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The Challenges of Making a Blastocyst-Stage Embryo: Impact of Heat Stress & Technical Factors Associated with IVP Procedures

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To the Graduate Council:

I am submitting herewith a thesis written by Estanislao Peixoto entitled "The Challenges of Making a Blastocyst-Stage Embryo: Impact of Heat Stress & Technical Factors Associated with IVP Procedures." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Lannett Edwards, Major Professor

We have read this thesis and recommend its acceptance:

Arnold Saxton, Neal Schrick

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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A Thesis Presented for
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Degree
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Abstract

It was hypothesized that technical factors associated with in vitro production (IVP) of embryos may influence rate of blastocyst development of oocytes matured at 38.5 or 41.0 C. To test this hypothesis, a retrospective meta-analysis was performed. Simple linear regression was performed to analyze continuous variables and ANOVA for categorical variables. Interactions among factors and maturation temperature on blastocyst development were analyzed using dummy regression for continuous variables, and using a factorial treatment design and ANOVA for categorical variables. Month of collection was the only variable that impacted responsiveness of ova to heat stress. Independent of maturation temperature, variables that explained most variation in blastocyst development included technician, total number of sliced ovaries per collection, ova number placed per well of oocyte maturation media, oocyte collection time, bull ID, sperm concentration added to ova, and ova age at IVF. The proportion of 8 to 16-cell embryos at time of cleavage assessment was the best predictor of blastocyst development. Results of model selection showed that development of ova matured at 38.5 C was associated with size of the collection, while development of ova matured at 41.0 C was mainly associated with ova age at fertilization. When data for ova matured at 38.5 and 41.0 C were combined, the effect of number of PZ per well on blastocyst development became evident. Use of these findings for optimizing efficiency of IVP procedures would effectively reduce experimental costs related to embryo production and increase laboratory productivity.

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Chapter I: Introduction

Heat stress detrimentally impacts fertility of female cattle during summer months (Badinga et al., 1985, Collier et al., 1982, Fuquay, 1981, Gwazdauskas et al., 1973, Gwazdauskas et al., 1975, Ryan et al., 1993, Wolfenson et al., 2000). A heat-stressed cow can exhibit elevated body temperatures that may reach or exceed 41°C (Badinga et al., 1985, Dunlap and Vincent, 1971, Ealy et al., 1993, Gwazdauskas et al., 1974, Gwazdauskas et al., 1973, Monty and Wolff, 1974, Turner, 1982, Ulberg and Burfening, 1967). Additionally, each degree increase in rectal temperature is correlated with a progressive decrease in pregnancy rates (Ulberg and Burfening, 1967).

Heat stress effects to decrease fertility are particularly problematic during estrus (Cavestany et al., 1985, Dutt, 1963, Dutt et al., 1959, Ingraham et al., 1976, Morton et al., 2007, Zakari et al., 1981). This is the time period that females are sexually receptive. When heifers are heat-stressed such that body temperatures are increased during estrus, embryos recovered seven days after insemination are developmentally retarded and of inferior quality compared to those obtained from non heat-stressed females (Putney et al., 1989). During estrus, the oocyte is undergoing meiotic maturation within the Graafian follicle. To determine the extent to which direct exposure of oocytes to heat stress results in reduction in embryo development, Edwards and Hansen (1996) removed ova from cattle ovaries and applied a 41.0°C heat stress to oocytes undergoing in vitro maturation. Heat stress exposure during maturation resulted

in 65% reduction in blastocyst development (Edwards and Hansen, 1996), which is similar to the 85% decline in pregnancy rates reported for heifers heat-stressed during the first 10 hours of estrus (Putney et al., 1989).

Further use of this in vitro model has identified potential mechanisms underlying heat-induced reductions in blastocyst development (Edwards and Hansen, 1997, Edwards et al., 2005, Lenz et al., 1983, Roth and Hansen, 2005, Schrock et al., 2007, Soto and Smith, 2008, Payton and Edwards, unpublished). However, the effect of heat stress to reduce blastocyst development varies from replicate to replicate. Effects of heat stress range from zero negative effect to 87.4% reduction in blastocyst development. For example in a single experiment, the developmental decrease in blastocyst rates ranged from 7 to 53% (Lawrence et al., 2004). Additionally, blastocyst development from ova matured at 38.5°C ranges from zero development to 46.1% (Lawrence, unpublished; Payton, 2009).

In vitro production of blastocyst stage embryos requires multiple steps: ovary collection and transport, collection, in vitro maturation and fertilization of oocytes, removal of cumulus (denuding) from putative zygotes (PZ), and culture of PZ. Multiple factors related to each step may account for variability in developmental and negative effect of heat stress including biological and technical variation. Among the biological factors that may have an influence on in vitro production (IVP) of embryos are the age of the animal (Revel et al., 1995), stage of the estrous cycle (Boediono et al., 1995), body condition (Ruiz et al., 1996, Snijders et al., 2000), reproductive status (Machatkova et al., 1996, Snijders et al., 2000), and bull ID (Avery and Quetglas, 1996,

Coelho et al., 1998, Kurtu et al., 1996, Larocca et al., 1996, Palma et al., 1996, Roses et al., 1999, Yang et al., 1995). Technical factors known to influence IVP of embryos include ovary age since removal from animal (Yang, 1990), ovary temperature (Yang, 1990), oocyte and embryo density per culture well (de Oliveira et al., 2005), sperm concentration (Ward et al., 2002), maturation time (Ward et al., 2002), and co-incubation time of ova with sperm (Rehman et al., 1994, Ward et al., 2002).

It was hypothesized that technical factors associated with IVP of embryos may influence rate of blastocyst development of oocytes matured at 38.5 or 41.0°C. To test this hypothesis, a retrospective meta-analysis was performed to identify technical factors that may affect blastocyst development of ova matured at 38.5°C or at 41°C, and to determine to what extent responsiveness of ova to certain technical factors among the two groups differs.

Chapter II: Literature Review

A long term goal of Dr. Edwards' laboratory is to improve fertility of cattle during the summer months when rectal temperatures meeting or exceeding 41.0°C (Dunlap and Vincent, 1971, Gaalaas, 1945, Monty and Wolff, 1974, Ulberg and Burfening, 1967) reduce fertility (Badinga et al., 1985, Collier et al., 1982, Fuquay, 1981, Gwazdauskas et al., 1973, Gwazdauskas et al., 1975, Ryan et al., 1993, Stott and Williams, 1962, Wolfenson et al., 2000). Heat-induced reductions in fertility are particularly problematic during estrus (Cavestany et al., 1985, Ingraham et al., 1974, Morton et al., 2007, Putney et al., 1989, Stott and Wiersma, 1976, Zakari et al., 1981), when the animal is receptive to breeding. It is during this time that the oocyte is undergoing meiotic maturation (Hyttel et al., 1997). In vitro studies have demonstrated that detrimental effects of direct heat stress are prominent on the maturing oocyte (Edwards and Hansen, 1996) and are responsible for some of the reduced fertility observed in heat-stressed cattle.

Examining Effects of Heat Stress on the In Vitro Maturing Oocyte

To study the direct effects of heat stress on ova during maturation, in vitro embryo production is used, encompassing many steps (ovary collection and transport, oocyte collection, in vitro maturation and fertilization, removal of cumulus and associated sperm from putative zygotes (PZ) and culture of PZ). This approach removes the influence of maternal environment and results in an average decrease in blastocyst development up to 65% when oocytes are exposed to 41°C during the first

12 hours of maturation (Edwards and Hansen, 1996, Lawrence et al., 2004, Roth and Hansen, 2004, Schrock et al., 2007), which is quite similar to the 85% decrease in embryonic development reported after heat-induced increases in elevated body temperature during the first 10 hours of estrus (Putney et al., 1989).

A decrease in the number of blastocysts after in vitro application of heat stress demonstrates direct effects on the oocyte during meiotic maturation. Reductions in blastocyst rates are not due to fertilization or cleavage failure (Edwards et al., 2005, Lawrence et al., 2004). Rather, a recent finding demonstrated that compaction rates on day 6 were reduced by heat stress application during maturation (Edwards et al., 2009).

Alterations Coincident with Heat-Induced Blastocyst Reductions

The use of in vitro embryo production has allowed discovery of potential mechanisms related to heat-induced blastocyst reduction. Levels of reactive oxygen species may be altered as a result of heat stress (Edwards and Hansen, 1997, Lawrence et al., 2004, Payton et al., 2003, Wang et al., 2009; Payton et al., unpublished), probably functioning as mediators of cell damage. Heat stress may accelerate cellular metabolism, resulting in an inability of mitochondria to properly reduce oxygen that remains in its radical state (Loven, 1988). Additionally, pool of proteins and transcripts are required in oocytes and embryos up through 8 to 16-cells until embryonic genome activation occurs (Memili and First, 1999, 2000). However, decreased protein synthesis (Edwards and Hansen, 1996) and possible decreases in polyadenylated RNA (Edwards et al., 2005) were reported for heat-stressed ova.

The maturation rate of the cytoplasm may also be altered by heat stress. During maturation, the arrangement of cortical granules, small membrane-bound secretory granules that undergo exocytosis to block ploypermy penetration (Wang et al., 1997), changes from an aggregated state throughout the cytoplasm (type I and II distribution) to a dispersed state along the oolemma (type III distribution). In bovine ova exposed to 41°C during the first 12 hours of maturation, there was a decrease in type II and increase in type III cortical granules compared to controls at 24 hours (Edwards et al., 2005). This suggests that the process of cytoplasmic maturation in heat-stressed ova may be faster than in controls.

The nucleus concurrently undergoes changes when the egg is exposed to elevated temperatures during maturation. Direct heat stress of murine oocytes at 40°C during maturation resulted in abnormal chromosome morphology and number (Fiorenza and Mangia, 1992). Alterations such as meiotic spindle abnormalities (Roth and Hansen, 2005, Wang et al., 2009) have been reported, which may impact progression of meiosis I for chromosome segregation and maturation promoting factor (MPF) inactivation (Brunet et al., 2003, Gallicano, 2001). Absence of polar bodies in matured bovine oocytes after heat stress suggested possible inhibition of meiosis (Lenz et al., 1983). However, the staining of nuclear material with Hoechst showed that heat-stressed bovine ova had not only the same rate of meiotic progression as controls, but reached metaphase I and metaphase II earlier than controls (Edwards et al., 2005). If nuclear maturation occurs faster in heat-stressed ova, fertilization at 24 hours would involve an aged egg (Edwards et al., 2005). When heat-stressed bovine ova (41.0°C,

first 12 hours of maturation) were fertilized earlier, there was an increase in the number of embryos that developed into blastocysts, with the best development occurring when IVF was performed 18 hours after the onset of maturation (Schrock et al., 2007).

Cumulus cells also play a key role in the development of the ovum during maturation (Zhang et al., 1995). Their connection with the oocyte through gap junctions allows for the transport of metabolites and regulatory factors (Buccione et al., 1990). Changes in the physiology of cumulus cells may impact the proper maturation of the oocyte. In essence, the capacity of response of a bovine oocyte to a heat stress through heat shock proteins is impaired without cumulus cells (Edwards and Hansen, 1996). Moreover, it is essential to document that a deterioration of cumulus expansion through a decreased incorporation of glucosamine in hyaluronic acid was induced with a 41°C stress in bovine oocytes (Lenz et al., 1983).

Variability in Blastocyst Development across Studies

Up to this point, the general trend for heat-induced reductions in blastocyst development after exposure to elevated temperature during oocyte maturation has been discussed (de Castro e Paula and Hansen, 2007, Edwards et al., 2009, Edwards and Hansen, 1996, 1997, Edwards et al., 2005, Lawrence et al., 2004, Roth and Hansen, 2004, b, Schrock et al., 2007) and represents an average response. Depending on the study, the responsiveness of oocytes to the heat stress may differ. For example, in two reports, heat-induced reductions in blastocyst development varied from 64.9% to 10.9% (Edwards and Hansen, 1996 versus Edwards and Hansen, 1997, respectively).

Moreover, not only the responsiveness of oocytes to heat stress differs among studies, but also variability is the rule for the blastocyst development of ova matured at 38.5°C. For example, among two random experiments, blastocyst development ranged from 25 to 46% (Roth and Hansen, 2004a versus Edwards and Hansen, 1997, respectively).

Variability in Blastocyst Development within an Experiment

If the effect of heat stress within replicates of an experiment is evaluated, heat-induced reductions in development may range from 0 to 53% of control values (Lawrence et al., 2004). Moreover, on some occasions, heat stress may produce higher blastocyst development than controls. Developmental potential of oocytes collected on a given day may also vary within experimental replicates. Development of ova matured at 38.5°C that on average reaches more than 20% (Moore and Thatcher, 2006) can range from 0.0% to 46.1% using similar protocols and laboratory conditions (Lawrence, unpublished; Payton, 2009).

Factors Potentially Responsible for Experimental Variability

High quality and quantity embryo production in an artificial environment depends on numerous biological and technical factors. In vitro embryo production allows researchers to obtain immature (i.e., germinal vesicle stage or GV-stage) oocytes from slaughterhouse derived ovaries and mature (IVM), fertilize (IVF) and culture (IVC) resulting embryos (reviewed by Moore and Thatcher, 2006). Each of these procedural parts has multiple steps where subtle differences may account for variability in

development. To obtain a blastocyst stage embryo, the acquisition of bovine oocytes from ovaries should be followed by 24 hours of maturation with gonadotropins (Hasler, 1992; 1998). Once oocytes are matured, successful in vitro fertilization will need a certain concentration of motile sperm (reviewed by Hasler, 1998). After this action, the embryos may be kept in laboratory conditions up to the hatched blastocyst stage previous to transfer to a recipient animal or cryopreservation (reviewed by Van Soom and de Kruif, 1996). These techniques yield 20 to 50% blastocysts of the oocytes fertilized (Moore and Thatcher, 2006).

Variability across and within experiments may be explained by biological differences such as the age of the animal (Revel et al., 1995), body condition (Ruiz et al., 1996, Snijders et al., 2000) and bull ID (Avery and Quetglas, 1996, Larocca et al., 1996). Technical factors such as the total time post mortem to obtain the oocytes, ovary temperatures (Nakatate et al., 2005, Yang, 1990), technician (Yang et al., 1995), number of oocytes per well (de Oliveira et al., 2005), sperm concentration (Ward et al., 2002), maturation time (Agung et al., 2006, Ward et al., 2002), gamete co-incubation time (Ward et al., 2002) and number of PZ per well (Fujita et al., 2006) may also influence blastocyst development. In an effort to explain what may be accounting for this variability, the following sections discuss the factors that are known to affect in vitro embryo production.

Biological Diversity

Oocytes collected from ovaries derived from abattoir cattle may differ in developmental potential because of biological diversity. This diversity may be related with factors such as genotypic differences, age of the animal, body condition, estrous cycle stage and reproductive status.

Age of the Animal

Studies of aged oocyte donors were mostly made in humans and showed an impairment of the quality of the oocytes mainly because of aneuploidy (reviewed by Armstrong, 2001, Zayed et al., 1997). Additionally, very young donors may be problematic, as oocytes from three month old calves (9% blastocyst formation rate) were less developmentally-competent than oocytes from adult cows (Armstrong, 2001, 20%; Revel et al., 1995).

Body Condition

Reported results from IVP of embryos using oocytes derived from cows of different body conditions scores suggest that malnutrition adversely affected embryo development (Ruiz et al., 1996, Snijders et al., 2000). Blastocyst rates from oocytes obtained from dairy cattle with body condition scores of 3 (21.1% morula and blastocyst per oocyte) was higher than from body scores of 1 (4.6%; Ruiz et al., 1996). However, another study found that body condition did not influence the capacity of oocytes to cleave or to reach blastocyst stage, but only scores greater than 3 were used (Fazio et

al., 1999). This is another factor that was unknown during routine IVP of embryos protocols using slaughterhouse derived ovaries.

Estrous Cycle Stage and Reproductive Status

The developmental competence of bovine oocytes collected from abattoir cattle ovaries on different stages of the estrous cycle or pregnancy may affect the developmental competence of bovine embryos. Ovaries possessing a corpus luteum (CL) in the luteal phase produce more competent oocytes as reflected with 24% blastocyst development with CL versus 13% without, and as reflected with 29% blastocyst development in the luteal phase versus 20% in other phases (Boediono et al., 1995). That may be a reason why oocytes obtained from pregnant cows may be more suitable for in vitro embryo production (Boediono et al., 1995). However, another study provided evidence that pregnancy status does not influence the capacity of oocytes to cleave or to reach blastocyst stage (Fazio et al., 1999). Examination of buffalo oocytes found that the best developmental rates were obtained when the oocytes came from paired ovaries with a corpus hemorrhagicum or CL and without a dominant follicle than when they came from paired ovaries with a CL with and a dominant follicle, regressing CL with dominant follicle, or ovaries with only small follicles (Manjunatha et al., 2007). The day of the cycle was also linked to the level of bovine oocyte competence, reaching a maximum at day 14 to 16 (Machatkova et al., 1996). In contrast, no differences on bovine in vitro embryo production between different estrous cycle stages and different follicle sizes were found, but the experiments were performed

during the summer, which is the season we have seen that the developmental competence would be impaired (Vlatka et al., 2008). Additionally, highly productive cows provided oocytes with less developmental competence than those from lower genetic merit cows (Snijders et al., 2000).

Ovary Collection

While biological origin is largely unknown when using abattoir-derived ovaries, other factors that can be controlled may play a role after ovaries are collected to account for developmental differences among experiments. During this first step, bovine ovaries are obtained from an abattoir and typically transported to the laboratory in insulated thermoses.

Total Time post Mortem to Obtain Oocytes

Several reports indicated that an increase in ovary age postmortem decreased blastocyst development (Blondin et al., 1997, Nakatate et al., 2005, Yang, 1990). According to Nakatate et al. (2005), when the bovine ovaries were kept at around 23.8°C, blastocyst development was less for transport times of 17 to 18 hours compared to transportation times of 1 to 1.5 hours. Additionally, Yang et al. (1990) reported that blastocyst development decreased from 23% to 12.6% if bovine ovaries were stored for 4 or 16 hours, respectively, using 325 ovaries.

Ovary and Oocyte Temperatures

Storage of bovine ovaries at 37 or 4°C for 4 hours demonstrated that blastocyst development was lower (10.6 and 0%, respectively) than when exposed to 25°C (23.0%; Yang, 1990). Blastocyst development after holding bovine ovaries for 24 hours in thermoses at different temperatures (15, 18 and 21°C) were all similar (Schernthaner et al., 1997), probably because the storage time was of 24 hours.

Bovine oocytes show high susceptibility to temperature changes. When oocytes were cooled to 30 (control), 20, 10 and 0°C for 30 minutes before IVM and then rewarmed to 30°C, the blastocyst development was 24.7, 26.3, 6.4 and 1%, respectively (Martino et al., 1995). Additionally, when they were cooled to 0°C for 0.5, 1, 10 or 30 minutes before IVM, development to blastocyst decreased to 16.3, 13.3, 7.0 and 1.2%, respectively (Martino et al., 1995). Furthermore, a decrease in bovine blastocyst development from 31 to 17% occurred with temperature fluctuations of 20 to 39°C, respectively (Schwartz et al., 1998).

Oocyte Collection

Oocytes are typically harvested from 3 to 8 mm follicles of selected ovaries and then washed in an oocyte collection medium. Using a stereomicroscope, oocytes are separated from debris in a search dish and then evaluated for having dark, evenly granulated ooplasm and compact cumulus prior to placement into maturation medium.

Technique

A technician's experience is a key factor towards achieving highly efficient embryo production. Research scientists, with experience ranging from two to ten years, were evaluated on their ability to produce bovine blastocysts and the difference among the highly and the least experienced technicians was astonishing (Yang et al., 1995). During handling and sudden temperature changes, the oocyte cytoskeleton function may be altered and lead to aneuploidy as shown with enhanced polarizing microscopy of spindles on living human oocytes (Eichenlaub-Ritter et al., 2002). Additionally, increased handling may accelerate metabolism resulting in an increased production of reactive oxygen species and subsequently intracellular damage (Leese et al., 2007).

Oocyte Morphology

The morphology of the cumulus-oocyte complexes (COCs) is important. Bovine COCs with slight expansion of the outer layers of cumulus cells (at the beginning of atresia stage) and slight granulation of the ooplasm provided a higher development to morula stages than COCs with compact cumulus and homogeneous ooplasm, but full expansion of the cumulus or no cumulus had the worst development (Blondin and Sirard, 1995).

Number of Cultured Oocytes per Well

The number of oocytes per drop of maturation medium (from one to four oocytes in a fixed volume) did not appear to modify the rates of embryo formation in humans

(Khamsi et al., 1999). In contrast, the culture of bovine oocytes in groups of 20 per 500 µl achieved better blastocyst development (37.4%) than single oocyte culture (10.1%) using co-culture with granulosa cells (Doherty et al., 1997). However, another study showed that when more bovine oocytes or embryos were cultured per 100 µl drop (from 5 to 30), less development to blastocyst was achieved (de Oliveira et al., 2005). It was also reported that culture of 30 instead of 3 bovine oocytes per 30 µl drop increased levels of ammonium concentration, but without detrimental effect on blastocyst development (Ikeda et al., 2000).

In Vitro Fertilization

After sperm straws at -196°C are removed from liquid N_2 and warmed for 45 seconds at 34°C, motile sperm may be isolated using discontinuous Percoll® column (Parrish et al., 1995). Once sperm are washed, concentration and motility are determined to calculate volume of sperm that should be added to the ova.

Bull Identification (ID)

The influence of bull, of the same or different breeds, on in vitro blastocyst development is well-established (Coelho et al., 1998, Katska-Ksiazkiewicz et al., 2009, Kurtu et al., 1996, Larocca et al., 1996, Parrish et al., 1986, Roses et al., 1999, Yang et al., 1995). For example, when semen from Jersey bulls was compared to semen from Holstein bulls, Jersey bulls consistently produced a greater number of blastocysts (Avery and Quetglas, 1996). Having knowledge of bull fertility may be predictive of the

performance of the sperm for IVF. German Simmental bulls with low non-return rates had significantly lower blastocyst development than bulls with normal non-return rates (Palma et al., 1996). When sperm from two different bulls with impaired fertility was used to study oocyte competence, significant differences in cleavage and blastocyst rates were found (Katska-Ksiazkiewicz et al., 2009). In another case, there was no correlation between bull fertility and blastocyst rates after IVF (Schneider et al., 1996).

Sperm Concentration

It has been suggested in the literature that a ratio of 1:5,000 oocyte/sperm (500,000 sperm/ml) is related to maximum blastocyst development (Long et al., 1994, Ward et al., 2002). For example, an assessment of the optimum bovine sperm concentration from 10,000 to 1,000,000 sperm/ml (1:100 to 1:10,000 oocyte:sperm, respectively) showed that 500,000 sperm/ml (1:5,000 oocytes/sperm) was the most effective level to achieve optimal (35.4% blastocyst yield) in vitro blastocyst development (24.9% blastocyst yield for 1:10,000 oocyte:sperm; 5.9% for 1:100; Ward et al., 2002). Concentrations of 2,000,000, 1,000,000 and 500,000 Holstein sperm/ml had similar rates of embryo cleavage, but blastocyst development was not reported (Kurtu et al., 1996). Increasing bovine sperm concentrations from 1:5,000 to 1:50,000 oocyte/sperm (Tanghe et al., 2000) produced similar fertilization and polyspermy rates but blastocyst development was not reported.

Maturation Time

The fertile lifespan of the in vitro matured oocyte is finite (4 to 8 hours; Ward et al., 2002). Holding matured mammalian oocytes in vitro before fertilization (i.e., aging) reduced subsequent blastocyst development (Agung et al., 2006, Takahashi et al., 2003, Tarin et al., 1998). Ward et al. (2002) reported that bovine blastocyst development was only affected when fertilization was performed at 16 (20.4% blastocyst yield) or 32 hours of IVM (24.8% blastocyst yield), and that the highest value of blastocyst development was obtained when IVF occurred at 24 hours of IVM (39.3% blastocyst yield; Ward et al., 2002). Others found that maximum development to bovine blastocyst was obtained at 22 hours of maturation and not at 16, 28, or 34 hours (Agung et al., 2006). It is important to mention that the extraction of oocytes from rabbit ovarian follicles was enough to trigger the process of maturation, and this can be a confounding factor to calculate the maturation time (Pincus and Enzmann, 1935).

Denuding

The purpose of the denuding step is to remove the associated cumulus cells and spermatozoa from PZ using the mechanical method of vortexing. This is done to avoid competition for nutrients from cumulus cells and release of toxins from the dead sperm. After washing and evaluation, PZ are placed in potassium simplex optimized medium (KSOM) for embryo culture.

Gamete Co-Incubation Time

A high concentration of sperm coincubated with ova in a small volume for a long period of time may be deleterious to in vitro embryo production (Rehman et al., 1994, Sumantri et al., 1996, Ward et al., 2002). Co-incubation of 15 to 20 matured bovine oocytes with 600,000 sperm/ml for 4, 8, 12, 16, 20, 24, or 28 hours did not alter blastocyst development (52.0 to 70.3% blastocysts of eight-cell embryos; Rehman et al., 1994). However, co-incubation for 10 hours was sufficient to maximize bovine blastocyst development (31.9 to 32.3% of total PZ cultured) while 1 (0%) and 5 hours (19.2%) were insufficient times for sperm to fertilize oocytes (Ward et al., 2002). Apparent disparity between Ward et al. (2002) and Rheman et al. (1994) may be related to different number of oocytes and the different embryo culture media used (Synthetic oviduct fluid vs. Chatot-Ziomek-Bavister with buffalo rat liver cells coculture, respectively). Additionally, the optimum time to keep the sperm with oocytes was reported to be different from breed to breed (Sumantri et al., 1996).

Number of Cultured Embryos per Well

In contrast to the reported trend for oocytes, most publications indicated that increasing the number of embryos in the culture media, increased development to the blastocyst stage (Fujita et al., 2006, Khurana and Niemann, 2000, Lane and Gardner, 1992, Pinyopummin et al., 1994). Development was significantly improved when bovine embryos from inferior quality oocytes were cultured in groups of 40 instead of groups of 20 per 500 µl of medium (Khurana and Niemann, 2000). Not only did higher number of

embryos increase the development to blastocyst, but also the transfer of media from high number embryos to single embryos, suggesting the presence of embryo development promoting factors that reach beneficial levels when bovine embryos are cultured together (Fujita et al., 2006).

Summary

For this thesis, it was hypothesized that technical and biological factors that are linked to an in vitro embryo production protocol may change responsiveness of ova to heat stress and alter their developmental competence. In retrospect, many IVP steps are confounded with factors such as biology of the sample, environmental conditions, and subtle technical differences. Variability in the blastocyst development and heat stress effect are not only found over time but also within a given experiment. Further analyses of the data were required to find confounding factors. The approach taken to test the hypothesis was to study the factors that affect development of ova matured at 38.5, or 41.0°C, and determine if responsiveness of oocytes matured at 38.5 and 41.0°C to certain technical factors differed.

Chapter III: Materials and Methods

In Vitro Production of Embryos

In vitro production of blastocyst stage embryos requires multiple steps: ovary collection and transport, oocyte collection, in vitro maturation and fertilization of oocytes, removal of cumulus (denuding) from putative zygotes (PZ), and culture of PZ. Multiple pieces of information recorded at each step were analyzed in an effort to explain variability in blastocyst development.

Data Source

Data collected from seven different experiments (112 different days of oocyte collections) by four different technicians (Edwards et al., 2009, Edwards et al., 2005, Lawrence et al., 2004, Payton, 2009, Schrock et al., 2007, Ward, 2008; Lawrence, unpublished; Table 1) were merged into a single dataset in order to perform a retrospective meta-analysis. For each source of data, number of replicates used, number of observations, number of oocytes, and duration of the experiment was reported (Table 1). Additionally, since efficiency of IVP should range from 20 to 50% blastocyst development (Moore and Thatcher, 2006), developmental competence was defined as more than 20% blastocyst development and developmentally-challenged ova defined as those with less than 20% (Table 1). All the efforts were to find the values of factors that enhance competent development. For each factor

Table 1. Data Sources for the Retrospective Meta-Analysis

Sources of Data	No. Replicates Used*	No. Competent**	No. Challenged***	No. Obs.	No. Oocytes	Starting Date	Final Date	Comments
Lawrence et al., 2004	10	7	3	20	946	03.13.02	06.04.02	Retinol Impact on Heat-Stressed Oocytes
Edwards et al., 2005; Lawrence, unpublished	16	13	3	32	1237	04.15.03	06.30.04	Impact of Heat Stress on Nuclear Maturation. Chemical Activation or IVF at 24 or 30 hours post-Maturation Impact on Heat Stressed Oocytes
Schrock et al., 2007	15	13	2	30	1674	11.10.05	04.11.06	Early IVF Impact on Heat Stressed Oocytes
Edwards et al., 2009	20	15	5	40	10140	06.08.06	03.22.07	Heat Stress During Oocyte Maturation Increases Susceptibility of Morulas to Heat Stress
Ward, 2008	28	17	10	55	5330	11.15.07	05.29.08	Culture of Bovine Oocytes at 38.5 or 41.0°C With or Without AL-8810
Payton, Chapter 2, 2009	22	14	8	44	2806	01.31.06	04.05.07	Total RNA and Transcript Abundance in Heat-Stressed Oocytes
Payton, Chapter 3, 2009	19	9	10	38	4231	04.19.07	03.25.08	Microarray Analysis of Heat-stressed Oocytes

*Number of replicates used
**Number of observations related with competent ova that achieved more than 20% blastocyst development
***Number of observations related with challenged ova that achieved less than 20% blastocyst development

(i.e., explanatory variable), number of observations based on days of collection, mean, standard error, range, mode and distribution type were reported in Table 2. The purpose of each factor in an IVP protocol is depicted in more detail in subsequent sections.

Ovary Source

Ovaries were obtained from an abattoir (Brown Packing Co., Gaffney, SC). Origins of the cattle were unknown, but most were from the southeast with some from Texas and Oklahoma. Genetic background of cattle was also unknown but less than 1% of the animals were *Bos indicus*. Of the more than 99% that were *Bos taurus*, 90 to 95% were beef (e.g., Angus, Hereford and Charolais) and 5 to 10% were dairy cattle (mainly Holsteins). In some cases, the animals were held for up to 48 hours with feed and water before processing.

Ovary Collection

To collect ovaries, cattle were euthanized with captive bolt and ovaries were collected beginning approximately at 7:15 AM ET, which was the beginning time used to calculate related processing times. After collection at the abattoir, ovaries were transported to the laboratory on Tuesdays or Thursdays (i.e., day of week; Table 2) via airplane or land vehicle (i.e., ovary transport type; Table 2). Collections were not performed during the months of July, August and September to minimize effects of elevated ambient temperature on oocytes (i.e., collection season; Table 2). Ovary age

Table 2. Description of Technical and Biological Factors Based on Ova Matured at 38.5°C

IVP Steps	Explanatory Variable	No. of Replicates*	Mean	se	Minimum	Maximum	Mode	Distribution Type
Ovary Collection								
	Day of Week	102						categorical
	Tuesday	49						
	Thursday	53						
	Collection Season	110						categorical
	Fall	20						
	Winter	33						
	Spring	57						
	Total No. Ovaries Collected	70	159.0	4.3	63.00	275.00	164.0	continuous
	Average No. Ovaries per Thermos	67	41.3	1.0	21.00	68.75	42	continuous
	Ovary Transport Type	111						categorical
	Airplane	76						
	Vehicle	35						
	Ovary Age at Laboratory Arrival (hr)	110	5.5	0.1	3.90	7.85	5.3	continuous
	Ovary Temperature (°C)	110	28.0	0.2	19.75	33.25	28.3	continuous
Oocyte Collection								
	Technician	112						categorical
	A	28						
	B	41						
	C	14						
	D	29						
	Total Slice Time (min)	101	40.2	1.5	10.00	75.00	41.0	continuous
	Total Sliced Ovaries	109	86.2	3.9	20.00	166.93	90.0	continuous
	Total Search and Evaluation Time (min)	105	82.6	2.1	25.00	130.00	83.0	continuous
In Vitro Maturation								
	M199 Age (days)	53	29.1	3.4	0.00	81.00	24.0	continuous
	Ova No. per Well	111	43.0	0.9	20.00	60.00	45.3	continuous
	Total Ova No. in OMM per Day	111	600.4	22.21	138.00	1433.00	607.0	continuous
	Total Ova No. in OMM per Ovary	110	7.8	0.2	2.48	15.08	7.4	continuous
	Oocyte Collection Time (min)	103	122.3	2.7	40.00	176.00	126.0	continuous
	Total Collection Time (hr)	111	7.9	0.1	5.88	10.58	7.8	continuous

Table 2. Continued

IVP Steps	Explanatory Variable	No. of Replicates*	Mean	se	Minimum	Maximum	Mode	Distribution Type
In Vitro Fertilization								
	Bull 1	25						categorical
	Bull 2	50						categorical
	Bull 3	4						categorical
	Bull 4	17						categorical
	Bull 5	11						categorical
	Bull 6	4						categorical
	Bull 7	2						categorical
	Bull 8	28						categorical
	Bull 9	71						categorical
	Bull 10	1						categorical
	Bull 11	5						categorical
	Centrifugation Method	112						categorical
	Centrifuge	55						
	Microcentrifuge	57						
	Sperm Assessment Method	112						categorical
	CASA	29						
	Hemocytometer	83						
	Sperm Concentration (Million Sperm/ml)	110	35.8	1.6	9.09	74.19	31.6	continuous
	Sperm Motility (%)	100	87.9	0.9	64.00	98.00	91.0	continuous
	Sperm Volume (µl)	99	9.1	0.4	2.30	18.30	8.6	continuous
	Sperm Concentration after Added to Ova (sperm/ml)	111						categorical
	500,000 (sperm/ml)	94						
	600,000 (sperm/ml)	7						
	700,000 (sperm/ml)	10						
	Sperm Preparation Time (min)	106	32.2	0.6	22.00	50.00	31.0	continuous
	Ova Age at IVF (hpm)	109	24.2	0.0	23.55	25.00	24.2	continuous
	Ova Age at IVF from Removal from Follicles (hr)	103	26.2	0.1	23.89	27.38	26.4	continuous

Table 2. Continued

IVP Steps	Explanatory Variable	No. of Replicates*	Mean	se	Minimum	Maximum	Mode	Distribution Type
Denuding								
	Gamete Co-incubation Time (hpi)	107	18.5	0.1	16.22	20.65	18.3	continuous
	Vortex Time (min)	96	4.4	0.0	3.50	5.00	4.5	continuous
	% Total PZ Recovered	110	96.0	0.5	84.00	118.18	96.9	continuous
	% Total PZ Lysed	112	2.5	0.2	0.00	13.00	2.0	continuous
	% Total PZ with Excessive Cumulus	110	3.1	0.4	0.00	22.44	1.8	continuous
	PZ No. per 500 µl KSOM	101	24.8	0.6	14.50	46.00	23.25	continuous
	Total Denuding Time (min)	110	24.5	0.7	10.00	69.00	23.0	continuous
In Vitro Culture								
	Cleavage Assessment Time (hpi)	103	71.7	0.2	66.35	77.22	71.9	continuous
	Blastocyst Assessment Time (hpi)	111	195.4	0.9	181.75	215.20	192.4	continuous
	% Cleaved	109	71.7	1.2	28.57	95.65	73.2	continuous
	% 2-Cells of Cleaved	109	7.9	0.6	0.00	28.57	6.4	continuous
	% 2-Cells of Total	109	5.5	0.4	0.00	17.24	4.7	continuous
	% 4-Cells of Cleaved	109	15.0	1.2	0.00	54.00	11.8	continuous
	% 4-Cells of Total	109	10.6	0.8	0.00	46.55	8.1	continuous
	% 8-16 Cells of Cleaved	109	77.0	1.4	28.57	100.00	80.0	continuous
	% 8-16 Cells of Total	109	55.5	1.5	15.79	86.11	57.1	continuous

* Based on number of different ovary/oocyte collection days

(from 7:15 AM when ovaries were collected from animals to time of arrival at the laboratory in hours) and temperature were recorded after arrival at the laboratory to study their effect on blastocyst development (Table 2). The number of ovaries collected was recorded and used as an indirect estimator of the collection size (Table 2). Additionally, with thermoses used to minimize fluctuations in ovary temperature, the average number of ovaries per thermos was calculated (Table 2).

Oocyte Collection

After temperatures were recorded, ovaries were washed with tap water equilibrated to the same temperature as the ovaries. Oocytes were harvested from 3 to 8 mm follicles of selected ovaries by performing a slicing technique, making checkerboard incisions of approximately 2 mm across the follicle surface. The number of sliced ovaries (Table 2), used as an estimator of collection size, and the time period required to slice ovaries (from beginning of ovary slice to beginning of oocyte search) were recorded (Table 2). Medium containing oocytes was filtered through an EmCon, washed with oocyte collection media, and remaining contents transferred to a search plate. Using a stereomicroscope, cumulus-oocyte complexes were separated from debris in a search dish and then evaluated. Only oocytes with compact cumulus and dark, evenly granulated ooplasm were selected for further culture. The time period when searching for oocytes began (after slicing was completed) until oocytes were placed in

maturation media (i.e., total search and evaluation time; Table 2) was also recorded to examine impact of processing time on blastocyst development.

Oocyte Maturation

Cumulus-oocyte complexes meeting evaluation criteria were transferred to oocyte maturation media (OMM) to be matured for approximately 24 hours at 38.5 (bovine physiological body temperature) or at 41.0°C but also at 41.5 or 42.0°C in some cases (typical body temperature of heat-stressed cattle; Gaalaas, 1945) with a CO_2 concentration of 5.5% and a O_2 concentration of approximately 21%. To prepare OMM, liquid base media M199 was used and its age recorded (Table 2). During this step, oocytes were cultured in Nunc 4-well plates (average number of COCs per 500 µl OMM in a well of a Nunc 4-well plate was recorded; i.e., ova number per well; if treatments were not reported by well then overall average was calculated; Table 2). Total ova collected per day (i.e., total ova in OMM per day; Table 2) was used as another direct estimator of collection size; and in combination with number of ovaries sliced, total ova in OMM per ovary was estimated (Table 2). As an additional effort to study the impact of processing times on blastocyst development, oocyte collection time (time period from when slicing began until oocytes were placed in OMM) and total collection time (defined as the time period from when collection of ovaries at the abattoir began until oocytes were placed in OMM at the laboratory) were documented (Table 2).

In Vitro Fertilization

Sperm from one bull or combinations of 2 or 3 bulls was selected from a pool of 11 different bulls (Beef: Senepol, (SE), Simmental, (SM), Red Angus, (AR), Angus, (AN), Braunveih) (BU)) and different collection dates. After removal from a liquid N_2 tank, selected sperm was layered on a Percoll® column (45/90%) and bull ID codes were registered. Motile and live sperm were separated from dead and immotile sperm on a discontinuous (45/90%) Percoll® column with two possible sperm centrifugation methods: a 640 to 760 *g* centrifugation for 10 to 16 minutes (4 ml column on 14 ml conical tubes) or a 2,000 *g* microcentrifugation for 10 to 12 minutes (1.5 ml column on 1.5 ml microcentrifuge tubes). Not only did the centrifugation *g* and exposure time vary, but also the plastic material, volume and surfaces of tubes used for sperm preparation. After Percoll® purification and Sperm-TALP wash, the pellet of predominant motile sperm was isolated and resuspended in IVF-TALP. Concentration and motility of the sample after Percoll® were assessed to calculate appropriate volume of sperm (µl per 500 µl of IVF-TALP well) to add to ova. To achieve this end, either a hemocytometer or computer assisted sperm analysis (CASA) was utilized (Table 2). The targeted sperm ratio for in vitro fertilization was 1:5,000 to 1:7,500 ova (500,000 to 750,000 motile sperm/ml, respectively). The time period from when sperm was thawed until addition to ova was also recorded as a measurement of the sperm processing time. Ova age at IVF was evaluated using two different approaches to clarify impact of age at sperm addition on blastocyst development: the total time in OMM until sperm addition, and the total time from ova removal from follicles until sperm addition (Table 2).

Denuding

Sperm and oocytes were coincubated for 16.2 to 20.7 hours before denuding (i.e., time between addition of sperm to ova and beginning of denuding; gamete co-incubation time; Table 2). Removal of associated cumulus and sperm from putative zygotes (PZ) was performed by vortexing for 3.5 to 5 minutes (i.e., vortex time; Table 2) in HEPES-TALP with 0.3% hyaluronidase. After washing, PZ were evaluated. Specifically, recovery (percentage of PZ recovered of total oocytes placed in OMM as indirect indicator of lysis), lysis (PZ recovered that no longer had an intact membrane; i.e., oolemma) and presence of excessive cumulus cells (percentage of PZ recovered with excessive cumulus) were assessed and recorded. Finally, the selected PZ were placed in KSOM for additional embryo culture (PZ number per 500 µl KSOM; Table 2). Total denuding time was also used to study impact of processing time on blastocyst development (time between beginning of denuding and placement of PZ in KSOM plates in the incubator; Table 2).

In Vitro Culture

Approximately, three days after IVF, ability of PZ to cleave was assessed (Table 2) and the stage of embryos present recorded (PZ cleaved, 2-cells of cleaved, 2-cells of total PZ, 4-cells of cleaved, 4-cells of total PZ, 8 to 16-cells of cleaved and 8 to 16-cells of total PZ per treatment; Table 2) to identify the best predictor of blastocyst development. Blastocyst assessment was performed approximately eight days after IVF (Table 2).

Statistical Analysis

Influence of different technical or biological factors on blastocyst development of ova matured at 38.5 or 41.0°C was assessed with single variable models. Simple linear regression was performed for continuous variables (PROC REG), ANOVA for categorical variables (PROC GLM), and ANOVA using multimember facility option for analyzing bull ID (PROC GLIMMIX). Quadratic polynomials and response surfaces were fit for appropriate variables. Interactions among factors and maturation temperature on blastocyst development were analyzed by comparing the estimated slope of each treatment using dummy regression for continuous variables (Blastocyst (%) = α + β*Factor + Treatment + Factor*Treatment + Error; PROC GLM) and comparing the estimated least squares means using a factorial treatment design and ANOVA for categorical variables (Blastocyst (%) = μ + Factor + Treatment + Factor*Treatment + Error; PROC GLM). Outliers were identified by values having higher than 1.5 interquartile range above 75^{th} or below 25^{th} percentile, or when significant data supported that observations were not a product of good laboratory practices, or when an atypical interval for a variable was confounded with only one technician. Confirmed outliers were deleted from relevant analyses.

For the development of complex models that included multiple variables, a model selection was performed using only those variables with more than 99 observations, to minimize removal of replicates from the analysis due to missing observations and to maximize use of variables. First, for continuous variables, a stepwise R^2 selection method was conducted. When the simplest model did not decrease its R^2 by more than

15% of the R^2 of the previous model or when a model with only 10 variables was obtained, the first selection step ended. To include categorical variables in the model, manual backwards selection was performed by combining the previously obtained model with the categorical variables, followed by discarding variables with the highest p-values one by one, until the model contained 5 variables. If the model R^2 decreased by more than 15% and contained less than 8 variables, the selection criteria was switched from elimination of higher p-values to elimination of variables whose removal produced less decrease in R^2. Data were analyzed with SAS 9.2 (2008, SAS Institute Inc., Cary, NC, USA.).

Chapter IV: Results and Discussion

Influence of Different Technical Factors Associated with IVP of Embryos on Blastocyst Development

It was hypothesized that technical factors associated with IVP of embryos may influence rate of blastocyst development of oocytes matured at 38.5 or 41.0°C. To test this hypothesis, a retrospective meta-analysis of data collected from March 2002 to May 2008 was performed to identify technical factors that may affect blastocyst development of ova matured at 38.5 or 41°C, and to determine the extent responsiveness of ova to certain technical factors may differ among the two different groups of oocytes.

Technical factors in this study included explanatory variables related to each step required to make a blastocyst stage embryo such as ovary collection and transport, oocyte collection, maturation and fertilization of oocytes, denuding and culture of putative zygotes (Table 3). For each explanatory variable, the number of observations, R^2, estimate (slope or mean, for continuous or categorical data, respectively), standard error of the estimate and P-value were reported. Information appearing under the heading of 38.5 or 41.0°C in Table 3 depicts results from simple linear regression or ANOVA analyses for continuous and categorical variables, respectively. Remaining columns, titled 'Interaction P' and 'Main Factor P', are the results derived after performing a dummy regression or ANOVA with a treatment factorial arrangement (i.e., combined) for continuous and categorical variables, respectively. Results for each factor are reported in more detail in subsequent sections.

Table 3. Influence of Different Technical or Biological Factors on Blastocyst Development after Ova were Matured at 38.5 and 41°C

		38.5°C					41.0°C					Combined Analysis				
IVP Steps	**Explanatory Variables**	**n**	**R^2**	**Estimates***	**se$^{\mp}$**	***P-Value***	**n**	**R^2**	**Estimates***	**se$^{\mp}$**	***P-Value***	**Interaction *P-Value*$^{\#}$**	**R^2**	***Main Effect Estimates*$^{\wedge,\flat}$**	**se$^{\mp}$**	***Main Effect P-Value*$^{\Theta}$**
Ovary Collection																
Day of Week			0.020			0.1594		0.031			0.0729	0.6988	0.025			**0.0237**
	Tuesday	49		24.710	1.253	A	50		22.895	1.372	A			23.794	0.902	A
	Thursday	53		22.246	1.205	A	53		19.429	1.333	A			20.837	0.933	B
Collection Season			0.013			0.4983		0.024			0.2790	0.5317	0.013			0.2477
	Fall	20		21.722	1.992	A	20		19.775	2.180	A			20.749	1.486	A
	Winter	33		24.385	1.551	A	34		19.565	1.672	A			21.939	1.148	A
	Spring	56		24.299	1.190	A	56		22.628	1.303	A			23.463	0.888	A
Total No. Ovaries Collected		71	0.107	-0.084	0.029	**0.0053**	70	0.004	-0.018	0.036	0.6164	0.1551	0.034	-0.052	0.023	**0.0283**
Average No. Ovaries per Thermos		68	0.121	-0.387	0.128	**0.0037**	67	0.039	-0.249	0.153	0.1094	0.4922	0.072	-0.319	0.100	**0.0017**
Ovary Transport Type			0.021			0.1274		0.005			0.4735	0.6195	0.011			0.1192
	Airplane	76		24.567	1.004	A	76		21.840	1.120	A			23.203	0.755	A
	Vehicle	34		21.793	1.501	A	35		20.405	1.651	A			21.089	1.121	A
Ovary Age at Laboratory Arrival (hr)		109	0.011	-0.938	0.855	0.2749	110	0.041	-2.008	0.930	**0.0331**	0.3981	0.024	-1.477	0.635	**0.0210**
Ovary Temperature (°C)		109	0.008	-0.311	0.332	0.3501	110	0.000	0.072	0.362	0.8433	0.4365	0.001	-0.118	0.247	0.6338
Oocyte Collection																
Technician			0.099			**0.0107**		0.024			0.4545	0.6601	0.050			**0.0107**
	A	27		21.768	1.632	BC	28		20.019	1.847	A			20.878	1.239	B
	B	41		21.509	1.325	C	41		20.439	1.526	A			20.974	1.015	B
	C	15		26.688	2.190	AB	15		21.302	2.523	A			23.995	1.678	AB
	D	28		27.558	1.603	A	28		23.803	1.847	A			25.677	1.228	A
Total Slice Time (min)		101	0.001	-0.022	0.059	0.7095	102	0.001	0.022	0.066	0.7375	0.6182	<0.001	0.001	0.044	0.9885
Total Sliced Ovaries		108	0.128	-0.077	0.020	**0.0001**	109	0.033	-0.043	0.022	0.0579	0.2478	0.070	-0.060	0.015	**<0.0001**
Total Search and Evaluation Time (min)		105	0.036	-0.075	0.038	**0.0523**	105	0.008	-0.038	0.042	0.3684	0.5226	0.018	-0.057	0.029	**0.0476**

Table 3. Continued

IVP Steps	Explanatory Variables	38.5°C n	38.5°C R^2	38.5°C Estimates*	38.5°C se‡	38.5°C *P-Value*	41.0°C n	41.0°C R^2	41.0°C Estimates*	41.0°C se‡	41.0°C *P-Value*	Combined Analysis Interaction *P-Value*[ж]	Combined Analysis R^2	Combined Analysis *Main Effect Estimates*[ת]	Combined Analysis se‡	Combined Analysis *Main Effect P-Value*[ɵ]
In Vitro Maturation																
	M199 Age (days)	52	0.010	-0.039	0.054	0.4751	52	0.000	-0.005	0.064	0.9381	0.6862	0.003	-0.021	0.042	0.6163
	Ova No. per Well	110	0.125	-0.316	0.081	**0.0002**	111	0.042	-0.212	0.096	**0.0300**	0.4065	0.074	-0.265	0.063	**<0.0001**
	Total Ova in OMM per Day	109	0.032	-0.007	0.004	0.0612	110	0.010	-0.004	0.004	0.2859	0.6339	0.038	-0.006	0.003	**0.0413**
	Total Ova in OMM per Ovary	109	0.061	0.848	0.321	**0.0094**	110	0.002	0.189	0.371	0.6108	0.1798	0.021	0.530	0.247	**0.0329**
	Oocyte Collection Time (min)	87	0.043	-0.106	0.054	**0.0552**	88	0.023	-0.077	0.054	0.1573	0.7154	0.050	-0.091	0.039	**0.0197**
	Total Collection Time (hr)	110	0.019	-1.042	0.719	0.1498	111	0.016	-1.000	0.763	0.1928	0.9675	0.017	-1.015	0.528	**0.0557**
In Vitro Fertilization																
	Bull ID		0.114			0.3194		0.118			0.2517	0.8843	0.092			**0.0343**
	Bull 1	25		36.276	6.066	A	24		38.142	6.726	A	49		37.280	4.505	A
	Bull 2	51		19.627	5.127	AB	51		9.932	5.646	DE	102		14.785	3.794	CD
	Bull 3	3		28.459	11.065	AB	4		33.521	10.911	ABCD	7		30.677	7.711	ABC
	Bull 4	17		24.010	4.462	AB	17		18.485	4.912	BCD	34		21.255	3.302	BC
	Bull 5	12		26.427	7.221	AB	12		17.539	7.953	ABCDE	24		21.988	5.344	ABC
	Bull 6	2		26.984	12.933	AB	2		48.101	14.246	AB	4		37.542	9.572	AB
	Bull 7	27		21.080	4.707	AB	28		13.059	5.152	CDE	55		17.043	3.473	CD
	Bull 8	71		23.498	3.730	B	72		27.725	4.108	ABC	143		25.605	2.760	BC
	Bull 9	4		11.245	9.558	B	4		-4.529	10.528	E	8		3.363	7.074	D
	Bull 10	5		23.880	10.462	AB	5		20.239	11.524	ABCDE	10		22.055	7.743	ABCD
	Bull 11	1		5.067	19.421	AB	1		32.862	21.392	ABCDE	2		18.971	14.374	ABCD
	Centrifuge method		0.088			**0.0015**		0.055			**0.0127**	0.7875	0.069			**<0.0001**
	Centrifuge	55		26.425	1.140	A	55		23.611	1.284	A			25.018	0.863	A
	Microcentrifuge	56		21.214	1.130	B	57		19.051	1.262	B			20.123	0.852	B
	Sperm Assessment Method		0.062			**0.0085**		0.022			0.1160	0.5549	0.038			**0.0035**
	Hemocytometer	28		27.550	1.621	A	28		23.803	1.831	A			25.677	1.230	A
	CASA	83		22.529	0.941	B	84		20.453	1.057	A			21.485	0.712	B

Table 3. Continued

IVP Steps / Explanatory Variables	38.5°C n	38.5°C R^2	38.5°C Estimates*	38.5°C se∓	38.5°C *P-Value*	41.0°C n	41.0°C R^2	41.0°C Estimates*	41.0°C se∓	41.0°C *P-Value*	Combined Analysis Interaction *P-Value*ж	Combined Analysis R^2	Combined Analysis *Main Effect Estimates*ת	Combined Analysis se∓	Combined Analysis *Main Effect P-Value*ө
In Vitro Fertilization (cont.)															
Sperm Concentration after Added to Ova (sperm/ml)		0.077			**0.0155**		0.092			**0.0062**	0.8563	0.099			**0.0001**
500,000 (sperm/ml)	93		23.047	0.917	B	94		20.691	0.990	B	187		21.868	0.675	B
600,000 (sperm/ml)	7		33.085	3.287	A	7		32.719	3.570	A	14		32.902	2.427	A
700,000 (sperm/ml)	10		24.234	2.750	B	10		20.345	2.986	B	20		22.289	2.030	B
Sperm Preparation Time (min)	105	0.008	0.129	0.146	0.3770	106	0.000	0.013	0.159	0.9356	0.5895	0.002	0.071	0.108	0.5123
Ova Age at IVF (hpm)	108	0.112	-9.547	2.612	**0.0004**	109	0.046	-6.699	2.946	**0.0249**	0.4705	0.073	-8.145	1.986	**<0.0001**
Ova Age at IVF from Removal from Follicles (hr)	103	0.104	-4.165	1.218	**0.0009**	104	0.047	-3.036	1.352	**0.0269**	0.5354	0.070	-3.608	0.917	**0.0001**
Denuding															
Gamete Co-incubation Time (hpi)	106	0.030	1.704	0.953	0.0766	107	0.005	0.752	1.004	0.4555	0.4939	0.013	1.172	0.696	0.0936
Vortex Time (min)	96	0.009	-2.368	2.575	0.3601	98	0.005	1.934	2.661	0.4689	0.2467	0.000	-0.160	1.867	0.9319
% Total PZ Recovered	109	0.002	-0.087	0.169	0.6078	110	0.000	0.015	0.199	0.9392	0.6950	0.000	-0.029	0.131	0.8265
% Total PZ Lysed	111	0.009	-0.301	0.301	0.3196	112	0.022	-0.453	0.289	0.1193	0.7169	0.015	-0.379	0.209	0.0712
% Total PZ with Excessive Cumulus	109	0.001	0.066	0.200	0.7415	111	0.002	-0.087	0.194	0.6549	0.5858	0.000	-0.029	0.140	0.8372
PZ No. per 500 µl KSOM	99	0.025	0.242	0.153	0.1166	100	0.050	0.394	0.173	**0.0249**	0.5111	0.038	0.322	0.115	**0.0056**
Total Denuding Time (min)	109	0.001	0.027	0.107	0.7980	110	0.003	-0.067	0.117	0.5663	0.5511	0.001	-0.038	0.079	0.6301
In Vitro Culture															
Cleavage Assessment Time (hpi)	102	0.010	0.430	0.424	0.3136	103	0.024	0.689	0.437	0.1175	0.6704	0.017	0.569	0.307	0.0651
Blastocyst Assessment Time (hpi)	111	0.022	-0.128	0.082	0.1199	112	0.006	-0.073	0.090	0.4182	0.6530	0.012	-0.110	0.061	0.0992
% Cleaved	108	0.182	0.284	0.059	**<0.0001**	109	0.183	0.314	0.064	**<0.0000**	0.7277	0.183	0.302	0.044	**<0.0001**
% 2-Cells of Cleaved PZ	108	0.106	-0.480	0.135	**0.0006**	109	0.166	-0.479	0.104	**<0.0000**	0.9982	0.151	-0.496	0.080	**<0.0001**
% 2-Cells of Total	108	0.044	-0.472	0.213	**0.0290**	109	0.080	-0.513	0.168	**0.0029**	0.8835	0.074	-0.533	0.129	**<0.0001**
% 4-Cells of Cleaved PZ	108	0.010	-0.073	0.069	0.2965	109	0.005	-0.058	0.077	0.4558	0.8860	0.009	-0.074	0.052	0.2075
% 4-Cells of Total	108	0.000	-0.014	0.095	0.8819	109	0.000	0.013	0.106	0.9019	0.8483	0.000	-0.013	0.071	0.8557

Table 3. Continued

		38.5°C					41.0°C					Combined Analysis				
IVP Steps	**Explanatory Variables**	**n**	**R^2**	**Estimates***	**se^{Ŧ}**	***P-Value***	**n**	**R^2**	**Estimates***	**se^{Ŧ}**	***P-Value***	**Interaction *P-Value*^{ж}**	**R^2**	***Main Effect Estimates*^{ת}**	**se^{Ŧ}**	***Main Effect P-Value*^{Θ}**
In Vitro Culture (cont.)																
	% 8-16 Cells of Cleaved PZ	108	0.046	0.128	0.056	**0.0256**	109	0.087	0.199	0.062	**0.0018**	0.3956	0.074	0.172	0.041	**<0.0001**
	% 8-16 Cells of Total	108	0.175	0.235	0.049	**<0.0001**	109	0.214	0.294	0.055	**<0.0001**	0.4176	0.204	0.270	0.036	**<0.0001**

*Blastocyst development slopes for continuous data or least squares means from ANOVA for categorical data
^{Ŧ}Standard error
^{ж}Interaction P-value: maturation temperature X explanatory variable interaction
^{ת}Main Effect Estimates: Blastocyst development slopes for continuous data or least squares means from ANOVA for categorical data when ova matured at 38.5 and 41.0°C combined
^{Θ}Main Effect P-value: P-value of technical factor when data for ova matured at 38.5 and 41.0°C were combined
A-D Within a variable means with no common letter differ (P<0.05)

Ovary Collection

Bovine ovaries were obtained from an abattoir and transported to the laboratory in insulated thermoses. For this IVP step of embryos, the influences of day of the week, collection season (month), total number of ovaries collected, average number of ovaries per thermos, ovary transport type, ovary age at laboratory arrival and ovary temperature on blastocyst development were examined.

Day of the Week

For the studies included in this retrospective meta-analysis, ovaries were collected predominantly on Tuesdays and Thursdays. Collection on these days allowed for the majority of intensive laboratory work to be conducted during the work week (i.e., ovary and oocyte collections, maturation and fertilization). Ovary collections on Tuesday rather than Monday, avoided possible concerns with possible differences in the holding time of cattle at the abattoir (i.e., held over the weekend versus not). In circumstances where biweekly collections were required, Thursday was preferred as a second collection day to limit procedural overlaps. ANOVA did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C between Tuesdays and Thursdays (Table 3). Because an interaction between maturation temperature and day of week was not observed, data were combined to examine main effect of day of the week (Table 3). Blastocyst rates were higher (P=0.0237) when ovaries were collected on Tuesday (23.79 ± 0.90%) versus Thursday (20.84 ± 0.93%).

Review of the literature failed to find research investigating the effect of day of the week for ovary collection and subsequent evaluation of blastocyst development using an IVP system. One explanation for effect could be due to holding times. According to the abattoir staff, cattle are held a maximum of 48 hours before slaughter. Since the abattoir services different regions of the Southeast, including Texas and Oklahoma, this may result in different transport and holding times of cattle. The extent of this factor to account for reduced blastocyst development remains unclear as information was not available to ascertain the status or type of cattle in which ovaries are collected for laboratory use. Another explanation for effect of day of the week could be due to Thursday collections requiring procedures to be performed on Saturday, introducing “human” factor by extending the normal work week.

Collection Season of the Year

Ovaries were collected in fall, winter and spring of each year to avoid collecting developmentally-challenged ova during summer months related to environmental heat stress effects (Rutledge et al., 1999). ANOVA was used to examine seasonal influences and did not reveal differences in blastocyst development for ova matured at 38.5 or 41.0°C (Table 3). Data were also examined by individual month of collection. Since visual assessment of individual month did not seem to follow a linear trend, a quadratic polynomial was fit and a significant effect of month on blastocyst development from ova matured at 41°C was noted (Figure 1; P=0.0455). When data were combined, a significant maturation temperature X month interaction was noted (P=0.0490). Heat-

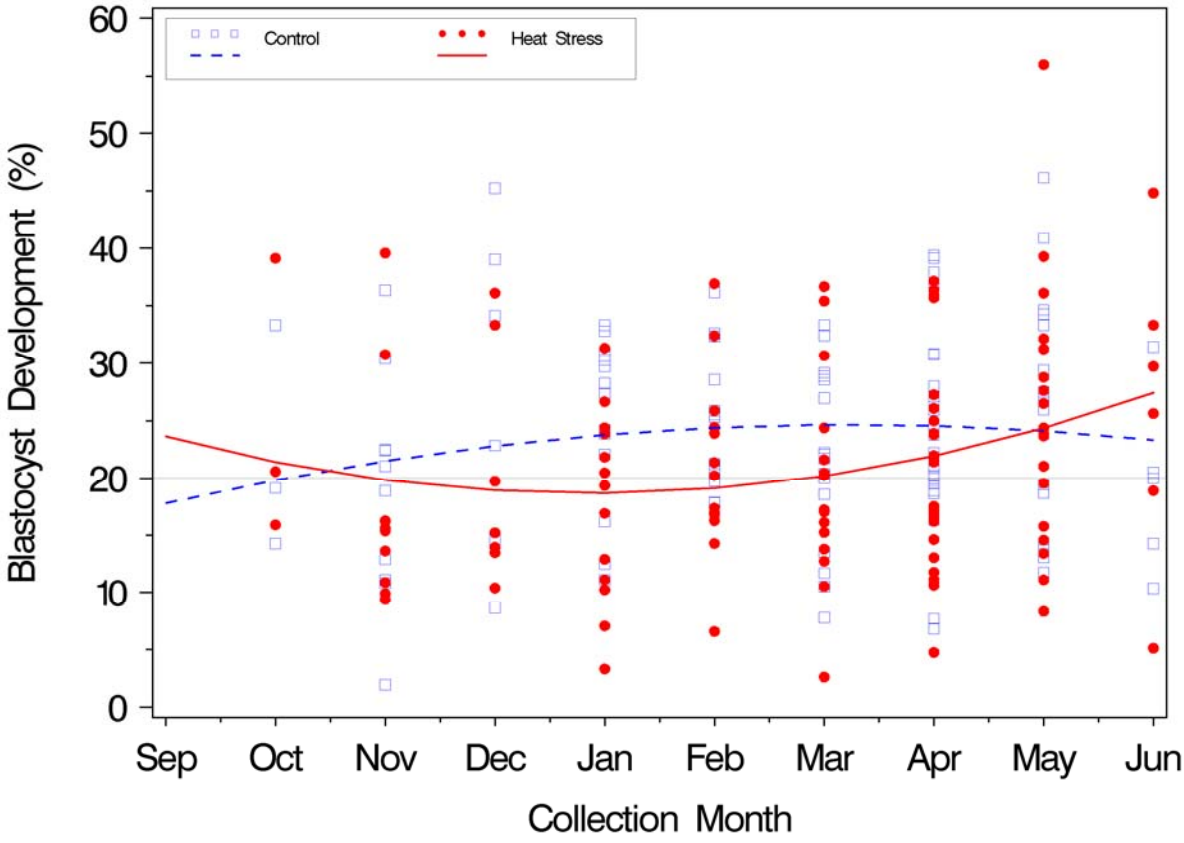

Figure 1. Influence of collection month on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Quadratic equations of the model were $14.96+2.70X-0.19X^2$ (P=0.3401) and $27.77-3.62X+0.36X^2$ (P=0.0455) for control and heat stress, respectively.

induced reductions in blastocyst development were more prominent during the months of December through March. Interestingly, heat stress effects were not as evident in October, November, April and May (Figure 1). In other words, responsiveness of ova to heat stress differed depending on month of year in which oocytes were collected. This variable explained 2.5% of the variation in blastocyst development.

Although difficult to explain, the loss of a negative heat stress effect during April and May may be due to seasonal differences, such as increased forage resources and longer photoperiods. It is important to point out that oocytes were obtained predominantly from grazing beef cattle for which increased forage resources and quality are important to increase nutritional body weights and body condition scores, as shown in a grazing comparison for British Friesian cattle between spring and autumn (Marsh, 2006). Better body condition scores positively affects developmental competence of bovine oocytes from dairy cattle (Snijders et al., 2000). The decrease in detrimental heat stress effect during October and November may be the result of carryover heat stress effects in cattle that were exposed to elevated ambient temperatures during summer to decrease developmental competence of ova matured at 38.5°C (Rutledge et al., 1999).

Total Number of Ovaries Collected

Total number of ovaries collected on a given day was used as an indirect estimate of collection size and ranged from 63 to 275 ovaries. Utilizing a dummy regression model on the combined data, a significant effect of total number of ovaries

was noted (Table 3; P=0.0283). As total number of ovaries collected increased, there was an associated decrease in blastocyst development of 0.52 ± 0.23% for each 10 ovary increase. This variable explained 3.4% of the variation in blastocyst development (Figure 2). To obtain at least 20% blastocyst development, the model predicted that fewer than 209 ovaries should be collected (Y=30.88-0.052X).

A review of the literature revealed no research regarding the effect of total number of ovaries collected on blastocyst development. Collecting more ovaries requires longer storage times prior to processing as well as increased processing time. Increased age of bovine ovaries prior to oocyte collection impaired development of oocytes to blastocyst stage (Nakatate et al., 2005, Yang, 1990).

Average Number of Ovaries per Thermos

At the abattoir, ovaries are collected and packed into thermoses to minimize possibility of extreme fluctuations in ovary temperatures. Number of ovaries per thermos ranged from 21 to 68 ovaries. Utilizing a dummy regression model on the combined data, a significant effect of average number of ovaries per thermos was noted (Table 3; P=0.0017). As total average number of ovaries per thermos increased, there was an associated decrease in blastocyst development of 3.19 ± 1.00% for each additional 10 ovaries. This variable explained 7.2% of the variation in blastocyst development (Figure 3). Packaging of more than 48 ovaries per thermos was predicted to average less than 20% blastocyst development (Y=35.63-0.32X).

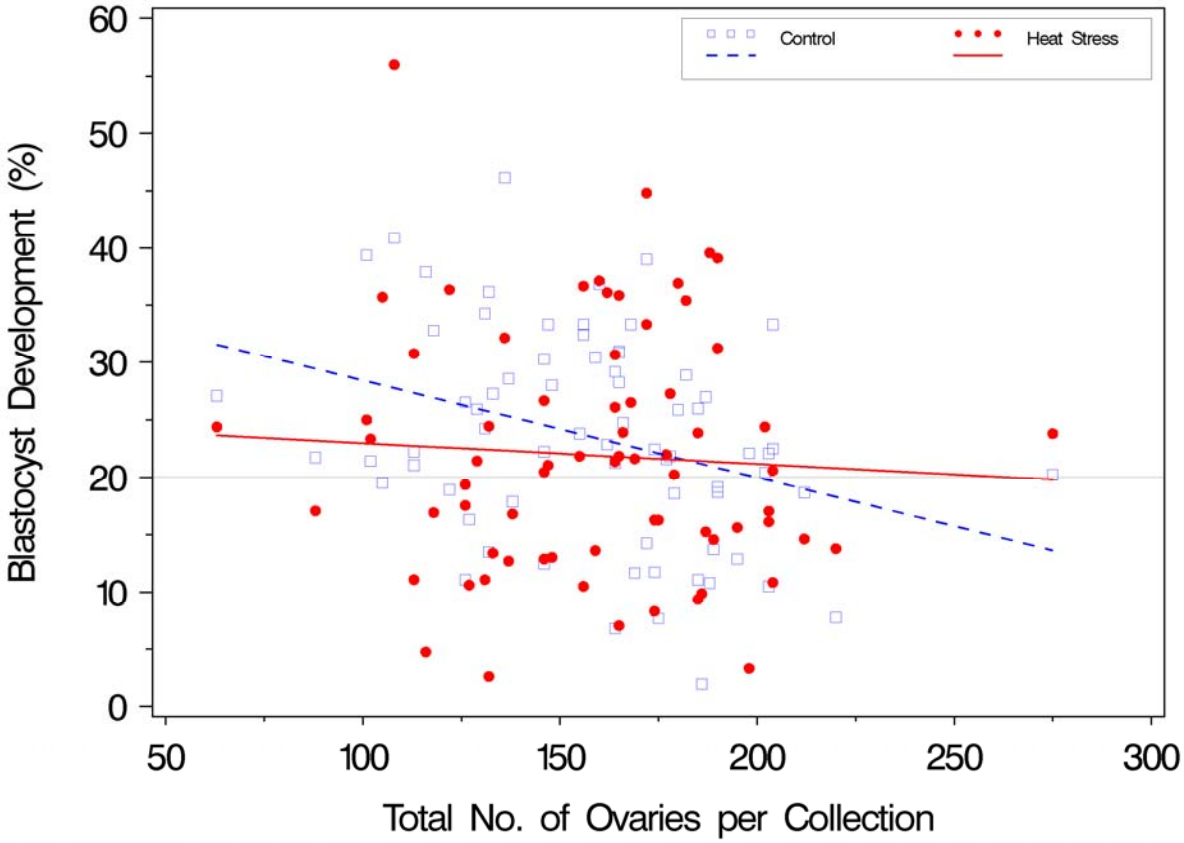

Figure 2. Influence of total number of ovaries obtained per collection on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=36.87-0.084X (P=0.0053) and Y=27.78-0.018X (P=0.6164) for control and heat stress, respectively.

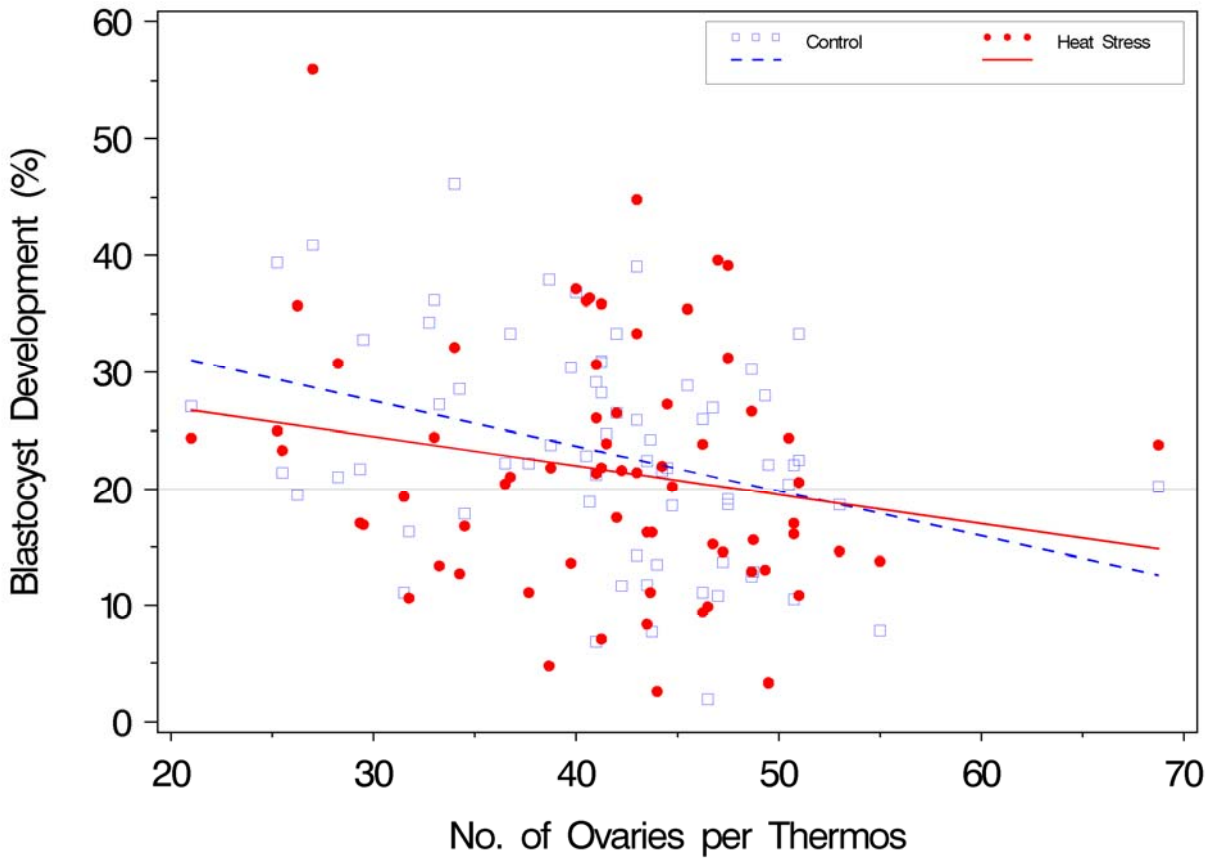

Figure 3. Influence of average number of ovaries per thermos obtained per collection on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=39.16-0.39X (P=0.0037) and Y=31.97-0.25X (P=0.1094) for control and heat stress, respectively.

This appears to be the first report on the effect of number of ovaries per thermos on rate of blastocyst development. Packaging more ovaries per thermos may result in decreased blastocyst rates by increasing age of ovary due to increased time to fill each thermos or subsequent processing time (Nakatate et al., 2005, Yang, 1990).

Ovary Transport Type

After collection at the abattoir, ovaries were transported to the laboratory by airplane or automobile. ANOVA analysis did not reveal any effect of transport type on blastocyst development for ova matured at 38.5 or 41.0°C (Table 3). Additionally, no effect of transport type was noted when utilizing an ANOVA model on the combined data (Table 3).

A review of the literature revealed no research examining effects of ovary transport type on blastocyst development. The lack of effect is important since many IVP laboratories are not located close to abattoirs and are limited by transport options. This factor was of particular interest as transport by plane occurred in the cargo hold, where temperatures are not controlled, and could have negatively impacted blastocyst development of oocytes derived from those ovaries.

Ovary Age at Laboratory Arrival

The time that ovaries were collected from the cow to time of arrival at the laboratory ranged from 3.9 to 7.8 hours. Utilizing a dummy regression model on the combined data, a significant effect of ovary age at laboratory arrival was noted (Table 3;

P=0.0210). Within the reported range, as ovary age at laboratory arrival increased, there was an associated decrease in blastocyst development of 1.47 ± 0.63% for each hour delay. This variable explained 2.4% of the variation in blastocyst development (Figure 4). Arrival times of more than 7.25 hours were predicted to average less than 20% blastocyst development (Y=30.66-1.4X).

It is important to note that results are restricted to conclusions regarding 3.9 to 7.8 hours of ovary age. Negative consequences of increased ovary age are not surprising as other authors have shown that an increase in ovary age postmortem decreases blastocyst development (Blondin et al., 1997, Nakatate et al., 2005, Yang, 1990). According to Nakatate et al. (2005), when transportation times were 17 to 18 hours and the bovine ovaries were kept at around 23.8°C, blastocyst development was inferior to when transport time was 1 to 1.5 hours. Additionally, Yang et al. (1990) reported that blastocyst development decreased from 23% to 12.6% if bovine ovaries were stored for 4 or 16 hours, respectively, using only 325 ovaries. It can be speculated that longer ex vivo times may be detrimental for cells because of lack of nutrients and necrosis.

Ovary Temperature

The temperature of ovaries within the thermoses was collected immediately upon arrival in the laboratory and ranged from 19.8 to 33.3°C. Simple linear regression did not reveal any impact of temperature on blastocyst development of ova matured at 38.5

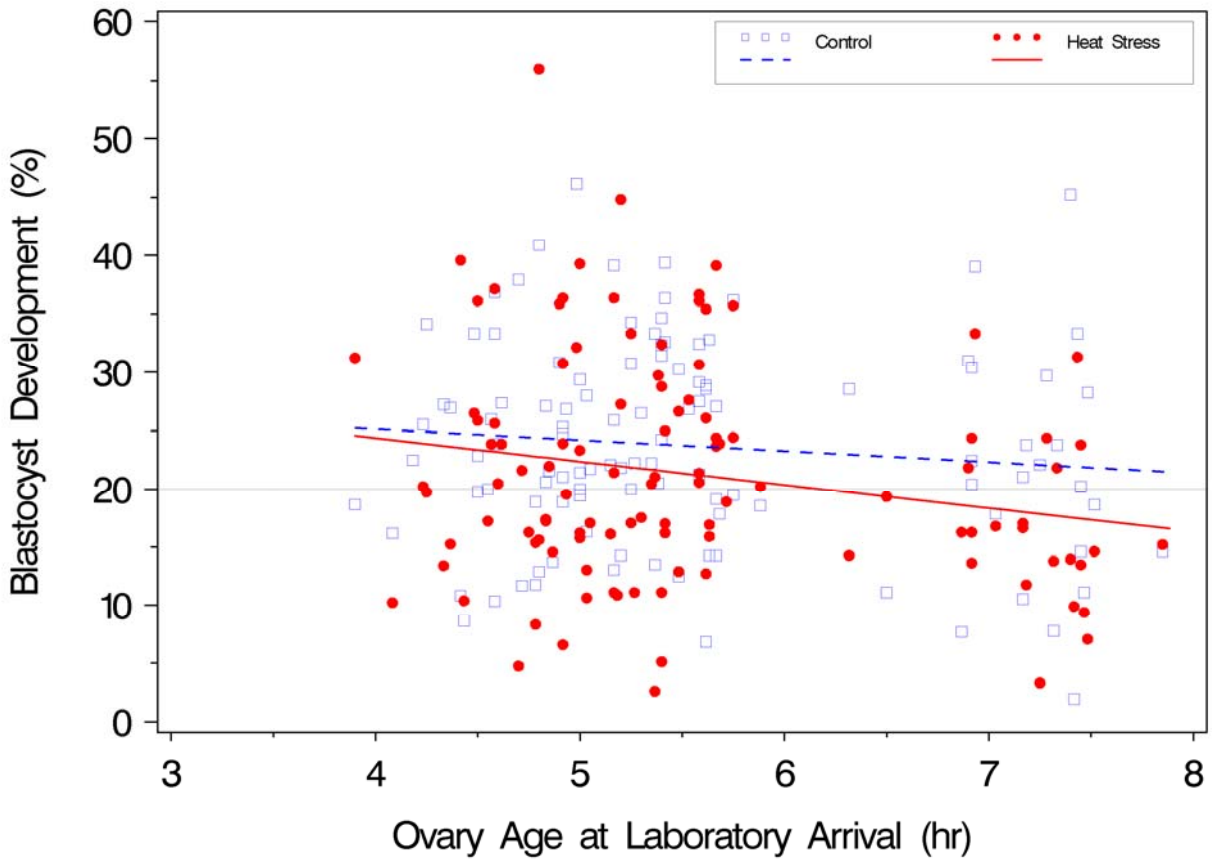

Figure 4. Influence of ovary age at laboratory arrival on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=28.89-0.94X (P=0.2749) and Y=32.41-2.01X (P=0.0331) for control and heat stress, respectively.

or 41.0°C. Utilizing a dummy regression model on the combined data, no effect of the ovary temperature was noted (Table 3; Figure 5).

The use of thermoses was required to minimize temperature fluctuations during ovary transport to laboratory. It is important to note that results were restricted to conclusions for temperatures 19.8 to 33.3°C and for this narrow range in the study did not negatively impact blastocyst development. Development becomes problematic when bovine ovaries are exposed to temperatures beyond our range. For example, with temperatures of 37 or 4°C for 4 hours, blastocyst development was lower (10.6 and 0%, respectively) than when exposed to 25°C (23.0%; Yang, 1990). Additionally, these results are associated with previous results of total ovaries collected and number of ovaries per thermos, which may corroborate that age of the ovary is more important than transport temperature (~19.8 to 33.3°C) to account for decreased blastocyst development.

Oocyte Collection

Oocytes were harvested from 3 to 8 mm follicles of selected ovaries and then washed in oocyte collection media. Using a stereomicroscope, oocytes were separated from debris in a search dish and then evaluated for dark, evenly granulated ooplasm and compact cumulus prior to placement into a maturation medium. For this step of IVP of embryos, influences of technician, total slice time, total number of sliced ovaries and total search and evaluation time on rate of blastocyst development were evaluated.

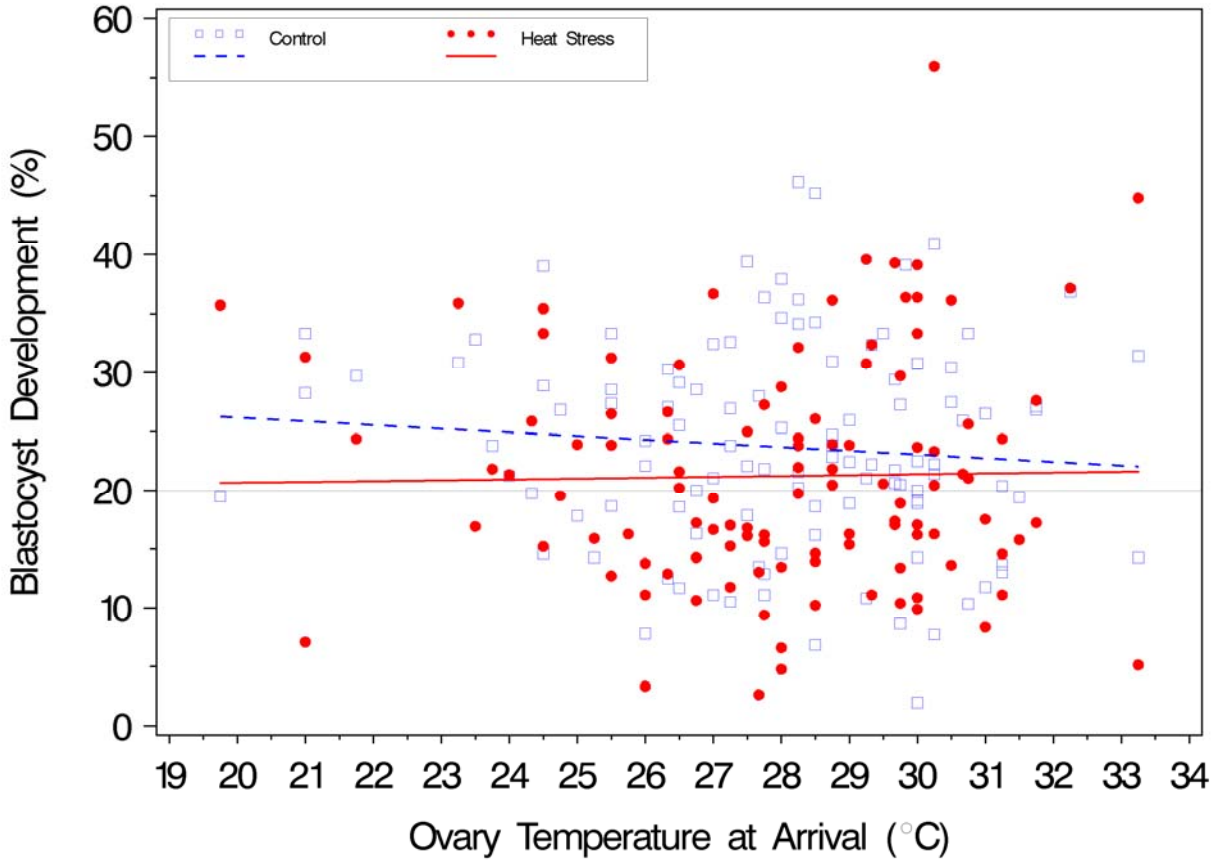

Figure 5 Influence of ovary temperature at arrival on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=32.40-0.31X (P=0.3501) and Y=19.42+0.07X (P=0.8433) for control and heat stress, respectively.

Technician

Data for the meta-analysis were derived from efforts of 4 different graduate students (i.e., technicians) who provided primary oversight of other individuals assisting during each step listed above. Utilizing an ANOVA model on the combined data, a significant effect of technician was noted (Table 3; Figure 6; P=0.0107). Blastocyst development was highest for technicians C and D and lower for A and B, ranging from 25.68 ± 1.23% to 20.87 ± 1.24%.

Results may reflect level of expertise with IVP of embryos of individual technicians. Using similar protocols as this study, Yang et al. (1995) observed that technicians having more experience with IVP procedures obtained higher rates of blastocyst development than less experienced researchers. In contrast, our results do not reflect level of experience since all technicians began their IVP training without any previous practical knowledge of the technique. Thus, results likely reflect individual differences between each technician's skills to obtain blastocysts.

Total Slice Time

Oocytes were removed from ovarian follicles by making checkerboard incisions approximately 2 mm in length on the follicular surface. Within a given day, the time period required to slice ovaries ranged from 10 to 75 minutes. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, there was neither an

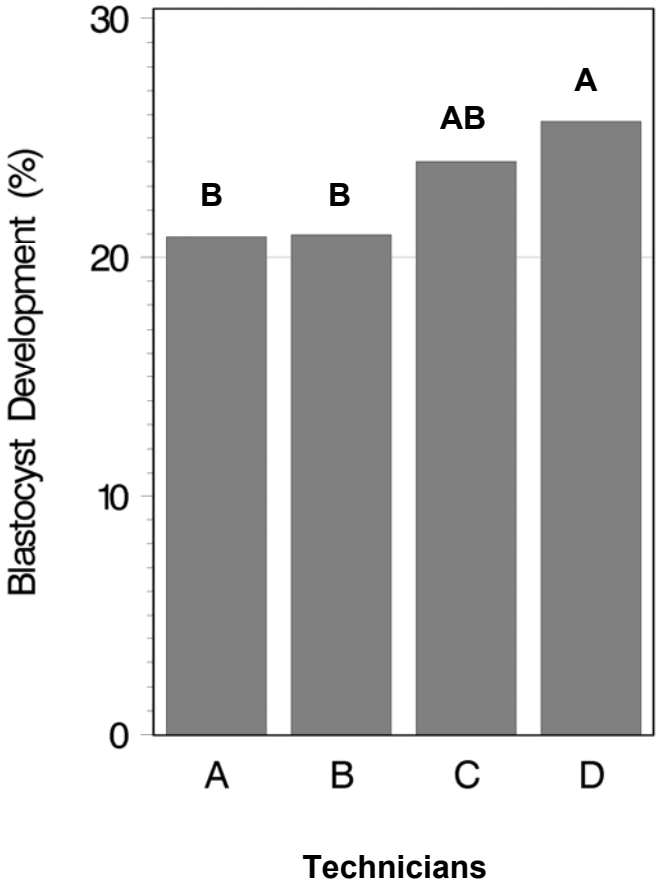

Figure 6. Influence of technician who performed the experiment on blastocyst development for ova matured at 38.5 and 41.0°C combined. $^{A-B}$ means differ (pooled SEM=1.29; P=0.0107).

interaction of maturation temperature X total slice time nor main effect of total slice time to influence blastocyst development (Table 3).

After an extensive review of the literature, no references were found examining the effect of ovary slicing time on blastocyst development. However, these results are of practical importance given the wide range in slicing time, indicating that this is not a critical period to impair oocyte developmental potential.

Total Number of Sliced Ovaries

Total number of ovaries sliced on a given day was recorded and used as an estimate of collection size (range of 20 to 200). Utilizing a dummy regression model on the combined data, a significant effect of total number of ovaries sliced was noted (Table 3; $P<0.0001$). As the total number of sliced ovaries within a given day increased, there was a progressive decrease in blastocyst development of 0.60 ± 0.15% for each 10 additional ovaries sliced. This variable explained 7.0% of the variation in blastocyst development (Figure 7). Achieving less than 20% development was coincident with slicing more than 127 ovaries ($Y=27.65-0.060X$).

Results provide supportive evidence that a larger collection may be counterproductive to producing blastocyst development greater than 20%. However, smaller collections are not always possible depending on the project. Larger collections are coincident with longer process times for oocytes collection and, in general, results in longer time periods for oocytes to be held in media outside of follicle. This is concerning as Pincus and Enzmann (1935) reported that removal of rabbit oocytes from antral

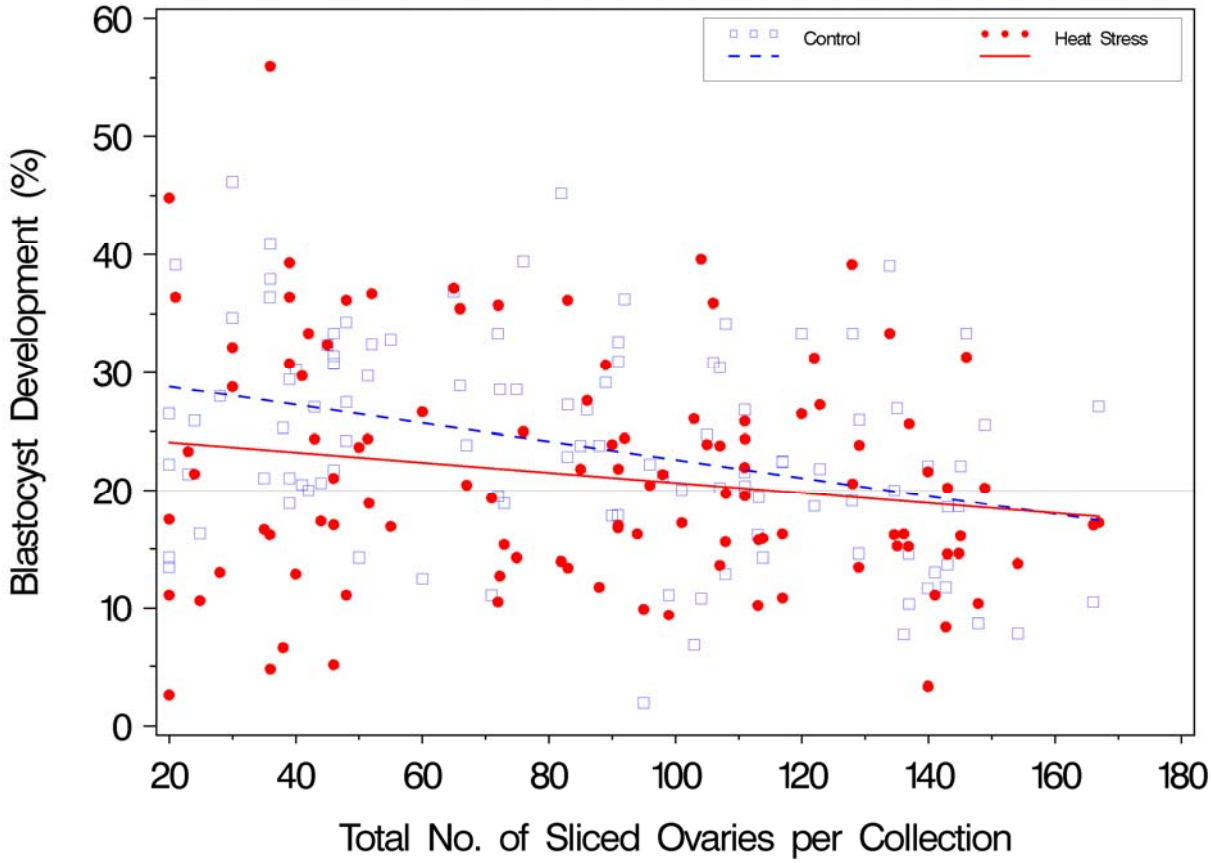

Figure 7. Influence of total number of ovaries per collection on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=30.35-0.077X (P=0.0001) and Y=24.95-0.043X (P=0.0579) for control and heat stress, respectively.

follicles induced resumption of meiosis per se as cultured in ringer solution without gonadotropins. If oocytes resume meiotic maturation before placement in OMM, then ova would be older than expected at time of sperm addition. It is also possible that holding oocytes in media after slicing may decrease blastocyst development because of increased exposure to light and ambient temperature, with possible changes in pH of media. For example, during handling and manipulation of oocytes, the spectral composition and intensity of ambient and microscope light to which oocytes and embryos are exposed (Ottosen et al., 2007) may damage DNA as noted in one-cell hamster embryos exposed to visible light for 30 minutes (Takahashi et al., 1999). Light exposure has also been linked to decreased blastocyst development in hamster one-cell embryos exposed to 1600 lux light conditions for 30 minutes (380 to 760-nm wavelengths; Umaoka et al., 1992). Addition of a physiological amount of 7.5 mM of urea to maturation media is associated with blastocyst decrease, suggesting that there is susceptibility to pH change during oocyte maturation (Ocon and Hansen, 2003). Furthermore, reports show that cumulus-oocyte complexes are susceptible to changes in temperature, as a decrease in blastocyst development from 31 to 17% occurred with temperature fluctuations of 20 to 39°C, respectively (Martino et al., 1995, Schwartz et al., 1998).

Total Search and Evaluation Time

Total search and evaluation time was defined as the time period when searching for oocytes began (after slicing was completed) until when oocytes were placed in

oocyte maturation media (OMM) and ranged from 25 to 130 minutes. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, a significant effect of search and evaluation time was noted (Table 3; P=0.0497). As the search and evaluation time increased, there was a progressive decrease in blastocyst development of 0.57 ± 0.29% for each additional 10 minutes. This model explained 1.8% of the variation in blastocyst development (Figure 8). Development of less than 20% was coincident with times longer than 122 minutes (Y=26.84-0.057X).

The significance of this finding is that the longer it takes to search and evaluate ova, the worse blastocyst development is expected to be. As discussed previously, it can be speculated that oocytes may resume meiosis before placement into maturation media. Reduced blastocyst rates with lengthier search and evaluation times may also be due to exposure to light and ambient temperature, and/or possible changes in media pH (Martino et al., 1995, Ocon and Hansen, 2003, Ottosen et al., 2007, Schwartz et al., 1998, Takahashi et al., 1999, Umaoka et al., 1992). In accordance with this hypothesis, DNA damage was noted in one-cell hamster embryos after only 30 minutes (Ottosen et al., 2007), a time close to the fastest search and evaluation time recorded in this meta-analysis. Therefore, the use of light filters on the microscopes, especially for the blue and violet spectra (Ottosen et al., 2007) may be beneficial to improve blastocyst development.

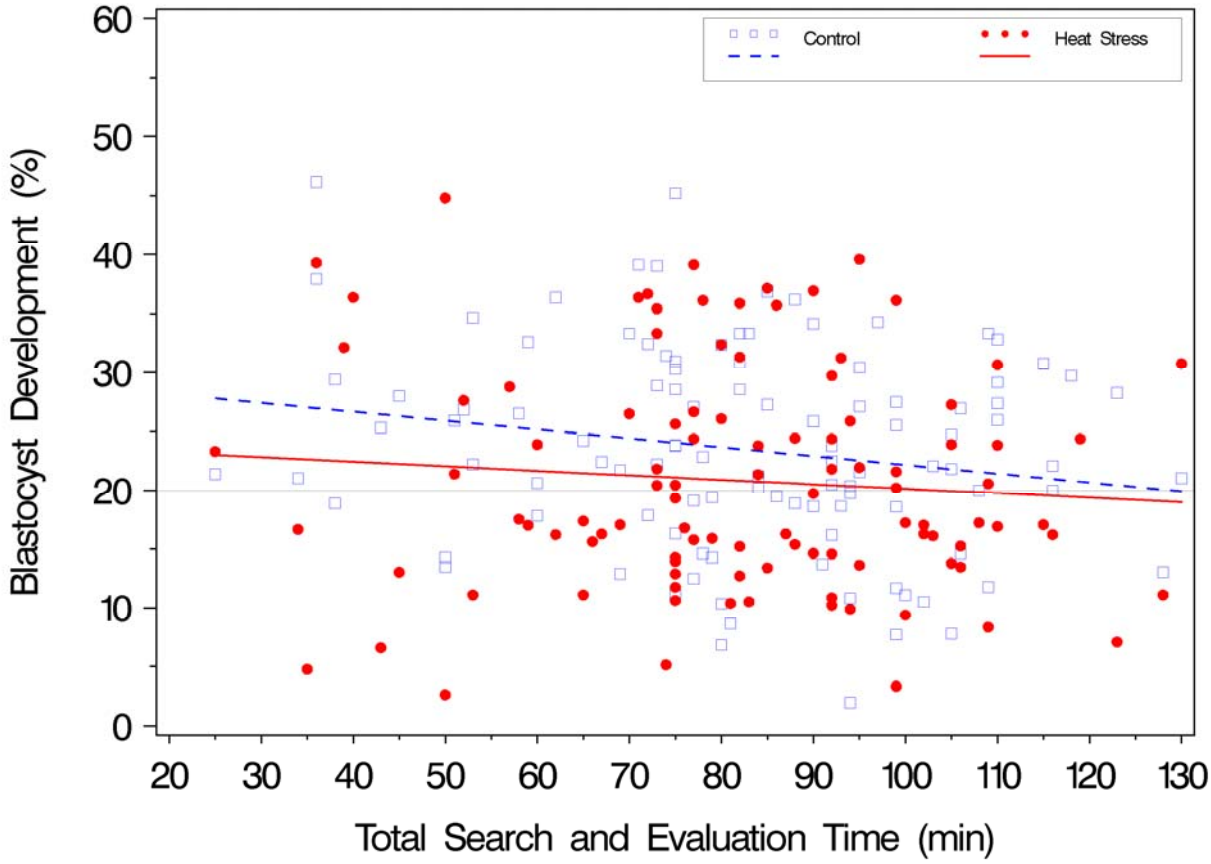

Figure 8. Influence of total search and evaluation time on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=29.66-0.075X (P=0.0523) and Y=23.98-0.04X (0.3684) for control and heat stress, respectively.

In Vitro Maturation

After oocytes were collected, washed, and evaluated, they were placed into a maturation medium for culture at 38.5 or 41.0°C (during the first 12 hours of maturation only) for approximately 24 hours. For this step of IVP of embryos, the influences of age of media (M199 stock age), ova number placed per well, total ova in OMM per day, total ova in OMM per ovary, time between beginning of ovary slice time and placing in OMM (oocyte collection time), and time between slaughter time and placing in OMM (total collection time) were examined.

M199 Stock Age

The liquid base media used for supplementation to produce the oocyte maturation medium was M199 and was stored up to 90 days at 4°C. For data described herein, liquid stock age ranged from 0 to 81 days. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, there was no interaction of maturation temperature X age of M199 stock, or influence of age of M199 on blastocyst development (Table 3). Age of stock was important to investigate because it is known that media constituents can break down over time making them unavailable or producing toxic byproducts (Freshney, 1989).

Ova Number per Well

Average number of cumulus-oocyte complexes placed per well of 500 µl of OMM ranged from 20 to 60. Utilizing a dummy regression model on the combined data, a significant effect of number of ova per well was noted (Table 3; $P<0.0001$). As total number of ova per well increased, there was a progressive decrease in blastocyst development of 2.65 ± 0.63% for each additional 10 ova per well. This model explained 7.4% of the variation in blastocyst development (Figure 9). Less than 20% blastocyst development was coincident with more than 54 oocytes placed per well ($Y=33.97-0.26X$). For additional clarity, data were broken down into categories that included 20 to 29, 30 to 39, 40 to 49 and 50 to 60 oocytes per well. Blastocyst rates were higher for 20 to 29 and 30 to 39 oocytes per well (26.04 ± 1.64% and 26.01 ± 1.64%, respectively) than 50 to 60 oocytes per well (20.17 ± 0.91%; Figure 10; $P=0.0031$).

The significance of this finding is that the more oocytes were placed in an OMM well, the less development to blastocyst was achieved. Similar results have been reported by others. For instance, Oliveira et al. (2005) reported that placing of 5, 10 or 20 oocytes per 100 µl of oocyte maturation medium under mineral oil achieved better blastocyst rates (24.1, 18.9, 20.4% blastocyst for 5, 10 and 20 oocytes per drop, respectively) than placing 30 oocytes per drop (12.6%; de Oliveira et al., 2005). During maturation, cumulus cells provide nutrients and other factors to the oocyte (Buccione et al., 1990, Liu, 2007). The metabolic activity of cumulus is related to changes in pH of the media, depletion of nutrients and negative conditioning though accumulation of embryo-toxic products such as ammonium (Bavister, 1995, Ikeda et al., 2000). In

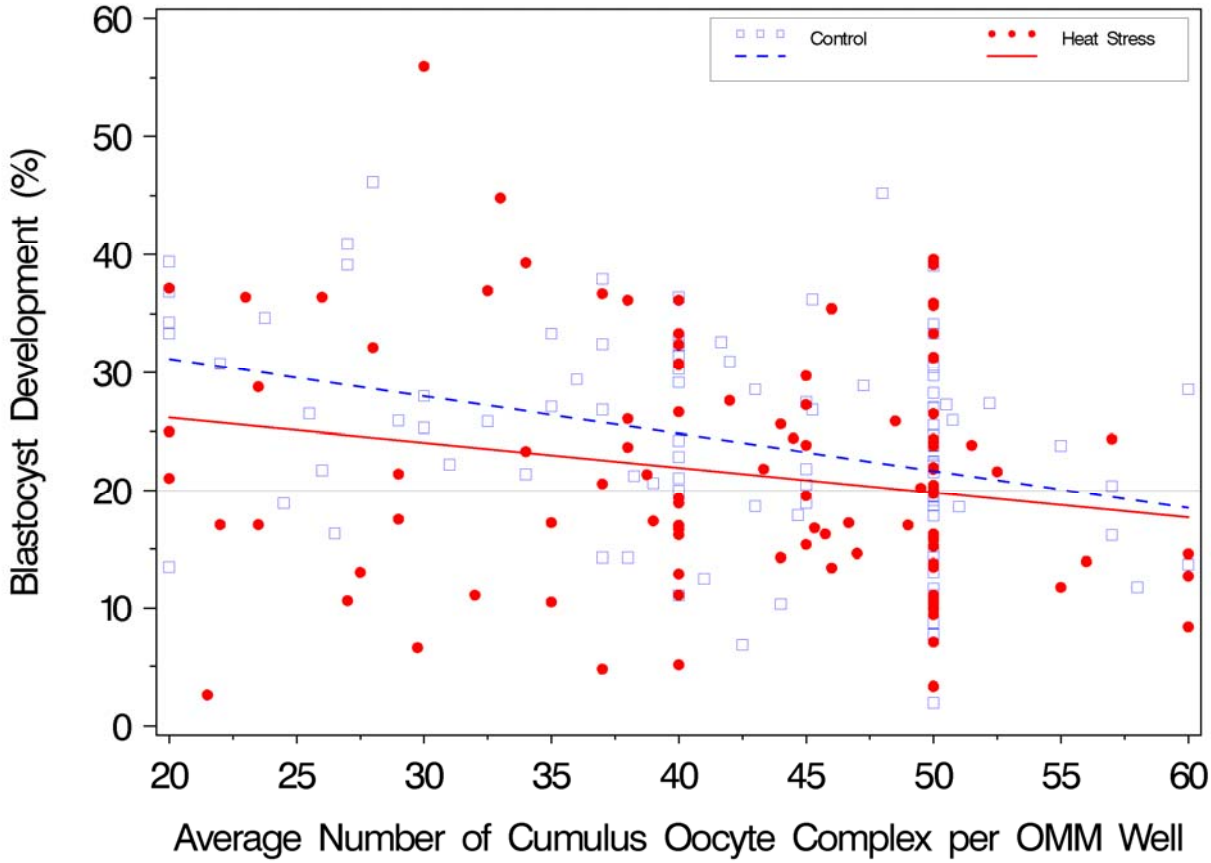

Figure 9. Influence of cumulus-oocyte complexes per well of OMM (500 µl) on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=37.47-0.32X (P=0.0002) and Y=30.42-0.21X (P=0.0300) for control and heat stress, respectively.

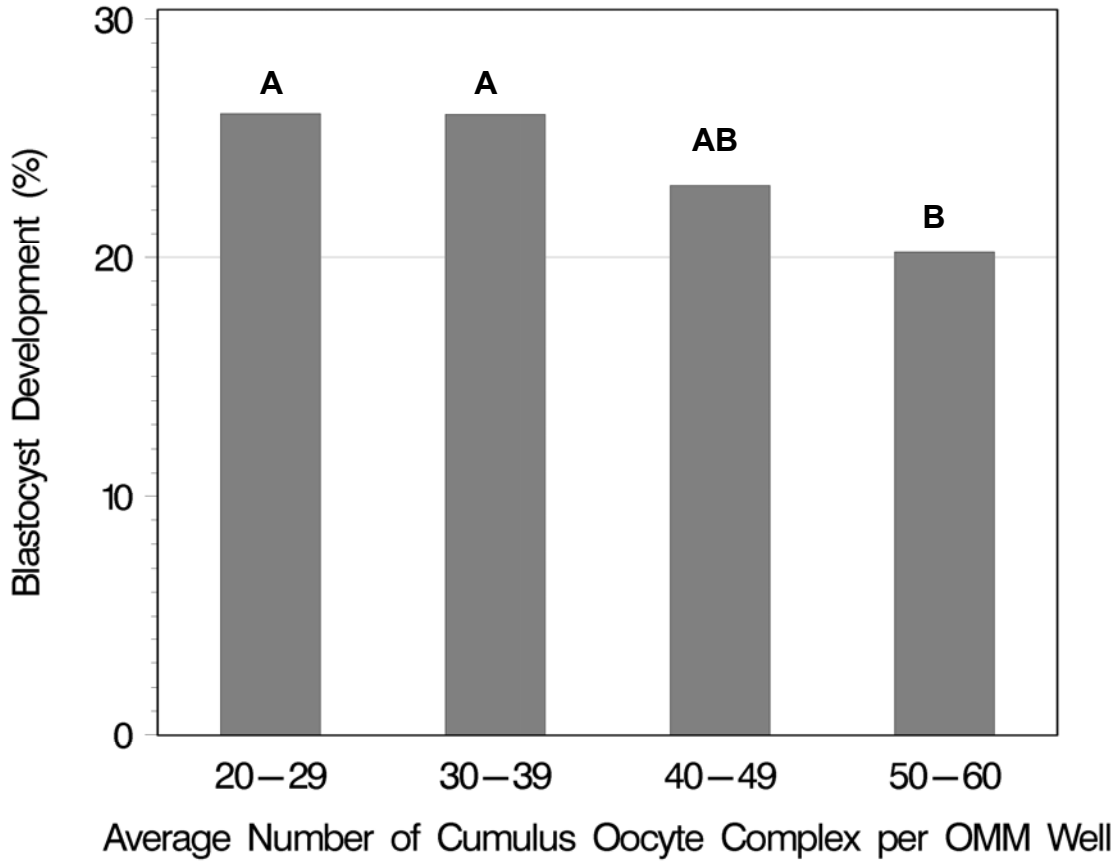

Figure 10. Influence of cumulus-oocyte complexes per well of OMM (500 µl) on blastocyst development for ova matured for 24 hours at 38.5°C (Control) and 41.0°C during the first 12 hours (Heat Stress). $^{A-B}$ **means differ (pooled SEM=1.34; P=0.0031).**

support of this, the use of phenol red as a color indicator of pH allows for visualization of pH changes (Yamaguchi et al., 1997). Specifically, the media becomes more acidic as maturation progresses with the change of color from red at a pH of 7.4 to an orange color at a pH less than 7.4 and changes of pH are related with decreased embryo development (Ocon and Hansen, 2003).

Total Ova in OMM per Day

Total number of oocytes having a dark, evenly granulated ooplasm and compact cumulus mass collected on a given day was recorded and used as an estimate of collection size (ranged from 138 to 1433 cumulus-oocyte complexes). A few collection days yielded more than 1200 cumulus-oocyte complexes but were considered outliers because they occurred rarely. Utilizing a dummy regression model on the combined data without those outliers, a significant effect of total number of oocytes per day was noted (Table 3; P=0.0413). As the total number of oocytes collected within a given day increased, there was a progressive decrease in average blastocyst development of 0.58 ± 0.28% for each additional 100 oocytes cultured. This variable explained 3.8% of the variation in blastocyst development (Figure 11). Less than 20% development was coincident with collecting more than 1032 oocytes (Y=25.99-0.0058X).

These results reflect the fact that larger collections appear to be counterproductive to producing blastocyst development greater than 20%. To get more oocytes, more ovaries must be collected and sliced, leading to longer processing times related to collection, evaluation and searching before placement into maturation

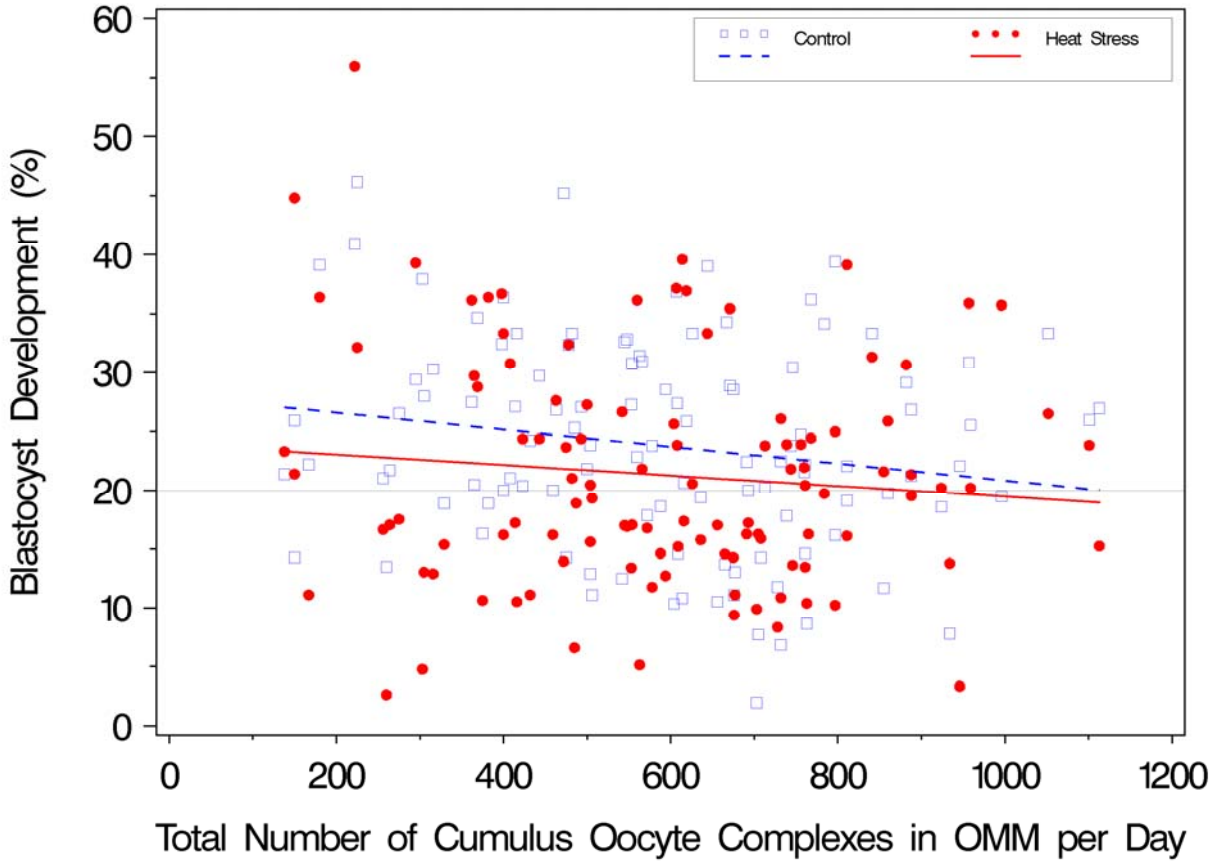

Figure 11. Influence of total number of cumulus-oocyte complexes in OMM per day on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=28.03-0.0072X (P=0.0612) and Y=23.97-0.0045X (P=0.2859) for controls and heat stress, respectively.

medium in incubator. As discussed previously, oocytes held in collection media are not only exposed to ambient light and temperature, but also may resume meiotic maturation prior to placement in maturation media (Pincus and Enzmann, 1935). Both scenarios lead to decreased blastocyst development (Martino et al., 1995, Ottosen et al., 2007, Schwartz et al., 1998, Takahashi et al., 1999, Umaoka et al., 1992). Thus, collection size should be minimized as much as possible for optimal development.

Total Ova in OMM per Ovary

The number of "culturable" oocytes collected per ovary ranged from 5.1 to 14.7. Utilizing a dummy regression model on the combined data, a significant effect of total ova in OMM per ovary was noted (Table 3; P=0.0329). As total ova in OMM per ovary increased by one, blastocyst development decreased by 0.53 ± 0.25%. This variable explained 2.1% of the variation in blastocyst development. Less than 20% development was coincident with obtaining less than 3.2 oocytes per ovary (Y=18.32+0.53X). Since the highest number of oocytes per ovary did not seem visually to be achieving the highest blastocyst development suggested by simple linear regression, a quadratic polynomial was fit and explained 3.2% more variation in rate of blastocyst development (Figure 12; quadratic slope P=0.0068). Maximum percentage of blastocyst development would be achieved with approximately 9.9 oocytes per ovary, whereas, culture of less than 5.1 or more than 14.7 oocytes per ovary was predicted to average less than 20% blastocyst development (Y=4.96+3.97X-0.20X2).

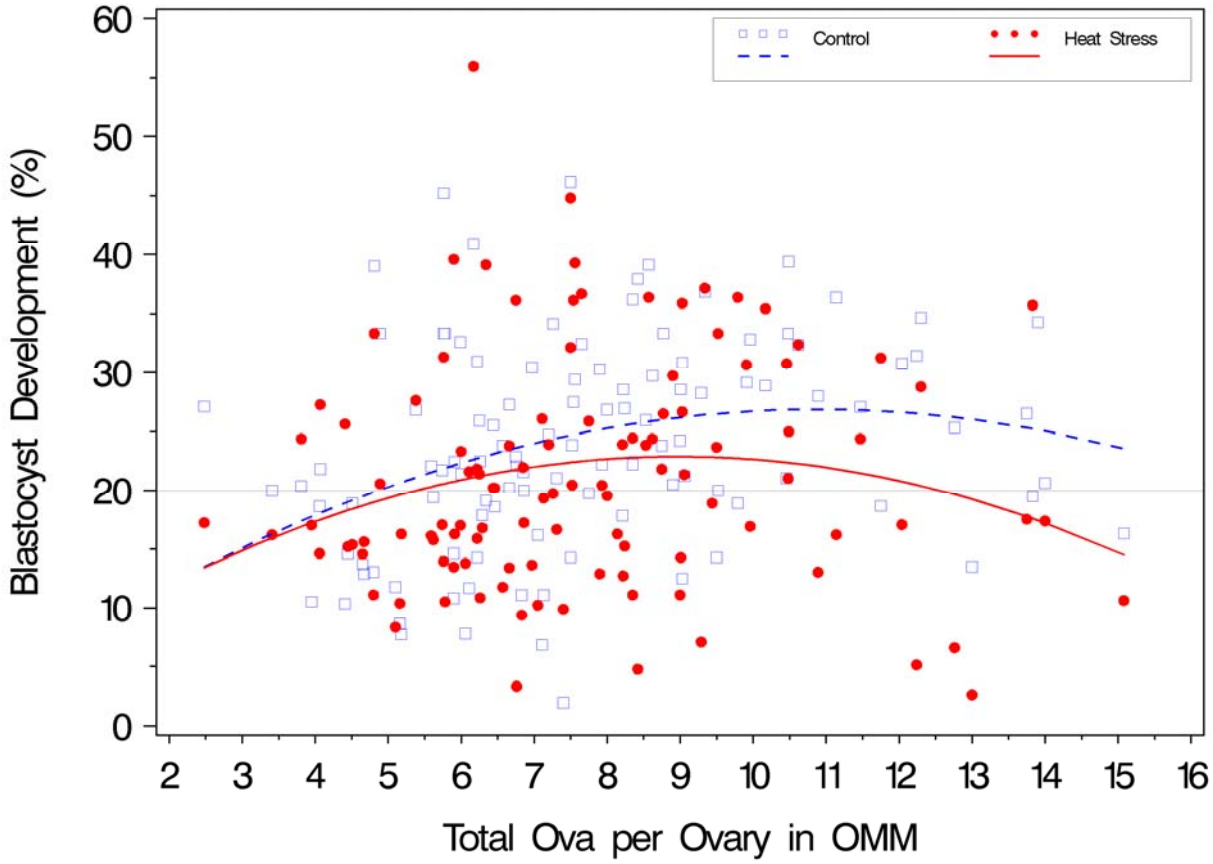

Figure 12. Influence of total ova per ovary in OMM on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Quadratic equations of the model were $Y=4.45+4.11X-0.19X^2$ (P=0.0051) and $Y=4.82+4.03X-0.22X^2$ (P=0.1131) for control and heat stress, respectively.

Oocyte number per ovary may be an indirect indicator of the number of follicles on an ovary. It has been reported that when young adult cattle were subjected to serial ultrasonography, animals with a consistently high (more than 25 follicles that were ≥3 mm in diameter) antral follicle counts were associated with an increased number of morphologically healthy follicles with an intact basal membrane, organized granulose cell layers and an intact oocyte and nucleus per gram of ovary (Ireland et al., 2008). The fact that ovaries with less than 5.1 follicles were related to decreased blastocyst development may be related with the age of the cattle. Cushman et al. (2009) observed that after 5 years of age, beef cattle antral follicle number began to decline. Additionally, oocytes collected from women older than 40 years were less developmentally competent due to aneuploidy and degenerative cytoplasmic defects (reviewed by Armstrong, 2001, Zayed et al., 1997). Unfortunately, the age of cattle from which ovaries were obtained was unknown.

Oocyte Collection Time

Oocyte collection time was defined as the time period from when slicing began (when oocytes were released from ovarian follicles) until oocytes were placed in OMM and ranged from 40 to 176 minutes. Since times less than 100 minutes were confined to one technician, these values were not included in the analysis. When a dummy regression model on the combined data was utilized, a significant effect of total oocyte collection time was noted (Table 3; P=0.0197). As oocyte collection time increased, there was an associated decrease in blastocyst development of 0.91 ± 0.39% for each

additional 10 minutes. This variable explained 3.1% of the variation in blastocyst development (Figure 13). Less than 20% development was coincident with oocyte collection times longer than 154.5 minutes (Y=34.06-0.091X).

Longer collection times (i.e., 3 hours) may be reflective of larger collection size which was previously shown to be counterproductive to embryo production as the number of sliced ovaries increased. Given that the oocyte may resume meiotic maturation upon removal from the follicle (Pincus and Enzmann, 1935), it would be accurate to account for oocyte collection time as part of maturation period. Therefore, for 3 hours of oocyte collection during which meiotic resumption may occur, the real oocyte age after 24 hours of culture may be up to 27 hours. As previously stated, oocytes may also be experiencing prolonged exposure to ambient light and temperature with possible pH changes of the media (Martino et al., 1995, Ocon and Hansen, 2003, Ottosen et al., 2007, Schwartz et al., 1998, Takahashi et al., 1999, Umaoka et al., 1992).

Total Collection Time

Total collection time was defined as the time period from when collection of ovaries at the abattoir began (7:15 AM) until oocytes were placed in OMM at the laboratory and ranged from 5.9 to 10.6 hours. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, a significant effect of total collection time was noted (Table 3; P=0.0557). Since visual assessment of total collection time did

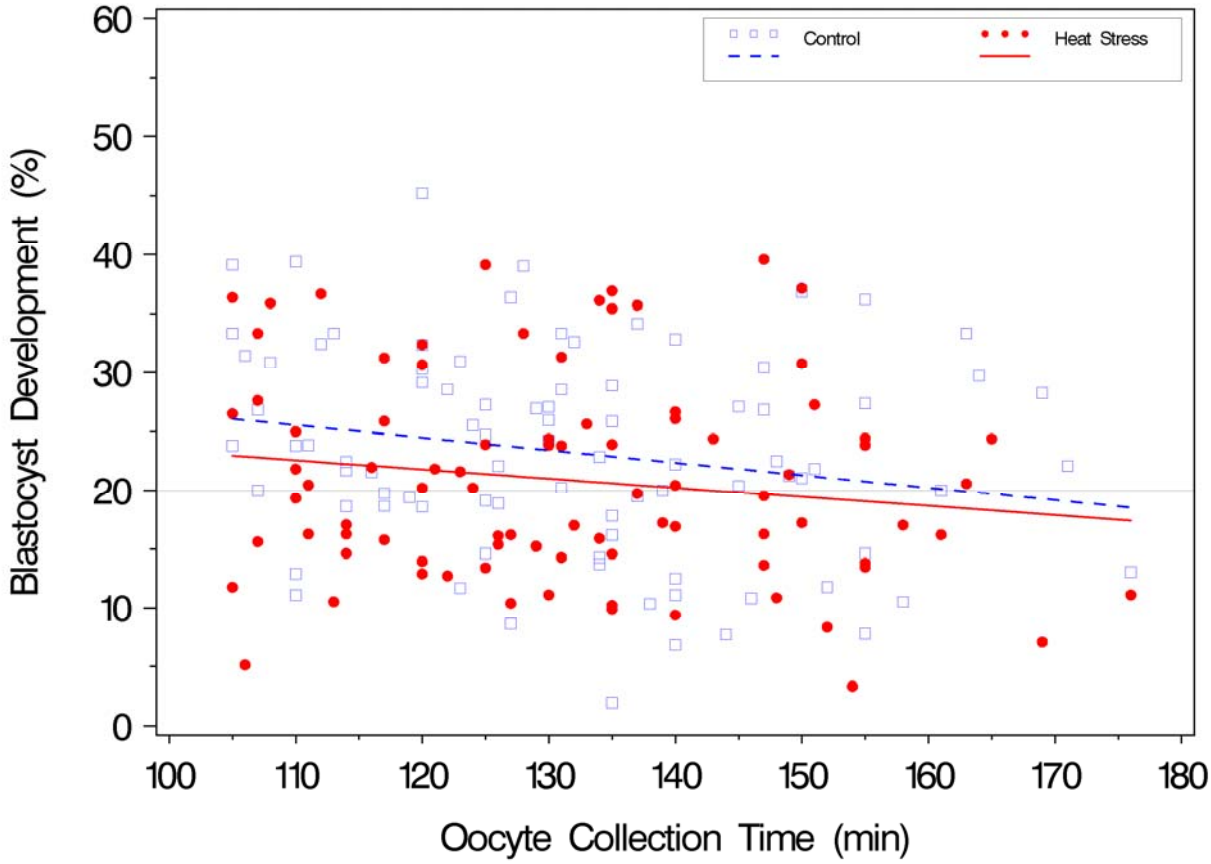

Figure 13. Influence of oocyte collection time on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=37.21-0.11X (P=0.0552) and Y=31.14-0.08X (P=0.1573) for control and heat stress, respectively.

not seem to follow a linear trend, a quadratic polynomial was fit and explained 4.8% more variation in rate of blastocyst development (Figure 14; quadratic slope P=0.0383). Maximum percentage of blastocyst development would be achieved with total collection time of approximately 7.7 hours post slaughter', whereas, total collection times of less than 5.7 and more than 9.6 hours were predicted to average less than 20% blastocyst development ($Y=-29.95+13.94X-0.91X^2$).

Total collection time depends on the transport time between laboratory and abattoir and the time to collect oocytes. Negative consequences of aged ovaries and of longer oocyte processing times are not surprising given results for other variables (ovary age at arrival and oocyte collection time). Total collection times of more than 9.6 hours in the retrospective meta-analysis may be long enough times to obtain less than 20% blastocyst development, possibly due to lack of nutrients and necrosis of the tissues. Additionally, oocytes may be resuming meiosis (Pincus and Enzmann, 1935) before placing in maturation media and/or may be experiencing prolonged exposure to ambient light and temperature with possible pH changes of the media (Martino et al., 1995, Ocon and Hansen, 2003, Ottosen et al., 2007, Schwartz et al., 1998, Takahashi et al., 1999, Umaoka et al., 1992).

In Vitro Fertilization

After semen straws stored at -196°C were removed from liquid N_2 tank and warmed for 45 seconds at 34°C, motile and live sperm were separated from dead and immotile sperm on a discontinuous Percoll® column (Parrish et al., 1995) and then

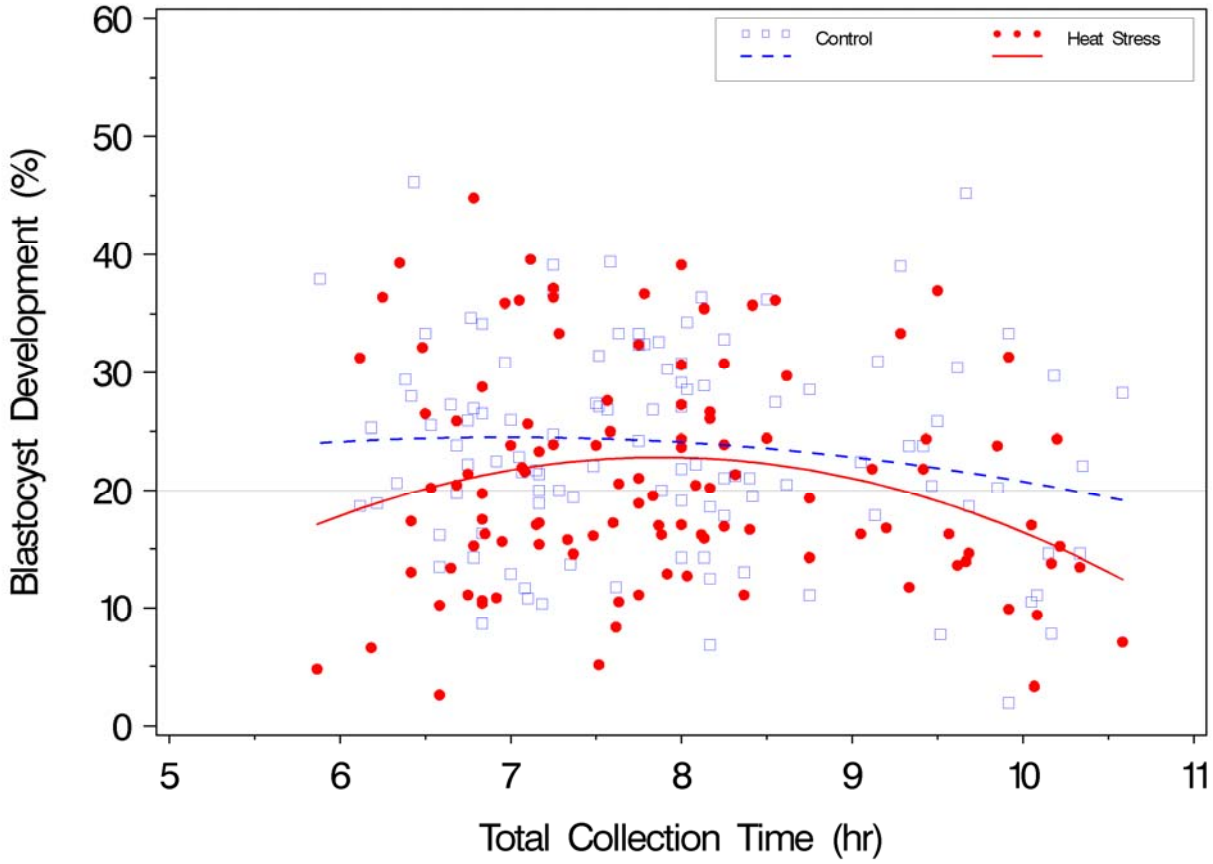

Figure 14. Influence of total collection time on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Quadratic equations of the model were $Y=4.57+5.73X-0.41X^2$ (P=0.2807) and $Y=-65.42+22.41X-1.42X^2$ (P=0.0350) for control and heat stress, respectively.

washed in sperm-TALP. Once sperm were washed, concentration and motility were determined in order to calculate volume of sperm that should be added to the ova. For this IVP of embryos step, the influences of bull ID, centrifuge method, sperm assessment, sperm concentration, sperm motility, sperm volume, sperm concentration after added to ova, ova age at IVF and ova age at IVF from removal from follicles were examined.

Bull ID

Only sperm from one bull or combinations of 2 or 3 bulls' were selected from a pool of 11 different bulls. Because no interaction between maturation temperature and bull ID was noted, data were combined to examine main effect (Table 3; P=0.0343). Rate of development spanned a wide range of 3.36 ± 7.07% to 37.28 ± 4.50% (Figure 15). Effect of bull ID explained 9.2% variation of rate of blastocyst development.

The influence of bull, of the same or different breeds, on in vitro blastocyst development is well-established (Coelho et al., 1998, Katska-Ksiazkiewicz et al., 2009, Parrish et al., 1986) and is not surprising given that fertility of bulls after natural service differs (Farrell et al., 1998, Palma et al., 1996, abstract, Schneider et al., 1996, abstract). For example, Coelho et al. (1998) reported that blastocyst rates ranged from 4.1% to 18.8% depending upon the Nellore bull used.

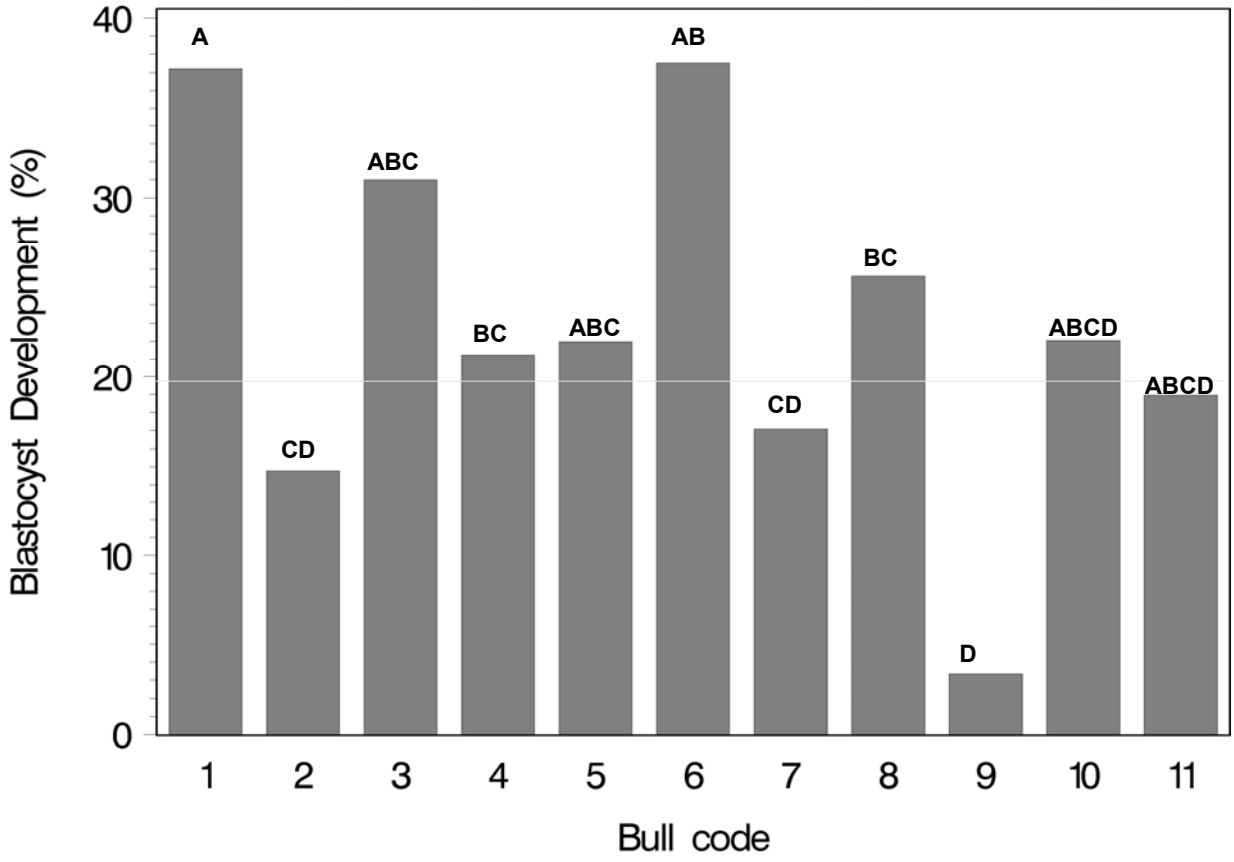

Figure 15. Influence of bull's sperm source used for IVF on blastocyst development for ova matured at 38.5 and 41.0°C combined. $^{A-D}$ means differ (pooled SEM=6.32; P=0.0343).

Centrifugation Method

For the meta-analysis described, motile and live sperm were separated from dead and immotile sperm on a discontinuous (45/90%) Percoll® column with two possible methods: a 640 to 760 *g* centrifugation for 10 to 16 minutes (4 ml column) or a 2,000 *g* microcentrifugation for 10 to 12 minutes (1.5 ml column). Because an interaction between maturation temperature and centrifugation was not observed, data were combined to examine main effect of centrifugation method (Table 3). Blastocyst rates were higher when a centrifuge was used (25.02 ± 0.86%) versus a microcentrifuge (20.12 ± 0.85%; Figure 16; $P<0.0001$).

It can be speculated that utilization of different centrifuges at different *g* forces may mechanically damage the sperm. Additionally, it can be speculated that the greater the *g* force and the smaller volume when microcentrifuge was used may be pulling down sperm's decapacitating factors from the extender (Aires et al., 2003, Amirat et al., 2004) to the pellet that may not occur at lower *g* forces and higher volumes. However, not only did the centrifugation *g* and exposure time vary, but also the plastic material, volume and surfaces of tubes used for sperm preparation (i.e., from 1.5 microcentrifuge to 14 ml conical tubes) which are confounded with centrifugation method.

Sperm Assessment Method

After Percoll® purification and Sperm-TALP wash, the pellet of predominant motile sperm was isolated and resuspended in IVF-TALP. Concentration and motility of the sample after Percoll® were then assessed to calculate the number of sperm to add

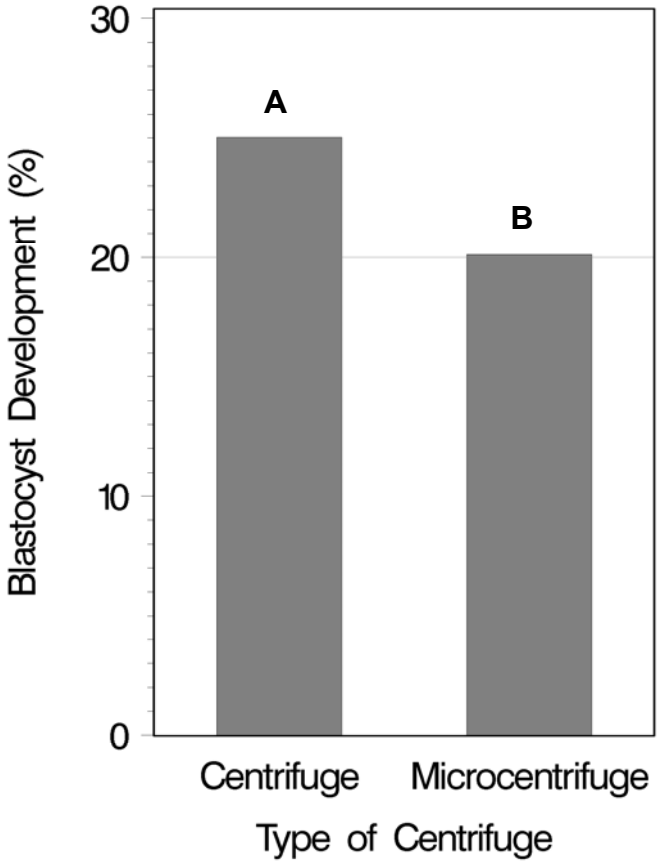

Figure 16. Influence of type of centrifuge on blastocyst development for ova matured at 38.5 and 41.0°C combined. $^{A-B}$ means differ (pooled SEM=0.86; P<0.0001).

to ova. To achieve this end, either a hemocytometer or computer assisted sperm analysis (CASA) was utilized. Use of hemocytometer only allows for calculation of total number of sperm while the CASA assesses concentration and motility. Because an interaction between maturation temperature and sperm assessment method was not observed, data were combined to examine the main effect of assessment method (Table 3). Blastocyst rates were higher when hemocytometer was utilized (25.68 ± 1.23%) versus CASA (21.49 ± 0.71%; Figure 17). When centrifuge type was added to the ANOVA model, the impact of the type of assessment method on blastocyst development was no longer apparent (P=0.4386). In general, the sperm assessment performed the experiment. Since these variables were no longer significant when centrifugation was accounted for, its impact on blastocyst development was minimal.

Sperm Sample Characteristics after Percoll® Preparation (Concentration, Motility, Volume)

Concentration and motility of sperm sample were assessed before addition of sperm in order to calculate volume needed to add ova. The targeted ratio was 1:5,000 to 1:7,500 ova per sperm.

Sperm Concentration before Addition to Oocytes

Sperm concentration before addition to oocytes ranged from 9.1 to 74.2 million sperm/ml. Utilizing a dummy regression model on the combined data, a significant effect of sperm concentration before addition to oocytes was noted (Table 3, P=0.0055). As

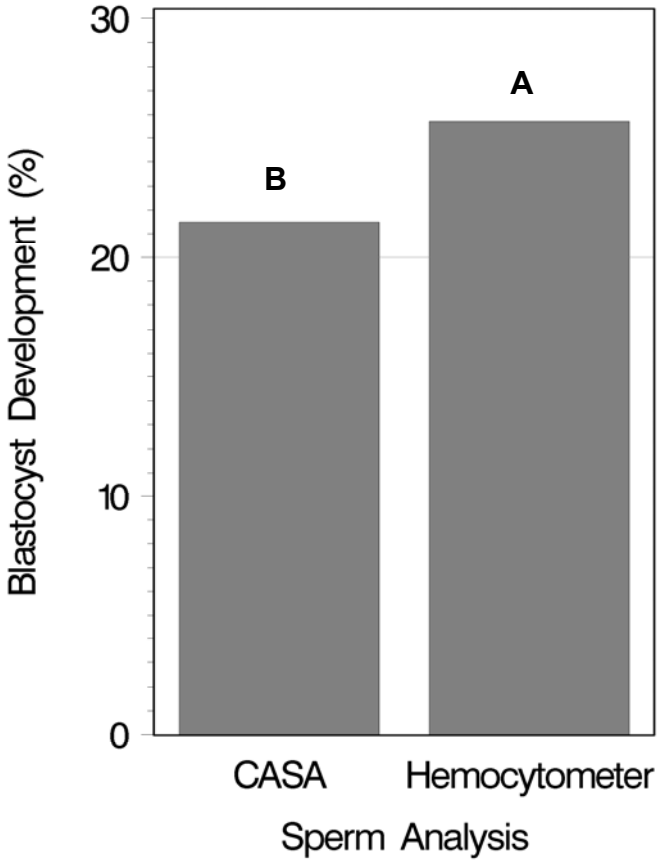

Figure 17. Influence of sperm concentration analysis method on blastocyst development for ova matured at 38.5 and 41.0°C combined. $^{A-B}$ means differ (pooled SEM=0.97; P=0.0035).

the sperm concentration increased, there was an associated decrease in blastocyst development of 1.05 ± 0.38% for each 10 million sperm/ml increment. This variable explained 3.5% of the variation in blastocyst development (Figure 18). Achieving less than 20% development was coincident with concentrations higher than 58.2 million sperm/ml (Y=26.11-0.11X).

Sperm Motility before Addition to Oocytes

Sperm motility before addition to oocytes ranged from 64 to 98%. Utilizing a dummy regression model on the combined data, a significant effect of sperm motility was noted (Table 3; P=0.0004). As sperm motility increased, there was an associated decrease in blastocyst development of 2.51 ± 0.70% for each 10% increase in motility. This variable explained 6.2% of the variation in blastocyst development (Figure 19). Motilities greater than 98.6% were predicted to average less than 20% blastocyst development (Y=44.66-0.25X). However, when low motility values were considered outliers, sperm motility greater than 85% was not detrimental for blastocyst development (P=0.1805).

Volume of Sperm Added to Ova

The volume of sperm added to ova depended on sperm motility and sperm concentration after resuspension and ranged from 2.3 to 18.3 µl. Simple linear regression did not reveal any differences in blastocyst development of ova matured at

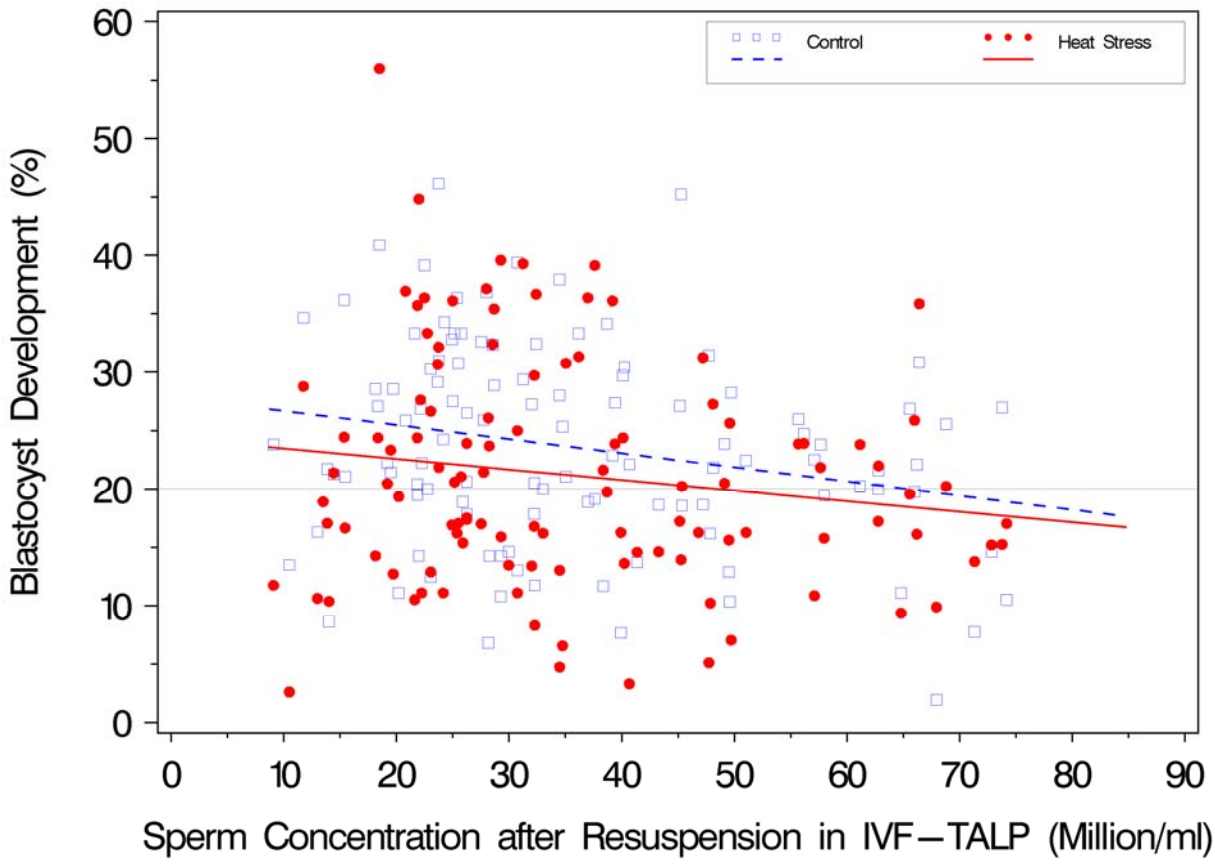

Figure 18. Influence of sperm concentration after resuspension in IVF-TALP on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=27.89-0.12X (P=0.0163) and Y=24.35-0.09X (P=0.1098) for control and heat stress, respectively.

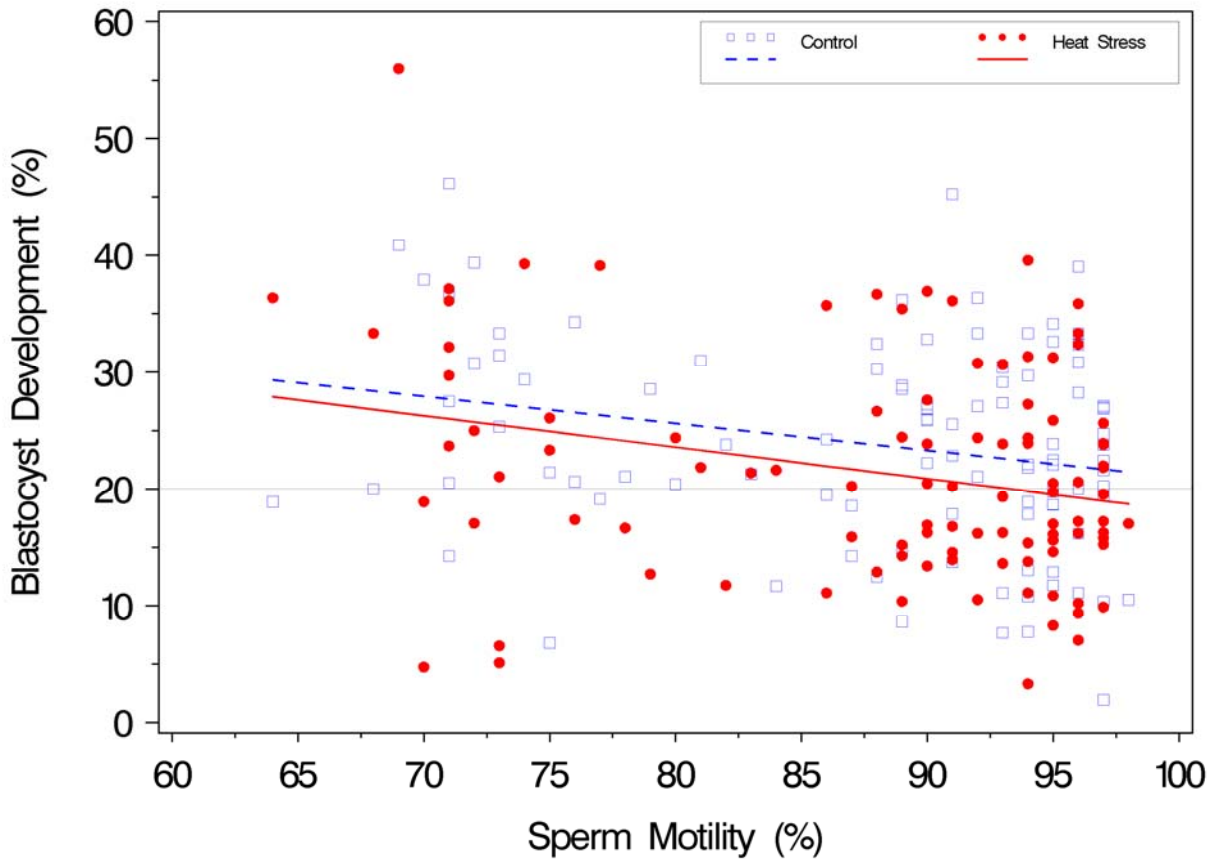

Figure 19. Influence of sperm motility for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=44.21-0.23X (P=0.0163) and Y=45.15-0.27X (P=0.0085) for control and heat stress, respectively.

effect of sperm volume was noted (Table 3; P=0.0002). As the sperm volume increased, there was an associated increase in blastocyst development of 5.62 ± 0.15% for each10 µl increase in volume. This variable explained 6.6% of the variation in blastocyst development (Figure 20). Sperm volumes of less than 4.2 µl sperm were predicted to average less than 20% blastocyst development (Y=17.64+0.56X).

A model that included sperm volume, concentration and motility showed that an increase in sperm concentration (P=0.0213) and motility (P=0.0479) was related to a decrease in blastocyst development, explaining 12.4% of the variation. This may be due to possible pipetting error associated with volumes less than 4.2 µl. Combining all three variables, the volume of sperm (P=0.4439) was not related to blastocyst development.

One explanation for the reported effect of sperm concentration on blastocyst development could be agglutination of highly concentrated sperm in the presence of heparin (Parrish and Susko-Parrish, 1989), hampering motility and fertilization to produce low developmental rates after addition to ova. This is unlikely since penetration rates using this IVP system are acceptable (60 and 75% at 10 and 16 hours after IVF, respectively) and routinely produce blastocyst rates of at least 20% (Edwards et al.,unpublished). It can also be speculated that increased motility may be related to sperm attrition. However, when the concentration or motility of sperm increased with a decrease in sperm volume added, development to blastocyst decreased. Addition of lower volumes of sperm to oocytes may be associated with increased pipetting error particularly when using a 20 µl micropipette. Thus, volumes less than 5 µl should be avoided by additional dilution of the sperm sample before adding to oocytes.

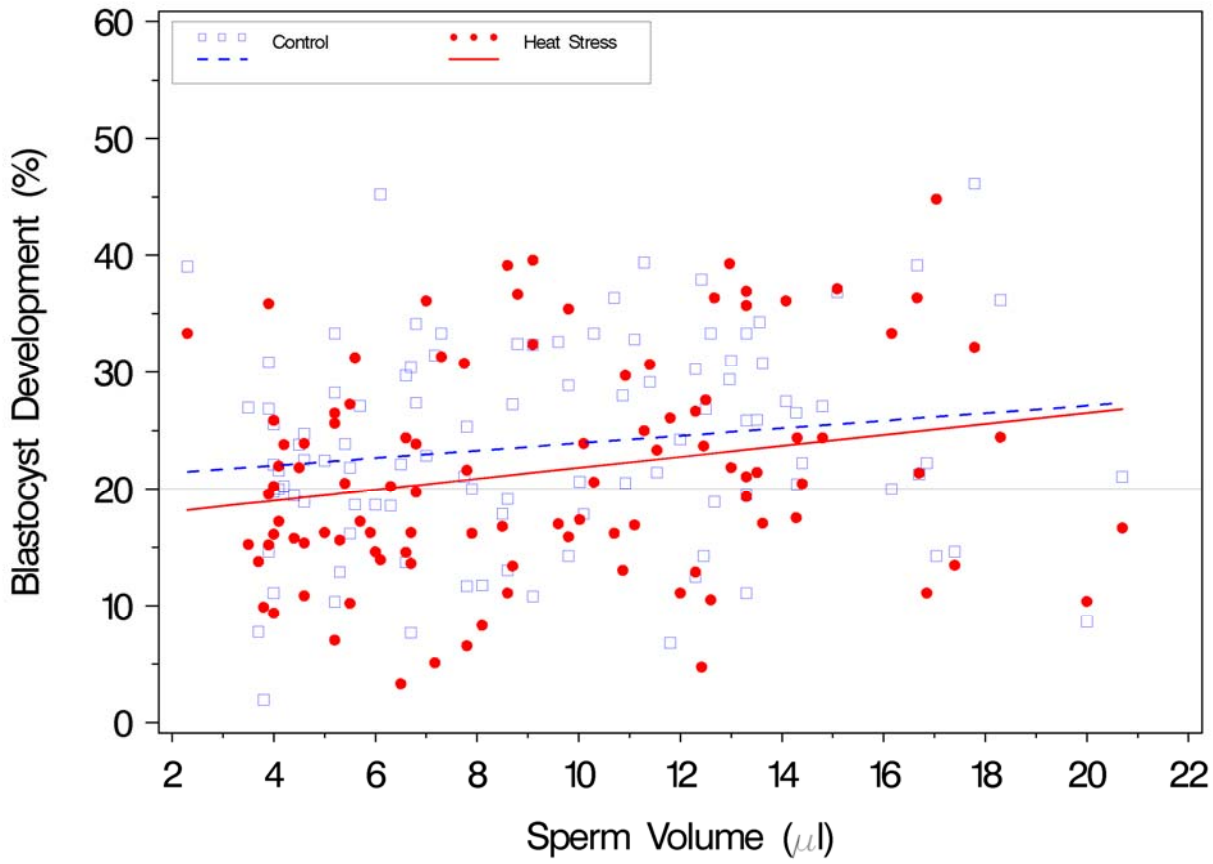

Figure 20. Influence of sperm volume added to ova on blastocyst development for ova matured at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=19.51+0.48X (P=0.0228) and Y=15.82+0.64X (P=0.0039) for control and heat stress, respectively.

Sperm Concentration after Addition to Ova

Final sperm concentration after addition to ova ranged from 500,000 to 750,000 sperm/ml, with a mode of 500,000 sperm/ml. This concentration range translates into a ratio of 1:5,000 to 1:7,500 oocytes per sperm. Because there was not an interaction between maturation temperature and sperm concentrations, data were combined to examine main effect (Table 3). Blastocyst rates were higher when sperm concentrations were 600,000 sperm/ml (n=28; 32.90 ± 2.41%) versus concentrations of sperm of 500,000 and 750,000 sperm/ml (500,000: n=181; 21.87 ± 0.67%; 750,000: n=60; 22.28 ± 2.03%; Figure 21; P=0.0001).

Results disagree with existing literature demonstrating that 1:5,000 oocyte/sperm (500,000 sperm/ml) produces maximum blastocyst development (Long et al., 1994, Ward et al., 2002). It is fundamental to point out that the data for those results were not necessarily corrected for sperm motility and 1:6,000 ova to sperm ratio was not evaluated. For example, Ward et al. (2002) reported that 1:5,000 oocyte/sperm (500,000 sperm/ml) had better development (35.4%) than 1:10,000 oocyte:sperm (24.9%).

Ova Age at IVF

To clarify the impact of ova age at sperm addition on blastocyst development, it was defined and evaluated using two different approaches: the total time in OMM until sperm addition, and the total time from ova removal from follicles until sperm addition.

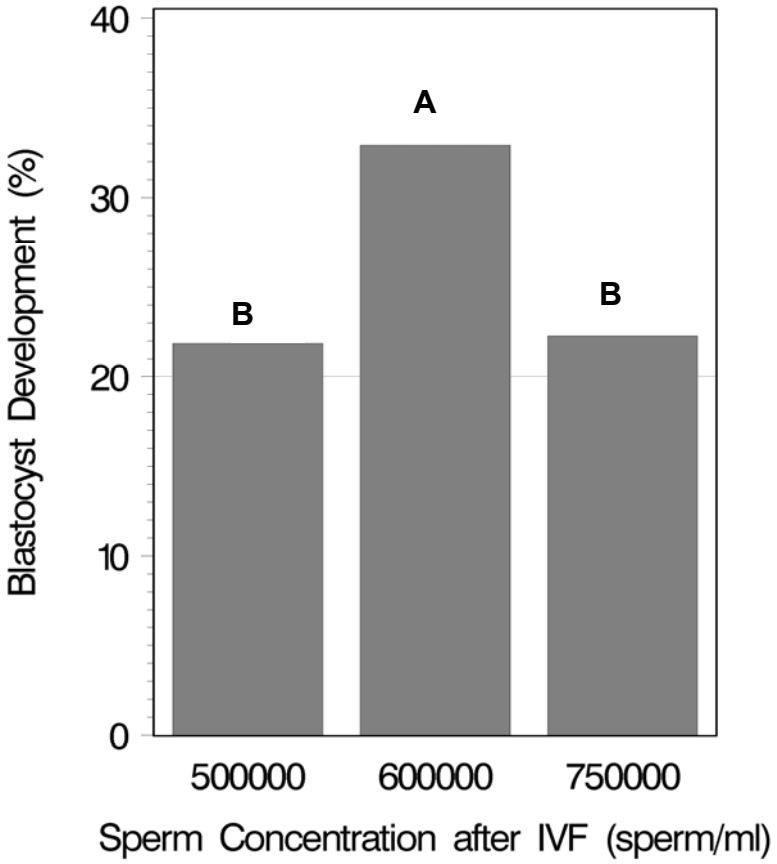

Figure 21. Influence of sperm concentration after IVF on blastocyst development for ova matured at 38.5 and 41.0°C combined. [A-B] means differ (pooled SEM=2.51 ; P=0.0062).

Total Time in OMM

Total time in OMM corresponded to the time period when oocytes were placed in OMM until IVF, and ranged from 22.62 to 25.00 hours. Outliers less than 23.3 hours were eliminated. Utilizing a dummy regression model on the combined data, a significant effect of the ova age at the addition of sperm was noted (Table 3; $P<0.0001$). As the ova age at IVF increased, there was an associated decrease in blastocyst development of 8.15 ± 1.98% for each additional hour in ova age. This variable explained 7.3% of the variation in blastocyst development (Figure 22). Ova ages of more than 24.5 hours were predicted to average less than 20% blastocyst development ($Y=219.65-8.14X$).

Total Time from Oocyte Removal from Follicles until Addition of Sperm

The total ova age at IVF from follicle removal was defined as the difference between onset of ovary slicing and sperm addition to ova and ranged from 23.89 to 27.38 hours. Utilizing a dummy regression model on the combined data, a significant effect of ova age at the addition of sperm from removal of follicles was noted (Table 3; $P=0.0001$). As the ova age from ovary slicing increased, there was a progressive decrease in blastocyst development of 3.61 ± 0.92% for each additional hour in ova age. This variable explained 7.0% of the variation in blastocyst development (Figure 23). Less than 20% blastocyst development was predicted to occur when IVF took place after 26.9 hours ($Y=116.88-3.61X$).

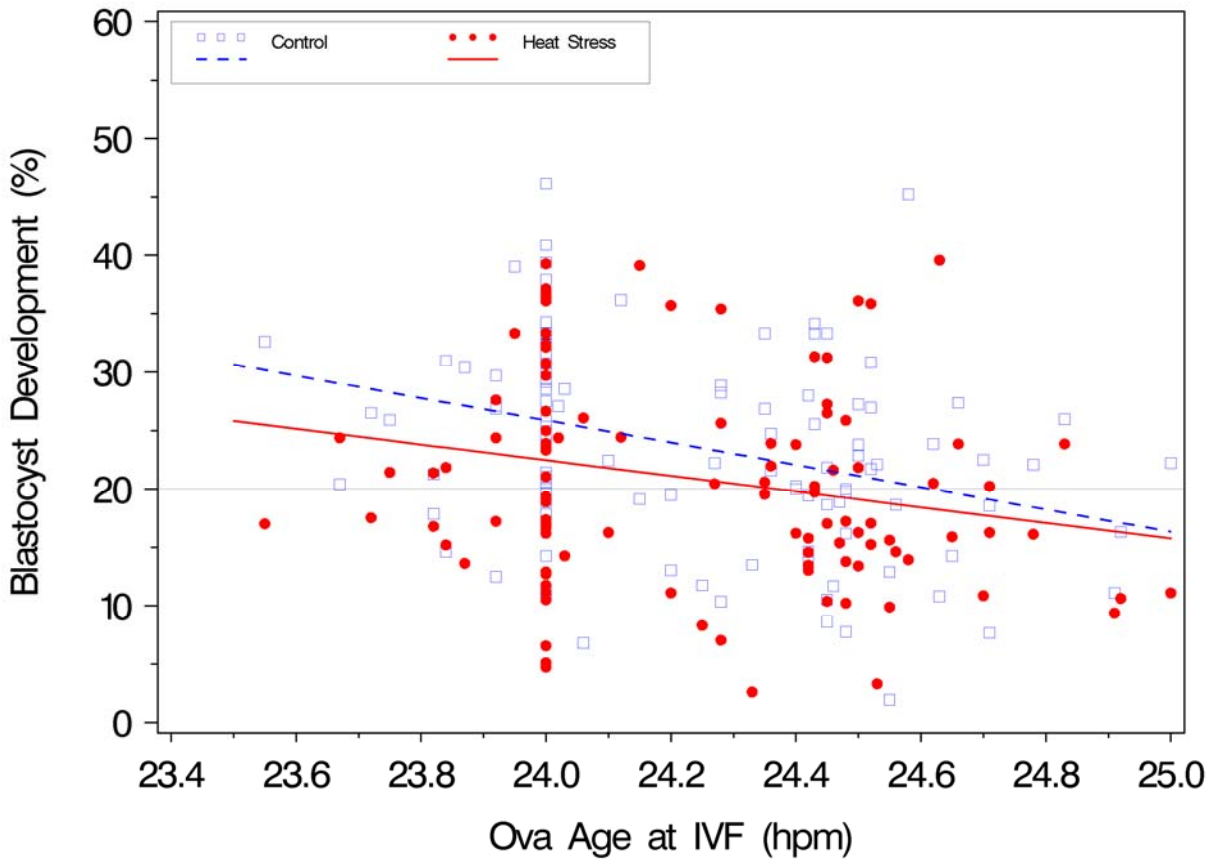

Figure 22. Influence of ova age at IVF on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=255.00-9.54X (P=0.0004) and Y=183.24-6.70X (P=0.0269) for control and heat stress, respectively.

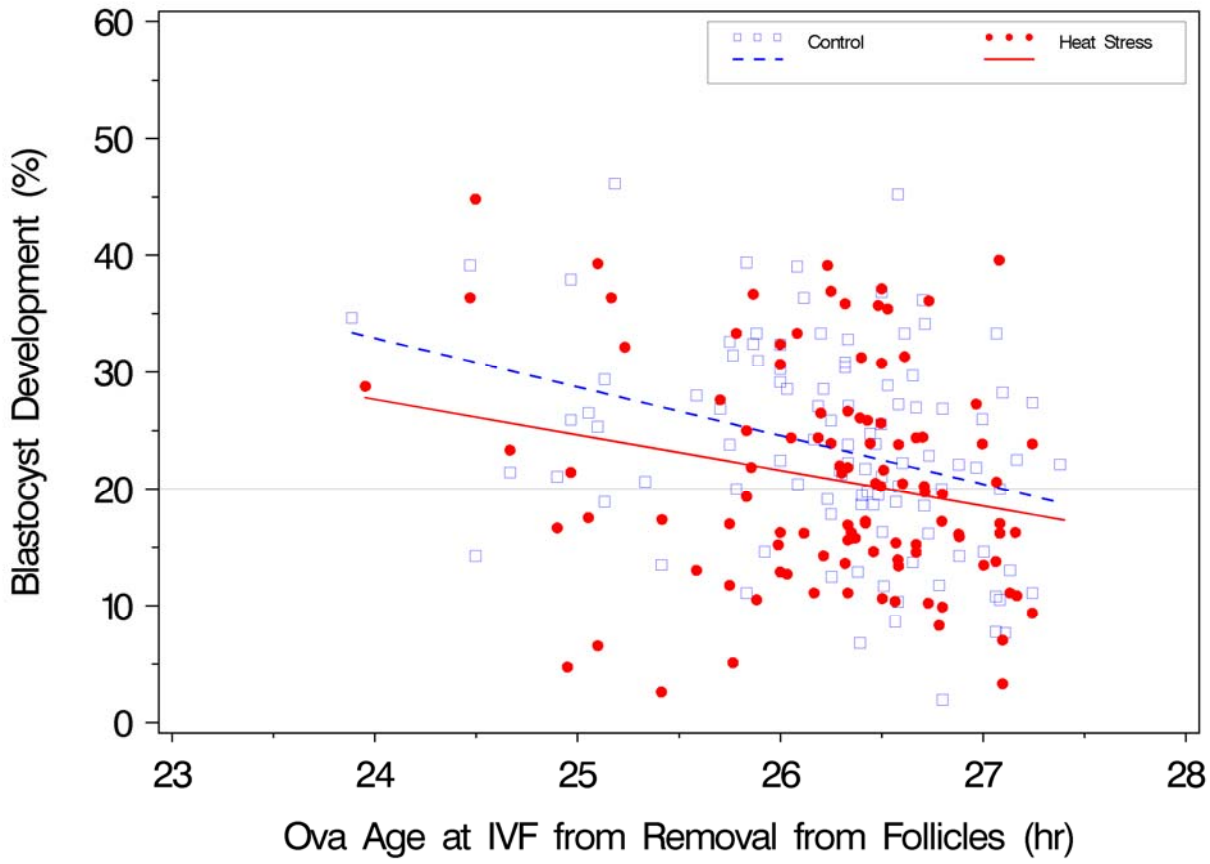

Figure 23. Influence of ova age at IVF from removal from follicles on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=132.87-4.17X (P=0.0009) and Y=100.52-3.04X (P=0.0269) for control and heat stress, respectively.

The fertile lifespan of the matured oocyte is finite, both in vivo (8 to 10 hours; Hunter, 1985) and in vitro (4 to 8 hours; Ward et al., 2002). In agreement with the results, holding matured mammalian oocytes in vitro before fertilization (i.e., aging) reduces subsequent blastocyst development (Agung et al., 2006, Takahashi et al., 2003, Tarin et al., 1998). It was previously hypothesized that removal of oocytes from ovarian follicles may cause oocytes to resume meiosis (Pincus and Enzmann, 1935). However, when the total time from removal from follicles to addition of sperm was compared with just total time in OMM, it was apparent that these variables were explaining the same amount of variation. Therefore, premature initiation of meiosis of oocytes in holding media is likely minimal. Taking results together, the duration of maturation from placement of oocytes into OMM until IVF had more influence on blastocyst development than total collection time.

Denuding

The purpose of denuding step was to remove the associated cumulus cells and spermatozoa from the putative zygotes using the mechanical method of vortexing. This is done to avoid competition for nutrients from cumulus cells and release of toxins from dead sperm. After washing and evaluation, putative zygotes were placed in KSOM for embryo culture. For this IVP step, the influences of gamete co-incubation time, vortex time, PZ recovered per treatment, PZ with excessive cumulus per treatment, PZ lysed per treatment, total PZ number per 500 µl KSOM, and total denuding time were examined.

Gamete Co-Incubation Time

The time between addition of sperm to ova and beginning of denuding ranged from 16.2 to 20.7 hours and was defined as gamete co-incubation time. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no significant effect of gamete co-incubation time was noted (Table 3; P=0.0936). Additionally, this result was independent of the bull ID effect when gamete co-incubation time and bull ID were combined in a dummy regression model (interaction P=0.2102).

This result agrees with others (Rehman et al., 1994; Ward et al., 2002). Specifically, co-incubation of 15 to 20 matured bovine oocytes with 600,000 sperm/ml for 4, 8, 12, 16, 20, 24, or 28 hours did not alter blastocyst development (52.0 to 70.3% blastocysts of eight-cell embryos; Rehman et al., 1994). However, shorter times of co-incubation than our reported interval may be problematic. Co-incubation for 10 hours was sufficient to maximize percentage of bovine blastocyst development (31.9 to 32.3% of total PZ cultured) while 1 (0%) and 5 hours (19.2%) were inadequate durations to allow for sperm to fertilize oocytes (Ward et al., 2002). The fact that Ward et al. (2002) reported disparate effects compared to Rheman et al. (1994) may be related with the higher number of oocytes used (n=712 versus 513, respectively) and the different embryo culture media used (Synthetic oviduct fluid vs. Chatot-Ziomek-Bavister with buffalo rat liver cells co-culture, respectively).

Vortex Time

The time between beginning and ending of vortexing ranged from 3.5 to 5.0 minutes and was defined as vortex time. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no significant effect of vortex time was noted (Table 3; P=0.9319).

This variable was thought to be problematic although no data were found to support this premise. Vortexing may stress PZ because of mechanical stress (Zeringue et al., 2005), light exposure and temperature fluctuations, but blastocyst rates from the aforementioned data range do not support this theory.

PZ Evaluation Characteristics after Vortexing

After vortexing and washing, putative zygotes (PZ) were assessed. Specifically, recovery, lysis and presence of excessive cumulus cells were evaluated and recorded.

PZ Recovered

The percentage of PZ recovered of the total oocytes placed in OMM ranged from 82.6 to 118.2%. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no impact of the percentage of the PZ recovered on blastocyst rates was noted (Table 3; P=0.8265).

PZ Recovered that were Lysed

The percentage of PZ recovered that were lysed was defined as those PZ that no longer had an intact membrane (i.e., oolemma). Lysis ranged from 0 to 18.5%. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, a significant trend of the PZ lysed on the blastocyst development was noted (Table 3; P=0.0712). As PZ lysis increased by 10%, blastocyst development decreased by 3.79 ± 2.09%. This variable explained 1.5% of the variation in blastocyst development (Figure 24). When more than 9 PZ were lysed at evaluation, blastocyst development was predicted to be less than 20% (Y= 23.47-0.38X). An additional analysis revealed that lysis was independent of collection month (interaction P=0.5003).

PZ with Excessive Cumulus Cells at Recovery

The percentage of PZ recovered with excessive cumulus ranged from 0 to 28.1%. Simple linear regression did not reveal any differences in blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no significance of PZ with excessive cumulus was noted (Table 3; P=0.8372).

Exposure of oocytes to elevated temperatures may induce lysis but there was only a trend for the physiologically-relevant temperature of 41.0°C to increase lysis. However, recovery, an indirect indicator of lysis, had no impact on blastocyst development. The slight indication that heat stress may increase oolemmal lysis should

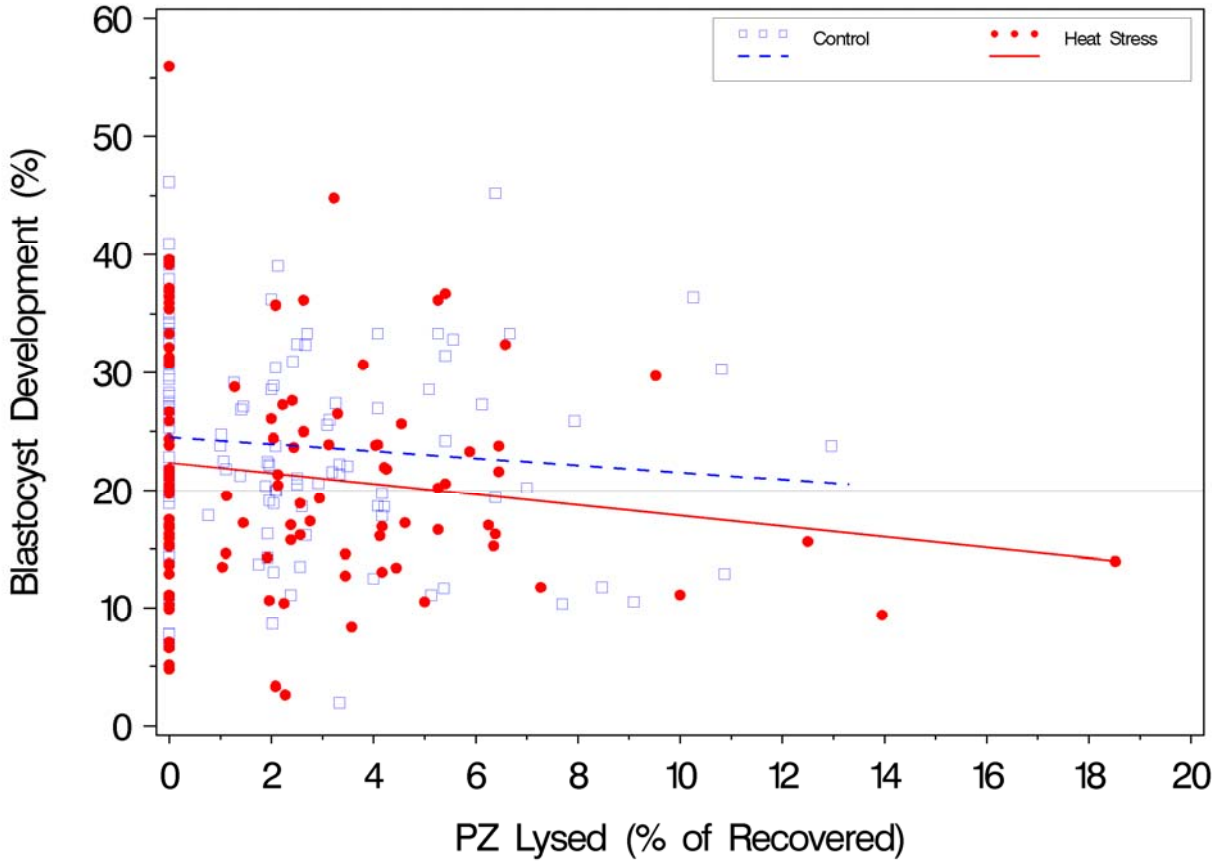

Figure 24. Influence of PZ lysed of recovered on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=23.66-0.066X (P=0.7415) and Y=21.61-0.087X (P=0.6549) for control and heat stress, respectively.

be further evaluated in future efforts. The presence of cumulus cells was also a concern because it may be related to maturation temperature. For example, heat-induced changes in the hyaluronic acid precursors that allow for expansion of the cumulus mass have been reported (Lenz et al., 1983). However, it did not impact blastocyst development.

PZ Number per 500 µl KSOM

After evaluation, selected PZ were placed in KSOM for additional embryo culture. Average number of PZ per 500 µl KSOM ranged from 14 to 46. Utilizing a dummy regression model on the combined data, a significant effect of the average number of PZ per 500 µl KSOM was noted (Table 3; P=0.0056). As PZ number per well increased, there was an associated increase in blastocyst development of 3.22 ± 0.12% for each 10 additional PZ. This variable explained 3.8% of the variation in blastocyst development (Figure 25). Fewer than 18 PZ per well was predicted to produce less than 20% blastocyst development (Y=14.28+0.32X). For additional clarity, data were separated into categories of 10 to 19, 20 to 29 and 30 to 49 PZ per well. Blastocyst rates were lower for 10 to 19 PZ per well (18.02 ± 1.53%) than 30 to 49 PZ per well (24.29 ± 1.71%; Figure 26). In addition, utilizing a response surface model on the combined data, the maximum predicted blastocyst yield was achieved with approximately 22 oocytes per OMM well and 33 PZ per KSOM well (R^2=0.131; overall model P<0.0001).

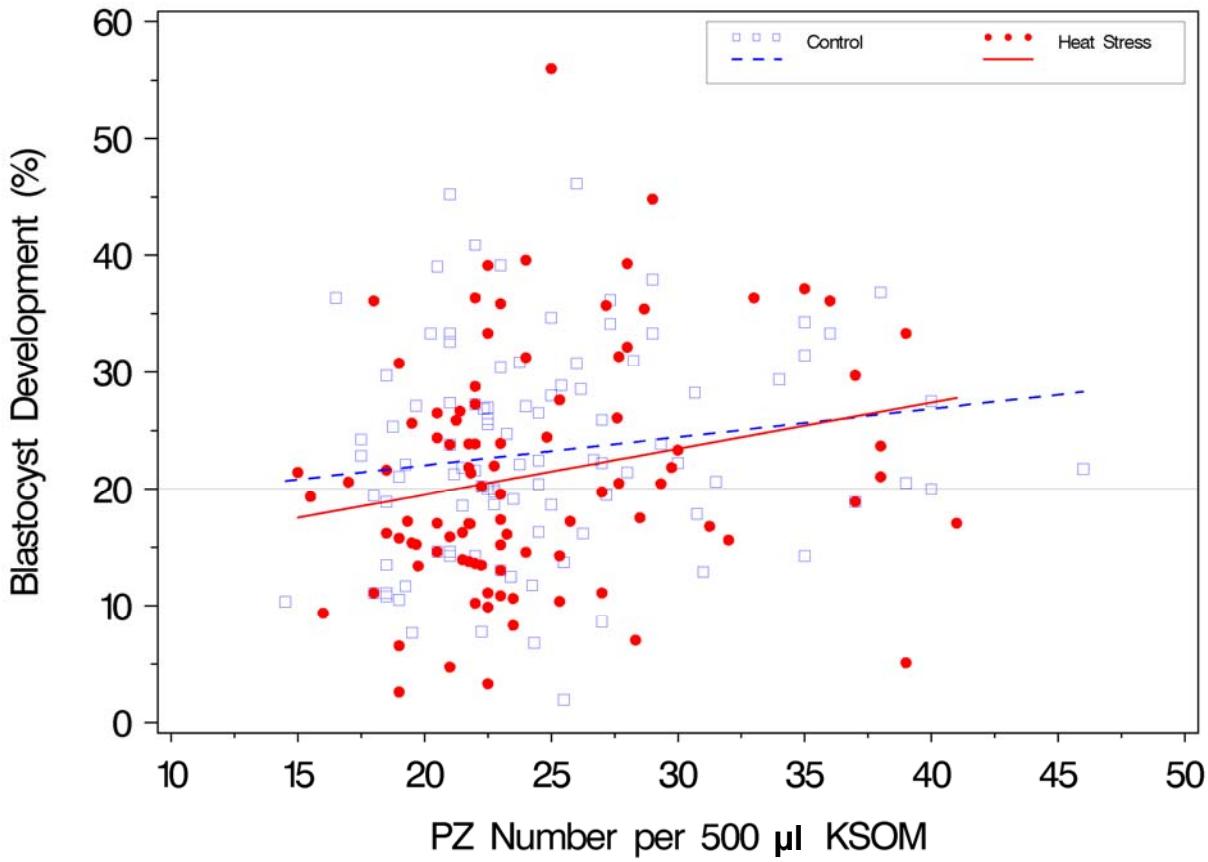

Figure 25. Influence of PZ number per 500 µl of KSOM on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=17.18+0.24X (P=0.1166) and Y=11.64+0.39X (P=0.0249) for control and heat stress, respectively.

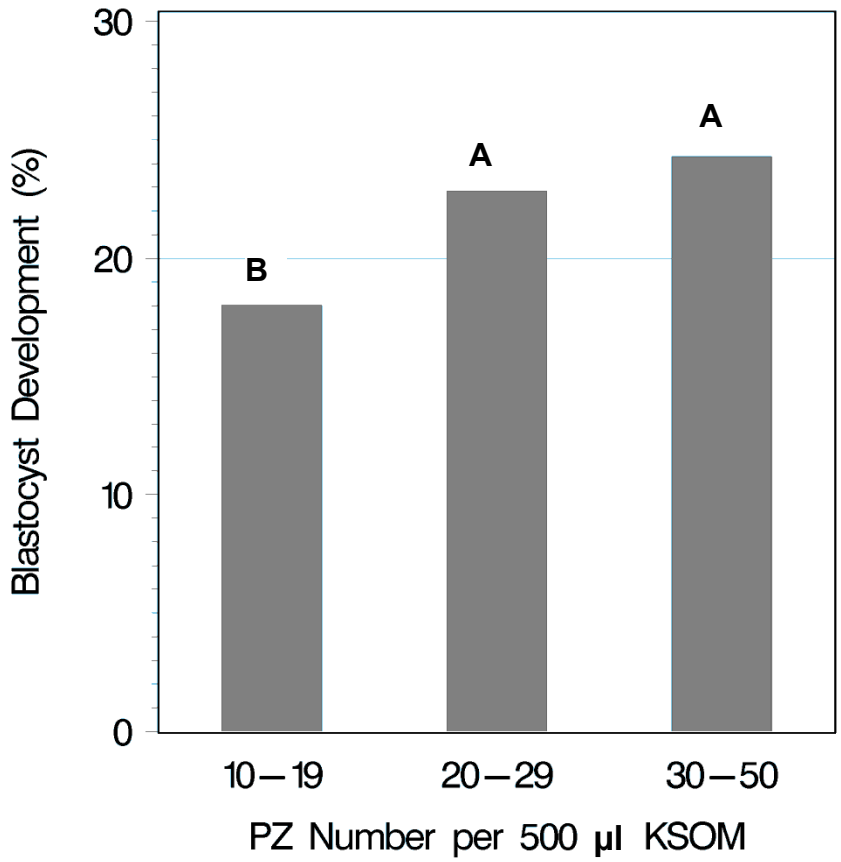

Figure 26. Influence of PZ number per 500 µl of KSOM on blastocyst development for ova matured at 38.5 and 41.0°C combined. $^{A-B}$ means differ (pooled SEM=1.35; P=0.0094).

The significance of this finding is that culturing more PZ per well produced better blastocyst rates, which is in agreement with existing literature for mammalian embryos (Ferry et al., 1994, Fujita et al., 2006, Khurana and Niemann, 2000, Lane and Gardner, 1992, Paria and Dey, 1990, Wiley et al., 1986). For example, development of one cell embryos from poor quality bovine oocytes (PZ from oocytes with fewer than three layers of cumulus cells or partially denuded but having a homogeneous evenly granulated cytoplasm) was improved by increasing the embryo density in culture from 20 (13.6% blastocyst development) to 40 embryos (22.7%) in 500 µl of media (Khurana and Niemann, 2000). This was in direct contrast to results for cumulus-oocyte complexes per OMM well, where fewer was better. Disparity is likely due to lack of cumulus cells on PZ that can deplete the media nutrients as discussed previously (see ova number per well section). It has also been reported that increased number of PZ per well may facilitate release of factors that improve blastocyst development (Fujita et al., 2006).

Total Denuding Time

Total denuding time was defined as the time between beginning of denuding and placement of PZ in KSOM plates in the incubator, including PZ removal from fertilization wells, vortexing, washing, and evaluation of PZ from ova matured at 38.5 or 41.0°C. The time it took to do so depended on experiment and ranged from 10 to 69 minutes. Simple linear regression did not reveal any impact of total denuding time on blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no significant effect of the denuding time was noted (Table 3;

P=0.6301). Since one-cell embryos are less tolerant to other stressors (Edwards et al., 2001) this result was not expected and difficult to explain.

In Vitro Culture

Three days after IVF, ability of PZ to cleave was assessed and type of embryos recorded: one-cells, two-cells, four-cells and 8 to 16-cells. For this IVP step, influences of cleavage assessment time, PZ cleaved, 2-cells of cleaved , 2-cells of total PZ, 4-cells of cleaved, 4-cells of total PZ, 8 to 16-cells of cleaved and 8 to 16-cells of total PZ per treatment on blastocyst development were analyzed. Blastocyst assessment was performed at approximated eight days after IVF.

Cleavage Assessment Time

The time difference between addition of sperm to ova and average cleavage assessment ranged from 65.0 to 77.2 hours. Simple linear regression did not reveal any impact of this variable on blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, a significant trend for cleavage assessment time to alter blastocyst rates was noted (Table 3; P=0.0651). As cleavage assessment time increased, there was an associated increase in blastocyst development of 5.69 ± 3.07% for each additional 10 hours. This variable explained 1.7% of the variation in blastocyst development (Figure 27). Assessment times of less than 67.47 hours were predicted to average less than 20% blastocyst development (Y=-18.46+0.57X).

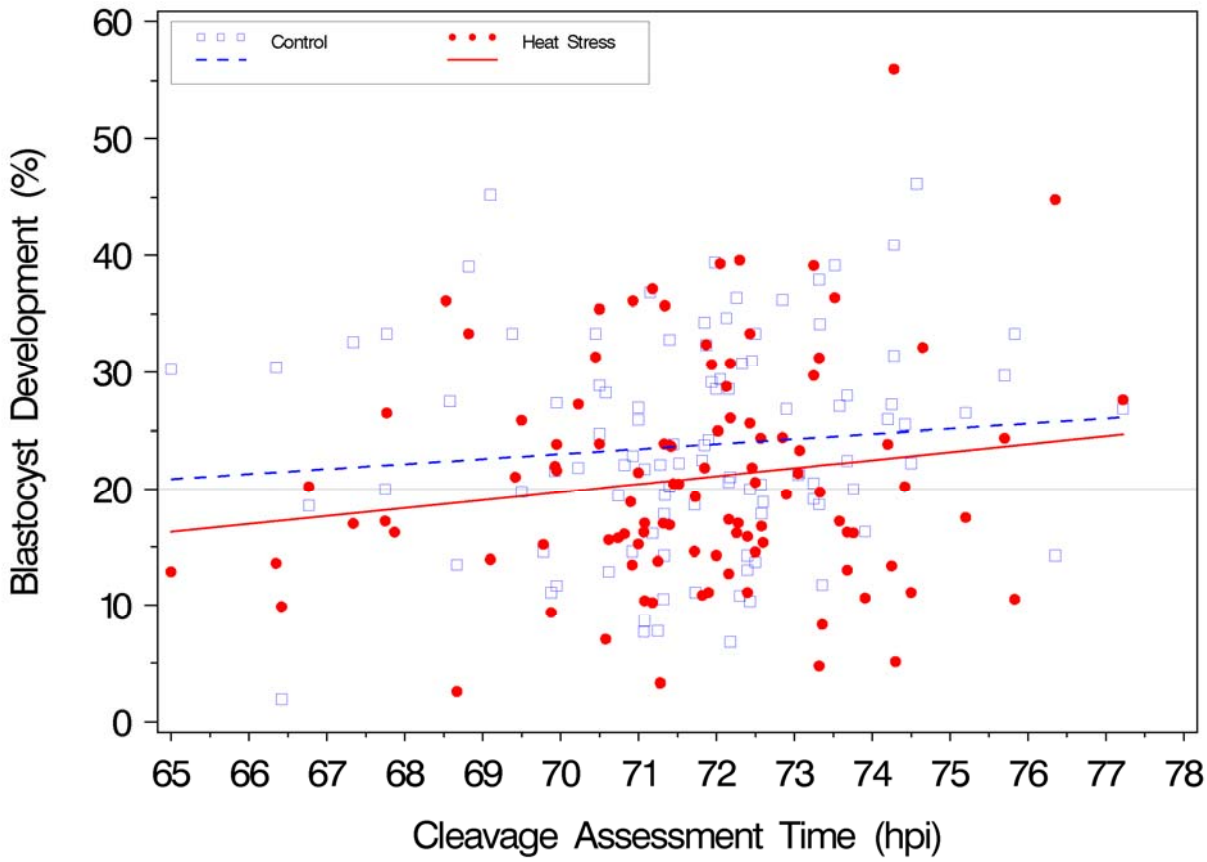

Figure 27. Influence of cleavage assessment time on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=-7.05+0.42X (P=0.3136) and Y=-28.50+0.69X (P=0.1175) for control and heat stress, respectively.

The particular interest in cleavage was related to the fact that the earlier embryos are stressed, the more they may be susceptible to perturbations in factors such as temperature and pH (Martino et al., 1995, Ocon and Hansen, 2003, Schwartz et al., 1998). For example, earlier stages of embryos are more susceptible to elevated temperatures compared to later stages (Edwards et al., 2001). In association with that result, embryos observed at 65 hours may not be as tolerant to withdrawal from the incubator as embryos observed at 77.2 hours to assess cleavage, but there was only a trend to decrease development.

Blastocyst Assessment Time

The time difference between addition of sperm to ova and assessment of blastocyst development ranged from 181.75 to 215.20 hours. Simple linear regression did not reveal any impact of blastocyst assessment time on blastocyst development of ova matured at 38.5 or 41.0°C (Figure 28). Utilizing a dummy regression model on the combined data, no significant effect of blastocyst assessment was noted (Table 3; P=0.0992).

If blastocyst assessment was performed too early, embryos may not have developed to blastocyst stage resulting biased blastocyst yields. Because blastocyst development was not different from 181.75 to 215.20 hours, this would suggest that assessment times were sufficiently accurate for blastocyst evaluation.

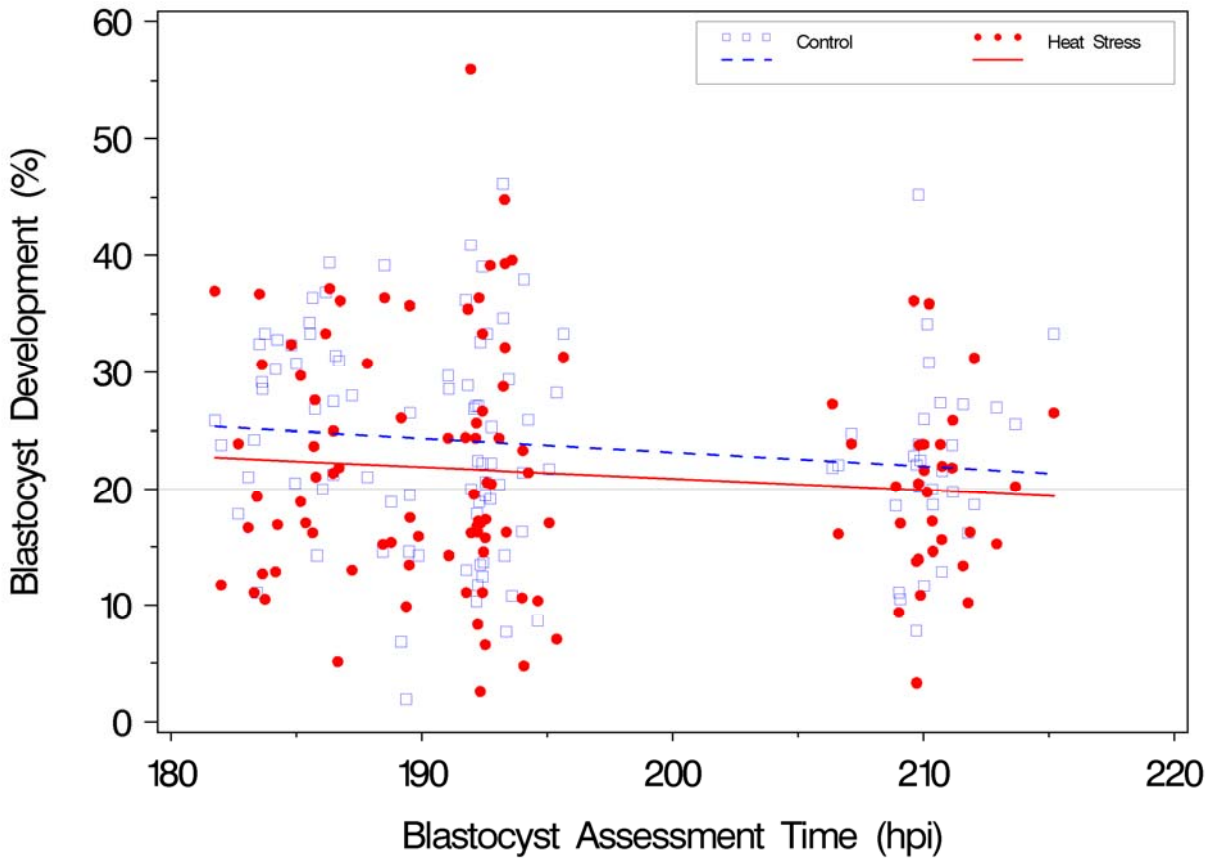

Figure 28. Influence of blastocyst assessment time on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=48.76-0.13X (P=0.1199) and Y=35.58-0.07X (P=0.4182) for control and heat stress, respectively.

Cleavage Characteristics

Cleaved

The percentage of PZ that cleaved ranged from 21.05 to 95.65%. Utilizing a dummy regression model on the combined data, a significant effect of percentage cleaved was noted (Table 3; $P<0.0001$). As the percentage of PZ that cleaved increased, there was an associated increase in blastocyst development of 3.02 ± 0.44% for each 10% increase in cleavage rate. This variable explained 18.3% of the variation in blastocyst development (Figure 29). Less than 63.5% cleavage was predicted to average less than 20% blastocyst development ($Y=0.93+0.30X$).

Two-Cells

The percentage of PZ that became two-cell embryos of cleaved ranged from 0 to 50%. Utilizing a dummy regression model on the combined data, a significant effect of cleaved embryos that were two-cells at assessment was noted (Table 3; $P=0.0001$). As percentage of two-cell embryos of cleaved increased, there was an associated decrease in blastocyst development of 4.96 ± 0.80% for each 10% increase in two-cells. This variable explained 15.1% of the variation in blastocyst development (Figure 30). Presence of more than 15% two-cell embryos was predicted to average less than 20% blastocyst development ($Y=27.10-0.50X$).

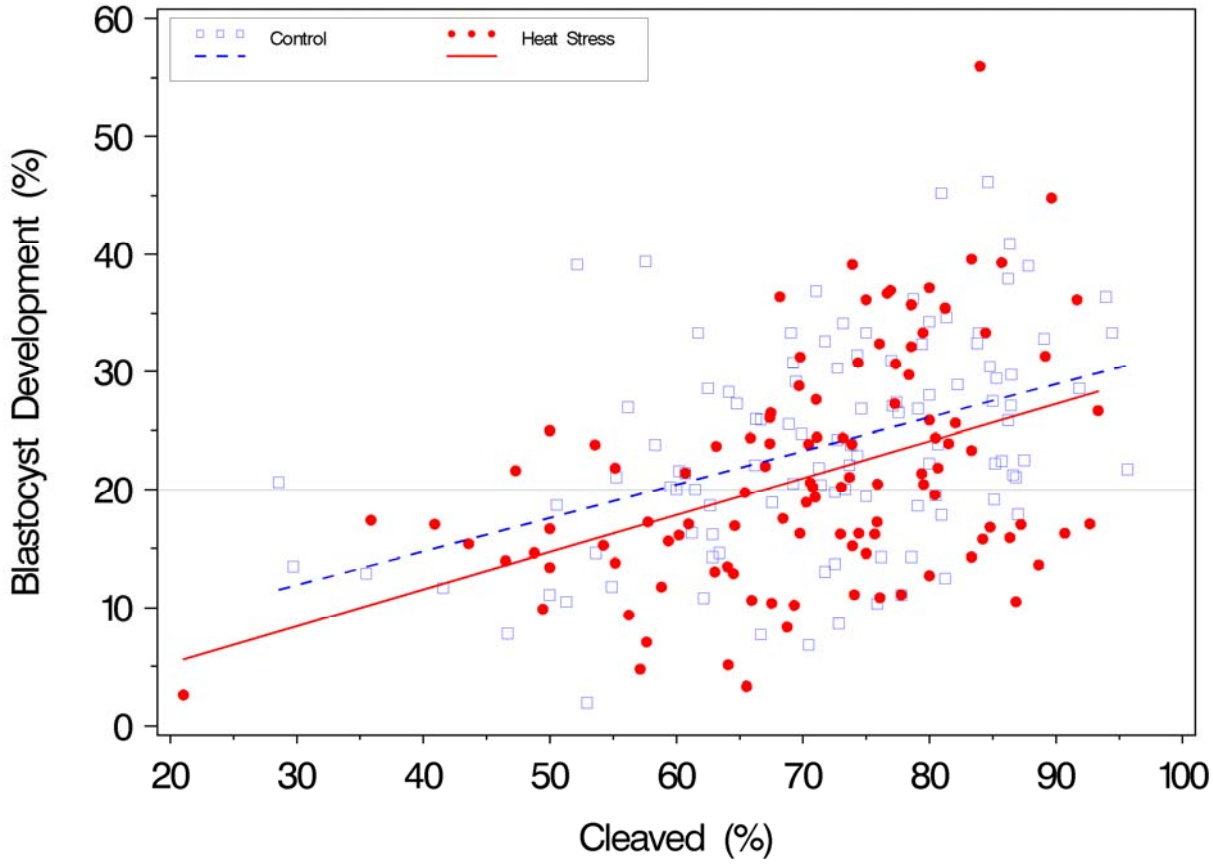

Figure 29. Influence of percentage of PZ cleaved on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=3.40+0.28X (P<0.0001) and Y=-1.01+0.31X (P<0.0001) for control and heat stress, respectively.

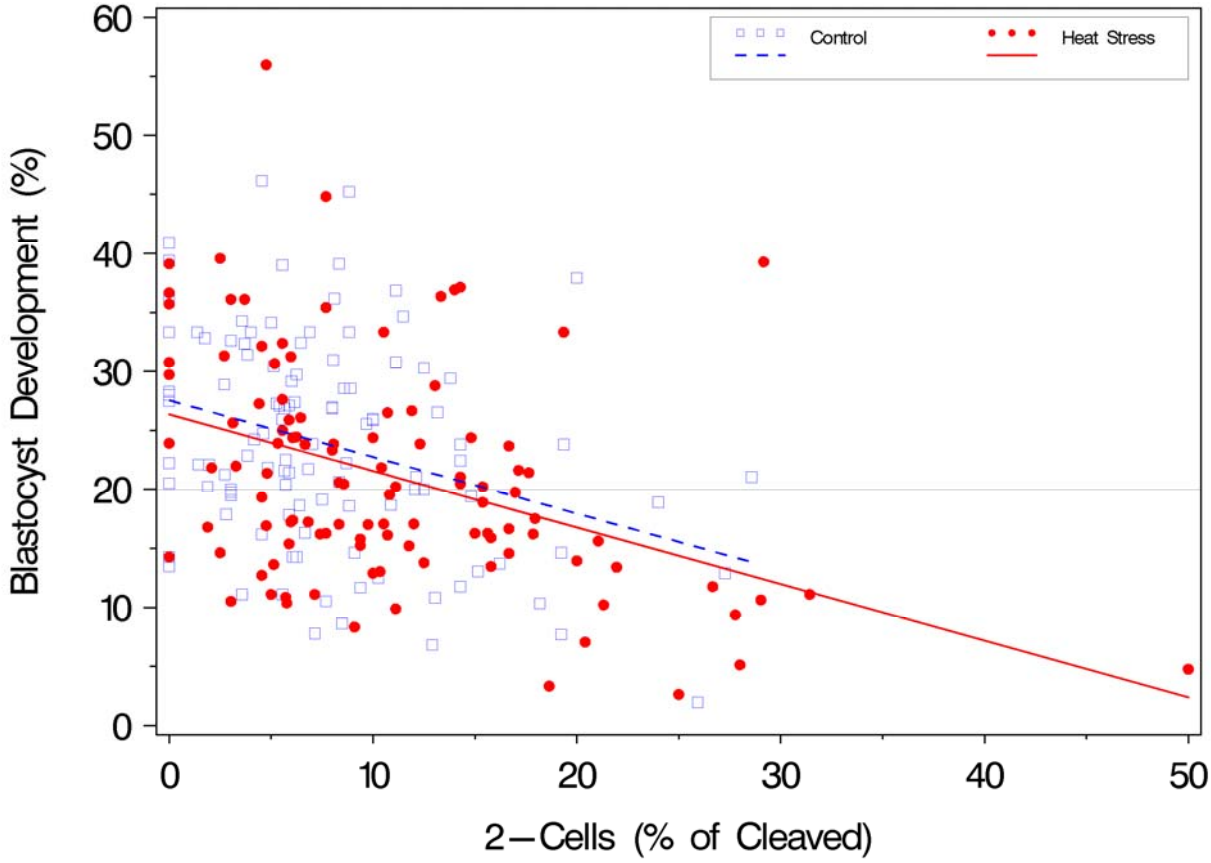

Figure 30. Influence of percentage of 2-cells of cleaved PZ on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=27.54-0.48X (P=0.0006) and Y=26.35-0.48X (P<0.0001) for control and heat stress, respectively.

The proportion of total PZ cultured that were two-cell embryos at cleavage assessment ranged from 0 to 28.57%. Utilizing a dummy regression model on the combined data, a significant effect of PZ that reached two-cell embryos was noted (Table 3; P=0.0002). As percentage of two-cell embryos increased, there was an associated decrease in blastocyst development of 5.33 ± 1.29% for each 10% increase in two-cells. This variable explained 7.3% of the variation in blastocyst development (Figure 31). Presence of more than 11.1% two-cells at cleavage assessment was predicted to average less than 20% blastocyst development (Y=25.86-0.53X).

Four-Cells

The proportion of PZ that reached four-cell embryos of cleaved embryos ranged from 0 to 54.0%. Simple linear regression did not reveal any impact of 4-cell embryos on development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no effect of cleaved embryos that reached the four-cell stage was noted (Table 3; P=0.1547).

The proportion of total PZ cultured that reached four-cell embryos ranged from 0 to 46.55%. Simple linear regression did not reveal any impact of this variable on blastocyst development of ova matured at 38.5 or 41.0°C. Utilizing a dummy regression model on the combined data, no effect of total PZ that were four-cell embryos at cleavage assessment was noted (Table 3; P=0.8557).

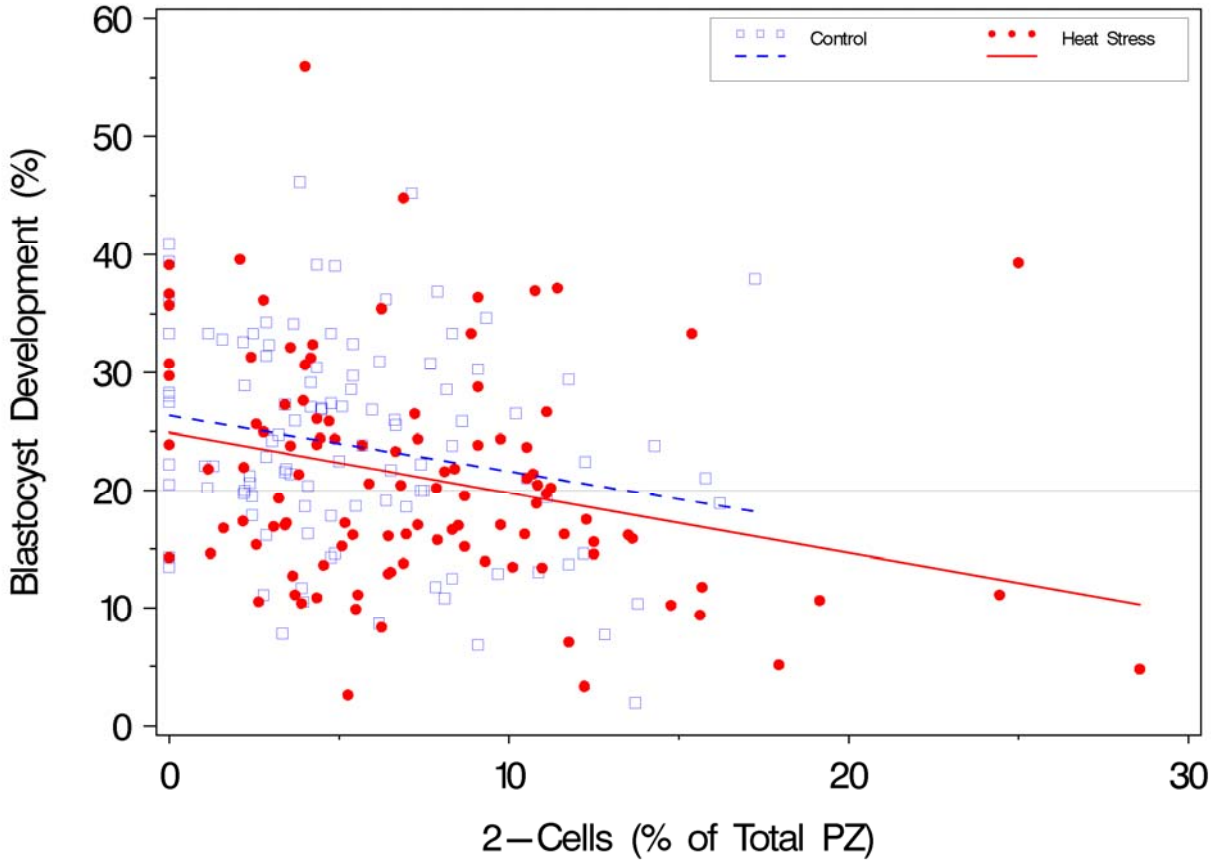

Figure 31. Influence of 2-cells of total PZ on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=26.34-0.47X (P=0.0290) and Y=24.92-0.51X (0.0029) for control and heat stress, respectively.

Eight to Sixteen-Cells

The proportion of cleaved embryos that reached the eight-cell stage ranged from 28.57 to 100%. Utilizing a dummy regression model on the combined data, a significant effect of eight-cell embryos of cleaved was noted (Table 3; $P<0.0001$). As the percentage of eight-cell embryos of cleaved increased, there was an associated increase in blastocyst development of 1.72 ± 0.41% for each additional 10% eight to sixteen-cells. This variable explained 7.4% of the variation in blastocyst development (Figure 32). The presence of less than 60.8% eight to sixteen-cells was predicted to average less than 20% blastocyst development ($Y=8.09+0.27X$).

The proportion of total PZ cultured that reached eight to sixteen-cell embryos ranged from 15.79 to 86.11%. Utilizing a dummy regression model on the combined data, a significant effect of eight to sixteen-cell embryos of total PZ was noted (Table 3; $P<0.0001$). As percentage of eight to sixteen-cell embryos increased, there was an associated increase in blastocyst development of 2.70 ± 0.36% for each 10% increase in eight to sixteen cells. This variable explained 20.4% of the variation in blastocyst development (Figure 33). The presence of less than 44.1% eight to sixteen-cells at cleavage assessment was predicted to average less than 20% blastocyst development ($Y=8.09+0.27X$).

When cleavage assessment was performed three days after fertilization, the proportion of PZ developing to the blastocyst stage was significantly and positively influenced by higher cleavage rates, increased eight to sixteen-cells, and decreased two-cell embryos. This indicates that a high proportion of cleaved embryos and eight to

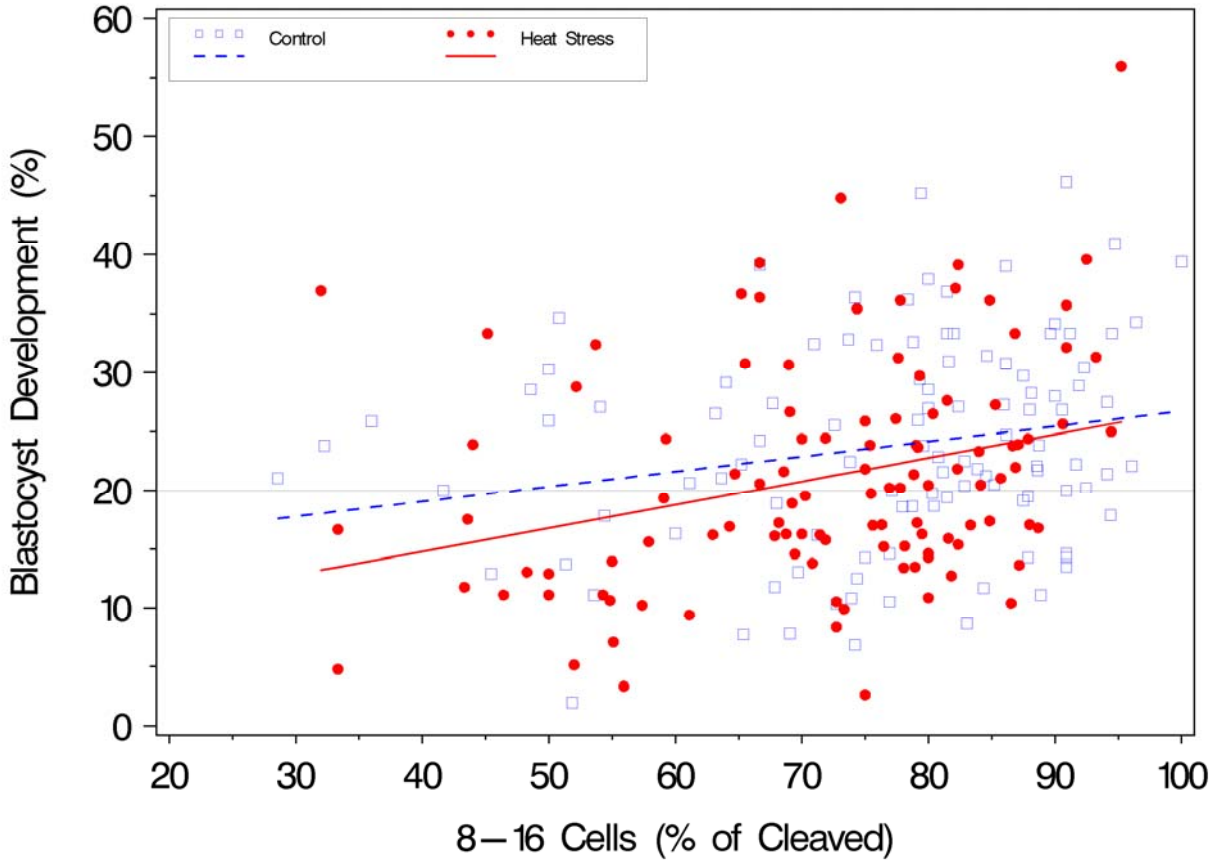

Figure 32. Influence of percentage of 8 to 16-cells of cleaved PZ on blastocyst development for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=13.95+0.12X (P=0.0256) and Y=6.84+0.20X (P=0.0018) for control and heat stress, respectively.

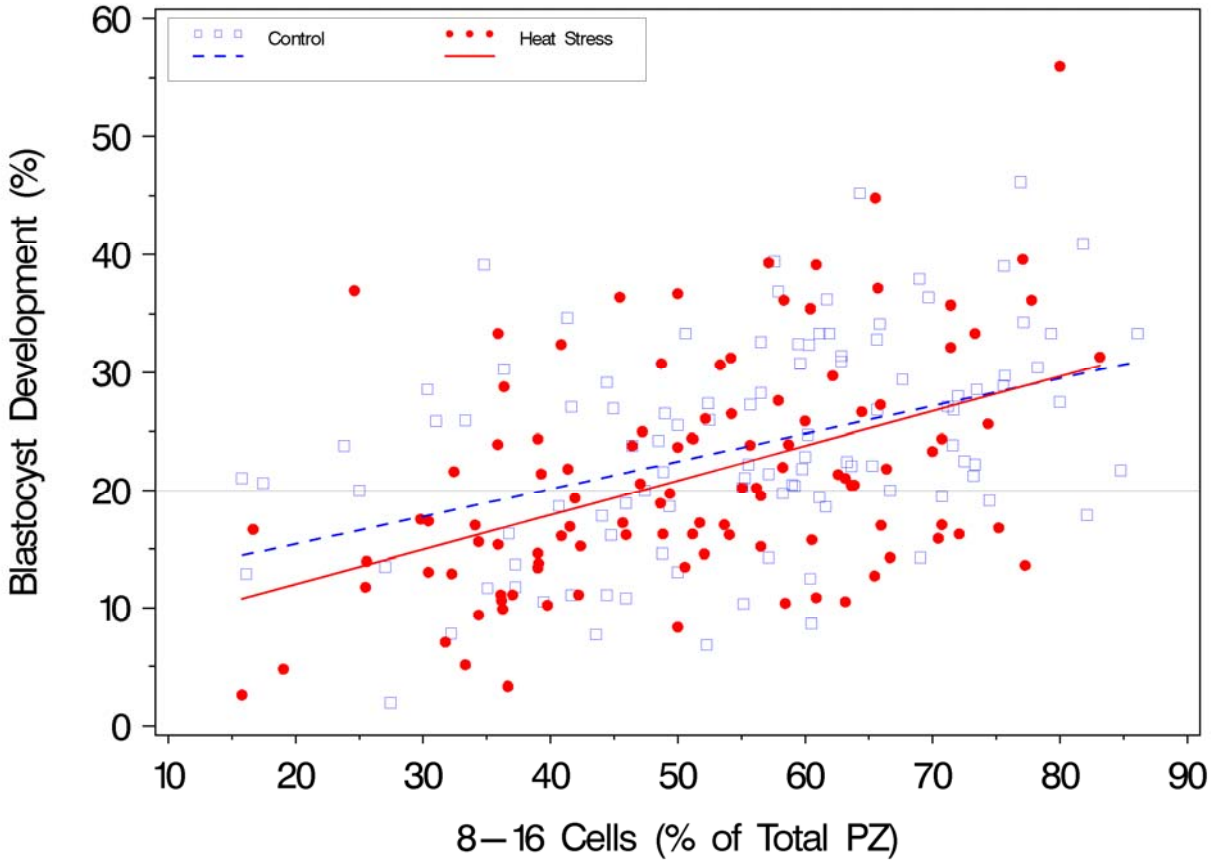

Figure 33. Influence of 8 to 16-cells of total PZ on blastocyst for ova matured for 24 hours at 38.5°C (Control) or 41.0°C during the first 12 hours (Heat Stress). Linear equations were Y=10.74+0.23X (P<0.0001) and Y=6.13+0.29X (P<0.0001) for control and heat stress, respectively.

sixteen-cell embryos are indicative of developmental competence. In contrast, a high proportion of two-cell embryos may depict developmentally challenged oocytes and retarded development when noted three days after fertilization. This is not surprising since an increased number of eight-cell human embryos was related to increased blastocyst formation (Ebner et al., 2003, Jones et al., 1998, Racowsky et al., 2000). Moreover, human 4-cell embryos yielded 0.024 blastocyst/embryo and 8-cell embryos yielded 0.751 blastocyst/embryo when cleavage was assessed at 72 hours post fertilization on a retrospective analysis (Shapiro et al., 2000).

When two-cell embryos were considered, the variation in the data were better explained by the proportion of 2-cells of cleaved than of total PZ. Additionally, variations in the data were better explained by the proportion of 8 to 16-cells of total PZ than the percentage of 8 to 16 cells of cleaved.

Use of Model Selection to Determine IVP Explanatory Variable Combinations Most Influential on Blastocyst Development

The analysis of single technical factors related to each IVP step only explained from less than 0.1% to 9.2% of the variation in blastocyst development (Table 3). Thus, additional efforts were made to find more complex models to explain more of the variation in blastocyst development. To achieve this end, a backward model selection was performed to identify the combinations that most successfully explained the variation in blastocyst development for ova matured at 38.5, 41.0°C or in combination (Table 4). This approach also allowed for evaluating the effect of the selected variables

Table 4. Model Selection of Different Technical Factor Combinations That were Most Influential on Blastocyst Development

In Vitro Maturation Temperature	R^2	Model P-Value	Explanatory Variables Included in Model	*Variable P-Value*[Ŧ]
38.5°C	0.251	**0.0013**	Collection Month	0.2472
			Total Sliced Ovaries	**0.0041**
			Ova Age at IVF	**0.0328**
41.0°C	0.302	**0.0047**	Collection Month	0.2732
			Technician	**0.0041**
			Ova No. per Well	**0.0008**
			Sperm Preparation Time	0.2069
			Ova Age at IVF from Removal from Follicles	**0.0021**
Combined (38.5 + 41.0°C)	0.175	***<.0001***	Technician	**0.0021**
			Ova No. per Well	**0.0175**
			Ova Age at IVF from Removal from Follicles	**<.0001**
			PZ No. per 500 µl KSOM	**0.0002**

[Ŧ]P-value of each technical factor given the other factors included in the model

on blastocyst development given other variables in the selected model. For each in vitro maturation temperature model, R^2, P-value, explanatory variables, and P-values for each variable included in the model were reported (Table 4). Results for each model are reported in more detail later in this section.

Ova Matured at 38.5°C

When collection month, total sliced ovaries and ova age at addition of sperm were included in a generalized linear model, 25.1% of the variation in blastocyst development was explained (Table 4; P=0.0013). Specifically, this model predicted that, for a given month of the year, blastocyst development decreases as the number of ovaries sliced and/or ova age at addition of sperm increases. Addition of the bull ID to the model increased the R^2 to 29.5% (P=0.0302).

Of the factors that were unique to this model, total number of sliced ovaries was most significant, highlighting the importance of minimizing collection size to maximize blastocyst development. The addition of bull ID increased both the P-value and only slightly the R^2 of the model, suggesting that bull ID effect is not as important as the other variables included in explaining variation in blastocyst development of ova matured at 38.5°C.

Ova Matured at 41.0°C during First 12 hours of Maturation

A generalized linear model including collection month, technician, cumulus-oocyte complexes per OMM well, sperm preparation time and ova age from slicing at addition of sperm explained 30.2% of the variation in blastocyst development (Table 4; P=0.0047). Specifically, this model indicated that for a given month of the year, technician and sperm preparation time, blastocyst development was predicted to decrease as the number of cumulus-oocyte complexes per well and/or ova age from slicing at addition of sperm increased. Addition of bull ID to the model increased the R^2 to 39.1% (P=0.0117).

The factors that differentiate this model from the model of ova matured at 38.5°C were technician, oocyte-cumulus complexes per OMM well, ova age at removal from follicles and sperm preparation time. This suggested that blastocyst development of ova matured at 41.0°C was more dependent on processing times, highlighting the importance of good technique by the researcher. The increased susceptibility of heat-stressed oocytes to aging is in agreement with previous results indicating that ova matured at 41.0°C may be aged at the time of fertilization compared to controls (Schrock et al., 2007). The addition of bull ID increased both the P-value and the R^2 (by an 8.9%) of the model, suggesting that for heat-stressed ova, the sperm source is more important to explain blastocyst development than for controls.

Combined Data Analysis (38.5 + 41°C)

When technician, cumulus-oocyte complexes per OMM well, ova age from slicing to addition of sperm and PZ per KSOM well were included in a generalized linear model, 17.5% of the variation in blastocyst development was explained (Table 4; $P<0.0001$). Specifically, this model predicted that for a given technician, blastocyst development should decrease as the number of cumulus-oocyte complexes per well and/or ova age from slicing to addition of sperm increase, while blastocyst development was predicted to increase as the PZ number per KSOM well increased. Addition of bull ID to the model increased the R^2 to 26.6% ($P<0.0001$).

Unexpectedly, the factor that differentiates this model from the other two is the presence of PZ number per KSOM well. While it is unclear why this variable was not selected in the previous models, PZ number per well is important because of embryotrophic factors released to enhance development (discussed in PZ number per KSOM well section). Since all three models contained a variable related to the ova age, this may be the most important variable to consider for improving blastocyst development of ova matured at 38.5 and 41.0°C. The addition of bull ID increased the R^2 by 9.1% without increasing the P-value, reflecting the importance of the bull effect on an IVP protocol.

Limitations of Model Selection Approach

None of the models explained more than 39.1% of the variation in blastocyst development. However, if the variables within those models could be controlled,

variability could be minimized. Reduced variation would result in more consistent blastocyst development, requiring fewer experimental replicates to demonstrate treatment differences. On the other hand, the fact that the models were explaining less than 40% of the total variation in blastocyst development may be a result of not including the impact of biological and environmental factors on the model. Factors that may increase our level of explanation, because they were able to also explain changes in blastocyst development in previous reports, may include genotypic differences (Rocha et al., 1998), age of the animal (Revel et al., 1995; Armstrong, 2001), body condition (Ruiz et al., 1996; Snijders et al., 2000), stage of the estrous cycle and reproductive status (Boediono et al., 1995). Unfortunately, information about those factors was not available.

Chapter V: Summary and Conclusions

The present study revealed that a variety of technical factors may affect embryo development, resulting in variation of both blastocyst rates and responsiveness of oocytes to heat stress. Optimization of the reported technical sources of variation shown to alter blastocyst development may allow for more efficient in vitro embryo production.

The month of the collection was the only factor that impacted responsiveness of ova to heat stress. Combination of variables showed that percentage of blastocyst development from ova matured at 38.5°C is particularly susceptible to the size of collection. In contrast, percentage of blastocyst development from ova matured at 41.0°C is especially susceptible to the age of ova at fertilization. Several single technical factors explained from 0% to 10% of the overall variability in blastocyst development. For ova matured at 38.5 and 41.0°C, bull ID and concentration of sperm added to ova were the two variables that individually explained the greatest amount of variation in blastocyst development. These were followed by the number of ova per OMM well, ova age at IVF, technician and variables related to the collection size such as the total number of sliced ovaries and oocyte collection time. Additionally, variables such as day of the week, total ovaries collected, ovary age at laboratory arrival, total search and evaluation time, total ova in OMM per ovary and total collection time were related to blastocyst development but explained less than 3.5% of the variation. Single variables that explain up to 10% of blastocyst variance may not be sufficient to optimize an IVP protocol by reducing overall variability. However, when combined, they may significantly

reduce variability in blastocyst rates among replicates, which is directly related to IVP optimization. Since variability explained by one factor may overlap with that explained by another factor, they are not truly additive. Thus, combination of variables using a model selection was the preferred method, because this approach adjusts and selects variables to minimize confounded factors. Utilization of this approach produced models that explained 17.9 to 39.1% of the variability in blastocyst results. While these values are still relatively low, they can make a pronounced difference in an experimental outcome. The models obtained through the selection process may reveal which variables are most important for IVP and whose modification would most improve blastocyst production.

These results will allow us to optimize production of blastocysts and decrease the amount of variation by modifying the protocol. Specifically, experiments examining effect of heat stress should be performed in December through March to maximize effect of heat stress to reduce blastocyst development. Since analysis of single variables allowed for predicting maximum percentage of blastocyst development, ovaries should arrive at the laboratory with 7.25 hours of removal from animals and no more than 120 ovaries should be sliced per collection. Changes and points to consider would include the increase of the number of human resources available during oocyte collections to minimize oocyte processing time, especially on search and evaluation time that showed to be the most susceptible processing interval that impacts blastocyst development and can be controlled. A potential indicator for successful blastocyst development from an oocyte collection is the presence of more than 5.1 and less than

14.7 oocytes per ovary. The selection of the bull is a key factor to achieve an efficient IVP system. Therefore, sperm of unknown fertility should be tested before any project to determine blastocyst rates. Additionally, during in vitro fertilization, the use of microcentrifugations should be avoided and centrifugation preferred under the depicted protocol conditions. Further recommendations would include the use of 1:6,000 oocyte:motile sperm ratio to fertilize, and addition of less than 4.2 µl of sperm sample to the ova should be avoided to maximize blastocyst development. Because of the strong effect on blastocyst development, adding sperm no later than 24.5 hours after the onset of oocyte maturation is a must. Response surface results indicated that 22 ova and 33 PZ should be cultured per well to maximize blastocyst developmental rates. The best predictor of blastocyst development at cleavage assessment was the proportion of 8 to 16-cell of total PZ cultured, explaining 20.3% of the variation in blastocyst development. Since in vitro embryo production is an expensive technique (approximately $1250 per replicate), single and combined factors analysis results predicting maximum percentage of blastocyst development may allow for increased efficiency and fewer replicates per experiment to reduce costs and increase laboratory productivity.

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Vita

Estanislao Peixoto was born in Buenos Aires, Argentina on April 5, 1980. He graduated from “Nuestra Señora de Guadalupe” High School in 1997. Peixoto earned his Bachelor of Science degree in Biology with an emphasis in Molecular Biology from the University of Buenos Aires in 2005. Estanislao began graduate school in fall 2007 at the University of Tennessee, Knoxville under the direction of Dr. Edwards. In summer semester 2010, Estanislao received a Master of Science degree in Animal Science.