62
	15	31.40	60
	16	31.15	41
	17	31.40	38
	18	34.70	45
	19	35.55	40
	20	33.95	48
	21	33.65	41
	22	32.20	38
	23	31.15	58
	24	31.65	55
	25	31.95	55
	26	29.65	43
	27	29.50	30
	28	31.40	27
	29	29.70	35
	30	32.50	43

Table A.12. Mean temperatures and relative humidity for the exercise period (1300-1500 h) for the months studied during 2005

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
May	15	27.80	40
	16	23.60	58
	17	28.30	44
	18	29.15	52
	19	28.60	55
	20	29.45	41
	21	33.60	40
	22	33.90	49
	23	31.70	49
	24	30.55	46
	25	31.40	84
	26	24.45	41
	27	30.30	61
	28	29.15	69
	29	26.30	65
	30	26.95	55
	31	30.00	51

Table A.12 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
June	1	27.25	60
	2	30.85	53
	3	30.25	59
	4	32.25	52
	5	31.95	52
	6	32.75	52
	7	32.75	55
	8	32.75	52
	9	32.50	47
	10	32.75	48
	11	31.95	45
	12	32.50	47
	13	31.70	55
	14	33.30	45
	15	34.15	46
	16	34.15	41
	17	34.15	45
	18	32.75	41
	19	33.05	52
	20	32.25	37
	21	32.50	35
	22	32.50	35
	23	32.50	35
	24	33.05	36
	25	32.75	41
	26	33.60	39
	27	33.70	41
	28	33.05	39
	29	34.15	40
	30	33.85	47

Table A.12 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
July	1	35.25	44
	2	34.70	44
	3	35.20	44
	4	35.55	40
	5	35.55	43
	6	35.00	35
	7	36.40	35
	8	36.40	50
	9	32.50	46
	10	32.80	44
	11	34.15	41
	12	34.20	45
	13	33.60	50
	14	33.60	48
	15	33.60	61
	16	30.85	79
	17	26.95	80
	18	27.35	67
	19	30.40	57
	20	32.75	57
	21	33.05	60
	22	32.65	49
	23	33.30	55
	24	32.25	53
	25	32.75	48
	26	33.05	61
	27	31.15	57
	28	31.40	53
	29	32.75	47
	30	33.05	39
	31	33.85	31

Table A.12 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
August	1	32.75	44
	2	34.15	48
	3	32.75	52
	4	32.20	50
	5	32.80	50
	6	33.05	45
	7	33.60	45
	8	31.55	52
	9	26.90	77
	10	24.00	94
	11	32.30	55
	12	33.30	52
	13	33.60	50
	14	29.30	72
	15	33.05	55
	16	31.80	59
	17	31.95	52
	18	32.80	59
	19	32.50	54
	20	33.85	47
	21	33.60	50
	22	33.60	46
	23	35.00	46
	24	34.75	45
	25	33.90	49
	26	33.05	47
	27	34.70	44
	28	35.00	47
	29	33.30	47
	30	33.60	36
	31	34.75	31

Table A.12 continued

Month	Day	Mean Temperature (ºC)	Mean Humidty (%)
September	1	35.25	36
	2	33.60	37
	3	34.75	41
	4	34.15	31
	5	33.85	28
	6	32.75	34
	7	32.75	31
	8	33.60	34
	9	33.05	34
	10	29.45	57
	11	33.05	43
	12	32.00	46
	13	33.60	49
	14	34.20	44
	15	35.40	46
	16	32.75	55

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