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USE OF PENETRATION OF ZONA-FREE HAMSTER EGGS BY BOVINE SPERM AS AN ESTIMATION OF FERTILITY

The Ohio State University

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USE OF PENETRATION OF ZONA-FREE HAMSTER EGGS BY BOVINE SPERM AS AN ESTIMATION OF FERTILITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

William C. Baird, III, B.A., M.S.

****

The Ohio State University

1982

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Capacitation of Sperm

Maturation of Ova
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INTRODUCTION

Since 1959, when M.C. Chang (1959) fertilized rabbit eggs in vitro, reimplanted them into recipient animals and produced live young, much work has employed the technique of in vitro fertilization. All aspects of reproductive biology have been studied: biochemical, sperm-egg interaction, fertility evaluation, fertility improvement, heterospermic insemination and cloning experiments. As spermatozoa first leave the male reproductive tract they are functionally incapable of achieving fertilization. According to Chang (1951) and Austin (1952) spermatozoa must undergo capacitation, physiological (anatomical) and biochemical changes in the female reproductive tract prior to penetrating an ovum. While Chang (1959) fertilized in vitro, he had retrieved capacitated sperm from the female tract. Yanagimachi and Chang (1964) were the first to fertilize rodent eggs in vitro using epididymal sperm, achieving both sperm capacitation and fertilization in a single in vitro system.

The first penetration of zona-free eggs by spermatozoa of a different species was reported by Hanada and Chang (1972). At present, in vitro capacitation and cross-species fertilization (Gwatkin, 1977) has been developed for at least eleven different mammalian species, including human (Yanagimachi, 1976) and bovine (Calcote, 1979) using the zona-free hamster penetration technique.

With the artificial insemination industry involved to such a significant extent, (49% dairy and 20% beef), it is advantageous to be able to predict bull fertility. Laboratory parameters are used to evaluate semen quality of young bulls as well as each collection of the older established bulls. Motility (M) and percent intact acrosome (PIA) are evaluated four hours post-thaw for each collection and the collection is either retained or discarded depending upon pre-determined...
standards (30% M; 50% PIA). While PIA is highly correlated with non-return rate (Marshall, 1976), it does not give direct evaluation of the spermatozoa's ability to fuse with the vitellus, undergo decondensation and form male pronuclei.

Bedford (1974) suggests that the morphological features of sperm penetration are essentially similar in several species examined: rat, hamster, rabbit and human. Bedford described the morphological changes observed in the spermatozoon as it approaches and traverses the outer egg investments which protect the vitellus. Yanagimachi et al. (1976) suggest that zona-free animal ova could be substituted for human ova in the preliminary assessment of human sperm fertilizing capacity. In the bovine, bovine eggs would make a more acceptable in vitro test system. However, the difficulty of obtaining large numbers of bovine ova displaying the same maturational age is not economically feasible at this time. The zona-free hamster system lends itself to a laboratory test to further evaluate bovine sperm fertility as exhibited by fusion with the membrane, sperm head decondensation and pronuclear development.

Saacke and White (1972) suggest that post-thaw PIA is more closely related to fertility than either sperm motility or abnormal cell count of the ejaculate. They also suggest that acrosomal cap retention accounted for 65% of the variation in fertility among bulls. Since 35% of the variation in fertility among bulls is not accounted for by PIA analysis, further fertility evaluation methods are needed. The acrosomal cap releases enzymes that are necessary for the sperm to fertilize the egg. The PIA test determines the number of spermatozoa that still have an intact acrosome after four hours incubation, and are therefore capable of releasing enzymes in the area of the egg, leading to fertilization. However, the PIA test does not evaluate how many of the spermatozoa will ever undergo the acrosome
reaction and become capable of ovum fertilization. The in vitro penetration test using zona-free hamster eggs does give a comparable evaluation of the number of eggs fertilized when spermatozoa from several bulls are tested in the same experiment, (Calcote, 1979).

Calcote (1979) shows a high correlation between PIA and penetration of zona-free hamster eggs for a group of high fertility dairy bulls using both thawed and fresh semen.

In the present experiments, dairy bulls from three categories of PIA were evaluated: 40%, 60% and 80%. The bull sperm were evaluated at different concentrations (sperm/ml), numbers of eggs/dish, and variable capacitation times. These experiments employed a media of BFF (bovine follicular fluid)/BSA (bovine serum albumin). However an evaluation was also made using a specific chemically defined media of BSA (no BFF) in an attempt to increase fertility, as well as the reproducibility of the results. Nine beef bulls previously used in a heterospermic insemination field trial will also be compared using the zona-free hamster penetration test in an attempt to rank them in relation to number of young produced.
Incubation time, an indication of the time required for spermatozoa to undergo capacitation in anticipation of the acrosome reaction, varies dramatically depending upon species studied and/or media employed. Capacitation, as described by Austin et al. (1973), is an endogenous, physiological change that spermatozoa must undergo prior to fertilization. However, since its original description by Austin (1952) and Chang (1951) capacitation has remained poorly understood with its exact mechanism(s) unknown.

In review, Yanagimachi (1973) discussed the involvement of the mammalian sperm head in fertilization. Suggesting that although the size and shape of sperm heads vary amongst species, the basic spatial arrangement of the structural elements of the sperm head is remarkably constant.

Membrane vesiculation during the acrosome reaction is responsible for the release of enzymes. The two enzymes representing the greatest investigative interest are hyaluronidase and acrosin (zona lysin). Hyaluronidase is found in the apical region of the acrosome. It dissolves the hyaluronic acid matrix that holds the cumulus oophorus cells together. Acrosin, (a trypsin-like enzyme) is located within the equatorial segment (acrosomal cells) and is released as the sperm passes through the zona pellucida.

Barros (1974), citing earlier electron microscopic analysis of the acrosomal membrane, suggested that hyaluronidase is released through vesiculations between the plasma membrane and the outer acrosomal membrane. No visual change was observed in the acrosomal region under phase contrast even though hyaluronidase was increasing in the incubation medium. In agreement with others, Gwatkin (1977) suggested that vesiculation occurs at the start of the acrosome reaction and
releases hyaluronidase, which facilitates passage of sperm through the cumulus oophorus. An increase in hyaluronidase levels in vitro indicates completion of capacitation in the rabbit and hamster, as well as onset of the acrosome reaction in guinea pig spermatozoa (Rogers and Yanagimachi, 1975a).

Rogers and Yanagimachi (1975a) described the acrosome reaction as multiple fusions between the outer acrosomal membrane and the overlying sperm plasma membrane. In this manner the acrosomal enzymes become accessible for digestion of the cumulus matrix and other egg envelopes as capacitated spermatozoa pass through these barriers. Rogers and Yanagimachi (1975a) suggested that measurements of hyaluronidase release as an index of the completion of capacitation might be applicable for bull, rabbit or human spermatozoa, which have acrosome reactions that are difficult to observe. However, Saacke and Marshall (1968), using differential interference-contrast microscopy, suggested that the acrosome reaction of the bull is not that difficult to observe.

Gwatkin (1977) reported that when employing a hamster: hamster in vitro system the acrosome separates, leaving behind the intact cap as the sperm penetrates the zona pellucida. This would indicate that for successful fertilization, loss of the acrosomal cap occurs on the surface of the zona pellucida. Once the hamster sperm loses its acrosomal cap it rapidly becomes infertile. Gwatkin (1977) reported that without the acrosomal cap, sperm would not penetrate the hamster zona pellucida. He suggested that acrosin is released at the surface of the zona pellucida.

Utilizing electron microscopy to specifically study the zona pellucida and the activity of acrosin, Yanagimachi and Noda (1970b) examined hamster spermatozoa embedded in the zona pellucida and described vesiculation of the equatorial segment of the acrosome. They further postulated that acrosin, found in
the equatorial segment, is responsible for penetration through the zona pellucida.

Barros and Yanagimachi (1972) demonstrated, by electron microscopy, that at two hours and five hours of incubation the vesiculation of the equatorial segment of the acrosome increases from slight to extensive. They showed that at four hours incubation the spermatozoa were no longer capable of penetrating the zona pellucida, but could still penetrate the zona-free vitelline membrane. From this, Barros and Yanagimachi (1972) suggested that the location of the zona lysin is the equatorial segment.

Spermatozoa are suggested to have receptors on their plasma membranes, specific for binding cites on the zona pellucida. Spermatozoa have been observed (SEM) undergoing the acrosome reaction at the surface of the zona pellucida using cumulus-free oocytes in the mouse (Saling and Storey, 1979) and domestic pig (Szollosi and Hunter, 1978).

The role of acrosin in zona pellucida penetration has been discussed by many authors (review, Meizel, 1978). It has also been suggested to have properties similar to bovine trypsin, principally because bovine trypsin is capable of freeing the zona pellucida from the egg.

Morphological studies have suggested that acrosin is associated with the equatorial segment and the inner acrosomal membrane. Srivastave et al. (1979) reviewed the above information and suggested that penetration of hamster zona by hamster sperm is not affected by acrosin-trypsin inhibitors once the initial association of the sperm:zona has taken place. They further hypothesized that acrosomal proteinases, derived from acrosomal extractions, in addition to acrosin may be involved with zona pellucida penetration. These proteinases were also effective in the absence of Ca\(^{2+}\), in destroying the zona pellucida.
Chang et al. (1971), comparing and contrasting various biological incubation media, compared fertilizability of rabbit spermatozoa collected 14-18 hours after mating from the uterus and vagina. In an in vitro system, they determined that spermatozoa recovered from the vagina fertilized more eggs than uterine spermatozoa. No observation was made as to the percent intact acrosomes in either collection. It is possible that the spermatozoa from the uterus had already undergone their acrosome reaction, therefore they would be unable to penetrate through the zona pellucida to fertilize the egg (Barros, 1974).

Citing in vivo research, Barros (1974) described how many mammalian spermatozoa undergo capacitation in the uterus, with some species exhibiting the greatest degree of capacitation completion in the oviduct. In review, Barros (1974) concluded that capacitation and the acrosome reaction are endogenous phenomena which can proceed spontaneously in a chemically defined in vitro media. The complexity and intermingling of an in vivo environment may require a more complex system, due to the apparently precise chronology between coitus, ovulation, capacitation and fertilization.

It has been demonstrated in the hamster system in vitro (Yanagimachi and Chang, 1964; Barros and Austin, 1967) that fluid from the oviduct of recently ovulated hamsters and follicular fluid (prior to ovulation) were both more effective than uterine fluid in capacitation. Barros and Austin (1967) suggested that follicular fluid is more effective than oviduct fluid.

According to Yanagimachi (1969b) a fraction of BFF (dialyzable and heat stable at 90°C) could, after incubation with hamster spermatozoa, induce such vigorous sperm motility that it became difficult to analyze their qualitative motility. Other authors (Yanagimachi, 1969a; Gwatkin and Anderson, 1969; Barros and Gnavigo, 1970) have demonstrated that factors responsible for capacitation
are present in bovine follicular fluid. Yanagimachi (1969b) suggests that BFF contains two fractions involved with capacitation: a spermatozoa activation fraction, previously described, and one responsible for the initiation of the acrosome reaction (non-dialyzable, heat labile at 90°C). Heated blood serum, when analyzed in the above experiment, also contained two fractions with similar properties. Yanagimachi (1969) tested follicular fluid of four other species for its ability to induce capacitation in hamster spermatozoa. The results showed hamster, mouse and rat in descending effectiveness, with rabbit follicular fluid being ineffectual in producing the capacitation response in hamster spermatozoa.

Induction of capacitation was achieved in the hamster using hamster follicular fluid and oviduct fluid independently and synergistically by Barros and Austin (1967). They observed that progesterone is present in even greater amounts as ovulation approaches, and that induction of the acrosome reaction increased in follicular fluid from follicles as ovulation approached. Ovulation occurs some 11 hours after HCG administration in the hamster. Barros and Austin (1967) incubated spermatozoa with follicular fluid at four hours and 10 hours post-HCG treatment and found spermatozoa with reacted acrosomes at 43% and 97% respectively. They also observed that tubal fluid with eggs present produced the acrosome reaction in 84% of hamster spermatozoa, while medium 199 with washed tubal eggs only produced the acrosome reaction in 8% of spermatozoa.

Triana et al. (1980) showed that exposure to follicular fluid caused influx of Ca^{2+} and subsequent release of acrosomal contents. Postulating the effect of follicular fluid, Triana et al. (1980) suggested a modifying effect on spermatozoa in vitro that resembles or is normally supplied by the egg or its vestments.

Hanada and Chang (1976a,b) found follicular cells, blood serum, BSA or even polyvinylpyrrolidone to be capable of supporting capacitation and the acrosome
reaction of hamster spermatozoa. They also demonstrated a positive correlation
between increased binding affinity of spermatozoa to the vitellus and state of
sperm capacitation in a rat sperm:zona-free hamster egg system.

Gwatkin (1977) also suggested that blood serum and follicular fluid are able
to capacitate hamster spermatozoa in vitro. However Mahi and Yanagimachi (1973)
stated that blood serum and follicular fluid are incapable of producing penetration
in a hamster:hamster in vitro system in all eggs. Therefore they do not produce a
reliable medium for morphological studies of capacitation.

Toyoda et al. (1971) along with Miamoto and Chang (1973) demonstrated the
need for serum albumin in artificial media to obtain capacitation of mouse sperm
in vitro. They both agree that albumin may supply the specific agent required for
capacitation to occur. Bavish and Murtan (1974) suggest that BSA (bovine serum
albumin) possesses "acrosome reaction-inducing" activity.

Barros (1974) found penetration of zona-free hamster eggs with guinea pig
spermatozoa in a media devoid of albumin or any other macromolecules. Miyamoto
and Chang (1973) also described capacitation in vitro in a defined media with
mouse spermatozoa. Austin et al. (1973), using a defined protein free medium,
demonstrated that capacitation in vitro does not depend on specific biological
factors, but occurs when conditions are optimum for sperm survival. When studying
fertilization of human oocytes, they suggested that capacitation can be achieved
when sperm are incubated with washed oocytes in chemically defined media.

Mahi and Yanagimachi (1973) attempted to determine proper environmental
conditions for hamster spermatozoa capacitation. The proper osmolarity and pH
were determined to be 230-240 mOsm and 7.2-7.8 respectively. The hamster
acrosome reaction did not occur at 16°C or at 20°C, but, at 23°C or above, the time
for reaction to occur decreased with increased temperatures up to 40°C.
Removal of peripheral proteins from sperm plasma membrane is partially responsible for capacitation (Gwatkin, 1977), enabling the plasma membrane to fuse with the outer acrosomal membrane. Gwatkin (1977) believes that cumulus cells contain the active component of capacitation and that cumulus cells capacitate hamster sperm, enabling all cumulus-free eggs added to an in vitro medium to be fertilized. He demonstrated that hamster sperm in vitro with hamster cumulus cells, remained in contact with cumulus oophorus cells (2-3 hours) and were released in a capacitated state. In comparison, sperm incubated in medium 199M2 lost motility in two hours, but in cumulus oophorus cell solutions rapid motility was maintained for eight hours or more (Gwatkin, 1977).

Glucose in the incubation media will retard the acrosome reaction in guinea pig spermatozoa, according to Rogers and Yanagimachi (1975b). However, these authors suggested that pyruvate and lactate are required. Miyomoto and Chang (1973) also found that pyruvate increased motility of sperm lacking acrosomal caps. Toyoda and Chang (1974) determined that with no lactate a decrease in egg penetration was obtained. Rogers and Yanagimachi (1975b) found that culture media containing lactate and pyruvate were more effective in guinea pig spermatozoa than if glucose was used as an energy source, in culture media specifically formulated for capacitation and acrosome reaction.

Blood serum contains lactate and pyruvate as well as cumulus cells, which according to Bavish and Yanagimachi (1977) can produce substantial amounts of pyruvate in vitro. This could be an argument for leaving the cumulus cells intact during in vitro homospecific fertilization, but during in vitro heterospecific fertilization the pyruvate must be added to the media because the cumulus cells and the zona pellucida are removed prior to addition of the sperm.
Incubating guinea pig spermatozoa in calcium-free and calcium available media, Yanagimachi (1972) found very little acrosome reaction of the sperm incubated in the calcium-free media. Extrapolating to mammals in general, he suggests the acrosome reaction is highly dependent on Ca$^{2+}$ ions.

Yanagimachi and Usui (1974) determined that Ca$^{2+}$ will initiate capacitation and the acrosome reaction within 10 minutes when added to a preincubated Ca-free media. Hyaluronidase went from 32% of maximum level to 64% in 10 minutes after addition of calcium chloride. They suggested that this was a synchronous release of hyaluronidase in response to the acrosome reaction and not due to the death of the spermatozoa and lysis of membranes.

For the acrosome reaction of guinea pig spermatozoa, extracellular Ca$^{2+}$ was required, according to Santos-Sacchi and Gordon (1980). A rise in the cGMP/cAMP ratio triggers Ca$^{2+}$ influx and the acrosome reaction. Gwatkin (1977), citing unpublished data, suggested that lanthanum (which blocks transmembrane calcium movement) inhibited the acrosome reaction in guinea pig spermatozoa. Gwatkin (1977) defended the role of Ca$^{2+}$ involved in membrane fusion in somatic cells.

Triana et al. (1980), studying fresh bovine spermatozoa, found that although the ejaculation concentration of Ca$^{2+}$ was high, there was a protein component of seminal plasma which interacted with the sperm surface. This protein decreased plasma membrane permeability of Ca$^{2+}$. Triana et al. (1980) stated that the molecular details of these regulatory interactions remain unknown. Likewise, little information is available concerning mechanisms whereby environmental conditions affect the intracellular concentrations and distributions of Ca$^{2+}$ in spermatozoa.

Chang (1957) suggested that there exists in seminal plasma a detrimental factor capable of decreasing the fertilizing capacity of sperm. He was capable of
producing sperm decapacitation by addition of 5% seminal plasma before tubal insemination. Bedford and Chang (1962) reported that the decapacitation factor associated with seminal plasma may have its mode of action on the acrosomal membrane, causing a stabilizing effect that would bind to the sperm surface. Bedford and Chang (1962) were capable of removing the decapacitation factor from seminal plasma by high-speed centrifugation.

Oliphant and Brackett (1973) employed antiserum against seminal plasma proteins to show that it was capable of agglutinating ejaculated spermatozoa, but not incubated (12 hour) in vitro spermatozoa. This suggests that seminal plasma is capable of inhibiting or even reversing capacitation. Austin (1975), investigating membrane fusion events in fertilization, theorized that sperm capacitation involves the removal of a sperm surface inhibiting factor.

Capacitation time differed in two strains of mice, according to Fraser (1977). By extending incubation time from 20 minutes to two hours prior to egg addition, the fertilization rate was significantly increased for both strains. Fraser (1977) defined penetration as fusion with the vitellus, with penetration of vitellus usually occurring within 30 minutes.

The specific conditions for initiation of the acrosome reaction have not been fully elucidated. However, spermatozoa must first undergo an as yet undefined process of biochemical changes described as capacitation. Although extracellular Ca$^{2+}$ may not be required for capacitation, in an in vitro system Ca$^{2+}$ has been shown to initiate the acrosome reaction (Yanagimachi and Usui, 1974; Miyamoto and Ishibashi, 1975).

Hyne and Gurbers (1981), working with guinea pig spermatozoa, found that BSA or serum incubated in a media at pH 7.4 could contribute to capacitation of
the spermatozoa by removing any inhibitory factor from the spermatozoa. After capacitation, extracellular Ca$^{2+}$ can initiate the acrosome reaction in the absence of any biological specific acrosome reaction inducing factors (Hyne and Garbers, 1981).

A morphological modification, the acrosome reaction, occurs before or during passage of mammalian spermatozoa between the cells of the cumulus oophorus surrounding the ova or at the surface of the zona pellucida. (Austin, 1975; Yanagimachi and Mahi, 1976).

In some species, capacitation and the acrosome reaction can occur in vitro in the absence of oocytes, although serum albumin or other biological factors appear to be required: human (Yanagimachi et al., 1976), dog (Mahi and Yanagimachi, 1978), rabbit (Oliphant, 1976) and hamster (Yanagimachi, 1969, 1970) to name a few.

In the artificial insemination industry semen evaluation consists of concentration determinations, qualitative motility, quantitative motility, live:dead, morphology and alterations of the acrosomal cap. The desired goal of semen evaluation techniques is to estimate the relative fertility of bulls at each collection. According to Wells et al. (1970), alterations in the acrosome are associated with tailless and abnormal spermatozoa and to a lesser degree with normal sperm. This work was carried out with fresh bovine spermatozoa.

Measuring the percent intact acrosome (PIA) post-thaw has become a reliable and acceptable method of evaluating freeze-thaw damage (Saacke and Marshall, 1968; Saacke and White, 1972; Marshall and Frey, 1976; Saacke et al., 1980). PIA is of predominate importance in evaluation of collections for culling.
Marshall and Frey (1976), agreeing with earlier work by Saacke and White (1972), confirm that PIA in post-thaw evaluation of frozen semen is highly correlated to bull fertility (non-return rate).

Evaluating abnormal morphology, Fleming (1976) suggests that differential interference contrast (DIC) microscopy increases the reliability of monitoring semen production problems in their bulls. Saacke and White (1976) found abnormal sperm head morphology and presence of protoplasmic droplets were of equal importance to fertility, a negative correlation, while in the same study, abnormal tail morphology demonstrated no correlation with fertility. In conclusion, Saacke and White (1972) demonstrated acrosomal cap retention to be more closely related to fertility than either sperm motility or abnormal cell counts. Marshall and Frey (1976), using four hour post-thaw incubation at 37°C prior to sperm evaluation, demonstrated acrosomal integrity as a more accurate indicator of subsequent non-return rate than could be achieved by motility evaluation at 0 hour or four hours. They demonstrated a correlation of .70 between four hour intact acrosomes and non-return rate. Marshall and Frey (1976) tested various post-thaw incubation times and found four hours to give the highest correlation with acrosome integrity and non-return rate. Fleming (1976) performed semen evaluation at 0 hour and three hour post-thaw, looking at motility, PIA and abnormals (if there appeared to be elevated numbers). Agreeing with earlier authors (Saacke and White, 1972; Marshall and Frey, 1976), Fleming (1976) found acrosome retention to be more highly correlated with fertility than was motility.

The early work in evaluating significance of alterations in the acrosomal cap of live unstained bull spermatozoa was done by Saacke and Marshall (1968) using differential-interference contrast microscopy. They observed that acrosomal cap changes occurred either after cell death or immediately prior to cell death,
because only spermatozoa having lost motility showed acrosomal alterations. Saacke and Marshall (1968) further stated that in fresh ejaculates, unaltered acrosomal caps predominated, and that unaltered acrosomes were even present on immotile cells, perhaps suggesting that these may again resume motility.

Foulkes and Watson (1975), evaluating fresh bull spermatozoa, found an increase in seminal plasma hyaluronidase with increased sperm damage. This can be related to previously discussed information demonstrating in laboratory animals that as the percentage of intact acrosomes decrease in a sample, an increase in hyaluronidase can be observed. Visual evaluation of intact acrosomes is much faster than quantitative measurement of a specific enzyme concentration. The presence of an apical ridge is used to differentiate an intact acrosome from damaged (age or freeze-thaw) spermatozoa in bulls (Saacke and White, 1972; Fleming, 1976; Marshall and Frey, 1976). Saacke and White (1972) further elucidated the relationship by reporting that the most critical alteration of the bovine acrosomal cap in quantitating the rate of sperm aging was the loss of the apical ridge. In reviewing the effect of long term storage on sperm aging and fertility, Salisbury and Hart (1970) found an association between aged gametes and decreased fertility with increased embryo mortality. Foote (1972) demonstrated no impairment of sperm fertilizing capability or ability to sustain pregnancy when recently frozen semen was compared with semen stored at least two years.

Questioning the importance of semen quality tests for predicting fertility, Saacke et al. (1980) employed the technique of heterospermic inseminations as a method of comparing the ranking of bulls by their ability to produce calves. Heterospermic insemination is defined as mixing semen from more than one male for a single insemination in vitro or in vivo. The technique of heterospermic insemination has been employed for several species, a few are; chickens (Martin et
Employing heterospermic insemination, Stewart et al. (1974) demonstrated using four bulls of the same breed (two bulls/insemination) with fresh semen the ratio of calves sired per bull was 1:1:1:1. However when frozen semen was employed, one bull sired 50% of the calves. This experiment suggested that heterospermic insemination may be an excellent method of determining fertility of bull sperm following freezing. Beatty et al. (1969), using heterospermic insemination, suggested that the heterospermic technique requires \( \frac{1}{170} \) - \( \frac{1}{340} \) the number of inseminations to distinguish differences in producing calves between two bulls than if homospermic insemination comparisons are made. Beatty also evaluated semen quality by morphology and staining affinity but showed a non-significant relationship to homospermic and heterospermic non-return rate.

In contrast, Saacke et al. (1980) found significant correlations between freeze-thawed semen quality parameters (PIA and motility) with a competitive index generated from a heterospermic insemination experiment. The competitive index was determined from the actual sire of 260 offspring produced from 25 semen combinations using 10 beef bulls.

Yanagimachi (1972) first used zona-free hamster eggs for a cross-species penetration study, in this case using guinea pig spermatozoa. The use of zona-free hamster eggs to evaluate spermatozoa fertilizing capacity has primarily been limited to man (Yanagimachi et al. 1976, Barros et al. 1978, Rogers et al. 1979, Overstreet et al. 1980). Yanagimachi et al. (1976) used zona-free hamster ova and found fresh human sperm would not penetrate eggs after two hours of
preincubation. However, after seven hours preincubation followed by two hours incubation with zona-free eggs 95% were penetrated. Yanagimachi et al. (1976) further found that no attachment or penetration occurred if zona intact eggs were used.

Barros et al. (1978) advanced the work performed by Yanagimachi et al. (1976) by evaluating sperm from fertile and suspected infertile men using the zona-free hamster assay. Of the fertile group 76% (31/40) while in the suspected infertile group only 34% (13/38) were capable of penetration. Barros et al. (1978) employed a preincubation of one hour followed by three hour egg:sperm incubation. No mention was made as to number of egg evaluated and sperm concentration/dish could not be determined.

Rogers et al. (1979) reported use of the zona-free hamster assay for evaluation of clinically defined fertile and infertile populations. In this study sperm were preincubated for 18 to 20 hours prior to addition of 20-25 zona-free eggs; the sperm concentration was $1 \times 10^7$ sperm/ml. The group clinically defined as fertile demonstrated penetration of 14 to 100% of the eggs, while the infertile group penetrated 0 to 10% of the eggs. Hall (1981) demonstrated a 66% (20-100%) penetration of zona-free hamster eggs by "fertile" human sperm, and 33% (0-100%) penetration using sperm from "infertile" men. In the group of men classified as infertile, 19 of 43 (44%) displayed hamster egg penetration below 20%. In Hall's study none of the fertile group demonstrated zona-ree hamster penetration below 20%. Overstreet et al. (1980) evaluated fertile and infertile men with a combination of human immature eggs and zona-free hamster eggs. Their conclusions were that the test system was helpful in diagnosing fertilization dysfunction in several cases of unexplained infertility, when all other sperm parameters appeared normal.
Lorton and First (1979) used the zona-free hamster egg assay with bovine spermatozoa to test the effectiveness of in vitro capacitation of fresh spermatozoa in high ionic strength (HIS) medium. They demonstrated that HIS-sperm penetrated 57% (28/49) of zona free hamster ova, but failed to penetrate zona-intact hamster ova or bovine follicular oocytes. Calcote (1979) compared penetration using the zona-free hamster egg assay with PIA of twelve dairy bulls using both fresh and frozen sperm, and a total of 825 eggs. The range of PIA for the twelve bulls was 60-87% while the percent of eggs penetrated for fresh and frozen-thawed sperm was 63-83.9 and 60-81.8 respectively. While evaluating 8-37 eggs/dish and 12-89 eggs/bull a significant correlation was found for both fresh sperm (r=.61; P <.05) and frozen sperm (r=.81; P <.01) when compared with percent intact acrosome.

Bousquet and Brackett (1982) and Brackett et al. (1982) discussed the use of zona-free hamster ova with frozen and fresh bull sperm, respectively. Brackett et al. (1982) used 70 to 105 minutes preincubation in HIS medium to assess the relative fertilizing ability of fresh ejaculates from two dairy bulls. Using hamster ova from normally ovulating animals and fresh semen they found penetration of 53.8% and 84.9%, compared with non-return rates (with frozen semen) of 69.3% and 66.3% respectively. Brackett et al. (1982) ran nine experiments (39 total eggs) for one bull and 14 experiments (53 total eggs) for the other. Bousquet and Brackett (1982) evaluated frozen sperm from two Holstein bulls with non-return rates of 68.2% and 64.3%. Penetration of zona-free hamster eggs by sperm from these two bulls averaged 94.5% and 68.2% respectively.

Many artificial insemination units (e.g. Curtiss & Select Sires) use acrosome retention and motility to indicate cell injury during processing. PIA and motility are also used for research evaluation when altering processing procedures, extender
composition, freezing rate and special treatments.

The hypothesis examined in this research is that semen evaluation could also benefit by using penetration of zona-free hamster eggs. This evaluation procedure can be used to generate information concerning the ability of sperm to bind with the vitellus, undergo decondensation along with male pronuclear development following freezing and/or other manipulations. The above stages of cell fusion and pronuclear development are important steps toward development. As Yanangimachi (1973), using electron microscopy, describes fertilization, the sperm enters the previtelline space and approaches the egg, numerous microvilli from the vitelline surface surround the spermatozoa, and the postnuclear cap is incorporated into the cytoplasm. The nuclear material starts to decondense (swelling) and the next step is formation of the pronucleus by cytoplasmic vesicles that surround the nuclear material to form the continuous nuclear envelope.

If in vitro penetration of zona-free hamster eggs can be demonstrated to provide repeatable evaluation of spermatozoa fertility, it may prove beneficial to the animal industry.
MATERIALS AND METHODS

Preparation of Culture Equipment:

Whittingham (1971) stressed the importance of glassware being of high quality (Corning, Pyrex), heat resistant and low in sodium, potassium and other metallic ions which may leach into media and stock during storage. A proper and consistent cleaning regime is essential for in vitro ova fertilization and culture. All glassware was presoaked in 7X tissue culture soap (Limbro, Inc.) prior to brush washing. The glassware was then heated to 80-85°C in a 1% 7X solution in tap water to remove adhering oil from previous experimental runs. The glassware was then rinsed in tap water followed by 1-2 hours soak in 10% 7X soap in tap water, and then rinsed in hot tap water. This glassware was then placed in 3X distilled water for 1-2 hours followed by extensive (six times) rinsing in 3X distilled water. The equipment was then wrapped in cloth towels and paper and autoclaved (30 minutes at 121°C at 17-18 psi).

Whittingham (1971) and others (Yanagimachi and Noda, 1970; Toyoda and Chang, 1974; Calcote 1979) stressed the importance of using suitable plastic equipment. Many manufacturers prepare disposable plasticware for tissue culture, but it may still be toxic for mammalian embryos in culture. Pre-sterilized 35x10 mm petri dishes (Falcon Plastics) were used.

In order to flush or prick the oviduct, 30 g, 1" hyperchroms stainless needles were used, attached to a 1 cc glass tuberculin syringe filled with the hyaluronidase medium. The tip of the needle was cut off to remove the sharp bevelled tip, exerting care not to occlude the opening. The needle was then bent at a 60° angle to enhance manipulation inside the petri dish, checked for fluid flow and prepared
along with the other equipment.

For transferring eggs from dish to dish, 9" long disposable pipets (Scientific Products) were lengthened over a gas flame until the inside diameter was only slightly larger than an egg (80-100 mcg). The end was broken off, leaving a smooth edge, and the tip was bent at a 90° angle to increase manipulability within the petri dish. All pipets were examined under a dissecting scope to assure that the tip was without sharp edges (see Calcote, 1979 for extensive instructions).

When the petri dishes were out of the incubator for egg collection under a dissecting scope or egg transfer under a light scope (40X), they were kept in water baths (Technicon) at 37°C at all times except when on the light scope stage.

All medium solutions prepared and used in these experiments were covered by paraffin oil (Soybolt, viscosity 125/135, Fisher Scientific Co.). The paraffin oil is used to protect culture medium from evaporation, temperature fluctuation and loss of CO₂ during incubation and removal of dishes from the incubator for egg and sperm manipulation (Toyoda and Chang, 1974). The culture medium (minus BSA) was added to sterilized paraffin oil 1:10 v/v and then 5% CO₂ was bubbled through the media and up through the oil for 20 minutes. The equilibration was performed approximately 12-25 hours prior to use and the media were stored in the incubator at 37°C, 5% CO₂ until use (Forma Scientific model #3157).

Preparation of Culture Medium:

The culture medium employed for these experiments was that used by Calcote (1979). The medium was essentially that of Toyoda and Chang (1974), except for the addition of BFF in the fertilization and incubation media. It was a modified Krebs-Ringer bicarbonate solution containing 94.6 mM NaCl, 4.78 mM
KCl, 1.71 mM CaCl$_2$·2H$_2$O, 1.19 mM KH$_2$PO$_4$, 1.19 mM MgSO$_4$·7H$_2$O, 25.07 mM NaHCO$_3$, to which 21.58 mM sodium lactate (60% syrup), 0.5 mM sodium pyruvate, 5.56 mM glucose, 27.75 mM fucose, 4 mg/ml essentially fatty-acid-free crystalline bovine serum albumin (BSA), 50 µg/ml streptomycin sulphate and 75 µg/ml potassium penicillin-G were added (see appendix V for source of all reagents). According to Toyoda and Chang (1974), the first five reagents are dissolved in a liter volumetric flask with 3X distilled water and phenol red indicator (0.2 ml/liter), then refrigerated at 4°C (Stock Solution #1). Sodium bicarbonate (0.154M) was dissolved in 3X distilled water and gassed 5% CO$_2$ in air through a filter unit (Swinnex-25) for 15 minutes. Phenol red indicator was added and the solution (Stock Solution #2) was stored at 4°C.

Glucose, sodium pyruvate, fructose and the antibiotics were dissolved in a mixture of 83.35 ml Stock #1, 16.28 ml Stock #2 and 0.37 ml sodium lactate (60% syrup) immediately before use (Primary Stock Solution).

To the above mixture was added BSA (4 mg/ml) and adjusted to a pH of 7.4-7.5. An equal volume was then passed through a filter unit (Swinnex-13) into an oil-filled (prepared paraffin) petri dish (35x10 mm, Falcon Plastics Inc.) containing an equal volume of heat-treated BFF to form a pooled suspension. This BFF/BSA media was thoroughly mixed to obtain proper uniformity and transferred (0.4 ml aliquots) to prepared oil-filled petri dishes for use as fertilization and incubation dishes in the experiments using both BFF/BSA.

Culture dishes requiring BSA media are prepared by adding 4 mg/ml of BSA to the primary stock solution, adjusting pH to 7.4-7.5, filtering (Swinnex-13) and adding to oil-filled petri dishes. This solution is used for all washes and as a fertilization and incubation media in the appropriate experiments.
A 0.1% hyaluronidase (Type I, 460 NF units/mg) solution was prepared from the primary stock solution (no BSA). Hyaluronidase served to loosen the cumulus oophorus cells and release the eggs from this cell mass as they are recovered from the ampullae. The hyaluronidase was adjusted to a pH of 7.4-7.5, filtered (Swinnex-13) and placed under oil.

A 0.1% bovine pancreatic trypsin (Type III) solution (1 mg/ml) was prepared with a modified Ca⁺⁺/Mg⁺⁺ - free bicarbonate buffer solution (Appendix II) previously prepared and refrigerated at 4 C. Trypsin is used to dissolve the zona pellucidae from the hyaluranidase cleansed eggs. Due to the instability of the trypsin solution, it was prepared and used within 10-15 minutes. The mixture was filtered (Swinnex-13) into an oil-filled petri dish, to which the eggs were then added for 1-2 minutes, to dissolve the zona pellucidae. The denuded eggs were then passed through two BSA washes before being introduced into the proper fertilizing media containing sperm.

Bovine follicular fluid was obtained at the slaughter house by aspirating the cavity of 0.5-2.0 cm follicles, and passing the needle into the follicular cavity by way of the ovary (not through the exterior follicular wall). The follicular fluid was pooled as it was collected, and maintained in a ice bath until it was returned to the laboratory. The BFF was centrifuged twice at 1200 g for 10 minutes to remove blood cells and other debris. The decanted fluid was again pooled and divided into 2-3 ml aliquots, heated in a water bath (56° C) for 30 minutes, and stored by freezing at -20 C. On the day of experimentation the fluid was thawed and passed through a sterilized filter (Whatman #1) into a petri dish.

**Preparation of Spermatozoa:**
All sperm was supplied by Select Sires, Inc. (Plain City, Ohio), packaged in 0.5 ml straws and stored in liquid nitrogen (-192°C). Select Sires, Inc. collected all bulls and determined concentration, pre-freeze parameters and four hour post-thaw parameters as described by Marshall (1976). On the day of experimentation the straws were removed from the liquid nitrogen and placed in a 37°C water bath for one minute. The contents were then placed under oil into a 35x10 mm petri dish and placed in 37°C water bath for 20 minutes to allow equilibration. At this time the sperm were counted using an improved Neubauer Bright-Line hemacytometer (American Optical) employing micropipets (Clay Adams) for dilution into a sperm counting solution (Appendix II). In most of the experiments, after the 20 minutes, 10-20 µl of the concentrated sperm were added to 0.4 ml of the proper culture medium. It was returned to the incubator for the designated pre-incubation time until the eggs were added. This gave a final concentration of 1 x 10^6 cells/dish (2.4 x 10^6 cells/ml).

Collection of Eggs and Insemination:

Mature female Syrian hamsters (2-6 months old) were purchased from Charles Rivers (New Jersey). All animals were kept four to a cage in air conditioning (22°C) with 12 light/12 dark. As described by Orsini (1961) and Harvey et al (1961), animals were examined between 10-12 a.m. three days before the planned experiment, for the presence of a post-estrous discharge (a sticky-elastic mucus) emanating from the vulva of the animal. Enough animals (displaying the post-estrous discharge) were injected with 30 I.U. of PMSG (Sigma Chemical Co.) i.p. in order to recover adequate numbers of eggs for the planned experiment. Fifty to 56 hours after PMSG, the animals were given 60 I.U. of HCG (W.A. Butler
Co.) i.p. in order to collect eggs 18 hours post-HCG. According to Greenwald (1979) ovulation in the hamster occurs 11-14 hours post-HCG and eggs remained in the ampullary region for several hours longer. Between 20-40 eggs were collected per hamster. It was observed that more consistent egg retrieval was obtained when animals were left in their original cages throughout the injection schedule. Therefore animals were marked with picric acid for identification.

Hamsters were anesthesized with ether, and killed by cervical dislocation. The oviducts were immediately separated from the ovary and uterus and placed in BSA medium. When several oviducts had been collected, they were placed one at a time into the hyaluronidase dish and either flushed or pricked. This step was performed as fast as possible in order to reduce exposure to hyaluranidase. After the eggs were removed from several oviducts and the cumulus oophoros cells had been digested 10-20 minutes, the eggs were removed using a mouth pipet and twice passed through a BSA wash. The eggs were left in the final BSA wash until the eggs had been collected from all of the hamsters used in the experiment. The trypsin was then prepared (for removal of the zona pellucidae), the eggs added and left from 2-5 minutes followed by two more BSA washes. The final step was to add the required number of eggs to the previously prepared sperm. The concentration, as well as the period of incubation for the sperm varied with experimental design. In all experiments, the time from sacrifice of the hamsters until combination with the sperm cells was between 1-2 hours. After three hours of incubation with the sperm, the eggs were removed, washed twice in BSA and added to the proper incubation media for 13 hours. At this time they were added to slides and fixed in formalin for 24-48 hours, prior to evaluation.
Evaluation of Eggs:

After 13 hours of incubation in the proper culture medium the eggs were pipeted onto a clean microscope slide and placed into a drop of cold 2.5% glutaraldehyde (see Appendix II). A cover slip (22x40 mm Erie Scientific) was prepared by placing small drops of inert stopcock grease at the four corners, then pressing the cover slip down onto the eggs (under 100X power) until the eggs had been gently flattened out. The slides were then placed into 10% formalin (J.T. Baker Chemical Co.) for 24-48 hours until evaluated. All slides were stained by rinsing with 3X distilled water, dehydrated with 95% ethanol (1-2 minutes), and stained with 0.25% laemoid in 45% acetic acid (Appendix II) for 10-15 minutes. The stain was drawn off with 0.25% acetoglycoine (Appendix II) as described by Calcote (1979) and Toyoda and Chang (1974). The slides were immediately examined under 200 and 400X light microscopy, and scored as penetrated or non-penetrated. For penetration, swollen sperm heads or male pronucleus with female pronucleus and polar body had to be observed (See Appendix IV for form used during evaluation).

Statistical Analysis:

Each bull was randomly assigned a given trial and evaluated with at least two different bulls, employing the same experimental design. A total of 90-120 eggs (3-4 trials) were evaluated for penetration/bull/treatment/experiment. Analysis of variance was performed on all experiments (except the heterospermic evaluation) using percent penetration for each dish. Chi square was used to analyze eggs penetrated/total eggs evaluated for all treatments and bulls. Correlation coefficients were determined for PIA or competitive index against
percent penetration when applicable. All of the data described for a given bull in these experiments were determined using frozen samples (straws) from one day's collection of that bull.

**Experimental Design:**

Several experiments were carried out with slight deviation from the procedure described thus far. The following lists each individual project and how it differed:

I. Six bulls representing three different PIA levels were evaluated at four different sperm concentrations: $10^6$, $10^5$, $10^4$, and $10^3$/ml (see Appendix III). The sperm were thawed and incubated for one hour prior to addition of eggs for three hours. BSA/BFF media were used during sperm incubation, three hour sperm-egg incubation and 13 hour egg maturation.

II. Three bulls representing three different PIA levels were evaluated at five different capacitation times; 0 hour, 0.5 hour, one hour, two hour, and four hour. The various incubation times were all followed by three hours of sperm:egg culture. BSA/BFF media were used during sperm incubation, three hour sperm-egg incubation and 13 hour egg maturation.

III. Three bulls representing three different PIA levels were evaluated at $10^6$ concentration of spermatozoa, incubated for one hour prior to addition of 5, 15, or 30 eggs/dish for the three hour culture period. BSA/BFF media were employed during sperm incubation, three hour sperm-egg incubation and 13 hour egg maturation.

IV. Three bulls representing three different PIA levels were evaluated using BSA media instead of a 50:50 BSA/BFF, which was used in the other experiments.
The bulls were run using four different concentrations: $10^6$, $10^5$, $10^4$, and $10^3$. All incubations and dilutions were similar to the other experiments.

V. Nine bulls of various PIA levels were evaluated using BSA/BFF media, $10^6$ concentration, 30 eggs/dish and 1–2 hour pre-incubation. These bulls were also combined into five groups, so that two bulls were mixed and represented in each dish.
The purpose of conducting laboratory evaluations of semen samples is to estimate fertility. In these experiments PIA and competitive index are used as estimates of fertility for comparison with zona-free hamster penetration data. An actual value of fertility (non-return rate) was not available for either the dairy or beef bulls. PIA has been shown by Saacke and White (1972) and Marshall and Frey (1976) to be highly correlated with actual fertility. Competitive index (Saacke et al., 1980) while being an in vivo evaluation, only determines how sperm perform in vivo with competitive heterospermic inseminations. Therefore, competitive index and PIA are only relative estimates of fertility.

Calcote (1979) demonstrated that fresh and frozen bull spermatozoa could penetrate zona-free hamster eggs in a medium containing BSA/BFF. He demonstrated that a high correlation existed between PIA and percent penetrated hamster eggs (fresh r=0.61 and frozen r=0.81), by evaluating 12 dairy bulls.

The experiments described in this dissertation attempted to verify and extend previous studies completed in our laboratory (Calcote, 1979). The goals were to:

1) Replicate experiments conducted by Calcote and to extend the range of apparent fertility of bulls examined. (We used samples with 41–90% PIA, compared with 60–87% PIA used by Calcote).

2) Examine the effects of varying concentrations of sperm on percent
penetration.

3) Examine the effect of different numbers of eggs/dish at a constant concentration of sperm.

4) Evaluate different preincubation times allowed the sperm prior to egg addition.

5) Compare BSA and BSA/BFF as the media for preincubation, fertilization and incubation.

6) Apply the zona-free hamster egg assay to a group of bulls used in a competitive heterospermic insemination field trial and to compare results from these diverse systems.

**Eggs/Dish:**

The affect of varying the number of eggs/dish while maintaining a constant sperm concentration was evaluated in this experiment. When examining the current research employing the zona-free hamster in vitro assay technique a large variation (3-40) in eggs/dish within and between experiments is observed (Oversteet and Henbree 1976; Hammond et al. 1982). This experiment examined the difference in number of eggs penetrated at three levels of eggs/dish 5, 15 and 30. Each level was analyzed in triplicate for three bulls representing three distinct PIA and motility levels.

As shown in Table 1, percent of penetrated ova was 72±9, 61±10 and 60±9 for dishes containing 5, 15 and 30 eggs respectively (N.S.). The percent penetration of hamster ova by the bulls used in this experiment, disregarding number of eggs/dish, was 75±10, 63±8 and 55±10 for bulls which had 90, 63 and 43 PIA respectively (Table 1, Figure 1). Analysis of variance of the results shown in Table
Table 1. Penetration rates compared with percent intact acrosome and motility as varied egg concentration.

<table>
<thead>
<tr>
<th>Eggs/Dish</th>
<th>Bulls PIA (motility)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>90 (50)</td>
<td>63 (25)&lt;br&gt;43 (5)</td>
</tr>
<tr>
<td>5</td>
<td>78±17 &lt;sup&gt;a&lt;/sup&gt; (11/14)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>63±14 (13/20)</td>
</tr>
<tr>
<td>15</td>
<td>76±20 (22/29)</td>
<td>66±13 (49/75)</td>
</tr>
<tr>
<td>30</td>
<td>71±17 (62/83)</td>
<td>59±14 (67/112)</td>
</tr>
<tr>
<td>Total</td>
<td>75±10 (95/126)</td>
<td>63±8 (129/207)</td>
</tr>
</tbody>
</table>

<sup>a</sup>LSMean±STD E R Mean of 3 dishes/bull/egg concentration.
<sup>b</sup>Total eggs penetrated/eggs evaluated/bull/egg concentration.
<sup>c</sup>Both PIA and Motility data obtained from Select Sires, Inc., Plain City, Ohio.
Figure 1. Penetration of zona-free hamster with motility and PIA. Each point represents the total eggs penetrated/total egg evaluated for each bull, disregarding eggs/dish.

Motility  ▲——▲

PIA  ●——●
% Penetration vs. % Motility

% Penetration

% Motility

PIA
demonstrated no significant differences among egg groups or among bulls, when dishes were used as the experimental unit. When penetration of individual eggs was analyzed for the three bulls by $X^2$ analysis, disregarding eggs/dish, a significant difference ($P < 0.01$) was found, whereas $X^2$ analysis of eggs/dish revealed no significant effect.

While no significant difference existed between 5, 15, and 30 eggs/dish, the 5 egg/dish tends to exhibit a higher penetration than either 15 or 30 eggs/dish. Rogers et al. (1979) and Hall (1981) controlled the numbers of eggs/dish at 20-25 and 20-30 eggs/dish respectively.

**Capacitation Time:**

In order to evaluate the effect of varying the time that sperm were preincubated prior to egg addition, 5 time intervals were examined; 0, 30, 60, 120 and 240 minutes. Calcote (1979) evaluated frozen and fresh bull sperm using the zona-free hamster egg technique and preincubated the fresh sperm for seven hours and the frozen sperm for one hour. Lorton and Firt (1979) and Brackett et al. (1982) examined fresh bull sperm with preincubation times of 20 minutes and 19-25 hours respectively. Bousquet and Brackett (1982) using frozen sperm had a preincubation time of 60 minutes after sperm preparation.

Three bulls representing three PIA levels were used. The first four time intervals were each analyzed in triplicate (30 eggs/dish), for each bull, while the 240 minute preincubation was analyzed using each bull once.

As shown in Figure 2 and Table 2, when incubation time was 0, 30, 60, 120 and 240 minutes the percentage of hamster eggs penetrated was $32 \pm 3$, $40 \pm 3$, $44 \pm 3$, $55 \pm 3$ and $55 \pm 6$ respectively. Using analysis of variance the 60 minute interval
Table 2. Penetration rates compared with percent intact acrosome and motility for different capacitation times.

<table>
<thead>
<tr>
<th>Capacitation Time (min.)</th>
<th>Bulls PIA (Motility)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.90 (50)</td>
<td>43 (5)</td>
</tr>
<tr>
<td>0</td>
<td>26+6 (^{a}) (21/81)(^{b})</td>
<td>31+6 (26/83)</td>
</tr>
<tr>
<td>30</td>
<td>35+6 (24/69)</td>
<td>45+6 (36/82)</td>
</tr>
<tr>
<td>60</td>
<td>49+6 (35/71)</td>
<td>36+5 (39/107)</td>
</tr>
<tr>
<td>120</td>
<td>54+6 (40/74)</td>
<td>52+6 (49/95)</td>
</tr>
<tr>
<td>240</td>
<td>44+10 (12/27)</td>
<td>53+10 (16/30)</td>
</tr>
<tr>
<td>Total</td>
<td>42+3 (132/322)</td>
<td>51+3 (177/370)</td>
</tr>
</tbody>
</table>

\(^{a}\)LS Mean+STD ERR LS Mean of 3 dishes/bull/capacitation time.

\(^{b}\)Total eggs penetrated/eggs evaluated/bull/capacitation time.

\(^{c}\)Both PIA and Motility data obtained from Select Sires, Inc., Plain City, Ohio.
Figure 2. Penetration of zona-free hamster ova with five different preincubation time. Each bar represents the LS Mean ± STD ERR LS Mean of nine dishes, representing triplicate samples for three bulls.
Figure 3. Penetration of zona-free hamster eggs for three bulls as preincubation time is increased. Each point represents the total eggs penetrated/total eggs evaluated for each bull.

PIA

43 O---O

63 ▲---▲

90 ★---★
appears greater ($P<0.10$) than the 0 minute interval and less ($P<0.10$) than the 120 minute preincubation period. The percent penetration of zona-free hamster eggs, disregarding preincubation time, was 48, 41 and 41% for bulls with 63, 90 and 43 PIA respectively (N.S.).

Figure 3 demonstrates the bull:capacitation time interaction. Although increased capacitation time shows a trend towards ($P<.10$) an increase in percent penetration, no significance can be determined between bulls at any of the five capacitation times. When total eggs penetrated/total eggs evaluated is analyzed with $X^2$, a significant ($P<.001$) difference is found between capacitation times, while no significant difference is found between bulls. Further extension of capacitation time should be evaluated to find where the decrease in fertility begins.

**Concentration Study I:**

Sperm from six bulls were examined at four concentrations. Percent penetration at each concentration was compared with PIA and motility. This experiment was conducted to determine whether an optimum sperm concentration would accentuate a difference between bulls in ability to penetrate zona-free hamster eggs. The bulls represented three distinct PIA groups, each bull was evaluated in triplicate at four sperm concentrations ($10^3, 10^4, 10^5$ and $10^6$). A medium containing BSA/BFF was used for preincubation (sperm), fertilization (sperm and eggs) and incubation (eggs).

As shown in Table 3 and Figure 4, percent penetration was $27 \pm 4$, $34 \pm 4$, $38 \pm 4$ and $42 \pm 4$ overall for the $10^3, 10^4, 10^5$ and $10^6$ sperm/ml concentrations. A difference ($P<.10$) was found between the greatest concentration ($10^6$) and the
Table 3. Concentration Study I. Penetration compared with percent intact acrosome and motility at different sperm concentrations.

<table>
<thead>
<tr>
<th>Bulls PIA(M)</th>
<th>Concentration (sperm/dish)</th>
<th>Total penetration</th>
<th>Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$10^6$</td>
<td>$10^5$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>90 (50)</td>
<td>45±9 (52/117)</td>
<td>57±9 (60/106)</td>
<td>40±9 (41/101)</td>
</tr>
<tr>
<td>82 (25)</td>
<td>41±10 (33/82)</td>
<td>43±10 (33/77)</td>
<td>30±12 (26/87)</td>
</tr>
<tr>
<td>65 (30)</td>
<td>52±10 (38/77)</td>
<td>37±10 (32/87)</td>
<td>22±10 (19/90)</td>
</tr>
<tr>
<td>63 (25)</td>
<td>44±9 (52/119)</td>
<td>31±12 (17/54)</td>
<td>44±10 (37/84)</td>
</tr>
<tr>
<td>43 (5)</td>
<td>46±10 (40/86)</td>
<td>36±10 (27/79)</td>
<td>34±10 (28/87)</td>
</tr>
<tr>
<td>41 (10)</td>
<td>23±10 (20/86)</td>
<td>23±10 (21/85)</td>
<td>36±10 (42/90)</td>
</tr>
<tr>
<td>Total</td>
<td>42±4 (235/567)</td>
<td>38±4 (190/488)</td>
<td>34±4 (193/539)</td>
</tr>
</tbody>
</table>

*a LS Mean ± STD ERR  LS Mean of 3 dishes/bull/sperm concentration.

*b Total eggs penetrated/eggs evaluated/bull/sperm concentration.

*c Both PIA and Motility data obtained from Select Sires, Inc., Plain City, Ohio.
Figure 4. Penetration of zona-free hamster ova with four different sperm concentrations, using a media composed of BSA/BFF. Each bar represents the LS Mean ± Std. Err. of 18 dishes representing triplicate samples for six bulls.
Penetration

Concentration (sperm/ml)

% Penetration

$10^3$  $10^4$  $10^5$  $10^6$
Figure 5. The linear relationship between percent penetrated zona-free hamster eggs and either PIA ($r=.81, p<.05$) or motility ($r=.85, p<.05$). Twelve dishes/bull were evaluated in determining percent penetration.
least \((10^3)\), when analyzed using analysis of variance. When examining the percent penetration for the six bulls, the bull with 90 PIA had 41 ± 4% ova penetrated while the bull with 41 PIA penetrated 28 ± 5% (N.S.). No significant difference existed between bulls at any of the concentrations, when analysis of variance was used.

\(\chi^2\) analysis of eggs penetrated/eggs evaluated disregarding bulls showed a significant difference \((P < .001)\) between concentrations. Again comparing eggs penetrated/eggs evaluated using \(\chi^2\) a significant difference \((P < .05)\) was found between bulls. When bulls were examined combining all concentrations a significant \((P < .05)\) correlation (Figure 5) was found between percent penetrated and both PIA \((r=.81)\) and motility \((r=.85)\).

As shown by Figure 5 a high correlation \(.81\) exists between percent penetration and PIA. This confirms the work of Calcote (1979) and suggests that the zona-free hamster egg assay can give a fertility estimate.

**Concentration Study II:**

When the zona-free hamster assay has been used for prediction of fertility in man (Rogers et al., 1979; Overstreet et al., 1980; Hall, 1981), female tract or follicular fluid has not been used. The above experiments involved the use of BSA as the sole protein source. Calcote (1979) found high correlations between penetration of zona-free hamster eggs and PIA using BFF/BSA, but Whittingham et al. (1974) and Bavister and Martar (1974) claim that variation encountered in biological fluid is difficult to account for. Moreover, collection and processing of BFF is time consuming and difficult.

Therefore, this experiment was run to compare penetration of zona-free hamster eggs using either BSA or BSA/BFF.

The experiment was similar to concentration study I, except BSA was used
Table 4. Concentration Study II: Penetration compared with percent intact acrosome and motility at different sperm concentrations.

<table>
<thead>
<tr>
<th>Concentration (Sperm/Dish)</th>
<th>Bulls PIA (Motility)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>M</td>
</tr>
<tr>
<td></td>
<td>90 (50)</td>
<td>63 (25)</td>
</tr>
<tr>
<td>$10^6$</td>
<td>98+8</td>
<td>97+8 (84/86)</td>
</tr>
<tr>
<td>$10^5$</td>
<td>94+9 (56/60)</td>
<td>72+8 (59/83)</td>
</tr>
<tr>
<td>$10^4$</td>
<td>65+8 (57/83)</td>
<td>58+8 (44/75)</td>
</tr>
<tr>
<td>$10^3$</td>
<td>49+8 (41/84)</td>
<td>40+9 (25/63)</td>
</tr>
<tr>
<td>Total</td>
<td>77+4 (215/289)</td>
<td>67+4 (212/307)</td>
</tr>
</tbody>
</table>

aLS Mean±STD ERR LS Mean of 3 dishes/bull/sperm concentration.
bTotal eggs penetrated/eggs evaluated/bull/sperm concentration.
cBoth PIA and Motility data obtained from Select Sires, Inc., Plain City, Ohio.
Figure 6. BSA concentration study of penetration of zona-free hamster ova with four different concentrations of sperm/ml. Each bar represents the LS Mean ± Std ERR of nine dishes, representing triplicate samples for three bulls.
% Penetration

Concentration

$10^3$ $10^4$ $10^5$ $10^6$
instead of BSA/BFF in the media for preincubation, fertilization and incubation. Three bulls representing three distinct PIA groups were analyzed in triplicate at four sperm concentrations ($10^3$, $10^4$, $10^5$, $10^6$).

As shown in Table 4 and Figure 6 percent penetration was $40 \pm 5$, $59 \pm 4$, $77 \pm 5$ and $96 \pm 4$ combining bulls for the $10^3$, $10^4$, $10^5$ and $10^6$ sperm/ml concentrations. A significant difference ($P < .05$) was found between all concentrations, when analyzed using analysis of variance. When examining the percent penetration for the three bulls, the bull with 90 PIA had $77 \pm 4\%$ ova penetrated while the 63 PIA bull had $67 \pm 4\%$ (N.S.) penetrated ova and the bull with 43 PIA penetrated $61 \pm 4\%$ ($P < .05$).

When penetration of individual eggs was analyzed for the three bulls by $X^2$ analysis disregarding concentration, a significant difference ($P < .001$) was found. However, when analyzed individually a significant difference was not found between all individual bulls. $X^2$ analysis of concentration revealed significance ($P < .001$) as well as a significance ($P < .001$) between each concentration level, Figure 6 and 7.

The use of BSA instead of BSA/BFF resulted in higher percent penetration along with greater variation between bulls and concentrations.

**Heterospermic Comparison:**

Saacke et al. (1980) ranked nine beef bulls using a competitive index (C.I.) following hesterospermic inseminations. Comparings PIA at 0 hour and 4 hour with competitive index Saacke's group found significant ($P < .01$) correlation of $r = .90$ and $r = .81$ respectively. In this experiment these nine bulls were evaluated using the zona-free hamster system. The test conditions were as described previously, with
Figure 7. Penetration of zona-free hamster ova with four different sperm concentrations, showing performance of three different bulls at each concentration level. Each bar represents the total egg penetrated/total eggs evaluated for each bull at the various concentrations.

<table>
<thead>
<tr>
<th>PIA</th>
<th>M</th>
</tr>
</thead>
<tbody>
<tr>
<td>43</td>
<td>5</td>
</tr>
<tr>
<td>63</td>
<td>25</td>
</tr>
<tr>
<td>90</td>
<td>50</td>
</tr>
</tbody>
</table>
each bull evaluated individually in triplicate.

As shown in Table 5, percent penetration ranged from 43 ± 10 to 83 ± 10 while C.I. ranged -46.1 to 24.5 and PIA 48 to 82. Figure 8 demonstrates the relationship between percent penetration and competitive index.

A lack of correlation (r = 0.14) exists when all bulls are evaluated. It will be noted, however, that the two Simmental bulls used in this study had high PIA and CI, but low percent penetration. Fraser (1977) suggested that different strains of mice exhibited significantly reduced fertility when several preincubation times were studied. While one strain exhibited high fertility at all preincubation times, the other demonstrated high fertility only at the longest preincubation period. If Simmental bulls were removed from the analysis the correlation would be improved to r = .32 (N.S.).

Fraser (1977) and Fraser and Drury (1976) suggested that capacitation and subsequent fertilization will occur to a greater extent in concentrated semen than if the semen were diluted. They found using mice that when sperm remained in the concentrated form for 20 minutes prior to a dilution for a two hour preincubation the fertilization rate was significantly (P < 0.001) decreased from sperm diluted after two hours in concentrated suspension. In our study, all of the semen was diluted after 20 minutes.

When the Simmental bulls are removed the competitive index will change. Therefore it may be more helpful to regard the numerical rankings of the bulls, Table 5. An obvious trend becomes apparent between penetration and competitive index, only An 14 appears not to fit.

It is difficult to distinguish between actual difference in bull performance and variation due to experimental design. Between the initial heterospermic
Table 5. Percent penetration of 9 bulls compared with percent intact acrosome and competitive index.

<table>
<thead>
<tr>
<th>Bull</th>
<th>Penetration % (#/#)</th>
<th>C.I. Index</th>
<th>PIA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hf29</td>
<td>83+10a (70/84)b</td>
<td>6.4c</td>
<td>59c</td>
</tr>
<tr>
<td>Hf44</td>
<td>78+10 (66/84)</td>
<td>4.3</td>
<td>74</td>
</tr>
<tr>
<td>Hf36</td>
<td>75+10 (62/84)</td>
<td>-19.1</td>
<td>48</td>
</tr>
<tr>
<td>An20</td>
<td>74+10 (66/89)</td>
<td>2.1</td>
<td>72</td>
</tr>
<tr>
<td>An29</td>
<td>71+10 (55/79)</td>
<td>-7.8</td>
<td>67</td>
</tr>
<tr>
<td>Sm1</td>
<td>59+10 (51/87)</td>
<td>23.8</td>
<td>82</td>
</tr>
<tr>
<td>Hf27</td>
<td>51+10 (43/86)</td>
<td>-46.1</td>
<td>54</td>
</tr>
<tr>
<td>An14</td>
<td>47+10 (37/73)</td>
<td>11.9</td>
<td>73</td>
</tr>
<tr>
<td>Sm147</td>
<td>43+10 (35/82)</td>
<td>24.5</td>
<td>79</td>
</tr>
</tbody>
</table>

aLS Mean+STD ERR LS Mean of 3 dishes/bull.
bTotal eggs penetrated/eggs evaluated/bull.
Figure 8. Comparison of percent penetration and a competitive heterospermic field trial (C.I.) employing nine beef bulls, evaluated in triplicate.
competitive field trial and the analysis with zona-free hamster ova, two years had elapsed. A superior method of evaluation of these two tests would have been to analyze both parameters at the same location and time. Another method to compare in vitro and in vivo results would be to use fluorescent dyes to "mark" sperm, in order to perform competitive heterospermic inseminations in a single petri dish. Overstreet and Hembree (1976) and Blazak (1980) used fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC) in man and found no deleterious affect on sperm motility or fertility.

General Discussion:
Calcote (1979) demonstrated that bull sperm could be capacitated in vitro and penetrate zona-free hamster eggs using a BSA/BFF media. This observation was confirmed during these experiments, as well as bull sperm capacitation and penetration occurring in the absence of BFF - or other female tract fluids or serum, when BSA was used. Lorton and First (1979) used fresh bull sperm to demonstrate penetration in BFF media only when sperm had been preincubated in a high ionic strength medium. In conclusion, it would appear that either BSA is capable of supporting capacitation in vitro, or frozen bull sperm do not need to undergo capacitation to penetrate zona-free hamster eggs.

Figure 9, demonstrates the relationship between percent penetration with semen from three bulls at four concentrations using either BSA or BSA/BFF media. The BSA medium clearly increases percent penetration and appears to increase variation among bulls. However, the concentration studies were not performed simultaneously (BSA/BFF March-May; BSA June-August) and variation between experiments has been observed overtime as will be discussed below. Figure 9,
Figure 9. Comparison of BSA and BSA/BFF as assay media. Evaluating percent penetration of zona-free hamster eggs with four different concentrations of sperm/ml. Each bar represents the combination of the same three bulls (three dishes/bull/concentration) evaluated for each medium.

BSA

BSA/BFF
strongly suggests that BSA is at least equivalent to BSA/BFF. Therefore, it appears reasonable that by eliminating BFF from future experiments, efficiency and consistency may be increased.

When percent penetration of three bulls is depicted (Figure 10) over time, it appears that differences exist between mean penetration. The relationship between percent penetration and PIA may be linear during an experiment, (see results concentration I and II) however experiments over time cannot be compared. The difference observed in Figure 10 may be related to different batches of BFF collected from cows and heifers throughout the year.

The concentration studies evaluated the effect of varying numbers of sperm cells. Recently, Pace et al. (1981) and Hall (1981) have examined the parameter of total motile cells. Pace et al. (1981) showed that higher concentrations of motile cells-straw post-thaw showed significantly superior fertility (non-return rate). Sullivan (1970) used three distinct PIA levels of bulls and increased fertility of lower PIA bulls by increasing the dose of motile sperm given. This observation is confirmed by the concentration study employing BSA as a media. Even when more than enough sperm are present to penetrate eggs a disparity in percent penetration was observed at all levels. It is observed that the low PIA bull penetrated considerably higher percentage of eggs at $10^6$ concentration than did the high PIA bull at any of the lower concentrations.

Bovine eggs are obviously more acceptable for an in vitro test system of bull fertility. However, the difficulty of obtaining large numbers of bovine ova displaying similar maturation is not economically feasible at this time. Salt stored eggs as a replacement for living ova has been discussed for use in the human (Overstreet and Henbree, 1976), but their availability is limited. Unlimited
Figure 10. Percent penetration of zona-free hamster eggs is compared overtime for three bulls, at $10^6$ sperm/ml, 30 eggs/dish, and 60 minute preincubation. Experiment I (capacitation time) was conducted between December 19, 1980 and February 10, 1981; Experiment II (concentration study I) was conducted between March 10, 1981 and May 22, 1981; Experiment III (eggs/dish) was conducted between June 23, 1981 and August 3, 1981.
Experiments

% Penetration

Experiments

I  II  III
numbers of salt stored or fresh bovine eggs could conceivably be obtained from the
slaughterhouse, but no conclusive results have as yet been demonstrated as to their
application in an in vitro test system assaying bull fertility.

In men where comparisons of fertility have been made using the zona-free
hamster egg assay, Hall (1981) and Rogers et al. (1979) both endorse its ability to
screen for subfertile men. It has been emphasized (Yanagimachi et al. 1976 and
Barros et al. 1978), however, that the use of the zona-free hamster egg assay may
have greatest application in the interpretation of negative results. Assessing
fertilizing ability may be most meaningful in cases where sperm cannot penetrate
the hamster vitellus, suggesting an inability to undergo capacitation and the
acrosome reaction, or a lack of sperm viability. These spermatozoa would be most
likely incapable of fertilization in vivo. In man (except for AI donor programs) a
"fertile or nonfertile" result is considered adequate when analyzing sperm
fertilizing ability. However, in bulls a predicted estimate of fertility in relation to
other bulls is of primary importance.

The zona-free hamster egg assay was demonstrated by Calcote (1979) and in
the present study to be highly correlated with PIA and motility. While the zona-
free hamster assay system may be beneficial to the evaluation of various
manipulations (freezing, extender, storage, thawing) performed on semen, we are
still only correlating it with various semen parameters. The laboratory procedure
which seems to be best correlated with actual fertility in bulls is the PIA assay,
however this assay accounts for only 65% of the differences in non-return rates,
leaving 35% unaccounted for. The zona-free hamster egg assay takes semen
evaluation one step beyond current methods since it gives an indication of sperm
ability to bind to the vitellus, undergo decondesation and pronuclear formation.
Therefore, the zona-free hamster egg assay alone, but most likely in conjunction with established estimators of semen fertility may eventually provide greater confidence in laboratory testing of bull fertility. Acceptance of this technique by the industry will only come when a field trial evaluating non-return rate can be combined with penetration data from a zona-free hamster egg assay.
SUMMARY

In order to ascertain the effectiveness of using the zona-free hamster (ZFH) egg penetration assay as an estimate of fertility six bulls (frozen straws) representing three distinct levels of percent intact acrosome (PIA) and 6,320 ZFH eggs were evaluated. Employing a modified Krebs-Ringer bicarbonate medium combined with either bovine follicular fluid (BFF)/bovine serum albumin (BSA) or BSA in an incubator at constant temperature (37°C) and atmosphere (5% CO₂ in air) a determined number of ZFH eggs were combined with known concentrations of preincubated sperm for three hours. The ZFH eggs were removed, washed and incubated for an addition 13 hours prior to evaluation for penetration. For penetration, swollen sperm heads or male pronucleus with female pronucleus and polar body had to be observed. No significant differences existed between 5, 15 and 30 eggs/dish as PP was 72±9, 61±10 and 60±9 respectively. X² analysis demonstrated a significant difference (P < .01) between bulls with PIA of 90, 63 and 43 exhibiting PP of 75, 63 and 55 respectively when total eggs penetrated/eggs evaluated were analyzed. The length of sperm incubation prior to ZFH egg addition was evaluated at 0, 30, 60, 120 and 240 minutes with corresponding PP of 32±3, 40±3, 44±3, 55±3 and 55±6 when combining the results of 3 bulls of 3 distinct PIA levels. When total eggs penetrated/eggs evaluated are analyzed with X², a significant difference (P < .001) is found between capacitation times, while no difference was demonstrated between bulls. Sperm from 6 bulls were examined at 4 concentrations 10³, 10⁴, 10⁵ and 10⁶ in BFF/BSA media exhibiting PP of 27±4,
34±4, 38±4 and 42±4 respectively. X² analysis of total eggs penetrated/eggs evaluated showed a significant difference between concentration (P<.001) and bulls (P<.05). A significant (P<.05) correlation was found between PP and both 4 hr. post-thaw PIA (r=.81) and motility (r=.85). A concentration study was also run with BSA media with 3 bulls (PIA 90,63,43) at 4 concentrations 10³, 10⁴, 10⁵ and 10⁶ with PP of 40±5, 59±4, 77±5 and 96±4 respectively. X² analysis of total eggs penetrated/eggs evaluated demonstrated a significant difference (P<0.001) between each bull and each concentration level. Nine beef bulls (Angus, Hereford and Simmental) PP ranged from 43±1 to 83±10 while PIA ranged from 48 to 82. A lack of correlation existed when all bulls are evaluated. If the Simmental bulls are removed the correlation improves, possibly suggesting a breed variation in response to ZFH egg assay. These data demonstrate that penetration of ZHF eggs by bull sperm is highly correlated with PIA and motility. While PP appears unaffected by eggs/dish, both increased sperm concentration and lengthened preincubation time increase PP. BSA media expands the range of PP compared with BFF/BSA media and appears to increase ZFH egg penetration. The ZFH egg assay could advance laboratory evaluation of bull sperm, by examining its ability to bind the vitelline membrane and form pronuclei, but comparison of the ZFH assay with actual fertility (non-return rate) is needed.
REFERENCES


APPENDIX I. Incubation Medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>MW</th>
<th>g/ℓ</th>
<th>mM/ℓ</th>
<th>mM/83.35 ml</th>
<th>Final Culture Media (100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>58.44</td>
<td>6.633</td>
<td>113.497</td>
<td>94.6</td>
<td>Stock</td>
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<tr>
<td>KCl</td>
<td>74.56</td>
<td>0.4276</td>
<td>5.735</td>
<td>4.78</td>
<td>solution #</td>
</tr>
<tr>
<td>*CaCl₂ 2H₂O</td>
<td>147.02</td>
<td>0.3017</td>
<td>2.052</td>
<td>1.71</td>
<td>I--83.35 ml</td>
</tr>
<tr>
<td>*KH₂PO₄</td>
<td>136.09</td>
<td>0.1943</td>
<td>1.428</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>*MgSO₄ 7H₂O</td>
<td>246.48</td>
<td>0.3520</td>
<td>1.428</td>
<td>1.19</td>
<td></td>
</tr>
<tr>
<td>**NaHCO₃</td>
<td>84.01 (see below)</td>
<td>25.07</td>
<td>--</td>
<td>II--16.28 ml (stock)</td>
<td></td>
</tr>
<tr>
<td>Na Pyruvate</td>
<td>110.00</td>
<td>0.055</td>
<td>0.500</td>
<td>--</td>
<td>0.0055g</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>180.16</td>
<td>5.00</td>
<td>27.75</td>
<td>--</td>
<td>0.500g</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>180.16</td>
<td>1.002</td>
<td>5.56</td>
<td>--</td>
<td>0.1005g</td>
</tr>
<tr>
<td>(60%) Na Lactate</td>
<td>112.00</td>
<td>--</td>
<td>21.58</td>
<td>--</td>
<td>III--0.37 ml</td>
</tr>
<tr>
<td>Streptomycin-S</td>
<td>(50 μg/ml)</td>
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<td>K±Penicillin-G</td>
<td>(75 μg/ml)</td>
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<td></td>
<td></td>
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<tr>
<td>**B o (fat free)</td>
<td>(4 mg/ml)</td>
<td>0.1g/25 ml</td>
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<tr>
<td>***0.1% Hyaluronidase</td>
<td>(460 NF/mg)</td>
<td>(25 mg/25ml)</td>
<td>0.025g/25ml</td>
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<tr>
<td>***0.1% Trypsin</td>
<td>(25mg/25ml)</td>
<td>0.025g/25ml</td>
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</tr>
<tr>
<td>Colcemid (.5 μg/ml)</td>
<td>(Stock Solution: 0.5 x 10⁻⁴g/ml)</td>
<td>0.2 ml + 9.8 ml media</td>
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<tr>
<td>1% Phenol Red</td>
<td>(0.2 ml/1000 ml media; 0.1 ml/500 ml media; 0.05 ml/250 ml media)</td>
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</tbody>
</table>

* -- Dissolve separately in = 100 ml 3x dist. H₂O and add to final solution.

** -- 0.154M NaHCO₃ (Stock) - 12.938g/ℓ 94 3.2345g/250 ml 3x dist. H₂O. Gas mixture 15 minutes with 5% CO₂ through a Swinnex-25 millipore filter.

*** -- Add each ingredient separately to individual 25 ml aliquots of stock medium with antibiotics.

† -- Adapted from Calcote, 1979.
1) Trypsin solution:

Trypsin (1 ng/ml) is dissolved in 1 ml of 0.001N HCl; 9 ml 
Ca$^{++}$/Mg$^{++}$ - free medium is then added.

Ca$^{++}$/Mg$^{++}$ - Free Bicarbonate Buffer Solution:

<table>
<thead>
<tr>
<th></th>
<th>g/100 ml 3x distilled H$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.68</td>
</tr>
<tr>
<td>KCl</td>
<td>0.04</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>0.22</td>
</tr>
<tr>
<td>glucose</td>
<td>0.10</td>
</tr>
<tr>
<td>phenol red</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

Sperm Counting Solution:

50 ml 3x distilled water + 2 ml % eosin + 1 ml 3% NaCl.

Lacmoid Staining Solution:

Stock solution 0.55 g lacmoid in 100 ml glacial acetic acid. 
Diluted solution 1 part stock:2.2 parts 3x distilled H$_2$O (v/v).

0.25% acetoglycerine:

Stock solution 0.55 ml glycerine + 99.45 ml glacial acetic acid. 
Diluted solution, 1 part stock: 2.2 parts 3x distilled water (v/v).

0.1M Phosphate Buffer:

17.8g (NaHPO$_4$.2H$_2$O) + 20 ml HCl. Dilute to 1 liter 3x distilled 
H$_2$O. Adjust pH 7.2 - 7.4.

2.5% Glutaraldehyde:

10 ml 25% glutaraldehyde + 90 ml 0.1M Phosphate Buffer.

* Adapted from Calcote (1979).
APPENDIX III: Concentration Experiment

Stock A: 0.4 ml incubation media (BFF & BSA)  
+ 0.01 ml conc semen

Stock B: 0.45 ml incubation media (BFF & BSA)  
+ 0.05 ml stock A

Conc. 1 - $10^6$: 0.40 ml incubation media + 0.02 ml conc. semen.
Conc. 2 - $10^5$: 0.32 ml incubation media + 0.10 ml stock A.
Conc. 3 - $10^4$: 0.41 ml incubation media + 0.01 ml stock A.
Conc. 4 - $10^3$: 0.41 ml incubation fluid + 0.01 ml stock B.
APPENDIX IV: Evaluation Form

Date:

Experiment Fixed Evaluated

Sperm Head/Ova

<table>
<thead>
<tr>
<th>BULL #</th>
<th># Ova</th>
<th># Ova Pen.</th>
<th># Ova Not Pen.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>&gt;6</th>
<th>Pronuc*</th>
<th>% Pen</th>
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</thead>
<tbody>
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COMMENTS:

* To be counted as male pronucleus; a female pronucleus and a polar body had to be present with the male pronucleus.
**APPENDIX V: Reagents**

<table>
<thead>
<tr>
<th>Reagent and Supplies</th>
<th>Producer (OSU Store)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>KCl</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>CaCl$_2$·2H$_2$O</td>
<td>OSU 25</td>
</tr>
<tr>
<td>KH$_2$PO$_4$ (Potassium, Phosphate Monobasic)</td>
<td>MCB Reagents</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>Na pyruvate</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>D-fructose (levulose)</td>
<td>Eastman Kodak Co.</td>
</tr>
<tr>
<td>D-glucose</td>
<td>Mallinckrodt</td>
</tr>
<tr>
<td>Na Lactate (60% syrup)</td>
<td>Pfanstiehl Lab.</td>
</tr>
<tr>
<td>Streptomycin-S</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>K + Penicillin-G</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>BSA (fat free)</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>Hyaluronidase (type I, 460 NF units/mg)</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>Trypsin (bovine pancreatic, type III)</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>MCB Reagents</td>
</tr>
<tr>
<td>3x distilled H$_2$O</td>
<td>OSU 45</td>
</tr>
<tr>
<td>Filter Units</td>
<td>Millipore Co.</td>
</tr>
<tr>
<td>Paraffin Oil</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Falcon Pelt</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>PMSG</td>
<td>Sigma Chemical Co.</td>
</tr>
<tr>
<td>HCG</td>
<td>OSU Vet. Pharmacy</td>
</tr>
</tbody>
</table>