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## The Use of Assisted Reproductive Technologies to Improve Pregnancy Rates in White-tailed Deer

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THE USE OF ASSISTED REPRODUCTIVE TECHNOLOGIES TO  
IMPROVE PREGNANCY RATES IN WHITE-TAILED DEER

A Dissertation

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

The Interdepartmental Program in  
the School of Animal Sciences

by

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## ABBREVIATIONS USED IN DISSERTATION

ABP – androgen binding protein  
AI – artificial insemination  
AR – acrosome reaction  
BIVF – bovine *in vitro* fertilization  
BSA – bovine serum albumin  
cAMP - cyclic adenosine monophosphate  
CA – capacitation agent  
Ca<sup>2+</sup> - calcium  
CIDR – controlled internal drug release device  
CIDR-G – controlled internal drug release device for goats  
CIDR-B - controlled internal drug release device for bovine  
CL – corpus luteum  
CO<sub>2</sub> – carbon dioxide  
CUB – clean-up buck  
DF – dominant follicle  
DHT - 5-dihydrotestosterone  
DISP – disposition  
DMSO – dimethyl sulfoxide  
DSOF – deer synthetic oviductal fluid  
eCG – equine chorionic gonadotropin  
FITC-PNA - fluorescein isothiocyanate conjugated with *Aechis hypogaea* (peanut)  
Agglutinin  
FSC - forward-scattered light  
FSH – follicle stimulating hormone  
FT – frozen-thawed  
FTAI - fixed-timed artificial insemination  
GAGS - glycosaminoglycans  
GnRH – gonadotropin releasing hormone  
HDL – high-density lipoprotein  
IACUC – Institutional animal care and use committee  
ID – insemination depth  
II – insemination interval  
IT – insemination time  
IU – international units  
IVC – *in vitro* culture  
IVF – *in vitro* fertilization  
IVM – *in vitro* maturation  
IVP – *in vitro* production  
LC - lysophosphatidylcholine  
LH – luteinizing hormone  
LN<sub>2</sub> – liquid nitrogen  
M540 – merocyanine 540  
MOET – multiple ovulation embryo transfer  
NADeFA – North American Deer Farmer Association

P4 – progesterone  
PC – phosphocholine  
PE - phosphoethanolamine  
PGF<sub>2α</sub> – prostaglandin F<sub>2α</sub>  
PI - phosphatidylinositol  
PKA - protein kinase A  
PM – plasma membrane  
PrI – propidium iodide  
PS - phosphatidylserine  
RD – red deer  
sAC - soluble adenylyl cyclase  
SOF – synthetic oviductal fluid  
SOFaaBSA – synthetic oviductal fluid with amino acids and bovine serum albumin  
SM – sphingomyelin  
SS – 20% sheep serum  
SSC - side-scattered light  
TALP - Tyrode's albumin lactate pyruvate  
WT – white-tailed deer  
ZP – zona pellucida

## ABSTRACT

Detection of estrus in white-tailed (WT) deer is laborious and often missed, as there are few visual cues presented during the 24 h period in which females are receptive to mating. Reproductive management of WT operations is improved by the use of fixed-timed artificial insemination (FTAI) protocols. Fixed-timed AI removes the need for estrus detection and ensures the breeding of an entire herd, as well as the ability to schedule fawning within predetermined timeframes. Two studies evaluated two FTAI regimens and their ability to synchronize estrous in WT. The first study implemented the administration of eCG 200 IU (im) at progesterone device removal (CIDR-g), which had been present for 14 d, followed by FTAI 60 h post-CIDR-g removal. Findings from this study demonstrated that eCG given at CIDR removal did not affect pregnancy rates, nor did the fecundity rates of does pregnant to FTAI differ from does pregnant from natural mating. Does that were inseminated  $\geq 60.5$  h after CIDR-g removal were 22 times more likely to become pregnant following FTAI than does which were inseminated  $\leq 60.5$  h post-CIDR-g removal. The second study evaluated the effect of GnRH administered at insemination on pregnancy rates following a 14 d CIDR-g regimen. Similar to the previous study, does inseminated beyond 60.5 h post-CIDR-g withdrawal were four times more likely to become pregnant to FTAI. The odds of pregnancy also increased 5 times when does were treated with GnRH. However, the pregnancy rates from this study were lower than those when eCG was given at CIDR-g withdrawal. In addition to improving WT estrous synchronization, FTAI can lead to further improvements in WT *in vitro* production and propagation of desired genetics throughout a herd. The third study evaluated the

chemical components of two *in vitro* fertilization media (BIVF and DSOF) and their ability to induce capacitation and the acrosome reaction in WT epididymal spermatozoa. Induction of lipid disorder was more likely when spermatozoa were in the presence of BIVF than DSOF. Additionally, when compared to supplementation of sheep serum, heparin in BIVF was more likely to improve capacitation rates. However, higher levels of calcium concentrations in DSOF were twice as likely to induce the acrosome reaction (AR).

## CHAPTER 1 INTRODUCTION

Cervid farming is one of the newer avenues in agriculture, and its prevalence across the United States has increased in the last several decades. Similar to all other agricultural divisions, cervid farming is composed of both producers and consumers. Producers consist of breeding facilities, trophy hunting preserves, commercial venison producers, and commercial scent collection (Anderson et al., 2007). According to the North American Deer Farmer Association (NADeFA), there were an estimated 7,828 cervid farms across the nation in 2007. Approximately 34% of those farms were classified as hunting preserves (Anderson et al., 2007). Hunting preserves maintain their genetic standards by either breeding from within their own stock or by purchasing cervids of desired genetic quality from breeding facilities to phenotypically enhance their current stock. Coinciding with NADeFA estimations, Texas A&M University generated a summary of the economic impact of the cervid farming industry. Results from this study indicated that cervid operations had a direct impact on the U.S. economy of \$893.5 million, indirect industry impacts of \$2.3 billion, and hunter contributions of \$757 million. In total, \$3.0 billion has been generated annually from both farming and hunter constituents (Anderson et al., 2007).

The *Cervidae* family consists of 42 species; 16 species are vulnerable and nine species are considered to be endangered, critically endangered, or extinct in the wild (IUCN 2011 Red List of Threatened Species). Members of the *Cervidae* family commonly farmed in North America include: white-tailed deer (WT), elk, red deer (RD), fallow, axis, reindeer, and sika. One of the more prominently farmed cervids is the WT,

due to their ability to acclimate to a vast array of climates and terrain, with their geographical distribution ranging from Canada to southern Peru, while sustaining their prolific capabilities (Jabbour et al., 1997). Ensuring genetic preservation of natural inhabitants of a reduced population, as well as ensuring genetic advancement and avoidance of inbreeding depression of captive herds has elevated the importance of reproductive studies and use of assisted reproductive techniques in cervids. Despite lineage similarities, there is not a single species considered to be a representative of all cervids for reproductive characteristics (Asher et al., 1999). Expansion of reproductive knowledge of farmed deer does not only aid in increasing future economic impact, but also allows captive species to act as systemic research subjects for *ex situ* populations. Such data attained from farmed populations includes habitat usage and preservation, further understanding of reproductive mechanisms, and application of reproductive technologies for enhancement of genetic and population management (Pukazhenthii et al., 2006).

To date, minimal data have been published regarding reproductive technologies in WT. This is due to the dichotomy of the perceived value of this species. White-tailed deer are considered to be a “pest” species due to overpopulation in some geographical locations, a necessity for game ranchers due to trophy hunting, or as a useful reproductive model for *ex situ* deer populations and endangered cervidae. For this farmed species, which does not adapt to human management systems as well as other species (Asher, 2011) and are prone to being highly stressed if handled too frequently (Willard et al., 2002), the importance and value of defining a fixed-timed artificial insemination (FTAI) program increases. Manipulation of the female reproductive cycle

allows for FTAI, decreases animal stress due to reduced handling of the animals, as well as eliminating the need for estrus detection. The most common tool for estrous synchronization in smaller cervidae is the implementation of a vaginally inserted progestin device (CIDR-g) for a period of 14 d (Asher et al., 1993; Morrow et al., 1995).

Incorporation and use of prolonged exogenous progestin synchronizes estrous; however, it does not synchronize follicular waves. Previous studies, in cattle, have reported the effects of administering exogenous gonadotropins, estrogen, and prostaglandins and their roles in follicular wave control when administered concurrently with progestin devices (Fricke et al., 1998; Ambrose et al., 2001; Martinez et al., 2002; Richardson et al., 2002; Kasimanickam et al., 2006; Kim et al., 2006). Results reported have stated that the time needed for pre-synchronization and synchronized follicular recruitment and corpus luteum (CL) regression is reduced when incorporating exogenous hormones to a progesterone synchronization protocol (Thatcher et al., 2001). However, utilization of some of these multi-exogenous hormone estrous synchronization protocols requires additional human to animal contact, which is not conducive within a WT operation. Therefore, the first objective of the studies presented herein was to determine if administration of exogenous gonadotropins (eCG or GnRH) administered within a FTAI regimen, each protocol requiring minimal animal handling, would improve pregnancy rates in WT.

When using a domestic species to obtain knowledge that may be applied to endangered species, it is imperative that assisted reproductive technologies are not completely focused on a single sex, but rather male and female gametes be taken into consideration. Information attained from improved estrous synchronization protocols

will lead to successive *in vitro* studies in which properly formulated *in vitro* environments are essential for gamete maturation, fertilization and embryo culture. To ascertain requirements for *in vitro* production (IVP) of WT embryos, the medium components required to induce sperm capacitation and the acrosome reaction (AR), which are necessary for fertilization to occur, must first be identified.

Capacitation is a maturational process which sperm must undergo before they become capable of fertilization. Capacitation research has been thoroughly conducted in both domesticated bovine and ovine. These applications have been applied to a few cervid species (red deer, fallow, and sika) and yielded variable results (Berg and Asher, 2003). Currently, we have found no capacitation studies in WT in the scientific literature. For an *in vitro* protocol to be successful in inducing capacitation, the medium environment should mimic the oviductal fluid and its ability to remove seminal plasma proteins from the sperm surface.

Established IVP protocols are species specific in regards to their media composition and capacitating agents (CA). The bovine IVP system is applicable to development of IVP embryos in wapiti (Pollard et al., 1995); however, this system does not translate to all cervid species (Chapman et al., 1999; Harnal et al., 2000; Berg and Asher, 2003). Comparison of the bovine and red deer systems reveals a distinct difference in the CA used (heparin and 20% sheep serum, respectively), as well as the amount of bicarbonate and calcium necessary for fertilization to occur (Berg and Asher, 2003). Red deer estrual oviductal fluid also has reduced levels of phosphate, as well as a low presence of glucose, when compared to bovine oviductal fluid (Berg and Asher, 2003). Currently, the composition of WT oviductal fluid is unknown and obtaining estrual

oviductal samples is difficult, as a result of seasonality and ability to identify females in estrus. Therefore, the objective of the third study evaluated the capacitation inducing ability of two CA (heparin and 20% sheep serum) in either a standard bovine or red deer (DSOF) fertilization medium on WT epididymal spermatozoa.

Establishment of proper *in vitro* fertilization (IVF) and *in vitro* culture (IVC) media for WT IVP can aid in proliferation of desired genetics throughout a farmed herd, as well as utilization of all does within a herd, as those with less desirable genetics could be used as surrogates. In addition, basic reproductive and *in vitro* knowledge obtained from WT studies are applicable to endangered species with goals of maintaining genetic diversity and proliferation of wild populations.

## **CHAPTER 2 REVIEW OF LITERATURE**

Cervids have been classified into two categories: Old World and New World species. These groups are distinguished based on ranges of chromosomal number, anatomical differences, antler regrowth intervals, estrous cycle length, and fecundity. Of the seven species commonly farmed in North America, both red deer (Old World species) and WT (New World species) have the highest herd inventory for breeding and hunting (Anderson et al., 2007). Red deer tend to be larger bodied, more seasonal as their habitats are more defined, exhibit a longer breeding life, and are monotocous (Asher, 2011). White-tailed deer, however, are small-bodied, have a shorter breeding life (<10 years) and produce multiple progeny each season (Asher, 2011). A unique aspect of this species is its ability to habituate across diverse geographical regions, from arctic forests to temperate lowlands. Although rut (breeding season) typically occurs during the fall and winter months when day length is shorter, it can continue year-round for the populations in tropical climates.

### **The Estrous Cycle Of White-Tailed Deer**

The reproductive timing of cervids is reliant on their endogenous circannual clock, genetics, and photoperiod. During periods of reduced day length the pineal gland is triggered to produce melatonin, which stimulates gonadotropin release and induction of gamete development and maturation (Malpaux et al., 2001). In the female, hypothalamic release of pulsatile gonadotropins initiates follicular development and recurrent estrous cycles. In larger species, such as cattle and elk, transrectal manipulations and ultrasonography of female reproductive tracts have been employed

to track follicular development. However, reproductive information cannot be obtained from WT does in the same manner due to their size and inability to properly visualize their ovaries via ultrasound. Thus far, studies have used ovaries from random, hunter-killed does from wild populations.

White-tailed deer ovaries collected during anestrus have revealed that the majority of antral follicles present at this time tend to be atretic (Harder and Moorhead, 1980). Follicular presence in WT, during transition months (August through October) and during the initial portion of the breeding season (November), remains fairly constant (2 to 5 follicles > 3 mm) (Harder and Moorhead, 1980). The percentage of atretic follicles is higher during transition months and during mid-November in comparison to the proportion of atretic follicles present at the onset of the breeding season (80% and > 90% vs 46%, respectively) (Harder and Moorhead, 1980). Follicular observations revealed an inverse relationship between the proportion of atretic follicles and the occurrence of regressing and/or active corpus lutea. Early in the breeding season does average one to two corpus lutea (regressing and/or active) with little variation, representing the occurrence of multiple ovulations. Coincident with the commencement of the breeding season, follicular atresia decreases and the occurrence of synchronized ovulation increases (Harder and Moorhead, 1980).

Ovarian follicular dynamics incorporates three phases: recruitment, selection and regression. It is these phases that encompass procurement of small follicles into a cohort for maturation with gonadotropin support to aid in progression towards ovulation. Most domestic species have two, three or four waves of synchronous follicular development during each estrous cycle and it is within each of these periods of follicular

growth that these phases occur (goat: Ginther and Kot, 1994; cattle: Ginther et al., 1996; sheep: Evans et al., 2000; wapiti: McCorkell et al., 2006; red deer: Asher et al., 1997). Similar to domestic species, WT have been reported to have an estrous cycle of 21 to 28 d in length, suggesting the presence of three to four follicular waves per cycle (Harder and Moorhead, 1980). Although no data have been reported, to this author's knowledge, on the details of white-tailed deer follicular development, estrus synchronization protocols have demonstrated similar ovarian responses to hormonal manipulation, suggesting similarities in follicular dynamics.

In species in which ovarian assessments have been conducted, most follicular growth occurs in waves of seven days in length. During the first half of the initial wave (Day 1 to 4 of cycle), gonadotropin dependent antral follicles (1.8-3.4 mm, 2-4 mm and 4-6 mm, in red deer, sheep and cattle, respectively) are recruited (Asher et al., 1997; Evans, 2003; Driancourt, 2001). In concert with follicle stimulating hormone (FSH) release, these follicles continue cellular growth (3-5 mm and 6 -9 mm, in sheep and cattle respectively) (Ravindra and Rawlings, 1997; Driancourt, 2001) until mid-wave (Day 4 of cycle), when a large, healthy dominant follicle (DF) is selected. By Day 5 of the estrous cycle, the DF secretes both estradiol and inhibin during development, separating itself and inhibiting growth of remaining subordinate follicles via a negative feedback on FSH (Forde et al., 2011). Although complete follicular data in WT are absent, hormonal data of the first follicular wave is similar to that of cattle, with estrogen rising (7 pg/ml) around Day 4, suggesting similarities in follicular development (Plotka, 1980; Kaneko et al., 1995; Evans, 2003). During Days 5 to 7 of the estrous cycle, the non-ovulatory DF continues to develop (>5 mm, 5-7 mm and 10-15 mm, in WT, sheep

and cattle, respectively) due to high frequency pulsatile luteinizing hormone (LH) secretion, which is released from the pituitary in response to hypothalamic gonadotropin secretion (Evans, 2003; Curtis et al., 2008; Asher, 2007). During the early and mid-luteal phase, LH pulses are insufficient (20-30 pulses/24 h and 6-8 pulses/24 h, respectively) to induce final maturation and the subsequent ovulation of the DF (Forde et al., 2011). As a result, the DF commences atresia; the negative feedback block of FSH on the hypothalamus/pituitary axis is removed, therefore allowing FSH secretion and initiation of a new follicular wave.

During DF growth and development within each wave, the number of small, recruited follicles increases. Assessment of red deer ovaries, revealed approximately 27 follicles  $\geq 2$  mm in diameter present at any point in time during the estrous cycle, with up to 12 follicles  $\geq 4$  mm and at least one DF ( $\geq 7.5$  mm) (McLeod et al., 2001). This is comparative to ultrasonography data in cattle, which have 3 to 10 follicles  $\geq 4$  mm in diameter in the ovaries at any given point in time (Evans, 2003). Following DF atresia, these newly recruited follicles repeat the development and selection process during the next 7 to 9 days (second follicular wave, Days 10 to 18 of estrous cycle). Recruitment, selection and regression continue in subsequent waves until an ovulatory DF (6-7 mm, 7 mm, 8.5-12.5 mm, and 12-20 mm, in sheep, red deer, wapiti and cattle respectively) is formed (Evans, 2003; Asher et al., 1997; McCorkell et al., 2006). During the follicular phase of the estrous cycle, when progesterone (P4) levels are basal, the estradiol produced by the ovulatory DF is greater, which induces a GnRH surge from the hypothalamus and expression of behavioral estrus (Forde et al., 2011). This GnRH

surge initiates an LH surge, which occurs at an amplitude and frequency that induces DF maturation and subsequent ovulation.

Behavioral estrus in WT is commonly exhibited when P4 levels are low (0.5 ng/ml), plasma estradiol concentrations increase (6-12 pg/ml) and the pre-ovulatory LH surge occurs (35.0 to 60.1 ng/ml in WT), which peaks approximately 24 h prior to ovulation (Plotka et al., 1980; Knox et al., 1992; Asher, 2007; Asher, 2011). Within 24 h post-behavioral estrus, LH concentrations decrease to basal levels ( $\leq 1.0$  ng/ml) (Knox et al., 1992). Following the LH surge and rupture of the DF, progesterone levels remain below 1 ng/ml until 72 h post-estrus when levels increase to around 5 ng/ml, which is indicative of corpus luteum formation and function (Plotka et al, 1980).

### **Synchronization Of Estrus In White-Tailed Deer**

Implementation of FTAI eliminates variability associated with estrus detection. Fixed -time AI allows producers to inseminate all synchronized females at a specified time, as well as improve herd genetics by inseminating with frozen-thawed (FT) semen from genetically desired sires outside of the herd. Fixed-time AI protocols aim to synchronize ovulation by synchronizing wave emergence and the pre-ovulatory LH surge (Martinez et al., 2002b). Progesterone has been the primary hormone used in controlling the female reproductive cycle, as it has proven to reliably inhibit estrus and ovulation by inhibition of gonadotropin release and suppression of follicular development (Ulberg et al., 1951). Further studies have shown that daily administration of exogenous P4 yielded satisfactory synchrony, yet low fertility rates. This advancement in ovarian control left the want for reduced management time and need for increased fertilization rates.

The amount of endogenous P4 produced by the CL differs between species. Therefore, the efficiency of exogenous P4 for hormonal control is dependent on species and dosage amounts. This variation creates a challenge when identifying proper P4 dosage levels for estrous cycle manipulation. When P4 dosage levels fall below the species-specific threshold it results in shorter intervals from the cessation of exogenous P4 treatment to estrus, continuation of follicular growth during treatment, as well as a persistent cystic state (Savio et al., 1993; Wehrman et al., 1993).

Administration of persistent exogenous progestins lengthens the estrous cycle, enhances ovarian control by suppressing the release of endogenous LH and therefore, minimizes the window for insemination or mating. The use of a controlled internal drug releasing devices (CIDR) has become commonplace in livestock management and has provided multiple avenues for advanced research into the usefulness and efficiency of CIDR protocols. As observed in sheep, seasonal species, like fallow deer, exhibit a reduced and delayed response when exogenous P4 is applied outside of the traditional breeding season (Morrow et al., 1995). With seasonal species or non-seasonal individuals that exhibit prolonged periods of anestrus, the quiescent period of follicular growth can be lessened by the use of hormonal treatments.

However, as the breeding season progresses, the interval from P4 removal to onset of behavioral estrus shortens. CIDR protocols have been applied to cattle (CIDR-B: 1.9 g (10% w/w) P4), goats, sheep and fallow deer (CIDR-G: 0.3 g (9% w/w) P4), as well as multiple non-domestic species, for periods ranging from 4 to 20 d (Fennessy et al., 1994; Jabbour et al., 1994; Morrow et al., 1995; Hosack et al., 1999). Supporting cattle and sheep data, deer studies agree that 12 or 14 d CIDR protocols inhibit estrus

and ovulation during treatment, with estrus and ovulation occurring shortly after CIDR withdrawal (Nash et al., 2012; Wheaton, et al., 1993; Asher et al., 1988; Morrow et al., 2009). Following the withdrawal of CIDR-G devices, the proportion of females which exhibit behavioral estrus increases and the interval from CIDR-G device removal to estrus shortens (Asher et al., 1993; Macmillan et al., 1993). In the Eld's deer, estrus occurred 45-52 h post CIDR-G removal, a range similar to that reported in cattle, with ovulation occurring 50-60 h following the end of P4 treatment, which is comparable to ovulation times in both ewes and does (Hosack et al., 1999). Fallow deer have demonstrated acceptable conception rates (~65%) when inseminated 48 h after CIDR removal (Asher et al., 1988).

### **Synchronization Protocols**

For a reduction in animal handling and time spent detecting natural estrus, steroids, prostaglandins and/or gonadotropins have been used, in cattle, in conjunction with exogenous P4 (Lane et al., 2001; Niasari-Naslaji et al., 2001; Peters et al., 1977; Busch, et al., 2007; Schafer et al., 2007; Dobbins et al., 2009; Kasimanickam et al., 2012; Twagiramungu et al., 1995; Beal, 1998; Kasimanickam et al., 2006; Lamb et al., 2006; Larson et al., 2006). Combination of CIDR-B with estradiol, GnRH, or porcine LH has provided suitable synchronization of follicular wave emergence and similar pregnancy rates (62%, 65%, and 56%, respectively) in beef heifers (Martinez et al., 2002). Comparable pregnancy rates have been reported in RD, fallow deer, and WT (Asher et al., 1993; Gentry et al., 2008).

Estradiol used in conjunction with a CIDR-B protocol has been employed to treat cows that are in anovulatory anestrus or in seasonal females at the start of the breeding

season. Administration of exogenous P4, in combination with estrogen, inhibits premature ovulation by preventing the estrogen-induced LH surge (Lane et al., 2001). When estradiol is administered at the beginning of the synchronization regimen it decreases the period (9 d from 14 d) for persistent P4, without decreasing the number of females that exhibit estrus (Beal, 1998; Lane et al., 2001). However, estrogen treatments have provided a varying range of luteolysis due to treated animals being in different stages of follicular development at treatment initiation (Bo et al., 1995). When estrogen is used in conjunction with both exogenous P4 and prostaglandin in beef cattle FTAI regimens, the induced synchronous ovulation consistently results in higher pregnancy rates in both heifers and cows (Lammoglia et al., 1998; Martinez et al., 2000).

Prostaglandin ( $\text{PGF}_{2\alpha}$ ) is a hormone that is luteolytic to females that have a CL present (d7 through d15 of the estrous cycle). To ensure ovulation synchrony, two doses of  $\text{PGF}_{2\alpha}$  are used so that the majority of the females undergo luteolysis (9 d apart and 11 d apart for sheep and goats, respectively) (Tamassia, 2011). Following ovulation, insemination or breeding should occur 42-54 h following the last injection in both sheep and goats (Tamassia, 2011). However, as this method requires multiple occasions of animal handling, it is not conducive to deer estrous synchronization regimes.

In females, which are enduring a prolonged anestrus, gonadotropin treatments, such as equine chorionic gonadotropin (eCG), have been applied to initiate estrus and ovulations (Leyva et al., 1998; Rhoades et al., 2003). Administration of eCG at CIDR-B removal has shown to stimulate behavioral estrus in treated cows (Macmillan and

Peterson, 1993). Red deer that received eCG (1200 IU) 72 h prior to CIDR removal had a decreased duration of onset to estrus and time to ovulation in comparison to hinds that only received exogenous P4 (16 h to 43 h and 31 h to 71 h, respectively) (Jabbour et al., 1994). Additionally, the administration of eCG (600 IU) increased the percentage of cows that ovulated (89% vs. 77%, respectively) however; it did not increase the percentage of females in estrus (73% vs. 68%, respectively). The incorporation of eCG in a short CIDR protocol in dairy cattle resulted in shorter inter-estrus intervals compared to non-treated females (9 d and 14 d, respectively), but produced below standard pregnancy rates (39%) (Xu et al., 1997).

In order to improve reproductive performance following estrus synchronization, protocols need to eliminate the presence of the dominant follicle at the beginning of a CIDR treatment with subsequent synchronization of new follicular development. Final stages of preovulatory follicular development are influenced by endogenous LH pulse frequencies (Ginther et al., 2012; Lindsey et al., 2002). Maintenance of LH pulses is reliant upon endogenous release of a hypothalamic peptide (gonadotropin-releasing hormone (GnRH)) pulses and the interactions within the hypothalamic-pituitary-adrenal axis. Treatment with exogenous GnRH induces follicular atresia and stimulation of a new follicular wave by artificially generating LH pulses (Morrow et al., 2009; McLeod et al., 1991; Sato et al., 2005). In a study evaluating follicular wave emergence in Holstein cows, treatments of GnRH and estradiol were compared and CIDR + GnRH protocols yielded new follicular activity within 2-4 d, proper follicular growth, synchrony of ovulation and increased pregnancy rates in comparison to estradiol treated females (65% and 35%, respectively) following FTAI (Kim et al., 2005). Calving rates were also

improved in Holstein-Friesian cows when GnRH is given at insemination in comparison to cows not treated with GnRH (57% and 49%, respectively) (Nakao et al., 1983).

In sheep, the use of GnRH 24 h post-CIDR withdrawal benefits FTAI protocols as it reduces the interval between CIDR removal and estrus (45-48 h vs 57-60 h in ewes without GnRH and in ewes receiving GnRH 36 h post-CIDR removal, respectively), as well as initiating the LH peak to occur 10 to 21 h earlier than the natural peak (34.5-45.5 h post-CIDR removal (Walker et al., 1989). However, administration of GnRH at 24 h or 36 h following progesterone removal did not improve pregnancy rates in ewes (Control: 62% vs 37% and 66%, respectively) (Walker et al., 1989). Similar results of reduced interval from progesterone removal to LH surge and less variability in ovulation timing were reported in Pygmy and Nigerian Dwarf goats (Pierson et al., 2003).

McLeod et al. evaluated the effect of intermittent or continuous GnRH injections in anoestrus Péré David's deer and demonstrated that both exogenous gonadotropin treatments increased LH concentrations, shortened the interval to the onset of estrus, and induced ovulation in more than 80% of treated females (1991).

### **Male Reproductive Physiology**

The male reproductive system produces, matures and stores spermatozoa, as well as deposits semen into the female tract during copulation. Production of spermatozoa (spermatogenesis) occurs in the testes, which are suspended by a spermatic cord and located within a scrotal sac. The scrotum reduces testicular temperature compared with the core body temperature via a testicular venous network known as the pampiniform plexus. This reduction in temperature is needed for optimal

sperm production to ensue. Each testis is composed of two types of tissues: an inner tubular compartment of seminiferous tubules and an outer layer of interstitial tissue.

The seminiferous tubules consist of seminiferous epithelium that contains somatic Sertoli cells and germinal cells within the adluminal region and a basement membrane that separates each tubule from the interstitial tissue. In the prepubertal male, immature Sertoli cells continuously divide. The quantity of Sertoli cells governs testicular size, the mass of germ cells per testis and final spermatozoa production (Walker et al., 2005). When the male reaches puberty the proliferation of Sertoli cells ceases, they elongate and form tight junctions between adjacent Sertoli cells (Walker and Cheng, 05). These cells, and their junctions, contribute to the formation of the blood-testis barrier, which is an impermeable barrier (to anything larger than 1000 daltons) that separates the seminiferous epithelium into the previously mentioned adluminal and basal regions (Kopera et al., 2010; Walker and Cheng, 05). Germ cells pass through the blood-testis barrier via tight junctions and become dependent upon nutrients and growth factors, supplied by surrounding Sertoli cells (Walker and Cheng, 05).

Interstitial tissue is comprised of steroid-producing Leydig cells, blood vessels, lymphatics, connective tissue, and nerves, of which the quantity and morphology varies between species (Noakes, et al., 2001). The components of the interstitial tissue have a marked role during the breeding season, when blood flow increases and the Leydig cells undergo hypertrophy (Hochereau-de Reviers and Lincoln, 1978).

Spermatogenesis begins within the seminiferous epithelium near the basement membrane. Sertoli cells are dispersed throughout the seminiferous epithelium and the

plasmalemma of these cells is in contact with maturing germ cells. Immature spermatozoa are then released from the seminiferous tubules, traverse through rete tubules, which are ducts within the testicular connective tissue, into the lumen of a cylindrical organ known as the epididymis.

The epididymis is divided into three sections: caput (head), corpus (body), and cauda (tail). The epididymal duct is enveloped by smooth muscle that provides rhythmic contractions of the duct, triggering spermatozoa passage from the caput to cauda epididymis. As sperm travel the epididymis they mature from immotile germ cells in the caput region to motile gametes with fertilizing capability in the corpus region. To obtain fertilizing potential, the sperm plasma membrane is altered by the addition of surface glycoproteins (Tulsiani et al., 1993; Gadella and Luna, 2014; Xin et al., 2014). The cauda epididymis serves as a reservoir for these mature sperm. The cauda epididymis extends parallel to the testis and widens into the vas deferens until it reaches the pelvic urethra. Recurring epididymal and vas deferens contractions move spermatozoa from the cauda epididymis to the pelvic urethra. The thickness of the vas deferens increases as it nears the pelvic urethra. The thickening of this duct forms another, temporary, spermatoc storage structure known as the ampulla. Fluid from the accessory sex glands contributes to the seminal plasma (Noakes et al., 2001). To avoid a build up of pressure within the testes, spermatozoa are periodically flushed from the epididymis, to the vas deferens, and into the pelvic urethra where they are removed through urination.

During sexual excitation, the neuropeptide oxytocin is released which causes epididymal and vas deferens contractions, moving spermatozoa to the pelvic urethra until ejaculation occurs. During the process of ejaculation, secretions from the vesicular

glands, provide the citrate and fructose components to semen. Prior to ejaculation, the bulbourethral gland, located between the pelvic urethra and anus, secretes seminal plasma necessary for coagulation of semen following ejaculation.

### **Hormonal Regulation Of Spermatogenesis**

Like female WT does and other deer species (Godfrey et al., 1990), WT buck reproductive hormone concentrations are affected by photoperiod (Suttie et al., 1989). As day length decreases, melatonin increases and hypothalamic gonadotropin release initiates hormonal stimulation of somatic and germinal cell lines within the testes. Male testicular function is regulated by pulsatile gonadotropin control (Finnerty et al., 1998), which catalyzes steroidogenesis (Huhtaniemi and Toppari, 1995). Luteinizing hormone (LH) release fluctuates in seasonal breeding species, with more frequent lower amplitude pulses occurring during the breeding season in comparison to higher amplitude, less frequent pulses through the non-breeding periods (Sanford et al., 1977). These variations in the pattern of gonadotropin release appear to be associated with photoperiod shifts (Sanford et al., 1977). These photoperiod shifts enable Leydig cells of seasonal species to undergo annual developmental and regression cycles (Johnson, et al., 1987). During the breeding season, LH pulses induce morphological changes in the cytoplasm of developing Leydig cells (Bilinska et al., 2006) where, through adenylate cyclase, the episodic hypothalamic release regulates the conversion of cholesterol into a testosterone precursor (pregnenolone) (Svechnikov et al., 2010). In most species, the release of testosterone occurs within 40-60 min following an LH peak, which recurs every 2-4 h (Muduuli et al., 1979; Noakes et al., 2001). Proper development, maturation,

and mobility of spermatozoa are dependent upon this androgenic conversion (Dohle et al., 2003).

Within the seminiferous tubules, testosterone is converted into 5-dihydrotestosterone (DHT) (Kolasa et al., 2011). This is a non-aromatizable, irreversible conversion, which yields a metabolite with greater potency than testosterone and is crucial for development of the male reproductive tract (Noakes et al., 2001; Walker and Cheng 2005; Meachem et al., 2007). Testosterone is important for male sexual differentiation and functions as the primary androgen in the testis, which regulates spermatogenesis (Walker, 2009). In the absence of testosterone, germ cell degeneration has been reported to occur in the later stages of spermatogenesis (Walker and Cheng, 05). Both testosterone and DHT are bound within the lumen of the seminiferous tubules by androgen-binding protein (ABP), a secretory product of the Sertoli cells (Dohle et al., 2003).

Sertoli cells are targeted by FSH, which, unlike LH secretion, maintains a higher frequency, lower amplitude pulsatile release (Melson et al., 1986). This gonadotropin initiates the secretion of glycoproteins from Sertoli cells such as androgen-binding protein (ABP), transferrin, production of inhibin, lactate and aromatization of testosterone into estrogens (Peterson and Soder, 2006; Hansson et al., 1975; Sofikitis et al., 2008). Aromatization and inhibin control the rate of sperm production throughout the breeding season via negative feedback on the pituitary release of LH and FSH. Estrogen inhibits Leydig cells and their role in steroidogenesis (Svechnikov et al., 2010). In response to FSH, sertoli cells increase intracellular  $Ca^{2+}$  and the production of cAMP,

with higher concentrations of cAMP being present in the later cycles of spermatogenesis (Walker and Cheng, 2005; Gorczynska-Fjalling, 2004).

## **Spermatogenesis**

Spermatogenesis incorporates all mitotic germ cell divisions (spermatocytogenesis), meiotic reductions, and morphological transformations of maturing gametes (spermiogenesis) that take place within the seminiferous tubules of the testes. Seminiferous tubules are composed of a basement membrane, in which Sertoli cells are anchored, and a layer of germinal epithelium where developing male gametes are maintained temporarily. Following puberty and under the influence of increased frequency of LH pulses (Godfrey et al., 1990; O'Donnell et al., 2011), previously arrested immature spermatogonia begin to undergo mitotic divisions into three maturational classes (A, intermediate, and B). A-spermatogonia are the least differentiated of the maturational classes and serve as a continual source of stem cells for future mitotic divisions (Noakes et al., 2001). B-spermatogonia undergo the final mitotic division, yielding primary spermatocytes, which undergo subsequent meiotic reduction yielding secondary spermatocytes and spermatids (Cheng et al., 2010). Meiotic divisions ensure that the sperm cells maintain genetic heterogeneity. As meiotic divisions progress, germ cells initiate a change in Sertoli cell gene expression causing an alteration in the surrounding microenvironment, allowing the diploid germ cells to move toward the seminiferous lumen producing the secondary spermatocytes (Griswold, 1995). These secondary spermatocytes proceed through the second meiotic division resulting in spherical haploid spermatids, marking the completion of meiosis (Cheng et al., 2010).

Spermiogenesis, transformation of post-meiotic cells into spermatozoa, occurs immediately after the second meiotic division. Spermiogenesis incorporates four phases: Golgi, cap, acrosomal, and maturation (Tulsiani et al., 1998). Initial spermatids contain a highly developed Golgi apparatus, located above the nucleus, which gives rise to Golgi and acrosomal vesicles. During the Golgi phase, the implantation apparatus and axoneme, necessary for proper motility, form due to centriole migration around the nucleus. In addition, the acrosomal vesicles attach to the nuclear envelope. These membranous vesicles elongate and flatten to form an acrosomal cap, which consists of an outer and inner acrosomal membrane, over the anterior portion of the nucleus. A primitive flagellum forms and extends from the distal centriole and projects toward the lumen of the seminiferous tubule. During the acrosomal and maturation phases, the Golgi apparatus repositions to the cell pole opposite the acrosome (Moreno et al., 2000). As the acrosomal cap extends over the anterior portion of the nucleus, chromatin within the nucleus condenses and elongates, and the spermatids becomes further implanted within the Sertoli cells. The elongation of spermatids and translocation through seminiferous epithelium is associated with a second junction of the Sertoli cells known as ectoplasmic specialization junctions (O'Donnell et al., 2011).

In last phase of spermiogenesis, the final stages of flagellum formation occur with mitochondrial migration from the nucleus followed by condensation around the flagellum to form the midpiece and plasma membrane that envelops the entire spermatozoan. Lastly, spermiation commences with fully formed spermatozoan released from the Sertoli cells and ectoplasmic specialization adhesion into the lumen of the seminiferous tubule (O'Donnell et al., 2011). The duration of spermatogenesis, from production of

spermatogonia to spermiation, is species-specific and is 61 days for the bull (Johnson et al., 2000), 74 days in man (Johnson et al., 2000), 40 days for the boar (Zeng et al., 2006), 48 days in the ram (Zeng et al., 2006), and greater than 100 days for the red deer stag (Lincoln 1971).

### **Capacitation And Acrosome Reaction**

Capacitation is a prerequisite for fertilization for all mammalian sperm.

Capacitation is a poorly understood maturational process that begins with the transit through the epididymis and ends *in vivo* within the female reproductive tract where the ability to undergo the acrosome reaction (AR) and fertilize an egg is obtained (Visconti et al., 1999). During spermatozoan maturation cell signaling pathways are enabled and epididymal sperm go through variations of membrane sterol content (Sherriff and Ali, 2010). The alterations of sperm plasma membrane (PM) sterols are species-specific, with human sperm having twice the cholesterol fraction as bovine and ovine sperm (Cross, 1998). However, following ejaculation, sperm come into contact with high bicarbonate concentrations (> 15 mM) inside the female genital tract (Flesch and Gadella, 2000), indicating the potential importance of bicarbonate in sperm fertilization preparation.

Capacitation within the female environment appears to be triggered by a ligand-receptor-G-protein second messenger system which involves an efflux of potassium and cholesterol from the sperm PM thus increasing membrane fluidity (Shoeb et al., 2004; Gadella et al., 2008; Tulsiani et al., 1998) and allowing  $\text{Ca}^{2+}$  and bicarbonate ions to enter the cell. Estrogen-dependent oviductal glycosaminoglycans (GAGS), such as chondroitin sulfite A, hyaluronic acid, and heparin (Kawakami et al., 2000), serve as

ligands for sperm PM receptors and aid in  $\text{Ca}^{2+}$  influx, cAMP synthesis and subsequent sperm motility (Cormeir and Bailey, 2003; Dapino et al., 2006). Sperm require the presence of extracellular  $\text{Ca}^{2+}$  for capacitation and subsequent acrosome reaction. Bicarbonate and  $\text{Ca}^{2+}$  enter the cell via ion channels in the PM ( $\text{Cl}^-/\text{HCO}_3^-$ ,  $\text{Na}^+/\text{HCO}_3^-$ ,  $\text{Na}^+/\text{Ca}^{2+}$  exchangers, voltage dependent  $\text{Ca}^{2+}$  channel, and  $\text{Ca}^{2+}$ -ATPase) (Flesch and Gadella, 2000; Shoeb et al., 2004). Bicarbonate ions bind to a soluble adenylyl cyclase (sAC) that initiate the cAMP/protein kinase A (PKA) signaling pathway which lead to the phosphorylation of tyrosine residues of several proteins essential to capacitation (Sherriff and Ali, 2010; Visconti, 2009). This influx of intracellular bicarbonate and  $\text{Ca}^{2+}$  ions is correlated with PM integrity and appears to be controlled via translocation of phospholipids between the inner and outer leaflets of the PM. In general, mammalian sperm PM contains 70% phospholipids, which is categorized into either phosphoglycerolipids or sphingomyelin (SM) (Flesch and Gadella, 2000). Buck (*Capra hircus*) sperm cells contain mostly phosphocholine glycerides (PC) and phosphoethanolamine glycerides (PE) with the remainder of the phospholipids composed of phosphatidylserine (PS) and phosphatidylinositol (PI) (Sundhey et al., 1992). Phospholipid scrambling of capacitated sperm results in increased PC and SM movement from the outer membrane leaflet to the inner leaflet and decelerated inward movement of PE and PS (Flesch and Gadella, 2000). Bicarbonate and  $\text{Ca}^{2+}$ -induced phospholipid scrambling enable the PM to fuse with the outer acrosomal membrane to begin the AR, as well as initiate sperm motility (Abou-haila and Tulsiani, 2009).

Immotile spermatozoa become hyperactivated once in the presence of bicarbonate and  $\text{Ca}^{2+}$  concentrations in female oviductal fluid and are regulated by

protein phosphorylation (de Lamirande et al., 1997; Urner and Sakkas, 2003; Visconti, 2009). Hyperactivation of spermatozoa is necessary for proper release of sperm from oviductal tissue, improved ability to pass through fluids of the female genital tract, and ability to penetrate the zona pellucida (de Lamirande et al., 1997). During capacitation of most mammalian sperm, protein tyrosine phosphorylation is limited to minute portions of both the principle and midpiece of spermatozoa, however, following zona pellucida binding phosphorylation is stimulated in the midpiece to yield 100% phosphorylation of the entire flagellum (Urner and Sakkas, 2003). Binding to the oocyte initiates changes in protein phosphorylation that correlate with the AR.

Subsequent to molecular alterations of the sperm PM, sperm surface receptors chemotactically recognize the extracellular matrix of cumulus cells surrounding the recently ovulated oocyte. Sperm-egg interaction encompasses loosely and reversibly bound spermatozoa to the egg's extracellular cumulus layer (Bleil and Wassarman, 1983). Hyperactivation, in combination with hyaluronidase enzyme on the outer surface of the sperm cell, facilitate a digestive path through the cumulus cell matrix. The second phase of interaction involves the binding of complimentary ligands on the surface of the oocyte's zona pellucida (ZP) to sperm surface receptors.

The ZP of most mammals is composed of three structural glycoproteins (ZP1, ZP2, and ZP3), with the exception of the pig, cow, and dog, which exhibit ZP4 instead of ZP1 and rats, hamsters and humans, which have all four glycoproteins (Tulsiani et al., 1997; Gupta and Bhandari, 2011). Glycoproteins ZP2 and ZP3 form a cross-linked polymer matrix that is bound by disulfide-linked homodimers of ZP1 (Monne and Jovine, 2011). Currently there is debate as to the roles of ZP2 and ZP3 in regards to sperm

binding (Clark, 2011). *In vitro* murine and human fertilization studies have shown that ZP3 maintains the receptor necessary for acrosome-intact sperm binding to the ZP (Bleil and Wassarman, 1980), but ZP2 serves as a secondary receptor for acrosome-reacted sperm binding to the ZP (Bleil and Wassarman, 1983; Tsubamoto et al., 1999).

Following progression through the cumulus cell matrix, successive adhesion of spermatozoa to the ZP leads to additional molecular modifications of the acrosome. ZP3 binds to sperm receptors and crosslinks sperm galactosyltransferase and carbohydrate residues of ZP3. This binding activates G-proteins and stimulates  $Ca^{2+}$ -mediated exocytosis of the acrosomal vesicle (Leyton and Saling, 1989). When  $Ca^{2+}$  increases in response to gamete binding, actin depolymerizes causing the sperm PM to lessen its distance from the outer acrosomal membrane (Tulsiani et al., 1998). The process of exocytosis of acrosomal contents requires multiple steps. First, the sperm PM establishes multiple fusion sites with the outer acrosomal membrane, a process known as vesiculation. Following vesicle formation, the acrosomal contents are dispersed in a time-dependent manner (Abou-haila and Tulsiani, 2009). Acrosin, an enzyme released during vesiculation, hydrolyzes ZP proteins and aids in the ability of sperm to lyse the ZP so it may pass through the ZP and perivitelline space to the PM of the oocyte for fertilization.

### ***In Vitro* Capacitation And Acrosome Reaction**

*In vitro* studies have demonstrated that capacitation of spermatozoa is facilitated when a defined medium is used (Singh et al., 1978; Andrews and Bavister, 1989; Tajik et al., 1994; Emiliozzi and Fenichel, 1997; Tardif et al., 2003; Wertheimer et al., 2008). The addition of calcium ionophore A23187 promotes rapid capacitation, exocytosis of

acrosomal contents, and increases *in vitro* fertilizing ability of mouse spermatozoa (Fraser and McDermott, 1992). Fraser (1982) demonstrated that mouse spermatozoon incubated with A23187, in the presence of  $\text{Ca}^{2+}$ , had reduced time to capacitation, increased hypermotility, and higher fertilization rates. Similarly, Alm and others (2001) reported that fresh equine spermatozoa treated with calcium ionophore A23187 have higher penetration and *in vitro* fertilization rates.

Other researchers have evaluated movement of transmembrane glycoproteins of boar spermatozoa in a  $\text{Ca}^{2+}$  enriched environment and reported displacement of transmembrane glycoproteins of the sperm head membrane when in the presence of both  $\text{Ca}^{2+}$  and A23187 ionophore (Aguas and Silva, 1989). Flesch and colleagues (1999) evaluated the capacitation ability of Tyrode's medium supplemented with or without bicarbonate and  $\text{Ca}^{2+}$  ionophore and reported that increased intracellular  $\text{Ca}^{2+}$  concentrations and bicarbonate presence are needed in capacitation media in order for protein phosphorylation to occur.

Although variations of the chemical makeup of *in vitro* fertilization media exist based on species (Berg et al., 2002; Berg and Asher, 2003; Tajik and Esfandabadi, 2003; Siriaroonrat et al., 2010), most media consist of both bicarbonate and  $\text{Ca}^{2+}$  levels that mimic estrual fluid. In the mouse, rat, hamster and guinea pig, research has shown beneficial properties of estrual fluids, however, the necessity of its inclusion in capacitation media is not required provided that progesterone, non-physiological  $\text{Ca}^{2+}$  ionophores, GAGS, ionomycin inducers, and/or increased K/Na ratio are present (Toyoda and Chang, 1974; Lukoseviciute et al., 2005). *In vitro* induction of the AR by stimulation of intracellular  $\text{Ca}^{2+}$  release,  $\text{Cl}^-$  influx and subsequent membrane

depolarization, and the downstream induction of protein tyrosine phosphorylation are progesterone-mediated events that have been reported in human, porcine and murine spermatozoa (Baldi et al., 1995; Melendrez and Meizel, 1995; Turner and Meizel, 1995; Espinosa et al., 1998; Linares-Hernandez et al., 1998).

Mammalian follicular fluid at the time of ovulation contains a high concentration of progesterone (~6 µg/ml) (Flesch and Gadella, 2000). Moseley and colleagues (2005), reported progesterone supplementation in *in vitro* fertilization medium was more effective in inducing the AR in human spermatozoa compared with a standard capacitation medium. However, for bovine spermatozoa to be influenced by progesterone supplementation, the capacitation medium must consist of either bovine seminal proteins or heparin (Therien and Manjunath, 2003). Extensive research has focused on heparin for capacitation initiation (Parrish et al., 1988; Parrish et al., 1989; Kawakami et al., 2000; Comizzoli et al., 2001; Berg et al., 2002; Cormier and Bailey, 2003; Dapino et al., 2006). According to Parrish et al. (1988), heparin increases the prevalence of capacitation and penetration of oocytes by sperm when supplemented in formulated media. It has been reported that heparin induces the AR in capacitated hamster spermatozoa, but has a delayed effect on capacitation or AR if the spermatozoa are incubated under non-capacitating conditions, such as the presence of glycolyzable substrates which acidifies intracellular pH (Parrish et al., 1989). Therefore, a proper balance of bicarbonate, Ca<sup>2+</sup>, albumin (a sterol acceptor), energy substrates, and steady pH are factors that must be considered before the addition of heparin to a fertilization medium to induce capacitation (Andrews and Bavister, 1989; Tajik et al., 1994).

## **Epididymal Sperm In Assisted Reproductive Technology**

Use of epididymal sperm, *in vivo* or under suitable culture conditions, allows producers to maximize the progeny from genetically desirable sires. Caput and corpus epididymal spermatozoa located within the testes are incapable of fertilizing a female gamete, however, caudal epididymal spermatozoa have reached a level of maturation where they are capable of fertilizing ova (Igboeli and Foote, 1968; Hoppe, 1975). As sperm move through the epididymis they gain motility, ability to undergo capacitation and the acrosome reaction and the ability to bind and penetrate ova.

Barker (1954) was the first to obtain pregnancies (63.6%) from cryopreserved (-79°C) epididymal sperm from a proven Jersey sire. Shortly thereafter, Barker and Gandier attempted similar inseminations in the mare (1957). Epididymal spermatozoa were recovered from two castrated Belgian stallions, cryopreserved, and stored (-79°C) for 1-2 months prior to inseminations. Of the seven females inseminated, one pregnancy (14%) occurred and resulted in the birth of a live foal.

Toyoda and Chang (1974) reported that rat oocyte penetration rates were higher (96%) when caudal epididymal spermatozoa were preincubated in a defined medium with increased KCl/NaCl ratio compared with penetration rates from sperm preincubated in modified Krebs-Ringer bicarbonate solution (89%). Toyoda and Chang (1974) also noted that KCl/NaCl ratio of uterine fluid of pro-estrus rats was equivalent to the high KCl/NaCl presented in this study.

Niwa et al. (1983) demonstrated that caudal epididymal spermatozoa, when cultured in chemically defined media, undergo capacitation and are capable of

penetrating rabbit cumulus oocyte complexes (92%) and denuded rabbit oocytes (99%), resulting in fertilization (95% and 93%, respectively).

Rath and Neimann (1997) compared the fertilizing capability of FT epididymal sperm to both fresh and frozen ejaculated spermatozoa from boars. Following IVF sperm preparation, FT spermatozoa had motility rates similar to fresh ejaculated spermatozoa (72.2% and 76.4%, respectively). Fertilizing ability was higher for FT epididymal sperm in comparison to fresh and FT ejaculated semen (23%, 8.9%, and 2.2%, respectively).

Santiago-Moreno et al. (2006), collected caudal epididymal spermatozoa from Spanish Ibexes within 2-24 h post-mortem, cryopreserved utilizing an egg-yolk extender, and stored in liquid nitrogen (LN<sub>2</sub>). Following six months of storage, both homologous and heterologous intrauterine inseminations were carried out. Of the six females inseminated, one became pregnant (16.7%) and resulted in the first documented live ibex birth from epididymal spermatozoa. Conception rates obtained from the heterologous artificial inseminations only occurred in goats inseminated with epididymal sperm that was collected within 8 h of sire death (25%, 9/36).

Chatiza et al. (2013) assessed the fertilization potential of springbok, impala and blesok caudal epididymal spermatozoa using heterologous IVF. Results showed that the antelope post-thawed epididymal spermatozoa were capable of fertilizing cattle oocytes.

## Media Used In *In Vitro* Embryo Production

*In vitro* production of WT embryos has potential to overcome restrictions of artificial insemination (AI) and multiple ovulation embryo transfer (MOET) by efficient use of FT semen and repeated collection of oocytes from donors. Caveats which arise from FT semen and oocyte collection are a) cost limitations, as semen from genetically superior bucks is expensive, b) seasonality constraints on semen and oocyte collection, c) need to conserve top breeding sires and dams for natural mating, and d) anesthetization of semen donor restricts number of collections per sire. Due to WT being seasonal breeders, the number of progeny from genetically elite breeding females is limited to only 2-3 offspring per season. Berg and Asher (2003) compared the efficiencies of producing cervid offspring using AI, MOET, and IVP. Their results showed that IVP is 40 times more proficient than AI and generated 24 times more offspring than MOET. In order to develop a proficient IVP system, it is important to establish the media components necessary for both fertilization and early embryonic development.

Most difficulties encountered in defining IVF media are obtaining sperm capacitation and mimicking oviductal and uterine environments *in vitro*. For most farmed species, heparin is the most used GAGS that stimulates adequate capacitation (Niwa and Ohgoda, 1988; Parrish et al., 1989; Cox et al., 1995). However, when heparin was applied to the RD *in vitro* system, fertilization rates dropped dramatically and no embryo development occurred beyond the 16-cell stage (Bainbridge et al., 1999). Similar findings were reported when day 1.5 deer serum was used (Berg et al., 1995). However, the use of 20% (v/v) day 1.5 sheep serum as a capacitating agent has

been shown to be successful in both RD and sika deer fertilization medium (77% and 56% fertilized, respectively) (Berg et al., 2002).

Due to seasonality or availability constraints that accompany collection of female gametes from rare or wild species, ascertaining the fertilizing ability of spermatozoa of these species can be challenging. To counter this issue, heterologous IVF has been tested among several species (Cox et al., 1994; Nelson et al., 1999; Roth et al., 1999; Comizzoli et al., 2001; Zhao et al., 2002; Soler and Garde, 2003; Garcia-Alvarez et al., 2009; Stoops et al., 2007) and has been shown to be an advantageous method for understanding the chemical requirements of *in vitro* media, as well as measuring sperm penetrability and polyspermic rates *in vitro*.

Previous cervid IVP studies have focused primarily on the utilization of established bovine and sheep systems. Thus far, cervid IVP research has been conducted using RD, sika deer, wapiti, and reindeer gametes (Krogenaes et al., 1994; Bainbridge et al., 1999; Comizzoli et al., 2001; Berg et al. 2003). Most cervid IVP research has used modifications of the established bovine IVP system; however, the results have varied dependent on the deer species at hand. The North American wapiti and reindeer have been two deer species that have responded favorably to the bovine IVP procedure (Krogenaes et al., 1994; Berg et al., 2003). However, results from other deer analyses have varied from *in vitro* embryos blocked at the 8- and 16-cell stage (Fukui et al., 1991; Bainbridge et al., 1999), less than 10% of embryos actually developing to blastocysts (Berg and Asher, 2003), and low blastocyst rates (22 to 26%) of *in vivo* zygotes cultured *in vitro* regardless of culture conditions (Berg et al., 1995b). Use of these systems has made it difficult to compare results among cervid species due

to differences in culture media, presence or absence of somatic cell culture, serum supplementation, and species differences among gametes (Berg et al., 2003). These outcomes highlight the need for basic knowledge of the *in vitro* media component requirements for deer embryos. Currently, the most defined cervid system is that of RD (Berg et al., 2003), although an *in vitro* maturation (IVM) media was recently defined for WT (Siriaroonrat et al., 2010).

The chemical makeup of WT oviductal fluid is unknown; however, the evaluation of RD oviductal fluid revealed the composition differs from that of cattle and sheep oviductal fluid. Significant differences were found in calcium, phosphate, and glucose concentrations (Berg et al., 2003). These variations led to the successful formulation of a cervid-specific IVF and two-step IVC media termed DSOF (deer synthetic oviductal fluid). The presence of exogenous calcium has been extensively studied, as it is required for initiation of capacitation, which is necessary for the AR. Without the occurrence of these events, fertilization will not take place, as neither gamete is properly primed. Phosphate and glucose have been reported to be involved in early embryonic developmental blocks by preventing oxidative phosphorylation, known as the Crabtree effect (Tsuji et al., 2004). However, unlike the majority of domestic livestock, oviductal fluid of RD has twice the calcium concentration (2 mM and 5 mM, respectively; Berg et al., 2003) and low concentrations of both phosphate and glucose. Due to the additional calcium concentrations in deer SOF (DSOF) the amount of BSA has been reduced to minimize the amount of calcium chelation that occurs. Deer SOF also differs from bovine IVF media in that it includes more of the essential amino acids, which are regulators of early embryonic development. Establishment of proper *in vitro* fertilization

(IVF) and *in vitro* culture (IVC) media for WT IVP can putatively reduce costs, proliferate desired genetics throughout a farmed herd, as well as use all does within a herd, as those with lesser genetics could be used as surrogates.

## CHAPTER 3

# THE EFFECT OF eCG ON PREGNANCY RATES OF WHITE-TAILED DEER FOLLOWING FIXED-TIMED ARTIFICIAL INSEMINATION

### Introduction

The popularity of WT (*Odocoileus virginianus*) as a farmed species has increased dramatically over the last decade, with these operations now common throughout North America (Census of Agriculture, 2009). However, the physiological control of the white-tailed doe's reproductive cycle is not well documented. Clearly, a better understanding would enhance genetic improvement in breeding programs and new information could be used in assisted reproductive technologies for endangered cervids.

The goal at most WT breeding facilities is production of large antler sets on breeding bucks, which are used to assist in marketing cryopreserved semen. However, to use cryopreserved semen from genetically superior bucks in AI programs, development of successful, repeatable estrus synchronization protocols followed by FTAI is essential. Although FTAI has been incorporated into commercial beef and dairy operations, very few studies have reported FTAI protocols for WT (Haigh, 1984; Magyar et al., 1988).

A protocol for FTAI of WT does could be more easily and economically incorporated into the overall management of this species than protocols using either laparoscopic AI or a more traditional AI program using detection of estrus. Regarding the latter, since these animals do not exhibit homosexual mounting activity (in contrast to cattle), teaser animals combined with visual estrous detection would have to be

employed. A successful FTAI program would yield pregnancy and subsequent fawning rates which are comparable to those produced by natural matings. Most FTAI protocols that have been successful in goats (Oliveria, et al., 2001; Motlomelo, et al, 2002), sheep (Ainsworth and Downey, 1986; Wheaton, et al. 1993), cattle (Macmillan and Peterson, 1993; Kim et al., 2006), fallow deer (Morrow et al., 1992), wapiti (McCorkell et al., 2007), and RD (Fennessy et al.,1990; Fennessy et al., 1991; Asher et al., 1992) have used a CIDR device as a progesterone source. Equine chorionic gonadotropin (eCG) is routinely administered at CIDR removal in RD to improve frequency and synchrony of ovulation, but has not been recommended for other cervidae, because of ovulation failure, multiple ovulations and reduced fertility (Asher and Smith, 1987; Jabbour et al., 1993b). Although there have apparently been no studies evaluating the effectiveness of eCG administration at CIDR removal in WT does, our impression is that is commonly used in estrus synchronization protocols.

In our preliminary studies (unpublished), WT does synchronized with an EAZI-BREED CIDR Sheep and Goat device for 14 days and transcervically inseminated 60 h post CIDR removal had similar pregnancy rates for chilled (60%) or FT (57%) semen from the same buck. The fecundity rate for does pregnant to AI was 1.3 fawns per doe compared with 1.8 fawns per doe for those which subsequently became pregnant to natural service (clean-up bucks). Therefore, the objective of the present study was to evaluate the effects of a synchronization protocol utilizing a CIDR device, in conjunction with eCG, on pregnancy and fecundity rates of WT following FTAI.

## **Materials And Methods**

### **Animal Subjects**

This experiment was approved by IACUC and conducted at the Bob R. Jones Idlewild Research Station near Clinton, LA, USA (30°86' N, 91°02' W) and was replicated over two breeding seasons (late November or early December). Mature WT does (Year 1: n = 36; Year 2: n = 38) used in this study had a mean age of 4.6 yrs (range: 2.5–8.5 yrs) and mean weight of 62.1 kg (range, 45–75 kg) (Table 3.1). Does were maintained on 20% deer pellets (Professional Sportsman's Choice Record Rack, Cargill Animal Nutrition, Minneapolis, MN, USA) and native grasses. Does were rotated among pens that ranged in size from 0.6 to 1.6 ha.

### **Experimental Design**

Each year, does were stratified by weight, age and last fawning date into two groups. Estrus synchronization protocols were then randomly assigned to each group. Thirty does were used both years; after stratification into groups and random assignment of treatment to groups, 18 does received the same treatment both years and 12 does received a different treatment across years. Also, six does were used in Year 1 that were not used in Year 2 and eight does were used in Year 2 that were not used in Year 1.

### **Estrus Synchronization And Fixed-Timed Artificial Insemination**

All does were synchronized with an EAZI-BREED CIDR Sheep and Goat device (0.3 g progesterone, Agtech Inc., Manhattan, KS, USA) on Day 0 and the CIDR remained in place for 14 days.

Table 3.1 Number, age and weight of White-tailed does involved in eCG FTAI synchronization trials.

Treatment	Does ( <i>n</i> )	Age <sup>a</sup>	Weight (kg) <sup>a</sup>
Experiment 1			
Control	20	4.0 ± 0.4	58.2 ± 1.3
GnRH	16	3.8 ± 0.4	60.8 ± 1.4
Experiment 2			
Control	20	5.4 ± 0.4	64.3 ± 1.4
GnRH	18	5.2 ± 0.4	64.4 ± 1.4
Combined			
Control	40	4.7 ± 0.3	61.2 ± 1.0
GnRH	34	4.6 ± 0.3	62.6 ± 1.0

<sup>a</sup> Mean ± SEM.

Treatments consisted of estrus synchronization for FTAI (adapted from Schenk and DeGrofft 2003) to occur, on average, 60 h post-CIDR removal across all animals (no eCG: Year 1, n = 20; Year 2, n = 20) or 200 IU (im) injection of eCG (G4877, Sigma-Aldrich, St. Louis, MO, USA) at CIDR removal, followed by FTAI to occur on the average at 60 h post CIDR removal across all animals (eCG: Year 1, n = 16; Year 2, n = 18). The exact time of CIDR removal was recorded for each doe.

Does were inseminated with FT semen ( $10 \times 10^7$  progressively motile sperm preefreeze) from a fertile buck that was collected via electroejaculation on-farm and frozen in 0.5-mL straws by a commercial bull stud facility (Genex Cooperative, Baton Rouge, LA, USA). Before AI, a single straw from each collection was thawed and motility was determined to be 65%. Time of insemination was recorded for each doe and the interval (h) from CIDR removal to AI was calculated. All inseminations were performed by a single technician.

Before insemination each doe received 1 mg (iv) detomidine hydrochloride (Dormosedan; Pfizer Animal Health, New York, NY, USA). Transcervical insemination was attempted using a special speculum, fitted with a light source. The speculum was inserted into the vagina until the cervical os was visualized. The insemination gun was inserted into the cervical os and manipulated through the cervical canal until it entered the uterine body (pipette passage of 3 cervical rings and forward progress of insemination pipette was no longer impeded) or no further progress could be attained. Each insemination was limited to no more than 3 min, regardless of insemination pipette location. The speculum was then retracted and semen was slowly expelled.

At insemination, disposition (DISP; 1 = calm with no movement, 2 = calm with some kicking, 3 = moderate kicking, 4 = frequent kicking and 5 = vocalizing and kicking), time of insemination, insemination depth (ID; 0 = vaginal, 1 = cervical and uterine) and vulva assessments (vulva color and vulva edema) were recorded for each doe. Fifty-four days following insemination, intact bucks were introduced into each group to naturally breed does which had not become pregnant to FTAI.

### **Pregnancy Diagnosis**

Pregnancy was determined via transrectal ultrasonography using an ALOKA SSD, 500 V scanner (Aloka Ltd, Tokyo, Japan) using a 5 MHz linear-array rectal transducer equipped with modified plastic PVC tubing (38 cm long) to aid in transducer manipulation. Ultrasonography was conducted 33 days post-insemination and confirmed 48 days post-insemination. Final pregnancy rates for AI and natural mating were derived from fawning data and fawning date.

### **Statistical Analysis**

Pregnancy rates were determined based on fawning rates and were analyzed by  $\chi^2$  analysis using the frequency procedure (Proc Freq; SAS Institute, Inc., Cary, NC, USA). Time interval from CIDR removal to FTAI was calculated for each doe and graphed using the chart procedure (Proc Chart; SAS Institute, Inc.) using the midpoint option. Based on the frequencies of pregnant and non-pregnant females inseminated by 57, 58, 59, 60 and 61 h post CIDR removal, insemination time (IT) was categorized into two groups (1 = females inseminated < 60.5 h and 2 = females inseminated  $\geq$  60.5 h). Logistic regression was used to determine the odds of pregnancy after FTAI using the following variables: ID, IT, treatment, age, year, DISP, vulva color and vulva edema.

Differences in doe age and number of fawns born per doe were analyzed using general linear model procedures (Proc GLM, SAS Institute, Inc.). All mean values are expressed as the mean  $\pm$  SEM.

## Results

Pregnancy rates did not differ between years ( $P = 0.35$ ) and overall, 50% of the does became pregnant by FTAI (Year 1: 44%; Year 2: 55%). Administration of eCG at CIDR removal did not affect pregnancy rate ( $P = 0.16$ ) (Table 3.2). The average interval from CIDR removal to AI was  $59.7 \pm 0.14$  h. More does ( $P = 0.02$ ) became pregnant to FTAI when insemination occurred at or beyond 60.5 h post-CIDR removal (19/28; 68%) compared with those does inseminated at  $< 60.5$  h post-CIDR removal (18/46; 39%) (Figure 3.1). Similarly, does inseminated at 60.5 h after CIDR removal or later were 22 times more likely ( $P = 0.002$ ; Table 3.3) to become pregnant to FTAI than does inseminated  $< 60.5$  h after CIDR removal.

When frozen-thawed semen was deposited in the cervix or uterus, does were 17 times more likely ( $P = 0.005$ ; Table 3.3) to become pregnant to FTAI compared with does for which semen was deposited in the vagina. However, deposition of semen into the uterus occurred in only 11% (8/74) of the does inseminated across both years. Age, IT and ID increased the odds of becoming pregnant to FTAI (Table 3.3).

Table 3.2 Effect of eCG at CIDR-g removal on FTAI pregnancy rates of WT deer.

	Treatment	Does (n)	FTAI Pregnancy rate (%)	P-Value
Year 1	Control	20	6/20 (30)	0.0512
	eCG	16	10/16 (63)	
Year 2	Control	20	11/20 (55)	0.9726
	eCG	18	10/18 (56)	
Years Combined	Control	40	17/40 (43)	0.1616
	eCG	34	20/34 (59)	

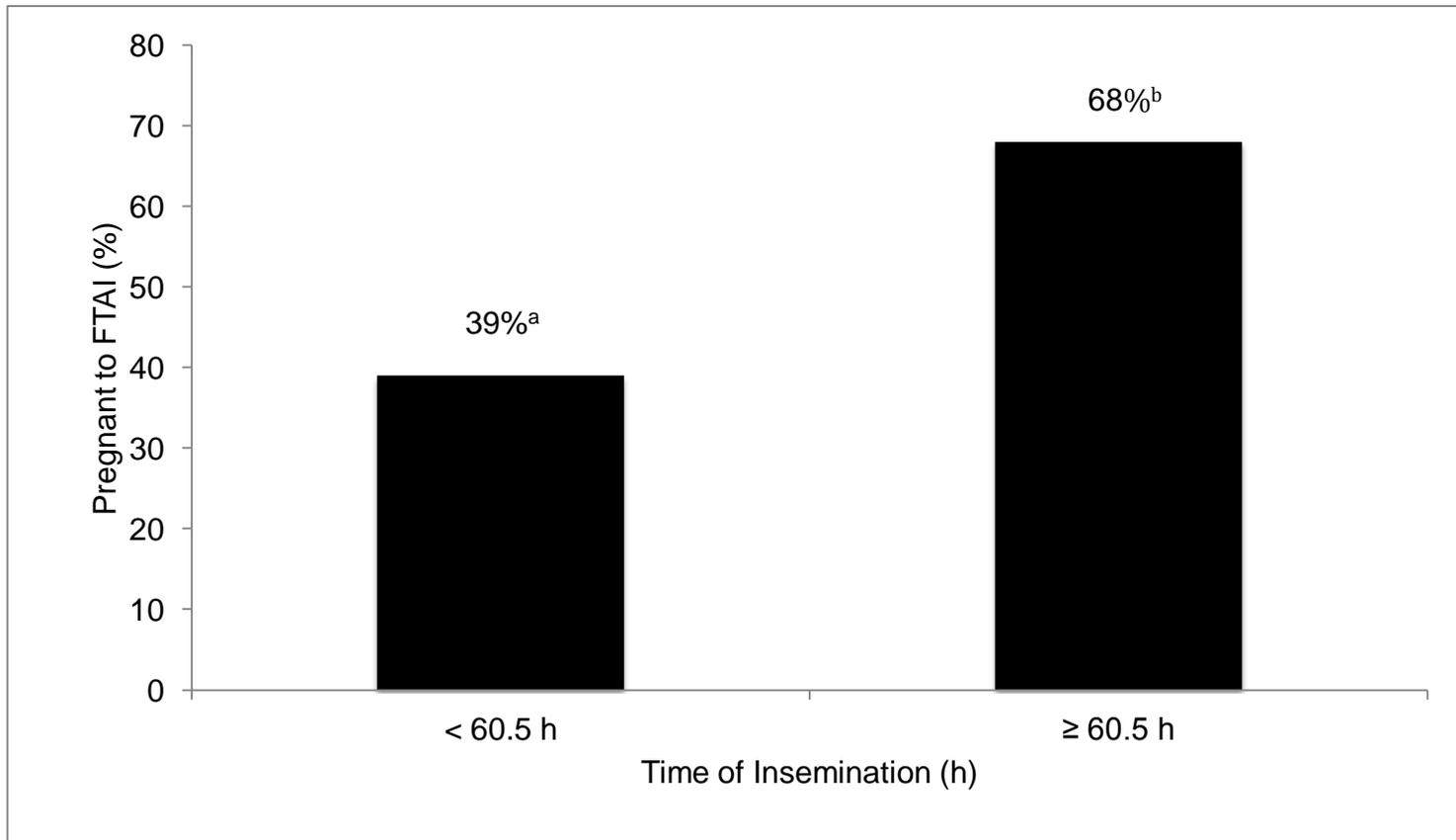


Figure 3.1 Pregnancy rate in White-tailed does based on time of insemination following CIDR-g removal.  
<sup>a,b</sup> Bars with different superscripts are different (P=0.02).

Table 3.3 Logistic regression analyses of 74 White-tailed doe fixed-timed artificial inseminations.

Predictor	$\beta$	SE $\beta$	Wald's $\chi^2$	df	P	$e^\beta$ (odds ratio)
Intercept	-5.7929	2.1153	7.4996	1	0.0062	0.003
Insemination Depth	2.8251	0.9941	8.0766	1	0.0045	16.862
Insemination Time	3.0842	0.9973	9.5628	1	0.0020	21.849
Treatment	0.6666	0.5951	1.2543	1	0.2627	1.948
Age	-1.6374	0.6516	6.3151	1	0.0120	0.194
Year	-0.5087	0.8459	0.3616	1	0.5476	0.601
Disposition	0.4675	0.4480	1.0888	1	0.2967	1.596
Vulva Color	0.2420	0.6683	0.1312	1	0.7172	1.274
Vulva Edema	0.6911	0.6415	1.1608	1	0.2813	1.996
Test			$\chi^2$	df	P	
Overall model evaluation						
Likelihood ratio test			25.4520	8	0.0013	
Score test			21.7425	8	0.0054	
Wald test			16.0102	8	0.0422	

Does that were  $\leq 4$  years of age were more likely ( $P = 0.01$ ) to become pregnant to FTAI than does  $> 4$  years of age (Table 3.3). Pregnancy rates were not affected by vulva color, vulva edema or DISP. All does that were confirmed pregnant via ultrasonography fawned within the reported gestation range of 187 to 222 days (Montone, 1996).

Fecundity was not different ( $P = 0.73$ ) between treatment groups ( $1.6 \pm 0.11$ ; no eCG vs.  $1.7 \pm 0.10$ ; eCG). Fecundity of does pregnant to FTAI was not different ( $P = 0.72$ ) compared with does pregnant to clean-up bucks ( $1.7 \pm 0.08$  AI does vs.  $1.7 \pm 0.09$  Clean-up bucks).

Does exhibited a heavier ( $P = 0.007$ ) mean body weight in Year 2 (64 kg) compared with Year 1 (60 kg), however, there was no difference in the average body weight of does in eCG and no eCG groups in Year 1 ( $P = 0.17$ ) and Year 2 ( $P = 0.87$ ). Likewise, there was no treatment by year interaction ( $P = 0.37$ ) for body weight across years. The average doe weight in this study across years was 62 kg and there was no difference ( $P = 0.49$ ) in the pregnancy rate of does weighing  $\leq 62$  kg (46%) compared to does weighing  $> 62$  kg (54%).

## **Discussion**

Although FTAI is now a common practice in beef and dairy herds, pregnancy rates following these protocols remain variable (Lambe, 2010). It is often advantageous to streamline FTAI protocols to reduce labor and animal stress. This could not be truer for the WT doe. Handling stress disrupts the endocrine system responsible for reproduction in most domestic livestock species (Echternkamp, 1984) and animal

temperament or flightiness has been associated with differences in pregnancy rates (Cooke et al., 2009). Therefore, management strategies for inseminating large numbers of does following detection of a natural estrus would likely not result in acceptable fertility following AI. Not only would this require the use of a teaser animal, the movement of non-synchronized estrual and non-estrual animals to and from the working facility for insemination would likely decrease pregnancy rates because of animal stress. Consequently, it is imperative that an estrus synchronization and FTAI protocol be developed for WT does that keeps human interaction to a minimum.

Based on the present study, an estrus synchronization and transcervical FTAI protocol could be successfully implemented over 17 days, with each doe entering the handling facility three times and restrained for a total of no more than 9 min, resulting in an acceptable pregnancy rate (50%). This pregnancy rate seemed similar to those reported for naturally cycling WT deer (Magyar et al., 1988; Jacobson et al., 1989), white-tailed does synchronized for FTAI (Saenz, 2007), and similar synchronization protocols and insemination methods for RD (Schenk and DeGrofft, 2003; Aller et al., 2009) and goats (Salvador et al., 2005).

Pregnancy rates following FTAI can be affected by factors that are either inherent to the animal or that can be controlled through management. To date, only a few studies have reported AI pregnancy rates of WT using FT semen following a natural cycle (Jacobson et al., 1989; Magyar et al., 1989). The pregnancy rates from our study seemed similar to those reported by Magyar, et al. (1989), but slightly lower than those reported by Jacobson, et al. (1989).

According to Asher, et al. (1993), the addition of eCG at or near CIDR removal in red deer has become part of the standard recommended synchronization protocol to increase the incidence of ovulation when administered in conjunction with an exogenous progesterone source. However, unlike results in other domestic species, such as sheep (Husein and Haddad, 2006) and RD (Asher et al., 1993), we concluded that administration of eCG following CIDR removal did not increase pregnancy rates in white-tailed does in this study. However, the present study had limited statistical power to detect an effect of eCG on pregnancy rate and the apparent difference may have been significant if more does had been included in the study.

Willard, et al. (1996) reported that sika hinds receiving 150 IU of eCG exhibited lower pregnancy rates compared with hinds receiving 100 IU (14.3 vs. 42.1%). In fallow deer, administration of 100 IU of eCG or greater resulted in an increased incidence of multiple ovulations which were associated with lower pregnancy rates and a higher incidence of embryonic mortality (Asher et al., 1990). Because pregnancy and fecundity rates in this study were not significantly different for does receiving 200 IU eCG compared with no eCG does, this phenomenon does not appear to occur in white-tailed females. Saenz (2007) reported increased pregnancy rates in WT does receiving 200 IU eCG compared with does not receiving eCG; however, does in that study were subjected to two different synchronization protocols, one incorporated a 7 days CIDR (no eCG) and the other a 14 days CIDR (eCG). Therefore, more studies are necessary to determine if higher doses of eCG would be advantageous to pregnancy rates in WT.

In the present study, does that were synchronized for FTAI and inseminated at 60.5 h or later post-CIDR removal exhibited a higher pregnancy rate compared with

does which were inseminated before 60.5 h. Our pregnancy rates seemed somewhat lower than those reported by Jacobson, et al. (1989) for WT does inseminated transcervically following a natural cycle with FT semen (80%), but higher than those reported for WT does transcervically inseminated with FT semen at 0, 6, 12, 18, 24 or 30 h after estrus detection (40%).

Asher et al. (1988) reported similar pregnancy rates (65%) in fallow deer transcervically inseminated with FT semen at 48 h post-CIDR removal; however, fallow deer inseminated at 61 h following a CIDR and prostaglandin administration had a 41% pregnancy rate (Jabbour et al., 1993b). The effect of insemination time on subsequent pregnancy rates may be species-specific, and the WT doe may physiologically require a longer interval from CIDR removal to AI than 60 h to exhibit increased pregnancy rates. In this study, we did not record ovulation or characterize concentrations of reproductive hormones, so this is merely speculation. More studies should be conducted to determine ovulation times in relation to CIDR removal in WT does to determine better insemination timings.

In this study, transcervical insemination resulted in deposition of semen into the uterus only 11% of the time. These results seemed lower than passage rates reported in red deer (74%) (Aller et al., 2009) and more similar to those reported in goats (17.5%) (Salvador et al., 2005). However, in sheep, the process of transcervical insemination has been replaced by laparoscopic intrauterine insemination because of reduced pregnancy rates (Anel et al., 2005). Some studies have indicated that even though transcervical passage rates in sheep can be acceptable using new techniques, fertility remains low (Rodriguez et al., 1988) and (Wulster-Radcliffe et al., 2004). The cause of

this phenomenon is unclear at this time, but may be due to the release of a spermicidal compound at insemination (Wulster-Radcliffe et al., 2004) or a release of PGF<sub>2</sub>α that negatively affects early embryonic development in sheep (Flint et al., 1975). It remains unclear whether this phenomenon is present in the WT doe. In this study only 8/74 inseminations resulted in deposition of semen into the uterus.

In RD, Aller, et al. (2009) reported no difference in pregnancy rates of hinds inseminated either intrauterine or intracervical following an FTAI protocol, and pregnancy rates were 43 vs. 20%, respectively. We concluded from our study in WT does that intrauterine or intracervical insemination resulted in higher pregnancy rates compared with intravaginal insemination. This was expected because of deposition of the semen deeper into the reproductive tract. However, too few inseminations resulted in deposition of semen in the uterus to make a valid statistical comparison between intrauterine and intracervical inseminations.

Behavioral signs of estrus in goats and sheep include swelling of the vulva and vaginal discharge. In the present study, neither vulvar inflammation nor color was associated with increased pregnancy rates. Because these factors were variable within both treatment groups, we hypothesized that the estrus response of does may not have been as tightly synchronized as anticipated resulting in a variable response of these two indicators. However, since estrous behavioral responses were not evaluated in our study, more investigations are needed to evaluate signs of estrus in WT does.

Future studies are needed in WT to investigate the effect of interval from CIDR removal to insemination and the effects of varying doses of eCG on FTAI pregnancy

rates. Also, incorporation of other hormones into the FTAI synchronization protocol to synchronize follicular wave emergence (GnRH) or to control the lifespan of the CL (or prostaglandin) may improve AI pregnancy and fawning rates.

In conclusion, we inferred that administration of eCG at CIDR removal did not enhance pregnancy rates. Additionally, with minimal human handling, estrus synchronization and FTAI of WT was successful in producing acceptable pregnancy rates, especially in does inseminated at 60.5 h post-CIDR removal or later.

## **CHAPTER 4**

### **EFFECT OF GnRH ON FIXED-TIMED ARTIFICIAL INSEMINATION PREGNANCY RATES OF WHITE-TAILED DEER**

#### **Introduction**

Popularity of White-tailed deer (WT) farming has increased in the last four decades and continues to grow and implement assisted reproductive techniques regularly used in domestic livestock operations. For this farmed species, which is prone to being highly stressed if handled too frequently, the importance and value of defining a fixed-time artificial insemination (FTAI) program increases. Manipulation of the female reproductive cycle allows for FTAI, decreased animal stress due to reduced handling of the animals, as well as eliminating the need for estrus detection. The most common tool for estrous synchronization in smaller cervidae is the implementation of a vaginally inserted progestin device (CIDR-g) for a period of 14 d (Plante, et al., 2006).

Although, estrous synchronization is achieved by prolonged administration of exogenous progesterone, adequate synchrony and increased pregnancy rates cannot be obtained without follicular wave control. Previous investigations, in cattle, have evaluated the effects of administering exogenous gonadotropins, estrogen, and prostaglandins and their roles in follicular wave synchronization when given concurrently with progestin devices (Fricke et al., 1998; Ambrose et al., 2001; Martinez et al., 2002; Richardson et al., 2002; Kasimanickam et al., 2006; Kim et al. 2006). Use of these hormones has reduced the time needed for pre-synchronization and synchronized follicular recruitment and CL regression (Thatcher et al., 2001). However, the use of these exogenous hormones requires additional human to animal contact,

which is not conducive within a WT operation. Therefore, the objective of this study was to determine if administration of exogenous GnRH given at FTAI would improve pregnancy rates in WT.

## **Materials And Methods**

### **Animal Subjects**

A captive herd of White-tailed deer was maintained at the Bob R. Jones Idlewild Research Station in Clinton, LA (30°86'N, 91°02'W). Two experiments, approved by IACUC, were conducted across two breeding seasons using mature does (Experiment 1: n=31; Experiment 2: n=40) having a mean age of 5.0 years (range: 1.5 to 9.5 years of age) and weighing between 44.5 and 79.8 kg (mean: 62.2 kg) (Table 4.1).

Does were maintained on 20% deer pellets (Professional Sportsman's Choice Record Rack, Cargill Animal Nutrition, Minneapolis, MN, USA) and native grasses. Does were rotated among enclosures ranging from 0.61 to 1.62 hectares.

### **Ovulation Control And Fixed-Time Artificial Insemination**

For each experiment, does were stratified by age, weight, and last fawning date and then randomly allocated to one of two estrous synchronization treatments. All does were synchronized with an EAZI-BREED™ CIDR® Sheep and Goat device (0.3 g progesterone, Agtech Inc., Manhattan, KS, USA) for a duration of 14 d (Experiment 1: November 5-19; Experiment 2: December 1-15). Fixed-timed AI with FT semen ( $10 \times 10^7$  progressively motile spermatozoa pre-freeze) commenced approximately 60 h after CIDR removal with a simultaneous injection of saline (Control: 2 ml i.m.; Experiment 1: n=14; Experiment 2: n=20) or GnRH (Treatment: 200 µg Cystorelin i.m.; Reproduction

Table 4.1 Number, age and weight of White-tailed does involved in GnRH FTAI synchronization trials.

Treatment	Does ( <i>n</i> )	Age <sup>a</sup>	Weight (kg) <sup>a</sup>
Experiment 1			
Control	14	6.2 ± 0.5	67.6 ± 1.9
GnRH	17	5.5 ± 0.5	65.5 ± 1.7
Experiment 2			
Control	20	4.6 ± 0.6	58.8 ± 1.9
GnRH	20	4.3 ± 0.6	59.0 ± 1.6
Combined			
Control	34	5.2 ± 0.4	62.4 ± 1.5
GnRH	37	4.8 ± 0.4	62.0 ± 1.3

<sup>a</sup>Mean ± SEM.

Resources, Walworth, Wisconsin; Experiment 1: n=17; Experiment 2: n=20) at insemination. Time of CIDR removal was recorded for each doe.

Frozen-thawed semen was obtained from two fertile bucks located at Idlewild Research Station. Semen was collected by electroejaculation and frozen in 0.5 cc straws by a commercial bull stud facility (Genex Cooperative, Baton Rouge, LA). Inseminations, conducted by the same technician, were done transcervically using a speculum, which was fitted with a light source. Use of the speculum provided visualization of the cervical os, as well as aided in guiding the insemination pipette to the cervical os. Once into the cervical os, the insemination pipette traversed the cervical rings until it reached the uterine body or until passage of the canal was no longer possible. Semen deposition occurred at three minutes regardless of location within the cervical canal. Time of insemination was recorded for each doe, which allowed the interval from CIDR withdrawal to semen deposition to be determined (insemination interval (II)).

At insemination, multiple variables were recorded for each doe: insemination interval (II), insemination depth (ID; 0: vaginal to 3: uterine body), mucous evaluations (viscosity and color), vulva assessments (edema and color), and sperm motility (1:  $\leq 30\%$  motile, 2: 31-60% motile, 3:  $\geq 61\%$  motile). Twenty-eight days subsequent to insemination, intact bucks were introduced to all does to allow for natural cover of females not pregnant to FTAI.

### **Pregnancy Diagnosis**

Pregnancy was determined by transrectal ultrasonography with an Aloka SSD-550 (Aloka Inc, Wallingford, CT) with a 5 MHz inlear-array rectal transducer fitted with a

modified plastic PVC tubing (38 cm in length) to assist in transducer manipulation. Ultrasonography was conducted 28 d following insemination and re-confirmed 75 d post-insemination. Pregnancy rates from FTAI and natural mating with clean-up bucks (CUB) were determined by fawning dates and an average gestation length of 195 d (range: 187 to 222 d).

### **Statistical Analysis**

Pregnancy rates to FTAI were assessed by Chi-square analysis using the frequency procedure of SAS 9.1 (SAS Institute, Inc. Cary, NC, USA). Calculated II for each doe was graphed by the chart procedure (Proc Chart; SAS Institute, Inc.). The II ranged from 58 h to 63 h post-CIDR withdrawal for both pregnant and open does, allowing the II being categorized into two groups (1: does inseminated < 60.5 h post-CIDR removal and 2: does inseminated  $\geq$  60.5 h). Insemination depth was also further categorized into two groups (1: vaginal deposition of semen and 2: cervical or uterine deposition of semen). Logistic regression determined the odds of pregnancy following FTAI using the variable collected at insemination, as well as treatment, age, and experiment. Differences in age of does and fecundity were assessed using general linear model procedures (Proc GLM, SAS Institute, Inc.).

### **Results**

Addition of GnRH at insemination increased the percentage of does pregnant to FTAI when compared to does in the control group (43% and 18%, respectively;  $p=0.0198$ ) (Table 4.2). Odds of pregnancy were five times higher when does were treated with GnRH ( $p=0.0084$ ) (Table 4.3). Evaluation of GnRH effects on each breeding season revealed a tendency to increase the number of females pregnant to

Table 4.2 Effect of GnRH given at FTAI on pregnancy rates in White-tailed deer.

Treatment	Does ( <i>n</i> )	Does Pregnant to FTAI (%)	P-value <sup>a</sup>
Experiment 1			
Control	14	4/14 (29)	0.1712
GnRH	17	9/17 (53)	
Experiment 2			
Control	20	2/20 (10)	0.0583
GnRH	20	7/20 (35)	
Combined			
Control	34	6/34 (18)	0.0198
GnRH	37	16/37 (43)	

<sup>a</sup> P < 0.05 denotes significance.

Table 4.3 Logistic regression analyses of White-tailed does following FTAI.

Predictor	$\beta$	SE $\beta$	Wald's $\chi^2$	df	P	$e^\beta$ (odds ratio)
Intercept	-3.3771	2.4525	1.8962	1	0.1685	0.034
Insemination Depth	-0.2364	0.7299	0.1049	1	0.7461	0.789
Insemination Interval	1.3665	0.6800	4.0377	1	0.0445	3.921
Treatment	1.6816	0.6385	6.9367	1	0.0084	5.374
Age	-0.4390	0.7439	0.3482	1	0.5551	0.645
Year	-1.5545	0.7764	4.0092	1	0.0453	0.211
Mucous	0.2754	0.2442	1.2719	1	0.2594	1.317
Vulva	0.2339	0.2685	0.7587	1	0.3837	1.264
Test			$\chi^2$	df	P	
Overall model evaluation						
Likelihood ratio test			16.4017	7	0.0217	
Score test			15.1424	7	0.0342	
Wald test			12.1871	7	0.0946	

FTAI when bred in December in comparison to controls (35% and 10%, respectively;  $p=0.0583$ ), however no effect was seen among does bred during the initial days of the breeding season (53% and 29%, respectively;  $p=0.1712$ ) (Table 4.2). The odds of pregnancy occurring were five times higher for does bred earlier in the breeding season in comparison to those bred later ( $p=0.0453$ ) (Table 4.3).

Comparison of the two breeding seasons yielded no difference in pregnancy rates ( $p=0.0790$ ), however, in contrast to rates previously reported by our laboratory (50%; unpublished), the overall pregnancy rate was lower (31%; Experiment 1: 42%, Experiment 2: 23%). In review of putative causes for reduced pregnancy rates, it was found that does had lost an average of 5% of their body weight (yearlings excluded) between the two breeding seasons, with fewer does weighing over 62 kg in Experiment 2 than in Experiment 1 (77% and 35%, respectively;  $p=0.0004$ ). However, assessment of weight did not affect the combined pregnancy rates ( $p=0.08955$ ; yearlings excluded) or rates when evaluated separately (Experiment 1:  $p=0.4533$ , Experiment 2:  $p=0.5568$ ).

Analysis of variables recorded at time of semen deposition (ID, vulva appearance, and spermatozoan motility) presented no effect on pregnancy rates between the control and GnRH treated groups. Does with opaque, viscous cervical mucous tended to have an increased pregnancy rate to FTAI than does which had clear, thin mucous (43% and 38%, respectively;  $p=0.0844$ ) (Table 4.4).

Table 4.4 Mucosal properties at FTAI in relation to FTAI pregnancy rates in White-tailed deer.

Treatment	Does ( <i>n</i> )	Mucosal Score	Does pregnant to FTAI (%)
Experiment 1	12	1 <sup>a</sup>	2/7 (29)
		2 <sup>b</sup>	4/5 (80)
Experiment 2	15	1 <sup>a</sup>	3/6 (50)
		2 <sup>b</sup>	2/9 (22)
Combined	27	1 <sup>a</sup>	5/13 (38)
		2 <sup>b</sup>	6/14 (43)

<sup>a</sup> Clear, non-viscous mucous

<sup>b</sup> Opaque, viscous mucous

Age also increased the odds of deeper ID, with older does (>4 years of age) one times more likely to be inseminated within the cervix or uterine body (Table 4.3). Does had a mean II of 60.2 h (range: 58.2-63.0 h) and while no differences were found among does inseminated before or after 60.5 h post-CIDR removal ( $p=0.3005$ ) (Figure 4.1), does inseminated beyond 60.5 h post-CIDR withdrawal were four times more likely to become pregnant to FTAI ( $p=0.0445$ ) (Table 4.3).

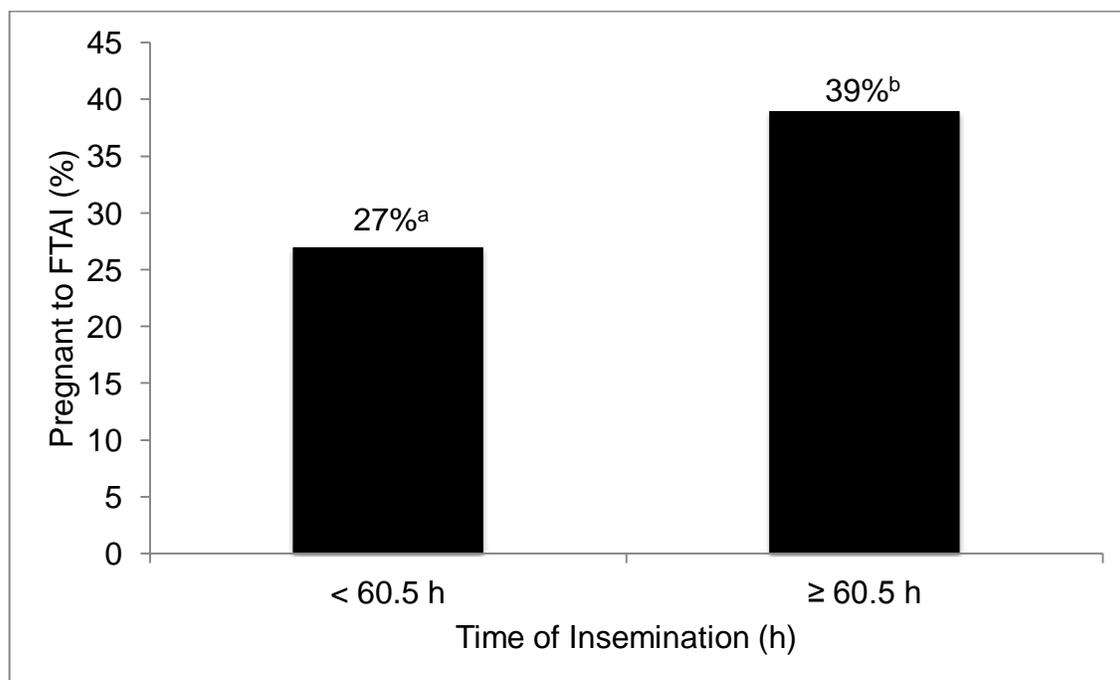


Figure 4.1 Percentage of White-tailed does pregnant to FTAI based on timing of insemination following CIDR withdrawal. <sup>a</sup> Bars with same subscripts are not different ( $p=0.3005$ ).

Pregnancies from FTAI and intact bucks were differentiated by fawning dates and ultrasonography. Placentomes were visualized and measured in FTAI pregnancies

(range: 32.7 to 56.2 mm in length), whereas pregnancies derived from intact bucks presented no identifiable placentomes (Figure 4.2). However, crown-rump measurements were obtainable from intact buck pregnancies (range: 13.4 to 21.7 mm) (Figure 4.3).

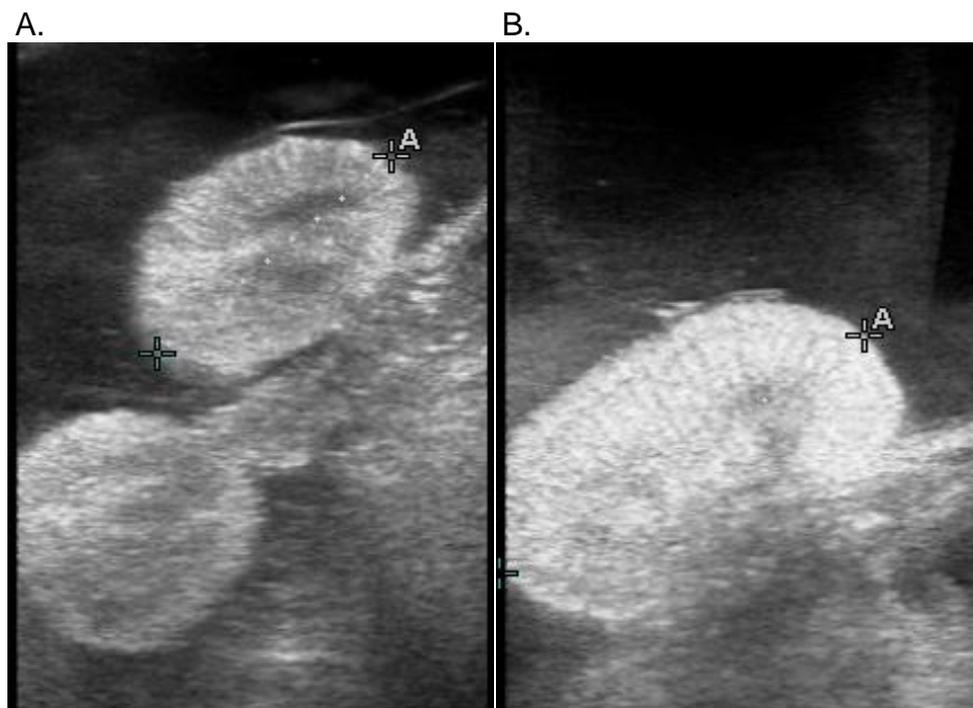


Figure 4.2 Placentomes in fixed-time artificially inseminated White-tailed does at day 75 of gestation. A. Top placentome measures 50.0 mm in length. B. Placentome measures 35.8 mm in length.

Comparison of fecundity rates between treatment groups yielded no difference ( $p=0.9537$ ) (Control:  $1.58 \pm 0.30$ ; Treatment:  $1.56 \pm 0.16$ ). Additionally, fecundity of WT does pregnant to FTAI did not differ ( $p=0.9611$ ) from fecundity rates of does pregnant to CUB (FTAI does:  $1.58 \pm 0.15$ ; CUB:  $1.85 \pm 0.13$ ).

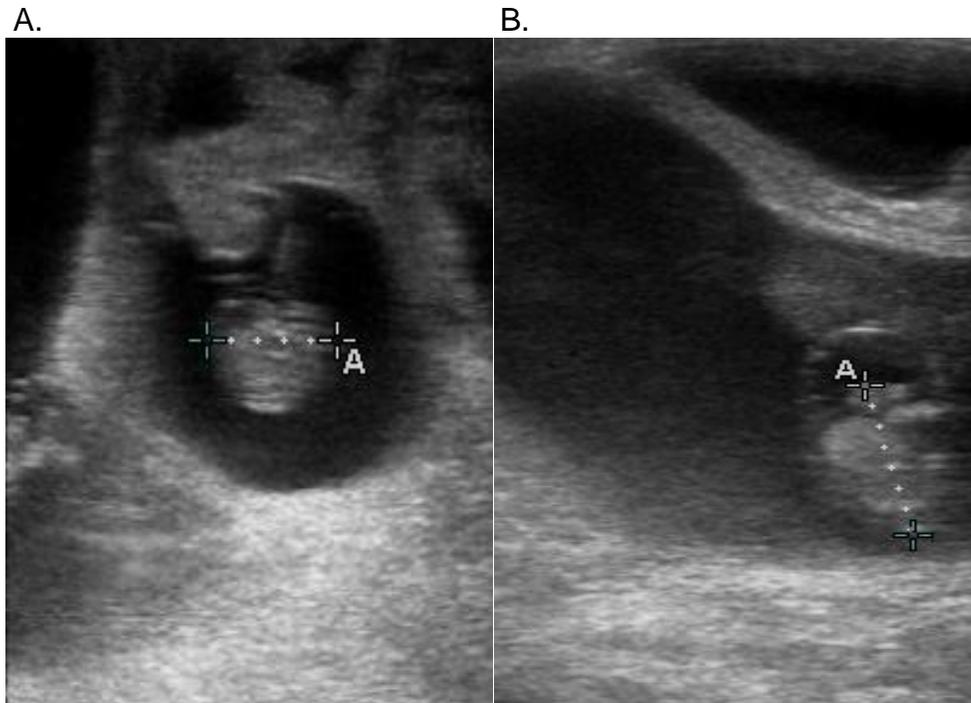


Figure 4.3 Fetal measurements from White-tailed does pregnant to intact bucks. A. Crown measurement of 13.5 mm, indicating a fetus of approximately 67 d of age based on data reported by Armstrong (1950). B. Crown-rump measurement of 16.5 mm, indicating a fetus of approximately 36 d of age based on data reported by Armstrong (1950).

## DISCUSSION

Spontaneous estrus detection and artificial insemination in farmed deer requires intensive labor and multiple, sporadic inseminations. The variability associated with estrus detection can be eliminated with the incorporation of a FTAI program that coordinates the timing of follicular development, synchronizes estrus, and yields similar, if not improved, pregnancy rates.

Administration of exogenous progestins, at any point during the estrous cycle, have been used extensively to stimulate estrous cyclicity, synchronize estrus, reduce labor and handling of animals during breeding seasons, enhance pregnancy rates, and

synchronize parturition in farmed species (Brown et al., 1988; Macmillan and Peterson, 1993; Stevenson, 2008; Leitman et al., 2009). The use of exogenous progestins lengthens the estrous cycle and postpones ovulation by suppressing the LH surge (Morrow et al. 2009). Treatment with exogenous GnRH induces follicular atresia and stimulation of a new follicular wave by artificially generating LH pulses (McLeod et al., 1991; Sato et al., 2005; Morrow et al., 2009). In CIDR-treated dairy heifers, GnRH is effective in inducing new follicular development, LH release, and synchronization of ovulation, as well as yielding a 61% pregnancy rate (Ambrose et al., 2001).

Very few cervidae studies have analyzed low doses of GnRH and its effect on CIDR controlled estrus synchronization regimes (McLeod et al., 1991; Jabbour et al., 1993). McLeod et al. evaluated the effect of intermittent and continuous GnRH injections in anoestrus Père David's deer (1991). Both exogenous gonadotropin treatments increased LH concentrations, shortened the interval to the onset of estrus (62 h vs 91 h), and induced ovulation in more than 80% of treated females. In sheep, the use of GnRH 24 h post-CIDR withdrawal benefits FTAI protocols as it reduces the interval between CIDR removal and estrus (45-48 h vs 57-60 h in ewes without GnRH and in ewes receiving GnRH 36 h post-CIDR removal, respectively), as well as initiates the LH peak to occur 10 to 21 h earlier than the natural peak (34.5-45.5 h post-CIDR removal) (Walker et al. 1989). However, administration of GnRH at 24 h or 36 h following progesterone abstraction did not improve pregnancy rates in ewes (Control: 62% vs 37% and 66%, respectively) (Walker et al., 1989). Similar results of reduced interval from progesterone removal to LH surge and less variability in ovulation timing were reported in Pygmy and Nigerian Dwarf goats (Pierson et al., 2003).

In this study, in order to initiate LH pulses, which influence the final stages of preovulatory follicular development (Baird, 1978), and induce ovulation following CIDR-G removal, exogenous GnRH was given at FTAI. Although hormonal data and ovulation occurrence were not evaluated, we inferred that the addition of exogenous GnRH at FTAI improved pregnancy rates in WT. While our data showed increased pregnancy rates, these rates decreased from those previously reported by our lab and from commercial standards (Gentry et al., 2012). It is speculated that there was less synchrony present in year 2 of this study, due to FTAI occurring slightly later in the breeding season, which deters comparison to results previously reported by our lab. Prior reported effects of GnRH on pregnancy rates have shown to approach commercial standards more so than those reported in this study (Ambrose et al., 2001). It is speculated that the higher pregnancy rates are due to females in previous studies having undergone pre-synchronization with prostaglandins in addition to the use of GnRH and FTAI. When European fallow does were inseminated with FT European fallow deer spermatozoa 65 h after CIDR removal, conception rates (75%) met the commercial standard (Jabbour et al., 1993). The data from this study are not in concert with the fallow deer study in regards to semen deposition, as the fallow deer females were inseminated laparoscopically and too few inseminations occurred intrauterine (11/70) to make a valid comparison as to the ability of this FTAI + GnRH regimen to produce pregnancy rates that would satisfy commercial standards.

It has been postulated that WT have an extended time from exogenous progesterone decline to LH peak and ovulation in comparison to domestic livestock (Gentry et al., 2012). However, Plotka et al. reported that normally cycling WT does

have progesterone fluctuations in concentration prior to the onset of estrus similar to goat, sheep and cattle (1977). There are also specie similarities in LH release and its temporal relationship to both estradiol and progesterone, with the LH peak occurring simultaneously with the onset of estrus (Plotka et al., 1980).

Within traditional (non-timed) cattle AI programs, when AI occurs beyond 12 h following the initiation of estrus, greater fertilization rates occur, but there is reduced embryo quality when compared to embryos from inseminations which were closer to the onset of estrus (Sales et al., 2011). Following FTAI in beef cows, ovulation has been reported to occur between 64 and 76 hours post-progestin removal (Hanlon et al., 1997; Carvalho et al., 2008; Sa Filho et al., 2010) and that there is a tendency for increased pregnancy rates when FTAI occurs at > 60 h in comparison to 54 h following CIDR withdrawal (Busch et al., 2008; Sales et al. 2011). Our results are comparable to data detailed by Busch et al. (2008) where it was reported that beef cows which underwent a CO-Synch + CIDR protocol with FTAI at 66 h had a higher pregnancy rate compared with cows bred at 54 h post-CIDR withdrawal. Similarly, transcervical insemination of WT does with FT semen  $\geq$  60 h post-CIDR removal has yielded a 68% pregnancy rate (Gentry et al., 2012). Data reported in the current investigation are supportive of increased pregnancy rates resulting from FTAI, which occurred beyond 60.5 h post-CIDR-g removal.

During the estrous cycle, the physical and rheologic properties of cervical mucous vary from transparent, non-viscous fluid when under estrogenic influence to opaque, viscous fluid when progesterone levels are increased (Tsiligianni et al., 2001). The penetrability of cervical mucous is of significance as spermatozoa must be able to

traverse the cervical fluid prior to fertilization. Cervical mucous aids in sperm selection by removing seminal plasma from the surface of spermatozoa and acts as a barrier to morphologically abnormal and immotile sperm (Suarez and Pacey, 2006). Data reported from this study support the theory that hydrated cervical mucous allows for increased spermatozoan penetration which leads to an increased likelihood of pregnancy occurrence.

In conclusion, data from this study show that administration of GnRH at FTAI increased pregnancy rates in WT does that underwent a 14 d CIDR-g estrous synchronization protocol and produced fecundity rates similar to does bred by naturally mating. It is hypothesized that pre-synchronization will program follicular waves of does to be in a more favorable stage of the estrous cycle prior to implementing a FTAI program which includes GnRH given at insemination. Therefore, it is recommended that the effects of prostaglandins prior to FTAI and GnRH administration be further investigated in WT. Additionally, further investigation into the appropriate insemination interval following estrous synchronization should be conducted.

To date there is a lack of continuous follicular data reported in WT. As follicular waves are consistently present during estrous synchronization regimens, it would also be beneficial to monitor hormonal concentrations, as well as the presence and lengths of follicular waves throughout synchronization to provide information on follicular responses to pre-determined concentrations of exogenous hormones. Following follicular growth and regression would assist in establishing proper timing of insemination following progestin withdrawal.

## CHAPTER 5

### ***IN VITRO* FERTILIZATION MEDIA AND CAPACITATION AGENT EFFECTS ON EPIDIDYMAL WHITE-TAILED DEER SPERMATOZOA**

#### **Introduction**

To ascertain the requirements for *in-vitro* production (IVP) of whitetail deer (WT) embryos, identification of media components necessary for the induction of sperm capacitation and the acrosome reaction (AR) must be obtained. Capacitation is a maturational process that sperm undergo within the female reproductive tract prior to fertilization. This process is a prerequisite for fertilization in mammalian sperm, including guinea pig, mouse, ram, buffalo, and cattle (Yanagimachi and Usui, 1974; Fraser, 1982; Florman and First, 1988; Boccia et al., 2013; Luna et al., 2015).

During natural mating, spermatozoa encounter albumin, a uterine and oviductal protein that serves as a cholesterol acceptor. Efflux of cholesterol from the sperm plasma membrane (PM) increases phospholipid scrambling on the surface of the spermatozoa, thus increasing membrane permeability and fluidity, which allows calcium and bicarbonate ions to enter the cell. These ions promote cyclic AMP (cAMP; adenosine 3', 5'-cyclic monophosphate) metabolism through adenylyl cyclase activity (Abou-haila and Tulsiani, 2009; Visconti, 2009; Sherriff and Ali, 2010). Downstream stimulation of protein kinases by cAMP yields increased protein tyrosine phosphorylation that is necessary for sperm hyperactivation, the AR and binding of spermatozoa to the zona pellucida (Visconti et al., 1999; Urner and Sakkas, 2003; Salicioni et al., 2007; Abou-haila and Tulsiani, 2009). However, the effectiveness of this mechanism is species specific. For example, the cAMP-dependent process in hamster

spermatozoa is activated by increased calcium ions, whereas boar spermatozoa require only bicarbonate ions to initiate the same process (Harrison, 2003).

Capacitation is mimicked *in vitro* by the use of defined media, which aids in the sperm's ability to initiate the AR and subsequently gain the capacity for fertilization (Visconti et al., 1999). The occurrence of tyrosine phosphorylation *in vitro* is dependent on the presence of albumin, bicarbonate ions, and calcium ions within the media; absence of any of these components from the media will cease the progression of capacitation (Visconti and Kopf, 1998, Harnal et al., 2000).

Epididymal spermatozoa, when compared to ejaculated spermatozoa, have a reduced amount of PM cholesterol, due to lack of contact with seminal plasma, resulting in lower sterol efflux (Matas et al., 2010). When immotile epididymal spermatozoa are exposed to *in vitro* capacitating conditions (bicarbonate, calcium, albumin, and HDLs), an efflux of cholesterol from the sperm PM and flagellum occurs, initiating hyperactivation and disrupting the cholesterol/phospholipid ratio (Carlson et al., 2007; Abou-haila and Tulsiani, 2009).

*In vitro* studies indicate that capacitated spermatozoa undergo the AR when they are incubated with zona pellucida glycoproteins, follicular fluid, and non-physiological calcium ionophore, ionomycin inducers (Lukoseviciute et al., 2005). Heparin, a glycoprotein, benefits the rate of capacitation and subsequent AR when added to a defined fertilization medium (Parrish et al., 1988). Brief exposure of epididymal spermatozoa to bovine seminal proteins *in vitro* is not sufficient to initiate capacitation in the absence of heparin or HDLs (Manjunath and Therien, 2002). However, the

interaction of bovine seminal proteins, heparin and LC increases acrosomal reactions of bovine epididymal spermatozoa (Manjunath and Therien, 2002).

Supplementation of heparin to bicarbonate buffered fertilization medium maintains the destabilized sperm PM of FT bovine spermatozoa, and induces an increase of sperm intracellular pH, calcium concentration, and protein phosphorylation (Lukoseviciute et al., 2005; Dapino et al., 2006). Kawakami et al. (2000) noted that in addition to heparin stimulating sperm motility and capacitation, that bovine oviductal fluid induces changes in the bovine sperm PM and activation of cyclic AMP. In capacitated hamster spermatozoa, heparin induces the AR; however, it has no effect on capacitation or the AR if the spermatozoa are incubated under non-capacitating conditions, such as in the presence of glucose (Parrish et al., 1989; Cormier and Bailey, 2003). The use of bovine IVF (BIVF) with heparin for sperm treatment in the North American wapiti has also been successful for inducing capacitation (Pollard et al., 1995; Berg and Asher, 2003).

Although the bovine IVP system has been successfully applied to develop IVP embryos in other species (Varner et al., 1993; Pollard et al., 1995; Roth et al., 1998; Carrell and Liu, 2002; Lukoseviciute et al., 2005), this system does not translate to all cervid species (Chapman et al., 1999; Harnal et al., 2001; Berg and Asher, 2003). The use of heparin as a CA for RD spermatozoa was inadequate for the induction of capacitation (5-11%) (Berg et al., 2002). Additionally, the use of heparin in reindeer evaluations has yielded low fertilization rates with epididymal sperm (36%) (Krogenaes et al., 1994). However, the use of 20% sheep serum (SS) increased sperm capacitation

and monospermic penetration in both RD and sika deer (77% and 49%, respectively) (Comizzoli et al., 2001; Berg et al., 2002).

Macromolecules, such as serum, are commonly used in culture media to mimic oviductal proteins. Serum and bovine serum albumin (BSA) promote capacitation by removing cholesterol from the sperm PM, therefore increasing the fluidity of the membrane (Harnal et al., 2000; Herrick et al., 2004; Salicioni et al., 2007). When heparin was compared to estrous deer serum, day 1.5 deer serum, or SS, results indicated that SS was more effective in inducing capacitation and yielded higher red deer fertilization rates (11%, 24%, and 73%, respectively; Berg et al., 1995).

To determine the requirements for a RD defined culture system, Berg and Asher (2003) evaluated the chemical composition of RD oviductal fluid and noted the need for higher calcium levels within a SOF-based fertilization and culture medium. The resulting DSOF reportedly produces higher fertilization rates in comparison to SOFaaBSA media (64% and 47%, respectively; Berg and Asher, 2003). Comparison of the bovine and RD systems revealed a distinct difference in the CA used (heparin and 20% sheep serum, respectively), as well as the amount of calcium concentrations necessary for fertilization to occur (Berg and Asher, 2003).

Currently, there is no research in regards to the chemical requirements necessary for inducing capacitation and the subsequent AR in WT sperm. Harnal et al. (2001) stated, based on a personal communication, that a modified Tyrode's medium supplemented with heparin was effective in inducing capacitation in FT WT spermatozoa. Therefore, the aim of this study was to compare and evaluate the ability

of bovine and RD fertilization media to provide an environment conducive to capacitation and induction of the AR of WT epididymal spermatozoa.

## **Materials And Methods**

### **Media**

Epididymal spermatozoa incubations occurred in modified Tyrode's media, with species-specific differences (Table 5.1).

### **Epididymal Spermatozoa**

Epididymal spermatozoa were obtained from fifteen hunter-killed bucks in 2011. Following epididymal collection, semen was extended with a commercial extender, Triladyl® (Minitub, Germany). The extended semen was then placed into 0.50 ml plastic straws (48 – 147 x 10<sup>6</sup> sperm/ml) and sealed. The straws were then placed in a room temperature water bath for 30 minutes, followed by a minimum of 2h at 4°C, and then frozen in LN<sub>2</sub> vapor.

### **Preparation Of Spermatozoa**

Frozen-thawed semen from each buck was layered over 2 ml of Bovipure® Bottom Layer Medium (Nidacon Laboratories AB, Gothenborg, Sweden) and centrifuged for 15 minutes at 1200 rpm to remove dead cells. The supernatant was removed; sperm TALP was added to the sperm pellet and centrifuged (10 minutes at 1200 rpm) to remove any remaining extracellular debris. The resulting sperm pellets were diluted to a final sperm concentration of 55 x 10<sup>6</sup> spermatozoa/ml fertilization media. Diluted sperm was then dispersed into 72 vials, with either BIVF (n=36) or DSOF (n=36), for a total of 1 x 10<sup>6</sup> spermatozoa/200 µl. Vials from each treatment were further supplemented with heparin (10 µg/ml), 20% sheep serum, or with no CA. Each

Table 5.1 Composition of bovine (BIVF) and red deer (DSOF) *in vitro* fertilization media.

Component	IVF-TALP (mM)	
	Bovine	Cervid (DSOF) <sup>a</sup>
NaCl	114	100
KCl	3.2	6
NaHCO <sub>3</sub>	25	25
NaH <sub>2</sub> PO <sub>4</sub>	0.34	0
Hepes	10	-
Ca lactate	-	1.3
Lactic Acid	10	-
CaCl <sub>2</sub> + 2H <sub>2</sub> O	2	1.9
MgCl <sub>2</sub> + 6H <sub>2</sub> O	0.5	-
MgSO <sub>4</sub> + 7H <sub>2</sub> O	-	0.6
Na <sub>2</sub> SO <sub>4</sub>	-	0.9
Na pyruvate	-	0.25
Glucose	-	0.1
Glutamine	-	0.25
Glycine	-	1.2
Alanine	-	0.6
Taurine	-	0.1
Proline	-	0.15
Asparagine	-	0.05
Histidine	-	0.09
Serine	-	0.075
P/S (µl/ml)	10	50
BSA (mg/ml) FAF	6	0.1
BME eAA (µl/ml)	-	20

<sup>a</sup> Modified from Berg and Asher, 2003.

supplemented sample was duplicated and incubated in humidified 5% CO<sub>2</sub> air at 39°C for 10 minutes (T0), 2 h (T2), or 4 h (T4).

### **Plasma Membrane Lipid Disorder**

To determine the state of the sperm PM, merocyanine 540 (M540, 2.7µM; Sigma Aldrich, St. Louis, MO), a hydrophobic dye, was applied to each aliquot at each specified time point (T0, T2, and T4) and analyzed with a flow cytometer. M540 intensely stains membrane lipid components that are in disorder, indicating capacitated sperm (Matas et al., 2010). The addition of YO-PRO®-1 Iodide (25 nM solution in DMSO; Life Technologies, Grand Island, NY), a membrane-impermeable fluorescent nucleic acid probe, was used assess PM permeability. Live, membrane-intact cells do not fluoresce, whereas dead, membrane-scrambled cells fluoresce green when labeled with YO-PRO®-1.

### **Initiation And Assessment Of Acrosome Reaction**

The AR was artificially induced using lysophosphatidylcholine (LC; 100 µg/ml) and evaluated each hour of incubation (T0, T2, and T4). Acrosomal integrity and sperm viability were examined by labeling aliquots for each time point with fluorescein isothiocyanate conjugated *Aechis hypogaea* (peanut) agglutinin (FITC-PNA, 5 µg/ml; Sigma Aldrich, St. Louis, MO), a marker for acrosomal leakage, and propidium iodide (Prl, 2.4mM), a marker for cell death. Post-labeling, samples were incubated at room temperature for 10 minutes and then analyzed by flow cytometry. Spermatozoa that presented green fluorescence of the acrosomal cap were considered to have an intact acrosomal membrane; whereas acrosome reacted sperm cells have disrupted labeling of the acrosomal cap.

### **Flow Cytometry**

Measurements were taken using a BD Accuri™ C6 flow cytometer (Becton, Dickinson and Company, San Jose, CA) at the end of each incubation period. The four fluorescent probes were excited by an Argon 488 nm laser. FITC-PNA and YO-PRO®-1 fluorescence were detected on detector FL1 and Pri and M540 signals were detected on detector FL3.

Sperm sorting and analyses were completed using CFlow software (Becton, Dickinson and Company, San Jose, CA), with a minimum of 10,000 events recorded per sample. Correlated measurements of forward-scattered light (FSC) and side-scattered light (SSC) allowed for differentiation of the spermatozoa population from the background within each sample. The heterologous population was gated for each sample and two subpopulations were analyzed for each pair of probes: (1) viable (Pri negative) sperm cell events that displayed an intact acrosome and viable sperm that underwent the acrosome reaction (PNA positive); (2) viable cell events (YO-PRO®-1 negative) that exhibited lipid disorder within the PM (M540 positive) and viable cells which did not uptake M540, indicating the lack of capacitation (Figure 5.1).

### **Statistical Analysis**

The GLIMMIX procedure of SAS 9.4 (Cary, NC, 2012) was used to analyze the data as a 2 x 3 factorial design with duplicate samples run within each buck. The media (BIVF and DSOF) and treatment applied (heparin, 20% sheep serum, no CA supplement) were considered the main variables. Time was included as the repeated factor. Saxton's macro algorithm was applied to determine differences between each variable. Backward stepwise regression was used to determine the odds of acrosome

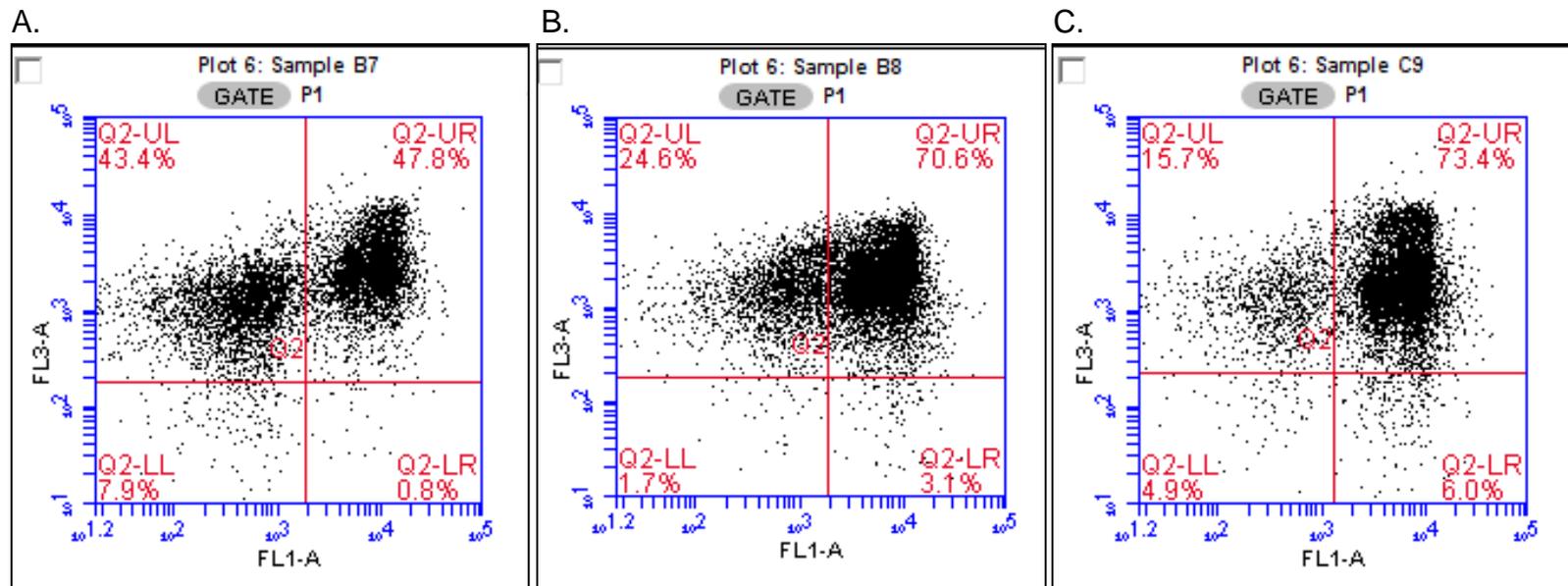


Figure 5.1 Representative flow cytometric dot plots of merocyanine 540/ YO-PRO®-1 -stained epididymal WT spermatozoa. The above flow cytometric dot plots are representatives of plots obtained in this study. A) Merocyanine 540 (FL3-A) and YO-PRO®-1 (FL1-A) stained epididymal WT spermatozoa when incubated in BIVF supplemented with heparin for 10 minutes (T0); B) Merocyanine 540 (FL3-A) and YO-PRO®-1 (FL1-A) stained epididymal WT spermatozoa when incubated in BIVF supplemented with 20% SS for 10 minutes; C) Merocyanine 540 (FL3-A) and YO-PRO®-1 (FL1-A) stained epididymal WT spermatozoa when incubated in BIVF alone (no supplementation with a capacitating agent) for 10 minutes.

reaction and lipid disruption occurrences when WT epididymal spermatozoa were incubated in two IVF media and two CA.

## **Results**

### **Lipid Disorder**

The odds of capacitated spermatozoa were 2.81 times more likely to occur from sperm incubated in reduced calcium levels within the BIVF than those incubated in DSOF. The probability of sperm having increased lipid disorder in BIVF is 20% compared to 8% in DSOF, which was indicative of a significant difference between media ( $P < 0.05$ ) (Table 5.2).

In evaluation of heparin increasing capacitation associated lipid disorder, it was found to be 64% higher than SS (Table 5.3). However, the odds of lipid disorder occurring were 0.706 times less likely in the presence of SS when compared to the absence of CA supplementation (Table 5.3). Although there was no difference between the probabilities for increased lipid disorder due to heparin and the absence of a CA (15% and 14%, respectively), these did differ from the use of SS for induction of capacitation (10%).

Inclusion of heparin in the BIVF was 1.70 times more likely to induce lipid disorder when compared to BIVF with SS and each of the DSOF treatments (heparin: 122% higher, SS: 251% higher, and no CA: 281%, respectively) (Table 5.4). No difference was indicated between probabilities of the occurrence of capacitation in BIVF with heparin and BIVF without a CA (21% and 26%, respectively); however, these did differ from BIVF with SS (14%) (Table 5.5). When the interactions of media and CA

Table 5.2 Modeling the probability of lipid disorder occurrence per variable.

Variable	$\beta$	SE $\beta$	Mu	MS Group <sup>a</sup>
Media				
BIVF	-1.4073	0.2084	0.1967	A
DSOF	-2.4408	0.2087	0.0801	B
CA				
Heparin	-1.7100	0.2149	0.1532	A
20% SS	-2.2051	0.2151	0.0993	B
No CA	-1.8570	0.2150	0.1351	A

<sup>a</sup> Differences seen in groups with different lettering within each variable sub-category.

Table 5.3 Modeling the odds of lipid disorder occurrence. Differences of media and capacitation agents least square means.

Variable	_Variable	$\beta$	SE $\beta$	Df	P	Odds ratio
Media						
BIVF	DSOF	1.0335	0.1045	238	<0.0001	2.811
CA						
Heparin	20%SS	0.4952	0.1281	238	0.0001	1.641
Heparin	No CA	0.1470	0.1279	238	0.2514	1.158
20%SS	No CA	-0.3481	0.1281	238	0.0071	0.706

Table 5.4 Modeling the odds of lipid disorder per supplemented media.

Variable	CA	_Variable	_CA	Df	P	Odds ratio
BIVF	Heparin	BIVF	20% SS	238	0.0035	1.703
BIVF	Heparin	BIVF	No CA	238	0.1773	0.784
BIVF	Heparin	DSOF	Heparin	238	<0.0001	2.221
BIVF	Heparin	DSOF	20% SS	238	<0.0001	3.512
BIVF	Heparin	DSOF	No CA	238	<0.0001	3.801
DSOF	Heparin	DSOF	20% SS	238	0.0123	1.581
DSOF	Heparin	DSOF	No CA	238	0.0034	1.711

Table 5.5 Modeling the probability of lipid disorder occurrence per supplemented media.

Media	Capacitation agent	$\beta$	SE $\beta$	Mu	MS Group <sup>a</sup>
BIVF	No CA	-1.0677	0.2328	0.2558	A
BIVF	Heparin	-1.3110	0.2329	0.2123	A
BIVF	20% SS	-1.8431	0.2332	0.1367	B
DSOF	Heparin	-2.1089	0.2335	0.1082	BC
DSOF	20% SS	-2.5671	0.2336	0.0713	CD
DSOF	No CA	-2.6463	0.2337	0.0662	D

<sup>a</sup> Differences seen in media/capacitation agent interactions in groups with differing MS Group lettering.

supplementations were plotted, BIVF was higher in ability to promote capacitation and an interaction occurred when sperm were in the presence of BIVF alone (Figure 5.2).

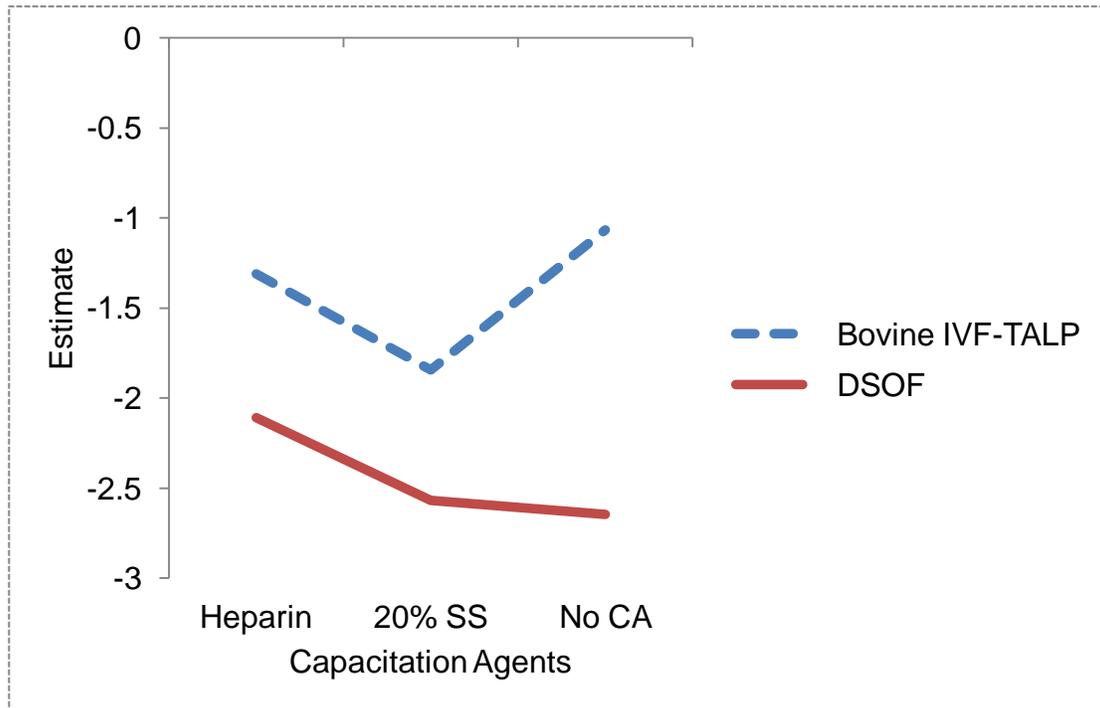


Figure 5.2 Fertilization media and capacitation agent interactions.

The presence of heparin in DSOF also yielded higher odds of lipid disorder in comparison to DSOF with SS or with no CA (58% and 71%, respectively) (Table 5.4). The probability of capacitation occurrence did not differ between DSOF with heparin and BIVF with SS (11% and 14%, respectively) (Table 5.5). The probability of increased capacitation rates did not differ between DSOF with heparin and DSOF with SS (11% and 7%); however, the DSOF with heparin had a higher probability of capacitation induction than DSOF with no CA (11% and 6.6%) (Table 5.5).

Although BIVF with heparin yielded higher odds of capacitated spermatozoa, the interaction of media, heparin and time did not produce significantly higher odds of lipid disorder. However, minimal incubation (0 and 2 hours) generated higher probabilities of lipid disorder (15% and 14%, respectively) when compared to spermatozoa incubated 4h (10%).

### **Acrosome Reaction**

The higher calcium levels in DSOF were twice as likely to induce the AR in comparison to BIVF (Table 5.6). The probability of sperm undergoing the AR was high in both DSOF and BIVF (99% and 98%, respectively) and significantly different (Table 5.7).

When the acrosomal reaction was assessed by the capacitation agent alone, heparin was nearly four times more likely to induce the reaction than SS and nearly twice as likely than when there was no capacitation agent present (Table 5.6). However, the presence of heparin, SS or lack of a capacitation agent in combination with either media treatment did not yield significant results.

Evaluation of the AR by time revealed that spermatozoa incubated for 10 minutes had higher odds than those incubated for 2 h and 4h (63% and 85%, respectively) (Table 5.8). A difference was present between T0 compared to T2 and T4 (99%, 98% and 98%, respectively) (Table 5.9). The interaction of media, CA, and time did not generate significant odds or increased probabilities in regards to the percentage of acrosome reacted spermatozoa.

Table 5.6 Modeling the odds of acrosome reaction occurrence per variable.

Variable	_Variable	$\beta$	SE $\beta$	Df	P	Odds ratio
Media						
BIVF	DSOF	-0.6657	0.1564	227	<0.0001	0.514
CA						
Heparin	20%SS	1.3558	0.1917	227	<0.0001	3.880
Heparin	No CA	0.5759	0.1924	227	0.0031	1.779
20%SS	No CA	-0.7798	0.1905	227	<0.0001	0.458

Table 5.7 Modeling the probability of acrosome reaction occurrence per variable.

Variable	$\beta$	SE $\beta$	Mu	MS Group <sup>a</sup>
Media				
BIVF	3.9280	0.3275	0.9807	B
DSOF	4.5937	0.3278	0.9900	A
CA				
Heparin	4.9047	0.3375	0.9926	A
20% SS	3.5490	0.3363	0.9720	C
No CA	4.3288	0.3368	0.9870	B

<sup>a</sup> Differences seen in groups with different lettering within each variable sub-category.

Table 5.8 Modeling the odds of acrosome reaction occurrence. Differences of incubation times least square means.

Time	_Time	$\beta$	SE $\beta$	Df	P	Odds ratio
0	2	0.4898	0.1896	227	0.0280	1.632
0	4	0.1947	0.1947	227	0.0050	1.851
2	4	0.1258	0.1938	227	0.5169	1.143

Table 5.9 Modeling the probability of the acrosome reaction occurrence per incubation period.

Variable	$\beta$	SE $\beta$	Mu	MS Group <sup>a</sup>
T0	4.6293	0.3365	0.9903	A
T2	4.1395	0.360	0.9843	B
T4	4.0137	0.3388	0.9823	B

<sup>a</sup>Differences seen in groups with different lettering within each incubation period.

## Discussion

It is known that spermatozoa must undergo biochemical and physiological changes prior to obtaining fertilizing ability. Destabilization of the sperm PM sterol levels, a part of the capacitation process, is a prerequisite for the initiation of the AR (Iborra et al., 2000). Components that induce sterol depletion from the sperm PM, enable sperm PM fluidity, increase transmembrane ion exchange, and lead to the occurrence of the AR are present in defined IVP media.

With the knowledge that the implementation of the bovine IVP system is not universally translated to all cervid species (Berg and Asher, 2003), this study assessed the ability of two *in vitro* fertilization media (BIVF and DSOF) and CAs (heparin and SS) to induce capacitation and subsequent AR in epididymal WT spermatozoa. To measure the capacitating ability of the chemically different media, M540/ YO-PRO®-1 were used to identify lipid disruption of the sperm PM and FITC-PNA/PI were employed for the identification of the AR.

Unlike red deer, WT deer spermatozoa do not require higher levels calcium to induce capacitation, as the odds of capacitation occurring were higher when incubated in BIVF than when incubated in DSOF. Although WT deer oviductal fluid composition has yet to be determined, these data suggest that spermatozoa come into contact with calcium concentrations *in vivo* that are similar to bovine oviductal fluid concentrations and lower than red deer oviductal fluid levels.

The molecular modulations involved in sperm capacitation are associated with fast and slow events. Activation of sperm motility, a fast event, is a result of increased

sperm PM fluidity and transmembrane movement of bicarbonate and calcium (Visconti, 2009). The CAs that were supplemented in each treatment were capable of inducing sperm PM fluidity, however, heparin exceeded SS in its ability to induce capacitation when combined with BIVF. Like in the North American wapiti, cattle, and goats, these data support that heparin is the most effective sulfated glycosaminoglycan for inducing capacitation in WT deer spermatozoa (Parrish et al., 1986; Cox et al., 1994; Pollard et al., 1995).

Slow events associated with sperm capacitation involve the occurrence of sperm hyperactivation and prolonged incubation with sterol acceptors that increases tyrosine phosphorylation, which correlate with prerequisites for the AR (Visconti, 2009). Results demonstrated that the ability to increase the actions necessary to raise the percentage of AR, which was present in both media, was more likely to occur when in the presence of higher calcium concentrations, as well as when incubated with heparin. Supplementation of LC to a defined fertilization medium with heparin will induce the AR in bovine spermatozoa within 15 minutes and the percentage of AR will increase over time (Parrish, 1988; Lukoseviciute et al., 2005). Unlike bovine spermatozoa, WT epididymal spermatozoa did not exhibit a time-dependent effect when incubated with LC in a defined medium.

Results from this study demonstrate the effectiveness of the defined bovine fertilization system for inducing capacitation in WT epididymal spermatozoa. Interestingly, there appears to be a need for higher calcium concentrations for higher rates of AR to occur, which is not required during the pre-requisite capacitation steps. Additional AR analyses could be obtained from *in vitro* heterologous and homologous

sperm/egg interaction studies. Heterologous fertilization of intact cattle and sheep oocytes by goat spermatozoa incubated in both heparin and SS have demonstrated high penetration rates (87% and 72%, respectively), indicative of high AR rates (Cox et al., 1994). Defining the chemical composition of WT oviductal fluid would also provide insight as to what WT spermatozoa are influenced by *in vivo*, thus providing further information for creating a WT defined *in vitro* fertilization and culture system.

## CHAPTER 6 SUMMARY AND CONCLUSIONS

The objectives of these studies were two-fold. First, the ability of FTAI protocols to reduce animal handling, eliminate estrus detection, and synchronize estrous in WT does was evaluated. Information attained from improved estrous synchronization protocols and FTAI programs will improve herd management and potentially lead to the development of successive IVP studies. Second, assessments of *in vitro* fertilization media components were made to determine if WT epididymal spermatozoa require a species-specific defined fertilization medium for inducing capacitation and the subsequent AR required for fertilization to occur. Establishment of improved FTAI protocols and defining properly defined IVP media will both aid in the proliferation of desired genetics throughout farmed WT herds.

In Experiment 1, a 14 d CIDR protocol with administration of exogenous eCG at CIDR removal was assessed in its ability to improve ovulation synchrony and yield pregnancy and fawning rates comparable to natural mating. Results showed higher pregnancy rates for WT does that were inseminated at or beyond 60.5 h post-CIDR withdrawal. Odds of does becoming pregnant to FTAI following an eCG treatment were increased for older does (>4 years of age), does inseminated beyond 60.5 h, and does that had semen deposited in the cervix or uterus. Fecundity rates of treated WT does were similar to rates of does that become pregnant due to natural mating. Administration of eCG following a 14 d progestin protocol provides minimal animal handling, synchronization of ovulation and pregnancy and fecundity rates that are similar to those of naturally bred WT deer.

In Experiment 2, evaluation of exogenous GnRH administration at FTAI, following a 14 d progestin protocol, was conducted, and its capability in improving pregnancy rates in WT does was assessed. Results showed no increase in pregnancy rates in treated WT does. Additionally, these rates were lower than those reported in Experiment 1. White-tailed does were also more likely to become pregnant when inseminated less than 60.5 h post-CIDR withdrawal, which is in contrast to results from Experiment 1. It is suggested that pre-synchronization of the estrous cycle with prostaglandins in addition to the GnRH protocol be studied in WT does. Additionally, further research into insemination intervals should be conducted.

In Experiment 3, different *in vitro* media components were assessed on their ability to induce capacitation and the subsequent AR in epididymal WT spermatozoa. Results showed that BIVF was more likely to induce lipid disorder in comparison to DSOF. Supplementation of heparin to BIVF also showed an increased likelihood of stimulating the steps toward capacitation when compared to supplementation with SS, however it did not differ from BIVF with no CA. Induction of the AR was twice as likely to occur when sperm were incubated in DSOF in comparison to BIVF, suggesting that higher levels of calcium concentrations are needed to complete the process of the AR. No interactions between the main variables were significant for inducing the AR; however, heparin was more likely to aid in the AR than SS or no CA, and incubation of sperm for 10 minutes was also more likely to result in higher percentages of AR sperm than if incubations were for 2 h or 4 h. Further research in regards to defining the chemical composition of WT oviductal fluid is suggested in order to create a defined WT

*in vitro* fertilization and culture system, as well as studies investigating *in vitro* heterologous and homologous sperm/egg interactions.

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## VITA

Jaclyn Christine Lambe-Steinmiller was born in Grand Prairie, Texas, to Tom and Jana Lambe. Jaclyn is the older sibling to Jordan Thomas Lambe and Payton David Lambe, both of which are firemen in the DFW area.

Following high school graduation in 2000 from South Grand Prairie High School, Jaclyn enrolled at Stephen F. Austin State University, Nacogdoches, Texas. She received her Bachelor of Science degree with a major in Animal Science and a minor in biology in May 2005 from Stephen F. Austin State University. In the interim between receiving her Bachelors and Masters degrees, Jaclyn worked as a hematology and parasitology technician for Antech Diagnostics, Irving, Texas.

In September 2006, Jaclyn enrolled in the Graduate School of Texas A&M University-Commerce. Under the direction of C. Pat Bagley, Ph.D., and in cooperation with the Dallas Zoo, she was awarded a Master of Science degree in Agricultural Sciences, with an emphasis in Animal Reproduction, in August 2008.

In August 2008, Jaclyn initiated her pursuit of a doctoral degree in Animal Sciences, and enrolled at Louisiana State University, Baton Rouge, Louisiana. Her research was conducted between 2008-2012 at the LSU Reproductive Biology Center and Bob R. Jones Idlewild Research Station.

In 2011, Jaclyn married Eric Aron Steinmiller. In 2012, Jaclyn moved to Chicago, Illinois, to begin her career as an embryologist. She is currently working for Northwestern Fertility and Reproductive Medicine, Chicago, Illinois.