ASSESSMENT OF EMBRYO VIABILTIY BASED ON MORPHOKINETICS IN CATTLE AND HORSES

by

Sydney Louise Hickerson

B.S., West Texas A&M University, 2019

A Thesis Submitted in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

Major Subject: Animal Science

West Texas A&M University

Canyon, Texas

August 2022

ABSTRACT

In both bovine and equine reproduction, fertility is a lowly heritable trait that has an exceedingly large financial impact and may be a limiting factor on the ability to grow genetic lines. In order to increase pregnancy rates using Assisted Reproductive Techniques (ART) such as Embryo Transfer (ET), in vitro fertilization (IVF), and cryopreservation, producers must have access to accurate decision-making tools. Traditionally, the embryo grading system accepted by industry standards is based on morphological evaluation, and is a method that has remained relatively unchanged for over four decades that uses subjective evaluation that varies with exposure of technicians to training and experience. Inconsistency in grading among embryologists can ultimately lead to decreased conception rates through increased potential in discarding viable embryos.

Seventeen In Vivo derived (IVD) morula stage fertilized equine embryos, 5 cloned equine embryos, 19 IVD blastocyst stage fertilized equine embryos, and 73 IVD, fertilized bovine embryos were collected from donor animals to be evaluated before transfer into recipient mares and cows. Embryos were graded according to the International Embryo Transfer Society (IETS) manual guidelines (IETS, 2009), that is the accepted industry standard of grading. Of the embryos graded, those that were given a quality score of Grade 1 or 2 were chosen for morphokinetic evaluation. The objective of this study was to discern whether IETS quality Grade 1 or 2 embryos could be measured based on their morphokinetic activity to determine if this activity was indicative of embryo viability, enforce embryos that would establish pregnancies, and that would fail to establish pregnancies Both equine and bovine embryos were filmed within 3 h of

transfer with the complete embryo in view of the camera. Videos were then amplified using video motion magnification (VMM) resulting in 300x amplification of the videos and revealed previously hidden components of these embryos.. Embryo morphokinetic activity became humanly perceptible and measurable by changes in morphological dimensions following the VMM step. Data measurements were taken on the embryo inner cellular mass area (ICM), complete embryo and ICM area, and on the vertical, diagonal, and horizontal axis of the sub-zonal (perivitelline) space, zona pellucida (ZP), and trophectoderm (TE).

Of the 72 bovine embryos transferred, as Grade 1 and 2, 19% failed to form a pregnancy as affected by morphokinetic activity. Of the 41 equine embryos transferred, 37% of the Grade 1 and 2 embryos failed to form a pregnancy. Morula stage equine embryos presented differences on the overall axis ($P \leq 0.01$), ZP thickness ($P < 0.05$), and perivitelline space shift ($P < 0.01$), as well as tendencies when measuring the area of the ICM ($P < 0.09$). Once bovine embryos were analyzed and evaluated as range of overall morphokinetic activity per section measured, the effect of pregnancy due to that activity was most significant when measuring the complete embryo area ($P < 0.03$) and embryo complete change in diameter along the X-axis and Y-axis ($P < 0.02$) with average changes of 1242.66μm^2 and 8.41μm, respectively, in embryos resulting in pregnancies. Both species demonstrate and exemplify that there is more to embryo grading than just morphology based on the observed effect on pregnancy status and morphokinetic values. By evaluating all of these factors, there are a wider range of parameters to rely on for the selection of viable embryos outside of the morphology.

ACKNOWLEDGEMENTS

Thank you from the bottom of my heart to all those that have been part of my journey to making the thought of getting my graduates degree into an actualization.

Thank you to Dr. Cara Wells of EmGenisys, Inc., and to Dr. Russell Killingsworth of EmGenisys, Inc., for allowing me to use your equipment, to be part of your research team for this project, for answering my numerous questions no matter the time, for the milkshakes and sandwiches at an Old Route 66 Diner, and finally for becoming my friends through this research.

Thank you to Dr. Gregg Veneklasen of Timber Creek Equine Hospital for pushing me to never settle for basic, for encouraging my passion for reproductive sciences, and for being the initial catalyst for a world in equine reproduction, cloning, agriculture, and and embryology that I didn't know I needed. Your enthusiasm for the work you do is contagious, and I love being part of that work.

Thank you to the Team at Timber Creek Equine Hospital for all the help with my research, as well as for the laughs and good times along the way. Thank you to Eryah Mora for all the times and talks spent in a newborn foal's stall, and to soon to be Dr. Elisa Vornkahl for introducing me to Dr. Veneklasen, and for being the most incredible support system, despite the miles apart.

Thank you, to my advisor Dr. Perkins and my thesis committee. Dr. Perkins: thank you for being willing to take on a horse girl into a realm of cow calf, and for encouraging me to take on a world I was initially intimidated by. I am grateful for the

chats in your office, whether they were about me panicking over deadlines or about horses we thought were cool, you always made me feel important and heard and for that I am so grateful. Thank you as well for the confidence in allowing me to have greater freedom in setting my schedules, getting work done, and allowing me to choose when the time was to step up to the plate. Finally, thank you for all of your endless patience with me as I figured all of these things out. I have discovered a lot about myself through this process with your help, and I am so grateful for every moment of this journey. Dr. Pipkin: you always knew the right questions to ask and your attention to detail and how I was doing outside of school was always appreciated. You asked the hard questions that made me reflect on myself and my endeavors, encouraged me to go above and beyond in all that I do, and you always brought enough banana bread around to put a smile on anyone's face. To you, I am grateful. To the final member of my committee (last, but definitely not least!), thank you to Dr. Baker: thank you for always offering a good laugh as well as good advice, for always being excited to see me and chat no matter the day you are having, for encouraging me to always be confident in my knowledge, and for assuring me that I know more than I think I do… I just need to breathe… and to then simply work the issue out. I am lucky to have you in my corner.

To the Perkins' Posse:

Thank you to all of the Posse for making my time at WTAMU special. You have all been so encouraging and kind, and I could not imagine a better lab group to complete my graduate program with. Noah: thank you for answering all of my random (and sometimes frantic) phone calls about stats I did not understand, and for doing everything with a smile. Thank you for being so light hearted and kind when at times that's what we

all needed. Katy Jo: There are not enough words to describe the gratitude I feel towards you. You have worn a number of hats through this graduate school process, whether acting as the schedule keeper reminding me of important dates and events that always seemed to slip my mind, the listener of all my woes and tribulations, the tutor for the (many) times I was lost, and most importantly, the role of a friend I did not realize how much I needed through this whole process. You have the kindest heart and so many wonderful accomplishments headed your way, and our friendship is one I know will last and one I will always cherish.

Thank you to the entire WT Ag Department and the graduate students that were a part of this shared journey. I am grateful for all of you and our time together, whether we began our journey in 2014 when I first arrived at WTAMU as a gangly soccer girl who was still finding her way, or whether our paths have just crossed, I will treasure all of the memories spent together. WTAMU and the WT Ag Department is a place I have been more than proud to call my home.

Finally, thank you to my fiancé, Tanner Pena, your unending encouragement and belief in me means more than I am capable of describing. You always push me to pursue my dreams and never let me take the easy route, all while holding my hand every step of the way. I am so grateful for you and can't wait for all the times ahead as your wife.

There are so many more I could thank, from my whole family one by one, to each person that has helped me or shown me kindness in the Ag Department, but as a whole, I would once again like to say from the bottom of my heart… thank you all for everything.

DEDICATION

I would like to dedicate this graduate's thesis to my Mom, Dad, and Pop. Mom and Dad, thank you for raising me to love and appreciate all creatures of this world, from sea creatures swirling around busy reefs, to fat cattle grazing in green pastures. Thank you for teaching me to live fearlessly and independently so that I may experience all life has to offer. Thank you for pushing me to be competitive in all that I do, whether in athletics, academia, or as a functioning member of society that puts my best foot forward every day. Mom: thank you for being one of the strongest and bravest women I know, a role model, a dreamer, and my number one fan. Dad: thank you showing me what exceptional character is made of, for letting me know I am never alone in anything that I do, and in the words of Baxter Black, to "like being a person to whom a horse matters". Finally, thank you to my Pop, who despite living an ocean away in Australia, has always encouraged curiosity in the scientific world, the natural world, and the world outside our back door. I dedicate this thesis to all of you, and would not be the person I am today without your love and guidance. Thank you.

APPROVAL

Approved:

Dr. Tommy Perkins, Thesis Committee Chair	Date
Dr. Lance Baker, Thesis Committee	Date
Dr. John Pipkin, Thesis Committee	Date
Dr. Lance Kieth, Department Head of Agricultural Sciences	Date
Dr. Kevin Pond, Dean of the Paul Engler College of Agriculture and Natural Sciences	Date
Dr. Angela Spaulding, Dean of Graduate School	Date

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE PAGE

LIST OF TABLES

TABLE PAGE

Chapter 4

I.

INTRODUCTION

In present society and the realm of reproduction, continuing to advance and promote Assisted Reproductive Techniques (ART) standards remains a top priority in order to compete on an economic as well as industry wide level. Selection of top quality progeny to fulfill the requirements for an ever growing world is key to success of producers. All of these top tier genetics begin in one tiny life form: the embryo. Traditionally based on visual morphological assessment according to reproductive industry standards, the current method of embryo evaluation is widely accepted. Nevertheless, this subjective evaluation has remained relatively unchanged for over four decades.

Evaluation of embryos can vary depending on exposure of technicians to training and experience, and the inconsistency in grading among embryologists can lead to losses from decreased conception rates through increased potential in discarding viable embryos. The subjectivity of the industry accepted manual of the IETS (2009) grading method led to the objective of this research. By providing a higher knowledge of embryo quality based not only on morphology, but the morphokinetics of morula and blastocyst stage embryos in cattle and horses, IETS quality Grade 1 or 2 embryos could be measured based on their morphokinetic activity to determine embryo viability to distinguish. These embryos would be evaluated according to those that establish pregnancies, and that would fail to establish pregnancies. As a whole, morphokinetics ("morpho," form/shape and "kinetics," movement) refers to time specific morphological changes during embryo development, proving dynamic information outside of pure

morphologic assessment. The purpose of this evaluation of activity was to discern whether a "good" embryo by industry standards was truly worth transferring or freezing for future use. More selective, non-invasive, and non-subjective methods of embryo selection through Embryo Transfer (ET) could be maximized, made more consistent, and profitable than might be achieved by natural fertilization. From this research, the aim was to prove that embryo morphokinetics (within certain boundaries) influence development of a viable pregnancy. More diagnostic technology of embryo assessment could potentially make an already growing market for embryo transfer technology more efficient and reliable over time.

II.

REVIEW OF THE LITERATURE

2.1. Embryonic Development

If a species is to survive, a mechanism must exist for successive production of new generations of that species (Hamilton et al., 1956). In order to maximize the attainment of new generations, marked stages of growth in embryonic development prove to be essential to the advancement of reproduction. Most losses of pregnancy occur during the peri-implantation stage of pregnancy, in the first 50 d (McCue and Squires, 2015) and is likely due to deficiencies in oocyte quality and early embryo development (Lonergan et al., 2019). These progressive changes from maturation, fertilization, cellular division, cleavage, implantation and more are all essential for the development of a healthy and viable fetus.

2.1.1. Oogenesis and Maturation

The production of ova, known as oogenesis, by the ovaries is a cyclic process, characterized by the formation, growth, and maturation of the ovum in an ovarian follicle (Hamilton et al., 1956). At human birth, each of the embryonic ovaries is initially populated by about three million oocytes. Through apoptosis, or cell death, this number reduces to about one million oocytes and only an exceedingly small fraction of these viable oocytes will develop fully into mature egg cells (Hadley and Levine, 2007). According to Hadley and Levine (2007), 99.9% of these oocytes are destined for atresia throughout the years of ovarian activity. In cattle, germ cells begin to arrive at the female gonad at approximately 35 d of gestation while in horses and humans, the arrival begins

at 22 to 28 d, respectively (Pepling, 2013). Beginning at 75 to 82 d in cattle, and 60 d of gestation in horses, germ cells begin meiosis, a gradual and prolonged process as the germ cells divide to form germ cell cysts. As these cysts cumulate and grow they become surrounded by somatic cells to form ovigerous cords. As these germ cells enter meiosis, they are referred to as oocytes. Oocytes in germ cell clusters eventually separate, breakdown, and become enclosed in primordial follicles consisting of one oocyte and several somatic granulosa cells that exhibit a flattened shape and remain dormant for varying amounts of time until activated to grow (Pepling, 2013). Following this process, cells die by programmed cell death, with only a third of the total cells surviving. The programmed cell death is repeated until a few individual oocytes remain.

At approximately 90 to 102 d following gestation in cattle and horses, respectively, follicles begin to form and the oocyte and associated granulosa cells change morphologically from flattened to cuboidal, indicating follicular activation into primary follicles (Pepling, 2013). As the follicle grows, the oocyte will also grow, increasing in size as the granulosa cells of the primary follicles divide into multiple cell layers, forming secondary follicles as they multiply and surround the oocyte. Fibroblast-like cells known as theca cells and granulosa cells support the oocytes while also producing hormones. The preantral, or secondary follicle gains a fluid-filled space and are then classified as antral follicles. Not all follicles survive past the secondary follicle stage, however the follicles that do survive are classified as preovulatory follicles and will proceed through meiosis into meiotic rest until fertilization (Pepling, 2013).

Follicular activation and development can be divided into the initial recruitment phase, meaning a continuous process referring to the activation of groups of primordial

follicles, and the cyclic recruitment phase, referring to the selection of only a few follicles to reach the preovulatory stage, according to McGee and Hsueh (2000). Although primordial follicles form at 90 d in cattle and 102 d in horses, a delay is observed until the first appearance of primary follicles. Most primordial follicles remain in an arrested state if they are not selected for further differentiation and development (Hadley and Levine, 2007). Antral follicles are recruited to grow by a small increase in follicle stimulating hormone (FSH) levels and after several days, a dominant follicle will become larger than the other subordinate follicles to sustain its gametogenic potential, until the subordinate follicles eventually become atretic (Pepling, 2013).

The follicle that eventually matures and ovulates is thought to be the one whose granulosa cells most rapidly acquire high levels of aromatase and luteinizing hormone (LH) receptor in response to an FSH rise (Hadley and Levine, 2007). This dominant follicle will then synthesize estradiol, decreasing FSH levels and signaling for dominant follicle selection. A surge of LH generates the resumption of meiosis I, mitochondrial localization, and ovulation (Pepling, 2013). Depending on the species, usually only a few, or even just one of these dominant follicles will reach maturity, culminating in ovulation of a fertilizable oocyte (Hadley and Levine, 2007). According to Hadley and Levine (2007), the purposes of the ovarian follicle are to (1) preserve the resident oocyte; (2) mature the oocyte at the optimal time; (3) produce a hormonal milieu that will develop a lush proliferative endometrium; (4) yield well-timed release of the oocyte; (5) provide for high-quality corpus luteum (CL) function, leading to implantation; and (6) preserve the hormonal conditions required for gestation until the fetoplacental metabolism is adequate.

2.1.2. Fertilization

In order to form a viable zygote, fertilization of the ovum is necessary and selection of fertilization method varies between producers. In cases of natural service, semen is deposited in the anterior vagina whereas with Artificial Insemination (AI), deposition of semen into the uterus or in the anterior cervix results in higher conception rates. Spontaneous ovulators can be divided into two major groups; (1) animals with nonfunctional corpora lutea and short (4 to 5 d) ovarian cycles; and (2) animals with functioning corpora lutea and long estrous cycles (e.g. cattle, horses).

In cattle, ovulation occurs 12 to 16 h after the end of 13 to 14 h of estrus. In horses, ovulation occurs 1 to 2 d before the end of estrus (Hadley and Levine, 2007). Post ovulation in both species, the oocyte released travels down the infundibulum to the site of fertilization, the oviduct. Fertilization of the oocyte normally occurs in the ampulla section of the oviduct close to the junction with the isthmus, and sequentially enters the uterus 4 to 5 d after ovulation (Ball and Peters, 2004). Upon entering the female reproductive tract, the process of capacitation is stimulated to modify the structural and enzymatic properties of the acrosome and anterior part of the sperm head membrane. This acrosome reaction following capacitation is necessary to allow penetration of the ovum by the sperm, and involves the fusion of the sperm cell membrane, acrosome, and formation of gaps that the acrosome contents can diffuse. The fusion of the sperm and ovum cell membranes begins as the sperm head becomes engulfed by the ova with the loss of the tail. Penetration of the fertilizing sperm therefore stimulates the resumption of the second meiotic division of the oocyte (Ball and Peters, 2004). Fertilization is

completed with the fusion of the haploid male and female pronuclei through a process termed syngamy.

2.1.3. Embryonic Divisions

Gestation is often divided into three stages: (1) the ovum from 0 to 13 d post ovulation, (2) the embryo from 14 d when germ layers begin to form until (3) at 45 d the fetus develops (Ball and Peters, 2004). Through a process known as cleavage, the ovum divides mitotically once fertilization is complete. Division continues from 1-cell, 2-cell, 4-cell, 8-cell, and 16-cell at 1, 2, 3, 4, and 5 d, respectively, until a cluster of blastomeres collectively known as a morula are formed by 5 or 6 d. Following the development into a morula at about 7 d after fertilization, the morula becomes a blastocyst and consists of a single spherical layer of cells known as the trophoblast, that will develop into the placenta and provide exchange of nutrients, waste, and gas exchange, as well as a group of cells known as the inner cell mass (ICM) that will develop into the embryo proper and ultimately the fetus. At about 8 d, the zona pellucida will begin to fragment, and the blastocyst “hatches”. The expanding hatched blastocyst undergoes a period of blastocyst elongation, and the development of germ layers begins about the fourteenth day and characterizes the beginning of the embryo phase (Ball and Peters, 2004). Three germ layers arise from the ICM and are termed the ectoderm, mesoderm, and endoderm, giving rise to eternal structures such as skin, hair, hooves, mammary glands from the ectoderm; heart muscles, and bones from the mesoderm; other internal organs from the endoderm. By 16 d, the embryo is sufficiently developed to signal its presence to the maternal system and prevent luteolysis (Ball and Peters, 2004; Forde and Lonergan, 2012; McCue

and Squires, 2015). By 45 d, formation of the primitive organs are complete and the fetal phase commences.

2.1.4. Maternal Recognition and Implantation

The uterus is a thick-walled, muscular organ that serves as a site for fetal development and as an endocrine organ during pregnancy (Hadley and Levine, 2007) and is the site of implantation of a fertilized egg and fetal development, and therefore the site of connection for all exchange in the form of waste, gas exchange, and nutrients between the conceptus and dam. Maternal recognition of pregnancy refers to a sequence of events that embryo-derived signals prolong luteal function, ultimately assuring ongoing progesterone secretion by the CL beyond its normal lifespan of the estrous cycle until approximately 100 d of gestation when the developing placenta assumes the role of secreting progestogens.

Maternal recognition of pregnancy in cattle occurs approximately 16 d post ovulation (Forde and Lonergan, 2012). The implantation process is characterized by elongation of a hatched blastocyst that has broken out of the zona pellucida followed by apposition, attachment, and adhesion of the trophectoderm to the uterine luminal epithelium (LE). In cattle, if an oocyte is successfully fertilized in the oviduct of the cow, the resulting embryo enters the uterus on approximately 4 d of pregnancy (Forde and Lonergan, 2012). Changes in endometrial secretions, such as progesterone receptors, are critical to the likelihood of a pregnancy to survive. In order for maternal recognition to occur in cattle, the conceptus trophectoderm, that differentiates eventually into the placenta, must secrete adequate levels of Interferon Tau (IFNT) in order to inhibit production of luteolytic prostaglandin F2α by the endometrium.

Maternal recognition of equine conceptus occurs between 12 to 16 d (McCue and Squires, 2015) following the migration of the embryo to the uterus between 5 and 6 d post ovulation. This migration of the equine embryo is facilitated by production of prostaglandin E_2 by the conceptus in order to successfully transport passage through the uterotubal junction (Klein, 2016). The mobile phase of the embryo is one of the unique occurrences of equine pregnancy, as this migration of the embryo through the uterine segments is essential for maternal recognition of pregnancy as premature regression of the CL is inhibited by embryonic vesicle mobility through those segments (McDowell et al., 1988; Meira et al., 2012). Following the cessation of embryonic mobility, implantation of the conceptus transpires in the caudal portion of one of the uterine horns between 15 and 17 d post ovulation (Ginther, 1998). The second unique factor of the equine embryo is the formation of an acellular "capsule" that replaces the zona pellucida as the embryo hatches. The capsule becomes first noticeable 6.5 d after ovulation between the trophectoderm and the zona pellucida (Klein, 2016) coinciding with the entry of the developing embryo into the uterus. As the embryo grows, the zona pellucida ZP) thins and is replaced by the capsule as the sole covering of the embryo and is therefore essential to survival of the embryo.

2.1.5. Early Embryonic Mortality

In both equine and bovine pregnancies, the highest rate of pregnancy loss is in the early stages of pregnancy. In cattle, the majority of pregnancy loss can be attributed to early embryonic loss, that occurs prior to maternal recognition of pregnancy 16 d post ovulation (Day 0; Forde and Lonergan, 2012). In mares if this loss is to occur, oocytes and low quality embryos remain trapped in the oviduct for several months where they

degenerate (Betteridge, 2000). The pre-implantation period of pregnancy is when most embryonic mortality occurs (Forde and Lonergan, 2012).

2.2. Embryo Transfer

The scientific and technological advances during the past decades in animal reproduction have resulted in the development of a variety of tools commonly referred to as ART. These techniques manipulate reproductive-related events such as fertilization, ovulation, and embryo development with the final goal of producing heathy offspring (Velasquez, 2008). One of these technologies, Embryo Transfer (ET), refers to the removal of an embryo from the reproductive tract (i.e. the uterus) of one donor animal and the transfer of that embryo into the reproductive tract of a recipient animal (McCue and Squires, 2015). Many advantages are offered with these technologies, the main advantage resulting in a higher number of embryos as well as pregnancies per unit of time (Ferré et al., 2020). Utilization of ET allows for greater control over the genetic makeup of a herd as well as improved pregnancy rates (ASAS, 2020). The number of reproductive centers, breeding farms, and veterinary clinics specializing in embryo transfer has increased due to advances in collection procedures, transfer techniques, equipment, flush media, embryo shipment and cryopreservation improving the enhanced success and clinical use of ET in horses and cattle (McCue and Squires, 2015). According to McCue and Squires (2015), advantages of ET include, but are not restricted to:

- Valuable animals may have more than one progeny per year
- Medical risks associated with pregnancy and parturition are avoided for the donor animal
- Older animals can donate embryos to young recipients

- Animals with repeated history of pregnancy loss can donate embryos to recipients
- Performance animals can donate embryos to remain in performance
- Donors with reproductive problems can donate embryos to reproductively healthy recipients
- Embryos can be cryopreserved and transferred at a later date
- Embryos can be collected from donors that are too young to carry offspring themselves
- Embryo recovery can be used as fertility evaluation of both sire and dam

Successful ET in all species is reliant on proper management of donor and recipient herds, as well as selection of the most viable embryos for transfer.

2.2.1. Brief History of Embryo Transfer

The first successful production of live young by ET was performed in rabbits in 1890 (Heape, 1890), followed by the successful transfers of rat and mouse embryos in the 1930's. Heape transferred two ova from an Angora doe rabbit into the fallopian tube of a Belgian Hare recipient that gave birth to six young comprising of two with Angora phenotypes and four with Belgian hare phenotypes. Heape's procedure demonstrated that early rabbit embryos could be flushed from the oviduct with physiological saline and transferred to a foster (recipient) mother, where they would resume normal embryonic development and be born (Biggers, 1981). The first successful ET of sheep, pig, and cattle embryos were reported in the early 1950's, when surgical transfer of embryos into the uterus of the recipient was the most successful technique used in early large animal transfer. In 1972, mice offspring were born from embryos that had been frozen, thawed, and then transferred successfully. Following the birth of the cryopreserved mice embryos

was the report of the first calf born following transfer of a frozen-thawed embryo. Transfer of embryos over long distances was first accomplished by placing pig and sheep embryos into the oviducts of rabbits used as biological incubators in the early 1970's. In July of 1978, the first human "test tube" baby was born, an incredible scientific achievement for all species. In 2010 a Nobel Prize for Physiology or Medicine was awarded to Robert Edwards for this development (Biggers, 2012). The first calf produced from in vitro fertilization (IVF) was born in 1981, followed by the birth of IVF pigs in 1983 and lambs in 1984. The birth of the lamb "Dolly" occurred as the first animal born following the nuclear transfer, or cloning, occurred in 1986 (McCue and Squires, 2015).

The development of cryopreservation methods, for example cryoprotectants such as ethylene glycol, in bovine embryos made ET a much more efficient technology, no longer depending on the immediate availability of suitable recipient, and with pregnancy rates only slightly less than those achieved with fresh embryos (Mapletoft, 2013).

2.2.2. Current Methods of Embryo Transfer

During non-surgical embryo collection procedure, a sterile catheter with an inflatable cuff is used for the flush/lavage technique. Sterile "Y-Tubing", with clamps to regulate inflow and outflow, is used to connect the uterine catheter to a container of flush media and to an embryo filter. These filters are most commonly equipped with a 75 μm screen to prevent passage of the embryo from the container. The average equine morula stage embryo ranges from 125 to 200 μm, and bovine embryo from 120 to 190 μm (Nogueira et al., 2018) in size. Other varying stages of blastocysts range from 150 to 2,500 μm (McCue and Squires, 2015). Some filters may also be used as embryo search dishes. Flush media commercially prepared may be used as embryo flush medium.

Technique may vary between clinics or technicians; however, the standard method of collection is a nonsurgical, transcervical uterine lavage (McCue and Squires, 2015). Sterile catheters are inserted through the cervix, and into the caudal uterine body. Once inserted, the cuff is inflated with 60 to75 mL of air using a syringe to act as a "lock" against the internal cervical os. Although there are many techniques to successfully remove an embryo, a common technique is to lavage the uterus of the donor animal sequentially with prewarmed (30 to 37°C) or room temperature media. The amount of fluid used for each flush is dependent on the size of the uterus of the donor animal. Once full, the flush media is allowed to regress out of the catheter by gravity flow through the embryo filter. After collection of one or multiple embryos, the producer may choose to either freeze the embryos via cryopreservation for future use, or transfer them directly into approved recipient females. According to Wessels et al. (2017), cryopreservation techniques decrease embryo viability and pregnancy success rates by 15 to 20%, based on the following:

1. Intracellular ice crystal formation causing freeze fracture.
2. Toxicity and osmotic shock from cryoprotectants necessary to cryopreserve embryos.

On average, embryos are recovered from approximately 50 to 65% of collection attempts in mares (McCue and Squires, 2015), versus 80% in superovulated cattle, and utilization of follicle stimulating hormone (FSH) stimulates oocyte production for superovulation to facilitate multiple embryo recovery from one flush. In females with a history of reproductive complications, embryo recovery may average 20 to 30% per cycle (McCue and Squires, 2015). Embryos are flushed at approximately 7 d post-estrus, before

embryos undergo implantation to the wall of the uterus. Factors affecting embryo recovery from ET may result from donor health, age of donor, semen type, embryo collection day, flush technique, number of ET cycles per season, number of ovulations, or clinical expertise.

2.2.3. Bovine Embryo Transfer

In cattle, ET is now a commercial operation in many countries throughout the world. The adoption of Artificial Insemination (AI), or the veterinary procedure of injecting semen into the vagina or uterus of an animal, was the main reason for development of ET. These two techniques together increase genetic progress made possible by ART.

Normal reproductive practices include superovulating cows so that a great number of embryos can be collected at each flush. Superovulation in cattle is induced by administering repeated injections of follicle stimulating hormone (FSH), usually over a 5 d period, to overcome the natural mechanism that would normally only allow one follicle to become dominant and ovulate (Ball and Peters, 2004). Superovulation results in a number of ovulations induced at a predetermined time by the injection of a prostaglandin or an analogue to cause luteolysis, ranging from one ovulation to well over 20 (Ball and Peters, 2004). Following is an injection of a Gonadotropin Releasing Hormone (GnRH) to ensure a well synchronized, pre-ovulatory LH peak, in that AI could be carried out (Ball and Peters, 2004). According to Ball and Peters (2004), the fertilization rate of recovered eggs is around 80% following superovulation. Non-surgical methods of recovery of embryos via the cervix is normally carried out after 6 d of the estrous cycle following ovulation (Ball and Peters, 2004) and before implantation may occur. The

donor cow is restrained in a normal cattle chute, and may be tranquilized or given an epidural anesthetic injection, after that embryos are flushed from the uterus. In cattle, embryos are typically transferred into recipients around 7 d after estrus, when the embryo has reached the blastocyst stage (Hansen, 2020). Occasionally, less developed morula stage embryos are transferred when embryos are particularly valuable or there is a shortage of blastocysts (Hansen, 2020).

2.2.4. Equine Embryo Transfer

Mares usually spontaneously ovulate only one dominant follicle per cycle (McCue and Squires, 2015). Because of this single ovulation, embryo collection attempts are based on one potential embryo per cycle at a rate of approximately 50 to 65% per cycle (McCue and Squires, 2015). Variability of collection attempts depends on inherent fertility of the dam and sire as well as other reproductive factors such as type of semen used, set up of dam estrous cycle, etc. Pregnancy rate following transfer is approximately 70 to 90% (McCue and Squires, 2015), with variability depending on experience of technicians, embryo quality, and recipient factors. The rate of pregnancy loss is similar between ET recipients and mares bred to carry their own pregnancy, with approximately 8 to 10% of pregnancies lost between initial detection and expected due date (McCue and Squires, 2015).

Embryo recovery attempts may be successfully performed 6.5 to 9 d after ovulation (McCue and Squires, 2015). Small embryos collected on 6 to 7 d may be collected for cryopreservation technique. Embryo collection attempts after 9 d often result in larger embryos that require special equipment and may be damaged during handling as well as transfer due to size.

2.2.5. Cloned Embryo Effect on Pregnancy and Parturition

Despite the fact that cloned animals derived from somatic cells have been successfully generated in a variety of mammalian species, there are still many unsolved problems with current cloning technology, such as high rate of abortion during early gestation and increased perinatal death (Han et al., 2003). Somatic cell nuclear transfer is a powerful technique for multiplication of unique animal genotypes for a number of animals ranging from endangered species to livestock. Successful production of cloning from somatic cells has been achieved in a number of species such as sheep, cattle, mice, goats, pics, cats, horses, and rabbits (Han et al., 2003). The first cloned sheep, "Dolly," inherited shortened cell telomeres from her cell donor (Han et al., 2003).

Nuclear transfer involves a series of complex procedures including culture of donor cells, in vitro maturation (IVM) of oocytes, enucleation of oocytes, cell or nucleus injection, fusion, activation, in vitro culture (IVC) of reconstructed embryos, and finally ET (Han et al., 2003). This leaves much room for any of these steps to be suboptimal, resulting in the failure of production in these cloned embryos. According to a study performed by Forseberg et al. (2001), cattle species had an efficiency rate of 20% conception out of 3435 cloned embryos transferred. A serious impediment to the practical use of somatic cell nuclear transfer techniques is low viability of cloned embryos during embryonic development, with only a few percent of reconstructed oocytes developing to term (Wilmut et al., 1997).

2.3. Embryonic Evaluation

By accurately and effectively evaluating all embryo components such as developmental stage, quality, and size, the success of embryo evaluation can provide

valuable information as to the probability of embryo survival after transfer. Morphological evaluation aids in identifying embryos with significant physical abnormalities, differentiation in stage consistent with embryo age, and diagnosis of unfertilized oocytes (UFO).

According to McCue and Squires (2015), normal structures associated with the morula or blastocyst developmental stage embryos include:

- Blastocoele: fluid filled cavity surrounded by a single layer of trophoblast cells of the blastocyst stage embryo.
- Blastomere: one of the cells compromising the early embryo.
- Capsule (unique to equine): acellular glycoprotein envelope produced by trophoblast cells; initially located between the trophectoderm and zona pellucida; becomes the outer protective layer after the zona is shed.
- Inner cell mass: cluster of blastomeres that will differentiate to form the embryo proper and eventually the fetus.
- Perivitelline space: potential space between the blastomeres of a morula-stage embryo or the trophoblast cells of blastocyst stage embryo and zona pellucida.
- Trophoblast (TE): layers of extraembryonic ectodermal tissue that differentiates during formation of the blastocyst stage embryo and surrounds the blastocoele cavity; eventually forms the chorion and amnion.
- Zona pellucida (ZP): noncellular glycoprotein coat surrounding the oocyte and early embryo; eventually thins and is shed as the equine embryo expands.

Embryo evaluation is traditionally performed with a microscope and is essential for traditional embryo evaluation of developmental stage, quality score or grade, and size

(μm). Additional comments may be necessary to record for characteristics of debris in flush or attached to the embryo, as well as abnormalities of the embryo itself.

The developmental stage of an embryo is normally directly related to age or number of days after ovulation (McCue and Squires, 2015). Morula or blastocyst stage equine embryos enter the uterus between 130 to 142 h after ovulation, with transport through the isthmus and uterotubular junction dependent on production of prostaglandin E_2 (PGE_2) from a viable embryo (McCue and Squires, 2015).

In the 1970's, intense commercial activity in ET led to the development of the International Embryo Transfer Society (IETS) in order to share discoveries and progress the development of techniques in superovulation, embryo collection, transfer, and freezing (Betteridge, 2003). The IETS created a standardized embryo collection and processing method as an outline of guidelines for the best contemporary practices in the ET industry, and with these progressions became an international society in 1974. The standardized record keeping of IETS aimed at assuring accuracy and confidence that embryos were identified precisely, and in 1986, the IETS Board of Governors approved this basic system of record keeping and grading. Based on this system, a standardized coding system for use in describing the stage of development and quality of embryos became available. According to the manual of the IETS (2009), the code for stage of development is numeric, ranging from "1" (meaning an unfertilized oocyte or 1-cell zygote) to "9" (expanding hatched blastocyst). According to manual of the IETS (2009), the code for embryo quality would also be numeric, and is based on morphological integrity of the embryo cell mass. The codes identified manual of the IETS (2009) for embryo quality ranging from 1 to 4 are as follows (IETS, 2009):

- Code 1: Excellent or Good: Symmetrical and spherical embryo mass with individual blastomeres (cells) that are uniform in size, color, and density. This embryo is consistent with its expected stage of development. Irregularities should be relatively minor, and at least 85% of the cellular material should be an intact, viable embryonic mass. This judgment should be based on the percentage of embryonic cells represented by the extruded material in the perivitelline space.
- Code 2: Fair: Moderate irregularities in overall shape of the embryonic mass or in size, color, and density of individual cells. At least 50% of the cellular material should be an intact, viable embryonic mass.
- Code 3: Poor: Major irregularities in shape of the embryonic mass or in size, color, and density of individual cells. At least 25% of the cellular material should be an intact, viable embryonic mass.
- Code 4: Dead or degenerating: Degenerating embryos, oocytes, or 1-cell zygotes: nonviable.

As stated by manual of the IETS (2009), this visible evaluation of embryos is a subjective evaluation of a biological system and is not exact science. Factors such as environmental conditions, recipient quality, and technician capability are important factors in conception rates.

2.3.1. Standards for Embryo Grading

Although there has been standardization of morphological assessment of embryo grading developed and implemented by the International Embryo Transfer Society (IETS), few advances have occurred in the last few decades with regard to current embryo transfer procedure (Saravelos and Li, 2019). Success rates of ET are still low

today with few returns on investment. Research from controlled studies report pregnancy rates of 70-80% from ET (Wells and Killingsworth, 2022), but practitioners and producers encounter drastically lower rates of these procedures on farms and ranches, with average success rates using ART as low as 30% in North America (CDC, 2017; Bormann et al., 2020a; Mapletoft et al., 2020). While causes of embryonic failure post ET are multi-factorial, according to Wells and Killingsworth (2022) 20% of pregnancies of transferred embryos are likely non-viable at time of transfer and will never result in pregnancy.

To assess embryos based on simple grading, one grade is assigned that takes in the overall physical appearance of the cleavage-stage embryo. The techniques are highly subjective and often are time consuming. These non-invasive techniques of examining developmental stages include embryo metabolism analysis, cellular respiration measurement, evaluation by time-lapse monitoring (TLM), quality of in vitro growth, integrity of blastomeres membrane, and electron-microscopy analysis (Nogueira et al., 2018). Embryo assessment criteria for grading systems may vary with embryo stage. As well as overall physical appearance, cell symmetry and fragmentation are also taken into account (Racowsky et al., 2010). In order to be more successful in implantation and fetus likelihood, formulas have been put into action to predict pregnancy prospect according to the appearance and development of embryos being evaluated. At different stages, such as the blastocyst stage, grading frequently takes into account the ICM composition and TE, and degree of expansion into the blastocyst cavity (Racowsky et al., 2010).

All in all, embryo quality is traditionally determined by its morphology, and thus the pregnancy rate and therefore success rate is directly dependent on the quality of the

transferred embryos (Rocha et al., 2017b). According to Racowsky et al. (2010), this morphology and therefore embryo quality bears indicative relation to implantation and reproductive potential. By selectively choosing the top-quality embryos chosen by both traditional morphology standards as well as new material presenting how energy of individual embryos correlates to effective pregnancies, the end goal of this research is to reduce the overall number of embryos transferred. Only embryos that prove themselves to check both boxes of morphology and appropriate energy will be chosen. There are three potential growth phases that will be researched; cleavage, morula, and blastocyst; by using the Society for Assisted Reproductive Technology (SART) method, and a 3-point grading scale of "good, fair, and poor," developed in 2006 (Hossain et al., 2016). This may also be portrayed numerically as 1 for good, 2 for fair, and 3 for poor as well as alphabetically with A/a for good, B/b for fair, and C/c for poor (Hossain et al., 2016). The International Embryo Transfer Society (IETS) also has its own particular method of grading embryo quality. According to IETS, embryonic quality is based upon the number and appearance of cells based on the stage of development and actual embryo quality (Nogueira et al., 2018). In an ideal situation, the embryo would form a compact and spherical body with blastomeres of uniform size, color, and texture. The perivitelline space, or the space between the ZP and the membrane of the fertilized ovum, should be clear. The ZP should not be cracked or collapsed. According to the IETS code, embryo grades range from "1", meaning good or excellent when meeting all above requirements, "2" or fair, when the embryos irregularities are moderate in size and color, grade "3" meaning poor quality when there are distinguishable irregularities in the above parameters, and finally "4" meaning dead or degenerating embryos, signifying a non-

viable structure (Nogueira et al., 2018). The grading of embryos by both this method is directly affected by those observing the embryonic quality. This grading varies by grading experience, accuracy, judgement, and persona. Each individual embryologist may apply a different methodology and criteria solely based upon the previously listed exposures.

Although there has been standardization of morphological assessment of embryo grading system accepted by industry and implemented by the IETS, relatively few advances have occurred in the last few decades with regard to current embryo transfer procedure (Saravelos and Li, 2019). This grading may further be affected by grading experience, accuracy, judgement, and persona. Each individual embryologist may apply a different methodology and criteria solely based upon the previously listed exposures. Today there is an average success rate using ART of approximately 30% in the United States (Bormann et al., 2020a).

2.3.2. Non-Invasive Alternatives of Evaluation

Although traditional morphological analysis and grading is accepted by industry and has been around and has been trusted for many years, there is a subjectivity that varies with each individual embryologist that observes and grades embryo quality based on their own personal bias and experience. Traditionally, oocytes and embryos are examined based upon simple observation of morphology of the developmental stages. These external effects may have an impact on the actual grading of the embryo quality, for good or for bad.

There are several semi-automatized methods that are now being developed in order to evaluate embryo quality and improve the embryo classification process in order

to eliminate external effects. Time-lapse is a non-invasive technology that is mainly used for human embryos to measure morphokinetic parameters, such as timing of karyogamy, time intervals between cytokinesis, and abnormal events that result in inconsistent blastomere sizes (Nogueira et al., 2018). Traditional time-lapse monitoring (TLM) records regular time interval photographs over a period of several hours (Nasiri and Eftekhari-Yadzi, 2013). Embryos are monitored under a camera that captures images at timed intervals. By utilizing mathematical and statistical tools for evaluation of embryo quality, the computer assisted scoring system (CASS) is said to have a higher discriminatory power for embryo selection over the traditional scoring system that exhibits examiner variability. Time-lapse monitoring utilizes credible morphological criteria to improve IVF success. Nogueira et al. (2018) mentions that several methods have or are being developed, and many embryologists select oocytes/embryos using a non-invasive examination based on simple observation focused on morphology and kinetics of the developmental stage (usually on the third day of culture or blastocyst stage), including methods of embryo metabolism analysis, cellular respiration measurement, evaluation by TLM, the quality of in vitro growth of embryos, the integrity of blastomeres membrane, and electron-microscopy analysis.

As routine TLM systems in clinical embryology have only been available since 2008 (Petersen et al., 2016), tremendous opportunity in clinically applicable embryo evaluation is becoming possible. With continued improvements of software, image processing as well as scaling algorithm, embryonic images could be evaluated and the technology could be commercialized in order to one day create a more accurate, non-biased system of grading.

2.3.3. Embryo Metabolism and Morphokinetics

The preimplantation period of mammalian embryo development is a highly dynamic phase according to Gardner et al. (2000) from the time when fertilization occurs over the next 4 to 5 d as the embryo undergoes significant changes in carbohydrate and amino acid consumption and utilization. These changes that have a profound impact on the normal development, and therefore viability, of the embryo (Gardner et al., 2000).

After considering the nutrition of embryos and somatic cells, and the phenomenon of caloric restriction, a conclusion that preimplantation embryo survival is best served by a relatively low level of metabolism; a situation achieved by reducing the concentrations of nutrients in culture media and encouraging the use endogenous resources (Leese, 2002). Handled early stage embryos as well as embryos in vitro are subject to the stress of being placed in an artificial environment, and stress responses may include expression of stress responses such as up-regulation of glucose metabolism and AMP-activated protein kinase (Leese, 2002). According to Leese (2002), this trigger of side-effects of embryos may include nutritional imbalances, oxidative stress, and defective genomic printing. Expenditure of embryo metabolism and display of morphokinetics may come from (Leese, 2002):

1. Overall metabolism
2. Glycolytic rates
3. Amino acid turnover
4. Anti-oxidant enzymes
5. 5'AMP protein kinase

Leese suggests further that the most viable preimplantation embryos are those that exhibit the lowest in metabolism, glycolytic rates, and amino acid turnover, as well as highest levels in ani-oxidant enzymes and 5'AMP protein kinase.

2.4. Morphokinetic Activity as an Embryo Health and Viability Prediction

Animal production has undergone many changes since the first successful production of live young via ET in 1890 thanks to agricultural research. The transfer of embryos, including In Vivo derived (IVD) and in vitro produced (IVP), is a routine breeding strategy in agricultural operations that enables the perpetuation of individuals with high genetic merit, improves herd performance, decreases parturition interval, and increases fertility in animals under stress or that are repeat breeders (Wells and Killingsworth, 2022). As production evolves and changes, reliable selection of embryos with a potential for creating viable pregnancies is obligatory for ART success. Despite the traditional method of IETS embryo classification, the evaluation of embryos is directly affected by the embryologist's accuracy, experience, and mood (Nogueira et al., 2018). According to Nogueira et al. (2018), the reason for this affect is that morphological analysis does not measure any objective variables to determine the embryo classification, and human vision is subjectively able to extract information about an image, but some information may be ignored or not observed. Furthermore according to Kirkegaard et al. (2015), morphology and developmental competence is not firmly correlated, thus predictive value of morphological assessment is limited in the identification of the most viable embryos. This measurement is often based on a comparison among objects or images, thus, analyses by an embryologist may have low reproducibility (Nogueira et al., 2018). Two types of error are described by Farin et al.

(1995) that support this statement. Inter-evaluator error occurs when the same embryo is classified with different quality grades by different embryologists. Intra-evaluator error occurs when the same embryologist classifies the same embryo as different grades (Nogueira et al., 2108). This often occurs with borderline quality grades or inexperienced or tired evaluators, however this evaluation step is critical in the ET procedure and is strongly related to successful pregnancy. The variability and subjectivity of embryo evaluation may ultimately lead to less precise disposition decisions and discarding of viable embryos (Bormann et al., 2020b) that may deal a detrimental blow to advancement of the embryology and reproduction field overall. In a previous study performed by Borman et al. (2020b), 56 embryologists performed with a consistency of 52.4%, whereas field assisted reproduction of machine learning convolutional neural networks (CNN) outperformed embryologists with a consistency of 83.92% ($P < 0.05$). Time-lapse monitoring in combination with decision support algorithms based on morphokinetic embryo assessment and more frequent observation in time specific intervals may help to facilitate the implementation policy for evaluation that is not biased (Kirkegaard et al., 2015; Petersen et al., 2016).

2.4.1. Project Design

Improving the processing of the evaluation will improve the whole representation of the embryo. Image processing reveals information and features that have a scientific purpose, mainly due to computer algorithm's ability to quantify variables and establish numeric measures, contrasting visual analysis by humans based on comparison between images (Rocha et al., 2017a). Unlike the previous studies involving time-lapse technology and in vitro produced embryos, this project will be observing IVD embryos in

short, 35 sec observation times. The reliance on a subjective grading method, while practical and economical, fails to account for the complex factors attributing to an embryo's health and viability. Factors such as genetic defects, metabolic activity, acute stress, mitotic activity, and response to environmental factors all play roles in embryo health and viability (Wells and Killingsworth, 2022). By applying computational analyses as well as non-invasive and non-subjective technology such as morphokinetic measurement through time lapse imaging and video motion magnification (VMM), a better classification of embryos is obtainable and human subjectivity may be eliminated in order to improve pregnancy outcomes of IVF and ET. According to Wong et al. (2013), time lapse imaging does not appear to cause any observable, detrimental effect in embryo development, and thus can be used safely in the clinic as a tool to select the best embryo for transfer. From a pilot study, the objective was two-fold: (1) to determine if embryo morphokinetic activity can be evaluated in short observatory period and (2) to determine if embryo morphokinetic activity can be used as an indicator of embryo health and viability (Wells and Killingsworth, 2022).

2.4.2. Preliminary Research

Based on the Ted Talk, "Small Change. Big Difference," from the software company Lambda Vue that allows for VMM, the idea of determining if embryo morphokinetic activity could be used as an indicator of embryo health and viability was born in 2020 by Dr. Cara Wells and Dr. Russell Killingsworth. By diagnosing embryos though morphokinetic evaluation, as well as utilizing non-invasive and non-subjective technologies, increasing the productivity of each animal through genetics and selectively may be that much more attainable. In the pilot study done by Wells & Killingsworth in

2020, 94 IVD embryos were flushed and sequentially filmed for 35 sec using a Nikon CoolPix camera mounted in a trinocular stereo zoom scope by licensed veterinarian from cattle in the Texas Panhandle region. Post recording, videos were filtered with the VMM system, Lambda Vue, that according to Wells and Killingsworth (2022) takes a standard video sequence as input, applies special decomposition and temporal filtering at 300x amplification, allowing for the resulting signal to be amplified and reveal hidden information (Wu et al., 2012). To study the embryo morphokinetic changes over time, Image J Software developed by the National Institute of Health, was used to record measurements of embryo area, diameter, and perivitelline space collected at 5 sec intervals from 0 to 30 sec. All embryos that met the IETS morphological grading scale for Grade 1 or 2 requirements were recorded and transferred into eligible recipient females. While not fully elucidated, the hypothesized analysis of embryo video data and morphokinetic activity observed in embryos provides a non-invasive as well as non-subjective assessment of embryo metabolic activity (Wells and Killingsworth, 2022). Further research is required to confirm the validity and repeatability of these results.

2.5. References

ASAS Editional. 2020. The incompletely-fulfilled promise of embryo transfer in cattle-what can we do about it? J. Anim. Sci. 98(11):1

Ball, P., and A. R. Peters. 2004. Reproduction in cattle. Fertil., Conception and Preg. 5:56-67.

Betteridge, K. J. 2003. A history of farm animal embryo transfer and some associated techniques. Anim. Reprod. Sci. 79:203-244.

Betteridge, K. J. 2000. Comparative aspects of equine embryonic development. Anim. Reprod. Sci. 60:691-702.

Biggers, J. D. 2012. IVF and embryo transfer: historical origin and development. Reprod. Biomed. Online. 25:118-127.

Biggers, J. D. 1981. In vitro fertilization and embryo transfer in human beings. The New England J. Med. 304(6):336-342.

Bormann, C. L., M. K. Kanakasabapathy, P. Thirumalaraju, R. Gupta, R. Pooniwala, H. Kandula, E. Hariton, I. Souter, I. Dimitriadis, L. M. Boehnlein, and H. Shafiee. 2020a. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. eLife. 9:55301.

Bormann, C. L., P. Thirumalaraju, M. K. Kanakasabapathy, H. Kandula, I. Souter, I. Dimitriadis, R. Gupta, R. Pooniwala, and H. Shafiee. 2020b. Consistency and objectivity of automated embryo assessments using deep neural networks. Fertil Steril,. 113(4):781-787.

CDC. 2017. Assisted Reproductive Technology Fertility Clinic Success Rates Report: Centers for Disease Control and Prevention.

Farin, P. W., J. H. Britt, D. W. Shaw, and B. D. Slenning. 1995. Agreement among evaluators of bovine embryos produced in vivo or in vitro. Theriogenology. 339–349.

Ferré, L., M. Kjelland, L. Strøbech, P. Hyttel, P. Mermillod, and P. Ross. 2020. Review: Recent advances in bovine in vitro embryo production: Reproductive biotechnology history and methods. Anim. 14(5):991-1004.

Forde, N., and P. Lonergan. 2012. Transcriptomic Analysis of the Bovine Endometrium: What is Required to Establish Uterine Receptivity to Implantation in Cattle ? J. Reprod. Dev. 58(2):189–195.

Forseberg, E., J. Betthauser, N. Strelchenko, P. Golueke, L. Childs, and G. Jurgella. 2001. Cloning non-transgenic and transgenic cattle. Theriogenology. 55: 269.

Gardner, D. K., T. B. Pool, and M. Lane. 2000. Embryo nutrition and energy metabolis and its relationship to embryo growth, differentiation, and viability. Semin. Reprod. Med. 18(2):205-218.

Ginther, O. J. 1998. Equine pregnancy: physical interactions between the uterus and conceptus. Proc. Am. Assoc. Equine Pract. 44:73-105.

Hadley, M. E., and J. E. Levine. 2007. Endocrinology. Upper Saddle River, NJ: Pearson Prentice Hall.

Hamilton, W. J., J. D. Boyd, and H. W. Mossman. 1956. Human embryology (prenatal development of form and function). J. Med. Education. 38(9):1.

Han, Y. M., Y. K. Kang, D. B Koo, and K.K. Lee. 2003. Nuclear reprogramming of

cloned embryos produced in vitro. Theriogenology. 59:33-44.

Hansen, P. J. 2020. The incompletely-fulfilled promise of embryo transfer in cattle-what can we do about it? J. Ani. Sci. 98(11):1-20.

Heape, W. 1890. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster-mother. Proc. Royal Soc. London. 48:457-458.

Hossain, A., J. Phelps, A. Agarwal, E. Sanz, and M. 2016. Mahadevan. A review of the society for assisted reproductive technology embryo grading system and proposed modification. Fertil. Steril. 10(2):141-147.

Kirkegaard, K., A. Ahlström, , H. J. Ingerslev, and T. Hardarson. 2015. Choosing the best embryo by time lapse versus standard morphology. Fertil. Steril. 103:323-324.

Klein, C. 2016. Maternal recognition of pregnancy in the context of equine embryo transfer. J.Equine Vet. Sci. 41:22-28.

Leese, H. J. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. BioEssays. 24(9):845-849.

Lonergan, P. J., M. Sanchez, D. J. Mathew, C. Passaro, and T. Fair. 2019. Embryo development in cattle and interactions with the reproductive tract. Reprod. Fertil. Dev. 31:118-125.

Manual of the International Embryo Transfer Society. 2009. 4th ed. International Embryo Transfer Society, Champaign, IL.

Mapletoft, R. J. 2013. History and perspectives on bovine embryo tranfser. Anim. Reprod. 10(3):168-173

Mapletoft, R., K. McDermott, and C. Ness. 2020. Summary of Embryo Transfer

Activity in Canada for 2020. Canadian Embryo Transfer Association. 1-2.

McCue, P. M., and E. L. Squires. 2015. Equine Embryo Transfer (1st ed.). Teton NewMedia.

McDowell, K. J., D. C. Sharp, W. Grubaugh, W. W. Thatcher, and C. J. Wilcox. 1988. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. Biol. Reprod., 39:340-348.

McGee, E. A., and A. J. Hsueh. 2000. Initial and cyclic recruitment of ovarian follicles. Endocrinology Review. 21:200-214.

Meira, C., J. C. Ferreira, E. S. M. Silva, and F. S. Ignácio. 2012. Developmental aspects of early pregnancy in mares. Anim. Reprod. 9(3):166-172.

Nasiri, N., and P. Eftekhari-Yazdi. 2013. An overview of the available methods for morphological scoring of pre-implantation embryos in in vitro fertilization. Cell J. 16(4):392-405.

Nogueira, M. F. G., B. V. Guilherme, M. Pronunciate, P. H. Santos, D. L. B. Silva, and J. C. Rocha. 2018. Artificial intelligence-based grading quality of bovine digital images: direct capture with juxtaposed lenses of smartphone camera and stereomicroscope ocular lens. Sensors.18:4440

Pepling, M. 2013. Oocyte development before and during folliculogenesis. Oocyte physiology and development in domestic animals. Wiley-Blackwell. 1:1-19.

Petersen, B. M., M. Boel, M. Montag, and D. K. Gardner. 2016. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on day 3. Human Reprod. 31(10):2231-2244.

Racowsky, C., M. Vernon, J. Mayer, G. D. Ball, B. Behr, K. O. Pomeroy, D. Wininger, W. Gibbons, J. Conaghan, and J. E. Stern. 2010. Standardization of grading embryo morphology. J. Assist. Reprod. Genet. 27: 437–439.

Rocha, J. C., F. J. Passalia, F. D. Matos, M. B. Takahashi, M. P. Maserati, M. F. Alves, T. G. Almeida, B. L. Cardoso, A. C. Basso, and M. F. Nogueira. 2017a. A method based on artificial intelligence to fully automatize the evaluation of bovine blastocyst images. Scientific Reports. 7:7659.

Rocha, J. C., F. J. Passalia, F. D. Matos, M. B. Takahashi, M. P. Maserati, M. F. Alves, T. G. Almeida, B. L. Cardoso, A. C. Basso, and M. F. Nogueira. 2017b. Data descriptor: automatized image processing of bovine blastocysts produced in vitro for quantitative variable determination. Scientific Data. 4:170192.

Saravelos, S. H., and T. Li. 2019. Embryo transfer techniques. Best Practice and Research Clinical Obstetrics and Gynecology. 59:77-88.

Velasquez, M. A. 2008. Assisted reproductive technologies in cattle: applications in livestock production, biomedical research and conservation biology. Annual review of biomed. sci. 10:36-62.

Wells, C., and R. Killingsworth. 2022. Real-time embryo morphokinetic activity can be measured in short observatory periods to provide objective evaluation of embryo health. American Embryo Transfer Association.

Wessels, C., L. Penrose, and S. Prien. 2017. Current state of ART embryo selection techniques. Investigations in Gynecology Research & Women's Health. 1(1).

Wilmut, I., A. E. Schnieke, J. McWhir, A. J. Kind, and K. H. Campbell. 1997. Viable offspring derived from fetal and adult mammalian cells. Nature. 385:810.

Wong, C., A. A. Chen, B. Behr, and S. Shen. 2013. Time-lapse microscopy and image analysis in basic and clinical embryo development research. Reprod. BioMedicine Online. 26(2)120-129.

Wu, Y., M. Rubinstein, E. Shih, J. Guttag, F. Durand, and W. Freeman. 2012. Eulerian video magnification for revealing subtle changes in the world. ACM Transcription on Graphics. 31(4):1-8.

III.

ASSESSMENT OF EMBRYO VIABILITY BASED ON MORPHOKINETICS IN Cattle

3.1. Abstract

In bovine reproduction, fertility is a lowly heritable trait that has an exceedingly large financial impact on the ability to grow genetic lines. In order to increase pregnancy rates using Assisted Reproductive Techniques (ART) such as Embryo Transfer (ET), producers must have access to accurate decision-making tools. Traditionally, the embryo grading system accepted by industry is based on morphological evaluation, and is a method that has remained relatively unchanged for over four decades (Saravelos and Li, 2019) that uses subjective evaluation. This evaluation varies depending on exposure of technicians to training and experience, and inconsistency in grading among embryologists can ultimately lead to decreased conception rates through increased potential in discarding viable embryos. The objective of this study was to discern whether IETS quality Grade 1 or 2 embryos could be measured based on their morphokinetic activity to determine if this activity was indicative of embryo viability, enforce that embryos would establish pregnancies, and that would fail to establish pregnancies. Embryos were graded according to the International Embryo Transfer Society (IETS) manual guidelines (IETS, 2009), that is the accepted industry standard of grading. Of the embryos graded, those that were given a quality score of Grade 1 or 2 were chosen for morphokinetic evaluation. For this study, 73 In Vivo derived (IVD), fertilized bovine embryos collected via conventional flushing from donor cows by licensed veterinarian Dr. Russell Killingsworth in Shamrock, TX, and were filmed for a 35 sec video taken

with a Nikon CoolPix Camera mounted on a trinocular stereo zoom scope before transfer into approved recipient cows. Embryos were required to be filmed within 3 h of transfer with the entire embryo in view of the camera. Videos were then amplified using the video motion magnification (VMM) system Lambda Vue, that filters the frames of the video resulting in 300x amplification that reveal previously hidden components of these embryos. Embryo morphokinetic activity became humanly perceptible and measurable by changes in their morphological dimensions following the VMM step. Measurements of significance were taken on the embryo intercellular mass area (ICM), complete embryo area, X-axis and Y-axis diameter of the ICM and complete embryo, and on the vertical, diagonal, and horizontal axis of the sub-zonal distance, or the distance between the zona pellucida and outer edge of the ICM, also known as the perivitelline space. All measurements were taken in microns. At 30 d of pregnancy, pregnancy outcomes were determined by rectal palpation, ultrasound, or blood test, to determine embryo competency. Of the 73 embryos evaluated, 38 resulted in confirmed pregnancies, for a 52% conception rate. Data was analyzed in a complete randomized design, with embryo as experimental unit and pregnancy as treatment, using SAS 9.4 (SAS Institute Inc., Cary, NC) and PROC MIXED and FISHER'S test methods. Once analyzed and evaluated as averages in microns per section measured, the effect of pregnancy due to measured morphokinetic activity was most significant when measuring the complete embryo area ($P < 0.03$) and embryo complete change in diameter along the X-axis and Y-axis ($P < 0.02$) with average changes of 1242.66 μm^2 and 8.41 μm, respectively, in embryos resulting in pregnancies. When divided by high, medium, and low ranges of morphokinetic activity, the diameter of the complete Y-axis and X-axis and 0° position of

zona pellucida thickness measured on the embryo proved significant ($P < 0.03$, $P < 0.03$, $P < 0.05$, respectively). Of the 52% of positive pregnancy embryos measured at the Y Axis, majority at 33% fell within the medium range of morphokinetic activity, 18% within the low range, and 1% high range. At the X Axis, 25% of embryos were within a medium range of morphokinetics, 26% within a low range, and 1% within a high range. Finally for measurement of embryos resulting in positive pregnancy along the 0° thickness of the zona pellucida, 14, 34, and 4% fell within the high, medium, and low ranges of activity, respectively.

3.2. Introduction

Based on visual morphological assessment by industry standards, selection of top quality bovine embryos for transfer is vital to the success of future reproduction and generations of genetically differentiated cattle. Genetic improvement through ART provides a path to greater efficiency in reproductive techniques., as declining fertility is observed as a globally recognized problem that represents a major source of economic loss and culling in cattle (Khatib et al., 2009). With this path to greater efficiency must come a more fruitful method of embryo evaluation and viability selection.

Although there has been standardization of morphological assessment of embryo grading developed and implemented by the International Embryo Transfer Society (IETS), few advances have occurred in the last few decades with regard to current embryo transfer procedure (Saravelos and Li, 2019). This grading may further be affected by grading experience, accuracy, judgement, and persona. Each individual embryologist may apply a different methodology and criteria solely based upon the previously listed exposures. Today the average success rate using ART is approximately 30% in the

United States (Bormann et al., 2020a). This evaluation can vary depending on exposure of technicians to training and experience, and the lack of consistency in grading among embryologists can lead to decreased conception rates through increased potential in discarding viable embryos.

The subjectivity of industry accepted grading method led to the intention of this research, to provide a higher knowledge of embryo quality based not only on morphology, but morphokinetics and micromotions morula and blastocyst stage embryos in cattle and horses using time-lapse technology. This evaluation of activity was to discern whether a "good" embryo by industry standards was truly worth transferring or freezing for future use in a non-subjective manner, and whether morphokinetic activity can be indicative of embryo viability with the end goal to produce a live and healthy offspring, determine embryos would establish pregnancies, and fail to establish pregnancies.

By determining transfer worthy embryos through more selective and non-invasive methods such as these, the impact of genetic selection through Embryo Transfer (ET) could be maximized and made more consistent and profitable than might be achieved in nature. From this research, the aim was to prove that embryo micromotions and energy (within certain boundaries) influence development of a pregnancy. More diagnostic technology of embryo assessment could potentially make an already growing market for embryo transfer technology more efficient and reliable over time.

3.3. Materials and Methods

This project and all experimental protocols were performed under the approval of the West Texas A&M University International Animal Care and Use Committee (IACUC) # 2021.04.002.

3.3.1. Production of Embryos

All cows were bred and superovulated according to protocol based on producer requirement with the assistance of a licensed veterinarian. Conventional ET followed at 6 d post ovulation.

From the months May, September, October, and November of 2020, IVD embryos were flushed and sequentially transferred by licensed veterinarian, Dr. Russell Killingsworth, from cattle in the Texas Panhandle region. Embryo evaluation was performed based on IETS industry standards (Bó and Mapletoft, 2013) by Dr. Killingsworth, with only Grade 1 and 2 embryos eligible for transfer and evaluation. Donor and recipient cows were required to be part of ET services, undergoing previous reproductive evaluation. Of the embryos flushed, 73 were selected for evaluation.

3.3.2. Nikon CoolPix Camera Recording

Upon flushing from donor cattle, 6 or less embryos were arranged in a line in the field of view of the image capture. A 35 sec video of each embryo was recorded under standard embryo culture (Wells and Killingsworth, 2022) with a Nikon CoolPix Camera at 150x magnification mounted in a trinocular zoom scope. All videos were recorded within 3 h of transfer through a digital, inverted microscope. In order to be viable for research evaluation, the entire embryo was required to be in the field of view of the

CoolPix Camera. Once recorded, embryos were transferred into previously approved and selected recipient cattle.

3.3.3. Embryo Transfer and Diagnosis of Pregnancy

All embryos were transferred post filming and embryo evaluation by licensed veterinarian, Dr. Russell Killingsworth in the Texas Panhandle. Diagnosis of pregnancy outcomes were performed at 30 d post ovulation (Forde and Lonergan, 2012). Rectal palpation with ultrasound was used to determine pregnancy.

3.3.4. Lambda Vue Video Motion Magnification (VMM)

Once recorded, 35 sec videos of each embryo were filtered with the video motion magnification (VMM) platform, Lambda Vue. Lambda Vue takes a standard video sequence as input, applies special decomposition, followed by temporal filtering to the frames. According to Wu et al. (2012), the resulting signal is then amplified to reveal hidden information, such as undetected embryo morphokinetics. A comparison of VMM filtered versus unfiltered video data may be observed in Fig. 3.1. Time-lapse monitoring (TLM) in combination with decision support algorithms based on morphokinetic embryo assessment may help to facilitate the implementation policy for evaluation that is not biased (Petersen et al., 2016).

Based on the Ted Talk, “Small Change. Big Difference,” from the software company Lambda Vue that allows for VMM, the idea of determining if embryo morphokinetic activity could be used as an indicator of embryo health and viability was presented in 2020 by Dr. Cara Wells and Dr. Russell Killingsworth. By diagnosing embryos though morphokinetic evaluation, as well as utilizing non-invasive and non-subjective technologies, increasing the productivity of each animal through genetics and

selectively may be that much more attainable. Once filtered with VMM, movement in both the ICM and zona pellucida (ZP) were humanly perceptible and therefore measurable in the forms of protrusions, bulges, depressions, pulses and changes in embryo shape from this amplification step. Amplification imagery can be found in Fig. 3.1.

3.3.5. ImageJ Software

Developed by the National Institute of Health, ImageJ Software was used to record measurements of embryo area, diameter, and sub-zonal (perivitelline) space. Measurements were collected every 5 sec for 35 sec once videos were filtered using Lambda Vue VMM Technology. Parameters for measurement may be viewed in Fig. 3.2. Measurements were based on the methodology of Wells and Killingsworth (2022).

3.3.6. Statistical Analysis

A completely randomized design was used to analyze these embryos with individual embryo as the experimental unit and pregnancy as the treatment. Data were measured according to stage of embryo, and because all were in the morula stage of development, no further distinction or separation for analysis was needed. Continuous data were analyzed with the PROC MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Categorical data was analyzed using Fisher's Exact Text in SAS 9.4 (SAS Institute Inc., Cary, NC). Means were separated using PDIFF pairwise comparison option. Significance was declared at $P \leq 0.05$ and tendencies were declared between $P > 0.05$ and $P \leq 0.10$.

3.4. Results and Discussion

3.4.1. Overall Change in Morphokinetic Activity and Pregnancy Status

Of the 73 embryos evaluated, 38 resulted in confirmed pregnancies, for a 52% conception rate (Table 3.1). Once analyzed in SAS 9.4 using PROCMIXED, morphokinetic values were observed at the complete embryo Y-Axis ($P < 0.04$), complete embryo area ($P < 0.03$), at the 0° position of the ZP thickness (ZP to TE) ($P < 0.03$), and finally at the perivitelline space shift (TE to ICM) at the 210° and 240° position ($P < 0.01$, $P < 0.02$, respectively; Table 3.2, Table 3.3). According to Molinari et al. (2012), the ZP is associated with a high level of structural organization, and could be positively related to the developmental potential of the embryo. When all like measurements such as the overall axis of the complete embryo and ICM, complete area and ICM area, ZP to trophectoderm (TE), and TE to ICM were evaluated as averages in microns per section measured, the measured morphokinetic activity based on pregnancy outcome was most noteworthy when measuring the complete embryo area ($P < 0.03$) and embryo complete change in diameter along the X-axis and Y-axis ($P < 0.02$; Table 3.4) with average changes of 1242.66 μm^2 and 8.41 μm, respectively, in embryos resulting in positive pregnancies. When evaluated using Fisher's Exact Test, embryos were divided by high, medium, and low ranges of morphokinetic activity (Wells and Killingsworth, 2022), and the diameter of the complete Y-axis and X-axis and 0° position of zona pellucida thickness measured on the embryo proved significant ($P < 0.03$, $P < 0.03$, $P < 0.05$, respectively; Table 3.5, Table 3.8). Embryos were broken into these high, medium, and low range of morphokinetic activity based on the protocol of Wells and Killingsworth (2022). As observed in Table 3.6, the range of morphokinetic activity on

the Y-Axis by pregnancy status was broken into high, medium, and low range of morphokinetic activity based on this same protocol. As a result, 62% conception rate of these positive bovine pregnancies identified as 13 of the embryos in low range of activity category (0 to 7 μm), 56% conception rate of the 43 embryos in the medium range (7 to 14 μm), and 11% conception rate in the 9 embryos in the high range of activity category (14 to 21 μm; $P < 0.03$). Using these parameters, a probability of 97% of embryos measured along the X-Axis and Y-Axis were in the low and medium morphokinetic activity ranges and were therefore significant measurements of embryo viability assessment of forming a positive pregnancy (Table 3.6 and 3.7). Comparably, when ZP thickness was measured (ZP to TE), 38 embryos with a conception rate of 66% were identified as low range of morphokinetic activity (0 to 6 μm), 28 embryos at 36% conception rate were identified as medium range (6 to 12 μm), and 7 embryos identified at 43% as high range embryos forming positive pregnancies ($P < 0.05$). According to Wells and Killingsworth (2022), embryos with a high degree of change in these 35sec durations do not establish pregnancies as frequently as embryos with moderate degree of change, as is apparent in changes of the significantly fewer embryos in the high categories of morphokinetic activity (Table 3.6, 3.7, and 3.9). This percent of movement of embryos in subzonal, or perivitelline, space can indicate likelihood of an individual embryo's change of establishing pregnancy (Wells and Killingsworth, 2022). Embryos displaying tendencies as well as no significance may be observed in Table 3.10. The embryos falling in the lower and mid-range morphokinetic activity sections may be more viable because they do not have to waste energy attempting to survive, a theory coined as the "Quiet Embryo Hypothesis" (Leese, 2002; Wells and Killingsworth, 2022). Fewer

differentiations in range of morphokinetic activity can be observed in Table 3.1. Throughout the 35 sec recording periods resulting in positive pregnancies, embryos that did not survive underwent greater change in microns with fewer embryos falling into the high range of morphokinetic activity category, thus following the Quiet Embryo Hypothesis. While these observations of morphokinetic changes could have been improved with further numbers of embryos measured, this non-invasive and non-subjective method has demonstrated that of the 73 Grade 1 and 2 embryos recorded, only 57% were truly viable.

3.5. Conclusion

According to Bó and Mapletoft (2013), one of the most important factors associated with the success and widespread application of ET technology is evaluation of the embryos before freezing and/or transfer to a recipient. The lack of consistency among embryologists is of significant concern, as variable decisions can lead to the increase in potentially discarding viable embryos and decreasing cumulative pregnancy rates (Bormann et al., 2020b). Combatting these inaccuracies with TLM systems may surpass this increased potential for errors among embryologists in order to raise conception rates.

Though few measurements as a whole proved significance, those that did demonstrated follow similar tendencies to studies done in previous trials by Wells and Killingsworth (2022). Following the "Quiet Embryo Hypothesis" suggested by Leese (2002), most embryos fell in the low to moderate activity categories. Even though all embryos were of industry standard quality for transfer, following IETS guidelines, a decrease in truly viable embryos was observed, following functionality previously observed by Wells and Killingsworth (2022), that one can expect 20% of Grade 1 and 2

Embryos to be incompetent at time of transfer. Further study to collect more data from IVD embryos will aid in further understanding of the relationship between morphokinetic activity and developmental potential in order to improve performance for selection of truly viable embryos. Whereas morphological evaluation of embryos relies on the expertise and experience of the person that evaluates the embryos (Bó and Mapletoft, 2013), morphokinetic evaluation relies on image processing that reveals information and features that have a scientific purpose based on computer algorithms' ability to quantify variables and establish numeric measures (Rocha et al., 2017). By evaluating all of these non-invasive, non-subjective factors, as well as the universally accepted embryonic features, this technology offers a wider range of parameters to rely on for the selection of viable embryos, that should therefore result in a better embryo selection (Aparicio et al., 2013).

3.6. References

Aparicio, B., M. Cruz, and M. Meseguer. 2013. Is morphokinetic analysis the answer? Reprod. Biomed. Online. 27(6):654–663.

Bó, G. A., and R. J. Mapletoft. 2013. Evaluation and classification of bovine embryos. Anim. Reprod. 10(3):334-348.

Bormann, C. L., M. K. Kanakasabapathy, P. Thirumalaraju, R. Gupta, R. Pooniwala, H. Kandula, E. Hariton, I. Souter, I. Dimitriadis, L. M. Boehnlein, and H. Shafiee, 2020a. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. eLife. 9:55301.

Bormann, C. L., P. Thirumalaraju, M. K. Kanakasabapathy, H. Kandula, I. Souter, I. Dimitriadis, R. Gupta, R. Pooniwala, and H. Shafiee. 2020b. Consistency and objectivity of automated embryo assessments using deep neural networks. Fertil. Steril. 113(4):781-787.

Forde, N., and P. Lonergan. 2012. Transcriptomic Analysis of the Bovine Endometrium: What is Required to Establish Uterine Receptivity to Implantation in Cattle ? J. Reprod. and Development, 58(2):189–195.

Khatib, H., W. Huang, X. Wang, A. H. Tran, A. B. Bindrim, V. Schutzkus, R. L. Monson, and B. S. Yandell. 2009. Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. J. Dairy. Sci., 92:2238-2247.

Leese, H. J. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. 2002. Bioessays. 24(9):845

Manual of the International Embryo Transfer Society. 2009. 4th ed. International Embryo Transfer Society, Champaign, IL.

Molinari, E., F. Evangelista, C. Racca, C. Cagnazzo, and A. Revelli. 2012. Polarized light microscopy- detactable structures of human oocytes and embryos are related to the likelihood of conception in IVF. J. Assist. Reprod. Genet. 29:1117-1122

Petersen, B. M., M. Boel, M. Montag, and D. K. Gardner. 2016. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on d 3. Human Reprod. 31(10):2231-2244.

Rocha, J. C., F. J. Passalia, F. D. Matos, M. B. Takahashi, M. P. Maserati, M. F. Alves, T. G. Almeida, B. L. Cardoso, A. C. Basso, and M. F. Nogueira. 2017. Data descriptor: automatized image processing of bovine blastocysts produced in vitro for quantitative variable determination. Sci. Data. 4:170-192.

Saravelos, S. H., and T. Li. 2019. Embryo transfer techniques. Best Practice and Research Clinical Obstetrics and Gynecology. 59:77-88.

Wells, C., and R. Killingsworth, 2022. Real-time embryo morphokinetic activity can be measured in short observatory periods to provide objective evaluation of embryo health. American Embryo Transfer Association.

Wu, Y., M. Rubinstein, E. Shih, J. Guttag, F. Durand, and W. Freeman. 2012. Eulerian video magnification for revealing subtle changes in the world. ACM Transcription on Graphics. 31(4):1-8.

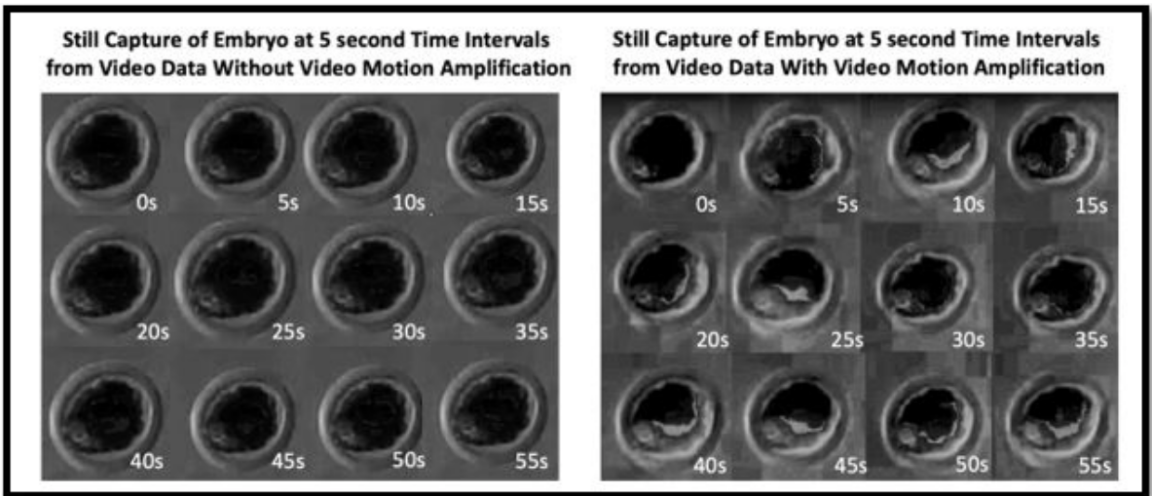

Figure 3.1. *VMM filtered versus unfiltered video data.* The raw video data (left) appears morphologically unchanged without Lambda Vue (VMM) compared to the video data (right) filtered with VMM. Morphological variations can be observed in the forms of protrusions, bulges, depressions, pulses and changes in embryo shape from this amplification step (Wells and Killingsworth, 2022).

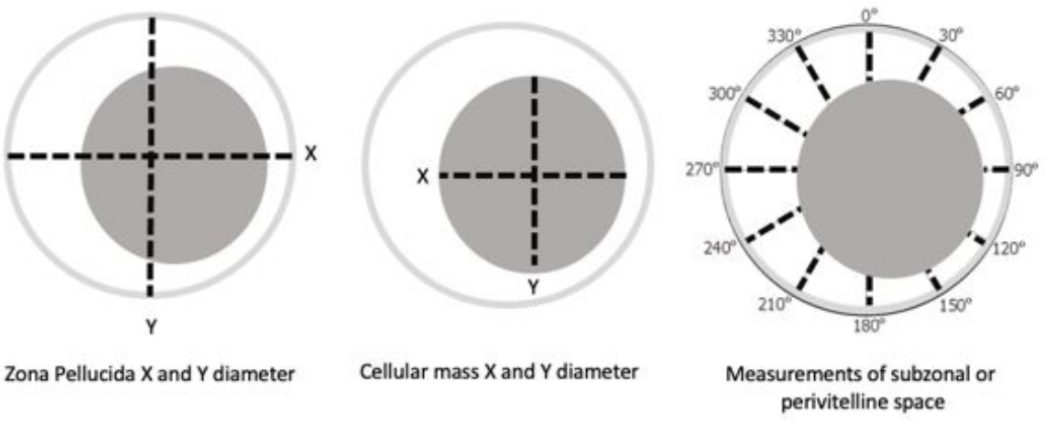

Figure 3.2. *Graphical representation of measurements collected on bovine and equine morula stage embryos*. Measurements include zona pellucida (ZP) area (not pictured), ICM area (not pictured), ZP X-axis and Y-axis diameter, ICM X-axis and Y-axis diameter, and distance between the ICM to the ZP to capture morphokinetic activity in the perivitelline space (Wells and Killingsworth, 2022).

Table 3.1. Descriptive statistics of morphokinetic range of IVD[1] bovine embryos

Item	IVD[1]	Item	IVD[1]
n	72.00	*n*	72.00
Conception rate, %	52.78	Conception rate, %	52.78
Complete axis[6]		ICM[2] area[7]	
Mean	8.23	Mean	960.68
Minimum	2.78	Minimum	222.21
Maximum	21.60	Maximum	3285.15
SD[5]	3.76	SD[5]	551.14
ICM[2] axis[6]		ZP[3] – TE[4]	
Mean	9.24	Mean	6.02
Minimum	2.86	Minimum	0.00
Maximum	32.73	Maximum	18.78
SD[5]	5.79	SD[5]	2.66
Complete area[7]		TE[4] – ICM[2]	
Mean	1458.27	Mean	7.25
Minimum	249.99	Minimum	0.00
Maximum	4890.69	Maximum	21.83
SD[5]	892.40	SD[5]	3.87

[1]In Vivo derived
[2]Inner cell mass
[3]Zona pellucida
[4]Trophecdtoderm
[5]Standard deviation
[6]Units (μm)
[7]Units (μm^2)

Table 3.2. Effect of morphokinetic values on pregnancy status of IVD[1] bovine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[5]				
Complete Y	8.53	10.47	0.66	0.04
Complete X	8.3	9.81	0.58	0.07
ICM[2] Y	12.55	12.18	1.06	0.80
ICM[2] X	13.72	12.82	0.90	0.47
Area[6]				
Complete	1242.66	1692.36	146.94	0.03
ICM[2]	851.87	1078.82	91.78	0.08
ZP[3] to TE[4,5]				
0°	5.91	7.50	0.50	0.03
30°	6.64	6.45	0.43	0.78
60°	5.96	6.37	0.47	0.52
90°	6.01	6.34	0.41	0.57
120°	5.29	5.76	0.39	0.39
150°	5.20	5.66	0.38	0.38
180°	6.13	7.14	0.54	0.27
210°	3.46	3.66	0.35	0.68
240°	6.73	6.67	0.43	0.91
270°	6.34	6.62	0.36	0.58
300°	6.15	6.08	0.44	0.91
330°	6.37	6.09	0.39	0.61

[1]In Vivo derived
[2]Inner Cell Mass
[3]Zona Pellucida
[4]Trophectoderm
[5]Units (μm)
[6]Units (μm^2)

Table 3.3. Effect of morphokinetic values on pregnancy status of IVD[1] bovine embryos, continued

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
TE[3] to ICM[2,4]				
0°	8.13	8.69	0.69	0.51
30°	8.08	6.78	0.59	0.11
60°	7.26	7.96	0.63	0.43
90°	6.85	6.96	0.69	0.91
120°	6.81	6.86	0.64	0.95
150°	5.41	5.57	0.6	0.85
180°	6.81	6.43	0.78	0.73
210°	7.76	5.32	0.64	<0.01
240°	6.76	8.44	0.51	0.02
270°	9.62	8.37	0.69	0.19
300°	7.37	6.97	0.56	0.61
330°	7.39	7.5	0.67	0.91

[1]In Vivo derived

[2]Inner Cell Mass

[3]Trophecdtoderm

[4]Units (μm)

Table 3.4. Effect of average morphokinetic values on pregnancy status of IVD[1] bovine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
n[7]			72	
Axis[5]				
Complete	8.41	10.14	0.51	0.02
ICM[2]	13.13	12.5	0.75	0.54
Area[6]				
Complete	1242.66	1692.36	146.94	0.03
ICM[2]	851.87	1078.82	91.78	0.08
ZP[3] to TE[4,5]				
	5.86	6.20	0.24	0.31
TE[3] to ICM[4,5]				
	7.04	7.47	0.26	0.25

[1]In Vivo derived

[2]Inner Cell Mass

[3]Zona Pellucida

[4]Trophectoderm

[5]Units (μm)

[6]Units (μm^2)

[7]Embryos total

Table 3.5. Comparison of range of morphokinetic activity on measured values of morula stage IVD[1] bovine embryos by pregnancy status

Item		Fisher's exact test *(P)*[2]
n[6]	72	
Axis[4]		
Complete X		0.03
Complete Y		0.03
ICM[3] X		0.93
ICM[3] Y		0.28
Area[5]		
Complete		0.14
ICM[3]		0.16

[1]In Vivo derived

[2]One-tailed *P* value

[3]Inner cell mass

[4]Units (μm)

[5]Units (μm^2)

[6]Embryos total

Table 3.6. Comparison of range of morphokinetic activity on the Y-Axis of morula stage IVD[1] bovine embryos by pregnancy status

	Range of morphokinetics by group[3,4]			
Item	0 to 7	7 to 14	14 to 21	Fisher's exact test[2]
Pregnancy status, *n* (%)				
Yes	13 (61.90)	24 (55.81)	1 (11.11)	$P < 0.03$
No	8 (38.10)	19 (54.29)	8 (88.89)	

[1]In Vivo derived
[2]One-tailed *P* value
[3]Unit (μm)
[4]Range based on Wells and Killingsworth (2022) Methodology

Table 3.7. Comparison of range of movement on the X-Axis of morula stage IVD[1] cattle embryos by pregnancy status

	Range of morphokinetics by group[3,4]			
Item	0 to 7	7 to 14	14 to 21	Fisher's exact test[2]
Pregnancy status, *n* (%)				
Yes	19 (70.37)	18 (43.90)	1 (20.00)	$P < 0.03$
No	8 (29.63)	23 (56.10)	4 (80.00)	

[1]In Vivo derived

[2]One-tailed *P* value

[3]Units (μm)

[4]Range based on Wells and Killingsworth (2022) Methodology

Table 3.8. Comparison of range of movement on measured values of morula stage IVD[1] bovine embryos by pregnancy status

Item measured along embryo (ZP[3] to TE[4])[5]	Fisher's exact test *(P)*[2]
n[6]	72
0°	0.05
30°	0.22
60°	0.09
90°	0.41
120°	0.51
150°	0.72
180°	0.50
210°	0.75
240°	0.93
270°	0.51
300°	0.74
330°	1.00

[1]In Vivo derived

[2]One-tailed *P* value

[3]Zona Pellucida

[4]Trophectoderm

[5]Units (µm)

[6]Embryos total

Table 3.9. Comparison of range of movement on the (ZP[3] to TE[4]) at 0° position of morula stage IVD[1] cattle embryos by pregnancy status

	Range of morphokinetics by group[5,6]			
Item	0 to 6	6 to 12	12 to 18	Fisher's exact test[2]
Pregnancy status, *n* (%)				
Yes	25 (65.79)	10 (35.71)	3 (42.86)	$P < 0.05$
No	13 (34.21)	18 (64.29)	4 (57.14)	

[1]In Vivo derived
[2]One-tailed *P* value
[3]Zona pellucida
[4]Trophectoderm
[5]Units (μm)
[6]Range based on Wells and Killingsworth (2022) methodology

Table 3.10. Comparison of range of morphokinetic activity on all measured values of morula stage IVD[1] bovine embryos by pregnancy status

Item measured along embryo (TE[3] to ICM[4])[5]	Fisher's exact test *(P)*[2]
n[6]	72
0°	0.27
30°	0.82
60°	0.13
90°	0.93
120°	0.93
150°	0.53
180°	1.00
210°	0.27
240°	0.06
270°	1.00
300°	0.93
330°	0.86

[1]In Vivo derived

[2]One-tailed *P* value

[3]Trophectoderm

[4]Inner cell mass

[5]Units (μm)

[6]Embryos total

I

ASSESSMENT OF EMBRYO VIABILTIY BASED ON MORPHOKINETICS IN HORSES

4.1. Abstract

In equine reproduction, fertility is a lowly-heritable trait that prevents progress in generations of progeny. In order to increase pregnancy rates using Assisted Reproductive Techniques (ART) such as Embryo Transfer (ET) and cryopreservation, producers must have access to accurate decision-making tools. Traditionally, the embryo grading system accepted by industry is based on morphological evaluation, and is a method that has remained relatively unchanged for over four decades that uses subjective evaluation that varies with exposure of technicians to training and experience. Inconsistency in grading among embryologists can ultimately lead to decreased conception rates through increased potential in discarding viable embryos. The objective of this study was to discern whether IETS quality Grade 1 or 2 embryos could be measured based on their morphokinetic activity to determine if this activity was indicative of embryo viability, select embryos establishing pregnancies, and embryos failing to establish pregnancies. Embryos were graded according to the International Embryo Transfer Society (IETS) manual guidelines (IETS, 2009), that is the accepted industry standard of grading. Of the embryos graded, those that were given a quality score of Grade 1 or 2 were chosen for morphokinetic evaluation. Of 22 total morula stage embryos, 17 overall were IVD cryopreserved from conventional flushing, thawed, and transferred, while 5 were cloned, thawed, and transferred morula-stage embryos. A total of 19 IVD blastocyst stage fertilized equine embryos were collected via conventional flushing from donor mares and transferred.

These embryos were filmed for a 35 sec video taken with an Iolight Microscope post conventional flush (blastocyst stage) or thaw from cryopreserved state (morula stage) or cryopreserved cloned embryo, before transfer into approved recipient mares. Embryos were required to be filmed within 3 h of transfer with the entire embryo in view of the camera. Videos were then amplified using the video motion magnification (VMM) system Lambda Vue, that filters the frames of the video resulting in 300x amplification that reveal previously hidden components of these embryos. Embryo morphokinetic activity became humanly perceptible and measurable by changes in morphological dimensions following the VMM step. Measurements were taken on the embryo intercellular mass area (ICM), embryo and ICM area, and on the vertical, diagonal, and horizontal axis of the sub-zonal (perivitelline) space. All measurements were taken in microns. At 35 d of pregnancy, when a fetal heartbeat could be detected, pregnancy outcomes were determined by rectal palpation and ultrasound, thus determining embryo competency. Of the 19 blastocyst embryos evaluated, 14 resulted in confirmed pregnancies, for a 74% conception rate. Of the 22 morula stage embryos evaluated, 12 resulted in confirmed pregnancies, for a 55% conception rate. Overall conception rate for the 41 embryos was 63%. Data was analyzed using SAS 9.4 with using PROC MIXED, FISHER'S EXACT, and PROC GLIMMIX test methods. In morula stage embryos, differences in measurements of significance occurred while measuring the range of morphokinetic activity at change on the overall axis and perivitelline space shift ($P \leq 0.01$), the zona pellucida (ZP) thickness ($P < 0.05$), and perivitelline space shift ($P < 0.01$) as well as showed tendencies when measuring the area of the inner cell mass (ICM;

$P < 0.09$), and therefore exemplifies that there is more to embryo grading than morphology.

4.2. Introduction

Mares usually spontaneously ovulate only one dominant follicle per cycle (McCue and Squires, 2015) and thusly embryo collection attempts are based on one potential embryo per cycle at a rate of approximately 50 to 65% recovery per cycle according to McCue and Squires (2015) with variability depending on inherent fertility of the dam and sire as well as other reproductive factors such as type of semen used, set up of dam estrous cycle, etc. Pregnancy rate following transfer is approximately 70 to 90% (McCue and Squires, 2015), with variability depending on experience of technicians, embryo quality, and recipient factors. The rate of pregnancy loss is similar between ET recipients and mares bred to carry their own pregnancy with approximately 8 to 10% of pregnancies lost between initial detection and expected due date (McCue and Squires, 2015).

The combination of poor embryo quality and poor endometrial receptivity could contribute significantly to the high percentages of implantation failure. The goal of this research was to assess the embryo and validate that viability is made more certain with morphokinetic activity measurements. Although there are many parameters that ART such as ET transpire from, this research focused primarily on embryo quality with the assumption of a well-balanced embryo-endometrium interaction. Both frozen and freshly flushed embryos were filmed, monitored, measured, and then transferred. In most studies comparing fresh and frozen embryo transfers, the best-quality embryos are chosen for the transfer (Roque et al., 2012) juxtaposing poor-quality embryos whose result may be more

varied. This research aims to eliminate the one-third of failures that are responsible for unsuccessful embryo transfers due to the embryo itself. For clarification, the other two-thirds of failure in embryo transfer is due to the impairment of the endometrial receptivity (Roque et al., 2012). By discretionary selection of the outstanding embryos, a higher survival rate may be achieved, and therefore a higher outcome of successful pregnancy rates.

Embryo quality is most commonly assessed by industry standard of morphologic evaluation (Wong et al., 2014). Although there has been standardization of morphological assessment of embryo grading accepted by industry and implemented by the International Embryo Transfer Society (IETS), relatively few advances have occurred in the last few decades with regard to current ET procedure (Saravelos and Li, 2019). This grading uses subjective evaluation that varies by technician grading experience, accuracy, judgement, and persona. Each individual embryologist may apply a different methodology and criteria solely based upon the previously listed exposures. Today the average success rate using ART is approximately 30% in the United States (Bormann et al., 2020). This evaluation varies depending on exposure of technicians to training and experience, and inconsistency in grading can ultimately lead to decreased conception rates that increase potential in discarding viable embryos.

The subjectivity of the industry accepted grading method led to the intention of this research, to provide a higher knowledge of embryo quality based not only on morphology, but morphokinetics and therefore viability of morula and blastocyst stage embryos in horses using TLM. This evaluation of activity was to discern whether a "good" embryo by industry standards was truly worth transferring or freezing for future

use in a non-subjective manner, and whether morphokinetic activity can be indicative of embryo viability with the end goal to enforce embryos that would establish pregnancies, and that would fail to establish pregnancies. According to Milewski et al. (2016), the use of TLM systems are related to better reproductive outcomes in comparison with conventional methodology because this technology offers the opportunity to continuously observe embryos and their development in a non-invasive method so that the precision of information acquired may be enhanced.

By determining worthy embryos through more selective and non-invasive methods such as these, the impact of genetic selection through Embryo Transfer (ET) could be maximized and made more consistent and profitable than might be achieved in nature. From this research, the aim was to prove that embryo morphokinetic activity (within certain boundaries) influences development of a viable pregnancy. More diagnostic technology of embryo assessment could potentially make an already growing market for ET technology more efficient and reliable over time.

4.3. Materials and Methods

This project and all experimental protocols were performed under the approval of the West Texas A&M University International Animal Care and Use Committee (IACUC) # 2021.04.002.

4.3.1. Breeding Procedure

Mares were bred in accordance with clinic procedures of Timber Creek Equine Hospital, and were checked via ultrasonographic pregnancy diagnosis. Mares were checked for pregnancy initially at 12 d post ovulation, when the embryonic vesicle may first be visible by ultrasound detection (McCue and Squires, 2015), and again at 35 d

when a heart-beat was observed within the embryo proper in order to confirm that the pregnancy was still viable.

4.3.2. Iolight Microscope Recording

Upon flushing from donor mares, each embryo was arranged in the field of view of the image capture by the Iolight Microscope (Fig. 4.1). A 35 sec video of each embryo was recorded under standard embryo culture (Wells and Killingsworth, 2022) with an inverted Iolight Microscope at 150x magnification. All videos were recorded within 3 h of transfer through the digital, inverted microscope. In order to be viable for research evaluation, the entire embryo was required to be in the field of view of the Iolight Camera. Once recorded, embryos were transferred into previously approved and selected recipient mares.

4.3.3. Embryo Transfer and Pregnancy Diagnosis

All embryos were flushed using conventional ET from donor mares and were transferred post filming and embryo evaluation by licensed veterinarian (Dr. Gregg Veneklasen, Canyon, Texas) into previously-approved recipient mares. A total of 41 embryos were analyzed, 22 morula stage embryos, and 19 blastocyst stage embryos. Pregnancy outcomes were determined initially by ultrasonographic pregnancy diagnosis via rectal palpation at 12 d post ovulation, when the embryonic vesicle may first be visible by ultrasound (McCue and Squires, 2015), and again at 35 d post ovulation when a heart-beat was observed within the embryo proper in order to confirm that the pregnancy was still viable. According to McCue and Squires (2015), 2.2% of ET pregnancies are lost between day of first detection (usually 11 to 12 d) and 16 d. The total

loss rate between 16 to 25 d rises to 5.7%, and then falls to 2.5% between 25 and 35 d when heartbeat could be detected (McCue and Squires, 2015).

4.3.4. Lambda Vue Video Motion Magnification (VMM)

Once 35 sec videos were recorded of each of the 19 blastocyst stage embryos, videos were then filtered with the video motion magnification (VMM) platform, Lambda Vue. Lambda Vue takes a standard video sequence as input, applies special decomposition, followed by temporal filtering to the frames to 300x amplification. According to Wu et al. (2012), the resulting signal is then amplified to reveal hidden information, such as undetected embryo morphokinetics. Morphology and developmental competence are not firmly correlated, thus predictive value of morphological assessment is limited in the identification of the most viable embryos (Kirkegaard et al., 2015), and leaves room for growth with time-lapse monitoring (TLM) in combination with decision supported algorithms based on morphokinetic embryo assessment. This TLM algorithm may help to facilitate the implementation policy for evaluation that is not biased (Petersen et al., 2016).

Based on the Ted Talk, “Small Change. Big Difference,” from the software company Lambda Vue that allows for VMM, the idea of determining if embryo morphokinetic activity could be used as an indicator of embryo health and viability was born in 2020 by Dr. Cara Wells and Dr. Russell Killingsworth. By diagnosing embryos though morphokinetic evaluation, as well as utilizing non-invasive and non-subjective technologies, increasing the productivity of each animal through genetics and selectively may be that much more attainable. Once filtered with VMM, movement in the inner cell mass (ICM), zona pellucida (ZP), perivitelline space, and trophectoderm (TE) were

humanly perceptible and therefore measurable in the forms of protrusions, bulges, depressions, pulses and changes in embryo shape from this amplification step. Amplification imagery can be found in Fig. 4.2.

4.3.5. ImageJ Software

Developed by the National Institute of Health, ImageJ Software was used to record measurements of embryo area, diameter, ZP thickness, TE thickness, and perivitelline space shift. Measurements were collected every 5 sec for 35 sec once videos were filtered using Lambda Vue VMM Technology. Parameters for morula measurement are presented in Fig. 4.3. Measurements on blastocyst stage embryos were of a similar method as represented in Fig. 4.3, measuring total embryo area, embryo diameter along the X-axis and Y-axis, and around the outside edges of the trophectoderm (TE) thickness at all degrees presented Measurements were based on the methodology of Wells and Killingsworth (2022).

4.3.6. Statistical Analysis

A completely randomized design was used to analyze these embryos with individual embryo as the experimental unit and pregnancy as the treatment. Data were measured according to stage of embryo. Because embryos were in the blastocyst stage transferred directly post conventional flush, and morula stage of development cryopreserved and thawed before ET, distinction or separation for analysis was necessary. A different method of measuring each type of embryo as well, thus further disabling the ability to run embryo results together. Continuous data were analyzed with the PROC MIXED procedure in SAS 9.4 (SAS Institute Inc., Cary, NC). Categorical data was analyzed using Fisher's Exact Text in SAS 9.4 (SAS Institute Inc., Cary, NC).

Frequency data was analyzed using the PROC GLIMMIX procedure of SAS 9.4 (SAS Institute Inc., Cary, NC). Least squares means were calculated with the LSMEANS option in SAS (SAS Institute Inc., Cary, NC) and means were separated using PDIFF pairwise comparison option all data considered significant at $P \leq 0.05$. Tendencies were declared between $P > 0.05$ and $P \leq 0.10$.

4.4. Results and Discussion

4.4.1. Blastocyst Stage Embryo Conception Rate

According to Ahlström et al. (2011), blastocyst transfer is one approach being used to achieve higher implantation and live birth rates compared with cleavage stage embryos. Based on descriptive statistics in the current trial (Table 4.1 and 4.2), blastocyst-stage embryos flushed by conventional techniques as fresh and sequentially transferred by ET into approved recipients had an overall conception rate of 74%. Between 2004 and 2013, studies showed an overall pregnancy rate at 16 d between 74.9 to 82% for transferred embryos (McCue and Squires, 2015).

Using Fisher's Exact Test, one measurement of significance among blastocyst stage embryos at position (TE) 240° ($P \leq 0.05$; Table 4.10 and 4.11) and two tendencies at the 0° and 270° positions ($P \leq 0.10$; Table 4.10 and 4.11), were observed as positions of effect, meaning that by measuring along these aspects of the TE, the probability of determining embryo viability would be 95% more significant of embryos measured along the 240° (TE; $P \leq 0.05$) position, and 90% measured along the (TE) 0° and 270° ($P \leq 0.10$) positions versus their non-significant counterparts (Table 4.9 and 4.10).

When evaluating with SAS 9.4 PROCMIXED, measurements of significance in blastocyst stage embryos were observed at the 90° (TE) position, therefore resulting in a

90% more accurate probability of determining embryo viability to form pregnancies when measured along this location on the TE ($P \leq 0.10$; Table 4.6 and Table 4.7) versus unlike measurements of no significance such as those observed in Table 4.8. According to Alhlström et al. (2011) in blastocyst selection for transfer in humans, the trophectoderm was the only statistically significant predictor of live birth outcome, the first time the predictive strength of TE graded over ICM for selection of superior blastocyst stage embryos. According to Thompson et al. (2013), TE morphology as well as embryo stage and patient age are highly significant independent predictors of both clinical pregnancy and live birth. The embryos falling in the lower and medium morphokinetic activity sections, such as at the 240° (TE; Table 4.11) position, may be more viable than other locations because they do not have to waste energy attempting to survive, a theory coined as the "Quiet Embryo Hypothesis" (Leese, 2002; Wells and Killingsworth, 2022). This Quiet Embryo Hypothesis can be observed in Table 4.11 that the combined lower and medium range (0 to 9 μm and 9 to 18 μm range, respectively) of morphokinetic shift in the TE (Wells and Killingsworth, 2022) movement, following the Quiet Embryo Hypothesis, throughout a 35 sec recording period resulted in more positive pregnancies, while fewer embryos overall underwent greater change in microns (18 to 27 μm). The inclusion of kinetic parameters into score evaluation may improve blastocyst selection criteria (Motato et al., 2016). With more frequent observations in TLM evaluation of embryo development, a potential benefit arises between TLM, embryo viability, and embryo selection.

4.4.2. Morula-Stage Embryo Conception Rate

Of the 22 total morula-stage embryos, a conception rate of 55% was observed (Table. 4.1). Of these 22 morula stage embryos, 17 overall were IVD cryopreserved from conventional flushing, followed by thawing and ET, while 5 were cloned embryos thawed and transferred, resulting in conception rates of 59% and 40.00% respectively (Table 4.16).

When evaluating with SAS 9.4 PROCMIXED, measurements of significance in morula stage embryos were observed at the complete X and Y axis ($P \leq 0.05$), 60° measurement of the ZP thickness ($P \leq 0.05$), as well as at 0°, 90°, 120°, and 210° perivitelline space shift of TE to ICM ($P < 0.03$; $P < 0.01$; $P < 0.03$; $P \leq 0.05$, respectively; Table 4.3). Tendencies were also observed in the ICM Y-Axis, ZP thickness, and perivitelline space shift (Table 4.3). Measurements of insignificance may be observed against those of significance in Table 4.4. When measurements of similarity were averaged, such as along each complete and ICM axis, ICM and complete area, ZP thickness, and perivitelline space shift, the average axis of the complete embryo had a probability of 99% ($P < 0.01$) significance when measured to predict embryo viability in forming a positive pregnancy. Morula stage embryos when measured along the ZP and perivitelline space shift also had a 95 and 99% probability, respectively, of predicting embryo viability in forming a positive pregnancy when measured (Table 4.5). According to Wells and Killingsworth, (2022), mean average changes in morphokinetics of embryo perivitelline space, morphokinetic changes in area of ICM, and embryos with moderate morphokinetic activity establish pregnancies at a greater rate than with high levels of morphokinetic activity. Wells and Killingsworth (2022) also reported that range of

embryo movement within the perivitelline space could be an important indicator of embryo competency (Table 4.2).

Finally, when using Fisher's Exact Test in SAS 9.4, while there were no measurements of significance, tendencies were observed along the ZP thickness at positions 0°, 30°, 60°, and 330° ($P < 0.10$; Table 4.13), as well as the measured shift in perivitelline space (TE to ICM) at the 150° and 270° position ($P < 0.10$; Table 4.14). Measurements of insignificance may be observed in Table 4.12.

4.4.3. Iolight Effect

Although not significant when comparing conception rates of morula stage embryos filmed on the Iolight Microscope protocol versus negative control morula stage embryos that were not filmed (Table 4.15), the conception rate of Iolight Protocol embryos versus the negative control was 56 and 48%, respectively ($P < 0.66$). There was an insignificant 34% probability of an Iolight effect on embryo viability according to these results.

4.5. Conclusion

Not every high-quality embryo will result in pregnancy due to varying factors that may cause a pregnancy to fail, including maternal effects, environmental effects, stress, and nutritional factors (Wells and Killingsworth, 2022). While many embryos will fail to form a positive pregnancy to term, according to Wells and Killingsworth (2022), one can expect 20% of Grade 1 and 2 embryos are incompetent at time of transfer, and the authors also suggested that embryos outside of 2 standard deviations (Table 4.1) of the mean can represent these incompetent embryos. By identifying these 20% with measurements of significance such as along the overall axis and perivitelline space shift

($P \leq 0.01$), the ZP thickness ($P < 0.05$) in equine morula stage embryos, the trophectoderm ($P \leq 0.05$) in blastocyst stage embryos, and the embryos falling in the lower to medium morphokinetic activity sections (Leese, 2002; Wells and Killingsworth, 2022), embryo dynamic developmental potential, competency and viability may be used to identify embryo health beyond traditional grading of morphological evaluation in a non-invasive, non-subjective manner. By evaluating all of these non-invasive, non-subjective factors, as well as the universally accepted embryonic features, this technology offers a wider range of parameters to rely on for the selection of viable embryos, that should therefore result in a better embryo selection (Aparicio et al., 2013).

4.6. References

Ahlström, A., C. Westin, E. Reismer, M. Wikland, and T. Hardarson. 2011. Trophectoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. Human Reprod, 26(12):3289–3296.

Aparicio, B., M. Cruz, and M. Meseguer. 2013. Is morphokinetic analysis the answer? Reprod. Biomed. Online. 27(6):654–663.

Bormann, C. L., M. K. Kanakasabapathy, P. Thirumalaraju, R. Gupta, R. Pooniwala, H. Kandula, E. Hariton, I. Souter, I. Dimitriadis, L. M. Boehnlein, and H. Shafiee. 2020. Performance of a deep learning based neural network in the selection of human blastocysts for implantation. eLife. 9:55301.

Kirkegaard, K., A. Ahlström, H. J. Ingerslev, and T. Hardarson. 2015. Choosing the best embryo by time lapse versus standard morphology. Fertil. Steril. 103:323-324.

Leese, H. J. 2002. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. BioEssays. 24(9):845-849.

Manual of the International Embryo Transfer Society. 2009. 4th ed. International Embryo Transfer Society, Champaign, IL.

McCue, P. M., and E. L. Squires. 2015. Equine Embryo Transfer (1st ed.). Teton NewMedia.

Milewski, R., A. J. Milewska, A. Kucyńska, B. Stankiewicz, and W. Kuczyński. 2016. Do morphokinetic data sets inform pregnancy potential? J. Assist. Reprod. Genet. 33:357-365.

Motato Y., M. J. Santos, M. J. Escriba, B. A. Ruiz, J. Remohí, and M. Meseguer. 2016.

Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. Fertil. Steril. 105(2):376-384.

Petersen, B. M., M. Boel, M. Montagand, D. K. Gardner. 2016. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on d 3. Human Reprod. 31(10):2231-2244.

Roque, M., K. Lattes, S. Serra, I. Solà, S. Geber, R. Carreras, and M. A. Checa. 2012. Fresh embryo transfer versus frozen embryo transfer in in vitro fertilization cycles: a systematic review and meta-analysis. Fertil. Steril. 99(1):156-162.

Saravelos, S. H., and T. Li. 2019. Embryo transfer techniques. Best Practice and Research Clinical Obstetrics and Gynecology. 59:77-88.

Thompson, S. M., N. Onwubalili, K. Brown, S. K. Jindal, and P. G. McGovern. 2013. Blastocyst expansion score and trophectoderm morphology strongly predict successful clinical pregnancy and live birth following elective single embryo blastocyst transfer (eSET): a national study. J. Assisted Reprod. and Genet. 30(12):1577–1581.

Wells, C., and R. Killingsworth. 2022. Real-time embryo morphokinetic activity can be measured in short observatory periods to provide objective evaluation of embryo health. American Embryo Transfer Association.

Wong, K. M., S. Repping, and S. Mastenbroek. 2014. Limitations of embryo selection methods. Semin. Reprod. Med. 32(02):127-133.

Wu, Y., M. Rubinstein, E. Shih, , J. Guttag, F. Durand, and W. Freeman. 2012. Eulerian video magnification for revealing subtle changes in the world. ACM Transcription on Graphics. 31(4):1-8.

Figure 4.1. *Iolight Microscope filming a blastocyst stage equine embryo.* The Iolight Inverted Microscope is depicted above recording an embryo at a magnification of 150x via Bluetooth connection to an iPhone.

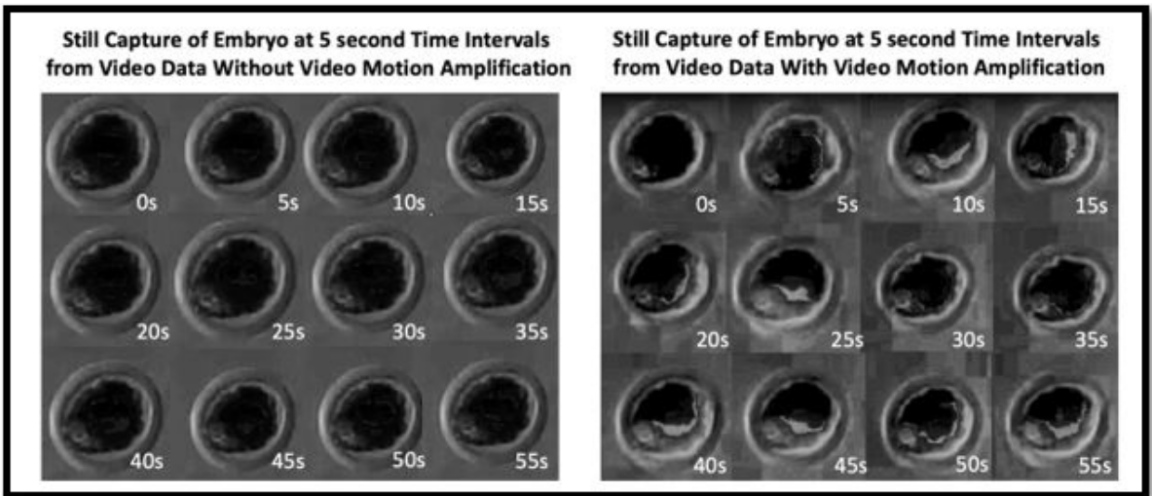

Figure 4.2. *VMM filtered versus unfiltered video data.* The raw video data (left) appears morphologically unchanged without Lambda Vue (VMM) compared to the video data (right) filtered with VMM. Morphological variations can be observed in the forms of protrusions, bulges, depressions, pulses and changes in embryo shape from this amplification step (Wells and Killingsworth, 2022).

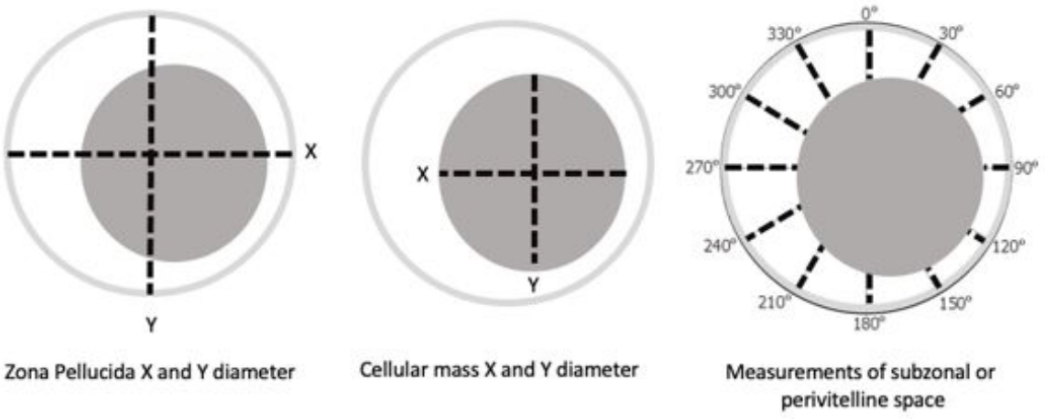

Figure 4.3. *Graphical representation of measurements collected on equine morula stage embryos*. Measurements include zona pellucida (ZP) area (not pictured), ICM area (not pictured), ZP X-axis and Y-axis diameter, ICM X-axis and Y-axis diameter, and distance between the ICM to the ZP to capture morphokinetic activity in the perivitelline space (Wells and Killingsworth, 2022).

Table 4.1. Descriptive statistics of morphokinetic change of IVD[1] equine embryos

Item	IVD[1] fresh[7]	IVD[1] cryopreserved-thawed[8]
n	19	22
Conception rate, %	73.78	54.55
Complete axis[5]		
Mean	13.54	17.77
Minimum	5.03	6.54
Maximum	32.28	33.65
SD[4]	6.37	7.27
ICM[2] axis[5]		
Mean	N/A	11.99
Minimum	N/A	4.04
Maximum	N/A	21.11
SD[4]	N/A	4.54
ZP[3] area[6]		
Mean	9.97	4660.88
Minimum	4.03	972.75
Maximum	22.05	11716.74
SD[4]	4.98	2641.74

[1]In Vivo derived
[2]Inner cell mass
[3]Zona pellucida
[4]Standard deviation
[5]Units (μm)
[6]Units (μm^2)
[7]Blastocyst stage
[8]Morula stage

Table 4.2. Descriptive statistics of morphokinetic change of IVD[1] equine embryos, continued

Item	IVD[1] fresh[8]	IVD[1] cryopreserved-thawed[9]
n[10]	19	22
Conception rate, %	73.78	54.55
ICM[2] area[7]		
Mean	N/A	2024.40
Minimum	N/A	477.83
Maximum	N/A	6501.24
SD[5]	N/A	1558.52
ZP[3] to TE[4,6]		
Mean	10.44	7.18
Minimum	2.30	0.00
Maximum	35.97	21.21
SD[5]	6.35	2.94
TE[4] to ICM[1,6]		
Mean	N/A	7.50
Minimum	N/A	0.00
Maximum	N/A	22.30
SD[5]	N/A	3.75

[1]In Vivo derived
[2]Inner cell mass
[3]Zona pellucida
[4]Trophectoderm
[5]Standard deviation
[6]Units (μm)
[7]Units (μm^2)
[8]Blastocyst stage
[9]Morula stage
[10]Embryos total

Table 4.3. Significant effects of morphokinetic ranges on pregnancy status of IVD[1] morula stage equine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[5]				
Complete Y	19.65	14.27	1.92	0.05
Complete X	21.34	14.72	2.38	0.05
ICM[2] Y	13.35	9.80	1.36	0.07
Area[6]				
Complete	5569.61	3570.40	789.79	0.08
ZP[3] to TE[4,5]				
90°	8.94	6.38	0.94	0.06
270°	9.25	6.35	2.20	0.09
60°	9.04	6.59	0.86	0.05
TE[4] to ICM[2.5]				
0°	8.00	4.82	0.96	0.03
90°	8.08	4.34	0.96	<0.01
120°	10.27	6.32	1.27	0.03
210°	8.93	5.30	1.27	0.05
240°	9.03	5.83	1.17	0.06

[1]In Vivo derived

[2]Inner cell mass

[3]Zona pellucida

[4]Trophectoderm

[5]Units (μm)

[6]Units (μm^2)

Table 4.4. Effect of pregnancy status on all morphokinetic ranges of IVD[1] morula stage equine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[5]				
Total Y	19.65	14.27	1.92	0.05
Total X	21.34	14.72	2.38	0.05
ICM[2] Y	13.35	9.80	1.36	0.07
ICM[2] X	11.96	12.60	1.49	0.76
Area[6]				
Total	5569.61	3570.40	789.79	0.08
ICM[2]	1970.10	2089.56	504.63	0.86
ZP[3] to TE[4,5]				
0°	7.79	6.97	1.13	0.60
30°	6.75	5.95	0.97	0.55
60°	9.04	6.59	0.86	0.05
90°	8.94	6.38	0.94	0.06
120°	7.18	6.46	0.43	0.23
150°	7.45	6.34	0.83	0.33
180°	9.03	6.77	1.01	0.11
210°	7.23	5.50	0.84	0.14
240°	7.63	6.41	0.80	0.27
270°	9.25	6.35	2.2	0.09
300°	7.15	6.02	0.71	0.26
330°	6.73	6.66	0.97	0.95
TE[4] to ICM[2,5]				
0°	8.00	4.82	0.96	0.03
30°	8.01	5.76	1.34	0.24
60°	8.22	7.58	1.42	0.74
90°	8.08	4.34	0.96	<0.01
120°	10.27	6.32	1.27	0.03
150°	7.86	6.55	1.01	0.35
180°	9.54	7.84	0.99	0.23
210°	8.93	5.30	1.27	0.05
240°	9.03	5.83	1.17	0.06
270°	9.54	7.84	0.99	0.22
300°	9.10	7.54	1.28	0.38
330°	7.54	6.25	0.86	0.28

[1]In Vivo derived
[2]Inner cell mass
[3]Zona pellucida
[4]Trophectoderm
[5]Units (μm)
[6]Units (μm2)

Table 4.5. Effect of averaged morphokinetic ranges on pregnancy status of IVD[1] morula stage equine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[5]				
Total	20.49	14.41	1.45	<0.01
ICM[2]	12.66	11.20	1.03	0.31
Area[6]				
Total	5569.61	3570.40	789.79	0.08
ICM[2]	1970.10	2089.56	504.63	0.86
ZP[3] to TE[4,5]				
	7.85	6.37	0.53	0.05
TE[4] to ICM[2,5]				
	8.54	6.24	0.62	0.01

[1]In Vivo derived
[2]Inner cell mass
[3]Zona pellucida
[4]Trophectoderm
[5]Units (μm)
[6]Units (μm2)

Table 4.6. Effect of morphokinetic ranges on pregnancy status of IVD[1] blastocyst stage equine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[3]				
Complete Y	12.78	14.07	2.65	0.68
Complete X	14.74	11.74	3.17	0.43
Area[4]				
Complete	9067.51	3594.07	2789.46	0.11
TE[2,3]				
0°	10.31	9.01	2.28	0.63
30°	10.68	6.69	3.05	0.28
60°	11.62	6.88	3.14	0.21
90°	13.70	7.44	3.11	0.10
120°	13.19	6.99	3.90	0.19
150°	10.59	8.60	3.15	0.59
180°	8.08	9.89	2.50	0.54
210°	10.66	8.26	2.69	0.46
240°	10.63	7.18	2.02	0.16
270°	11.82	9.38	2.93	0.48
300°	11.79	7.24	2.46	0.13
330°	11.52	8.47	2.62	0.33

[1]In Vivo derived

[2]Trophectoderm

[3]Units (μm)

[4]Units (μm^2)

Table 4.7. Significant effects and tendencies of morphokinetic ranges on pregnancy status of IVD[1] blastocyst stage equine embryos

	Pregnancy Status			
Item (TE[2,3])	Yes	No	SEM	*P* - Value
90°	13.70	7.44	3.11	0.10

[1]In Vivo derived

[2]Trophectoderm

[3]Units (μm)

Table 4.8. Effect of average morphokinetic ranges on pregnancy status on IVD[1] blastocyst stage equine embryos

	Pregnancy Status			
Item	Yes	No	SEM	*P* - Value
Axis[3]				
	13.76	12.90	2.44	0.77
Area[4]				
	9067.51	3594.07	2789.46	0.11
TE[2,3]				
	11.37	7.85	2.19	0.19

[1]In Vivo derived

[2]Trophectoderm

[3]Units (μm)

[4]Units (μm^2)

Table 4.9. Comparison of range of morphokinetic activity on measured values of blastocyst stage IVD[1] equine embryos by pregnancy status

Item	Fisher's exact test *(P)*[2]
n[5]	19
Y Axis[3]	0.49
X Axis[3]	0.80
Area[4]	0.15

[1]In Vivo derived

[2]One-tailed *P* value

[3]Units (μm)

[4]Units (μm^2)

[5]Embryos total

Table 4.10. Comparison of range of morphokinetic activity on measured values of blastocyst stage IVD[1] equine embryos by pregnancy status

Item measured (TE[3])[4]	Fisher's exact test *(P)*[2]
n[5]	19
0°	1.00
30°	0.93
60°	1.00
90°	0.50
120°	0.79
150°	0.64
180°	0.73
210°	0.82
240°	0.05
270°	1.00
300°	0.34
330°	0.80

[1]In Vivo derived

[2]One-tailed *P* value

[3]Trophectoderm

[4]Units (μm)

[5]Embryos total

Table 4.11. Comparison of range of morphokinetic activity (TE[3]) 240° position of blastocyst stage IVD[1] equine embryos by pregnancy status

	Range of morphokinetic activity by group[4,5]			
Item	0 to 9	9 to 18	18 to 27	Fisher's exact test *(P)*[2]
Pregnancy status, *n* (%)				
Yes	5 (50.00)	8 (100.00)	1 (100.00)	$P \leq 0.05$
No	5 (50.00)	0 (0.00)	0 (0.00)	

[1]In Vivo derived

[2]One-tailed *P* value

[3]Trophectoderm

[4]Units (μm)

[5]Range based on Wells and Killingsworth (2022) Methodology

Table 4.12. Comparison of range of morphokinetic activity on measured values of morula stage IVD[1] equine embryos by pregnancy status

Item		Fisher's exact test *(P)*[2]
n[6]	22	
Axis		
Complete X-Axis[4]		0.63
Complete Y Axis[4]		0.18
ICM[3] X-Axis[4]		0.28
ICM[3] Y-Axis[4]		0.42
Complete Area[5]		0.67
ICM[3] Area[5]		0.51

[1]In Vivo derived

[2]One-tailed *P* value

[3]Inner cell mass

[4]Units (μm)

[5]Units (μm^2)

[6]Embryos total

Table 4.13. Comparison of range of morphokinetic activity on all measured values of morula stage IVD[1] equine embryos by pregnancy status

Items (ZP[3] to TE[4])[5]	Fisher's exact test *(P)*[2]
n[6]	22
0°	1.00
30°	1.00
60°	1.00
90°	0.22
120°	0.62
150°	0.82
180°	0.66
210°	0.39
240°	0.82
270°	0.22
300°	0.66
330°	1.00

[1]In Vivo derived

[2]One-tailed *P* value

[3]Zona pellucida

[4]Trophectoderm

[5]Units (μm)

[6]Embryos total

Table 4.14. Comparison of range of morphokinetic activity on measured values of morula stage IVD[1] equine embryos by pregnancy status

Items (TE[3] to ICM[4])[5]	Fisher's exact test *(P)*[2]
n[6]	22
0°	0.48
30°	0.48
60°	0.27
90°	0.66
120°	0.48
150°	1.00
180°	0.21
210°	0.27
240°	0.19
270°	1.00
300°	0.39
330°	0.47

[1]In Vivo derived

[2]One-tailed *P* value

[3]Trophectoderm

[4]Inner cell mass

[5]Units (μm)

[6]Embryos total

Table 4.15. Conception rates of IVD[1] morula stage equine embryos analyzed with the Iolight Microscope and negative controls

	Iolight Microscope		
Item	Yes	No	*P* - Value
IVD[1], cryopreserved-ET[2]			
N[3]	22	21	0.66
Conception rate, %	55.55	47.62	

[1]In Vivo derived

[2]Embryo transfer

[3]Embryos total

Table 4.16. Embryo type versus conception rate

Item	IVD[1] Fresh[3,5]	IVD[1] cryopreserved-thawed[4,2]	Cloned cryopreserved-thawed[4]
n	19	17	5
Conception rate, %	73.78	58.82	40.00

[1]In Vivo derived
[2]Not including cloned embryos
[3]Conventional flushing
[4]Morula stage
[5]Blastocyst stage