Fertility of heterospermic semen in vivo and in vitro

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INTRODUCTION

The discovery of glycerol as a cryoprotectant (Polge et al., 1949) and that bovine sperm could be frozen, thawed, used for AI, and create pregnancies (Polge, 1952) were two of the crucial discoveries that led to development of the process of artificial insemination (AI) in cattle. Today, bovine semen is extended, frozen, and kept in liquid nitrogen for an indefinite amount of time. The use of AI and the bull as the primary vector of genetic change remains the most influential reproductive technology to date for cattle and with the advent of genomics for identification of genetic potential, will continue in this role into the future.

Artificial insemination benefits both beef and dairy producers by: 1) providing access to superior genetics, 2) reducing incidence of venereal disease, 3) removing the need to house bulls on the farm, and 4) supporting the application of ovulation synchronization and fixed-time AI programs (TAI). The use of AI and estrus synchronization in beef cattle results in: 1) more breeding opportunities per cow, 2) decreased duration of the breeding season and calving season, 3) increased age and weight of calves at weaning, and 4) subsequent increased revenue per calf (Johnson, 2005). The greatest success story of utilizing AI though is the dairy industry. The use of progeny-tested bulls has resulted in greater milk yield compared to non-progeny tested (+ 107 to 200 kg) and natural service (+ 366 to 444 kg) bulls and ultimately increased revenue for the dairy producer (Norman et al., 2003). Estrus synchronization and TAI have been adopted in the US dairy industry. However, TAI-pregnancy rate in lactating dairy cattle has not been as

successful as in beef cattle, due to reproductive deficiencies associated with lactation, post-partum anestrus, uterine health and embryo mortality (Macmillan, 2010).

The probability that a female conceives to a single AI is in part due to management, production, breed, optimizing the time of AI relative to ovulation, creating a favorable environment for embryo survival, and also, the bull. The bull's contribution to conception is through the production of sperm that are capable of traversing the female reproductive tract, fertilizing an oocyte, and creating a viable embryo. Differences among bulls in the ability of their sperm to complete this journey, due to enhancements or aberrations that may occur from the time of spermatogenesis through ejaculation, dictate in part, their observed fertility. Understanding the life cycle of sperm from early development through fertilization results in a better understanding of what bull fertility is and how to evaluate it. This review will begin by describing the life cycle of sperm, and building upon that knowledge, define what bull fertility is and how it can be estimated.

THE LIFE CYCLE OF A SPERMATOZOON

Understanding the structure and function of the sperm cell necessitates understanding its purpose and ultimate goal. A spermatozoon is a highly-specialized cell with one goal, traverse the female reproductive tract and fertilize an oocyte. The life cycle of sperm from spermatogenesis to fertilization is eloquently depicted in Fig. 1. Sperm are produced in the seminiferous tubules of the testis. Sperm are not able to fertilize oocytes immediately after release from the seminiferous tubules and must undergo a series of modifications starting in the epididymis and completing immediately prior to fertilization. Put simply, sperm that leave the seminiferous tubules are not the same as sperm in the ejaculate or those sperm that will

participate in fertilization in the oviduct. Ejaculated sperm are deposited in the vagina in some species and must overcome several obstacles before reaching the oocyte in the oviduct. These include physical and selective obstacles such as the cervix, immunological obstacles in the uterus, uterotubal junction (UTJ), and attaching to the oviduct. Sperm that overcome these obstacles can bind to oviductal epithelial cells (OEC) in the isthmic region of the oviduct, creating a reservoir of sperm capable of fertilizing an oocyte. Sperm that are released from the isthmus travel into the caudal ampulla to participate in fertilization. The fertilizing spermatozoon must penetrate the cumulus cells that surround the oocyte, bind to the zona pellucida (ZP), undergo the acrosome reaction, penetrate the ZP, fuse with the oolemma, incorporate into the cytoplasm of the egg, undergo nuclear decondensation, and ultimately result in creation of a viable embryo.

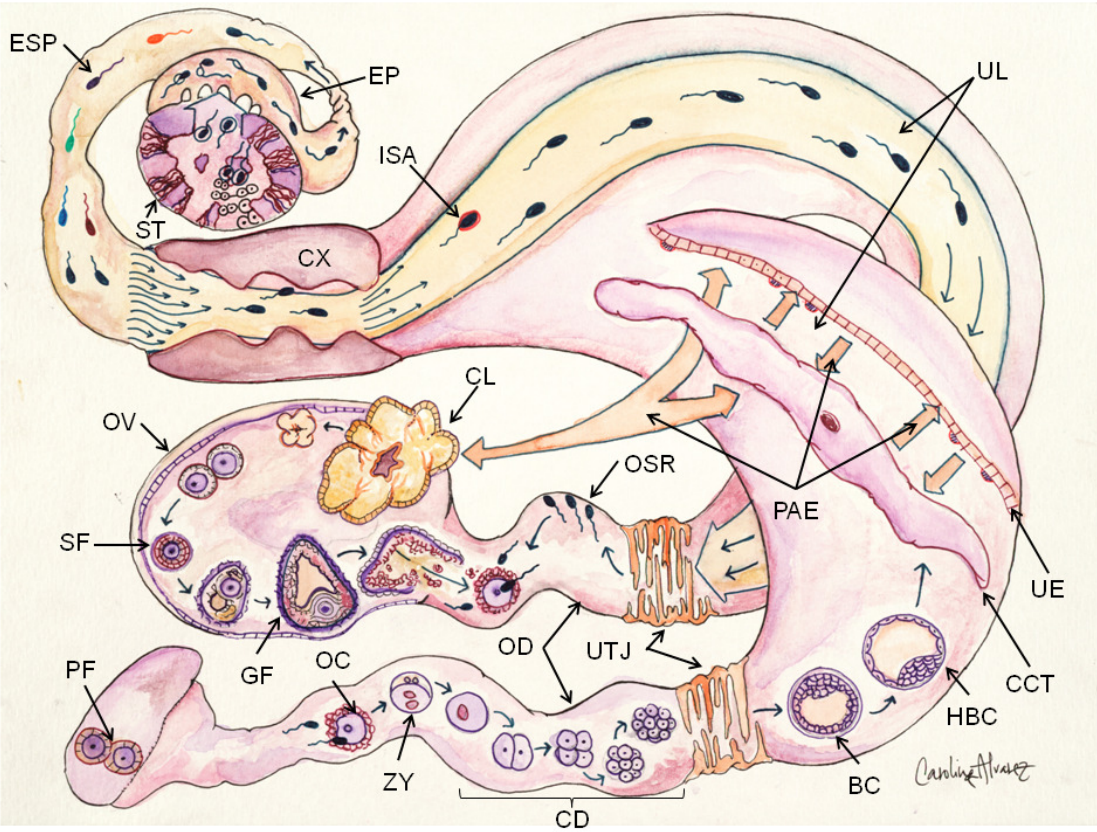

Figure 1. The events leading to creation of a sustainable embryo from gametogenesis to the peri-implantation period in cattle. Sperm are formed in the testes during the process of spermatogenesis and undergo continual modification up through ejaculation. Sperm must first traverse the uterus and are met in the female reproductive tract by immunological defenses before reaching the uterotubal junction (UTJ). The UTJ is a convoluted passageway that sperm must navigate before ultimately binding to oviductal epithelial cells in the oviductal isthmus and creating a sperm reservoir. Upon completion of capacitation and attainment of hyperactive motility, sperm are released and travel to the ampulla where fertilization will occur. Following fertilization, sperm and egg pronuclei will decondense, and come in proximity of one another prior to beginning mitosis. A series of cellular divisions occur eventually resulting in a morula,

which is released into the uterus where it develops into a blastocyst, and eventually hatches from the zona pellucida. In cattle, the embryo releases interferon-tau at approximately 15-17 d post ovulation to inhibit luteolysis. Implantation occurs shortly thereafter followed by progressive embryonic then fetal growth. The contribution of a bull to creation of a viable pregnancy can be realized anywhere throughout these series of events. (BC = blastocyst; CCT = conceptus; CD = cellular divisions; CX = cervix; EP = epididymis; ESP = ejaculated sperm; GF = Graafian follicle; HBC = hatched blastocyst; ISA = immune system attack; OC = oocyte; OD = oviduct; OSR = oviductal sperm reservoir; OV = ovary, PAE = paracrine, autocrine, and/or endocrine communication; PF = primary follicle; SF = secondary follicle; ST = seminiferous tubule; UE = uterine epithelium; UL = uterine lumen; UTJ = uterotubal junction; ZY = zygote).

Spermatogenesis

Puberty in the bull occurs around 9.5 to 12.5 mo of age, and its functional definition is the first ejaculate that contains 50 million sperm/mL and > 10 % motility (Wolf et al., 1965). The physiological and endocrine events that lead up to successful initiation of spermatogenesis have been reviewed (Rawlings et al., 2008). The stages of spermatogenesis and endocrine regulation in sexually mature bulls have been previously described by several authors (Amann and Schanbacher, 1983; Garner and Hafez, 2000; Senger, 2005; O'Donnell et al., 2006), and the descriptions below are a compilation thereof.

The testis is comprised of seminiferous tubules, Leydig cells, blood vessels, and connective tissue. The seminiferous tubules contain the Sertoli cells and developing germ cells. Seminiferous tubules are separated from the rest of the testes by a basement membrane. The basement membrane along with tight junctions between Sertoli cells form the blood-testis

barrier, which prevents immune cells from attacking the developing germ cells. The hypothalamic-pituitary-gonadal axis functions very much like that of the female, the one major exception being the lack of a hypothalamic GnRH surge center. Tonic pulses of GnRH elicit pulsatile release of LH from the anterior pituitary. Luteinizing hormone acts on Leydig cells, in the interstitium of the testis, resulting in production of testosterone and to a lesser degree, estradiol-17β. Testosterone is transported into Sertoli cells, which are under the influence of FSH, where it is converted to dihydrotestosterone (DHT) and estradiol-17β. All three steroid hormones exert negative feedback at the level of the hypothalamus, thus inhibiting GnRH and consequently LH secretion. Inhibin, a product of the Sertoli cells, inhibits FSH production at the level of the anterior pituitary gland. Both FSH and testosterone are important for Sertoli cell function and spermatogenesis, and these hormones act cooperatively or even synergistically to maximize spermatogenesis (O'Donnell et al., 2006). Therefore, the testis itself exerts a substantial regulatory mechanism on sperm production via steroid hormone and inhibin production. Spermatogenesis, the production of spermatozoa from diploid progenitor cells, takes approximately 61 d from start to finish in the bull (Amann and Schanbacher, 1983). The phenomenon of spermatogenic waves ensures a continual release of sperm into the lumen of the seminiferous tubules, resulting in production of approximately 10 billion sperm/d in mature Holstein bulls (Amann and Schanbacher, 1983). Mature sperm, released into the lumen of the seminiferous tubules, are collected in the rete testis, and flow into the efferent ducts followed by entry into the head of the epididymis.

Sperm, which are newly released from the testes, do not exhibit motility nor are they able to fertilize oocytes until having reached a certain point of epididymal transit (Amann et al., 1993;

Robaire et al., 2006). In the bull, total epididymal transit time is 6-10 d (Amann and Schanbacher, 1983). The acquisition of normal fertility is the consequence of several maturational changes of sperm including: loss of the cytoplasmic droplet, completion of chromatin condensation, exchange of plasma membrane proteins and modification of existing proteins, changes in cholesterol/phospholipids ratios, and surface antigen rearrangement (reviewed by Amann et al., 1993; Caballero et al., 2011 ENREF 38). The caudal epididymides of a Holstein bull store approximately 39 billion sperm (Amann and Schanbacher, 1983), a fraction of which are recruited during ejaculation. Considering a bull ejaculates approximately 4 billion sperm (Garner and Hafez, 2000), 8 – 10 successive ejaculations are necessary to deplete the sperm reserves of the epididymis. The majority of seminal plasma volume, fructose, and proteins are added by the seminal vesicles. The prostate gland produces the most fluid volume after the vesicular glands and contributes ions to the seminal plasma. The bulbourethral glands do not contribute a substantial volume to semen but instead flush the urethra prior to ejaculation.

Sperm in the female reproductive tract

During natural service, bulls deposit semen into the vagina, an acidic environment which is buffered by seminal plasma. Initially, sperm are rapidly transported through reproductive tract and into the oviducts, but these sperm do not participate in fertilization. Sperm that undergo sustained transport and colonize the oviduct are those that have the potential to participate in fertilization (reviewed by Saacke, 1982). The cervix is the first major barrier encountered by sperm (10-fold loss of sperm in cervix; Dobrowolski and Hafez, 1970) and has been proposed as the first method of selection by the female reproductive tract (Mullins and Saacke, 1989). Sperm that enter the cervical lumen encounter viscous sulfomucin whereas those that migrate

into the deeper, cervical folds encounter a less-viscous, sialomucin, resulting in privileged paths to the uterus (Mullins and Saacke, 1989).

The focus of this paper is from the context of intrauterine AI; thus, the uterus is the first obstacle encountered by sperm on the path to fertilization and presents immunological challenges to sperm. The immunological response to semen in the uterus has not been as thoroughly studied in cattle as in other species, but the loss of sperm by phagocytosis following insemination has been suggested (Hawk, 1987). Of the sperm that enter the uterus, 1% to 3% of those will make it to the UTJ (Dobrowolski and Hafez, 1970). Postulation that the UTJ is a formidable obstacle and selection tool of sperm is based on: 1) the ability to compress its lumen, limiting the time span in which sperm can pass; 2) mucosal folds with dead ends; 3) thick mucus could filter weakly-motile sperm; and 4) presence of specific proteins on the sperm surface that allow passage through the UTJ (Hung and Suarez, 2010). Sperm that make it through the UTJ colonize and bind to oviductal epithelial cell cilia in the isthmus and will form a sperm reservoir (reviewed by Suarez, 2002). The process of capacitation appears to play a role release of sperm cells from binding to oviductal epithelial cells (Lefebvre and Suarez, 1996; Ignotz et al., 2001) for the opportunity to participate in fertilization. Sperm are not capable of immediately fertilizing oocytes after ejaculation. They must undergo reorganization of the plasma membrane during their time in the female tract to prepare for the acrosome reaction, penetration of the ZP, and ultimately fertilization.

The section above highlights important events in the life cycle of a spermatozoon from spermatogenesis through fertilization and early embryonic development. Possibilities of sperm failure or success are numerous, and could arise anywhere during the time from spermatogenesis

through ejaculation. For example, increased testicular temperature negatively affects spermatogenesis and epididymal maturation, evident by presence of sperm abnormalities, and the ability of those sperm to create embryos in vitro. Variation in sperm quality among bulls can also occur post spermatogenesis as differences in epididymal and seminal plasma proteins among bulls have been related to both field fertility and the ability of sperm to interact with oocytes.

DEFINING AND PREDICTING BULL FERTILITY

The true fertility of a bull is difficult to evaluate due to factors, other than bull fertility, that influence the success or failure of conception or embryo survival. There are many sources of variation that can make bulls appear different in terms of conception rate. These include effects of farm, geographical region, heat stress and numerous sources of variation related to individual cows in which semen is deposited such as milk production, parity, body condition and uterine health.

Measures of fertility

Conception rate (animals pregnant/animals bred) is the most direct and best measurement of bull fertility. However, frequent collection of those data from AI services on a large scale was not common in the past. For many years, non-return rate (NRR) was used as an indirect measurement of fertility in US dairies, making the assumption that a lack of return to estrus by 30, 60, 90, or 120 days, for example, is indicative of conception. Within this definition, several possibilities exist for error. Dairy Records Management Systems (Raleigh, NC) previously reported sire fertility as Estimated Relative Conception Rate (ERCR), which is based on 70-d NRR. In 2006, the USDA Animal Improvement Programs Laboratory (AIPL) took over the role of monitoring bull fertility and implemented a new estimate of sire fertility, Sire Conception

Rate (SCR), in 2008. The following factors are included in the statistical modeling of SCR to account for differences among bulls including: inbreeding of bull and the resulting embryo, bull age, AI organization and year of mating, and the overall effect of bull. Nuisance variables that are accounted for to increase the accuracy of SCR are: herd, location, year of mating, parity of cow, service number, interval between matings, age and milk yield, and inclusion of cow as random error. The distribution of current SCR values for Jersey and Holstein AI sires are depicted in Figure 2 and 90 % of SCR data are within a range of -4.2 to 4.2 or -3.7 to 3.7 % for those breeds, respectively. These data indicate that SCR fertility depicts little variation among AI sires. The median number of inseminations per bull within breed for these data are 579 (Jersey) and 1316 (Holstein) with a range of 203 to 22,494 and 300 to 136,001, respectively. Meaningful comparison of bulls with 500 vs. 100,000 inseminations with less than a 10% point range is difficult at best considering the narrow range in fertility and wide range in number of AI.

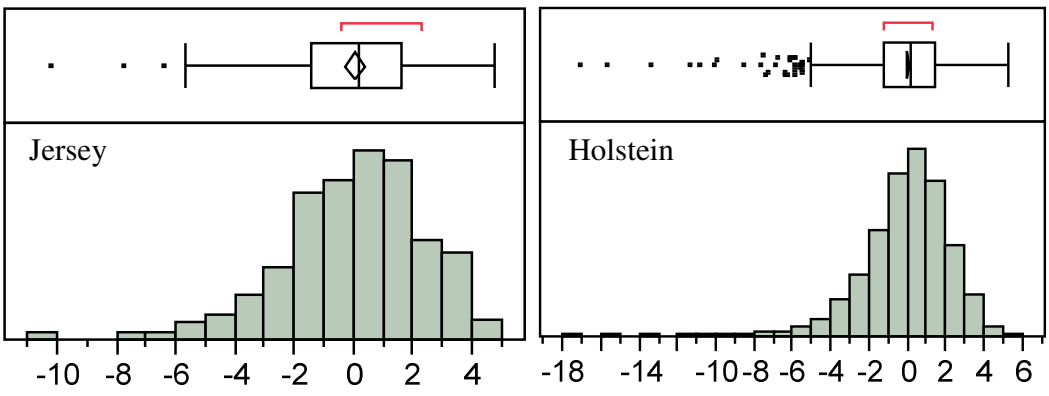

Figure 2. Distribution and box plot of Sire Conception Rate (SCR; USDA, August, 2012) values for Jersey (n = 221) and Holstein (n = 1917) sires.

Conception rate, NRR, ERCR, SCR, and other estimates of fertility are based on binomial distributions, and the inherent problems of these kinds of data in regard to bull fertility

have been discussed (Amann and DeJarnette, 2012). Binomial data consist of "yes" or "no" outcomes, and the more outcomes that are recorded, the more accurate the observed measure. Figure 3 depicts the actual conception rate of 2 theoretical Holstein bulls and the change in 90% confidence intervals with increasing numbers of AI. At 500 AI, the 90% confidence intervals no longer overlap for Bulls A (42 to 34%) and B (33 to 27%), but the range in the observed fertility differences could be as great as 15 or as little as 1 percentage points. Confidence in binomial data is based on both the sample size and extraneous sources of variation other than bull as mentioned previously. Each straw of semen is deposited into a unique environment (the female), which is subject to varying production and environmental conditions; therefore, the observed effect of bull fertility is influenced differentially among females. Thus, increasing the number of females inseminated using a given bull will dilute individual cow variation and increase confidence in the fertility estimate of that bull. One way to make a better comparison of bull fertility and eliminate the confounding effects of cow and the management she is provided is to compare bulls within the same environment. Heterospermic insemination is one approach to make comparisons among bulls in a common environment.

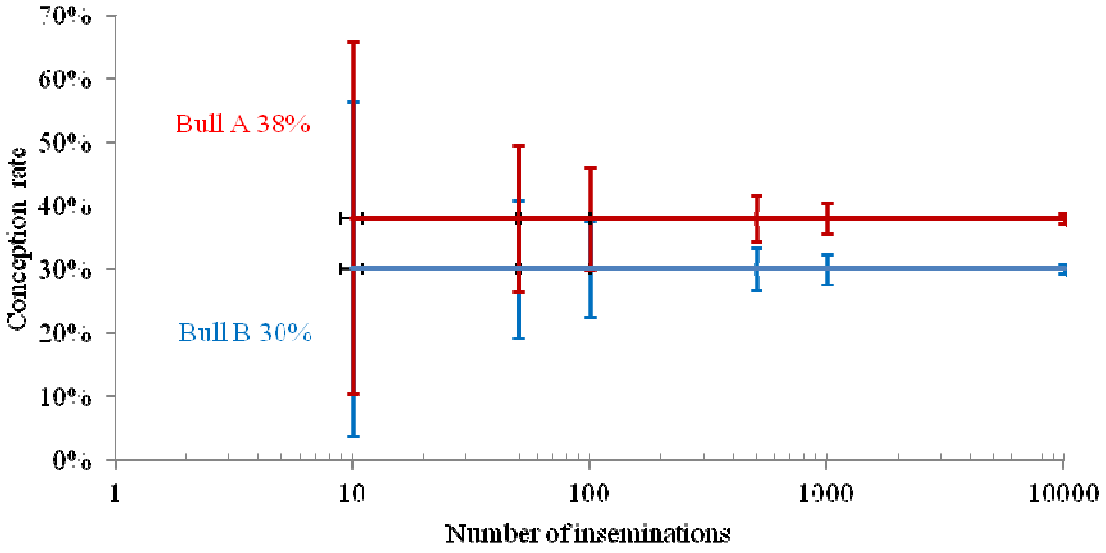

Figure 3. Example of bionomail variation expressed as 90% confidence intervals (vertical bars) of conception rate for two hypothetical bulls in a herd of cows.

The principles, techniques, and utility of heterospermic insemination were reviewed over 15 yr ago for variety of species (Dziuk, 1996), but this article is the only comprehensive summary of this technique to date. Heterospermic insemination utilizes a combined dose of semen from two or more males with equal numbers of sperm from each male. It creates a scenario in which sperm from both males have an equal opportunity to fertilize a single oocyte. If sperm from two males of equal fertility are combined in a 1:1 ratio, then the ratio of resulting offspring, in regard to sire, would theoretically be 1:1. For example, consider heterospermic doses of semen from Bulls A and B that are used to inseminate 100 heifers. If 60% of the heifers conceived and the bulls are of equal fertility, then 30 pregnancies would result from Bull A and 30 from Bull B. However, if 45 pregnancies are sired by Bull A and only 15 by Bull B, Bull A has a competitive advantage over Bull B. Previous research has indicated that heterospermic

insemination in fact results in ratios of greater or less than 1:1 across a variety of species including boars (Martin and Dziuk, 1977; Hammitt et al., 1989; Berger, 1995), bulls (Beatty et al., 1969; Stewart et al., 1974; Nelson et al., 1975; Beatty et al., 1976; Saacke et al., 1980; Berger, 1995; Flint et al., 2003; Kasimanickam et al., 2006), cockerels (Martin and Dziuk, 1977), rabbits (Beatty, 1960; Parrish and Foote, 1985; Robl and Dziuk, 1988; Berger, 1995), and rams (Choudhry et al., 1995), representing a potential approach to estimate relative fertility of different males more accurately.

One of the first studies that utilized heterospermic insemination in cattle was conducted at The Ohio State University in cooperation with Central Ohio Breeding Association, now C.O.B.A Select Sires, and compared the effect of single versus mixed (4-way) ejaculates of Holstein or Guernsey bulls on NRR (Hess, 1953). Mixed ejaculates resulted in an approximate 12 to 16% point increased in NRR across breeds compared to homospermic ejaculates. Nelson et al. (1975) reported an increase in first service pregnancy rate of beef cows inseminated with a mixture of 3 bulls compared to 2 bulls or single bull across 2 breeding seasons. A later study, however, reported no significant difference between homo- and heterospermic mixtures (Revell, 1993). A more recent experiment reported that conception rate from a heterospermic mix of 3 Holstein bulls was not different than the mean conception rate that was achieved with homospermic doses from each bull (DeJarnette et al., 2008). In production settings, heterospermic insemination is a viable approach for ensuring reasonable conception rates when using bulls of unknown fertility, especially when sire identification is not necessary. In a research setting, heterospermic insemination represents a potential tool for magnifying differences in fertility among bulls and understanding factors that influence fertility of sperm.

Relative fertility among bulls has been compared using heterospermic insemination in the past by calculating a competitive index (CI) for each bull based on the proportions of calves sired by a particular bull across all heterospermic mixes which contain that bull. The first report utilizing heterospermic insemination for comparison of bull fertility indicated that heterospermic insemination was >170-fold more efficient at resolving fertility differences among bulls than homospermic insemination, and that 112-d NRR and heterospermic CI were positively correlated ($r = 0.69$; Beatty et al., 1969). The effects of experimental treatments on sperm have also been tested using heterospermic insemination. Mixing semen of 4 bulls in a 1:1:1:1 ratio and then comparing fertility with fresh versus frozen semen resulted in 3:3:3:4 versus 5:1:1:4 ratio of calves, respectively, indicating variation among bulls in the effect of freezing on fertility (Stewart et al., 1974). Heterospermic performance of bulls was tested in single or superovulation scenarios, but relative fertility among the same bulls differed between the two scenarios (Flint et al., 2003). Some studies have utilized heterospermic insemination to investigate the importance of specific sperm attributes such as chromatin integrity, plasma membrane integrity, motility, and oxidative stress response for predicting bull fertility (Ballachey et al., 1988; Kasimanickam et al., 2006; Kasimanickam et al., 2007); those attributes were correlated to heterospermic CI.

Heterospermic experiments have been conducted in vitro as well as in vivo and will be discussed in more detail in a subsequent section. The most heavily-investigated heterospermic technique over the last 35 yr is the competitive ZP or oocyte binding assays that use fluorescent labeling of sperm to distinguish among bulls within heterospermic mixes (Davis et al., 1987; Henault and Killian, 1995; Braundmeier et al., 2002; Puglisi et al., 2010). These techniques have resulted in correlations to homospermic but not heterospermic field fertility data. In vitro

heterospermic assays have also been used to test the effect of seminal plasma from high or low fertility bulls on oocyte penetration rate (Henault et al., 1995; Henault and Killian, 1996). Those studies suggested seminal plasma from high fertility bulls in some instances improved heterospermic binding performance of sperm from low fertility bulls, but low fertility seminal plasma did not necessarily hinder performance of sperm from high fertility bulls. A more recent study investigated the relationships between homospermic fertility estimations, based on 56-d NRR, and a heterospermic ZP-binding or fertilization test (Puglisi et al., 2012), but no significant relationships to NRR were found.

Predicting bull fertility

Predicting bull fertility requires indentifying the most accurate measure of relative fertility differences among bulls, which was discussed in the previous section. Although natural service sires are evaluated as fertile or sub fertile, AI sires represent a subset of fertile bulls. However, this is a bit of an unfair comparison as the remainder of fertile bulls will never have their fertility evaluated on the scale of AI sires. Genetic selection pressure, most notably in the Holstein breed, and stringent semen quality control are the likely factors that set AI sires apart from other fertile bulls (DeJarnette et al., 2004).

Analysis of semen quality ranges from the most basic and rudimentary procedures for establishing minimum thresholds for breeding soundness to the most advanced laboratory procedures involving fluorescent staining, computer-assisted analysis of sperm motility, and in vitro fertilization techniques. The breeding soundness exam (BSE), a common evaluation for natural service bulls, includes a series of examinations that indicate whether or not a bull will be a “satisfactory breeder.” Several authors have reviewed the basic procedures of the BSE

(Parkinson, 2004; Barth, 2007; Kastelic and Thundathil, 2008), which include examination of: 1) overall physical soundness of the bull, 2) soundness of the external genitalia, and 3) semen quality. Barring no physical defects, satisfactory breeders will have > 30 % progressively motile sperm, > 70% morphologically normal sperm, and a scrotal circumference of greater than 30 cm at 1 yr of age and 35 cm at ≥ 2 yr of age. Sperm abnormalities have been characterized as primary or secondary based on whether the defect occurred during spermatogenesis or epididymal transit, respectively, or by major or minor, based on the effect of a defect on fertility (reviewed by Barth, 2007). An ejaculate or individual sperm may contain multiple sperm abnormalities. Some abnormalities such as tapered, asymmetrical, and long sperm heads are selected against in the female tract and are thus represented in a lesser percentage at the level of the oocyte compared to the inseminate (Saacke et al., 1998).

Semen quality parameters can be divided into compensable and uncompensable traits (Saacke, 2008). Compensable traits can be compensated for by increasing the number of sperm inseminated. Motility is an example of a compensable trait, as increasing total sperm number increases the number of motile sperm within an inseminate capable of participating in fertilization. Uncompensable traits are those which allow sperm to participate in fertilization but result in cessation of further embryonic development or result in embryonic loss, and these traits cannot be remedied by increasing the number of sperm inseminated. Compensable traits would be less detectable in natural service bulls, which deposit billions of sperm per mating, compared to millions of sperm deposited during AI. Therefore, increased sperm number per AI dose may be necessary for some sires to achieve optimal fertility (DeJarnette et al., 2004). The frequency of uncompensable traits determines the threshold fertility of a bull. Specific sperm

characteristics, not necessarily identified as abnormalities using light microscopy, which could affect the ability of sperm to traverse the female tract, fertilize, and/or create a viable embryo, can also be classified as compensable and uncompensable traits. Quantifying these characteristics may offer predictions of bull fertility prior to collection of field insemination data.

Identifying predictors of bull fertility has been an area of intense investigation since the introduction of AI. Throughout this period, there has been an emphasis to identify a single factor or set thereof, to rank bulls on relative fertility. The earliest factor that was proposed as a key predictor of fertility was motility (Cheng et al., 1949), and more recently, the use of genomics to identify genetic sources of infertility such as lethal haplotypes (VanRaden et al., 2011). Several authors have commented on the use of in vitro or laboratory procedures for prediction of fertility and cautioned on making interpretations of fertility using these measures (Amann, 1989; Amann and Hammerstedt, 1993, 2002; Foote, 2003; Moce and Graham, 2008; Amann and DeJarnette, 2012). Some common themes/questions among them are: 1) what definition of fertility was used?; 2) are measured differences in fertility realistic?; 3) what is the biological significance of the fertility test(s)?; and 4) consideration that multiple traits define fertility and may be correlated to one another. Predictors of bull fertility may differ throughout the life cycle of the sperm. These might be morphological and physiological traits evaluated by traditional microscopy or other more advanced techniques. There are a few important concepts to consider about analysis of semen before using various characteristics thereof for prediction of bull fertility. First, the ejaculate is a heterogeneous mixture of sperm with various traits in varying proportions. Secondly, no two ejaculates are exactly the same; therefore, bull fertility can fluctuate based on fluctuation in sperm characteristics by ejaculate. In vitro cleavage and

blastocyst rates were reported to vary among different ejaculates within bull using frozen-thawed semen (Zhang et al., 1997).

Evaluation of motility

Evaluation of motility is typically a subjective measurement of gross motility, accounting for swirling patterns of movement in semen, or individual motility, an estimation of progressive motility of individual sperm (Barth, 2007) . All subjective motility measures referred to henceforth will be individual motility. Degree of motility reflects sperm viability and thus, it is not surprising that motility is positively correlated to plasma membrane integrity (Kasimanickam et al., 2006). Motility is a characteristic of sperm that is intuitively correlated to fertility, as non-motile sperm cannot participate in fertilization. Correlation coefficients between motility and fertility from several older studies ranged from 0.21 to 0.84 (reviewed by Berndtson et al., 1981). Post-thaw motility has also been positively correlated to heterospermic performance of bulls (Saacke et al., 1980; Kasimanickam et al., 2006). Although intra-evaluator variation of a trained individual is likely minimal, there is more chance for variation among different evaluators. Photographic techniques have been employed in an attempt to objectively assess progressive motility and sperm track velocity (Katz and Dott, 1975; Wood et al., 1986). Photographic motility was correlated ($r = 0.93$) to heterospermic CI (Saacke et al., 1980). These innovative approaches eventually culminated in development of computer-assisted semen analysis (CASA), which is commonly used today in clinical and research laboratories (Pena, 2012).

The basic function of CASA is to recognize the heads of individual sperm and track their movement using an X and Y coordinate system (Budworth et al., 1987). Several

measurements of sperm velocity and path dynamics are calculated. A recent review described the CASA system, function, measurements, and other pertinent details (Kathiravan et al., 2011). Computer-assisted semen analysis measurements of motility have been related to both in vitro and in vivo fertility in addition to heterospermic performance. Total motile and progressively motile sperm were correlated (r = 0.34 and r = 0.39, respectively) to 75-d NRR but were more highly correlated to an in vivo heterospermic CI (r = 0.86 and r = 0.87, respectively; Budworth et al., 1988). A more recent experiment also reported a positive correlation between CASA progressive motility and a heterospermic CI in vivo (Kasimanickam et al., 2006). Percent motile sperm was a heritable trait ($h^2 = 0.785 \pm 0.302$) and moderately genetically correlated to SCR ($r = 0.302 \pm 0.293$), but the amount of error associated with these estimates limit their predictive capabilities (Epper-Yowell, 2011). Some caution is warranted when analyzing CASA measurements. Semen preparation, slide-chamber type, sperm concentration, duration from the dispensing of the sample and reading, accounting for debris, as well as device settings can substantially affect results (Contri et al., 2010).

Flow cytometry

Fluorescence microscopy allows for the examination of certain structures or quantification of the physiological status of sperm cells utilizing fluorescent markers that adhere to structural components of sperm. Flow cytometry allows for the evaluation of thousands of sperm within minutes. It also provides an ever-growing battery of tests for structural and functional soundness of sperm (Graham, 2001; Gillan et al., 2005; Sutovsky and Lovercamp, 2010; Petrunkina and Harrison, 2011).

An important flow-cytometric measure of sperm quality is DNA integrity. Differences in DNA fragmentation after thermal stress have been detected for high- and low-fertility Holstein bulls (Evenson et al., 1980). The degree of fragmentation was negatively correlated to percent normal morphology (r = -0.6; Ballachey et al., 1988), NRR (r = -0.5 to -0.7; Ballachey et al., 1987), and heterospermic competitive indices (r = -0.7 to -0.9; Ballachey et al., 1988; Kasimanickam et al., 2006).

Essentially any target on or in sperm, which can be labeled with a fluorescent probe, could conceivably be evaluated using flow cytometry. Plasma membrane integrity, acrosome integrity, and mitochondrial function have been investigated intensively using flow cytometry and have been highly correlated to microscopic evaluation (Graham et al., 1990; Thomas et al., 1997). Plasma membrane integrity was not correlated to NRR (Garner et al., 1986; Ericsson et al., 1993) but was highly correlated to a heterospermic fertility ranking (Kasimanickam et al., 2006). Acrosome integrity of frozen/thawed semen and acrosome reaction induction were predictive of 56-d NRR (Birck et al., 2010) but was not related to heterospermic fertility ranking (Kasimanickam et al., 2006).

Fertility Associated Antigen

It was originally found that some highly fertile bulls were more prone to undergo the acrosome reaction compared to their less-fertile counterparts in the presence of glycosaminoglycans (GAGs), such as heparin (Handrow et al., 1982; Ax et al., 1985; Ax and Lenz, 1987; Miller et al., 1990; Nass et al., 1990). Although multiple GAGs were present in a selected group of bulls, the presence of one 30-kDa GAG (see previous section on epididymal and seminal plasma proteins) in particular was present in highly fertile bulls (Bellin et al., 1996)

and would later be referred to as Fertility Associated Antigen (FAA; Bellin et al., 1998). Although there is a relationship between the presence of FAA and fertility to natural service or AI, neither the increase in fertility nor the number of bulls that are FAA-positive or negative were consistent (Bellin et al., 1998; Dalton et al., 2012; Sprott et al., 2000), thus making this test alone an inadequate predictor of fertility.

The FAA is a prime example of the postulate that fertility cannot be defined by a single factor but is, in fact, the result of a more complex combination of several sperm traits at varying levels or frequency (Amann and Hammerstedt, 1993). While many factors described above have been related to fertility, the best predictor remains the proportion of animals that conceive of those submitted to AI. In this scenario, the contributions of the multiple traits, which can be assessed in the laboratory, are allowed to be expressed and reflect the fertility of the bull. The challenges of collection of these data on a large number of bulls were previously described. A plausible alternative to making this determination in vivo is through use of in vitro bioassays that permit several important sperm traits to be expressed. This approach may be the best laboratory-based approach to predict field fertility. The use of in vitro culture of gametes, fertilization, and embryo culture has been used to distinguish differences in fertility among bulls.

In vitro gamete interactions, fertilization, and embryo culture

One of the initial components of the fertilization process is binding of sperm to the zona pellucida (ZP) and oocyte penetration. Mean number of sperm bound to the ZP was correlated (r = 0.50 to 0.73) to 56-d NRR (Zhang et al., 1998; Zhang et al., 1999). Two studies reported that bulls of greater in vivo fertility out-performed contemporaries of lesser fertility in competitive

ZP-binding assays (Henault and Killian, 1995; Puglisi et al., 2010). Specifically, 81% (13/16) of fertile/subfertile bull pairings resulted in greater numbers of sperm from fertile than subfertile bulls bound to the ZP (Henault and Killian, 1995). In contrast, others reported no correlation between ZP-binding ability and in vivo fertility, defined by either a heterospermic CI or NRR (Braundmeier et al., 2002). Competitive indices derived from heterospermic insemination of ZP-free hamster oocytes by bull sperm were correlated ($r > 0.86$) to 59-d NRR (Davis et al., 1987). Sperm penetration of oocytes at 3 h post insemination (hpi) was correlated to 150-d NRR (Ward et al., 2002). A sperm fertility index, calculated from homospermic binding to ZP-free hamster oocytes of 46 bulls, was correlated ($r = 0.77$) to 60-d NRR (Park et al., 2012).

In vivo data indicate that fertilization rate (~70 to 95%) is consistently high across bulls as long as sperm number is not a limiting factor (Kidder et al., 1954; Diskin and Sreenan, 1980; Sartori et al., 2004); however, the relationship between in vivo fertility and in vitro fertilization rate is not clear. Two studies indicated a weak relationship between in vitro fertilization rate and in vivo fertility (Marquant-Le Guienne et al., 1990; Ward et al., 2002) whereas others indicated no significant or discernible relationship (Eid et al., 1994; Puglisi et al., 2004). There is a trend that the absolute rate of zygote cleavage (see Table 1) and the timing of cleavage (Figure 4) are positively correlated with in vivo fertility although two studies reported no correlation between in cleavage rate and in vivo fertility (Schneider et al., 1999; Zhang et al., 1999). The timing of cleavage is directly affected by the preparation time for, and the execution of, the first mitotic division of the cell cycle. Bulls of greater in vivo fertility exhibited an earlier and longer S-phase (DNA replication) and shorter G1-and G2-phases (preparation for DNA replication and mitosis, respectively) of the first cell cycle compared to bulls of lower in vivo (Eid et al., 1994; Eid and

Parrish, 1995) or in vitro fertility (Comizzoli et al., 2000). In turn, these characteristics of the cell cycle were associated with earlier cleavage and greater blastocyst rates. However, the correlation between in vivo fertility and in vitro blastocyst production has not been as consistent as cleavage rate (Table 1) although bulls of greater fertility were reported to produce more blastocysts from cleaved zygotes than bulls of lesser fertility (Hillery et al., 1990). The greater correlation of NRR to cleavage rate compared to the ability of an embryo to development to a blastocyst suggests that fertilization rate and early division, which are encompassed by cleavage rate, are more accurate indicators of in vivo fertility.

Table 1. Correlations between non-return rate (NRR) to estrus and in vitro embryo cleavage and blastocyst rates from several studies.

Study	Bulls (n)	NRR range (NRR type)	Cleavage rate (%)		Blastocyst rate (%)[1]	
			Range	r	Range	r
Lonergan, 1994*	6	60 – 76 (na[3])	61 – 87[a]	0.92[+]	17 - 30	0.26
Schneider et al., 1999	9	63 – 75 (60-90-d)	na	0.38	na	-0.17
Shamsuddin and Larsson, 1993*	5	69 – 77 (58-d)	47 – 68[b]	0.76[+]	28 – 34[2]	0.35
Ward et al., 2001	6	57 – 78 (150-d)	56 – 83[a]	0.53[+]	9 - 27	0.36[+]
Zhang et al., 1997	20	51 – 75 (56-d)	29 – 82[a]	0.59[+]	2 - 32	0.35[+]
Zhang et al., 1999*	12	62 – 67 (56-d)	57 – 74[a]	-0.25	6 - 36	-0.28

[a,b]Cleavage rate defined as embryos ≥ 2 cells at 48 (a) or 72 h (b) post insemination.
[1]Blastocyst rate expressed as (blastocysts) / (total oocytes) unless specified otherwise.
[2]Blastocyst rate expressed as (morulae and blastocysts) / (cleaved zygotes).
[3]na = data not available.
[*]Correlation coefficients (r) not provided in study. Calculated post-hoc using individual bull data.
[+]Correlation coefficient (r) is significant ($P < 0.05$).

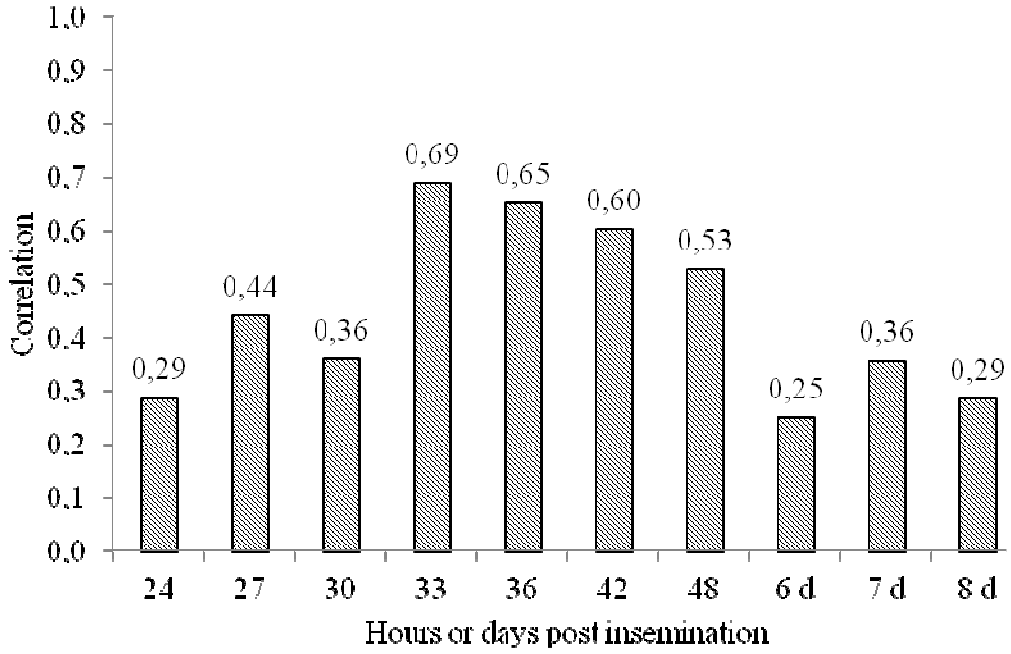

Figure 4. Correlation between 150-d non-return rate from 6 bulls and cleavage and blastocyst rates at 24 to 48 h or 6 to 8 d post insemination. All correlations are significant ($P < 0.004$; adapted from Ward et al., 2001).

SUMMARY

There has not been a lack of variety in the number of sperm quality tests and evaluations over the last 50 years, and many of these have been related to bull fertility. Although there is strong biological rationale for many of the tests mentioned above the lack of strong and consistent correlations of these tests to bull fertility across the literature is likely due to two factors:

- Measure of bull fertility – a narrow range in AI bull fertility, extraneous variation other than bull, and small sample sizes result in inaccurate estimates of fertility from NRR and conception rate data collected across multiple locations.
- Deficiencies of sperm within a single bull or among bulls are not likely the result of single factor given the hierarchy of multiple events leading to fertilization and creation of an embryo, but instead multiple factors.

The most predictive bull fertility test would result from a fertility measure that has the most power and precision to distinguish bulls in terms of relative fertility and using a predictor that encompasses several traits of sperm important for creating a pregnancy; heterospermic insemination and in vitro fertilization, respectively, intuitively fit those criteria. Combining heterospermic insemination with in vitro fertilization (IVF) may provide a laboratory-based estimation of bull fertility by combining accurate relative measure of fertility and the use of a biological assay, respectively. Therefore, we conducted a series of experiments to explore the relationships between in vivo and in

vitro heterospermic performance and the sperm traits that contributed to each. The overall hypotheses for these experiments were:

- Heterospermic in vivo and in vitro rankings would be similar, indicating that fertility differences observed in vivo are, in part, realized in an in vitro system.
- The heterospermic ranking of bulls would be explained by specific characteristics of sperm and/or the progression of embryonic development in vitro and not differences in number of motile sperm.
- Heterospermic rankings among bulls would be explained in part by homospermic IVF performance and several sperm characteristics.

These hypotheses were tested by designing a series of experiments with the following objectives:

- Determine the relationship between a previously-established (Kasimanickam et al., 2006) in vivo heterospermic ranking and a new in vitro heterospermic ranking using the same batch of frozen semen.
- Determine if the in vitro heterospermic ranking is altered by standardizing to equal number of motile sperm immediately prior in vitro fertilization.
- Investigate relationships between several sperm characteristics, homospermic IVF performance, and heterospermic performance among bulls.

Some of the findings from this comprehensive set of experiments will be included in the presentation that accompanies this paper.

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