# PREDICTION
# OF
# BOVINE MALE FERTILITY

Promotor: dr. ir. E.W. Brascamp
hoogleraar in de veefokkerij

Co-promotor: dr. ir. T. van der Lende
universitair docent departement dierwetenschappen

# PREDICTION OF BOVINE MALE FERTILITY

# VOORSPELLEN VAN VRUCHTBAARHEID VAN STIEREN

Nanke (J.H.G.) den Daas

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
dr. ir. C.M. Karssen
In het openbaar te verdedigen
op dinsdag 11 november 1997
des namidddags te 16.00 uur in de Aula
van de Landbouwuniversiteit te Wageningen

Prediction of Bovine Male Fertility / Nanke J.H.G. den Daas
Thesis Wageningen - With ref. - With summary in Dutch.
ISBN: 90-5485-757-9. bound.
Subject headings; fertility, cattle, spermatozoa.

Printed by: Drukkerij Tamminga Siegers te Duiven
Cover design: Jeannete Weijers

Humans have never experienced freedom as they have now,
De mens is nog nooit zo vrij geweest,

technology rules the world however.
maar de techniek is de baas.

(J.B.B. Ellul)

Voor mijn Boebedoeps en Poekedoos

"(....) By art likewise, we make animals greater or taller than their kind is; and contrariwise dwarf them, and stay their growth: we make them more fruitful and bearing then their kind is; and contrariwise barren and not generative. Also we make them differ in colour, shape, activity, many ways. We find means to make commixtures and copulations of different kinds; which have produced many newkinds, and them not barren, as the general opinion is.
(...) Neither do we do this by chance, but we know beforehand of what matter and commixtures what kind of those creatures will arise."

(Francis Bacon in New Atlantis - 1624)

Contents

Remark: Due to the fact that some of the chapters have been published as full papers, wording and abbreviations might be inconsistent between chapters.

## Abbreviations

AI = artificial insemination
AR = acrosome reaction
$a_{ASYM}$ = asymptotic value
BLUP = best linear unbiased prediction
$b_{RATE}$ = rate of increase to the asymptote
BU = breeding unit
cirtot = percentage circular moving cells of the total spermatozoa population
cNR56 = corrected non-return to day 56
COC = cumulus oocyte complexes
cpr = conception rate
cvr = calving rate
ECS = estrus cow serum
FCS = foetal calf serum
FSH = Follicle Stimulating Hormone
HF = Holstein -Friesian breed
HYS = herd-year-season
IVF = in vitro fertilization
LPC = lysophosphatidylcholine
$N_{BU}$ = number of spermatozoa per breeding unit
$N_{95}$ = number of spermatozoa per breeding unit needed to achieve 95% of the asymptotic NR
NR = non-return
NR28 = non-return to day 28
NR56 = non-return to day 56
MRY = Meuse-Rhine-Issel breed
$P_{CR}$ = estimated conception rate
$P_{CALVR}$ = estimated calving rate given conception
$p_T$ = probability of non-return by time t after insemination

Chapter 1

# GENERAL INTRODUCTION

J.H.G. (Nanke) den Daas

Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

History of artificial insemination in cattle

The history of artificial insemination (AI) starts with a report written around 1400 about an Arabic chief inseminating a mare by a cotton clough placed in the vagina which was drenched in the semen of an elite stallion. The next mention of insemination results is by the Italian priest, Spallanzani, who had success after depositing semen in the vagina of a bitch in oestrus in the year 1870 as cited by Siebenga (1937). Around 1800 the first attempts were made by Hunter to inseminate human sperm, followed by Sims in 1866 who tried this procedure in order to prevent childless marriages. By the end of the 19th century the Russian professor Elie Ivanov started his work in artificial insemination with spermatozoa retrieved from testis, epididymis or vas deferens. He was given a grant by the Russian government and in 1907 published the important report "De la fecondation artificielle chez les mammiferes" (Ivanov, 1907). These initial results were to a large degree obtained from horses in Russia. In the year 1912 semen of 41 stallions was used to inseminate 3,397 mares with a pregnancy result of 41.4% (Siebenga, 1937).

In Russia, in the early 1930s the number of cows inseminated had increased to 300,000 per year. In 1937, in contrast to what is generally believed, insemination in the bovine was mainly practiced to (a) use genetically superior males, (b) to overcome the cervical block and therefore to increase fertility and (c) to overcome the logistical problem of shipping animals. Prohibition of venereal diseases (d) comes last on the list of reasons mentioned at that time (Siebenga, 1937). Around 1930 in most European countries zootechnological animal production associations were formed (Politiek, 1990).

Before World War II progress in artificial insemination came mainly in the areas of improving logistics and methods of semen collection (Politiek, 1990). Of course, there was much interest in the possibility of storing semen for a longer period than the few days it could be used. The first successful deep freezing of spermatozoa by adding glycerol was reported by Polge et al. in 1949 and Smith and Polge in 1950. Until early 1960s, when freezing in pellets on dry ice was developed by Nagase and Niwa (1964), semen was mainly frozen in

glass ampules (Polge and Lovelock, 1950). Almost simultaneously freezing in straws was further developed by Cassou in France (1964) and the first results were reported at the International Conference on Animal Reproduction in Trento in 1964 along with the pellet freezing results. In the USA the first technical conference on artificial insemination of the National Association of Animal Breeders (NAAB) was held in 1966.

Current situation

As the use of artificial insemination increased, genetic improvement of the breeds by selection of the superior bulls became more important for the AI-Industry. Artificial insemination in cattle enables the industry to obtain reliable breeding values of a bull on traits which can only be measured by his daughters' performance. Over the past three decades a decrease has been observed in the number of AI-associations as a consequence of the merging of the breeding programs into larger genetic improvement programs. Driving forces behind the merge include the sophisticated data retrieval from milk recording, the centralized breeding value calculation and the intensity of selection on milk production needed to remain competitive.

A continuous paradox in the AI-industry is created and sustained by the need for high artificial insemination results per cow inseminated versus the number of inseminations performed per ejaculate. AI-industry constantly has to balance farmers' needs to get a cow in calf with its own needs to perform as many inseminations possible per ejaculate. The goal of AI-associations is to maximize the number of offspring produced by selected genetically superior bulls, thus disseminating genetic improvement to the cattle population as efficiently as possible. This is accomplished by maximizing semen collection and the number of breeding units per ejaculate without a reduction in fertility. The ability of spermatozoa to fertilize when inseminated depends upon the quality of semen produced and upon its subsequent processing (Saacke, 1983).

Since the start of artificial insemination there has been this desire to predict (dose dependent) fertility results under field conditions. However, the numerous attempts to do so

have remained fairly unsuccessful so far (Uwland, 1984; Hammerstedt, 1996). At Holland Genetics it is routine to determine a dose response curve using 5, 10, 15 and 20 million total spermatozoa per insemination for each proven bull. The first ejaculates collected after a bull has been proven are the ones used in this field test. Information on at least 1,000 first or second inseminations per concentration have been used to evaluate the dose response curve.

## Scope of this thesis

In order to be able to evaluate the relationship between insemination results and semen characteristics measured in vitro both have to be estimated accurate and repeatable. Therefore, when we tried to answer, once more, the 'million dollar question' of how to predict bull fertility, we started by considering the research approach which should be taken. The motivation of the chosen research approach is given in chapter 2. Insemination data as retrieved under field conditions in the Netherlands need to be evaluated for environmental influences. Results of this inventory are discussed in chapter 3. The situation in the Netherlands with regard to field data retrieval is rather unique in the sense that data concerning all inseminations performed by a technician are sent to one central database on the same day the inseminations are carried out. Insemination data includes herd, cow, age of the cow, technician, bull and batch number. In the Netherlands 85% of all cows are inseminated by a technician service. From these insemination data non-return (NR) rates are calculated as the proportion of cows that were inseminated and did not return for another registered AI service within a specified period of time, usually 60 to 90 days. In the Netherlands NR is calculated at 28 and 56 days after insemination. Non-return rates measure the decline in number of cows not returning to service over time. The non-return rates can be used to derive more elementary measures of reproductive efficiency, such as conception rate and calving rate. In chapter 4 the decline in non-return is used to model a multiphasic logistic function which is then used to estimate conception rate, calving rate given conception and characteristics of the first two estrus cycles.

The relationship between the number of spermatozoa inseminated and insemination results is discussed in chapter 5. Fertility per bull is characterized by, (a) maximal non return, (b) rate of increase to this maximum, (c) maximal conception rate, (d) rate of increase to maximal conception and (e) calving rate given conception. Two of these fertility estimates, b and d, can only be determined when inseminations are performed in the dose range where insemination results are dependent on the dose inseminated, while the estimates a, c and e are not influenced by the number of spermatozoa inseminated (chapter 5). In chapters 6, 7 and 8, we try to predict these fertility estimates using motility, capacitation and bovine oocyte penetration as in vitro measurements for spermatozoa of different bulls. Special emphasis has been placed on the possibility of prediction of the dose dependent estimates because they will give information on the number of breeding units which can be produced from an ejaculate without compromising pregnancy results. Finally the results of these studies are discussed against the background of our physiological and biological understanding of the fertilization process in chapter 9.

References

Cassou, R., 1964. La methode des paillettes en plastique adaptee a la generalisation de la congelation. Proc. 5th Int Congr. Anim. Reprod. & A.I., Trento, IV: 540-546.

Hammerstedt, R.H., 1996. Evaluation of sperm quality: Identification of the subfertile male and courses of action. Anim. Reprod. Sci., 42: 77-87.

Ivanov, E., 1907. De la fecondation artificielle chez les mammiferes.

Nagase, H. and Niwa, T., 1964. Deep freezing bull semen in concentrated pellet form. I. Factors affecting survival of spermatozoa. Proc.5th Int Congr. Anim. Repord. & A.I. Trento, IV: 410-415.

Polge, C. and Lovelock, J.E., 1950. Preservation of bull semen at -79$^0$ C. Vet. Rec., 64: 396-397.

Polge, C., Smith, A.U. and Parkes, A.S., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature, 164: 666.

Politiek, R.D., 1990. Markante ontwikkelingen. Nederlanse Zootechnische Vereniging 1930-1990. Handelsdrukkerij 1874, Leeuwarden, the Netherlands. ISBN: 90-9003780-2.

Saacke, R.G., 1983. Semen quality in relation to semen preservation. J. Dairy Sci., 66: 2635-2644.

Siebenga, J., 1937. Kunstmatige inseminatie bij runderen. ISN: 347696-02.

Smith, A.U. and Polge, C., 1950. Storage of bull spermatozoa at low temperatures. Vet. Rec., 62: 115-116.

Uwland, J., 1984. Possibilities and limitations of semen evaluation for the prognosis of male fertility. In: The male in farm animal reproduction. Edt. A. Courot, Martinus Nijhoff, Boston, Ma, USA.: 269-289.

Chapter 2

# MOTIVATION OF RESEARCH APPROACH

J.H.G. (Nanke) den Daas

Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

## The motivation

### Introduction

In the bovine, artificial insemination is used to disseminate genetic progress and to prevent the spread of venereal diseases. Improvement of genetic progress has only been possible by fully exploiting the opportunities bovine reproduction offers. Due to a combination of (1) oestrus expression in the cow; (2) the anatomy of the cow's reproductive tract and (3) the fact that an ejaculate of a bull can be successfully extended and cryopreserved in liquid nitrogen, artificial insemination is the method used in bovine reproduction.

Ad 1) The detection of oestrus in the cow is relatively easy, provided farm management and circumstances to show signs of heat are adequate. Cows are non-seasonal breeders and will express oestrus behavior every three weeks when cyclic. In larger herds, when production stress is low, feeding is adequate and heat detection is performed by watching the animals for twenty minutes three times during the day, a detection level of 70 to 80% of the cows can be obtained. It is, however, generally agreed that detection efficiency is lower than 50% in the normal farm situation (Senger, 1994). Detection efficiency is dependent upon human, cow and environmental factors.

Ad 2) In the cow intra uterine insemination is routine. The cervix stiffens during heat and the rectum is wide enough to enter and manually fixate the cervix. The insemination pipet can be guided through the cervix with ease and semen can be deposited in the uterus. In this respect the bovine differs from the other domesticated species. In goats and sheep intracervical inseminations can be performed by retraction of the cervix under vaginoscopical guidance. In the pig the semen is deposited intracervically using an insemination pipet with a cork-screw tip.

Ad 3) The technique for cryopreservation of bull semen has been developed through empirical studies during the past 4 decades. In the bovine the percentage of spermatozoa surviving freeze-thawing and remaining capable of fertilization is approximately 25%, which is enough to ensure reasonable fertilization rates (Hammerstedt et al., 1990). There has been a

large volume of scientific and empirical research to adapt these successful techniques for use in other mammalian species. However, until recently this has not yielded successful protocols.

Another feature which makes the bovine situation unique is the relatively low number of spermatozoa and the small volume of the inseminate needed for fertilization. This may be due to the fact that it is possible to inseminate in the uterus. Spermatozoal output per ejaculate is age, breed and bull dependent and also depends on the interval between the collection of ejaculates (Van Os et al., accepted). The material of twenty years of semen production in the Dutch-Friesian and Holstein-Friesian breeds was studied in a review by Van Os et al. (accepted). The mean output per ejaculate was 7.8 $x10^9$ spermatozoa when ejaculates were collected in a twice weekly routine. The average total number of spermatozoa inseminated per breeding unit can vary from 2 x $10^6$ in an insemination with liquid semen (Shannon and Vishwanath, 1995) to a maximum of 40 x $10^6$ after freeze-thawing. The number of breeding units processed from one ejaculate will vary from bull to bull and can be as high as 4,000. In cattle the volume inseminated is as low as 0.5 to 0.25 ml. At these volume levels inseminated there is no relationship between volume and fertility (Penfold et al., 1997). In the porcine, however, the volume inseminated intracervically should be as high as 80 ml.

Between the time of insemination and the onset of embryonic development there is a sequence of events that should occur and that are influenced by the spermatozoon population and/or the spermatozoon which penetrates the oocyte. In order to achieve fertilization the spermatozoa are 'bathed' in excretions and fluids from the testis, the epididymis and the secretory products of the accessory glands (Amann et al., 1993). For the purpose of motivation of the research in this thesis we will mainly focus on events after semen collection.

To motivate the research approach followed we will: (1) describe physiological and functional requirements of the spermatozoon during the process from collection to fertilization; (2) review field fertility data; and (3) relate the possibilities of in vitro evaluation to the sequential requirements in the process of sperm production and fertilization.

1) Physiological and functional requirements of spermatozoa after ejaculation

The ejaculate is composed of spermatozoa and seminal plasma. Addition of seminal plasma to the spermatozoa which leave the cauda epididymis activates progressive forward motility. Fructose from the seminal plasma is used by the spermatozoon as the anaerobic energy source. The plasma membrane of the spermatozoon is also altered by compounds of seminal plasma which adhere to or are trapped by this membrane. At copulation the ejaculate is expelled through the penis into the caudal part of the vagina in case of the bovine. The environment in which the spermatozoa are deposited has a low pH and a low oxygen concentration. Seminal plasma contains prostaglandin and serotonin which act as stimulants of smooth muscle contractions. Some researchers suggest that these may play a role in transport of spermatozoa through the uterine lumen (Harper, 1988). Seminal plasma also contains proteolytic and nucleolytic enzymes, enabling it to dispose of the nucleoproteins and enzymes from decaying spermatozoa. The spermatozoa need to swim actively through the cervix in the cervical mucus. Motility of the spermatozoa is a prerequisite to reach the uterine environment. In the uterus leucocytes are present to clean up dead and immotile cells.

When ejaculates are collected for artificial insemination, a vial connected to an artificial vagina is used. Within a few minutes after collection an egg yolk or skimmed milk extender is added to the semen. This diminishes effects of longer storage in seminal plasma as certain compounds of seminal plasma are thought to have negative effects on the performance of the spermatozoa (Martinus et al., 1991). At artificial insemination in the bovine the spermatozoa are deposited directly in the uterus, hence bypassing the barrier formed by the cervix.

Interaction between the male and the female in fertility

'Compensable' factors

The uterus seems to be a quite hostile (harsh) environment for the spermatozoa. Especially in the bovine, numerous leucocytes are present at oestrus to eliminate foreign

bodies such as spermatozoa. After insemination we find that these leucocytes are filled with spermheads (Howe, 1967). To quote Ohno (cited by Cohen, 1984) "each copulation is a tissue transplant". Some researchers hypothesize that this second barrier acts as a selection for the right spermatozoa for fertilization (Cohen and Werrett, 1975; Cohen and Tyler, 1980). Others consider it just as a mechanism to get rid of dead or damaged cells and that vital spermatozoa escape the immunological reaction by either staying in the uterine folds or by having a surface protein composition that protects them from being phagocytized. During oestrus the uterine muscles show a certain contraction pattern and it is believed that these contractions, probably together with the residual motility of the spermatozoon itself, ensure transport to the oviduct. In the oviduct the spermatozoa adhere to the wall where they complete the maturation process needed to gain their fertilizing capacity. In cattle the first part of the oviductal isthmus is thought to be the sperm storage reservoir (Hunter and Wilmut, 1984).

Capacitation of a spermatozoon is defined as the acquirement of the ability to exhibit the acrosome reaction. During capacitation the negative surface charge of the plasma membrane is reduced (Eddy, 1988). The lipid and protein composition of the plasma membrane are altered during capacitation thus altering membrane fluidity. Changes in distribution of intramembranous particles are also noticed as are changes in lectin binding ability. Capacitation renders the membranes of the acrosome ready to fuse and release its contents during the acrosome reaction. After capacitation the spermatozoa become hypermotile and migrate through the oviduct to the ampulla (Suarez et al., 1990) and when they do not bind to the cumulus-oocyte complex they leave the reproductive tract into the peritoneal cavity. Capacitated spermatozoa are generally accepted to be fragile, unstable and short-lived (Hunter, 1987; Smith et al., 1987; Boatman, 1990).

In in vitro fertilization, capacitation in the bovine is mimicked by the addition of heparin. When spermatozoa are frozen, part of the same changes in the plasma membrane occurs because of membrane phase transitions and osmotic stress. Therefore bovine spermatozoa are easy to capacitate and acrosome react after freeze-thawing (Parrish and Foote, 1987).

The capacitated, hypermotile spermatozoa have to encounter the oocyte and bind to its

zona pellucida. This primary form of binding is species specific and is the final trigger for the true acrosome reaction. The acrosome is a specialized lysosome covering the rostral part of the spermatozoa. When proteases like acrosin are released during the acrosome reaction, they regionally alter the composition of the zona, enabling secondary binding of the internal acrosomal membrane and enabling the spermatozoon to penetrate the zona. The alterations of the region of the zona where the acrosome reaction took place, prohibits further primary binding of more spermatozoa (Florman and First, 1988). After penetrating the zona the spermatozo reaches the plasma membrane of the oocyte, and both membranes fuse. Immediately after fusion the oocyte protects itself by extrusion of the cortical granules, thus prohibiting multiple penetration. At any one time the number of capacitated hypermotile spermatozoa present in the oviduct appears to be limited (Pollard et al., 1991; DeJarnette, 1992).

Figure 1 shows the sequence of events spermatozoa need to undergo from ejaculation to fertilization. The number of spermatozoa progressively declines during the process from copulation or insemination to fertilization (fig. 1). After maturation and storage in the epididymis ejaculated spermatozoa form a very heterogenous population (Amann et al., 1993). In the ejaculate this population is thought to be heterogenous with regard to the time needed after insemination to fully mature and become capable to fertilize. An ejaculate ideally contains different subpopulations of sperm that are subsequently capable of fertilizing the oocyte over a wide time-span after insemination. At any one time only 30% of the bovine spermatozoa was found to be capable of acrosome reaction at zona binding (Florman and First, 1988). When this 'combined effective amount' (Amann et al., 1993) is large enough over time, the chance to fertilize the oocyte is maximal and insemination results are independent of the number of spermatozoa inseminated. Enough sperm are present to compensate all dose dependent factors (i.e. named 'extrinsic factors by Den Daas, 1992; 'compensable factors' by Saacke et al., 1994; Hammerstedt, 1996) . The compensable factors evaluated in this thesis are; 1) motility characteristics, 2) capacitation and acrosome reaction and 3) homologous oocyte penetration.

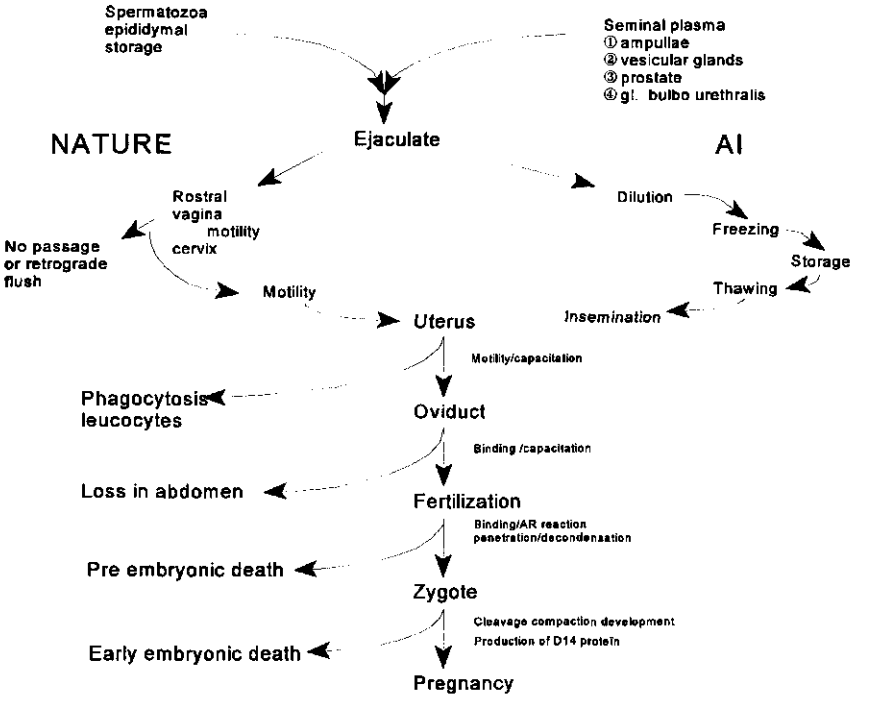



*Figure 1. Sequence of events from ejaculation to fertilization.*

'Uncompensable' factors

Once an oocyte is fertilized, development of the zygote into a full grown calf depends on: (1) the developmental potential of the oocyte, (2) the quality of the maternal environment and (3) the developmental potential of the fertilizing spermatozoon. In this introduction we will only focus on this last, paternal, contribution to a successful pregnancy. This paternal contribution is independent from the inseminated dose and referred to by Saacke et al. (1994) as 'uncompensable' factors, or 'intrinsic sperm quality' by Pace et al. (1981) and Den Daas (1992). During spermiogenesis the DNA of the sperm nucleus becomes highly condensed and the meiotic histones are replaced by a protamine rich of arginine. DNA in spermatozoa is packed with twice the density of the nucleosomes in a somatic cell. In the epididymis the majority of -S-S- (disulfide) cross-links are established between the protamine molecules.

Bedford and Calvin (1974) hypothesized that these -S-S- bridges serve to give sperm additional rigidity, which may be helpful in the penetration of the zona pellucida. However, part of the decondensation process appears to start during the passage of the spermatozoa through the reproductive tract of the female as increased SH levels were found in spermatozoa attached to the oocyte plasma membrane (Miller and Masui, 1982). The cytoplasm of ovulated mature oocytes has the capacity in their cytoplasm to reduce sperm nuclear disulfide bonds. This reducing capacity, although a prerequisite, is not sufficient for male pronucleus formation (Zirkin et al., 1985). Other factors in the oocyte cytoplasm are needed to exchange and remove the sperm specific protamines for full decondensation (Perrault, 1989). Uncompensable factors which might be evaluated in the spermatozo are the 'quality' of DNA condensation and the amount of damage to the DNA after freeze-thawing.

2) Review of field fertility data

The oestrus cycle of a cow is a cycle of 21 days on average. During this period there are waves of follicles growing (figure 2 from Ko et al., 1991). It is only after 18 days, when progesterone levels are decreasing (figure 3 from Ireland and Roche, 1987) that the dominant follicle can start its final growth towards the preovulatory stage. Oestrus behavior, the sign of receptiveness in the cow starts 25 to 30 hours before ovulation (O'Farrel, 1975; De Kruif, 1978; Walker et al., 1996). Oestrus is generally detected in the morning or afternoon when cows are intensively observed. Oestrus detection levels in Holstein Friesians range from 45% (USA) to 90% (NZ) (Macmillan et al., 1996). There are also marked differences between breeds with regard to oestrus detection levels (Macmillan et al., 1996). Oestrus behavior lasts for 14 to 15 hours (Macmillan and Watson, 1975). Insemination should be timed in such a way that the period spermatozoa are capable of fertilizing the oocyte completely covers the period of the penetrable oocyte present (figure 4). In practice this implies that insemination should be performed during late oestrus (Macmillan and Curnow, 1977).

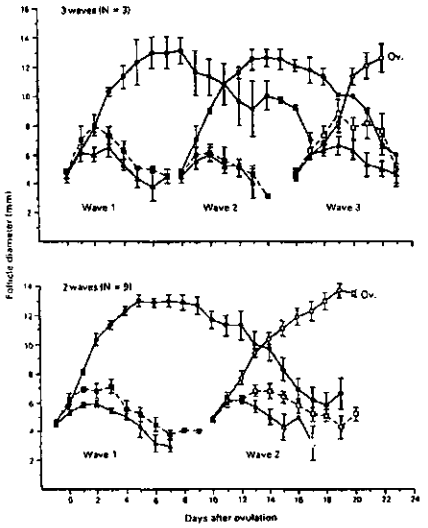



*Figure 2. Mean (sem) diameters of the dominant follicle and largest and second largest subordinate follicles during one oestrous cycle (from Ko et al., 1991).*

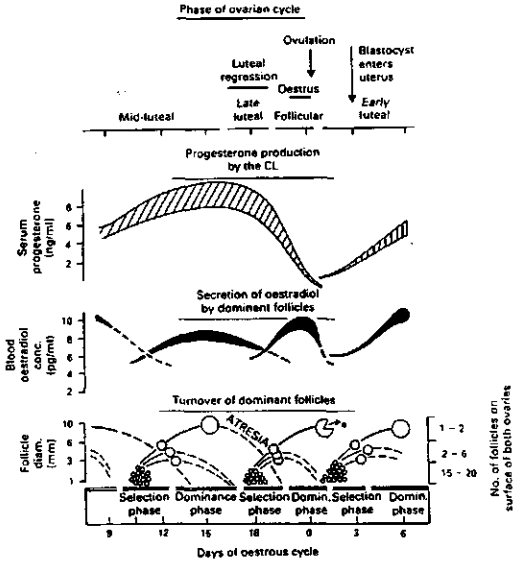



*Figure 3. Cycles of development of dominant follicles during the oestrous cycle of a cow (from Ireland and Roche, 1987).*

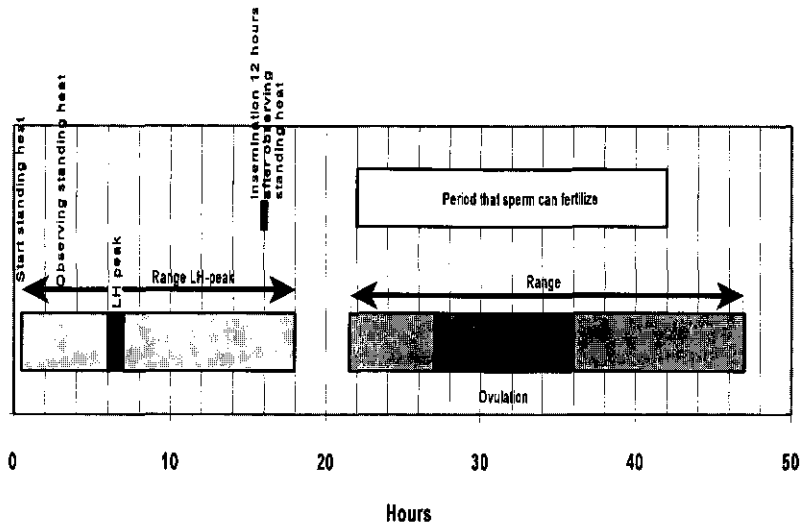



*Figure 4. Events related to time of insemination in relation to signs of standing heat.*

The insemination either leads to fertilization or to fertilization failure in which case the cow should return to oestrus in the following cycle 3 weeks after insemination. After fertilization the zygote passes the oviduct and reaches the tip of the uterine horn around 4 days after fertilization at the 32 cell to early morula stage. The embryo hatches around 10 days after fertilization when embryonic membranes start to form. Maternal recognition of pregnancy in the bovine occurs around 15 to 17 days post fertilization when the embryo produces and secretes bTP as a signal to the uterine cells and thus inhibits the production of prostaglandins by these uterine cells (King and Thatcher, 1993). Pre-signal embryonic failure does not show up as a prolonged oestrus interval. However, post-signal embryonic failure leads to an irregular and prolonged interval between heats.

The results of inseminations are generally scored as the number of cows not returning to service after a fixed number of days, generally noted as NR56 or NR 60-90. Non-return (NR) levels are influenced by differences in management. For instance, in a seasonal system like that found in New Zealand where all cows should have a one year calving interval, NR

rates are only informative during a six week period when every cow should be inseminated. After this period farmers generally tend to bring in a bull to cover the last animals returning to service. In management systems where the calving interval is not that strict, the pattern of cows returning to service is altered because the pressure on becoming pregnant is different (Barth, 1993). NR rates over-estimate the true rate of conception as natural service and culling are not taken into account (Oltenacu and Foote, 1976). Frozen extender without semen in the USA can produce an NR 60-90 days of 18% (Graham, 1968). Other known influences than herd management on NR rate are: parity of the cow, season, technician, bull, insemination number and day of the week. As a first step to measure NR rates more accurately a model to adjust for environmental effects is presented in chapter 3 of this thesis.

The distribution of return intervals has been studied by Macmillan and Curnow (1977) dividing the intervals into short (1-17 days) regular (18-24 days) and long (25-49 days). They showed that short intervals were related to the accuracy of heat detection, regular intervals to no conception and pre-signal embryonic death, and long intervals to accuracy of heat detection or post-signal embryonic death. Daily NR patterns were studied by Grossman et al. (1995) (chapter 4 of this thesis).

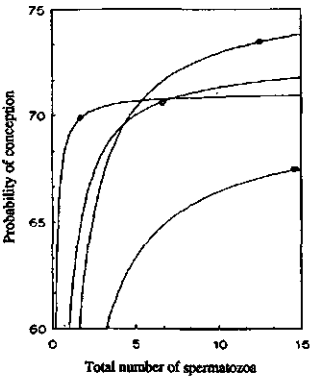
In chapter 4 we developed a model to monitor the decline in NR rate over time by estimating conception rate (cpr), calving rate (cvr) and characteristics of the first two oestrus cycles. From the proportion of cows that conceived but failed to complete gestation because of postsignal embryonic death, i.e. the irregular return intervals, (comparable to the long intervals of the Macmillan study) the model estimates cpr and cvr. From the proportion of cows that failed to conceive or that conceived but failed to complete gestation because of presignal embryonic death, (i.e. regular return intervals), the model estimates the proportion of return, or probability of detecting oestrus. This model might be more reliable for the evaluation of bull fertility than NR rates at arbitrarily chosen days after insemination since more fundamental information cattle reproduction is taken into account.

The number of inseminations needed for a reliable NR estimation is considerable. On a per cow basis gestation is an all or nothing phenomenon. Because of the binominal distribution of the data, estimated NR based on 1,000 inseminations will have a standard

deviation of 1.6 to 0.9 percent depending on the level of NR. For field studies on effects of a treatment, semen of several bulls and several ejaculates of each bull should be included and, most importantly, because of possible masking of effects, inseminations should be performed with sperm numbers below the dose where NR starts to become dose dependent (i.e. threshold level, (fig.5) of maximal fertility). Furthermore only when inseminations are well randomized over technicians and cows, conclusions can be drawn by evaluating NR for different treatments. In this research we wanted to focus on the prediction of bull fertility.

Variation in NR between bulls that are used for artificial insemination is generally in the order of 10 to 15% NR (Uwland, 1984). It is obvious that these bulls have been preselected, as bulls that do not have adequate semen characteristics after collection are not evaluated for field fertility. Moreover, each AI stud knows of some bulls with normal semen characteristics which give no or a very low number of offspring. The number of offspring of these animals is too low for a reliable estimation of the breeding value. These animals do not become a proven bull and therefore the number of inseminations is limited to the test inseminations. Because of these reasons the full range of NR is diminished from 70% to only about 15%. There have been numerous reports (around 13,000 references) in the literature of studies that tried to predict insemination results. Most of these studies however do not separate compensable (i.e. dose dependent) fertility factors from the uncompensable fertility factors (Den Daas, 1992; Saacke et al., 1994). In most studies only one concentration of spermatozoa above threshold level is considered (Figure 5). Inseminations with concentrations above this dose will mask differences between bulls or treatments either positive or negative (Hammerstedt, 1996).

In order to be able to evaluate compensable and uncompensable factors separately we inseminated dose rates from 2 to 17 x $10^6$ total spermatozoa (chapter 5 of this thesis). Insemination results were evaluated according to the methods developed for corrected NR (chapter 3 of this thesis) and estimated cpr and cvr given conception (chapter 4 of this thesis).




- *Threshold for dose dependence differ between bulls*

*Figure 5. Relationship between non-return rate and the total number of spermatozoa inseminated. Semen from different bulls differ in the maximum non-return rate and in the rate at which the asymptote is approached. Ranking of bulls changes with sperm dosage (from Den Daas, 1992).*

3) In vitro evaluation of bull semen: what do we measure

'Compensable' factors

Motility

Motility of the spermatozoon is the most used criterion to evaluate semen before and after processing in the laboratory. Microscopical evaluation of sperm motility is dependent on the diluent used for extending the ejaculate, the extension rate, the temperature at evaluation and holding time. As the repeatability of the subjective motility estimation by microscope was good within the evaluator but poor between them, much effort went into developing motility analyzing systems. These systems are based on videotaping of the spermatozoa and are reported to be precise, accurate and repeatable. Most of these systems not only give an estimate for percentage motile cells, they also characterize the motility pattern by estimation of progressive motility, velocity, lateral head displacement, beat cross frequency, et cetera. The possibility of this in depth analysis of motility gave rise to another flow of papers trying to predict fertility (Knuth et al., 1987; Rath et al., 1987; Budworth et al., 1988).

Spermatozoa need to be motile in order to reach the oocyte. Therefore they need to be able to express this motility in the uterine environment and in the oviduct where they become hyper motile after capacitation (see figure 1). Motility therefore appears to be a prerequisite for fertilization, but it is however only one of several. Motility is one of the obligatory events in the lock-step series of events needed for fertility as described by Hammerstedt (1996). Motility is one of the first in this series of events and in most cases is not limiting (Amann and Hammerstedt, 1993). Motility evaluation may be in reality only an infertility assay. Non motile sperm will not perform after insemination, although not all motile sperm are able to fertilize! Furthermore the population of sperm that are motile and able to fertilize changes over time after insemination. At a given time span after insemination this heterogeneity of the spermatozoa results in a population ready to fertilize, and therefore optimizes the chance of fertilization during this period (see fig 4).

Motility is, for the reasons mentioned above, one of the compensable factors as described by Saacke et al. (1994). When enough motile sperm are present at any one time during the window of possible fertilization, the relationship between motility and rate of increase to the threshold level is not expected to exist. This was the motivation to test whether motility, as evaluated by an objective videotaping system, is one of the limiting factors in the sequence of events. Results of this study on the relationship between estimated motility characteristics and field fertility data on sperm concentration are reported in chapter 6 of this thesis.

Capacitation and acrosome reaction

Capacitation prepares the sperm cell for its interaction with the oocyte. After capacitation spermatozoa are hypermotile and have acquired the ability to acrosome react. During capacitation mainly biochemical modifications take place, and it is amazing that no morphological changes have been observed. In the mouse a method was developed to monitor membrane changes during capacitation (Lee and Storey, 1985). Capacitation of spermatozoa can be measured by the ability of the cells to acrosome react. This ability is evaluated by microscope or by flowcytometry (Graham et al., 1990; De Leeuw et al., 1991).

De Leeuw et al. (1991) developed a method for simultaneous determination of viability and acrosomal status of bovine spermatozoa. Immediately following ejaculation over 85% of the spermatozoa are viable and acrosome intact. With incubation time there is an increase in the number of viable acrosome reacted cells. In vitro capacitation and acrosome reaction abilities vary amongst bulls (Ax and Lenz, 1987; Davis and Foote, 1987) and might therefore pick-up differences in (dose dependent) insemination results.

In the sequence of events leading to pregnancy capacitation starts immediately after ejaculation and finishes just before fertilization (fig 1). After insemination and therefore during the process of capacitation the number of spermatozoa declines dramatically (1). This decrease coincides however, with an increase of the fertilizing capacity of the cells still present. When capacitation is evaluated in vitro, the total population of spermatozoa is present. The natural selection of spermatozoa, either by chance or actively as far as the female tract is responsible, is omitted. So direct comparison of the situation within the animal is not possible. What we can evaluate however, is whether variation in in vitro capacitation can predict (dose dependent) insemination results from bulls.

*This formed the motivation to study semen capacitation of a bull measured as the ability* to acrosome react as one of possible predictors for fertilization results (chapter 7 of this thesis).

Oocyte penetration rate

Oocyte penetration rate in vitro is measured as the number of oocytes with a visible (decondensed) sperm head in the cytoplasm of the oocyte 18 hours after onset of fertilization. The limitation of this penetration test is that only one spermcel can be evaluated per oocyte. Per oocyte, penetration is a binomial all or non event. The number of oocytes tested per bull should therefore be in the order of several hundreds to measure differences between animals. Another problem in the oocyte penetration test is the variability of oocyte maturation between different oocyte batches which adds substantially to the variation between tests. Evaluation can therefore only be performed by comparing test results of bulls on the same group of oocytes.

For penetration of the oocyte in vitro spermatozoa need to be: motile (1), capacitated (2), able to bind to the zona pellucida (3), able to acrosome react (4), able to penetrate the zona (5), able to fuse with the oocyte (6) and able to show decondensation of the nucleus (7). After freeze-thawing and centrifugation viable spermatozoa are selected for oocyte penetration in vitro. This is a population of spermatozoa different from the in vivo situation. As can be seen in figure 1 only few of the spermatozoa that are inseminated reach the oviduct and are contributing to the chance of fertilization. In vitro oocyte penetration therefore might be the ultimate way to measure the effect of limiting sperm numbers (compensable factors) in fertilization. This was the motivation to study the possibilities as to whether or not variation in oocyte penetration would explain variation in (dose dependent) insemination results (chapter 8 of this thesis).

'Uncompensable' factors

Inseminations at dose rates above threshold give information about the paternal contribution to the developmental potential of the conceptus (Den Daas, 1992; Saacke et al., 1994). The reason is that maximal fertilization rates are obtained. This paternal contribution to embryo development given penetration varies between bulls, the variation being in the order of 10% of calving rate given conception (chapter 5 this thesis) or when measured as NR rates 10 to 15% (Uwland, 1984; Marquant-Le Guienne and Humblot, 1992). Prediction of this paternal contribution by evaluation of the compensable factors studied in this thesis is only possible when variation in intrinsic sperm quality is reflected in these compensable factors. Nevertheless we checked whether the uncompensable factors could be predicted motility characteristics, capacitation and bovine oocyte penetration. Prediction of the paternal contribution however, is not that important to AI-industry as it is not possible to influence this trait by changing the dose inseminated. Therefore we did not study other possibilities for prediction. It should be emphasized that possibilities to improve this paternal contribution, if possible at all, are most likely to be found in optimization of semen conservation.

In the final considerations, chapter 9, the motivation of this research will be discussed

against the background of the holistic versus reductionistic approach to Science. Additional information on the use of the results in daily AI-routine in the Netherlands by, amongst others Holland Genetics, will also be given in chapter 9.

References

Amann, R.P., Hammerstedt, R.H. and Veeramachaneni, D.V.N., 1993. The epididymis and Sperm Maturation a Perspective. Reprod. Fertil. Dev., 5: 361-381.

Amann, R.P. and Hammerstedt, R.H., 1993. In vitro evaluation of sperm quality: an Opinion. J. of Andrology, 14: 397-405.

Ax, R.L. and Lenz, R.W., 1987. Glycosaminoglycans as probes to monitor differences in fertility of bulls. J. Dairy Sci., 70: 1477-1486.

Barth, A.D., 1993. Factors affecting fertility with artificial insemination; Veterinairy clinics of North America: Food animal Practice, 9: 275-289.

Bedford J.M. and Calvin H.I., 1974. The occurrence and possible functional significance of -S-S- crosslinks in sperm heads, with particular reference to eutherian mammals. J. Exp. Zool., 188: 137-156.

Boatman, D.E., 1990. Oviductal modulators of sperm fertilizing ability. In: Fertilization in Mammals. Edt. B.D. Bavister, J. Cummins and E.R.S. Roldan, Serono Symposia, Norwell, Mass, USA: 223-238.

Budworth, P.R., Amann, R.P. and Chapman, P.L., 1988. Relationships between computerized measurements of motion of frozen-thawed bull spermatozoa and fertility. J. of Androl., 9: 41-54.

Cohen, J., 1984. Immunological Aspects of Sperm Selection and Transport. In: Immunological aspects of reproduction in mammals. Edt. D.B. Crichton, Butterworth's, London: 77-89.

Cohen, J. and Werrett, D.J., 1975. Antibodies and sperm survival in the female tract of mouse and rabbit. J. Reprod. Fert., 42: 301-310.

Cohen, J. and Tyler, K.R., 1980. Sperm populations in the female genital tract of the rabbit. J. Reprod. Fert., 60: 213-218.

Davis, A.P. and Foote, R.H., 1987. Relationship of Sire fertility to Acrosome Reacted and Motile Spermatozoa after Treatment with Liposomes. J. Dairy Sci., 70: 850-857.

DeJarnette, J.M., Saacke, R.G., Bame, J. and Vogler, G.C.J., 1992. Accessory sperm: Their importance to ferility and embryo quality, and attemps to alter their numbers in artificially inseminated cattle. J. Anim. Sci., 70: 484-491.

De Kruif, A., 1978. Factors influencing the fertility of a cattle population. J. Reprod. Fert. 54: 507-518.

De Leeuw, A.M., den Daas, J.H.G. and Woelders, H., 1991. The fix vital stain method for simultaneous determination of viability and acrosomal status of bovine spermatozoa. J. of Andrology, 12: 112-118.

Den Daas, J.H.G.,1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci 28: 87- 94.

Eddy, E.M., 1988. The spermatozoon: Modification of the sperm plasma membrane during ejaculation and capacitation. In: The Physiology of Reproduction, Edt. E. Knobel and J. Niel et al. Raven Press Ltd. New York USA 1: 28-41.

Florman, H.M. and First, N.L., 1988. The regulation of Acrosomal Exocytosis. Developm. Biology, 128: 453-463.

Graham E.F., 1968. The usefulness of useless data-field tests and responsibilities. Proc 2nd Tech. Conf. Artif. Insem. Reprod., NAAB.: 88-90.

Graham, J.K., Kunze, E. and Hammerstedt, R.H., 1990. Analysis of Sperm Cell Viability, Acrosomal Integrity and Mitochondrial Function Using Flow Cytometry. Biol. Reprod., 43: 55-64.

Grossman, M., Koops, W.J. and den Daas, J.H.G., 1995. Multiphasic analysis of reproductive efficiency of dairy bulls, J. Dairy Sci., 78: 2871-2876

Hammerstedt, R.H., Graham, J.K. and Nolan, J.P., 1990. Cryopreservation of mammalian sperm: What we ask them to survive. J. of Andrology, 11: 73-88.

Hammerstedt, R.H., 1996. Evaluation of sperm quality: Identification of the subfertile male and courses of action. Anim. Reprod. Sci., 42: 77-87.

Harper, M.J.K., 1988. Gamete and Zygote transport. In: The Physiology of Reproduction, Edt. E. Knobil and J.D. Neil, Raven Press Ltd. New York, USA., vol 1: 103-134.

Howe, G.R., 1967. Leukocytic response to spermatozoa in ligated segments of the rabbit vagina, uterus and oviduct. J. Reprod. Fertil.,13: 563-566.

Hunter, R.H.F. and Wilmut, I., 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Developm., 24: 597-608.

Hunter, R.H.F., 1987. The timing of capacitation in mammalian spermatozoa - a reinterpretation. Research in Reproduction 19: 3-4.

Ireland, J.J. and Roche J.F., 1987. Hypothesis regarding development of dominant follicles during a bovine estrus cycle. In: Follicular growth in ovulation rate in farm animals. Edt. J. Roche and D. O'Callaghan, Martinus Nijhoff, The Hague, The Netherlands.

King, G.J. and Thatcher, W.W., 1993. Pregnancy. In: Reproduction in Domesticated Animals Elsevier Sci. Publ., Amsterdam, The Netherlands: 264.

Knuth, U.A., Yeung, C.H. and Nieschlag, E., 1987. Computerized semen analysis: Objective measurement on semen characteristics in biased by subjective parameter setting. Fert. Ster., 48: 118-124.

Ko, J.C.H., Kastelic, J.P., Del Campo, M.R. and Ginther, J.O., 1991. Effect of a dominant follicle on ovarian follicular dynamics during the oestrus cycle in heifers. J. Reprod. Fertil., 91: 511-519.

Lee, M.A. and Storey, B.T., 1985. Evidence for Plasma Membrane Impermeability to Small Ions in Acrosome Intact Mouse Spermatozoa Bound to Mouse Zona Pellucida, Using Aminoacridine Fluorescent pH Probe: Time Course of the Zona-induced Acrosome Reaction Monitored van Both Chlortetracycline and pH probe Fluorescence. Biol. Reprod., 33: 235-246.

Macmillan, K.L. and Curnow, R.J., 1977. Factors influencing A.B. conception rate X. Seasonal trends in fertility and in the distribution of return intervals: differences between deep-frozen and liquid semen; New Zealand J. Exp. Agricul., 5: 273-278.

Macmillan, K.L. and Watson, J.D., 1975. Factors influencing AB conception rates V. Cow age and stage of estrus at insemination. New Zealand J. Exp. Agricul., 3: 29-35.

Macmillan, K.L., Lean, I.J. and Westwood, C.T., 1996. The effects of lactation on the fertility of dairy cows. Austr. Vet. J., 73: 141-147.

Marquant-Le Guienne, B. and Humblot, P., 1992. Test de fertilite chez l'animal domestique par fecondation in vitro. Ann Zootech., 41: 361-370.

Martinus, R.D., Molan, P.C., and Shannon, P., 1991. Deleterious effect of seminal plasma in the cryo-preservation of bovine spermatozoa. New Zealand J. Agric. Res., 34: 281-285.

Miller, M.A. and Masui, Y., 1982. Changes in the stainability and sulphydryl level in the sperm nucleus during sperm-oocyte interaction in mice. Gamete Res., 5: 167-179.

O'Farrel, 1975. The role of management in dairy herd fertility. Irish Vet. J.: 118-125.

Oltenacu, E.A.B. and Foote, R.H., 1976. Monitoring fertility of AI-programs: Can non-return rate do the job? Proc. 4th Tech. Conf. Artif. Insem. Reprod., NAAB: 61-68.

Pace, M.M., Sullivan, J.J., Elliot, F.I., Graham, E.F. and Coulter, G.H., 1981. Effects of Thawing Temperature, Number of Spermatozoa and Spermatozoal Quality on Fertility of Bovine Spermatozoa Packaged in 5-ml French Straws. J. Anim. Sci., 53: 693-701.

Parrish, J.J. and Foote, R.H., 1987. Quantification of Bovine Sperm Separation by a Swim-up method Relationship to Sperm Motility, Integrity of Acrosomes, Sperm migration in Polyacrylamide Gel and Fertility. J. Androl., 8: 259-266.

Penfold, L.M., Garner, D.L., Donoghue, A.M. and Johnson, L.A., 1997. Comparative Viability of bovine sperm frozen on a cryomicroscope or in straws. Theriogenology, 47: 521-530.

Perrault, S.D., 1989. Regulation of sperm nuclear reactivation during fertilization. In: Fertilization in Mammals. Edt. B. Bavister, J. Cummins and E. Roldan, Serono Symposia USA.: 285-296.

Pollard, J.W., Plante, C., King, W.A., Hansen, P.J., Betterigde, K.J. and Suarez, S.S., 1991. Fertilizing capacity of bovine sperm may be maintained by binding to oviductal epithelial cells. Biol. Reprod., 44: 102-107.

Rath, D., Weitze, K.F., Armbrecht, S. and Bielfeld, P., 1987. Einjarhige Erfahrungen mit einem Videomikrographiesystem (cellsoft) zur Spermabeurteilung. Deutsche Tierarzliche Wochenschrift, 94: 501-503.

Saacke, R.G., Nadir, S. and Nebel, R.L., 1994. Relationships of semen quality to sperm transport, fertilization and embryo quality in ruminants. Theriogenology, 41: 45-50.

Senger, P.L., 1994. The Estrus Detection Problem: New Concepts, Technologies, and Possibilities J. Dairy Sci., 77: 2745-2753.

Shannon, P., and Vishwanath, R., 1995. The effect of optimal and suboptimal concentrations of sperm in the fertility of fresh and frozen bovine semen and a theoretical model to explain the differences. Anim. Reprod. Sci., 39: 1-10.

Smith, T.T., Koyanagi, F. and Yanagimachi, R., 1987. Distribution and number of spermatozoa in the oviduct of the golden hamster after mating and artificial insemination. Biol. Reprod., 37: 225-234.

Suarez, S.S., Drost, M., Redfern, K. and Gottlieb, W., 1990. Sperm motility in the oviduct. In: Fertilization in mammals Edt. B.D. Bavister, J. Cummins and E.R.S. Roldan, Serono Symposia, Norwell, Mass., USA.: 111-124.

Uwland, J., 1984. Possibilities and limitations of semen evaluation for the prognosis of male fertility. In: The male in farm animal reproduction. Edt. A. Courot, Martinus Nijhoff Boston Ma. USA.: 269-289.

Van Os, J.L., de Vries, M.J., den Daas, J.H.G. and Lansbergen, L.M.T., Trends in spermcount of dairy bulls: accepted by Journal of Andrology.

Walker, W.L., Nebel, R.L. and Mcgilliard, M.L., 1996. Time to ovulation relative to mounting activity in dairy cattle. J. Dairy Sci., 79: 1555-1561.

Zirkin, B.R., Soucek, D.A., Chang, T.S.K., Perrault, S.D., 1985. In vitro and in vivo studies of mammalian sperm nuclear decondensation. Gamete Res., 11: 349-365.

Chapter 3

# EFFECTS OF AI SIRES AND TECHNICIANS ON NON-RETURN RATES IN THE NETHERLANDS

A. Reurink[1]

J.H.G. den Daas[1]

J.B.M. Wilmink[2]

[1] Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

[2] Royal Netherlands Cattle Syndicate

P.O. Box 454

6800 AL Arnhem

The Netherlands

Abstract

Fertility of AI bulls is evaluated by non return rates. Usually these NR rates are calculated as averages, uncorrected for effects of environments. A NR rate is measured on the cow, but is affected by the bull and by the technician who carried out the insemination. The objective of this paper was to develop a system to adjust NR rates of AI sires and insemination results of technicians for systematic environmental effects. Of 875 sires, who accounted in total for 1.8 million first inseminations during one year, NR rates were evaluated. NR rates were adjusted for the effects of herd-season, season of insemination, age of the inseminated cow and technician. The correlation between adjusted and unadjusted NR rates was 0.83 for the NR rate on 56 days. Large changes in NR rates after adjustment occurred by sires, who were frequently used on heifers. Technicians within AI stud differed more than 9% NR, corrected for other effects. It was concluded that evaluating systems of NR rates of AI sires and technicians should correct for the effects of herd-season, age of the inseminated cow, month of insemination, technicians and sires. In order to provide unbiased NR rates for management and research purposes.

(Keywords: AI; cattle; non-return; semen; technicians).

## Introduction

For many years the fertility of dairy bulls in artificial insemination (AI) has been evaluated by non-return (NR) rates (Oltenacu and Foote, 1976). A single NR measurement (0,1) is measured on the cow, but is affected by the fertility qualities of the cow herself and of the bull she is inseminated by, and also by the insemination qualities of the technicians who carried out the insemination. The most frequently used measure of male fertility is the rate of NR within some period of time (28 of 56 days) after first insemination (Vinson, 1982). NR rates overestimate the true rate of conception (Oltenacu and Foote, 1976). Until now,

however, no other system to monitor male fertility has been introduced on a large scale.

AI studs use NR rates in their management to evaluate the results of sires and of technicians. Selection on NR rates, to improve the fertility status of the herd, will have a positive efffect on farmers' income (Everett, 1975).

NR rates of AI sires are normally computed as averages, uncorrected for environmental effects. Recent research has shown that NR rates based on uncorrected averages are biased (Chavaz and Weber, 1985; Taylor et al., 1985; Distl et al., 1986; Jansen and Lagerweij, 1987). Ranking of sires based on uncorrected averages is influenced by systematic environmental factors, such as AI stud, herd-year-season (HYS), technician, month of insemination and age of the cow (Taylor et al., 1985; Jansen and Lagerweij, 1987). Sire effects for NR rates therefore should be estimated by mixed model methodology.

After adjustment for some systematic environmental factors, such as AI stud, herd-year-season etc., sire effects for NR rates are still a combination of some permanent environmental and genetic effects. Examples of permanent environmental effects include methods of diluting, freezing and thawing the semen by the AI centers, which are not always the same for every bull. Semen of heavily used sires may be diluted more than semen of other sires. It is possible also that these effects change with time.

For these reasons the NR rates of sires may be useful in fertility management, i.e. to monitor the effects of freezing, diluting, thawing, etc. of semen under field conditions. In additon, NR rates for the technicians, adjusted for other systematic influences such as HYS, sires, AI stud, etc., maybe useful to instruct technicians.

The purpose of this study was to develop a system to monitor the NR rates of sires and of technicians.

## Material

Data consisted of nearly 2 million first inseminations, carried out in the Netherlands by AI sires during the period from 1 September 1984 through 31 August 1985. All first inseminations were checked for a second insemination within a period of 28 or 56 days. This

resulted in two variables: non-return to day 28 (NR28) and non-return to day 56 (NR56). NR28 and NR56 were scored 1 if no second insemination occurred, 0 otherwise. If the interval between the two inseminations exceeded 200 days, the second insemination was considered as a first insemination.

Each first insemination was required to meet the following criteria: (1) carried out in a herd with at least 20 inseminations; (2) carried out by a technician with at least 1,000 inseminations; (3) from a sire with at least 350 inseminations.

After editing, the data set consisted of 1,843,888 first inseminations from 875 sires carried out by 599 technicians in 33,414 herds. To evaluate the results, average NR rates without correction for systematic environmental effects were compared with those NR rates after correction for these effects. The traits analysed were uncorrected (u) and corrected (c) NR rates within 28 or 56 days after first insemination. They are identified as $NR28_u$, $NR56_u$, $NR28_c$, and $NR56_c$.

## Method

### Analysis

The mixed model used for analysis considered the following effects.

(1) Herd-season. Within each herd, two seasons were created: 1 September through 31 March and 1 April through 31 August.

(2) Age × season. Because of the possible interaction between age of the inseminated animal and season of insemination (Jansen and Lagerweij, 1987), 36 classes were defined for age × season. Six classes were defined for age (<25 months, 25-36, 37-48, 49-60, 61-84 and >84 months) and six for season (Jan.-Feb., Mar.-Apr., May-Jun., Jul.-Aug., Sep.-Oct. and Nov.-Dec.).

(3) Technician. In the Netherlands, artificial insemination is carried out by technicians employed by the AI studs. Each technician works only for one AI stud. Therefore technicians were nested within AI studs. Dairy farmers are only member of one AI stud. One dairy farmer will only be supplied by technicians of one AI stud. As a result, in this

analysis AI stud effects were removed by technicians.

(4) Sire.

(5) Genetic Group. Sires in the data showed a wide variety in breed and genetic descendence. Therefore eight genetic groups were created (Table 1).

*Table 1. The genetic group of sires defined in this study.*

| Group | Breed[1] | No. of sires | Total inseminations |
|---|---|---|---|
| 1 | 100% MRY | 210 | 524 778 |
| 2 | Crossbred MRYXHF (red and white) | 43 | 25 567 |
| 3 | 100% DF | 70 | 95 578 |
| 4 | 25-50% HF (black and white) | 28 | 116 274 |
| 5 | 75% HF | 105 | 91 040 |
| 6 | 87.5% HF | 93 | 130 001 |
| 7 | 100% HF | 280 | 792 312 |
| 8 | Remaining breeds | 47 | 68 338 |

[1]*MRY = Meuse-Rhine-Issel, HF = Holstein-Friesian, DF = Dutch-Friesian*

The following general mixed model was applied:

$$Y = X\beta + Zu + e$$

where: Y = $NR28_c$ or $NR56_c$; β = vector with fixed effects; u = vector with random sire effects; e = error. X and Z are known design matrices relating fixed effects and random sire effects to the observations. $NR28_c$ and $NR56_c$ were calculated on the data as averages of the NR (0.1) observations. Y, u and e were considered random with the following expectations and variances:

$$E\begin{bmatrix} Y \\ u \\ e \end{bmatrix} = \begin{bmatrix} X\beta \\ 0 \\ 0 \end{bmatrix}$$

$$V\begin{bmatrix} Y \\ u \\ e \end{bmatrix} = \begin{bmatrix} ZZ^{1}\sigma_u^2 + I\sigma_e^2 & ZZ^{1}\sigma_u^2 & I\sigma_e^2 \\ & I\sigma_u^2 & 0 \\ & symm. & \\ & & I\sigma_e^2 \end{bmatrix}$$

Solutions for β and u were obtained by solving the MME equations:

$$\begin{bmatrix} X'X & X'Z \\ symm. & Z'Z+kI \end{bmatrix}\begin{bmatrix} \beta \\ u \end{bmatrix} = \begin{bmatrix} X'Y \\ Z'Y \end{bmatrix}$$

Where the ratio $k\left(\sigma_e^2 / \sigma_u^2\right)$ was assumed to be 225, corresponding to a heritability of about 0.018. When solving the normal equations, equations for herd-seasons were absorbed.

Non-return as a binomial trait

The Y variables, NR28 and NR56 (uncorrected and corrected), has a binomial distribution. For binomial traits, analysis with log-linear or non-linear models give optimal results (Meijering, 1984). Meijering and Gianola (1985) compared Best Linear Unbiased Predicition (BLUP) models with non-linear models to estimate breeding values for bulls for binomial traits. Non-linear models gave better estimates, especially with relatively high heritabilities (>0.20) and low frequencies (<0.05) in one of the binomial classes. Because of the low $h^2$ and the relative high frequency in one of the binomial classes of these traits, NR28 and NR56 were analysed with linear models.

Results and discussion

The effect of age and month of insemination

Least-squares constants for $NR28_c$ and $NR56_c$ by age and season classes are given in

Table 2. Evident interaction exists between age and season, this interaction is significant. Lowest NR rates were found in the winter season (Nov.-Feb.) and highest NR rates in late spring season (May-Jun.), which agrees with Jansen and Lagerwij (1987). In American and Canadian research % NR was lowest in winter, and highest in late summer and fall (Murray et al., 1983; Taylor et al., 1985). Opposite results, however, were reported from Israel (Ron et al., 1984). The existence of a seasonal pattern in % NR is clear.

Figure 1 shows the pattern of $NR56_c$ per age class. NR rates of yearlings (Age group 1) averaged higher than NR rates of older cows, which agrees with results of Oltenacu and Foote (1976), Ron et al. (1984), Taylor et al. (1985) and Jansen and Lagerweij (1987). Yearlings form a different population than older cows, probably because there is no stress owing to production, which influences the fertility status (Oltenacu and Foote, 1976). The different seasonal pattern between yearlings and older cows (Figure 1) suggests these reasons to be more important in the spring and summer seasons. This may be caused by the climatic circumstances in the Netherlands. In the winter season, yearlings are kept indoors and are therefore mostly bred artificially. In the spring and summer seasons, it is more common to use natural service on yearlings because they can be kept outside together with a farm bull. Repeat heifers are more likely to be bred naturally. These natural breedings could not be included in the analysis, because this information is not recorded in the AI data system and was therefore not available.

Also in Figure 1, the seasonal patterns in % NR were quite similar (only differences in level), except for cows in first lactation (Age class 2). In Jul.-Aug. NR rates decreased with increasing age class number. In November through April, % NR for first parity cows (Age class 2) were lower than for third parity cows (Age class 4). This phenomenon may be due to energy deficiencies of first parity cows in the winter and/or by inseminations with semen of test bulls. In the Netherlands, young bulls are mated at random to first lactation cows.

Effect of technician

Variation was found in the results of the technicians. The standard deviation of corrected NR rates in NR56 of technicians was 2.1% NR. Most AI studs have a difference

greater than 9% NR for top and bottom efficiency technician. Similar results were reported by Chavax and Weber (1985) and by Jansen and Lagerweij (1987). Despite continuous guidance and training, large differences between technicians still exist. Farmers and AI studs should be aware of these differences because lower NR rates have negative effects on farmers' income (Everett, 1975).

Differences between breeds

Table 3 shows the estimates for the different genetic groups. Only small differences in NR existed between groups. Purebred Dutch-Friesian (100% DF) had a higher NR rate, whereas purebred Holstein-Friesian (100% HG), HF crosses and Meuse-Rhine-Issel (100% MRY) sires had lower NR rates.

Differences between genetic groups cannot be directly interpreted as genetic differences, because groups were confounded with other effects. For example, sires of different groups (breeds) are not used in comparable groups of cows (breeds). Because of *differences between breeds and differences in breeding decisions taken by farmers within* these breeds, some breeds may have a different NR rate level.

A farmer is more patient in getting his high producing cows pregnant. Every time a cow is on heat, the farmer decides whether he will inseminate her or not. If not, the cow will be culled. For low producing cows, he might already with a first return, decide not to inseminate her. This means that low producing cows, only look pregnant (are non return). High producers therefore have a greater probability of being bred repeatedly. Bulls with a low breeding value for production will be used on lower producing cows. When these lower producing cows are not randomly distributed among sires, this may cause upward bias on NR rates of service sires, with lower breeding values for production traits.

*Table 2. Least squares constants for $NR28_c$[1] and $NR56_c$[1] by age and season.*

| Age (months) | Season | $NR28_c$ | $NR56_c$ |
|---|---|---|---|
| <25 | Jan.-Feb. | 2.19 | 3.45 |
| | Mar.-Apr. | 6.94 | 10.31 |
| | May-Jun. | 5.85 | 8.52 |
| | Jul.-Aug. | 6.91 | 9.92 |
| | Sep.-Oct. | 4.39 | 5.24 |
| | Nov.-Dec. | 1.83 | 2.28 |
| 25-36 | Jan.-Feb. | -1.55 | -2.26 |
| | Mar.-Apr. | -0.11 | -1.20 |
| | May-Jun. | 0.94 | 1.42 |
| | Jul.-Aug. | 0.64 | 1.77 |
| | Sep.-Oct. | -0.07 | 0.28 |
| | Nov.-Dec. | -2.13 | -2.84 |
| 37-48 | Jan.-Feb. | -0.79 | -0.50 |
| | Mar.-Apr. | 0.14 | -0.20 |
| | May-Jun. | 0.67 | 1.39 |
| | Jul.-Aug. | -0.02 | 0.46 |
| | Sep.-Oct. | -0.25 | 0.44 |
| | Nov.-Dec. | -1.22 | -1.72 |
| 49-60 | Jan.-Feb. | -0.51 | -0.63 |
| | Mar.-Apr. | -0.11 | -0.64 |
| | May-Jun. | 0.03 | -.71 |
| | Jul.-Aug. | -0.55 | -0.25 |
| | Sep.-Oct. | -0.03 | -0.29 |
| | Nov.-Dec. | -1.26 | -1.79 |
| 61-84 | Jan.-Feb. | -1.01 | -1.46 |
| | Mar.-Apr. | -0.90 | -2.15 |
| | May-Jun. | -0.27 | 0.02 |
| | Jul.-Aug. | -1.59 | -1.87 |
| | Sep.-Oct. | -1.15 | -1.64 |
| | Nov.-Dec. | -2.03 | -3.20 |
| >84 | Jan.-Feb. | -2.60 | -3.62 |
| | Mar.-Apr. | -2.20 | -4.24 |
| | May-Jun. | -1.75 | -2.79 |
| | Jul.-Aug. | -2.81 | -4.32 |
| | Sep.-Oct. | -1.91 | -3.40 |
| | Nov.-Dec. | -3.35 | -5.28 |

[1] *Adjusted for herd-season, technician and sire*

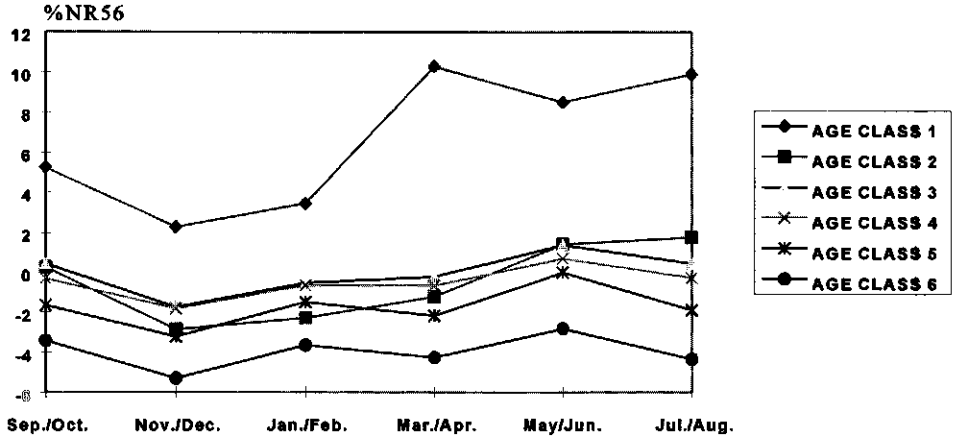



*Figure 1. Pattern of NR56 by age class.*

*Table 3. Least squares constants for $NR28_c$ and $NR56_c$ for different genetic groups.*

| Group[1] | $NR28_c$ | $NR56_c$ |
|---|---|---|
| 100% MRY | -0.47 | -0.48 |
| MRY x HG | 0.53 | 1.03 |
| 100% DF | 0.66 | 0.86 |
| 25-50% HF | 0.16 | 0.21 |
| 75% HF | -0.36 | -0.52 |
| 87.5% HF | -0.37 | -0.91 |
| 100% HF | -0.27 | -0.50 |
| Remaining breeds | 0.13 | 0.31 |

[1]*MRY = Meuse-Rhine-Issel, HF = Holstein-Friesian, DF = Dutch-Friesian*

Non-return rates of sires

Table 4 shows means, standard deviations, minima and maxima for the NR rates of the sires. As expected, the standard deviations decreased after correction.

Product-moment correlations were calculated between the different measures of NR rate (Table 5). The correlation between the uncorrected and corrected NR rates was 0.85 for NR28 and 0.83 for NR56. After correction for systematic environmental effects, ranking of sires changed. The average absolute change in NR rate after correction was 2%. The ranking

of sires based on corrected NR28 and NR56 was different (correlation 0.90), which indicates differences between these two traits.

To illustrate the effect of correction, $NR56_u$ and $NR56_c$ were compared, for the 20 most heavily used sires (Table 6). Despite the extremely large numbers of inseminations some sires (Sire 8, 15 and 17) changed 3-4% after correction. This is a change of more than one standard deviation, which is considerably high. These three sires were frequently used on yearlings, because they cause fewer calving difficulties. This illustrates the need to correct NR rates for age of the cow.

In sampling programs, semen of young sires is mostly used in a period of 3 months. Because of the seasonal pattern, uncorrected NR rates of bulls sampled in the summer period might be biased upwards compared to the results of bulls sampled in the winter period.

*Table 4. Means, standard deviation, minimums and maximums for NR rates of sires.*

| Trait | Mean | Standard deviation | Minimum | Maximum |
|---|---|---|---|---|
| $NR28_u$[1] | 80.84 | 3.02 | 66.47 | 89.71 |
| $NR56_u$ | 71.95 | 3.86 | 56.59 | 82.31 |
| $NR28_c$ | 0.00 | 1.84 | -9.51 | 6.27 |
| $NR56_c$ | 0.00 | 2.28 | -9.95 | 6.90 |

*[1] $NR28_u$, $NR56_u$ are uncorrected NR averages; $NR28_c$, $NR56_c$ are adjusted for herd-seasons, month of insemination and technicians.*

*Table 5. Product-moment correlation coefficients between the different measures of NR rate.*

| | $NR28_u$ | $NR28_c$ | $NR56_u$ | $NR56_c$ |
|---|---|---|---|---|
| $NR28_u$ | 1.00 | 0.85 | 0.93 | 0.78 |
| $NR28_c$ | | 1.00 | 0.75 | 0.90 |
| $NR56_u$ | | | 1.00 | 0.83 |

*Table 6. NR rates 56 days after first insemination, Uncorrected ($NR56_u$) and corrected ($NR56_c$), for the 20 most heavily used sires.*

| Sire | $NR56_u$[1] | $NR56_c$[1] | *n* first inseminations |
|---|---|---|---|
| 1 | -4 | -3 | 86 494 |
| 2 | -1 | -1 | 76 668 |
| 3 | -3 | -2 | 67 506 |
| 4 | -2 | -1 | 53 178 |
| 5 | -1 | -1 | 46 916 |
| 6 | 2 | 0 | 46 754 |
| 7 | -1 | -2 | 46 204 |
| 8 | 5 | 2 | 37 801 |
| 9 | 0 | 0 | 36 095 |
| 10 | 0 | 1 | 34 351 |
| 11 | -1 | -1 | 34 219 |
| 12 | -2 | -1 | 29 390 |
| 13 | 1 | 2 | 26 901 |
| 14 | -1 | -1 | 26 779 |
| 15 | 5 | 2 | 25 309 |
| 16 | -2 | -1 | 22 609 |
| 17 | -1 | -5 | 22 400 |
| 18 | -1 | 0 | 18 459 |
| 19 | 0 | 2 | 18 056 |
| 20 | 3 | 2 | 15 866 |

[1] *Figures are expressed as deviations from the mean. When mean is 72% NR after 56 days, Bull 1 had 68% NR based on uncorrected data, and 69% NR based on corrected data.*

Monitoring system

The results of this study demonstrate the need for an evaluating system for NR rates of AI sires, which are corrected for systematic effects. Uncorrected averages are influenced by several environmental factors and give a biased ranking of the sires. This is especially true for sires with low numbers of inseminations or with a relatively high percent of inseminations on heifers. In the practical situation, by management support to AI studs and dairy farmers, a NR monitoring system should be correct for the environmental effects used in this study. Because of the small sire variance related to the total variance of NR, sire evaluations should

be based on at least 500 first inseminations, to obtain repeatabilities of more than 70%.

Based on the results of this study an NR monitoring system will be developed to support management of the AI associations and the farmers in the Netherlands. In this system, corrected NR rates for technicians and sires are calculated every 2 months, based on the data of the last half year.

In addition, corrected NR rates for technicians are calculated also on the first inseminations of the last 2 months. These NR rates can be compared with the NR rates for technicians based on first inseminations during the last half year. These NR rates are useful for instructing the technicians. Corrected NR rates for sires can be used for optimising the treatment of semen of heavily used sires.

## Conclusions

Non-return rates of AI sires are influenced by the effects of herd-season, age of the inseminated cow, month of insemination and technician. Uncorrected averages of NR rates give biased information. Therefore in evaluating systems of NR rates of AI sires and technicians a correction needs to be carried out for systematic environmental effects.

## References

Chavax, J. and Weber, R., 1985. Eine neue Auswertungsmethode für die Non Return Rate. Mitteilungen des Schweizerischen Verbandes für Künstliche Besamung und der Interessengemeinschaft Schweiz. Besamungszüchter, 23: 58-59.

Distl, O., Krauslich, H. and Averdunk G., 1986. Zuchtwerdschätzung auf männliche Fruchtbarkeit. Tierzuecht., pp 241-243.

Everett, R.W., 1975. Income over investment in semen. J. Dairy Sci., 58: 1717-1722.

Jansen, J. and Lagerweij, G.W., 1987. Adjustment of Non-Return rates for AI technicians and dairy bulls. Livest. Prod. Sci., 16: 363-372.

Meijering, A., 1984. dystocia and still birth in cattle - a review of causes, relations and implications. Livest. Prod. Sci., 11: 143-177.

Meijering, A. and Gianola, D., 1985. Linear versus non linear methods of sire evaluations for categorical traits; a simulations study. Genet. Sel. Evol., 17: 115-132.

Murray, B.B., Schaeffer, L.R. and Burnside, E.B., 1983. Heritability of Non Return rate of Canadian Holstein Friesian bulls. Can. J. Anim. Sci., 63: 59.

Oltenacu, E.A.B. and Foote, R.H., 1976. Monotoring fertiltiy of AI-programs: Can non return rate do the job? Proc. 4th Tech. Conf. Artif. Insem. Reprod., NAAB p. 61.

Ron, M., Bar-Anan, R. and Wiggans, G.R., 1984. Factors affecting conception rate of Israeli Holstein cattle. J. Dairy Sci., 67: 854-860.

Taylor, J.F., Everett, R.W. and Bean, B., 1985. Systematic environmental, direct and service sire effects on conception rate in artificially inseminated Holstein cows. J. Dairy Sci., 68: 3004-3022.

Vinson, W.E., 1982. Selection for secondary traits in dairy cattle. Proceedings of the Second World Congress on Genetics Applied to Livestock Production, 5: 370-386.

Chapter 4

# MULTIPHASIC ANALYSIS OF REPRODUCTIVE EFFICIENCY OF DAIRY BULLS

M. Grossman[1]

W.J. Koops[2]

J.H.G. den Daas[3]

[1] Department of Animal Sciences

University of Illinois

Urbana 61801

[2] Department of Animal Husbandry

Agricultural University

P.O. Box 338

6700 AH Wageningen

The Netherlands

[3] Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

Abstract

Reproductive efficiency of dairy bulls is usually measured by non-return rate (NR). NR is a compound trait that is a result of two events, conception and gestation, that leads to calving. NR rates can be used to derive more elementary biological measures for reproductive efficiency, such as conception rate and calving rate, which separately might be more reliable than NR rate itself to evaluate the fertility of a bull or the performance of an AI technician.

The challenge of this study was to examine the decline in NR rate in light of the theory of multiphasic analysis. A multiphasic logistic function was developed to model decline in non return rate by estimating conception rate, calving rate, and characteristics of the first two estrous cycles. The model is illustrated with data on daily NR rates to 120 d.

From the proportion of cows that conceived but failed to complete gestation because of postsignal embryonic death, the model estimates conception rate and calving rate. From the proportion of cows that failed to conceive or that conceived but failed to complete gestation because of presignal embryonic death, the model estimates the proportion of returns, or probability of detecting estrus, duration of NR, and time of maximum decline in NR rate for the first two cycles. Using the proposed model, conception rate and calving rate estimated from daily NR rates might be more reliable for evaluation of performance of an AI technician and fertility of a bull than NR rates at arbitrarily chosen days after insemination.

(Key words: calving rate, conception rate, mathematical model, non-return rate)

## Introduction

Reproductive efficiency of dairy bulls is usually measured by NR rate, the proportion of cows that were inseminated and did not return for another service within a specified time, usually 60 to 90 d. NR is a compound trait that is a result of conception at or near time of

insemination, followed by gestation or, jointly, gestation after insemination. Conception at insemination depends on, among other factors, the availability of ova and their quality and on the population of spermatozoa inseminated and its characteristics. Gestation after insemination depends on conception, maternal and paternal contributions to developmental potential of the conceptus (den Daas, 1992). NR rates can be used to derive more elementary biological measures for reproductive efficiency, such as conception rate and calving rate, which separately might be more reliable than the NR rate itself to evaluate the fertility of a bull or performance of an AI technician (Koops et al., 1995).

The relationship of NR rate and time after insemination is nonlinear (Erb and Flerchinger, 1954; Salisbury and VanDemark, 1961). The greatest decrease in NR rate occurs during the first 90 d after inseminations; only a slight decrease occurs from 90 to 180 d (Foote and Bratton, 1952; Salisbury et al., 1952). This relationship can be described by a nonlinear function that relates increase in time after insemination to decline in NR rate. NR rates at 28, 56, and 84 d after insemination have been described by a logistic function, based on the logit and parameterized to estimate conception rate and calving rate (Koops et al., 1995).

Cows return to service after insemination for various reasons, including failure of ovulation, resulting in anovulatory estrus, and failure of conception (Lasley, 1962). Not all cows that return, however, fail to ovulate or to conceive. Some cows simply return during normal pregnancy (Lasley, 1962); others return because of failure of implantation, embryonic of early fetal death, or abortion (Salisbury and VanDemark, 1961). A large proportion of returns to service, however, is due to failure or conception (about 10%) and early embryonic death (about 20%) ( Linares, 1982; Sreenan and Diskin, 1986; King and Thatcher, 1993).

Two types of embryonic death have been distinguished. One type occurs when the conceptus does not survive long enough to provide the signal for maternal recognition of pregnancy, dying before about 15 to 17 d after insemination. This type, which we refer to as presignal embryonic death, results in the cow returning to estrus at the next regular 21-d cycle. The other type occurs when the conceptus long enough to provide signal for maternal recognition of pregnancy, dying after about 15 to 17 d after insemination. This type, which we

refer to as postsignal embryonic death, results in the cow being precluded from returning to estrus at the next regular cycle; thus the interval between inseminations is prolonged beyond normal cycle length (King and Thatcher, 1993).

Inspection of Figure 2 of Erb and Flerchinger (1954), using daily NR rates to 180 d, illustrates that decline in NR is nonlinear with time after insemination and that decline follows a cyclic, or phasic, pattern; phases correspond to the estrous cycles of cows. We concluded, therefore, that decline in NR rate after insemination can be described by multiphasic function, which would give detailed information about decline in NR rate, Multiphasic functions have been used to analyze growth of mice (Koops, 1986; Koops et al., 1987) and chickens (Grossman and Koops, 1988) and to analyze lactation of dairy cows (Grossman and Koops, 1988).

The challenge of this study was to examine decline in NR rate in light of the theory of multiphasic analysis. A multiphasic logistic function was developed to model decline in NR rate by estimating conception rate, calving rate, and characteristics of cycles. The model is illustrated with data obtained by inspection from Figure 2 of Erb and Flerchinger (1954).

## Materials and methods

### Theory

The development of the multiphasic model for NR uses the following notation: $p_T$ = Probability of non return by time t after insemination, or non return rate; cpr = probability of conception at time of insemination, or conception rate; and cvr = probability of completing gestation after insemination, or calving rate. From figure 1, total probability of unity for non-return rate at insemination can be partitioned as

$$1 = cvr + (cpr - cvr) + (1 - cpr). \qquad [1]$$

To model NR, we suppose that cows can be grouped on the basis of one of three conditions: $C_1$, the cow conceived at insemination and completed gestation, with probability

$P(C_1)$ = cvr; $C_2$, the cow conceived at insemination but failed to complete gestation because of postsignal embryonic death, with probability $P(C_2)$ = cpr - cvr; and $C_3$, the cow failed to conceive or conceived but failed to complete gestation because of presignal embryonic death, with probability $P(C_3)$ = 1 - cpr.

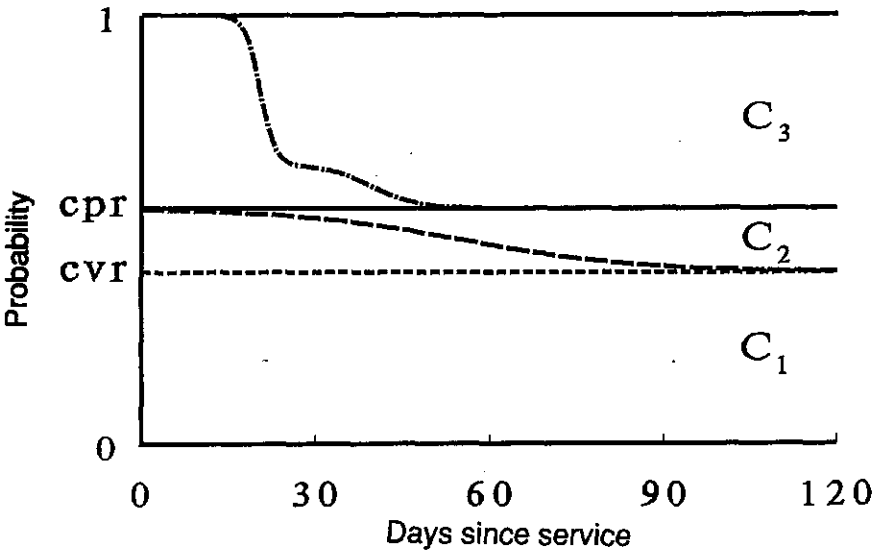



*Figure 1. Course of probability of non-return for conditions: $C_1$ (-----), completed gestation with probability (cvr); $C_2$ (_ _ _ ), conceived but failed to complete gestation with probability (cpr-cvr); and $C_3$ (_ . _ ) failed to conceive or conceived but failed to complete gestation with probability (1- cpr).*

The probability of non return by time t after insemination can be written as:

$$\begin{aligned} Pt &= \sum_{j=1}^{3} P(\text{non-return by time } t \text{ and condition } j) \\ &= \sum_{j=1}^{3} P(\text{non-return by } t / C_j) P(C_j) \\ &= \sum_{j=1}^{3} p_{t/C_j} \quad P(C_j) \end{aligned} \qquad [2]$$

Where $p_{t/C_j}$ is probability of NR by time t given condition j. Each of the three conditional probabilities is examined separately.

*Conception and Completed Gestation.* All cows that conceived and completed gestation ($C_1$) did not return and did calve. For cows that conceived and completed gestation, therefore, the probability of NR by time t is unity (figure 1):

$$p_{t/C_1} = 1 \qquad [3]$$

*Conception but Failure to Complete Gestation.* For cows that conceived but failed to complete gestation because of postsignal embryonic death ($C_2$), the probability of NR by time t has a negative relationship with time (Figure 1), which can be expressed as a logistic function (Koops et al., 1995):

$$p_{t/C_2} = 1 - \left( \frac{1}{1 + e^{-\frac{t-c}{d}}} \right) \qquad [4]$$

where c is time of maximum decline, or a measure of the mean of the distribution; 4 d is a measure of duration of NR, or a measure of the standard deviation and includes about 96 % of the distribution (Koops and Grossman, 1991); and e is the base of the natural logarithms.

Such a function has the property of constraining values for NR rate ($p_{t/C_2}$) within an upper asymptote of unity for t < c and a lower asymptote of zero at t > c. Because of this property, $p_{t/C_2}$ is not equal to unity at t = 0, which is the expected value for the probability of NR at time of insemination, but may be close to unity, depending on values of parameters c and d.

*Nonconception and Failure to Complete Gestation.* For cows that failed to conceive or that conceived but failed to complete gestation because of presignal embryonic death ($C_3$), the probability of NR by time t has a negative relationship with time (Figure 1), which can be expressed as a sum of logistic functions to characterize multiple estrous cycles, or phases:

$$p_{t/C_3} = 1 - \left[ \sum_{i=1}^{n} \frac{h_1}{1 + e^{-\frac{t-c_1}{d_1}}} \right] \qquad [5]$$

where, for each phase i, $h_i$ is proportion of cows in $C_3$ returning during that phase, subject to the constraint that $\sum_1^n h_i = 1$, so that $h_n = 1 - \sum_1^{n-1} h_i$; $c_i$ is time of maximum decline of non-returns during that phase; and $4d_i$ is a measure of duration of NR and includes about 96% of that phase (Koops and Grossman, 1991).

Finally, the probability of NR by time t can be modeled by substituting Equations [3] through [5] into Equation [2] to yield.

$$p_t = cvr + \left[1 - \left(\frac{1}{1 + e^{-\frac{t-c}{d}}}\right)\right](cpr - cvr) + \left[1 - \left(\sum_{i=1}^{n} \frac{h_j}{1 + e^{-\frac{t-c_1}{d}}}\right)\right](1 - cpr) \qquad [6]$$

Data

To illustrate the model in Equation [6], data were taken from Figure 2 of Erb and Flerchinger (1954), using a grid over enlarged smooth graphs, and were rounded to the nearest .005. Although NR were presented for each day, from d 0 through d 180 after insemination, we used data only from every 3rd d through d 120, for a total of 41 observations. Data were limited to 120 d after insemination was less than 1% of the total decline [Table 2 of Erb and Flerchinger (1954)]. Based on NR rates at 180 d, 40 bulls had been divided into four fertility groups. For the purpose of illustration, we used only the lowest fertility group (6,210 inseminations) and highest fertility group (17,164 inseminations).

Estimation of parameters

For each of the two fertility groups, 41 observations on NR rate were fitted by Equation [6]. Model parameters were estimated by nonlinear regression using an adaptive nonlinear least squares algorithm (Sherrod, 1994). A default value of $1 \times 10^{-10}$ was used for the tolerance factor, which specifies the convergence criterion for the iterative estimation

procedure.

We assumed that it was not possible to detect more than two phases. If conception rate defined as fertilization rate plus presignal embryonic death, is about .85 (Sreenan and Diskin, 1986), then probability of failure to conceive is about .15. One would expect, therefore, that 15% of all cows would return to estrus after insemination. If the probability of detecting estrus was 1, then those cows would be presented for service in the first cycle. The probability of detecting estrus, however, is at most .90 (Rasbech, 1986), so that probability of failure to detect estrus is at least .10. One would expect, therefore, that about .10 of 15% or 1.5%, of cows would return during the second cycle because of failure to detect estrus during the first cycle. Thus, percentage of cows returning during third or later cycles due to failure to detect estrus at earlier cycles, would be negligible.

For Equation [6], therefore, we assumed a diphasic (n=2) model, with $h_1 + h_2 = 1$, Which means that $h_1$ is the proportion of cows returning during cycle 1, and $h_2 = 1 - h_1$ is the proportion of cows returning during cycle 2 or later. Goodness of fit of the model was measured by residual standard error.

## Results

Estimates and standard errors for cvr, cpr, time of maximum decline in NR rate, duration of NR, proportion returning during cycle 1, time of maximum decline in cycle 1, duration of cycle 1, time of maximum decline in cycle 2, duration of cycle 2, and residual standard error by low and high fertility group are in Table 1. Each estimate was statistically significant ($P \leq .00001$).

The probability of completing gestation, or cvr, was .526 for the low fertility group and .670 for the high group (Figure 2). These results are consistent with 180-d (the latest observed) NR rates for the experiment of Erb and Flerchinger (Table 2 (1954)), which was .533 for the low fertility group and .670 for the high group.

*Table 1. Estimates (Est) and standard errors for calving rate (cvr), conception rate (cpr), time of maximum decline in NR rate (c), duration of NR (4d), proportion returning during cycle 1 ($h_1$), time of maximum decline in cycle 1 ($c_1$), duration of cycle 1 ($4d_1$), time of maximum decline during cycle 2 ($c_2$), duration of cycle 2 ($4d_2$), and residual standard error, by low and high fertility group.*

| Parameter | Fertility Group | | | |
|---|---|---|---|---|
| | Low | | High | |
| | Est | SE | Est | SE |
| cvr | .526 | .0019 | .679 | .0013 |
| cpr | .636 | .0140 | .762 | .0100 |
| c | 55.7 | 3.07 | 55.0 | 2.76 |
| 4d | 59.9 | 4.93 | 59.9 | 4.46 |
| h1 | .77 | .029 | .75 | .032 |
| c1 | 20.6 | .08 | 19.9 | .08 |
| 4d1 | 5.1 | .32 | 4.2 | .23 |
| C2 | 39.7 | .73 | 39.2 | .71 |
| 4d2 | 15.8 | 3.23 | 16.9 | 3.28 |
| Residual | ... | .00364 | ... | .00242 |

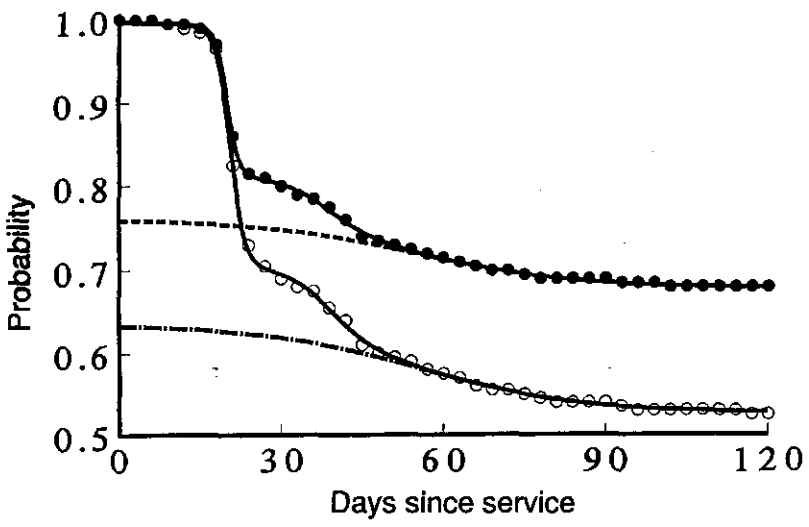



*Figure 2. Actual NR rates for low fertility group ( ○ ) and high fertility group ( ● ), by days since service; predicted probability of NR (___ ) for low fertility group and high fertility group; and predicted probability of NR for low fertility group (_ . _ ) and high fertility group (----), for cows that conceived but failed to complete gestation because of postsignal embryonic death.*

Probability of conception, or cpr, was .636 for low fertility group and .762 for the high group. Time of maximum decline in NR rate was about 55 d for the two fertility groups, which means that, because of properties of the logistic function, one-half of the decline was achieved by 55 d. An earlier time of maximum decline permits an earlier estimate of cvr, for a given duration. Duration of NR was 60 d for the two fertility groups, which means that about 96% of non returns occurred within 60 d.

For cows that failed to conceive or that conceived but failed to complete gestation because of presignal embryonic death, the proportion of cows that returned during cycle 1 was about .76. Under this diphasic model, therefore, 23% of cows returned during cycle 2 or later. For the two fertility groups, maximum decline was at about 20 d for cycle 1 and at about 39 d for cycle 2. Duration of cycle 1 was 5.1 d for the low fertility group and 4.2 d for the high group, and duration of cycle 2 was 15.8 d for the low group and about 16.9 d for the high group. Thus, about 96% of NR occured within about 5 d during cycle 1, and about 96% occured within about 16 d during cycle 2. The model fit data for the high fertility group best; the value for residual standard error was $2.42 \times 10^{-3}$, which was about two-thirds that for the low group.

## Discussion

The AI organizations use NR rates to evaluate performance of bulls and technicians. Koops et al. (Koops et al., 1995) suggested that an estimate for conception rate is a more reliable measure to evaluate performance than NR rate at some specified times after insemination. In this study, cpr from NR data on the high fertility group was about .76, which is lower than the .89 of Screenan and Diskin (Sreenan and Diskin, 1986), which might overestimate conception rate because of the possibility of excluding abnormal cows.

The .76 is also lower than the .90 estimated by Koops et al. (Koops et al., 1995) from data on NR by 28, 56, and 84 d. In addition to abvious dissimilarities in the data files because of differences in their source, one explanation for the difference between .76 en .90 is that .90 is based on a model that assumes that NR rate by 28 d includes cows that conceived but

failed to complete gestation only because of postsignal embryonic death.

Figure 2 shows the difference best. NR rates for 28 d are above predictions for cows that conceived but failed to complete gestation because of postsignal embryonic death, NR rates for 56 d are close to predicter lines, and NR rates for 84 d are on predicted lines. If data includes non-return rates before about 50 d, then the model of Koops et al. (Koops et al., 1995) tends to overestimate conception rate.

The probability to detect estrus at cycle 1 was about .76, which means that 76% of cows returned to service at cycle 1, which is consistent with results of Rasbech (Rasbech, 1986). This value of .76 is probably an underestimate, because cows that are detected in estrus are not always offered for service.

To obtain satisfactory estimates of parameters in Equation [6], it is important to determine the time after insemination necessary to achieve a specific decline to completed gestation. To determine the day by which, say, 95% of decline to completed gestation was achieved, the value for [1 - .95(1 - cvr)] was computed, substituting estimates for cvr from Table 1, which yielded .55 for the low fertility group and .70 for the high fertility group. By inspection of predicted values for Equation [6], we determined that, for each fertility group, 95% of the decline to completed gestation was achieved by about 75 d after insemination . Table 2 of Erb and Flerchinger (Erb and Flerchinger, 1954) shows that roughly 95% of total decline in NR rate by 180 d was achieved by about 60d after insemination. Koops et al. (Koops, et al., 1995) reported that about 95% of total decline in NR rate was achieved by approximately 85 d after insemination. To obtain satisfactory estimates of parameters in Equation [6], therefore, about 60 to 90 d are required to achieve about 95% of the decline. The robustness of the parameters in the model to differences in time after insemination requires further study.

## Conclusions

The AI organisations use NR rate to evaluate fertility of a bull or performance of a technician. Through the relationship of daily NR rate and time after insemination, the

mathematical function presented here is a model for reproductive efficiency of bulls. From the proportion of cows that conceived but failed to complete gestation because of postsignal embryonic death, the model estimates cpr and cvr. From the proportion of cows that failed to conceive or that conceived but failed to complete gestation because of presignal enbryonic death, the model estimates for each cycle the proportion of returns, or the probability of detecting estrus, duration of NR, and time of maximum decline. Using the proposed model, cpr and cvr estimated from daily NR rates might be more reliable for evaluation of performance of an AI technician and fertility of a bull than NR rates at arbitrarily chosen days after insemination.

Acknowledgments

The authors thank Lucia Lansbergen and Janneke van Wagtendonk, Holland Genetics, for their cooperation and helpful discussions in this research. The manuscript benefitted from a review by J.P. Gibson, University of Guelph, and from discussions with R.L. Fernando, D.J. Miller, and Yi Xu, University of Illinois.

References

Den Daas, J.H.G., 1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci., 28: 87.

Erb, R.E. and Flerchinger, F.H., 1954. Influence of fertility level and treatment of semen on non return decline from 29 to 180 days following artificial service . J. Dairy Sci., 37: 938.

Foote, R.H. and Bratton, R.W., 1952. The influence of antibiotics on delayed returns in artificial breeding. J. Dairy Sci., 35: 261.

Grossman, M. and Koops, W.J., 1988. Multiphasic analysis of growth curves in chickens. Poult. Sci., 67: 33.

Grossman, M. and Koops, W.J., 1988. Multiphasic analysis of lactation curves in dairy cattle. J. Dairy Sci., 71: 1598.

King, G.J. and Thatcher, W.W., 1993. Pregnancy. Page 264 in Reproduction in Domesticated Animals. Elsevier Sci. Publ., Amsterdam, The Netherlands,: 264.

Koops, W.J., 1986. Multiphasic growth curve analysis. Growth, 50: 169.

Koops, W.J. and Grossman, M., 1991. Applications of a multiphasic growth function to body composition in pigs. J. Anim., 69: 3265.

Koops, W.J., Grossman, M. and den Daas, J.H.G., 1995. A model for reproductive efficiency of dairy bulls. J. Dairy. Sci., 69: 3265.

Koops, W.J. Grossman, M. and Michalska, E., 1987. Multiphasic growth curve analysis in mice. Growth, 51: 372.

Lasley, J.F., 1962. Puberty, breeding season and estrous cycle. Reproduction in Farm Animals. E.S.E. Hafez, ed. Lea and Febiger, Philadelphia, PA.: 97.

Linares, T., 1982. Embryonic development in repeat breeder and virgin heifers seven days after insemination. Anim. Reprod. Sci., 4: 189.

Rasbech, N.O., 1986. Embryonic losses and the role of nutrition-statistical considerations. Page 223 in Embryonic Mortality in Farm Animals. J. M. Streenan and M. G. Diskin, ed. Martinus Nijhoff Publ., Dordrecht, The Netherlands,: 223.

Salisbury, G.W., Bratton, R.W. and Foote, R.H., 1952. The effect of time and other factors on the non return to service estimate of fertility level in artificial insemination of cattle. J. Dairy Sci., 35: 256.

Salisbury, G.W. and VanDemark, N.L., 1961. Physiology of Reproduction and Artificial Insemination of Cattle. 1st ed. W.H. Freeman and Co., San Francisco. CA.

Sherrod, P.H., 1994. Nonlinear Regression Analysis Program (NLREG), Version 3.2. Phillip H. Sherrod, Nashville, TN.

Sreenan, J.M. and Diskin, M.G., 1986. The extent and timing of embryonic mortality in the cow. Embryonic Mortality in Farm Animals. J.M. Sreenan and M.G. Diskin, ed. Martinus Nijhoff Publ. Dordrecht, The Netherlands,: 1.

Chapter 5

# THE RELATIONSHIP BETWEEN NUMBER OF SPERMATOZOA INSEMINATED and REPRODUCTIVE EFFICIENCY FOR INDIVIDUAL DAIRY BULLS

J.H.G. den Daas[1]

G. de Jong[2]

L.M.T.E. Lansbergen[1]

A.M. van Wagtendonk-de Leeuw[1]

[1] Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

[2] Dutch Cattle Syndicate

P.O. Box 454

6800 AL Arnhem

The Netherlands

Submitted to Journal of Dairy Science

Abstract

Semen from 20 proven bulls was split sample diluted and contained 2.1 to 17.3 million total spermatozoa per 0.25 ml French straw. The number of viable inseminated spermatozoa ranged from 1.1 to 11.8 million. The percentage viable cells after freeze-thawing was bull specific (P=0.03). 2430 to 5330 first or second inseminations per bull were carried out on the Dutch cattle population. The non-return rate (NR) at 56 days (NR56) after insemination was estimated for every dilution. Daily non-return rates of up to 180 days were used for the estimation of conception rate and calving rate at a given concentration using the multi phasic model developed by Grossman et al. (chapter 4). The estimations were used to determine the exponential curve describing the relationship between NR56, the multi phasic analysis of reproductive efficiency and the number of spermatozoa inseminated.

Bulls were found to differ significantly in their maximal NR at high sperm numbers per insemination and in their rate of approaching this maximum. No correlation was found between the maximum NR at high sperm numbers and the rate of approach. This implies that the optimal number of spermatozoa per insemination is bull dependent and rank of bulls by non-return rate 56 days after insemination is unrelated to the optimum sperm number required for a bull.

Multiphasic analysis of reproductive efficiency revealed that the relationship between estimated conception rate ($P_{CR}$) and the number of spermatozoa inseminated, as described for NR56, differed between bulls. The estimated calving rate given conception ($P_{CALVR}$) was found to vary from 82 to 90% and was not influenced by the number of spermatozoa inseminated. Multi phasic analysis of reproductive efficiency gives the best solution for the estimation of sperm numbers needed to obtain 95% of the estimated conception rate.

(Key words: fertility, non return rate, number of spermatozoa)

## Introduction

The number of spermatozoa needed per insemination to obtain acceptable fertility results is crucial to AI-industry. The goal is to optimize the use of the top bulls by maximizing total sperm out put and the number of spermatozoa per insemination, thus serving as many cows as possible without lowering the pregnancy rate.

When we study bull fertility we are in fact studying the bovine reproductive efficiency as a total of the contributions from the male, the female and the environment (Koops et al., 1995). Reproductive efficiency in AI bulls is usually measured by non-return rate (NR), which is commonly defined as the proportion of cows that were inseminated and did not return to service within a specified number of days. NRs usually over-estimate the true rates of non-return to service as natural breeding and culling are not taken into account. In addition, NRs are influenced by, and should be corrected for, systematic influences (Uwland, 1984; Taylor et al., 1985; Jansen and Lageweij 1987; Chapter 3 of this thesis).

The reproductive efficiency of a bull is a repeatable characteristic within certain limits. When a young bull is first sampled at an age of approximately one year, his semen characteristics may still be subject to rapid change because he is just in or just out of his puberty. Because of the relatively low numbers of breeding units (BU) used to approximately a hundred daughters, reproductive efficiency at that age is difficult to estimate. When a bull becomes a breeding bull he is approximately five years of age and sexually mature. In 1969 Beatty et al. concluded from hetero spermic experiments that the fertility of a male is virtually a constant for that male. Whenever a bull is allowed to age naturally it is generally accepted that fertility has a tendency to decrease (Uwland, 1984).

The relationship between the number of spermatozoa inseminated and NR has generally been studied by grouping bulls of low, medium and high fertility (Foote, 1970; Pace et al., 1981; Uwland, 1984). This implies that the traits conception rate and calving rate are intertwined. The relationship between the number of spermatozoa inseminated and NR has an exponential form with an asymptotic level when the number of spermatozoa inseminated

are above threshold level (Saacke, 1982). Some early studies indicate a decrease when an 'overdose' of sperm cells are inseminated (Foote, 1970; Foote and Oltenacu, 1980). However, these studies have not been confirmed recently. Schwartz et al. (1981) proposed a model to relate the number of spermatozoa to the probability of conception, based upon the Poisson distribution. Pace et al. (1981) found the same relationship by non-linear regression analysis techniques on field data to be $NR = a \times e^{-\frac{b}{x}}$ where x is the number of spermatozoa inseminated, a is the asymptotic (limiting) value and b is related to the rate of increase. If the correlation between a and b is strong the reproductive efficiency of bulls can be studied by estimation of the level of NR at high numbers of spermatozoa inseminated. However, if this correlation is weak or not existing at all, then reproductive efficiency should be studied by titration of every bull to his threshold level. NR is a compound trait that is a result of two events: conception and embryonic/fetal survival. Information on daily NRs can be used to derive more elementary biological measures for reproductive efficiency, such as conception rate and calving rate given conception. Grossman et al. (chapter 4 of this thesis) used literature to illustrate the developed multi phasic logistic function using data on daily NR rates. In this study field, data on daily NR, were used to calculate estimated conception rate (cpr) and calving rate (cvr) in order to determine their value for the evaluation of bull fertility.

The objective of this study is to describe the relationship between concentration and reproductive efficiency, to study the effects of this relationship on individual bulls and to estimate the number of spermatozoa needed per breeding unit ($N_{BU}$) to obtain optimal reproductive efficiency for the individual bulls. Reproductive efficiency is expressed as corrected NR at days after insemination (cNR56) (chapter 3 of this thesis) or as conception rate and calving rate given conception as predicted from data on daily non return by means of a novel multi phasic method described by Grossman et al. (chapter 4 of this thesis).

## Material and Methods

### Semen processing

Twenty-four proven bulls were selected based upon the expectation that 4,500 (BU)

from each bull would be used within a period of one year, in order to get sufficient numbers of inseminations for the trial. Bulls were housed on several Dutch AI stations. Twice weekly two ejaculates were collected. Ejaculates on the same day were pooled for processing. Except for volume and general aspects of the ejaculate, no restrictions were put on the quality of the semen. The semen was diluted to 50 ml at 15 minutes after collection with Tris-egg yolk extender containing 7% glycerol (Foote, 1970). The extender was produced in one batch and frozen (-20°C) until use. The diluted ejaculate was then cooled down to 5°C and transported to the laboratory where the semen was processed. The concentration was estimated using an electronic particle counter (Coulter industrial-D:70 µm orfice (electronics GB)). Split sample dilutions containing approximately 2.5, 3.75, 5, 7.5, 10 and 15 million total spermatozoa per BU (mini-straw) were frozen in liquid $N_2$ vapour after an equilibration time of 2.5 hours at 5°C. A batch number was printed on the straws to identify different concentrations and ejaculates.

Per bull approximately 7,200 BU were frozen. The semen was processed at one laboratory by one technician. To minimize seasonal influences, the ejaculates were frozen between September and December 1986. Ejaculates per bull were equally spread over this period of time. The frozen straws per bull were distributed randomly amongst technicians of the different AI-associations in the Netherlands. The number of straws per AI-association were weighted for the number of inseminations from a certain bull performed in the region. Technicians were unaware of the sperm quantity per straw, but they could identify straws from the trial by their colour. A total of 85,385 first and second inseminations were carried out in the Dutch cattle population from January to September 1987.

Concentration estimation

The total number of spermatozoa inseminated per straw was estimated in duplicate after freeze/thawing using the electronic particle counter. The standard error of the mean, calculated from concentrations measurement, was always less than 10% of the number estimated. After processing and freeze-thawing $N_{BU}$ were not divided into distinct concentration classes but ranged from 2 to 17 million per BU. For each bull the inseminations

performed were divided into 10 dilution classes with approximately equal numbers of inseminations per class.

The percentage viable spermatozoa per BU was estimated by using Hoechst 33258 according to De Leeuw et al. (1991). With this information the number of viable cells inseminated was calculated per BU and again the inseminations performed were divided into 10 dilution classes with approximately equal numbers of inseminations per class.

Calculations of corrected NR

The total data set to calculate cNR56 in the present trial included all 1,852,669 first and second inseminations performed by technicians between January and September 1987, in the Netherlands. The trial data set was a part of the total data. Non-return calculations were performed on the total data set because the additional relationships between herd, parity, technician, et cetera led to more reliable estimations. Data recorded included herd, cow, sire and technician identification, date of service, code number of the straw used and age of the cow in months. The data were edited to include:

1 Inseminations without return to service within 8 days.
2 Inseminations carried out in a herd with at least 10 inseminations per season. Two seasons were created: January 1 through April 30 and May 1 through August 31, 1987.
3 Inseminations carried out by a technician with at least 300 inseminations between January 1 and August 31, 1987.
4 Inseminations from a bull with at least 350 inseminations between January 1 and August 31, 1987.

About 5% of the cows were re-inseminated within 8 days after the first insemination. Due to restrictions 2, 3 and 4 another 5% of the data was lost. After editing, the data set consisted of 1,660,274 inseminations.

Additional editing criteria on the trial data set to ensure the reliability of the analysis results were:

A A minimum number of 200 inseminations per dilution class.

B A check on validity of the inseminations by comparing the bulls identity number with the registered code of the straw. 4% of the inseminations were lost due to administrative failures.

The number of bulls in the trial was reduced from 24 to 20, because 4 bulls did not meet the minimum number of 200 inseminations (criterium A) per dilution class.

The model for calculation of the cNR56 was:

$$NR56 = s_i d_J + (\mu + HS_K) + AM_L + D_M + I_N + T_O + e_{IJKLMNOP} \quad [1]$$

where

| | |
|---|---|
| NR56 | = non return rate 56 days after insemination |
| $s_i d_J$ | = fixed effect of ij-th sire-dilution group |
| $\mu$ | = overall uncorrected mean |
| $Hs_K$ | = fixed effect of k-th herd-season group |
| $Am_L$ | = fixed effect of l-th age-month group |
| $D_M$ | = fixed effect of m-th day of the week |
| $I_N$ | = fixed effect of n-th insemination-breed group |
| $T_O$ | = fixed effect of o-th technician |
| $e_{IJKLMNOP}$ | = error term |

The definitions of classification and numbers of classes of each case group in model [1] are in Table 1. Pre-analysis of NR per bull revealed no significant influence of ejaculates and therefore ejaculate effect was not included in the model.

*Table 1.* *The number of classes and the classification definition of each variable in the model* $NR56=s_J + (\mu + HS)_K + AM_L + D_M + I_N + T_O + e_{IJKLMNOP}$

| category | No. classes | Classification/grouping |
|---|---|---|
| sire-dilution | 795 | 200 dilution classes for bulls in trial 595 classes for additional bulls in regular use |
| herd-season | 62,461 | within herd 2 season classes:<br>Jan 1-Apr 30 and May 1-Aug 31 |
| age-month | 30 | 6 age classes (in months):<br><25, 25-36, 37-48, 61-84 and >84<br>5 months classes:<br>Dec-Jan, Feb-Mar, Apr-May, Jun-Jul and Aug-Sept |
| day of the week | 7 | 7 day of the week classes:<br>Mon, Tues, Wed, Thurs, Fri, Sat and Sun |
| insemination-breed | 4 | 2 insemination classes:<br>first and second inseminations<br>2 breed classes:<br>dairy bulls and beef bulls |
| technician | 612 | |

Grossman et al. (1995) (chapter 4 of this thesis) developed a multi phasic logistic function to model the decline in NR by grouping cows on the basis of three conditions:

1) the cow conceived at insemination and completed gestation (cvr)
2) the cow conceived at insemination and failed to complete gestation because of post signal embryonic death
3) the cow failed to conceive or conceived and failed to complete gestation because of presignal embryonic death

The final model estimates conception rate ($P_{CR}$), calving rate (cvr), characteristics of the first two oestrous cycles and the percentage correctly detected heat.

$$p_t = cvr + \left[1 - \frac{1}{1 + e^{-\frac{t-c}{d}}}\right](P_{cr} - cvr) + \left[1 - \left(\sum_{i=1}^{n} \frac{h_i}{1 + e^{-\frac{t-c_i}{d_i}}}\right)\right](1 - P_{cr}) \qquad [2]$$

where

$p_T$ = probability of non return at time t after insemination
$P_{CR}$ = probability of conception
cvr = probability of completing gestation after insemination, or calving rate
$h_i$ = probability to detect heat in cycle i
$c_i$ = time of maximum decline in non return rate at cycle i
$d_i$ = duration of cycle i

Conception rate ($P_{CR}$) and calving rate given conception ($P_{CALVR} = cvr / P_{CR}$) in relation to the number of inseminated spermatozoa were estimated according to the above given model. $P_{CR}$ is referred to by Grossman et al. (1995) as fertilization followed by embryonic survival up to the stage providing the signal for maternal recognition of pregnancy where as $P_{CALVR}$ is the proportion of cows that conceived and completed gestation. Differences in herd management, season and parity will be reflected in differences in percentage detected heat. For data on daily NR, the non-corrected intervals of cows returning to service within 180 days after first or second insemination, were used. Because of the relatively low number of inseminations per dilution class (approximately 450) inseminations were pooled per bull to determine the characteristics of the first and second oestrous cycle. These data were then used as input for the subsequent estimation of $P_{CR}$ and $P_{CALVR}$ per dilution class.

Relationship between cNR56, $P_{CR}$, $P_{CALVR}$ and number of spermatozoa

Linear and exponential curves were fitted per bull to determine the relationship between cNR56 (cNR56 = $\mu + s_i d_J$), $P_{CR}$ and $P_{CALVR}$ in relation to the number of spermatozoa inseminated using Genstat (manual, 1993). The results were compared on the percentage variation explained of the total sum of squares corrected for level and residual mean square error. The best results gave the exponential relation

$$y = a \times e^{-\frac{b}{x}} \qquad [3]$$

where
y = cNR56 or $P_{CR}$
x = total or viable number of cells inseminated
a = asymptotic level for cNR56 or $P_{CR}$
b = rate of increase
e = base of natural logarithm

As expected there was no influence of number of spermatozoa inseminated on $P_{CALVR}$ (chapter 4).

## Results

### Semen production

Table 2 gives the number of ejaculates per bull needed to obtain approximately 7200 BU. The mean volume of the ejaculate ranged from 6.1 to 19.7 ml. and the mean concentration of spermatozoa from 470 to 1427 $10^6$/ml. The bulls that needed a low number of ejaculates (5 to 6) had either a low concentration of the ejaculate and a high volume or a high concentration combined with low volume. The 6 bulls that needed 10 or more ejaculates to produce the quantity of spermatozoa needed all had a relatively low volume and a moderate concentration. The mean output of spermatozoa per ejaculate ranged from 5,220 $10^6$ to 14,495 $10^6$ for total number of cells. The correlation between total sperm output and volume was 0.65 ($p < 0.01$).

*Table 2. Sperm production characteristics for each of the 20 sires.*

| bull | Number ejaculates* | volume (ml) | | concentration ($10^6$/ml) | | total sperm output *$10^6$ mean | % viable cells postthaw |
|---|---|---|---|---|---|---|---|
| | | mean | range | mean | range | mean (# cells) | |
| 1 | 7 | 8.8 | 5.5 - 11 | 978 | 640 - 1347 | 8606 | 60 |
| 2 | 6 | 14.6 | 12 - 19 | 686 | 579 - 812 | 10016 | 70 |
| 3 | 7 | 18.1 | 15 - 20 | 470 | 410 - 529 | 8507 | 64 |
| 4 | 5 | 13.4 | 5 - 19 | 960 | 845 - 1130 | 12864 | 65 |
| 5 | 5 | 13.4 | 8 - 21 | 1007 | 656 - 1361 | 13494 | 66 |
| 6 | 7 | 11.1 | 8.5 - 14.5 | 776 | 514 - 1085 | 8613 | 61 |
| 7 | 11 | 8.8 | 7 - 9.5 | 658 | 467 - 869 | 5790 | 58 |
| 8 | 8 | 11.9 | 10 - 15 | 715 | 555 - 898 | 8508 | 72 |
| 9 | 5 | 12.4 | 9 -16 | 1169 | 970 - 1194 | 14495 | 55 |
| 10 | 6 | 8.9 | 5.5 - 12 | 1127 | 906 - 1565 | 10030 | 59 |
| 11 | 12 | 6.1 | 4 - 0 | 990 | 617 - 1292 | 6039 | 61 |
| 12 | 12 | 6.3 | 4.5 - 8 | 897 | 746 - 1120 | 5651 | 60 |
| 13 | 8 | 8.1 | 6.5 - 11 | 978 | 706 - 1257 | 7922 | 63 |
| 14 | 6 | 8.1 | 6.5 - 11 | 1427 | 1018 - 1988 | 11559 | 63 |
| 15 | 9 | 6.3 | 4 - 10 | 1261 | 975 - 1531 | 7944 | 58 |
| 16 | 5 | 19.7 | 11 - 23 | 680 | 514 - 789 | 13396 | 62 |
| 17 | 12 | 9.4 | 3 - 12.5 | 573 | 303 - 755 | 5386 | 63 |
| 18 | 10 | 8.6 | 5 - 11 | 820 | 585 - 1206 | 7052 | 57 |
| 19 | 6 | 10.0 | 5.5 - 17 | 1028 | 638 - 1345 | 10280 | 64 |
| 20 | 13 | 6.2 | 4 - 9.5 | 842 | 357 - 1208 | 5220 | 55 |
| mean | 8.0 ±2.7 | 10.5 | ±3.7 | 902.1 | ±233.2 | 9068 ±2822 | 61.8 ± 4.32 |

* *Number of ejaculates needed to produce 7,200 Breeding Units*

Concentration measurement and viability

The total number of spermatozoa inseminated per dilution class ranged from 2.1 to 17.3 ($x10^6$). The average viable number ranged from 1.1 to 11.8 ($x10^6$). The percentage of viable cells after freeze-thawing was sire specific (Table 2). The number ranged from 2,871 to 5,482 ($x10^6$) cells per ejaculate. No relationship between concentration and percentage viable cells after freeze-thawing was found. The correlation between the original volume of the ejaculate and percentage viable cells is 0.55 ($p \leq 0.01$).

Correction of NR56 for herd/season, age, day of the week, technician and breed only accounted for 10% of the total variation. The herd/season effect is as big as 7 out of the 10%. Considerable variation is left after correction. However, to be able to compare absolute levels between bulls the correction is essential.

Corrected NR56

Figure 1 shows the resulting curves for the number of viable and total cells inseminated for four different bulls. Bulls 1 and 20 show an increasing NR with concentration. The asymptotic value a is the maximal NR56 at high concentrations. The value for b is related to the steepness of the curve. Bulls 4 and 3 show no effect of number of viable or total cells inseminated for the tested concentrations. The percentage variance ($R^2$) explained by these curves was 66.4 and 63.6 for bulls 1 and 20 respectively. For bulls 3 and 4 the percentage variance explained was 0.0. It is to be expected that insemination with lower sperm levels would reveal a relationship similar to sires 1 and 20.

In Table 3 values for a and b and their standard deviation are given for the total and viable number of cells inseminated for each of the 20 bulls. The product-moment correlation between a-values for total and viable number of spermatozoa inseminated is 0.98 and between b-values 0.99. The rank correlation between the asymptotic level a and the rate of increase b is 0.30 ($p=0.20$) for total and 0.31 ($p=0.19$) for viable cells inseminated.

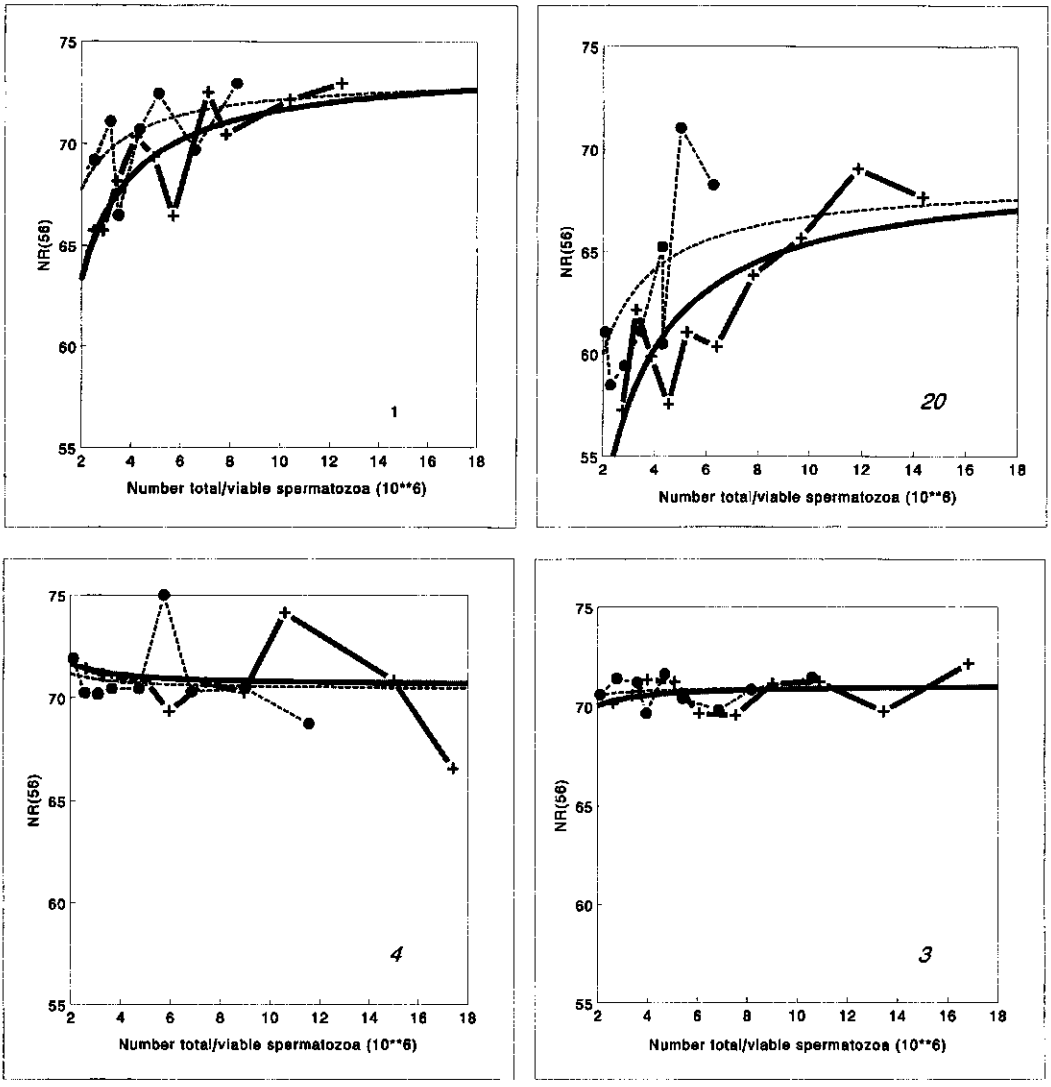



*Figure 1. The relationship between cNR56 and total or viable number of spermatozoa inseminated for bulls 1, 20, 3 and 4 (+ = number of total spermatozoa inseminated, ● = number of viable spermatozoa inseminated).*

*Table 3. Values for the asymptotic level (a), rate of increase (b) and percentage of variance explained ($R^2$) by equation: corrected non return rate 56 days* cNR56 = $a \times e^{-\frac{b}{x}}$, *calculated for the total and viable number of cells.*

| | a | | | | b | | | | $R^2$ | |
|---|---|---|---|---|---|---|---|---|---|---|
| bull | total mean | ± se | viable mean | ± se | total mean | ± se | viable mean | ± se | total | viable |
| 1 | 74.0 | 1.2 | 73.4 | 1.1 | 0.31 | 0.02 | 0.16 | 0.01 | 66.4 | 64.2 |
| 2 | 76.9 | 1.9 | 74.9 | 1.6 | 0.37 | 0.04 | 0.20 | 0.01 | 50.0 | 47.7 |
| 3 | 70.7 | 1.2 | 70.6 | 1.0 | -0.02 | 0.08 | -0.02 | 0.05 | 0.0 | 0.0 |
| 4 | 71.2 | 0.6 | 71.0 | 0.5 | 0.03 | 0.00 | 0.01 | 0.00 | 0.0 | 0.0 |
| 5 | 73.0 | 1.4 | 72.4 | 1.6 | 0.23 | 0.02 | 0.12 | 0.01 | 35.0 | 17.7 |
| 6 | 68.4 | 2.0 | 68.4 | 1.0 | -0.03 | 0.11 | -0.01 | 0.03 | 0.0 | 0.0 |
| 7 | 72.3 | 1.3 | 72.2 | 1.3 | 0.24 | 0.01 | 0.13 | 0.01 | 51.2 | 53.9 |
| 8 | 74.5 | 0.9 | 74.4 | 1.3 | 0.10 | 0.00 | 0.06 | 0.00 | 16.9 | 3.2 |
| 9 | 69.8 | 1.7 | 70.2 | 1.6 | 0.49 | 0.05 | 0.28 | 0.02 | 66.1 | 70.2 |
| 10 | 76.6 | 1.1 | 76.2 | 1.3 | 0.36 | 0.02 | 0.18 | 0.01 | 72.7 | 62.2 |
| 11 | 74.4 | 1.0 | 73.9 | 1.0 | 0.32 | 0.02 | 0.16 | 0.01 | 73.9 | 70.6 |
| 12 | 75.8 | 1.1 | 75.1 | 1.0 | 0.29 | 0.01 | 0.15 | 0.01 | 72.6 | 68.5 |
| 13 | 77.3 | 1.2 | 77.4 | 1.5 | 0.29 | 0.01 | 0.19 | 0.01 | 68.5 | 56.9 |
| 14 | 72.8 | 1.5 | 72.5 | 1.1 | 0.19 | 0.01 | 0.10 | 0.00 | 29.1 | 42.0 |
| 15 | 76.4 | 1.1 | 76.6 | 1.0 | 0.32 | 0.01 | 0.19 | 0.01 | 76.5 | 81.2 |
| 16 | 72.9 | 2.3 | 72.6 | 2.1 | 0.23 | 0.03 | 0.12 | 0.01 | 11.2 | 9.9 |
| 17 | 72.6 | 2.4 | 72.0 | 1.2 | 0.16 | 0.00 | 0.07 | 0.00 | 3.5 | 24.0 |
| 18 | 71.5 | 1.5 | 71.7 | 1.4 | 0.04 | 0.00 | 0.03 | 0.01 | 0.0 | 0.0 |
| 19 | 71.8 | 1.5 | 71.8 | 1.6 | 0.32 | 0.03 | 0.20 | 0.02 | 46.7 | 41.9 |
| 20 | 69.2 | 1.9 | 68.9 | 2.3 | 0.56 | 0.07 | 0.28 | 0.02 | 63.6 | 50.5 |
| Mean | 73.1 | ± 2.5 | 72.8 | ± 2.4 | 0.24 | ± 0.16 | 0.13 | ± 0.08 | | |

Estimated conception rate ($P_{cr}$)

The estimated conception rate is the closest possible approximation of the real penetration rate of oocytes. The curve with the best fit for day $P_{CR}$ was found to have the same shape as for cNR56;

$$P_{CR} = a \times e^{-\frac{b}{x}} \qquad [4]$$

where

$P_{CR}$ = estimated conception rate according to Grossman et al. (6)
x = total or viable number of cells inseminated
a = asymptotic value for estimated conception rate
b = rate of increase

Table 4 gives the estimated asymptotic $P_{CR}$ and rate of increase of the curve per bull for the total number of cells inseminated and their respective standard errors. Because of the high correlation between total and viable cells inseminated only results for total cells are given. The asymptotic $P_{CR}$ rates from 71.3 to 83.2% and the rate of increase ranges from .036 to .605 for the total number inseminated. The product-moment correlation between a-values for the total and viable number of cells inseminated is 0.97 and between b-values 0.96. The rank correlation between the asymptotic level a and the rate of increase b is 0.39 ($p= 0.09$).

Calving rate ($P_{CALVR}$)

Because the information on the calving rate is only relevant after conception, the calving rate given conception was calculated by dividing cvr through $P_{CR}$. For all but one bull the relationship between the number of spermatozoa inseminated and $P_{CALVR}$ was not significant. This bull showed a negative relationship between number of spermatozoa, both total and viable, inseminated and $P_{CALVR}$. $P_{CALVR}$ is given as the mean of the estimated $P_{CALVR}$ per dilution class. Table 4 gives the information on the mean calving rates given conception and their standard errors. The mean ranges from 82.3 to 90.3% for the total number of cells inseminated.

*Table 4. Values for the asymptotic level (a), rate of increase (b) and percentage of variance explained ($R^2$) by equation: estimated conception rate ($P_{CR}$) = $a \times e^{-\frac{b}{x}}$ as well as the mean calving rate given conception ($P_{CALVR}$) for each bull, calculated for the total number of cells.*

| | a | | b | | $R^2$ | $P_{CALVR}$ | |
|---|---|---|---|---|---|---|---|
| bull | mean | ± se | mean | ± se | | mean | ± se |
| 1 | 77.4 | 1.7 | 0.41 | 0.10 | 65.1 | 85.0 | 0.03 |
| 2 | 81.1 | 1.4 | 0.32 | 0.03 | 61.9 | 87.1 | 0.02 |
| 3 | 75.2 | 2.1 | 0.04 | 0.01 | 0.0 | 84.0 | 0.02 |
| 4 | 75.5 | 1.3 | 0.05 | 0.10 | 0.0 | 84.5 | 0.02 |
| 5 | 77.3 | 1.4 | 0.21 | 0.02 | 35.0 | 83.7 | 0.02 |
| 6 | 73.3 | 1.9 | 0.08 | 0.01 | 0.0 | 87.0 | 0.03 |
| 7 | 71.3 | 1.3 | 0.17 | 0.01 | 31.7 | 84.0 | 0.03 |
| 8 | 76.6 | 1.1 | 0.15 | 0.01 | 31.2 | 85.3 | 0.02 |
| 9 | 73.9 | 1.7 | 0.43 | 0.05 | 61.0 | 85.0 | 0.03 |
| 10 | 79.8 | 1.3 | 0.36 | 0.03 | 71.0 | 84.8 | 0.02 |
| 11 | 76.4 | 1.4 | 0.31 | 0.03 | 59.8 | 85.5 | 0.02 |
| 12 | 77.8 | 1.4 | 0.29 | 0.02 | 62.0 | 86.2 | 0.03 |
| 13 | 77.6 | 1.4 | 0.32 | 0.02 | 65.7 | 89.3 | 0.01 |
| 14 | 75.1 | 1.8 | 0.24 | 0.03 | 33.2 | 88.0 | 0.02 |
| 15 | 83.2 | 2.5 | 0.48 | 0.17 | 75.2 | 86.7 | 0.03 |
| 16 | 75.9 | 1.5 | 0.20 | 0.08 | 4.5 | 85.5 | 0.03 |
| 17 | 78.2 | 2.1 | 0.25 | 0.13 | 50.9 | 87.0 | 0.02 |
| 18 | 77.4 | 1.0 | 0.05 | 0.04 | 0.0 | 90.3 | 0.02 |
| 19 | 78.8 | 2.1 | 0.51 | 0.09 | 85.3 | 89.4 | 0.03 |
| 20 | 74.1 | 1.8 | 0.61 | 0.09 | 64.5 | 82.3 | 0.02 |
| mean | 76.8 | ± 2.7 | 0.27 | ± 0.16 | | 86.0 | ± 2.0 |

The relationship among cNR56, $P_{CR}$ and $P_{CALVR}$

Figure 2 A through D shows the relationship between $P_{CR}$, cNR56, $P_{CALVR}$ and the total number of spermatozoa inseminated for bull 7, 8, 15 and 20. Bull 7 shows a relatively small difference between the asymptotes of both curves. Bull 8 shows neither the effect of the number of cells inseminated on cNR56 nor on $P_{CR}$. Bull 15 combines a moderate asymptotic value for cNR56 with a high asymptotic level for $P_{CALVR}$. For bull 20 the asymptotic levels for cNR56 and $P_{CR}$ are only realised with high sperm numbers. There is a tenfold difference in accuracy between the point estimation for cNR56 and $P_{CR}$ or $P_{CALVR}$. The average values for the standard errors are in the order of 2% for cNR56 and 0.2% for $P_{CR}$ or $P_{CALVR}$.

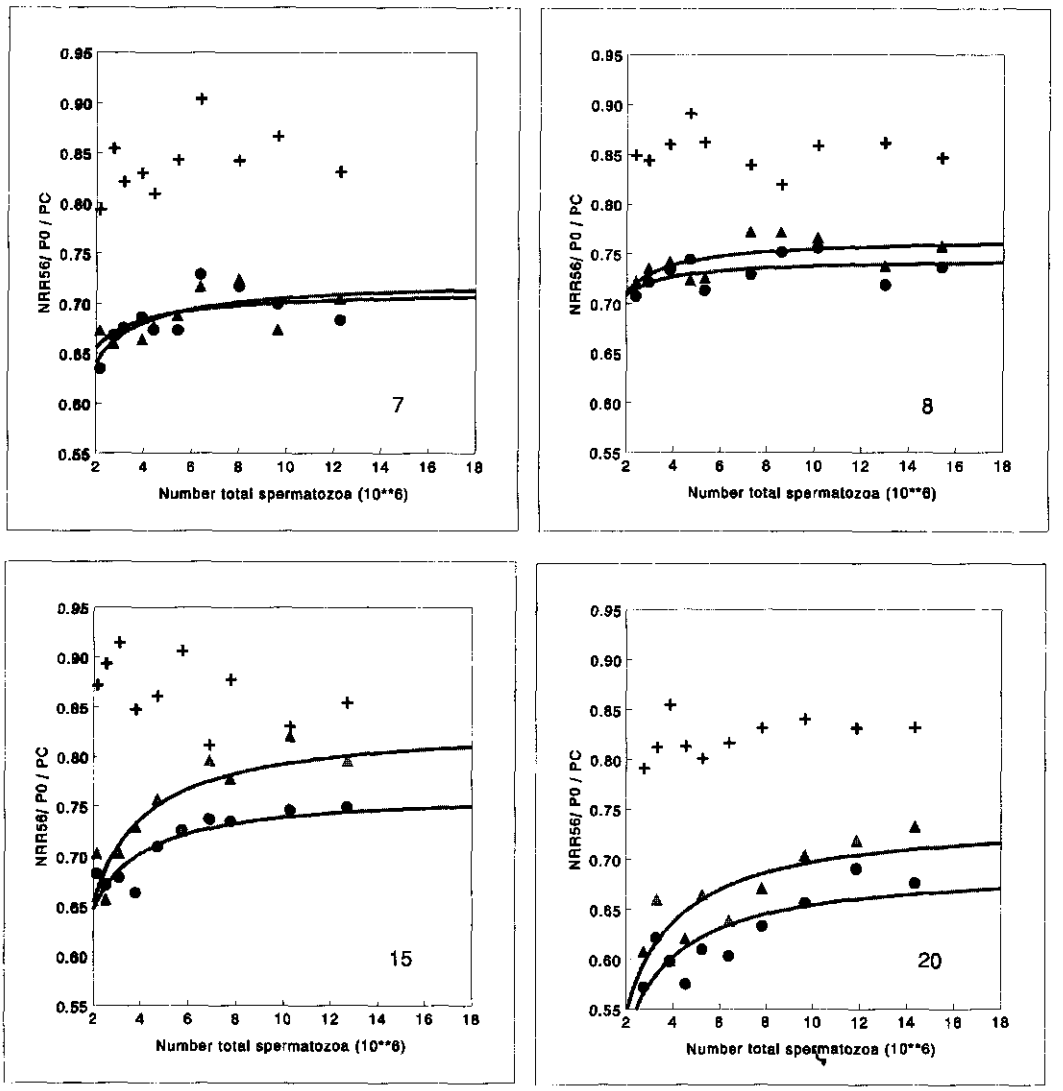



*Figure 2. The relationship between cNR56 (= ●), $P_{CR}$(= ▲), $P_{CALVR}$ (= +) and total number of spermatozoa inseminated for bulls 7, 8, 15 and 20.*

The total number of spermatozoa per breeding unit needed in order to achieve 95% of the estimated asymptotic values ($N_{95}$) for cNR56 and $P_{CR}$ and the difference between them are given in Table 5. For bulls numbered 3, 4, 6, 8 and 18, $N_{95}$ was out of the range of the tested concentrations, therefore these bulls were omitted for the rank correlation calculations. The difference between $N_{95}$ estimations range from -3.67 to 1.15 million spermatozoa (Table 5).

*Table 5. Total number of spermatozoa needed to inseminate to achieve 95% of the asymptotic values for corrected non-return rate ($N_{95}$cNR56) and for estimated conception rate ($N_{95}P_{CR}$). The difference between $N_{95}$cNR56 and $N_{95}P_{CR}$ is given.*

| bull | $N_{95}$cNR56 | ± sd (*$10^6$) | $N_{95}P_{CR}$ | ± sd (*$10^6$) | difference |
|---|---|---|---|---|---|
| 1 | 6.1 | 0.4 | 8.0 | 2.0 | -1.9 |
| 2 | 7.2 | 0.8 | 6.3 | 0.6 | 0.9 |
| 3* | -0.4 | 1.6 | 0.7 | 0.2 | |
| 4* | 0.6 | 0.0 | 1.0 | 2.0 | |
| 5 | 4.5 | 0.4 | 4.1 | 0.4 | 0.4 |
| 6* | -0.5 | 2.1 | 1.6 | 0.2 | |
| 7 | 4.7 | 0.2 | 3.3 | 0.2 | 1.4 |
| 8* | 1.9 | 0.0 | 2.8 | 0.2 | |
| 9 | 9.5 | 1.0 | 8.3 | 1.0 | 1.2 |
| 10 | 6.9 | 0.4 | 7.1 | 0.6 | -0.2 |
| 11 | 6.1 | 0.4 | 6.1 | 0.6 | 0.0 |
| 12 | 5.7 | 0.2 | 5.6 | 0.4 | 0.1 |
| 13 | 5.6 | 0.2 | 6.3 | 0.4 | -0.7 |
| 14 | 3.7 | 0.2 | 4.6 | 0.6 | -0.9 |
| 15 | 6.3 | 0.2 | 9.4 | 3.3 | -3.1 |
| 16 | 4.4 | 0.6 | 3.8 | 1.6 | 0.6 |
| 17 | 3.2 | 0.0 | 4.9 | 2.5 | -1.7 |
| 18* | -0.7 | 0.0 | 0.9 | 0.8 | |
| 19 | 6.2 | 0.6 | 9.7 | 1.8 | -3.5 |
| 20 | 10.8 | 1.4 | 11.8 | 1.8 | -1.0 |
| Mean | 4.59 | ± 3.1 | 5.32 | ± 3.1 | |

** sires outside detection range see text*

*Table 6. Product-moment correlations between asymptotic levels (a) and rate of increase (b) for corrected non-return rate cNR56, estimated conception rate $P_{CR}$ and $P_{CALVR}$ for viable and total number of cells inseminated, for 15 bulls.*

| | aNR56v | bNR56v | aNR56t | bNR56t | $aP_{CR}t$ | $bP_{CR}t$ | $P_{CALVR}t$ |
|---|---|---|---|---|---|---|---|
| aNR56v | 1.00 | | | | | | |
| bNR56v | -0.21 | 1.00 | | | | | |
| aNR56t | 0.99 | -0.27 | 1.00 | | | | |
| bNR56t | -0.29 | 0.96 | -0.32 | 1.00 | | | |
| $aP_{CR}t$ | 0.66 | 0.05 | 0.68 | -0.07 | 1.00 | | |
| $bP_{CR}t$ | -0.18 | 0.78 | -0.23 | 0.78 | 0.25 | 1.00 | |
| $P_{CALVR}t$ | 0.45 | -0.21 | 0.42 | -0.39 | 0.43 | -0.04 | 1.00 |

*v* = *viable*
*t* = *total*
*aNR56v* = *corrected non-return rate at 56 days after insemination*
*bNR56v* = *corrected non-return rate at 56 days after insemination*
*aNR56t* = *corrected non-return rate at 56 days after insemination*
*bNR56t* = *corrected non-return rate at 56 days after insemination*
$aP_{CR}t$ = *estimated conception rate*
$bP_{CR}t$ = *estimated conception rate*
$P_{CALVR}t$ = *estimated calving rate given conception*

Table 6, based on data for the remaining 15 bulls, gives the product-moment correlations between the asymptotic values for cNR56 and $P_{CR}$, $P_{CALVR}$ and the rate of increase to this level for cNR56 and $P_{CR}$. There is a significant rank correlation between the results for viable and total cells inseminated. The product-moment correlation between the a-values for NR56 and $P_{CR}$ is 0.68 for total and 0.66 for viable number of cells inseminated. The product-moment correlation between the b-values for NR56 and $P_{CR}$ is 0.78 for both total and viable number of cells inseminated. The rank correlation between the asymptotes and the rates of increase are not significantly different from zero.

## Discussion

The estimation of the number of spermatozoa needed per insemination to obtain acceptable fertility results is important to AI-industry. AI-industry aims to serve as many

farmers as possible with their top quality bulls and is therefore constantly seeking the balance between acceptable fertility results and the number of inseminations performed per bull over a certain period of time. The balance mentioned is influenced by the number of calves born sired by a superior bull (a), the costs to the farmer in terms of open days (b) and savings to the AI stud when fewer bull have to be housed an fewer ejaculates have to be collected and processed (c). The latter savings (c) are marginal, the costs of progeny testing are not altered and still the same number of bulls will be tested. So in a 'closed' cooperative system where the farmers own the AI-stud, the genetic gain in terms of number of offspring born has to balance the costs to the farmer in terms of open day's. In reality however, there is an open competion between the AI-studs. Moreover, because of the negative correlation between milk production and fertiilty, farmers want to get their cow in calf. The farmes also need to have enough replacement animals in their herds. This last aspect makes that the farmer, or client is seeking for almost maximal reproductive results.

Reproductive efficiency in the AI-industry is usually measured by non-return rates. These rates depend on the ability of a bull to fertilize the cow, thus initiating pregnancy, and on the ability of the cow and the conceptus to sustain pregnancy and complete gestation (Hawk, 1987; Koops, 1995). In this study we show that the relationship between corrected NR56 or $P_{CR}$ and the number of spermatozoa inseminated differs between bulls. The relationship between the number of spermatozoa inseminated and NR56 or $P_{CR}$ is not linear but has an asymptotic shape (Den Daas, 1992; Parrish and Foote, 1987; Saacke, 1982). When high numbers of spermatozoa are inseminated the NR or $P_{CR}$ reaches an asymptotic (limiting) value. The term 'critical number' (Uwland, 1984) or 'threshold value' (Saacke et al., 1994) of spermatozoa per insemination can be defined as the number needed to reach a percentage (for example 95 or 97%) of the asymptotic value. In this study $N_{95}$ was estimated using corrected NR56 and $P_{CR}$ (Table 5).

For five bulls the $N_{95}$ was out of the range of the tested concentrations. Apparently semen from some bulls is more affected by diluting and freezing than from others. Poorer results at low sperm numbers were expected for all bulls since the optimal number of spermatozoa inseminated is approximately 10 to 15 million (Foote, 1970; Uwland, 1984).

Schenk et al. (Schenk et al., 1987) reported a reduced fertility after inseminations with 11 million spermatozoa per straw. Sullivan and Elliot (1968) found optimal fertility by inseminating 10 million motile spermatozoa per BU using semen from high fertility bulls. Variations in results can be caused by the fact that in these studies, the mentioned bulls were grouped for NR calculations instead of being compared individually.

$N_{95}$ for the individual bulls studied ranges from 1 to 11 million. This wide range exists for both cNR56 as well as $P_{CR}$. The rank correlation between $N_{95}$ for NR56 and $P_{CR}$ is 0.80 ($P<0.001$). The method for estimation of $P_{CR}$ and $P_{CALVR}$ as described by Grossman et al. (chapter 4 this thesis) is based on daily NR. The percentage detected estruses (h) in this model absorbs the environmental differences between the populations of cows presented to be inseminated by a specific bull. Therefore it is possible to compare results for cNR56 and $P_{CR}$ between bulls. The theoretical model actually tries to split bull fertility into two characteristics. The first characteristic ($P_{CR}$) is related to the penetration of the oocyte and is therefore biologically related to the quality of the population spermatozoa inseminated. It is, however, impossible to detect the correct penetration rate of oocytes because in vivo only survival of the conceptus until day 15 will result in a prolonged cycle. This characteristic is referred to as extrinsic sperm quality (Den Daas, 1992) or the compensable factor (Saacke et al., 1994). The second characteristic, $P_{CALVR}$, is related to the paternal contribution of the developmental potential of the day 15 conceptus up to calving. Saacke et al. (1994) and den Daas (1992) refer to this characteristic as the 'incompensable' factor or 'intrinsic sperm quality'. In this study the range in $P_{CALVR}$ was 82.3 to 90.3% with a mean of 86.0% (table 4). $P_{CVR}$ seemed to be independent of the compensable effects as correlations between those effects were low (table 5). The concept of two independent effects; compensable and non-compensable factors both involved in determine the reproductive efficiency is supported by these results. Because of the ability to take bull differences in $P_{CR}$ and $P_{CALVR}$ into account the method of Grossman et al. (chapter 4 this thesis) is more accurate in the estimation of $N_{95}$.

The number of viable spermatozoa per breeding unit after freeze/thawing varies between bulls when the number of spermatozoa frozen is constant, as was also found by

Sullivan et al. (1968). The rank correlation between the estimated cNR56 for the number of viable cells and the number of total cells inseminated is however 0.98 ($P<0.05$). From Table 2 it can be seen that the average number of spermatozoa surviving freezing is around 60%. Insemination results using fresh semen instead of frozen however yield the same level of results when insemination are performed with 25 to 33% of the number of cells inseminated after freezing (Schenk et al., 1987). Therefore, even the viable cells after freeze thawing appear to be damaged. Within this study all breeding units were frozen under identical conditions. Using this procedure we found no relationship between concentration and survival rate after freezing at the concentration range studied.

Semen of each bull will react differently when his semen is processed as was shown by Shannon and Vishwanath (1995). This is another reason why $N_{95}$ is bull dependent. In the Dutch AI industry, usually one 'standard' way of semen processing is used, which means that, under standard conditions altering the limiting (parameter a) cNR56 level or $P_{CALVR}$ of a individual bull is not possible. Because correlation was low between the asymptotic level (a) and the rate of increase (b) attention should be paid to the prediction of the rate of increase. If laboratory methods could assess specific motility, or the ability of the spermatozoa to capacitate and acrosome react, or the ability to penetrate homologous oocytes, which can predict parameter b accurately, then AI-industry can make optimal use of the selected top bulls by using these methods to estimate $N_{95}$.

Acknowledgments

The authors wish to acknowledge B. Engel, J.B.M. Wilmink and W.J. Koops for assistance with statistical analysis and the Dutch AI-studs, J.D. Nieland and technicians for their excellent assistance. This research was funded by the Cooperative A.I. Associations in the Netherlands.

References

Beatty, R.A., Bennet, G.H., Hall, J.G., Hancock, J.L. and Stewart, D.L., 1969. An Experiment with Heterospermic Insemination in Cattle. J. Reprod. and Fertility, 19: 491-502.

Den Daas, J.H.G., 1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci., 28: 87- 94.

Foote, R.H., 1970. Influence of extender, extension rate and glycerolating technique on fertility of frozen bull semen. J. Dairy Sci., 53: 1478- 1482.

Foote, R. H. and Oltenacu, E.A.B., 1980. Increasing fertility in artificial insemination by culling bulls or ejaculates within bulls. Proc. 8th Natl. Assoc. Anim. Breeders; Tech. Conf. Artif. Insem. Reprod., Columbia, MO. USA : 6-12.

Genstat 5 committee, 1993. Genstat 5 release 3 reference manual. Edt. Payne, R.W. and Lane, P.W., Cralendon Press, Oxfort, UK.

Hawk, H.W., 1987. Transport and fate of spermatozoa after insemination in cattle. J. Diary Sci., 70: 1487-1503.

Jansen, J. and Lageweij, G.W., 1987. Adjustment of non-return rate for AI technicians and dairy bulls. Livest. Prod. Sci.., 16: 363-372.

Koops, W.J., Grossman, M. and den Daas, J.H.G., 1995. A model for reproductive efficiency in dairy bulls. J. Dairy Sci., 78: 921- 928.

Leeuw, A.M. de, den Daas, J.H.G. and Woelders, H., 1991. The fix vital stain method, simultaneous determination of viability and acrosomal status of bovine spermatozoa. J. Androl., 12: 112-118.

Pace, M.M., Sullivan, J.J., Elliot, F.I., Graham, E.F. and Coulter, G.H., 1981. Effects of thawing temperature, number of spermatozoa and spermatozoal quality on fertility of bovine spermatozoa packaged in .5 ml french straws. J. Anim. Sci.., 5: 693- 701.

Parrish, J.J. and Foote, R.H., 1987. Quantification of bovine sperm separation by a swim-up method. Relationship to sperm motility, integrity of acrosomes, sperm migration in polyacrylamide gel and fertility. J. Androl., 8: 259-266.

Saacke, R.G., 1982. Components of semen quality. J. Anim. Sci. 55:suppl. II: 1-13.

Saacke, R.G., Nebel, R.L., Karabinus, D.S., Bame, J.H. and Mullins, J., 1988. Spermtransport

and accessory sperm evaluation. Proc. 12th Natl. Assoc. Anim. Breeders; Tech. Conf. Artif. Insem. Reprod., Columbia, MO., USA : 7-14.

Saacke, R.G., Nadir, S. and Nebel, R.L., 1994. Relationships of semen quality to spermtransport, fertilization, and embryo quality in ruminants. Theriogenology 41: 45-50.

Schenk, J. L., Amann, R.P. and Allen, R.C., 1987. Effects of extender and insemination dose on post thaw quality and fertility of bovine sperm. J. Dairy Sci., 70: 1458- 1464.

Schwartz. D., MacDonald, P.D.M. and Heuchel, V., 1981. On the Relationship Between the Number of Spermatozoa and the Probability of Conception. Reprod. Nutr. Develop. 21 (6A): 979-988.

Shannon, P. and Vishwanath, R., 1995. The effect of optimal and sub optimal concentrations of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. Anim. Reprod Sci., 39: 1-10.

Sullivan, J.J. and Elliott, F.I., 1968. Bull fertility as affected by an interaction between motile spermatozoa concentration and fertility level in artificial insemination.6th Int. Congr. Anim. Reprod. Artif. Insem. Paris, France. Vol. II: 1307-1309.

Taylor, J. F., Everett, R.W. and Bean, B., 1985. Systematic enviromental, direct and service sire effects on conception rate in artificially inseminated Holstein cows. J. Dairy Sci., 68: 3004-3022.

Uwland, J., 1984. Possibilities and limitations of semen evaluation for the prognosis of male fertility. In: The male in farm animal reproduction. Edt. M. Courot Martinus Nijhoff , Boston, USA: 269-289.

Chapter 6

# MOTILITY CHARACTERISTICS OF SPERMATOZOA AND ITS VALUE FOR PREDICTING INDIVIDUAL BULL FERTILITY

J.H.G. den Daas[1]

P.J.F. Ursem[2]

B. Colenbrander[2]

L.M.T.E. Lansbergen[1]

[1] Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

[2] University of Utrecht

Veterinary Faculty

Department of Herd Health and Reproduction

P.O. Box 80.151

3508 TD Utrecht

The Netherlands

Submitted to Journal of Animal Science

## Abstract

The relation of motility characteristics of bull semen after different tests for resistance to incubation stress with corrected non-return rate 56 days (NR56), estimated conception rate ($P_{CR}$) and estimated calving rate given conception ($P_{CALVR}$) was studied. Semen from twenty bulls was used to study the relationship between concentration of frozen/thawed serially diluted ejaculates and motility characteristics. Motility characteristics were measured directly after thawing (treatment A), after incubation for 5 hours at 30°C (treatment B = thermo resistance test) and after 24 hours incubation at 5°C (treatment C = endurance test). The relationship between concentration (x) and percentage motile cells (y) was best described by the equation $y = c_{ASYM} \times e^{-\frac{d_{RATE}}{x}}$. The percentage of variance explained by this model was 66%, 51% and 52% for treatments A, B, and C, respectively. The relationship between concentration and motility characteristics had little predictive value for the relationship between concentration and fertility measures (NR56, $P_{CR}$ and $P_{CALVR}$) as found earlier for the same bulls under field conditions. Motility characteristics therefore have limited value for the prediction of the minimal number of sperm to be inseminated in order to achieve high non-return rates. For concentrations of sperm allowing maximal non-return rates, the correlation between percentage motile cells and predicted NR56 was 0,52 for treatment A ($p < 0.05$) and not significant for treatments B and C.


## Implications

Motility measurements may be useful in monitoring semen processing in the laboratory, but are of limited use in predicting fertility. Measurement of motility characteristics of serially diluted sperm after thawing or after thawing followed by a thermo resistance or endurance test cannot be used to predict individual bull fertility. At most, the motility of sperm directly after thawing at a concentration that allows maximal non-return for all bulls, can only be used to predict the individual maximal NR rate, and then only with relatively low accuracy (r=0.52).

## Introduction

The number of spermatozoa required per insemination to obtain acceptable fertility results is important for Artificial Insemination (AI)-industry. The goal is to optimize the use of the genetically superior bulls in order to serve as many cows as possible without a decrease in pregnancy rate.

In AI-practice laboratory methods are generally used to qualify semen prior to insemination. One of the frequently used laboratory tests for semen evaluation is the visual estimation of the percentage motile spermatozoa immediately after freeze-thawing or after a thermo resistance or endurance test (Uwland, 1984). These motility tests provide information about the semen itself and also about the processing procedure in the laboratory. They are in general considered to be predictive for the performance of the semen in the field (O'Connor et al., 1981). The predictive value of the subjective visual assessment for performance of the semen in the field varies between studies. Objective but labor intensive methods for evaluating sperm motility, like multi exposure photography, were developed in the mid-fifties (Rikmenspoel and van Herpen, 1957). In 1987 motility analyzing systems using videotaping of spermatozoa were described (Budworth et al., 1987; Knuth et al., 1987; Rath et al., 1987). These systems are reported to be precise and accurate for evaluating bull sperm motility.

The relationship between number of spermatozoa inseminated and reproductive efficiency for individual bulls was studied by den Daas et al. (chapter 5 of this thesis). That study included semen from 20 bulls, inseminated over a range of 2 to 17 million spermatozoa. The reproductive efficiency was measured by the proportion of cows that were inseminated and did not return to service within 56 d (cNR56) (chapter 3 of this thesis), the estimated conception rate ($P_{CR}$) and calving rate given conception ($P_{CALVR}$) (chapter 4 of this thesis).

As indicated by den Daas et al. (chapter 5) the relationship per bull between number of spermatozoa inseminated and cNR56 or $P_{CR}$ is exponential. From this exponential relationship den Daas et al. (chapter 5) estimated the number of spermatozoa required per breeding unit to obtain optimal reproductive efficiency. Optimal reproductive efficiency was defined as the number of spermatozoa required to obtain 95% of the asymptotic value of

cNR56 or $P_{CR}$ ($N_{95}$).

It is expensive and time consuming to determine the above described relationship for each individual bull by performing a field trial. Furthermore, reliable and accurate records are a prerequisite for this type of field trial and therefore this procedure is not feasible for every AI company. The question is whether or not a similar exponential relationship exists between the number of spermatozoa per AI dose inseminated and motility characteristics after freeze-thawing. If such a relationship exists then we would be able to predict the asymptotic value ($a_{ASYM}$) and the rate of increase ($b_{RATE}$) of fertility characteristics for each individual bull, using motility characteristics measured in the laboratory. The prediction of $b_{RATE}$ is of special interest because it will allow an estimation of $N_{95}$ and therefore obviate a field trail.

The aim of the present investigation is to study whether the variation in reproductive efficiency between individual bulls can be predicted by the motility characteristics of their sperm cells. More specifically, the aim is to assess the value of sperm motility characteristics for the prediction of $N_{95}$. These motility characteristics were assessed immediately after freeze-thawing, after a thermo resistance test and after an endurance test using different concentrations.

## Material and Methods

### Semen

Ejaculates from twenty proven bulls were split sample diluted to 10, 15, 20, 30, 40 and 60 million spermatozoa per ml. The same ejaculates were used to assess the relationship between the number of spermatozoa per insemination and fertility characteristics (chapter 5 of this thesis). The proven bulls in this trial were mildly selected for fertility, because they had already produced offspring in order to obtain data on the performance of their daughters. Ejaculates used in the study were selected for volume and general appearance like the presence of blood, sand or wood shavings. Semen was processed according to den Daas et al. (chapter 5). Three randomly chosen ejaculates per bull were used to determine motility characteristics. The different concentration classes of each ejaculate were studied

immediately after thawing (treatment A), after a thermo resistance test of 5 hours at 30°C (treatment B) and after an endurance test of 24 hours at 5°C (treatment C). A total of 20 (sires) x 3 (ejaculates) x 6 (concentration classes) x 3 (treatments) = 1,080 samples were analyzed for motility characteristics.

For thawing, straws were held in a water bath of 30°C for a duration of 30 seconds. Special slides were prepared according to Budworth et al. (1988). After a one minute equilibration time at 38°C on the object table of the microscope, per evaluation a minimum of 16 fields were recorded on videotape as a time laps serie of 12 frames. For recording and analyzing the Cellsoft semen system (Cellsoft, 1986) was used. Samples were recorded in duplicate.

Motility analyses

The parameter setting (see table 1) was slightly modified from that used by Budworth et al. (1988). The motility characteristics were measured at different concentrations and two slides/samples were analyzed with a minimal number of 8 fields/slides and with a minimal number of 400 spermatozoa per evaluation.

*Table 1. Parameter setting of the Cellsoft Semen Analyzer System.*

| | |
|---|---|
| number of frames per field | 12 |
| pixel scale | 0.67 µm |
| cell size | 20 - 70 pixels |
| minimal analyses motility | 2 frames |
| minimal analyses velocity | 2 frames |
| minimal analyses lateral head displacement | 7 frames |
| minimal analyses circular movement | 10 frames |
| maximal velocity | 200 µm/sec |
| minimal velocity | 20 µm/sec |
| minimal velocity lateral head displacement | 20 µm/sec |
| minimal velocity circular movement | 20 µm/sec |
| minimal linearity lateral head displacement | 3.5 |
| maximal radius for circular movement | 80 µm |

Motility characteristics

The motility characteristics estimated by the Cellsoft system are 1) the velocity of the cells in µm/sec; 2) linearity of the motion scored on a scale of 1 to 10 (a straight line = 10); 3) maximal and mean amplitude of the lateral head displacement in µm; 4) beat cross frequency of the head across the direction of motion in Hz; 5) the radius of the circular moving cells in µm; 6) the percentage motile cells; 7) the percentage circular moving cells of the motile and 8) of the total spermatozoa population (Cellsoft manual, 1986).

Reliability of cellsoft measurements at different concentrations; effect of number of frames.

The computer evaluates the digitized image of any object. From pixel area and luminosity, the computer determines whether an object is to be considered a spermatozoon and its centroid is calculated and stored. Based on the coordinates of the centroid and the maximum velocity (200 µm/sec), a circular area of possible location in the next frame of the time laps series is calculated for each spermatozoon. If a spermatozoon is detected within this area in the next frame it is assumed to be the same cell. If a second centroid is located in the same area of possible location, the computer assumes that the paths of the spermatozoa coincided and will exclude subsequent data, from that frame, from analysis (Budworth et al., 1988). The number of collisions and the motility and velocity of each individual cell were investigated for the extreme concentrations of 10 and 60 million. To evaluate whether spermatozoa which were traced only for a few frames had characteristics apparently differing from spermatozoa which were analyzed for 12 frames, the number of frames analyzed were counted per sperm. The extreme concentrations were investigated including all sires and the three treatments. Within concentration and between concentrations there was no effect of the number of frames a cell was analyzed on the motility characteristics of the cell.

Statistical analyses

The relationship between motility characteristics and concentration was estimated using the nonlinear regression procedure of Genstat (1993). The exponential equation used was:

$$y = c_{asym} \times e^{-\frac{d_{rate}}{x}} \qquad [1]$$

where:

y = motility characteristic
x = concentration spermatozoa
$c_{ASYM}$ = asymptotic level of motility characteristic
$d_{RATE}$ = rate of increase to asymptotic level of motility characteristic

This exponential relationship, similar to the relationship established by Pace et al. (1981) and used by den Daas et al. (chapter 5), to describe the effect of sperm concentration on fertility, was used in order to investigate whether concentration effects on motility in the laboratory could mimic field concentration effects on fertility.

To establish the predictive value of motility characteristics for the individual bull fertility parameters, the rank correlations between the motility characteristics and the fertility characteristics were determined, using the Spearman rank correlation procedure of Genstat (1993). The motility was described by all characteristics, measured by the Cellsoft system after treatments A, B and C. The fertility parameters concerned the asymptotic values ($a_{ASYM}$) for cNR56 and $P_{CR}$ as well as the rate of increase ($b_{RATE}$) to the asymptotic level of cNR56 and $P_{CR}$. Calving rate given conception was also considered as fertility parameter ($P_{CALVR}$). On the basis of a significant ($p < 0.10$) rank correlation of motility and fertility parameters, motility characteristics were selected to predict $N_{95}$.

## Results

### Evaluation of motility characteristics

Table 2 gives the mean percentage of variance explained by equation [1] for the different motility characteristics after treatment A, B and C. The criterion for a reasonable fit to the data was that at least 10 of the 20 bulls tested with 10% of the variance be explained by equation 1. The motility characteristics velocity, linearity, maximal and mean amplitude of

lateral head displacement, beat cross frequency and the percentage circular moving cells of the motile spermatozoa population did not fulfill this requirement. It was therefore concluded that the relationship between these characteristics and concentration was not of the type described by equation [1]. For these characteristics mean values were used to evaluate rank correlations with fertility parameters. For the remaining characteristics, percentage circular moving cells of the total spermatozoa population (cirtot) and percentage motile cells (permot), $c_{ASYM}$ and $d_{RATE}$ values were calculated according to equation [1].

*Table 2. Percentage of variance explained ($R^2$) by the equation [1] describing the relationship between motility characteristics and concentration in each treatment group per bull. $Y = C_{ASYM} \times e^{-d_{RATE} \times}$ [1]. Number of bulls tested = 20.*

| motility characteristic | treatment | mean $R^2$ | Number of bulls $R^2 > 10$ | mean $R^2$ $R^2 > 10$ |
|---|---|---|---|---|
| velocity | A | 10.4 | 8 | 25.6 |
| | B | 8.8 | 6 | 25.5 |
| | C | 6.5 | 5 | 23.3 |
| linearity | A | 6.8 | 6 | 19.0 |
| | B | 7.6 | 7 | 20.7 |
| | C | 8.5 | 6 | 26.7 |
| alhmax | A | 9.9 | 6 | 29.9 |
| | B | 6.1 | 5 | 20.8 |
| | C | 3.9 | 3 | 18.7 |
| alhmean | A | 9.6 | 6 | 28.4 |
| | B | 6.2 | 5 | 21.1 |
| | C | 3.7 | 3 | 17.8 |
| radius | A | 7.6 | 6 | 24.0 |
| | B | 9.4 | 7 | 25.2 |
| | C | 6.1 | 4 | 25.3 |
| bcfreq | A | 7.3 | 7 | 16.3 |
| | B | 5.2 | 4 | 24.1 |
| | C | 8.9 | 8 | 20.7 |
| cirmot | A | 4.2 | 3 | 17.0 |
| | B | 5.6 | 5 | 21.0 |
| | C | 4.2 | 4 | 20.9 |
| cirtot | A | 48.5 | 19 | 50.6 |
| | B | 24.8 | 14 | 34.3 |
| | C | 27.5 | 15 | 36.0 |
| permot | A | 66.0 | 20 | 66.0 |
| | B | 50.6 | 20 | 50.6 |
| | C | 51.7 | 20 | 51.7 |

*velocity = linear velocity of the cells in µm/sec.*
*linearity = linearity of the motion; a straight line = 10.*
*alhmax = maximal amplitude of lateral head displacement.*
*alhmean = mean amplitude of lateral head displacement.*
*bcfreq = beatcross frequency in Hz.*
*radius = average radius of the circular cells.*
*cirmot = percentage circular moving cells of the motile spermatozoa population.*
*cirtot = percentage circular moving cells of the total spermatozoa population.*
*permot = percentage motile cells.*

In table 3 the mean and range of the different motility characteristics for treatment A, B and C are given for those characteristics which did not show an exponential relationship with concentration. The values found immediately after thawing are the highest for velocity, linearity and radius of the circular movement. After an incubation time of 5 hours at 30 °C the velocity of the cells was reduced and the percentage of circular moving cells increased. The radius of the circular movement slightly decreased altough a holding time of 24 hours at 5 °C had no effect on velocity of the moving cells in comparison with treatment A (immediately after thawing) but resulted in the highest maximal lateral head displacement.

Figure 1 shows the average relationship, over all bulls, between percentage motile cells and concentration for treatment A, B and C. The percentage motile cells at each concentration is always the highest for treatment A and always the lowest for treatment C. For all treatments there is a strong significant correlation between percentage motile cells and concentration (see also table 2).

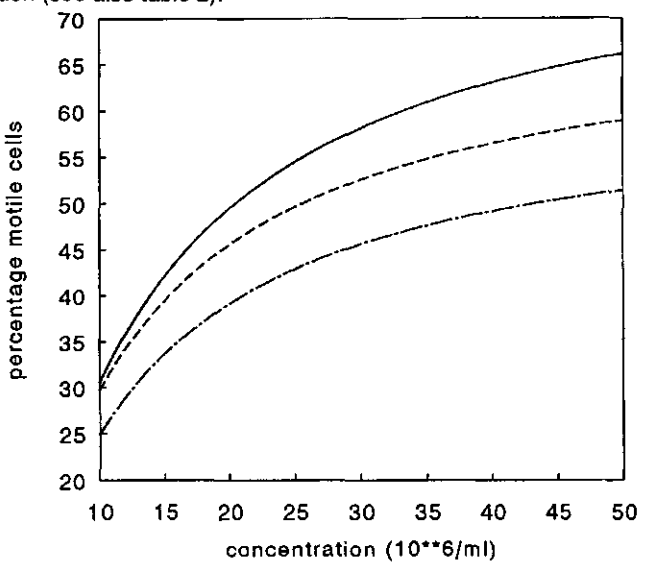



*Figure 1. Relationship (average of 20 bulls) between percentage motile cells and concentration for treatment (A) immediately after freeze-thawing (_____), (B) after a thermo-resistance test (-------) and (C) after an endurance test (_._._._).*

*Table 3. Mean and range of motility characteristics that showed no exponential relation to concentration in each treatment group. N = 20 bulls.*

| motility characteristic | treatment | mean | range (min.-max.) |
|---|---|---|---|
| velocity | A | 64.0 | 50.0 - 74.5 |
| | B | 44.7 | 37.3 - 54.0 |
| | C | 63.6 | 52.9 - 70.5 |
| linearity | A | 6.89 | 5.34 - 7.58 |
| | B | 5.96 | 4.95 - 7.24 |
| | C | 5.59 | 4.86 - 6.15 |
| alhmax | A | 2.39 | 1.77 - 2.92 |
| | B | 2.22 | 1.64 - 2.99 |
| | C | 3.36 | 2.78 - 4.02 |
| alhmean | A | 2.08 | 1.52 - 2.56 |
| | B | 1.92 | 1.38 - 2.61 |
| | C | 2.97 | 2.39 - 3.60 |
| radius | A | 16.2 | 15.4 - 16.9 |
| | B | 15.7 | 13.2 - 17.8 |
| | C | 14.5 | 12.6 - 16.4 |
| bcfreq | A | 26.0 | 19.8 - 29.8 |
| | B | 21.6 | 16.2 - 28.0 |
| | C | 22.3 | 16.5 - 26.4 |
| cirmot | A | 19.9 | 14.7 - 28.6 |
| | B | 30.8 | 22.7 - 36.9 |
| | C | 18.5 | 13.8 - 28.2 |

*velocity* = *linear velocity of the cells in µm/sec.*
*linearity* = *linearity of the motion; a straight line = 10.*
*alhmax* = *maximal amplitude of lateral head displacement.*
*alhmean* = *mean amplitude of lateral head displacement.*
*bcfreq* = *beatcross frequency.*
*radius* = *average radius of the circular cells.*
*cirmot* = *percentage circular moving cells of the motile spermatozoa population.*

In table 4 mean and range of the asymptotic value ($c_{ASYM}$) and the rate of increase to this asymptotic value ($d_{RATE}$) for cirtot and permot are shown. The influence of concentration on the percentage circular moving cells of the total spermatozoa population was moderate (see Table 2). The mean asymptotic level $c_{ASYM}$ of the percentage of motile cells decreased during incubation at 30°C, while the range remained constant. The mean velocity (table 3) of the spermatozoa decreased significantly during incubation at 30°C (B). During holding at 5°C (C) the velocity, range and mean (table 3) of the motile cells showed no change when compared with the values immediately after thawing. The asymptotic level $c_{ASYM}$ of the percentage motile cells, mean and range decreased further after holding the spermatozoa at 5°C for 24 hours.

*Table 4. Mean and range of motility characteristics that showed an exponential relation to concentration in each treatment group.*

| motility characteristic | treatment | mean | range (minimum-maximum) |
|---|---|---|---|
| $d_{RATE}$ cirtot | A | 0.12 | 0.05 - 0.25 |
| | B | 0.25 | 0.07 - 0.57 |
| | C | 0.25 | 0.06 - 1.03 |
| $c_{ASYM}$ cirtot | A | 16.8 | 12.4 - 22.2 |
| | B | 21.1 | 13.7 - 30.1 |
| | C | 11.4 | 6.9 - 16.6 |
| $d_{RATE}$ permot | A | 0.12 | 0.05 - 0.25 |
| | B | 0.15 | 0.05 - 0.45 |
| | C | 0.15 | 0.04 - 0.37 |
| $c_{ASYM}$ permot | A | 84.5 | 55.9 - 96.1 |
| | B | 74.3 | 55.9 - 91.3 |
| | C | 67.3 | 41.0 - 83.5 |

$d_{RATE}$ *cirtot* = *rate of increase to the asymptic value of percentage circular moving cells of the total spermatozoa population.*

$c_{ASYM}$ *cirtot* = *asymptotic value of percentage circular moving cells of the total spermatozoa population.*

$d_{RATE}$ *permot* = *rate on increase to the asymptotic value of percentage motile cells.*

$c_{ASYM}$ *permot* = *asymptotic value of percentage motile cells.*

Relationship between motility characteristics and fertility parameters

Spearman rank correlations were calculated between motility characteristics and fertility parameters. Only radius and $d_{RATE}$ of cirtot and permot showed at least one significant rank correlation ($P < .10$) with the rate of increase to the asymptotic level of the fertility parameter $b_{RATE}$. Table 5 reports the rank correlation coefficients with at least one significant ($P < .10$) rank correlation between motility characteristics and $b_{RATE}$. The rank correlations are calculated on data for 20 and 15 bulls respectively since for five bulls the concentration range tested revealed no relationship between the number of spermatozoa inseminated and cNR56 or $P_{CR}$ (chapter 5). Judging all results for radius (table 5) suggests that the significant correlation found is an incident. The rank correlation for $d_{RATE}$ of cirtot and $d_{RATE}$ of permot treatment B were more consistent. These $d_{RATE}$s were used to predict the number of spermatozoa required to obtain 95% of the asymptotic value of cNR56 or $P_{CR}$ in equation [1] ($N_{95}$) (chapter 5).

Table 6 gives the $N_{95}$ values calculated for $d_{RATE}$ of cirtot and $d_{RATE}$ of permot compared to the values found through evaluation of insemination results. The predictions via the motility characteristics lead to a smaller range in $N_{95}$ than when calculated directly from the relationship between concentration and fertility parameters. In general, for bulls with a high rate of increase to the asymptotic level (bulls 3, 4 and 18), and thus a low $N_{95}$, the number of cells to be inseminated was over estimated. For bull 9, which had one of the lowest values for the rate of increase to the asymptotic level, $N_{95}$ was under estimated.

*Table 5. Rank correlations between motility characteristics and the rate of increase to the asymptotic level ($b_{RATE}$) in the eqation describing the relationship between cNR56 or the estimated conception rate ($P_{CR}$) and concentration spermatozoa.*

| | treatm | number bulls | mean radius (P) | $d_{RATE}$ cirtot (P) | $d_{RATE}$ permot (P) |
|---|---|---|---|---|---|
| | A | 20 | 0.12 (0.62) | -2.11 (0.37) | -0.52 (0.02)* |
| $b_{RATE}$ | | 15 | -0.48 (0.07)* | 0.04 (0.90) | -0.31 (0.27) |
| cNR56 | B | 20 | 0.06 (0.80) | -0.55 (0.01)* | -0.51 (0.02)* |
| | | 15 | 0.29 (0.30) | -0.52 (0.05)* | -0.66 (0.01)* |
| | C | 20 | 0.09 (0.72) | -0.16 (0.50) | -0.13 (0.59) |
| | | 15 | -0.23 (0.40) | 0.13 (0.63) | -0.17 (0.54) |
| | A | 20 | 0.20 (0.40) | -0.18 (0.44) | -0.40 (0.08)* |
| $b_{RATE}$ Pcr | | 15 | -0.26 (0.36) | 0.08 (0.79) | -0.10 (0.73) |
| | B | 20 | -0.08 (0.74) | -0.44 (0.05)* | -0.29 (0.22) |
| | | 15 | 0.04 (0.99) | -0.34 (0.22) | -0.34 (0.22) |
| | C | 20 | 0.08 (0.75) | -0.20 (0.39) | -0.09 (0.70) |
| | | 15 | -0.14 (0.62) | 0.03 (0.92) | -0.14 (0.61) |

*radius* = *average radius of the circular cells.*
*cirtot* = *percentage circular moving cells of the total spermatozoa population.*
*permot* = *percentage motile cells.*
* = $P \leq 0.10$

*Table 6. Total number of spermatozoa needed for insemination to achieve 95%of the symptotic values for cNR56 and $P_{CR}$ from and their predictions when calculated sires cirtot or permot in equation [1].*

$$Y = c \times e^{-\frac{d}{x}}$$

| bull | | cNR56 | ± sd | $P_{CR}$ | ± sd | cNR56 dcirmot | cNR56 dpermot | $P_{CR}$ dcirmot | $P_{CR}$ dpermot |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | 6.1 | 0.4 | 8.0 | 2.0 | 5.6 | 4.9 | 6.2 | 5.5 |
| 2 | | 7.2 | 0.8 | 6.3 | 0.6 | 6.6 | 6.4 | 7.1 | 6.5 |
| 3 | * | -0.4 | 1.6 | 0.7 | 0.2 | 6.0 | 6.3 | 6.5 | 6.4 |
| 4 | * | 0.6 | 0.0 | 1.0 | 2.0 | 2.9 | 4.6 | 3.7 | 5.3 |
| 5 | | 4.5 | 0.4 | 4.1 | 0.4 | 4.4 | 3.4 | 5.1 | 4.5 |
| 6 | * | -0.5 | 2.1 | 1.6 | 0.2 | 1.5 | -0.5 | 2.4 | 1.8 |
| 7 | | 4.7 | 0.2 | 3.3 | 0.2 | 4.9 | 5.2 | 5.5 | 5.7 |
| 8 | * | 1.9 | 0.0 | 2.8 | 0.2 | 2.0 | 3.8 | 2.9 | 4.8 |
| 9 | | 9.5 | 1.0 | 8.3 | 1.0 | 6.0 | 5.9 | 6.5 | 6.2 |
| 10 | | 6.9 | 0.4 | 7.0 | 0.6 | 1.3 | 5.7 | 2.2 | 6.0 |
| 11 | | 6.1 | 0.4 | 6.1 | 0.6 | 6.3 | 6.0 | 6.9 | 6.2 |
| 12 | | 5.7 | 0.2 | 5.6 | 0.4 | 5.8 | 5.2 | 6.4 | 5.7 |
| 13 | | 5.6 | 0.2 | 6.3 | 0.4 | 4.9 | 6.2 | 5.5 | 6.4 |
| 14 | | 3.7 | 0.2 | 4.6 | 0.6 | 4.4 | 4.2 | 5.1 | 5.0 |
| 15 | | 6.3 | 0.2 | 9.4 | 3.3 | 5.3 | 5.1 | 5.9 | 5.6 |
| 16 | | 4.4 | 0.6 | 3.8 | 1.6 | 5.9 | 4.3 | 6.4 | 5.1 |
| 17 | | 3.2 | 0.0 | 4.9 | 2.5 | 4.9 | 2.4 | 5.6 | 3.8 |
| 18 | * | -0.7 | 0.0 | 0.9 | 0.8 | 2.5 | 4.4 | 3.3 | 5.2 |
| 19 | | 6.2 | 0.6 | 9.9 | 1.8 | 5.5 | 3.8 | 6.1 | 4.7 |
| 20 | | 10.8 | 1.4 | 11.8 | 1.8 | 6.6 | 6.1 | 7.1 | 6.3 |

* = *Sires outside detection range (see text).*
*NR56* = *total number of spermatozoa needed to achieve 95% of a NR56 in millions (2)*
$P_{CR}$ = *total number of spermatozoa needed to achieve 95% of a $P_{CR}$ in millions (3)*

Figure 2 shows the relationship between the rate of increase to the asymptotic value ($d_{RATE}$) of the percentage motile cells for treatment B and the rate of increase ($b_{RATE}$) to the asymptotic value of cNR56 for 20 and 15 bulls respectively. The greater the degree to which motile cells are affected by concentration (higher $d_{RATE}$), the lower the $b_{RATE}$ values are; low $b_{RATE}$ values give rise to lower $N_{95}$ estimations. The latter means that semen characterized by a large effect of concentration on percentage motile cells after incubation at 30°C for 5 hours, (semen, which is sensitive to dilution) needs a relatively low number of spermatozoa per insemination dose in order to achieve 95% ($N_{95}$) of the asymptotic value of cNR56.

Table 7 gives the rank correlations between motility characteristics and the asymptotic values for cNR56 and $P_{CR}$ and $P_{CALVR}$. The asymptotic values are from den Daas et al (chapter 5 of this thesis) from 20 bulls. Motility characteristics were tabulated on the basis of P<.10. The maximal and mean amplitude of lateral head displacement were not correlated to the $a_{ASYM}$ values or $P_{CALVR}$. The $c_{ASYM}$ values of percentage motile cells immediately after thawing (treatment A) showed a significant rank correlation of 0.5 to both $a_{ASYM}$ of cNR56 and $a_{ASYM}$ of $P_{CR}$. This correlation is comparable to those found in the literature (Saacke et al 1994). This relationship is absent however, after the stress (treatment B) or endurance test (treatment C). All other motility parameters failed to show consistent correlations with the asymptotic values or $P_{CALVR}$.

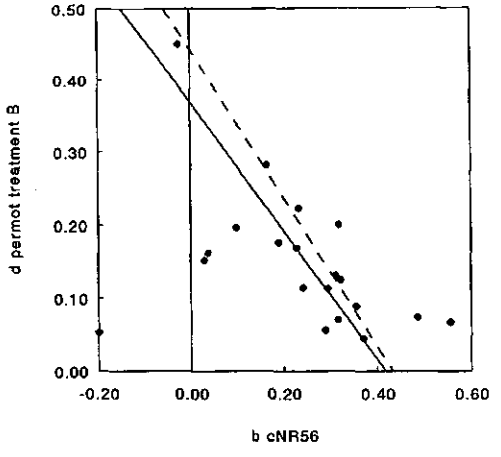



*Figure 2. Relationship between rate of increase to the asymptotic value of the percentage of motile cells for treatment (B) after a thermoresistance test (d permot) and the rate of increase to the asymptotic value of the corrected non-return rate 56 d after insemination (b cNR56) for 20 (_____) and 15 bulls (-----).*

*Table 7. Rank correlations between motility characteristics and the asymptotic level ($a_{sym}$) in the equation describing the relationship between cNR56 or the estimated conception rate ($P_{CR}$) or calving rate ($P_{calv}$) and concentration spermatozoa inseminated.*

| | treatm | velocity | linearity | radius | bcfreq | $c_{ASYM}$ totcir | $d_{RATE}$ permot | $c_{ASYM}$ permot |
|---|---|---|---|---|---|---|---|---|
| $a_{sym}$ cNR56 | A | -0.13 | -0.08 | 0.20 | 0.13 | 0.26 | -0.39 * | 0.52 ** |
| | B | 0.04 | -0.38 * | -0.10 | -0.27 | 0.06 | -0.24 | 0.33 |
| | C | 0.04 | -0.19 | -0.26 | -0.22 | -0.10 | -0.01 | 0.12 |
| $a_{sym}$ $P_{CR}$ | A | -0.17 | 0.01 | 0.07 | 0.28 | 0.53 * | -0.19 | 0.50 ** |
| | B | -0.26 | -0.28 | -0.20 | -0.38 * | 0.16 | -0.05 | 0.17 |
| | C | -0.01 | -0.13 | -0.22 | -0.37 | 0.01 | -0.09 | 0.23 |
| $P_{calv}$ | A | -0.27 | 0.11 | 0.01 | 0.03 | 0.37 | 0.15 | 0.11 |
| | B | -0.46 ** | -0.26 | -0.44 ** | -0.61 ** | 0.01 | 0.22 | -0.04 |
| | C | 0.06 | 0.05 | -0.25 | -0.20 | 0.01 | 0.36 | -0.01 |

*velocity* = *linear velocity of the cells in µm/sec.*
*linearity* = *linearity of the motion; a straight line = 10.*
*bcfreq* = *beatcross frequency.*
*radius* = *average radius of the circular cells.*
*cirmot* = *percentage circular moving cells of the motile spermatozoa population.*
*cirtot* = *percentage circular moving cells of the total spermatozoa population.*
*permot* = *percentage motile cells.*
* = *significant P< 0.1.*
** = *significant P < 0.05.*

Discussion

In this study there is a certain risk of over emphasizing significant results because of the number of correlations computed. When calculating 3 (treatments) x 11 (characteristics) x 5 (fertility estimates) to make a total of 165 correlations on data from 20 bulls, it is to be expected that a few correlations appear significant due to chance. Therefore in addition to the significance of the correlations, the theoretical biological background is also of importance when interpreting the results presented in this paper.

Prediction of the fertility of bulls is an item thoroughly discussed in many studies (Uwland, 1984; Foote., 1988; Budworth et al., 1988; Saacke, 1982). AI centers require methods to detect differences among males with a narrow range of fertility. Although many semen evaluation methods were developed and proven to be positively related with male fertility, the introduction of these methods did not result in an appreciable improvement of conception in practice (Uwland 1984). Most of the studies on the relationship between fertility and different laboratory tests to characterize semen are performed on one given concentration. The correlation found between percentage motile cells (estimated by eye) and fertility is generally in the order of 0.4 to 0.7 (O'Connor 81 et al., Saacke, 1982). In the present study we found at high concentrations a correlation between permot and non-return rate or conception rate of 0.5 (table 7). However, bull ranking for fertility alters with the number of spermatozoa inseminated (chapter 5). This implies that the correlation of laboratory tests with fertility will alter with concentration. This fact could be one of the reasons why inconsistent results have been reported.

In this study ejaculates of proven bulls were selected on the basis of general appearance before processing in the laboratory. No pre-selection of ejaculates or concentrations was made after freezing and before insemination. A range of concentrations from 2.5 to 15 million spermatozoa per breeding unit could be evaluated for motility characteristics immediately after freeze-thawing (A), after a thermo resistance test of 5 hours at 30°C (B) and after a endurance test of 24 hours at 5°C (C). To thaw, the straws were placed in a water bath at 30°C for 30 second. Thawing method and temperature are

important and should be uniform in order to be able to compare results (Pace et al., 1981) The relationship with fertility estimates according to den Daas et al. (chapter 5) could be established. A strong relationship between percentage motile cells and concentration was established. If generally accepted criteria to qualify semen for insemination had been used, a pre-selection would have taken place and about 40% of the breeding units with the lowest 2 concentrations (2.5 and 3.75 million) of spermatozoa would have been discarded (Stalhammar et al., 1994).

By adding caffeine, bovine serum albumine or seminal plasma the non- motile fraction of semen at low concentrations can be activated (den Daas unpublished observations). These observations are supported by reports (Woelders, 1991) using live dead dye exclusion staining techniques to indicate that there is a fraction of spermatozoa that is viable but non motile. In addition to these observations no relationship between concentration and percentage cells surviving freezing has been found with semen used in the study of den Daas et al. (chapter 5).

The reproductive efficiency of the bulls studied was characterized by the asymptotic values of cNR56 and $P_{CR}$, $P_{CALVR}$ and the rate of increase to the asymptotic value of cNR56 and $P_{CR}$ respectively. The motility characteristics, velocity of the cells, linearity of the motion, maximal and mean amplitude of the lateral head displacement, radius of the circular moving cells, beat cross frequency of the head and percentage circular moving cells of the motile spermatozoa population for treatments A, B and C, were not informative for the prediction of fertility. The exceptions to this were the percentage of motile cells and the percentage of circular moving cells of the total spermatozoa population after treatment A and B. The rate of increase to the asymptote for the percentage circular moving cells over all cells or percentage of motile cells (d value in equation 2) was negatively correlated (- .5) with the rate of increase to the asymptotic value of cNR56 (Table 5). When this relationship was studied in more detail (figure 2, 20 bulls) the best relationship was linear and explained about 25% of the variation present. The rate of increase to the asymptotic value of the percentage circular moving cells of the motile spermatozoa population or the percentage motile cells was used for the estimation of $N_{95}$. For bulls with extreme results for rate of increase to the asymptotic value of

cNR56 or $P_{CR}$, the number of sperm cells estimated to be required to obtain 95% of the asymptotic value of cNR56 or $P_{CR}$ was outside the confidence interval. Therefore motility characteristics in relation to concentration are of limited use for the prediction of $N_{95}$.

References

Budworth, P.R., Amann, R.P. and Chapman, P.L., 1988. Relationships Between Computerized Measurements of Motion of Frozen-Thawed Bull Spermatozoa and Fertility. Journal of Andrology, 9: 41-54.

Budworth, P.R., Amann, R.P. and Hammerstedt, R.H.A., 1987. Microcomputer-Photographic Method for Evaluation of Motility and Velocity of Bull Sperm. J. Dairy Sci., 70: 1927-1936.

Cellsoft User Manual, 1986. Version 3.0. Copyright CRYO Resources Ltd.

Foote, R.H., 1988. Preservation and fertility prediction of spermatozoa. International Congress on Animal Reproduction vol 5: 127-134.

Genstat 5 committee, 1993. Genstat 5 release 3 reference manual. Edt. Payne, R.W. and Lane, P.W., Cralendon Press, Oxfort, UK.

Grossman, M., Koops, W.J. and den Daas, J.H.G., 1995. Multiphasic Analysis of Reproductive Efficiency in Dairy Bulls. J. Dairy Sci. 78: 2871-2876.

Knuth, U.A., Yeung, C.H. and Nieschlag, E., 1987. Computerized Semen Analysis: Objective Measurement of Semen Characteristics is Biased by Subjective Parameter Setting. Fertility and Sterility, 48: 118-124.

O'Connor, M.T., Amann, R.P. and Saacke, R.G., 1981. Comparison of Computer Evaluations of Spermatozoal Motility with Standard Laboratory Test and Their Use for Predicting Fertility. J. Anim. Sci., 53: 1368-1376.

Pace, M.M., et al., 1981. Effects of Thawing Temperature, Number of Spermatozoa and Spermatozoal Quality on Fertility of Bovine Spermatozoa Packaged in 5-ml French Straws. J. Anim. Sci., 53: 693-701.

Rath, D.,Weitze, K.F., Armbrecht, S. and Bielfeld, P., 1987. Einjährige Erfahrungen mit einem Videomikrographiesystem (Cellsoft) zur Spermabeurteilung. Deutsche Tierärztliche Wochenschrift, 94: 501-503.

Rikmenspoel, R. and van Herpen, G., 1957. Photoelectric and cinematographic measurements of the motility of bull sperm cells Phys. Med. Biol. Ser I 1: 54-63.

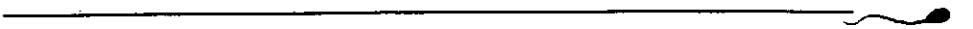
Saacke R.G., Nadir, S. and Nebel, R.L., 1994. Relationship of semen quality to sperm transport fertilization and embryo quality in ruminants. Theriogenology, 41: 45-50.

Saacke, R.G., 1982. Semen quality in relation to semen preservation. J. Dairy Sci, 66: 2635-2644.

Stalhammar, E.M., Janson, L. and Philipsson, J., 1994. The impact of Sperm Motility on Non-return Rate in preselected dairy bulls. Reprod Nutr. Dev. 34: 37-45.

Uwland, J., 1984. Possibilities and Limitations of Semen Evaluation for the prognosis of Male Fertility. In: The male in Farm Animal Reproduction. Edt: Courot, A. Martinus Nijhoff Boston MA. USA: 269-289.

Woelders, H., 1991. Overview of in vitro methods for evaluation of semen quality. Edt: Johnson, L.A. and Rath, D. Reproduction of Domestic Animals. Proceedings of the Second International conference Boar Semen Presentation, Beltsville, MD, 20-22 Aug. 1990, Paul Parey, Berlin, Suppl. 1: 145-164.



Chapter 7

# PREDICTION OF REPRODUCTIVE EFFICIENCY OF INDIVIDUAL BULLS BY IN VITRO MEASUREMENT OF VIABILITY AND CAPACITATION DURING POST THAW INCUBATION OF SPERMATOZOA

J.H.G. den Daas[1]

A.M. van Wagtendonk-de Leeuw[1]

L.M.T.E. Lansbergen[1]

and

H. Woelders[2]

[1] Holland Genetics

P.O. Box 5073

6892 EB Arnhem

The Netherlands

[2] Institute for Animal Science and Health (ID-DLO)

P.O. Box 65,

8200 AB Lelystad

The Netherlands

In preparation for Journal of Andrology

Abstract

A concentration range of 2.5 to 15 million total spermatozoa per insemination of semen of 20 individual bulls was used to study the relationship between in vitro capacitation and insemination results. In this study in vitro capacitation was defined as the competence of spermatozoa to perform an induced true acrosome reaction (AR) during post thaw in vitro incubation at 39°C. AR was induced at 0, 2 or 4 hours of incubation by adding 100 µg/ml lysophosphatidylcholine (LPC). For determination of the true AR, the fix-vital stain method (de Leeuw et al, 1991) was used. Insemination results of a bull in relation to concentration were characterized by an exponential curve (chapter 5 of this thesis). The rate of increase ($b_{RATE}$) and the asymptotic level ($a_{ASYM}$) of this exponential curve were calculated per bull for both corrected non-return rates at 56 days (cNR56) and estimated conception rate ($P_{CR}$). The relationship between calving rate given conception ($P_{CALVR}$) and in vitro capacitation was also studied. In vitro capacitation patterns were differed greatly between bulls. A high percentage of competent to AR intact cells after thawing combined with low percentage cells showing AR after LPC trigger at 4 hours of incubation was found for bulls who require a low number of spermatozoa per insemination to achieve 95% of the asymptotic value for cNR56 of $P_{CR}$. The prediction of $b_{RATE}$ by multiple regression on parameters for the in vitro capacitation pattern appears to be reliable since the correlation between estimated and measured $b_{RATE}$ was 0.55. If no information of performance of the bull in the field is available, determination of the percentage competent to AR intact cells direct post thaw and the percentage of cells showing AR upon addition of LPC at 4 hours of incubation can be used to predict the optimal semen dose for that bull.

## Introduction

Cattle breeding industry today selects the most valuable bulls based upon the production of their daughters. Once bulls are selected, their valuable genes need to be

disseminated into the population as efficiently as possible. Therefore maximization of the number of insemination doses per ejaculate of top bulls, without compromising pregnancy rates is required.

In the last two decades the relationship between the number of spermatozoa inseminated and insemination results was frequently studied. In general, when grouping bulls, an exponential relationship was established between the number of spermatozoa inseminated and insemination results, measured as the fraction of cows inseminated that did not return to service within a certain number of days ( Pace, et al., 1981; Uwland, 1984; den Daas, 1992). Den Daas et al. (chapter 5 of this thesis) studied this relationship for 20 bulls with at least 4500 inseminations each and a range of concentrations of 2.5 to 15 million total spermatozoa testing the bulls individually. Reproductive efficiency was measured either by the corrected non-return rate at 56 days (cNR56) (chapter 3 of this thesis) or by estimated conception rate ($P_{CR}$) and estimated calving rate given conception ($P_{CALVR}$), based on daily non-return rates up to 180 days after insemination (chapter 4 of this thesis). The relationship between the number of spermatozoa inseminated and cNR56 or $P_{CR}$ can be described by an exponential function, characterized by its asymptote, i.e. the maximum level at high sperm dosage ($a_{ASYM}$), and the rate of increase to this level ($b_{RATE}$). These parameters differed significantly between bulls and were used to estimate the number of spermatozoa required per insemination to obtain optimal reproductive efficiency, which was defined as the number of spermatozoa required to obtain 95% of $a_{ASYM}$. Field experiments for individual bulls to determine $a_{ASYM}$ and $b_{RATE}$ are expensive and time consuming. Therefore easy-to-perform laboratory tests to predict the parameters $a_{ASYM}$ and $b_{RATE}$ for cNR56 or $P_{cr}$ are desirable.

In a recent study (chapter 6 of this thesis) we have shown that various motility characteristics, determined by multi exposure video taping, have only a limited predictive value for $a_{ASYM}$ and $b_{RATE}$.

Spermatozoa need to undergo capacitation before they are competent enough to perform the acrosome reaction (AR) and to penetrate an oocyte (Florman and First, 1988). Capacitation normally takes place in the female reproductive tract and appears to be time related (Hunter and Wilmut, 1984). We can hypothesize that bulls that produce semen

capable of maintaining a competent population of spermatozoa at the site of fertilization over a long time after insemination, are the ones requiring relatively low numbers of spermatozoa per insemination for optimal reproductive efficiency. Thus, it could be that the time course of capacitation is related to the fertility characteristics of a bull.

The aim of this study is to test the hypothesis that the variation in in vitro capacitation pattern among bulls relates to the earlier observed variation in parameters $a_{ASYM}$ and $b_{RATE}$ for cNR56 and $P_{CR}$ which describe the relationship between sperm dose and reproductive efficiency. The in vitro capacitation pattern is defined as the competence of frozen-thawed viable spermatozoa to undergo the acrosome reaction (AR) induced by the addition of lysophosphatidylcholine (LPC) at 0, 2 and 4 hours of in vitro incubation at physiological temperature (39°C).

## *Materials and Methods*

### Semen

Semen from the same bulls and the same ejaculates was used as in the original study of den Daas et al. (chapter 5 of this thesis) describing the relationship between insemination dose and fertility per bull. In this original study ejaculates were split sampled in order to obtain concentrations of 2.5 to 15 million total spermatozoa per straw. Straws were frozen as described in chapter 5 of this thesis. To study in vitro capacitation patterns of individual bulls a total of six straws of two or three ejaculates were used (10 to 15 million total spermatozoa per straw). Straws were thawed in a waterbath at 37°C for 30 seconds. The straws were pooled and washed free of seminal plasma and extender as previously described (de Leeuw et al., 1991). Pellets were re-suspended in sperm-TALP (Parrish et al., 1988). The spermatozoa concentration was standardized at 50 x $10^6$ spermatozoa/ml.

### Preparation of lysophosphatidylcholine (LPC)

Egg yolk LPC (Sigma, Chemical Co., St Louis, MO) was prepared according to Parrish *et al.* (1988). Briefly, the egg yolk LPC was dissolved in methanol and stored at -20°C. At the

day of use an aliquot was removed and the methanol was evaporated using nitrogen gas. The LPC was re-dissolved in sperm-TALP (stock solution). The concentration of LPC in this stock solution was 10 mg/ml.

In vitro capacitation pattern

To study the capacitation pattern 100 µl aliquots of thawed washed spermatozoa were put into 1.5 ml capped polypropene tubes and incubated in a waterbath at 39°C for 0, 2 or 4 hours (three tubes per time/treatment). After 0, 2 and 4 hours of incubating, 1 µl of the stock, LPC solution was added to two of the three tubes (final concentration: 100 µg/ml). The third tube served as a control. After an incubation of 1, 2.5, 5, 7.5, 10 and 20 minutes for LPC-challenged tubes and 0 and 20 minutes for control tubes, 10 µl of cell suspension was removed and processed according to the fix-vital stain method (de Leeuw et al., 1991). Using this method, procedural loss, selection, or damage of cells is avoided. The method enables differentiation between viable acrosome intact sperm (A), viable acrosome reacted sperm (B), non-viable acrosome intact sperm (C) and non-viable acrosome reacted spermatozoa (D). Two hundred cells were evaluated per sample of the duplicate incubations and two times two hundred cells were evaluated for the control incubation. For three bulls the procedure was repeated on another day to study test day influences. True acrosome reaction is defined as the decrease in percentage viable acrosome intact cells after LPC induction of the acrosome reaction and a concomitant increase of viable acrosome reacted cells.

Data evaluation

Data were statistically evaluated using Genstat (1993). The Genstat stepwise regression analysis procedure Rselect (Goedhart and Thissen, 1992) was used in order to study the predictive value of the capacitation pattern of a bull for prediction of insemination results. Fertility parameters this study attempted to predict were: rate of increase ($b_{RATE}$) to the asymptotic level ($a_{ASYM}$) of the exponential curve describing the relationship between insemination results and sperm dose per bull. The insemination result is given as corrected non-return rate at 56 days (cNR56) while the estimated conception rate ($P_{CR}$) and calving rate

given conception was calculated according to Grossman et al. (chapter 4 of this thesis). Independent variables describing the in vitro capacitation pattern were: the percentage viable cells with an intact acrosome immediately after thawing ($A_0$); the decrease of this percentage after 2 or 4 hours incubation at 39°C (no LPC) and the absolute ($A_0$-Alpc) and relative percentage of viable and acrosome intact cells ($100/A_0$ X ($A_0$-Alpc) that reacted after LPC addition at different times of incubation. Independent variables were added or dropped on the basis of P values $\leq 0.05$.

## Results

### In vitro capacitation

Figure 1 shows the decrease in the percentage viable acrosome intact spermatozoa and the concomitant increase of percentage viable acrosome reacted cells for the various in vitro induction intervals averaged over bulls. Over the 4 hour incubation period, a loss of viable cells that have an intact acrosome (A) from 68 to 36% was observed. The decrease in percentage viable acrosome intact cells coincided with a concomitant increase of non-viable cells (C+D) from 28 to 42% and an increase of percentage viable acrosome reacted cells (B) from 3 to 22% (figure 1).

After addition of LPC the induction of true acrosome reaction was seen immediately. In all cases the maximum response was obtained within one minute after addition of LPC. The response in samples 1 to 20 minutes after addition of LPC were not significantly different ($P>0.01$). Therefore the mean values were calculated of the percentages of cells categorized as A, B, C or D obtained at the different time points, to characterize the reaction of the semen upon LPC trigger at 0, 2 or 4 hours after incubation.

There was no significant day to day variation. The average standard deviation of percentage of viable, acrosome intact spermatozoa of duplicate incubations was 3.5% on the same day and 4.6% on different days.

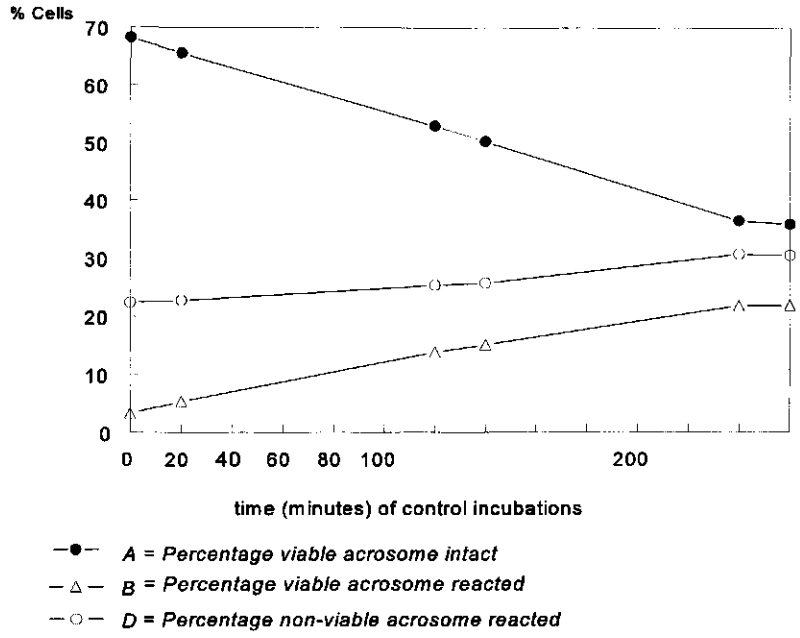



*Figure 1. The change in percentage viable and acrosome intact cells during control incubation. Average change over the 20 bulls tested.*

The average increase of viable acrosome reacted cells during the control incubation in the absence of LPC at 0, 2 and 4 hours of incubation was 2.4%, 1.0% and 0.1% respectively. This relatively small increase was consequently neglected for the rest of this study.

*Table 1. Percentage viable acrosome intact cells (cat. A) during incubation at 39° C for 0, 2 and 4 hrs and the relative percentage of these cells that react on LPC addition.*

| bull | percentage of cells of control | | | relative percentage reacting on LPC | | |
|---|---|---|---|---|---|---|
| | 0 h | 2 h | 4 h | 0 h | 2 h | 4 h |
| 1 | 63.0 | 47.0 | 24.3 | 23.6 | 19.7 | 25.6 |
| 2 | 73.5 | 59.2 | 41.0 | 25.5 | 13.3 | 54.2 |
| 3 | 66.0 | 40.0 | 30.5 | 19.3 | 10.3 | 31.5 |
| 4 | 80.2 | 63.0 | 39.0 | 26.4 | 34.9 | 21.4 |
| 5 | 70.0 | 50.5 | 33.7 | 10.2 | 5.9 | 22.8 |
| 6 | 75.5 | 54.0 | 49.5 | 12.9 | 15.4 | 28.2 |
| 7 | 76.0 | 59.2 | 30.5 | 8.3 | 15.1 | 54.7 |
| 8 | 69.0 | 51.0 | 37.5 | 15.9 | 22.7 | 37.1 |
| 9 | 70.2 | 54.5 | 46.2 | 13.0 | 20.3 | 50.3 |
| 10 | 64.0 | 50.5 | 31.8 | 9.0 | 40.9 | 22.3 |
| 11 | 66.5 | 37.0 | 31.8 | 14.8 | -3.9 | 26.6 |
| 12 | 64.0 | 57.0 | 41.7 | -0.5 | 20.7 | 46.3 |
| 13 | 67.2 | 60.0 | 50.7 | 10.3 | 20.3 | 59.0 |
| 14 | 77.0 | 67.7 | 34.0 | 11.0 | 18.8 | 66.3 |
| 15 | 65.5 | 43.2 | 41.7 | 16.4 | 18.8 | 61.8 |
| 16 | 56.2 | 54.7 | 25.0 | 14.1 | 47.8 | 29.3 |
| 17 | 68.5 | 54.0 | 36.5 | 14.9 | 22.0 | 56.5 |
| 18 | 69.5 | 56.0 | 34.0 | 19.2 | 16.7 | 30.4 |
| 19 | 72.2 | 59.5 | 41.2 | -1.1 | 4.5 | 37.6 |
| 20 | 51.0 | 40.5 | 28.5 | 12.7 | 17.1 | 62.4 |

The initial (post thaw) percentage viable acrosome intact cells, and the decrease of that percentage during incubation in vitro was bull specific (see table 1). During LPC treatment the percentage viable acrosome intact cells was markedly reduced, and this coincided with an increase in the percentage viable acrosome reacted spermatozoa (figure 2). After LPC addition the number of viable acrosome reacted cells increased after 0, 2 and 4 hours of incubation. The relative percentage of cells that react on LPC was defined as $(A_{CONTR}-A_{LPC}) \times 100/A_{CONTR}$ (table 1). During incubation the absolute and relative percentage of viable cells that acrosome react upon LPC addition increased. These relative percentages are on average 13.9, 19.6 and 42.2 at 0, 2 or 4 hours of incubation at 39°C respectively (table 1).

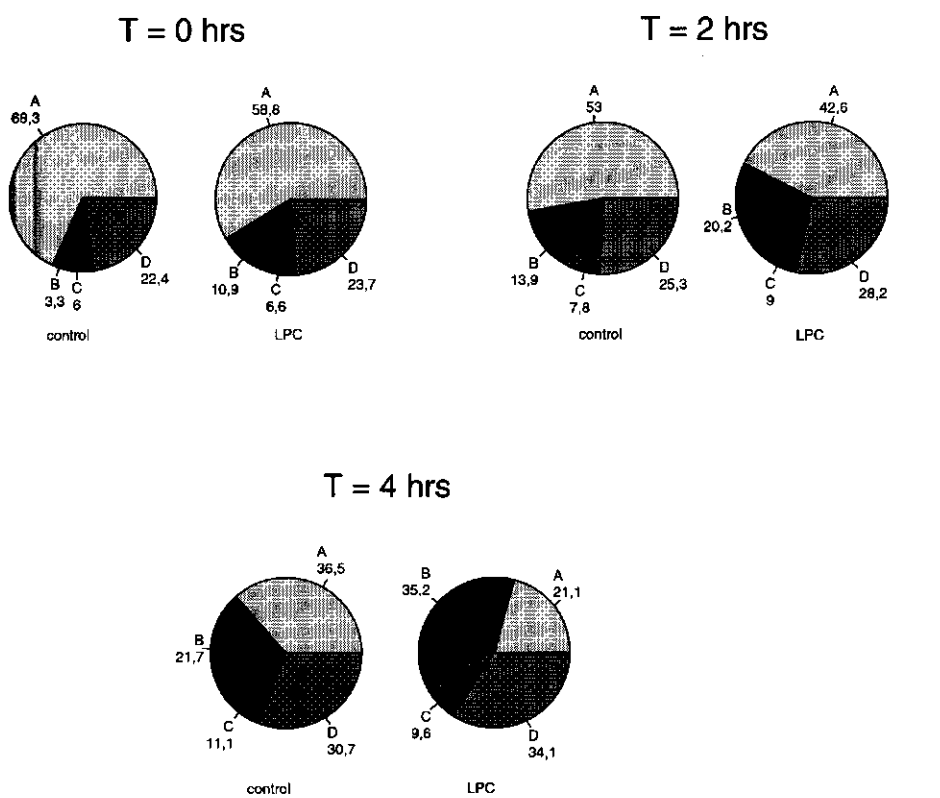



*Figure 2. Distribution of percentage viable, non-viable, acrosome intact and acrosome reacted cells before and after LPC addition after incubation at 39°C at 0, 2 and 4 hrs.*

*Table 2. Correlations between the percentage viable acrosome intact cells (A) at 0, 2 and 4 hrs after incubation at 39° C and after the LPC included AR relations and reproduction efficiency characteristics.*

| | percentage of cells of control | | | percentage of cells reacting on LPC | | | relative percentage of cells reacting on LPC | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0h | difference 0-2h | difference 0-4h | at 0h | at 2h | at 4h | 0h | 2h | 4h |
| acNR56 | -0.306 | -0.154 | -0.150 | -0.089 | 0.189 | 0.080 | -0.081 | 0.247 | 0.038 |
| $aP_{CR}$ | -0.283 | -0.173 | -0.258 | 0.034 | -0.002 | 0.085 | 0.065 | 0.120 | 0.008 |
| $P_{CALVR}$ | 0.237 | -0.352 | -0.258 | 0.053 | 0.121 | 0.435* | -0.011 | -0.050 | 0.302 |
| bcNR56 | -0.345 | -0.246 | -0.373 | -0.356 | -0.248 | 0.268 | -0.257 | -0.084 | 0.289 |
| $bP_{CR}$ | -0.373 | -0.281 | -0.495* | -0.388* | -0.208 | 0.335 | -0.298 | -0.024 | 0.36 |

** P<0.05*

*acNR56 = asymptotic value for cNR56*
*$aP_{CR}$ = asymptotic value estimated conception rate*
*$P_{CALVR}$ = calving rate given conception*
*bcNR56 = rate of increase to asymptote for cNR56*
*$bP_{CR}$ = rate of increase to asymptote for estimated conception*

The in vitro capacitation pattern in time after LPC triggering is bull specific. In figure 3 this is shown for ten bulls. Percentage viable acrosome intact cells ranged from 80.2 to 51.0 % at thawing and from 50.7 to 24.3% after 4 hours of incubation (table 1). The relative percentage of viable acrosome intact cells that performed the acrosome reaction after LPC addition ranged from 0 at the start of incubation to 66.3% after 4 hours of incubation (table 1). Figure 3 illustrates these capacitation patterns for ten bulls. The loss of acrosome intact cells during incubation as well as the capability to acrosome react after an LPC addition at different times of incubation is different between bulls (Anova, $P<0.05$).

Predictive value of in vitro capacitation parameters for reproductive efficiency

Because of the risk of confounding effects, only results pertaining to viable acrosome intact cells are taken into account in the remaining part of this study. Table 2 shows the correlations between in vitro capacitation pattern and insemination results. The correlation between change in % A, in control and LPC samples at 0, 2 and 4 hours after the onset of incubation and the values of $a_{ASYM}$ for, cNR56 and $P_{CR}$ were rather low and never significant. This was also true for correlations between, the absolute and relative percentages of viable cells that react after LPC addition and $a_{ASYM}$. There was a significant correlation between the percentage reacting cells on LPC addition after 4 hours of incubation and calving rate given conception ($P_{CALVR}$). The rate of increase to the asymptotic level of the estimated conception rate ($b_{RATE}P_{CR}$) is negatively correlated ($P<0.05$) to the decrease in the percentage of viable acrosome intact cells during incubation and to the decrease in percentage viable acrosome intact cells after LPC AR induction immediately after thawing.

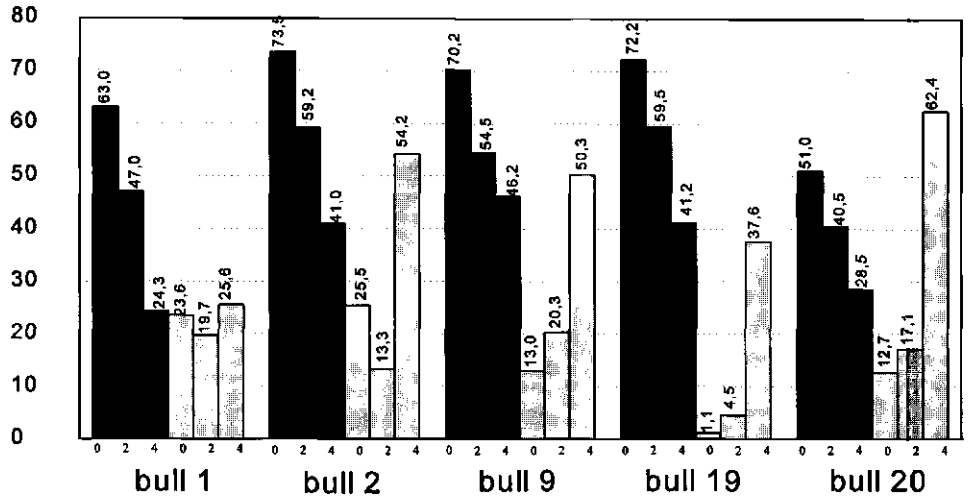



*Figure 3A.*

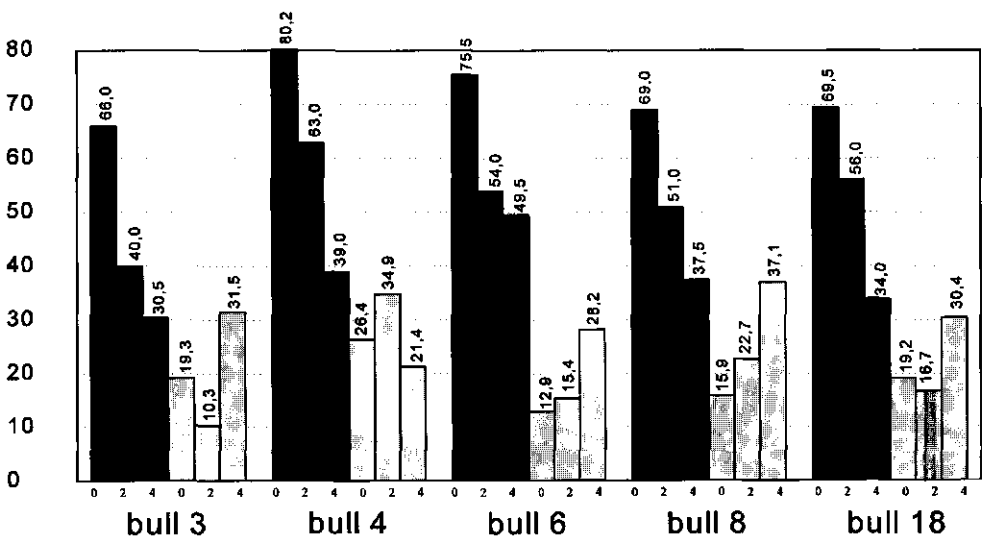



*Figure 3B.*

= Percentage viable acrosome intact cells at 0, 2, and 4 hours

= Relative reaction after LPC induction of AR at 0, 2, and 4 hours

*Figure 3. The percentage viable acrosome intact cells at 0, 2 and 4 hours and the relative reaction after LPC induction for 10 bulls. A: Bulls selected for high dose rate dependence. B: Bulls selected for low dose rate dependence.*

*Table 3. Regression models and correlation between estimated and measured fertility estimates*

| factors | P values |
|---|---|
| $a_{asymp}$cNR56 = 0.946 -0.675*$A_0$ +0.709*$A_4$ +1.700*$A_{4LPC}$ -0.006*$rA_{4LPC}$ | |
| $A_0$ | 0.004 |
| $A_4$ | 0.006 |
| $A_{4LPC}$ | 0.002 |
| $rA_{4LPC}$ | 0.003 |
| | R2adj=35.1 |
| $b_{RATE}$ cNR56= 0.930 - 1.210*$A_0$+ 0.886*$A_{4LPC}$ | |
| $A_0$ | 0.012 |
| $A_{4LPC}$ | 0.050 |
| | R2adj=32.1 |
| $b_{RATE}$ $P_{CR}$ = 0.971 - 1.2145*$A_0$+ 0.989*$A_{4LPC}$ | |
| $A_0$ | 0.011 |
| $A_{4LPC}$ | 0.031 |
| | R2adj=35.3 |

*$A_0$ = percentage cells of category A of control at 0 h*
*$A_4$ = difference between percentage cells of category A of control at 0 h and 4 h*
*$A_{0LPC}$ = percentage cells of category A reacting on LPC at 0 h*
*$rA_{4LPC}$ = relative percentage cells of category A reacting on LPC at 4 h*
*$A_{4LPC}$ = percentage cells of category A reacting on LPC at 4 h*
*$a_{ASYM}$cNR65 = asymptotic value for cNR56*
*$b_{RATE}$cNR56 = rate of increase to asymptote for cNR56*
*$b_{RATE}$Pcr = rate of increase to asymptote for estimated conception rate*

The RSELECT procedure of Genstat (1993) was used to establish estimated regression relationship between the reproductive efficiency and the in vitro capacitation parameters. Table 3 shows the regression models $b_{RATE}$ for cNR56 and $P_{CR}$ , respectively, and for $a_{ASYM}$ of cNR56. Only the variables which made a significant contribution to the model are shown ($P< 0.05$). Adjusted $R^2$ were 32.1 and 35.3 for $b_{RATE}$ to NR56 and $P_{CR}$ respectively. The adjusted $R^2$ is based upon the residual mean squares instead of the residual sum of squares and it is stated that this is usually a better guide to the fit of a model (Genstat 1993). Bulls

who require relatively few spermatozoa to reach 95% ($N_{95}$) of the asymptotic values for cNR56 or $P_{CR}$ (low $b_{RATE}$) are characterized by a high percentage of viable acrosome intact cells after thawing and low percentage of cells performing acrosome reaction after LPC treatment at 4 hours of incubation (see figure 3 b). The correlation between estimated and measured $b_{RATE}$ was 0.55 ($P \leq 0.01$) for both cNR56 and $P_{CR}$. The maximal percentage adjusted variance ($R^2$) estimated by the model to predict $a_{ASYM}$ cNR56 was 35.1. The asymptotic level for cNR56 was explained by the percentage of viable acrosome intact cells (A) after thawing, the difference between category A at 0 and 4 hours of incubation and the relative and absolute data after LPC treatment at 4 hours of incubation at 39°C. Bulls with semen which is characterized by a low number of viable acrosome intact cells after thawing, a high number of cells that either acrosome react or lose viability during incubation and a high percentage of cells reacting after LPC treatment at 4 hours of incubation are the bulls with a low asymptotic value for cNR56. The correlation between estimated $a_{ASYM}$ and measured $a_{ASYM}$ was 0.63 ($P \leq 0.01$) for cNR56. Examples of bulls which show the effects as described are bulls 9 and 20 in figure 3a.

## Discussion

The *in vitro* capacitation pattern, both spontaneous and induced differs significantly between individual bulls. Not only is the percentage of viable acrosome intact spermatozoa after thawing bull specific, but also its decrease during 4 hours in vitro control incubation at 39°C. Bulls also differ with regard to the percentage of viable intact cells that are able to acrosome react after LPC induction at 0, 2 and 4 hours after the onset of the incubation. Parrish et al. (1988) demonstrated that for fresh semen, incubation of spermatozoa with a capacitating agent like heparin is required before these cells are able to acrosome react upon a LPC trigger or are able to fertilize oocytes. Ax and Lenz (1987) also used other glycosaminoglycans as capacitating agents to evaluate differences in the frequency of acrosome reaction between bulls. These authors also highlighted differences between bulls in the % acrosome reacting cells from 1 to 20 minutes after LPC addition, again for fresh

semen. Davis and Foote (1987) showed a time related acrosome reaction of frozen-thawed sperm triggered by 7 or 15 minutes incubation with PC12 (dilaurylphosphatidylcholine). Our results indicate an immediate effect of LPC (maximum response 1 minute after addition of LPC) at each time of incubation for all bulls. Moreover, in contrast to the findings of Parrish et al. (1988) and Davis and Foote (1987), our results were obtained without using heparin or any other capacitating agent. The differences found between our results and those of Davis and Foote (1987) and Parrish et al. (1988) respectively, could be caused by the processing and freezing of semen.

Before freezing, semen is exposed for several hours to tris-egg yolk extender which might act as a capacitating agent. Egg yolk contains a wide range of proteins and all kinds of (lyso) lipids, probably including glycosaminoglycans. Glycosaminoglycans are also present in oviductal fluids which are known to have a capacitating effect (McNutt and Killian, 1991). When semen is frozen there is a strong dehydration effect caused by the freezing of the surroundings of the cells. Hypertonic solutions are known to have destabilizing effects on membranes and this may be another reason why spermatozoa seem to be more ready to perform the acrosome reaction after freeze-thawing (Hammerstedt et al., 1990).

Mammalian spermatozoa undergo capacitation in the female reproductive tract before they are fully competent to perform the acrosome reaction and complete fertilization (Florman and First wasserman, 1988). The istmic part of the oviduct is considered to be the compartment of the female reproductive tract where sperm cells are 'stored' for capacitation (Hunter and Wilmut, 1984) after which they become hyperactive and swim towards the ampulla where they may meet the ovulated oocytes (Suarez et al., 1990). We have attempted to mimic this situation in vitro by estimating the population of sperm cells that are viable and able to acrosome react spontaneously or after LPC induction at certain time periods during incubation at 39 C in sperm Talp. We refer to the profiles thus obtained as the capacitation pattern.

The individual bulls in this study differ in a) the number of spermatozoa that are viable after freeze thawing, b) the extent of a spontaneous AR during incubation and c) the number of spermatozoa that demonstrate true AR after LPC triggering. Saacke and White (1972)

reported a positive correlation between the percentage of spermatozoa with an intact acrosome and fertility of the bulls. Fertility was measured at the non-return rate at 60-90 days using a high number of spermatozoa per insemination. Ax et al. (1987) showed a positive correlation between NR rate at high dose rates and the effect of glycosaminoglycans on fresh semen. Whitfield et al. (1995) related a high percentage of frozen thawed cells that exhibited the acrosome reaction after calcium ionofoor (A23187) induction to a high 90-d non-return rate at high dose rates. In this study we could not confirm this relationship, the correlation between the asymptote for cNR56 and the percentage viable acrosome intact cells after freeze-thawing was -0.3 and not significant (see table 2). The conclusions of these respective studies are not supported by our present observations. Moreover, their findings cannot be easily explained by existing models of fertilization ( Pace et al., 1981; Schwartz et al., 1981; Saacke et al. 1994), since it is generally assumed that the maximum level of fertilization is not influenced by the percentage of 'fit' spermatozoa. Instead the asymptotic level is thought to be influenced by the non compensable (for number of sperm inseminated) sperm factors by Saacke et al. (1994) and by den Daas (1992) in which publication it is referred to as being influenced by intrinsic sperm factors.

The hypothesis that bulls with a capacitation pattern which is characterized by a high percentage of viable acrosome intact cells after thawing and low number of spermatozoa ready to show AR after LPC tigger at 4 hours of incubation, need a relatively low number of spermatozoa to be inseminated in order to obtain acceptable fertility results was proven by this study (see table 3 and figure 3b).

It is possible to predict rate of increase to the asympotic level ($b_{RATE}$) of the study of den Daas et al (chapter 5) for individual bulls through the assessment of the number of spermatozoa surviving freeze thawing and the number of spermatozoa showing true AR after LPC treatment at 4 hours of incubation (table 3). The correlations between $b_{RATE}$ and percentage viable intact cells after freeze-thawing and the percentage reacting upon LPC induction at 4 hours of incubation, respectively, were insignificant (see table 2).

From a practical point of view, the observed differences in in vitro capacitation patterns between bulls may be used to predict the minimal dose of semen necessary to achieve

optimal reproductive efficiency. This prediction is relatively reliable and if no information of performance in the field can be obtained through monitoring of the bulls' non-return results, it can be used for prediction of this minimal dose. If there is a possibility to monitor reproductive efficiency in the field the capacitation pattern of an individual bull can be used to choose the range of dosages to be screened in a field trial for that bull.

References

Ax, R.L. and Lenz, R.W., 1987. Glycosaminoglycans as Probes to Monitor Differences in Fertility of Bulls. J. Dairy Sci., 70: 1477-1486.

Davis, A.P. and Foote, R.H., 1987. Relationship of Sire Fertility to Acrosome-Reacted and Motile Spermatozoa After Treatment with Liposomes. J. Dairy Sci., 70: 850-857.

De Leeuw, A.M., den Daas, J.H.G., and Woelders, H., 1991. The Fix Vital Stain Method Simultaneous Determination of Viability and Acrosomal Status of Bovine Spermatozoa. J. of Andrology, 12: 112-118.

Den Daas, J.H.G., 1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci., 28: 87- 95.

Florman, H.M. and First, N.L., 1988. The Regulation of Acrosomal Exocitosis. Developmental Biology, 128: 453-463.

Genstat 5 committee, 1993. Genstat 5 release 3 reference manual. Edt. Payne, R.W. and Lane, P.W., Cralendon Press, Oxford, UK.

Hammerstedt, R.H., Graham, J.K. and Nolan, J.P., 1990. Cryopreservation of mammalian sperm: What we ask them to survive. J. of Andrology, 11: 73-88.

Hunter, R.H.F., and Wilmut, I., 1984. Sperm transport in the cow: peri-ovulatory redistribution of viable cells within the oviduct. Reprod. Nutr. Developm, 24: 597-608.

McNutt, T.L. and Killian, G.J., 1991. Influence of Bovine Follicular and Oviduct Fluids on Sperm Capacitation in Vitro. J. of Andrology, 12: 244- 252.

Pace, M.M., Sullivan, J.J., Eliot, F.I., Graham, E.F. and Coulter, G.H., 1981. Effects of Thawing temperature, Number of Spermatozoa and Spermatozoa quality on fertility of bovine spermatozoa packaged in .5 ml french straws. J. Anim. Sci., 53: 693-701.

Parrish, J.J., Susko-Parrish, J., Winer, M.A. and First, N.L., 1988. Capacitation of Bovine Sperm by Heparin. Biology of Reprod., 38: 1171-1180.

Saacke, R.G. and White, J.M., 1972. Semen quality tests and their relationship to fertility. Proc. IVth Tech. Conf. Anim. Reprod., NAAB: 22-27.

Saacke, R.G., Nadir, S. and Nebel, R.L., 1994. Relationships if semen quality to sperm transport, fertilization and embryo quality in ruminants. Therio., 41: 45-50.

Suarez, S.S., Drost, M., Redfern, K. and Gottlieb, W., 1990. Sperm motility in the oviduct. In: Fertilization in mammals Edt. B.D. Bavister, J. Cummins and E.R.S. Roldan. Serono Symposia USA, Norwell MA: 111-124.

Schwartz, D., MacDonald, D.P.M. and Heuchel, V., 1981. On the relationship between the number of spermatozoa and the probability of conception. Reprod. Nutr. Dev., 21: 979-988.

Uwland, J., 1984. Possibilities and limitations of semen evaluation for the prognosis of male fertility. In: The male in farm animal reproduction. Edt. A. Courot, Martinus Nijhoff Boston Ma. USA.: 269-289.

Whitfield, C.H. and Parkinson, T.J., 1995. Assessment of the fertilizing potential of frozen bovine spermatozoa by in vitro induction of acrosome reactions with calcium ionophore (A23187). Therio., 44: 413-422.

Chapter 8

# EFFECTS OF BOVINE SPERM AND OOCYTE CONCENTRATION ON HOMOLOGOUS OOCYTE PENETRATION IN VITRO: AN EVALUATION OF THE RELATIONSHIP WITH IN VIVO FERTILITY

J.H.G. den Daas[1]

Y.A. Wurth[2]

and

Th.A.M. Kruip[3]

[1] Holland Genetics

P.O. Box 5073

6892 AB Arnhem

The Netherlands

[2]Koekkoeksbloem 62

3984 CL Odijk

The Netherlands

[3] Institute for Animal Science and Health (ID-DLO)

P.O. Box 65

8200 AB Lelystad

The Netherlands

In preparation for Journal of Reproduction and Fertility

Abstract

The aim of this study was to develop an in vitro test for the prediction of fertility of bulls. Bovine oocyte penetration rate was assessed using different concentrations of sperm and oocytes. Bovine cumulus oocyte complexes retrieved from slaughterhouse ovaries were matured and incubated with spermatozoa in vitro. The oocytes were fixed overnight and stained with aceto-orcein after 18 hours of incubation with spermatozoa. Both oocyte and sperm concentration affected the number of oocytes penetrated. Five bulls of which the in vivo fertilization behavior with different insemination doses was known, were tested for in vitro penetration rates at 400 to 4,000 spermatozoa per oocyte. Bulls were found to vary in the total number of oocytes penetrated as well as in the increase of the penetration rate after raising the sperm oocyte ratio. The variation found in vivo and in vitro did not correlate. Therefore oocyte penetration rate in vitro cannot be used for the prediction of fertility results after insemination.

(Keywords: bovine semen, oocyte penetration, in vitro fertilization, field fertility)

## Introduction

In bovine AI-programs ejaculates are evaluated before further processing to breeding units. In the Netherlands selection criteria for ejaculates are: a minimal volume of 2 ml, a minimal concentration of 300 million spermatozoa per ml and 70% morphologically normal cells. An inventory in the Netherlands revealed that only 660 out of 75,238 ejaculates, from bulls in regular production, did not meet these criteria (van Os et al., accepted). Moreover, in order to be able to select on daughters' performance; a certain level of fertility (offspring) is a prerequisite. In the Netherlands about 7% of the bulls fail to fulfill this prerequisite.
The remaining variation in non-return (NR) rates of different bulls (from 10-15%) (Marquant-leguinne et al., 1990; Killian et al., 1993; den Daas et al., chapter 5) is the result of

compensable (i.e. dose dependent) and uncompensable factors in frozen semen (Den Daas, 1992; Saacke et al., 1994; Shannon and Vishwanath, 1995). Den Daas et al. (chapter 5) showed that the compensable factors form a major source of variation between bulls. The number of spermatozoa needed to obtain a level of 95% of the estimated maximal conception rate ranges from less than 2.5 million to over 11.7 million per insemination dose per bulls (chapter 5 of this thesis). The variation in calving rate given conception (Den Daas et al. chapter 5) is 82 to 90%, and can be used as an indicator of 'uncompensable' variance. In bovine AI-programs most interest for prediction of fertility is focussed on estimation of the dose dependent variation.

In Vitro Fertilization (IVF) could be a way of predicting the in vivo fertilizing capacity of bull spermatozoa. In the routine IVF procedure in cattle, bulls are found to vary both in the oocyte penetration rate and the production rate of blastocysts (Marquant-Le Guienne and Humblot, 1992; Den Daas and Merton, 1994). The penetration rate in cattle IVF has been found to depend on both the sperm and heparin concentration (Eyestone and First, 1989; Marquant-Le Guinne et al., 1990). In addition, Siddiquey and Cohen (1982) for the mouse showed, that the volume of the fertilization droplet, the oocyte concentration and the sperm oocyte ratio are also relevant for the penetration rate.

The objective of this study is to determine whether the in vitro penetration rate can be used to predict reproductive efficiency characteristics of bulls. The variation in the in vitro penetration rate of zona-intact homologous oocytes was studied by fertilizing oocytes with frozen thawed spermatozoa of different bulls under standardized conditions. The source of the in vivo fertilization data is a field trial on dose dependent insemination results from Den Daas et al. chapter 5.

## Materials and Methods

### Development of standardized IVM/IVF conditions:

1) Development of the oocyte maturation standard slaughterhouse ovaries were transported at a temperature of 30 °C to the laboratory. Cumulus oocyte complexes (COC)

were aspirated from 2-8 mm follicles using an 18 gauge hypodermic needle. For oocyte maturation COC's were selected according to the following criteria: at least two intact layers of cumulus cells; oocytes of the quality classes I, II and III according to de Loos et al. (1989). Four media were tested for nuclear maturation inducing capacity,: a) M199 with 20% oestrus cow serum (ECS), b) M199 with 20% fetal calf serum (FCS), c) M199 with 20% FCS plus 10µg/ml FSH and d) Menezo B2 plus 10 g/ml FSH. COCs were incubated for 24 hours in micro droplets (50 µl) under sterile oil (silicon oil, Wachen GmbH, Munchen, FRG) at 39°C under 5% $CO_2$ in air. The maturation response was measured as the percentage of oocytes that reached the metaphase II stage within 24 hours of incubation.

2) Development of the standard treatment for spermatozoa

In order to study the variation in semen quality between bulls, the same population of cells should be brought into contact with the oocytes in vitro as is used for insemination in vivo. Spermatozoa can be preselected by performing a swim-up procedure and subsequent standardization of the concentration of sperm cells as is generally used in IVF practice and will mask differences between bulls. Therefore an experiment was performed in which mixed semen of three HF bulls was used for incubation with oocytes, after different pre-treatments post-thaw. After thawing sperm was extended in a HEPES buffered medium with BSA and treated according to:

1) a one hour swim-up and a concentration of 10 µg heparin in the fertilization droplet. (Parrish et al., 1986).
2) a swim-up of one hour
3) without any pre-treatment.
4) pre-treated with hexokinase and ATP, in order to exclude side effects of carbohydrates in the extender and a concentration of 10 µg heparin in the fertilization droplet.
5) a wash in PBS and centrifugation at 4,000 G for 4 min. over silicon oil and adding 10 µg heparin to the fertilization droplet.

In all treatment groups 50,000 spermatozoa were added to groups of 10 oocytes in a

fertilization droplet of 50 µl. The final concentration of spermatozoa was $1.10^6$ per ml. The routine procedure of Parrish et al. (1986) was used as an internal standard throughout all experiments because of possible day to day variations. After 18 hours of incubation with spermatozoa in modified TALP medium, oocytes were stripped from their cumulus cells by vortexing. The oocytes were subsequently mounted on object slides and fixed in methanol:acetic-acid (3:1, v/v). After overnight fixation, staining followed in 1% aceto-orcein and specimens were evaluated for the presence of two pronuclei of synchronous development, with particular reference to the male pronucleus. At 18 hours after the start of incubation the male pronucleus should have reached at least stage 4 (Xu and Greve, 1988). Polyspermy, which occured in less than 3% of the oocytes, was neglected.

Repeatability of the penetration test

In order to study day-to-day and within-day variation between duplicates of spermatozoa from the same ejaculate, spermatozoa of one bull were used in four consecutive duplicate incubation experiments at a ratio of 2,000 and 4,000 spermatozoa per oocyte. Oocytes were matured over 24 hours in Menezo's B2 medium supplemented with 10 µg FSH. The penetration percentage was evaluated after 18 hours of incubation and orcein staining of the oocyte as described above.

Influence of oocyte number

Two experiments were carried out to evaluate the influence of oocyte number on the penetration rate. In the first experiment the volume of the IVF droplet was 10µl, containing 1, 3, 5 or 10 oocytes and 5,000 spermatozoa per oocyte. Thus the spermatozoa concentration varied from $0.5x10^6$ to $5x10^6$ per ml. In the second experiment the IVF drop was 10µl, with 1,3,5 and 10 oocytes respectively, and the concentration of spermatozoa was kept constant at $0.5x10^6$ /ml. Therefore the oocyte to sperm ratio declined from 5,000 to 500. In both experiments a control was included and every experimental group contained approximately 45 oocytes.

Bull differences in the final test

Five different HF bulls were evaluated for variation in the oocyte penetration rate. The bulls studied were those numbered 3, 6, 9, 12 and 20 of the study of Den Daas et al. (chapter 5). Bulls were selected in order to explore most of the variation found after insemination in vivo. Straws from three ejaculates were pooled and used for fertilization according to the following scheme:

| group | sperm concentration | oocytes per droplet | ratio spermatozoa per oocyte |
|---|---|---|---|
| A | $0.2x10^6$ | 10 | 400 |
| B | $0.2x10^6$ | 5 | 800 |
| C | $1.0x10^6$ | 10 | 2000 |
| D | $1.0x10^6$ | 5 | 4000 |

A volume of 20 µl per fertilization droplet was chosen to ensure adequate volume per oocyte. All 5 bulls were tested in 4 experiments. Each experiment was performed with 50 oocytes for each of the above mentioned fertilization conditions (A, B, C, D) and an extra 50 oocytes served as a control (Parrish). Experiments were randomized per bull per category. Penetration percentage was evaluated after orcein staining of the oocyte as described above.

Statistics: Results were evaluated using the T-test and $X^2$-test.

Results

Development of standardized conditions

1) Development of oocyte maturation standard.

Table 1 gives the results of the four different maturation mediums studied. The number of oocytes that developed to metaphase II during maturation was significantly lower in M199 complemented with 20% ECS ($P < 0.05$: paired t-test). For reasons of convenience and to

exclude batch to batch variation of FCS, B2 with 10 g of FSH was chosen for all further experiments.

*Table 1. Number and percentage of oocytes that reached metaphase stage II after incubation for 24 hours at 39° C under 5% $CO_2$ in air in four different media.*

| culture medium | metaphase II/total number of oocytes | % metaphase II |
|---|---|---|
| M199 + 20% ECS | 142/206 [a] | 68.9 |
| M199 + 20% FCS | 151/185 [b] | 81.9 |
| M199 + 20% FCS + 10 g FSH | 136/181 [ab] | 75.1 |
| B2 + 10 g FSH | 174/219 [b] | 79.5 |

*ECS= estrus cow serum (6 hrs after onset of heat)*
*FCS= fetal calf serum*
*FSH= follicle stimulating hormone*
*[a,b] $P < 0.05$*

2) Development of the standard treatment for spermatozoa

Table 2 gives the results for the different sperm pre-treatments. There is a distinct effect of the addition of heparin during fertilization. Both treatments without heparin show a significantly lower penetration rate. Adding spermatozoa in the extender to the fertilization droplet, even when extender effects are counteracted with the addition of hexokinase and ATP, gives lower penetration results. However, by washing the spermatozoa after thawing using PBS and centrifugation over silicon oil, the penetration rate of oocytes is at a level comparable with the routine procedure (Parrish et al., 1986). Selection of sperm cells is avoided by this procedure (De Leeuw et al., 1991). At a ratio of 10,000 spermatozoa per oocyte, a selection effect for viable spermatozoa is absent. The penetration rate is at its maximal level even for unselected spermatozoa, which implies an 'overdose' of sperm cells in the control procedure.

Because selection of spermatozoa is undesirable and because of the usefulness of the procedure, washing, spinning and heparin was used in all subsequent experiments, except

for the "Parrish" control.

*Table 2. Penetration rate of oocytes by spermatozoa comparing different treatments of spermatozoa after thawing*

| sperm treatment | number of penetrated oocytes/total | number of replicates | % penetration |
|---|---|---|---|
| swim-up +Hep | 308/436 [a] | 11 | 70.6% |
| swim-up -Hep | 18/116 [b] | 3 | 15.5% |
| direct | 4/57 [b] | 2 | 8.8% |
| direct +Hex +ATP +Hep | 40/116 [ab] | 3 | 34.5% |
| washing +Hep | 129/187 [a] | 4 | 68.9% |

a differs significantly from b: $P < 0.05$.

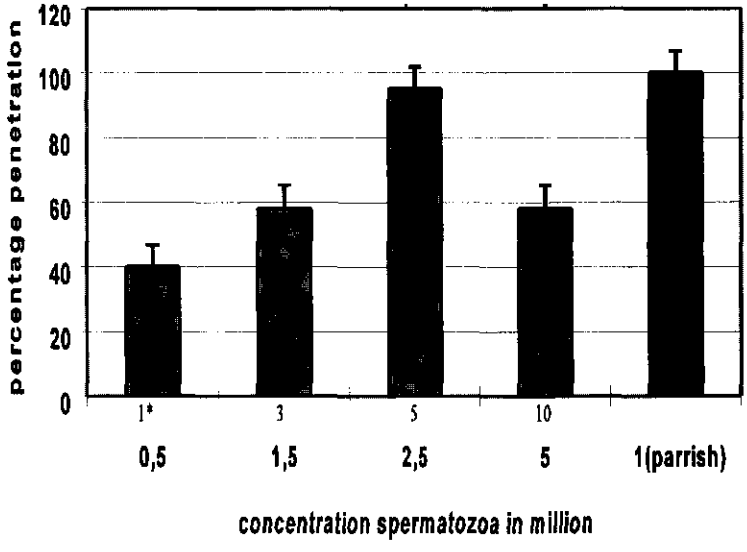



*Figure 1. Percentage penetration at a constant sperm to oocyte ratio of 5,000 by increasing the number of spermatozoa and oocytes per fertilization in a constant volume. Compared to control penetration rate = 72.7.*

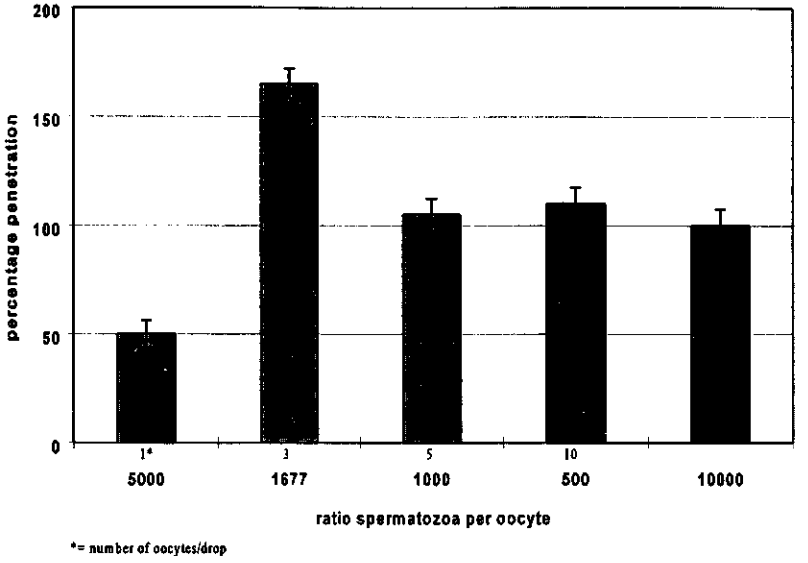



*Figure 2. Percentage at a constant spermconcentration of 0.5 x $10^6$ /ml decreasing the number of spermatozoa per oocyte by increasing number of oocytes. Compared to control penetration rate of 45.5.*

*Table 3. Repeatability of the penetration test*

| test day | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | $n_p/n_t$ | $n_p/n_t$ | $n_p/n_t$ | $n_p/n_t$ |
| Parrish (internal control) | 30/33 | 12/16 | 16/18 | 26/32 |
| 1) 2000 spermatozoa/oocyte | 26/35 | 9/16 | 10/19 | 12/15 |
| 2) duplo | 28/34 | 5/14 | 11/20 | 30/36 |
| 3) 4000 spermatozoa/oocyte | 17/31 | 9/19 | 10/19 | 23/31 |
| 4) duplo | 31/37 | 8/20 | 13/20 | 24/35 |

*$n_p$ = number of oocytes penetrated*
*$n_t$ = total number of oocytes tested for penetration*

Repeatability of the penetration test

Table 3 gives the results of the "Parrish" control and two duplicate measurements of penetration rates on four days. There was no significant difference between days. ($X^2$-test P > 0.05), nor between the duplicates ($X^2$-test P>0.05). It is therefore justifiable to regard the randomly selected slaughterhouse oocyte as the experimental unit. Day effects can be left out of the analyses.

Influence of the oocyte number

Oocyte number, sperm number and oocyte/sperm ratio results are shown in figs. 1 and 2. Penetration rate increases to five oocytes in 10 l after which it decreases (fig. 1). When the ratio of spermatozoa per oocyte is declining there is a strong effect of number of oocytes per drop. By increasing the number of oocytes per drop from one to three at a constant concentration of spermatozoa, thus decreasing the sperm oocyte ratio from 5000 to 1677, the penetration rate increased three fold (see figure 2). A number of 500 unselected spermatozoa per oocyte gives a penetration rate of around 50%.

Bull differences in the final test

Despite the results obtained for repeatability, a control was performed each test-day on approximately 50 oocytes using the method of Parrish (86) for sperm treatment and using straws of one ejaculate from one bull. This resulted in a penetration rate of 86.2 +/- 10.2 % (20 repeats, 760 oocytes tested in total). Table 4 and figure 3 give the penetration and pronuclear stage 4 (PN4) results of the 5 bulls tested for oocyte penetration. In general the percentage penetration and PN4 formation were low compared to the control. Bulls 9 and 12 show a low penetration rate at a ratio of 400 and 800 spermatozoa. For bull 9 the penetration rate rises to 30% for ratio's of 2,000 and 4,000 spermatozoa per oocyte. Penetration rates of

this bull (9) seems to be sperm concentration dependent instead of ratio dependent. For the other four bulls there is a gradual increase of penetration depending on the ratio. The penetration rate ranges from 7.5 to 27.9 % for bull 12 and from 15.2 to 64.8% for bull 20. At the lowest ratio (400 spermatozoa per oocyte) penetration rates were 7.5 to 32.2 ($P<0.01$) for bull 12 and 6 respectively. At the highest ratio (4,000 spermatozoa per oocyte) penetration rates were 27.9 to 65.9 ($P<0.01$) for bull 12 and 6. The percentage PN4 is generally somewhat lower but in the same range as the percentage oocytes penetrated.

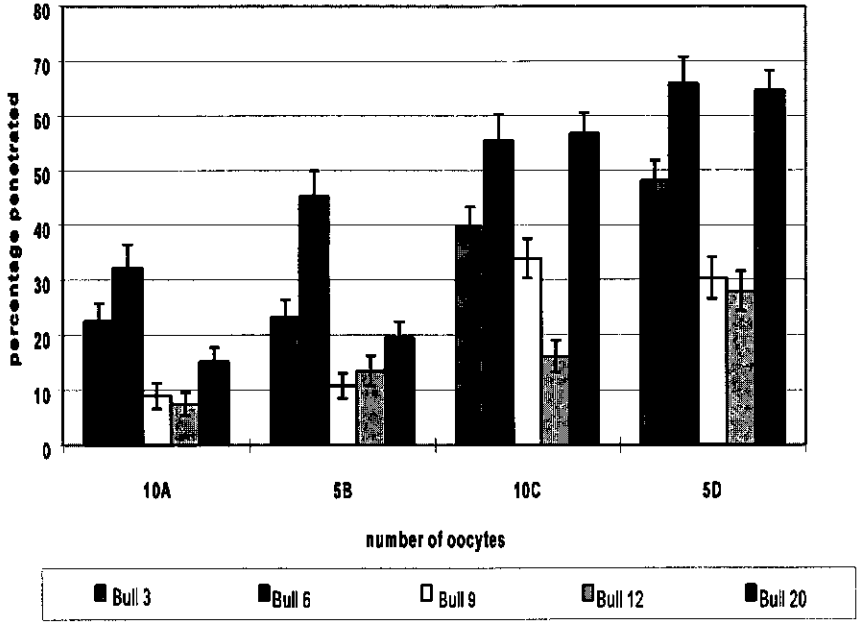



*Figure 3. Penetration rates of bovine oocytes in vitro by spermatozoa from 5 different bulls.*

*Tabel 4. Penetration rates and synchronous pronuclear development after oocyte penetration of five bulls.*

| bull | conc spermatozoa n oocytes ratio | total oocytes | oocytes penetrated | percentage penetrated | sd | PN4* | percentage PN4 | sd |
|---|---|---|---|---|---|---|---|---|
| 3 | 0.2 10 A 400 | 177 | 40 | 22.6 | 3.1 | 27 | 15.3 | 2.7 |
| 3 | 0.2 5 B 800 | 177 | 41 | 23.2 | 3.2 | 32 | 18.1 | 2.9 |
| 3 | 1.0 10 C 2000 | 169 | 67 | 39.6 | 3.7 | 50 | 29.6 | 3.5 |
| 3 | 1.0 5 D 4000 | 183 | 88 | 48.1 | 3.7 | 74 | 40.4 | 3.6 |
| 6 | 0.2 10 A 400 | 124 | 40 | 32.2 | 4.2 | 32 | 35.9 | 3.9 |
| 6 | 0.2 5 B 800 | 117 | 53 | 45.3 | 4.6 | 42 | 35.9 | 4.4 |
| 6 | 1.0 10 C 2000 | 108 | 60 | 55.5 | 4.8 | 47 | 43.5 | 4.8 |
| 6 | 1.0 5 D 4000 | 97 | 64 | 65.9 | 4.8 | 46 | 47.4 | 5.1 |
| 9 | 0.2 10 A 400 | 155 | 14 | 9.0 | 2.3 | 9 | 5.8 | 1.9 |
| 9 | 0.2 5 B 800 | 176 | 19 | 10.8 | 2.3 | 10 | 5.7 | 1.8 |
| 9 | 1.0 10 C 2000 | 174 | 59 | 33.9 | 3.6 | 42 | 24.4 | 3.2 |
| 9 | 1.0 5 D 4000 | 145 | 44 | 30.3 | 3.8 | 34 | 23.4 | 3.5 |
| 12 | 0.2 10 A 400 | 161 | 12 | 7.5 | 2.1 | 10 | 6.2 | 1.9 |
| 12 | 0.2 5 B 800 | 156 | 21 | 13.5 | 2.7 | 12 | 7.7 | 2.0 |
| 12 | 1.0 10 C 2000 | 161 | 26 | 16.1 | 2.9 | 23 | 14.3 | 2.7 |
| 12 | 1.0 5 D 4000 | 154 | 43 | 27.9 | 3.6 | 39 | 25.3 | 3.5 |
| 20 | 0.2 10 A 400 | 191 | 30 | 15.2 | 2.6 | 27 | 14.1 | 2.5 |
| 20 | 0.2 5 B 800 | 190 | 37 | 19.5 | 2.9 | 32 | 16.8 | 2.6 |
| 20 | 1.0 10 C 2000 | 176 | 100 | 56.8 | 3.7 | 82 | 46.6 | 3.8 |
| 20 | 1.0 5 D 4000 | 182 | 118 | 64.8 | 3.5 | 96 | 52.7 | 3.7 |

** = pronuclear stage 4 and further developed decondensed male pronucleus*

Table 5 gives the reproductive efficiency data from the same bulls using field data as found by den Daas et al (chapter 5). Bulls 3 and 6 show almost no effect of concentration spermatozoa inseminated on NR56 or on estimated conception rates. They are the ones with the highest penetration percentages at the lowest sperm oocyte ratio in vivo as well as in vitro. Bulls 9 and 20 show the strongest effect of concentration of spermatozoa inseminated in vivo (table 5). These bulls however, do not show the lowest penetration rates at a low gamete ratio neither do they show the highest increase of penetration with an increasing sperm/oocyte ratio in vitro. In vivo NR rate or estimated conception rates are highest for bull 12.

*Table 5. Reproductive data the bulls as estimated by den Daas et al. chapter 5.*

| bull | aNR56 | ± se | bNR56 | ± se | $a_{CRr}$ | ± se | $b_{CR}$ | ± se | $P_{CALVR}$ | ± se |
|---|---|---|---|---|---|---|---|---|---|---|
| 3 | 70.7 | 1.2 | -0.02 | 0.08 | 75.2 | 2.1 | 0.04 | 0.01 | 84.0 | 0.02 |
| 6 | 68.4 | 2.0 | -0.03 | 0.11 | 73.3 | 1.9 | 0.08 | 0.01 | 87.0 | 0.03 |
| 9 | 69.8 | 1.7 | 0.49 | 0.05 | 73.9 | 1.7 | 0.43 | 0.05 | 85.0 | 0.03 |
| 12 | 75.8 | 1.1 | 0.29 | 0.01 | 77.8 | 1.4 | 0.29 | 0.02 | 86.2 | 0.03 |
| 20 | 69.2 | 1.9 | 0.56 | 0.07 | 74.1 | 1.8 | 0.61 | 0.09 | 82.3 | 0.02 |

*aNR56* = *asymptotic value for non return rate at 56 days after insemination*
*bNR56* = *rate of increase to the asymptotic value for NR56*
$a_{CR}$ = *asymptotic value for estimated conception rate*
$b_{CR}$ = *rate of increase to the asymptotic value for conception rate*
$P_{CALVR}$ = *estimated calving rate given conception*

## Discussion

The objective of this study was to determine whether the in vitro penetration rate of oocytes can be used to predict in vivo reproductive efficiency characteristics of individual bulls. We will discuss this objective in two parts (1) the development of the in vitro test and (2) the comparison of the in vitro and in vivo fertilization rates, using different sperm/oocyte ratios.

1) Development of the in vitro test

In the bovine spermatozoa used for in vitro fertilization are preselected before fertilization. Selection criteria concern either motility (swim-up, Parrish et al., 1986) or density of the cell using percoll gradient centrifugation. The sperm concentration is established after preselection. Selection may mask differences between bulls of interest as we don't know whether it mimics the changes in the sperm population in the female reproductive tract before fertilization occurs. We therefore designed a system that avoided pre-selection; identical populations of spermatozoa were inseminated in vitro and in vivo.

In several papers, the semen capacitation effect of heparin in relation to oocyte penetration in vitro is reported for the bovine (Parrish et al., 1985; Marquant-Le Guinne et al., 1990; Leibfried Rutlegde et al., 1989). They all show a bull specific effect of heparin on the penetration ability of spermatozoa in vitro. (Marquant-Le Guinne et al., 1990 and 1992; den Daas and Merton, 1994). The effect of heparin is batch dependent. Average molecular weights for heparin batches are constant however, this average can hide differences in the underlaying distribution (data not shown). In order to focus on semen differences between bulls (and because of test costs) a heparin concentration of 10 µg was used. This concentration gives high penetration rates with low frequency of polyspermy with the reference bull.

Oocyte penetration rate might be affected by sperm concentration. It is known that low sperm concentrations are correlated with low motility rates (chapter 6 of this thesis). Therefore the collision frequency between spermatozoa and oocytes might be a limiting factor in oocyte penetration. Furthermore Heeres et al. (1996) showed that sperm cells seem to get trapped in the oil/fluid interface when sterile oil is used to create a micro environment for the fertilization droplet. In our experiments we did use sterile oil to cover fertilization droplets. This trapping of cells resulted in a reduced number of spermatozoa available for fertilization. However, increasing the sperm/oocyte ratio by decreasing the number of oocytes per fertilization droplet for bulls 9 and 3, when the sperm concentration is low, gives no increase in penetration frequency (this study). This can neither be explained by differences in motility, as the sperm concentration remained the same, nor by sperm trapping. The only

explanation is that the oocytes are more likely to get penetrated when present in higher numbers, 10 in stead of 5, in the fertilization droplet. In order to do so oocytes or the surrounding cumulus cells should stimulate spermatozoa in a dose related manner. The effect of oocyte number on the penetration rate was already found for the mouse by Siddequey and Cohen (1982). They showed, besides an effect of sperm concentration also effects of oocyte number and fertilization volume.

We expected to find a increase in penetration rate when sperm/oocyte ratio was increased and when the number of oocytes would not be the limiting factor. At the highest sperm/oocyte ratio the maximal penetration rate is only 64.8%, (bull 20). The penetration rate of the Parrish control was 86.2%, so oocyte quality or oocyte number does not seem to be the limiting factor. Increasing the spermatozoa concentration had a positive effect for all bulls. There is a bull times concentration interaction in the increase in penetration rate as can be seen in bulls 12 and 20 who showed a 10% and 40% increase respectively. When the sperm to oocyte ratio is increased the number of detectable penetrations is not increased accordingly. For fertilization to occur, the spermatozoon has to arrive at the zona, bind, acrosome react and subsequently penetrate the oocyte. At a ratio of 400 spermatozoa to one oocyte the achieved penetration rate is 32% for bull 6. So only one out of 1,200 spermatozoa is capable of penetration. When the number of spermatozoa is increased the ones that are capable of full penetration will be hindered by other spermatozoa binding and/or acrosome reacting at the zona. If spermatozoa acrosome react when bound to the zona pellucida the surrounding zona composition is altered and looses its binding capacity for acrosome intact spermatozoa (Bleil and Wassarman, 1983). This stearic and enzymatic hindering effect will only be overcome when several spermatozoa capable of full penetration are present per oocyte. This is the case in the control experiments where a preselected sample of spermatozoa is present at a ratio of 10,000 to one oocyte. This hindering effect might be the explanation for the non linearity of the relationship between sperm/oocyte ratio and penetration rate.

2) Bull differences comparing in vivo and in vitro

To compare results between field data for fertility and oocyte penetration in vitro an identical population of spermatozoa was used in both situations. In the in vivo situation, spermatozoa are brought into the uterine lumen. There sperm are progressively lost, through expulsion with mucus through the cervix (Hawk, 1987), through the peritoneal cavity by uterine contraction (Overstreet, 1983; Hunter, 1988) and by leucocyte ingestion (Mattner, 1968). A small fraction of the spermatozoa migrate to the oviduct and adhere to the columnar epithelial cells at the caudal 2 cm of the oviduct isthmus which is the sperm storage reservoir in cattle (Suarez et al., 1990) to ensure that the sperm oocyte ratio at the site of fertilization is close to unity (Saacke et al., 1994). The progressive loss can be considered to be bull dependent and therefore different doses of inseminated spermatozoa are needed to reach the critical number of spermatozoa in the oviductal reservoir. It still remains unclear whether the sperm selection in the female genital tract is mediated by properties of the spermatozoon only or whether the female plays an active roll (Cohen, 1975; Saacke et al., 1988), which, from the bull's perspective leaves room for environment (female) genotype interactions.

In vitro an undifferentiated population of spermatozoa is presented to the oocytes. Therefore the in vivo and in vitro situations are quite different. In vivo, only a selected population of spermatozoa is permitted at the fertilization site and capable of fertilization. In vitro however, an undifferentiated population of spermatozoa is present and a larger fraction of sperm cells may be capable of fertilization. Evaluation of the penetration rate gives an idea about fertilization capability per se. The differences between the in vitro and in vivo results support the hypothesis of the existence of two populations of spermatozoa. This is indirect evidence for the speculation that spermatozoa are selected, passively or actively in the female reproductive tract (Cohen, 1975).

References

Bleil, J.D. and Wassarman, P.M., 1983. Sperm-egg interaction in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida protein. Dev. Biol. 95: 317-324.

Cohen, J., 1975. Gamete redundancy-wastage or selection? In Gamete Competition in Plants and Animals, Edt. D.L. Mulchaly Elsevier Amsterdam,:99-112.

De Leeuw, A.M., den Daas, J.H.G. and Woelders, H., 1991. The Fix Vital Stain Method for Simultaneous Determination of Viability and Acrosomal Status of Bovine Spermatozoa. J. of Andrology, 12: 112-118.

De Loos, F., van Vliet, C., van Maurik, P. and Kruip, Th., 1989. Morphology of immature bovine oocytes. Gamete Res 24: 197-204.

Den Daas, J.H.G., 1992. Laboratory assessments of semen characteristics In: Clinical trend and Basic research in animal reproduction. Edt: Dieleman, S.J., Colenbrander, B., Booman, P. and van der Lende, T. Elsevier, Amsterdam,: 87-94.

Den Daas, J.H.G., and Merton, S., 1994. In vitro embryo production, Its Use, 10th reunion A.E.T.E.- Lyon, 9-10 September,: 117-124.

Eyestone, W.H. and First, N.L., 1989. Variation in bovine embryo development in vitro due to bulls, Theriogenology vol 31: 1, p 191.

Hawk, H.W., 1987. Transport and fate of spermatozoa after insemination of Cattle. Journal of Dairy Sci 70: 1487-1503.

Heeres A.A., Merton, J.S., Hazeleger, W., van Wagtendonk-de Leeuw, A.M., Kemp, B. Optimization of sperm/oocyte ratio during in vitro fertilization of bovine cumulus-oocytes-complexes. Theriogenology vol 45: 266.

Hunter, R.H.F., 1988. The fallopian tubes Their role in fertility and infertility Eds. R.H.F. Hunter. Springer Verlag New York.

Killian, G.J., Chapman, D.A. and Rogowski, L.A., 1993. Fertility-associated proteins in Holstein bull seminal plasma. Biol. Reprod. 49: 1202-1207.

Leifried-Rutledge, M.L., Critser, E.S., Parrish, J.J. and First, N.L., 1989. In Vitro maturation and fertilization of bovine oocytes. Theriogenology vol 31:1 page: 61-74.

Marquant-Le Guienne, B., Humblot, P., Thibier, M. and Thibault, C., 1990. Evaluation of bull semen fertility by homologous in vitro fertilization tests. Reproduction Nutrition Development 30, 259-266.

Marquant-Le Guienne, B. and Humblot, P., 1992. Tests de fertilite chez l'animal domestique par fecondation in vitro. Ann Zootech: 41 361-370.

Mattner, P.E., 1983. The distribution of spermatozoa and leucocytes in the female genetic tract in goats and cattle. J. Reprod. Fert. 17: 253-261.

Overstreet, J.W., 1983. Transport of gametes in the reproductive tract of the female mammal. In: Mechanisms and control of animal fertilization. Edt. J.F.Hartmann Academic Press, New York pp 499-543.

Parrish, J.J., Susko-Parrish, J.L. and First, N.L., 1985. Effect of heparine and chondroitine sulfate on the acrosome reaction and fertility of bovine sperm in vitro. Theriogenology 24: 537-549.

Parish J.J., Susko-Parrish J.L., Leibfried-Rutledge M.L., Critser E.S., Eyestone W.H. and First N.L., 1986. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25: 591-600.

Saacke, R.G., Bame, J., Karabinus, D.S., Mullins, J., Withman, S., 1988. Transport of abnormal sperm in the artificially inseminated cow bases upon accessory sperm in the zona pellucida, 11th Int'l. Cong . Anim. Reprod. and Artif. Insem. Dublin; 3: 292.

Saacke, R.G, Nadir, S. and Nebel, R.L., 1994. Relationships of semen quality to sperm transport, fertilization and embryo quality in ruminants. Theriogenology, 41:54.

Shannon, P. and Vishwanath, R., 1995. The effect of optimal en sub optimal concentration of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain differences. Anim. Reprod. Sci., 39 (1):1-10.

Siddiquey, A.K.S. and Cohen, J., 1982. In-vitro fertilization in the mouse and the relevance of different sperm/egg concentrations and volumes J. Reproduction and Fertility, 66: 237-242.

Suarez, S.S., Drost, M., Redfern, K. and Gottlieb, W., 1990. Sperm motility in the oviduct. In: Fertilization in mammals Edt. Bavister, B.D., Cummins, J. and Roldan, E.R.S. Serono Symposia USA, Norwell Mass,: 111-124.

Van Os, J., de Vries, M.J., den Daas, J.H.G. and Lansbergen, L.M.T.E., 1997. Trends in Sperm Count of Dairy Bulls, accepted in Journal of Andrology.

Xu, K.P. and Greve T., 1988. A detailed analysis of early events during in-vitro fertilization of bovine follicular oocytes, J. Reprod. Fert., 82: 127-134.

Chapter 9

# FINAL CONSIDERATIONS

J.H.G. (Nanke) den Daas

Holland Genetics

P.O. Box 5073

6802 EB Arnhem

The Netherlands

## Introduction

Results from the study in this thesis and subsequent implementation in AI-industry are discussed in this chapter. The concepts of holistic and reductionistic approaches to science are introduced and used to discuss the path of research followed in this thesis.

## Holism and reductionism

We try to answer most of the questions asked in Science by performing trials which study only a small part of the total question. In the philosophy of science this is called the reductionistic approach. From the seventeenth century on, we have diverted from the holistic approach to science. Holistic science studies every aspect of the question together and in relation to nature (Van der Wal, 1997). Asking the right questions is of utmost importance in science. Science is not so much about getting the right answers as it is about asking the right questions. Research has found many answers to irrelevant or unimportant questions as well as to those of importance. By studying Nature in our very deterministic way, we seem to have lost the knowledge gained from Nature's lessons. One of the most important lessons is that Nature never optimizes!!, as she wants to be prepared for unexpected events. Had we taken this lesson into account we would never have undertaken the research described in this thesis, or many other projects. We were trying to answer the wrong question, and we did not take the holistic approach. We all know that reductive science as has been performed in this thesis can corrupt our reality. There is a first reality world in which we participate. We see the sunset reflecting on the sea, we see the moon and stars following us and feel the sand on the beach passing quickly from beneath our feet. This experience is referred to by some of us as the child's perception. The second reality world is when we start to study, and we enter the world of objects. We learn that the earth turns and makes the sun set, we learn that moon and stars appear to follow us because of the distance et cetera. However, when we pass information from this second world to the first we should be very careful. We should not

make them equal. We should not loose the ability to ask the right questions as we might do if we make the two worlds equal. We have to study, however, after reduction because the real questions are too complex and cannot be handled by our science. The greatest challenge is to find the right balance between the two worlds.

## Balancing holism and reductionism

### What can we learn from the approach taken in this thesis?

The (holistic) question asked in this thesis is: is it possible to predict (dose dependent) bovine male fertility results after insemination using in vitro laboratory tests to study the different characteristics of spermatozoa. This main question has been broken into three smaller questions which were: 1) the evaluation of field fertility data, 2) the estimation of dose dependent insemination results in order to characterize variation in fertility of bulls and 3) the possibility to predict this (dose dependent) variation in fertility between bulls by studying different characteristics of spermatozoa.

In order to be able to evaluate the link between in vivo results of insemination and in vitro characteristics of spermatozoa both the in vivo and the in vitro tests have to be accurate, precise, sensitive and specific (Banks and Schmehl, 1989). The first reduction of the holistic question was to study the evaluation of the information obtained from inseminations performed under field conditions in the Netherlands (see figure 1). The information on inseminations is registered on a per herd and per cow basis and interval of returning or not returning to service is used to calculate non-return (NR) for technicians and bulls. In chapter 3 the adjustment of NR for environmental effects was studied. Even in the case of 100% registration of all insemination data as is the case in the Netherlands, information on culling and natural service will change by farm, breed, season and over the years, NR information will still be biased, even after adjustments. It was however, concluded that NR rates of bulls, calculated on the basis of insemination data , are influenced by effect of herd-season, age of the inseminated cow, month of insemination and technicians. Therefore, non-adjusted NR rate give biased information when it comes to compare bulls. This study was based on data

from the first insemination per cow. A routine system to adjust NR rates for these effects has been introduced in the Netherlands.

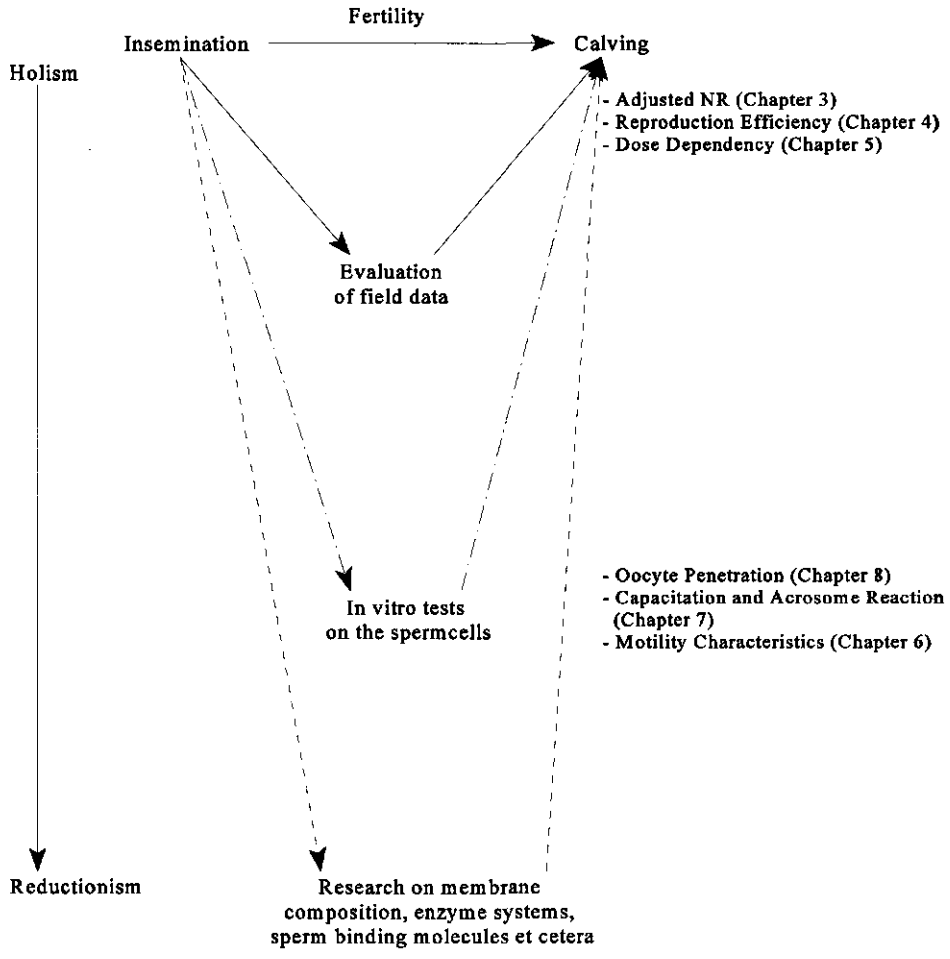



*Figure 1. Research in male reproduciton from holism to reductionism*

In chapter 4 we took a more holistic approach to the same quest of how to evaluate information obtained from insemination data. The approach was developed by combining the current knowledge in bovine reproduction with information from field inseminations. NR rates are the result of the combined effect of conception and gestation. To evaluate the link between in vitro characteristics of spermatozoa which are expected to be informative for conception it is important to unravel these combined effects in the NR information. A model to monitor the decline in NR rate over time was developed using information on the event leading up to a successful gestation. Using this model we can estimate conception rate, calving rate given conception and characteristics of the first two estrus cycles. From the proportion of cows that conceived but failed to complete gestation because of postsignal embryonic death, the irregular return intervals (Macmillan and Curnow, 1977), the model estimates conception rate (cpr) and calving rate (cvr). From the proportion of cows that failed to conceive or that conceived but failed to complete gestation because of presignal embryonic death, regular return intervals, the model estimates the proportion of return, or probability of detecting estrus. This model might be more reliable for the evaluation of bull fertility than non-return rates at arbitrarily chosen days after insemination. In this model we cannot adjust for the environmental factors as described in chapter 3. Only cow and herd effects are taken into account in the proportion of cows returning during cycle calculated from the model. We are currently evaluating whether the model is more fit for routine evaluation of a technician or a bulls performance than adjusted NR56. One of the problems is that the model was developed and tested on data on daily NR to 120 days after service, which is comparatively long for day to day management information on performance. In research however, the model might prove its value.

The next step in answering the main question was to evaluate insemination results of different number of spermatozoa inseminated. Special emphasis is put on the possibility to predict dose dependent estimates of insemination results, because they will give information on the number of breeding units which can be produced from an ejaculate without compromising pregnancy results. The answer to this question is of economic importance to

AI-industry. Results of insemination are independent of the insemination dose until this dose has declined to the threshold level of dose dependency. There is an exponential relationship between dose rate inseminated and insemination results. It is not possible however, to obtain information on very low doses inseminated as farmers of a cooperative will not allow such a field trail to be performed. Therefore the variance in NR information obtained from field data is limited. On the other hand one should realize that the proven bulls used to obtain the NR information are already pre-selected. The variation in NR is also reduced because to become a proven bull, production information from his daughters is needed. Here we find double reduction of full (holistic) information which could be obtained ( see figure 1). Although it is quite unusual to be allowed by an AI-cooperative to gather information on the low concentrations of spermatozoa inseminated in this study (chapter 5) of the 20 proven bulls for which dose dependency was studied only 15 were tested at a concentration range where the dose dependency could be estimated. The remaining 5 bulls should have been inseminated at an even lower dose range in order to obtain the information wanted. In order to study dose rate dependent ( i.e. compensable factors by increasing the sperm number per insemination) and uncompensable factors, dose rates from 2 to 17 x $10^6$ total spermatozoa were inseminated (chapter 5). The range of NR rates increased from 9% at 17 x $10^6$ to 18% at 2 x $10^6$ (calculated from table 3; chapter 5). The rate of increase to the maximal asymptotic level of insemination results altered with the dose inseminated. At Holland Genetics it has become routine to determine a dose response curve using 5 to 20 x $10^6$ total spermatozoa after freeze-thawing per insemination for each proven bull in order to obtain information on required number of spermatozoa required for optimal fertility results.

Towards reductionism

It is expensive and time consuming to determine the above described relationship for every individual bull with the possibility that at the dose rates tested no exponential relationship between insemination results and the number of spermatozoa inseminated is revealed. Furthermore, reliable and accurate records are a prerequisite for this type of field

trial and therefore this procedure is not feasible for every AI-company. The aim in reproduction research is to predict insemination results using laboratory tests in order to circumvent the field trials or make them superfluous. However, when we start to answer this question we reduce a lock step sequence of events (Hammerstedt, 1996) needed before a spermatozoa can fertilize (figure 2) to study of only one aspect of this sequence. Furthermore this aspect is studied under in vitro conditions which are quite different from the in vivo situation. Once again a double reduction of the question; only one aspect is studied and moreover, the conditions of this study are artificial (see figure 1). Of course we had realized this difficulty while performing the research in question. However, because of the high economic importance of a solution and because the research would add to our understanding of science in reproduction and fertility, we nevertheless decided to proceed.

In vitro tests evaluated for the ability to predict dose dependent insemination results in this thesis were; 1) motility characteristics of the spermatozoa, 2) capacitation and acrosome reaction and 3) homologous oocyte penetration. No tests were designed to study dose independent factors (i.e. uncompensable factors). However, each one of the three in vitro tests performed was evaluated for the possibility of prediction uncompensable factors as well. Changes in motility characteristics, capacitation and oocyte penetration of spermatozoa may reflect changes in the paternal contribution to the development potency of the zygote formed after fertilization and therefore may give information on the maximal insemination results.

Motility characteristics

In chapter 6 dose dependent motility characteristics of bulls were evaluated over a concentration range of 2 to 17 x $10^6$ spermatozoa per insemination. We found that the concentration of sperm cells had a strong effect on the percentage motile cells. Both the relationship between concentration and motility characteristics and the motility characteristics tested were found to have little predictive value for the relationship between dose inseminated and fertility measured under field conditions. Only 25% of the variation present in insemination results could be explained by dose dependency of percentage motile cells. In the sequence of events (figure 2) motility of the spermatozoa is one of the first and in most

cases not limiting (Amann and Hammerstedt, 1993). Therefore, although motility is a pre-requisite for fertility, motility estimates will only give information on infertility of a bull.

Subjective motility estimation by eye is still the daily routine in most laboratories where bovine semen is frozen. After this research we realized that motility after freeze-thaw provides information on the quality of processing in the laboratory only. The subjective estimation of motility by eye can be used to fulfill this objective of laboratory processing control.

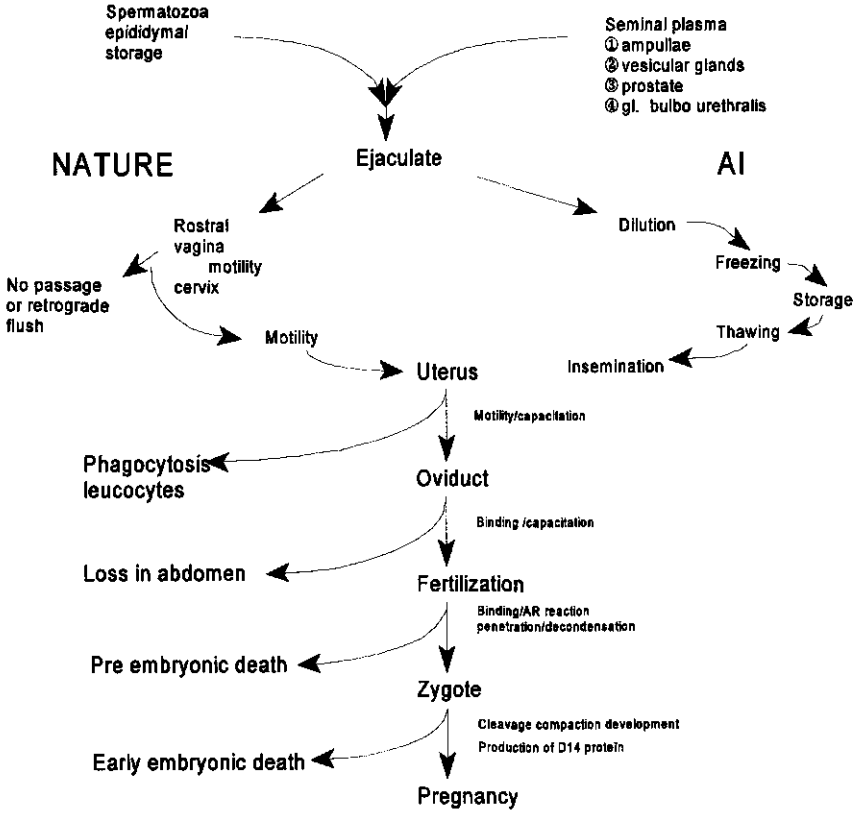



*Figure 2. Sequence of events during insemination and fertilization*

Capacitation and acrosome reaction

Capacitation prepares the sperm cell for its interaction with the oocyte and can be measured by the ability of the spermatozoa to perform the acrosome reaction. In the sequence of events leading to fertilization, capacitation starts immediately after ejaculation and finishes just before fertilization occurs. During capacitation spermatozoa are progressively lost from the female reproductive tract by retrograde flush through the cervix, phagocytosis in the uterus and sperm-aging (figure 2). However, the decrease in number of spermatozoa coincides with an increase in the fertilizing capacity of the cells still present. The reduction of the main question is again two fold: 1) the natural selection of spermatozoa either by chance or actively as far as the female tract is responsible is omitted and 2) capacitation is studied by monitoring acrosome reaction under non-physiological in vitro circumstances. In the sequence of series of events capacitation is completed just before fertilization. Therefore, we might be studying a limiting factor in one of the last steps before fertilization occurs. The results revealed that a high percentage of viable acrosome intact cells after freeze-thawing combined with a low percentage of cells showing acrosome reaction after lysophosphatidyl-choline (LPC) trigger at 4 hours of incubation was found for bulls who required a low number of spermatozoa per insemination to reach maximal insemination results (fig. 3 chapter 7). These results suggest that at any one time the number of capacitated hyper motile spermatozoa present in the oviduct can be compensated by increasing the number of spermatozoa inseminated. The period over which this critical number of spermatozoa in the oviduct can be realized is also important for obtaining adequate insemination results.

If no information on performance of the bull in the field is available, determination of the percentage spermatozoa competent to acrosome react post thaw and the percentage of cells showing acrosome reaction upon addition of LPC at 4 hours of incubation can be used to predict the optimal semen dose. We are, however, able to obtain information on insemination results. In this case the developed test gives us information on the dose range to be tested under field conditions. The power of prediction of this test is promising and we are studying whether it is possible to perform the evaluation of viability and acrosomal status by flow

cytometer. The flow cytometer has the advantage that the number of spermatozoa evaluated per test can be upgraded from 400, as in this study per sample, when using microscopical evaluation by eye, to several thousand.

*Oocyte penetration*

For the spermatozoa the ability to penetrate the oocyte is the last event in the sequence leading to fertilization. Therefore homologous oocyte penetration might be the best characteristic to study when we want to predict insemination results (figure 1). In the in vitro test spermatozoa need to be; motile, capacitated, able to bind to the zona pellucida, able to acrosome react, able to penetrate the zona, able to fuse with the oocyte and able to show decondensation of the nucleus (figure 2). In vivo the pre-requisites are the same; the population of spermatozoa evaluated however, is quite different as only a few of the spermatozoa inseminated reach the oviduct and are contributing to the chance of fertilization. Because we study the last step before zygote formation oocyte penetration might be the ultimate way to measure the effect of limiting sperm numbers. However, once again the question of predicting insemination results is reduced in several ways (figure 1). The study on oocyte penetration in vitro resulted in information on the effect of circumvention of the environmental influence of the female reproductive tract. When this environmental influence of the female reproductive tract is omitted, as is the case in fertilization in vitro, only few viable spermatozoa are capable of performing normal zygote formation. A sperm to oocyte ratio of 4,000 : 1 with semen of some bulls gives a penetration rate of only around 30% (chapter 8 of this thesis). Only one out of 12,000 spermatozoa of these bulls is capable of fertilization measured by oocyte penetration. Penetration of spermatozoa can be influenced by capacitating agents as heparin or chondroitin sulphate (Parrish et al., 1985). However, even when this artificial capacitation is used around 10,000 spermatozoa are needed for one to successfully penetrate the oocyte. The hypothesis that we can predict compensable factors of spermatozoa after insemination by the level of (homologous) oocyte penetration was rejected in this study. More information on oocyte penetration and subsequent development to blastocysts is now available from the in vitro production of bovine embryos, for several

bulls (Lansbergen et al., 1997). The results of bulls in routine in vitro production of embryos sustain the results found in this thesis when only oocyte penetration was evaluated.

Uncompensable factors

Insemination results obtained by insemination numbers of spermatozoa above threshold level gives information about the paternal contribution to the developmental potential of the conceptus. (den Daas et al., 1992; Saacke et al., 1994). This paternal contribution to embryo development varies between bulls. The variation is in the order of 10% of calving rate given conception (chapter 5 of this thesis). Maybe this variation in 'intrinsic' sperm quality (den Daas et al., 1992) is reflected in the sperm characteristics measured in vitro in this thesis. We should be aware however, that once again we take another step towards reductionism (figure 1). For motility characteristics the correlation between dose rate independent insemination results and percentage motile cells after thawing was .52 ($p=0.05$) (chapter 6). This correlation is comparable to results found in literature (Uwland, 1984; Hammerstedt 1996). The evaluation of capacitation and acrosome reaction of spermatozoa (chapter 7) was not significantly correlated to dose independent insemination results. Moreover, also the oocyte penetration test, where sperm head decondensation is evaluated in cytoplasm of the oocyte and which might therefore be expected to give information on this paternal contribution, showed no correlation with dose independent insemination results.

There may be other possibilities to study intrinsic sperm quality for instance by measuring DNA packaging of the spermatozoa. Evenson et al. (1980) related for the first time mammalian sperm chromatic heterogenity to fertility. The method they used was flow cytometry using dual fluorescent staining. Thus they measured the amount of single versus double stranded DNA in the population inseminated. Another possible way to measure DNA 'quality' was developed by Van Loon et al. (1991). This method quantifies DNA breakage points. The first report to relate this characteristic to fertility was by Van der Schans et al. (1996).

Embryo development, quality and number, after in vitro fertilization might be another way to measure the paternal contribution to the developmental potential of the conceptus.

Most reports do find variation between bulls with regard to the aspect of embryo development after fertilization in vitro (Hillery et al., 1990; Marquant-Le Guinne and Humblot, 1992; Lansbergen et al., 1997). However, not in all cases does this variation reflect the results obtained after insemination: once again, did the reduction go too far to be able to answer the question by performing the research proposed? The variation obtained in vitro avoids all selection of spermatozoa and therefore does not mimic the natural situation well. DeJarnette et al. (1992) showed a positive relationship between embryo quality in single ovulating cows and the median of accessory sperm number present in the zona. So passage of spermatozoa through, and competition in the genital tract, selects spermatozoa for fertilization. Moreover, this result suggests that either chance or competition among fertilizing spermatozoa may be favorable to embryonic development as well as fertilization (Saacke et al., 1994). This last hypothesis also needs to be researched further with respect to the timing of fertilization in relation to sperm age.

## Reductionistic research

Real reductionistic research in the study of male reproduction is the fundamental research at the level of detection of membrane composition, enzymes, signal transduction pathways, cellular and other mechanisms involved in fertilization. In this thesis we did not perform any fundamental research in this sense, as we always stayed at the level of the total spermatozoa and studied the reaction of this cell to time of incubation, temperature and other environmental changes and challenges.

## Conclusion

Results of the developed fertility assays so far are able to exclude most in- or subfertile males and eliminate wrong processing protocols. When one of the essential steps in the lock step series of events before fertilization fails to reach the critical level then fertility results will be impaired. Fertility assays, however, are not able to point out the males with superior

fertility or to predict the insemination dose at which maximal insemination results are obtained. Combining the in vitro parameters measured in order to predict fertility did not achieve an acceptable prediction of insemination results either (data not shown). This can be understood when the lock step series of events needed for fertilization are approached as by Hammerstedt in his review in 1996. Whenever fertility assays are interpreted, full knowledge and understanding of the biology of the system of interest should be taken into account. What is needed is reductive research driven by the right questions and incorporated into the holistic approach. More informative fundamental knowledge has to be developed on the molecular basis of, and physiology of the sequence in events before and during fertilization. The reason for this lack of knowledge is that we know little of what is going on and therefore we tend to think every little detail is as important as the next. We have certainly been studying many little details. Our research has been almost completely reductionistic from the year 1600 and since then we have been investigating details instead of trying to focus on the total picture (Mea-Wan Ho, 1997). In our research philosophy we have developed a way of thinking which prevents us from asking the right questions and keeping to the main track.
Investment in more philosophical thinking might develop our ability to take the holistic approach in order to find the right questions. Finally, with improvement of real knowledge therapeutic concept for the in- or subfertile male might be developed as well as new ideas around processing and storage of semen.

Note: The part above is a summary of my thinking and reading on the philosophy of Science, on discussions about perceptions and presumptions in research. Most of the ideas have been discussed in the conference "the future of DNA" organized by the If-gene (International Forum for Genetics Engineering) group in October 1996 at the Goetheanum in Dornach Switzerland. This conference has resulted in the book The Future of DNA by J. Wirz and E.T. Lammerts van Bueren edited by Kluwer Academic Publishers (ISBN 0-7923-4620-3). In my opinion research philosophy should be part of every training in science. Moreover, students should be confronted with this part of science early in their training.

References

Amann, R.P., Hammerstedt, R.H. and Veeramachaneni, D.V.N., 1993. The epididymis and Sperm Maturation a Perspective. Reprod. Fertil. Dev., 5: 361-381.

Banks, H.L. and Schmehl, M.K., 1989. Parameters for Evaluation of Viability Assays: Accuracy, Precision, Specificity, Sensitivity and Standardization. Cryo., 26: 203-211.

DeJarnette, J.M., Saacke, R.G., Bame, J. and Vogler, C.J., 1992. Accessory sperm: Their importance to fertility and embryo quality, and attempts to alter their numbers in artificially inseminated cattle. J. Anim. Sci 70: 484-491.

Den Daas, J.H.G., 1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci 28: 87-94.

Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R., 1980. Relation of mammalian sperm chromatic heterogeneity to fertility. Science, 210: 1131-1133.

Hammerstedt, R.H., 1996. Evaluation of sperm quality: Identification of the subfertile male and courses of action. Animal Reprod. Sci., 42: 77-87.

Hillery, F.L., Parrish, J.J. and First, N.L., 1990. Bull specific effect on fertilization and embryo development in vitro. Theriogenology 33: 249.

Lansbergen, L.M.T.E., Hanenberg, E.H.A.T. and van Wagtendonk-de Leeuw, A.M., 1997. Predictive Value of in Vitro Embryo Production Characteristics for in Vitro Fertility of Bulls. Theriogenology 47: 259.

Macmillan, K.L. and Curnow, R.J., 1977. Factors influencing A.B. conception rate X. Seasonal trends in fertility and in the distribution of return intervals: differences between deep-frozen and liquid semen; New Zealand J. of Exp. Agricul.., 5: 273-278.

Mae-Wan Ho, 1997. DNA and the new organism. In The Future of DNA; Edt. Wirz, J. and Lammerts van Bueren, E.; Kluwer Acad. Publ. Dordrecht The Netherlands: 78-93.

Marquant-Le Guienne, B. and Humblot, P., 1992. Tests de fertilite chez l'animal domestique par fecondation in vitro. Ann. Zootech., 41: 361-370.

Parrish, J.J., Susko-Parrish, L. and First N.L., 1985. Effect of heparin and chrondroitin sulfate on the acrosome reaction and fertility of bovine spermatozoa in vitro. Theriogenology, 24: 537-549.

Saacke, R.G., Nadir, S. and Nebel, R.L., 1994. Relationships of semen quality to sperm transport, fertilization and embryo quality in ruminants. Theriogenology 41: 45-50.

Uwland, J., 1984. Possibilities and limitations of semen evaluation for the prognosis of male fertility. In: The male in farm animal reproduction. Edt. Courot, A., Martinus Nijhoff Boston Ma. USA.: 269-289.

Van der Schans, G.P., Timmerman, A.J. and den Daas, J.H.G., 1996. An Immunochemical assay for the assessment of bovine sperm quality with respect to fertility efficiency. The 13th Int. Congr. on Animal Reproduction, Sydney, Australia., 3: 24-15.

Van der Wal, J., 1997. Back to the future- towards a spiritual attitude for managing DNA. In: The Future of DNA; Edt. J. Wirz and E. Lammerts van Bueren: Kluwer Acad. Publ., Dordrecht, The Netherlands: 43-54.

Van Loon, A.A.W.M., den Boer, P.J., van der Schans, G.P., Makcenbach, P., Grootegoed, J.A., Baan, R.A. and Lohman, P.H.M., 1991. Immunochemical Detection of DNA damage Induction and Repair at Different Cellular Stages of Spermatogenesis of the Hamster after in Vitro and In Vivo Exposure to Ionizing Radiation. Exp. Cell. Res., 193: 303-309.

## Summary

Cattle Breeding industry today selects the most valuable dairy bulls based upon the production performance and type classification of their daughters. Once bulls are selected the goal is to disseminate their genes into the population. Therefore it is important to maximize the number of insemination doses per bull without compromising pregnancy rates. AI-industry constantly has to balance the farmers needs, which is to get a cow in calf, with its own needs, which is to perform as many inseminations possible per bull. The goal of AI-industry is to maximize the number of offspring produced by selected genetically superior bulls, thus disseminating genetic improvement to the cattle population as efficiently as possible. This is accomplished by maximizing semen collection and the number of breeding units produced per ejaculate without compromising fertility. The aim of this thesis was to study the possibilities to predict (dose dependent) fertility of bulls by in vitro tests on their semen.

The motivation of the research approach taken in this thesis in order to study possibilities of the prediction of dose dependent insemination results is discussed (in chapter 2) against the background of our current knowledge of the biology of reproduction. In order to be able to monitor fertility results after artificial insemination, information on performance of the insemination is retrieved from the field and non-return rates are calculated. Non-return rates are defined as the proportion of cows that were inseminated and did not return for another service within a specified time after insemination, usually 60 to 90 days and in the Netherlands 28 and 56 days. In chapter 3 of this thesis fertility evaluation by non-return rates was studied with the objective to develop a system to adjust non-return rates of bulls and insemination results of technicians for systematic environmental effects. We concluded that under current circumstances in the Netherlands non-return rates should be adjusted for effects of herdseason, season of insemination, age of the inseminated cow, technician and sire, in order to provide unbiased non-return rates for management and research purposes.

In order to see whether we could extract more elementary information on reproductive performance of a bull from non-return rate data when knowledge on the biology of conception

and gestation is taken into account we studied daily decline of non-return rates in chapter 4. A multiphasic logistic function was developed to model decline in non-return rate by estimating conception rate, calving rate and characteristics of the first two cycles.

In chapter 5 the effect of number of spermatozoa inseminated on fertility results is studied. Semen from 20 proven bulls was split sample diluted and each dose contained 2.1 to 17.3 x $10^6$ total spermatozoa. The exponential relationship between dose rate inseminated and non-return rates (chapter 3) or conception rate or calving rate (chapter 4) was evaluated for each bull. For 5 of the 20 bulls in this trial there was no observed dose dependent decline in fertility results. The expected decline was for these bulls outside the tested dose range. The optimal number of spermatozoa per insemination varied between bulls and moreover, ranking of bulls for non-return rate 56 days altered with the insemination dose. It is however, expensive and time consuming to determine the above described relation for each individual bull by performing a field trial. Furthermore, reliable and accurate records are a prerequisite for this type of field trial and therefore this procedure is not feasible for every AI company. Therefore, the aim is to predict the dose dependency of field fertility results using laboratory tests in order to circumvent these field trials.

Around 1985 several motility analyzing systems using video taping and tracking of spermatozoa were developed. These systems are objective and repeatable in comparison to the subjective motility estimation by eye which had been performed so far. In chapter 6 the dose dependence of motility characteristics from bull spermatozoa after freeze-thawing and different tests for resistance to incubation stress was evaluated. The information was subsequently used for prediction of the relationship between dose and corrected non-return rate at 56 days, estimated conception rate and estimated calving rate given conception. The motility characteristics had limited predictive value for the relationship between dose inseminated and fertility. The highest correlations found were in the order of -0.5 to -0.6. The correlation between maximal non-return rates and percentage motile cells immediately after thawing, being the most informative predictor, was 0.52. Capacitation and subsequent acrosome reaction was studied by simultaneous determination of viability and acrosomal status of the spermatozoa using microscopical evaluation in chapter 7. Determination of the

percentage of intact cells that are competent to acrosome react directly post thaw and the percentage of cells showing acrosome reaction upon addition of lysophosphatidylcholine at 4 hours of incubation can be used to predict the optimal semen dose when no information on field performance is available. In the case where we are able to retrieve field fertility data this test will give us information on the dose range to be tested under field conditions. This is where this test might prove its value for every AI laboratory. In chapter 8 the homologous oocyte penetration was studied for only five of the twenty bulls evaluated in chapter 5. It was quite difficult to design a repeatable test which measured only penetration as there was an influence if the concentration of sperm present altered, while sperm oocyte ratios stayed unchanged. Also the number of oocytes in test had its influence on penetration rates. The variation found in vitro and in vivo insemination results did not relate for the bulls tested. In the final considerations the research performed in this thesis is discussed against the background of the concept of holism and reductionism in science philosophy (chapter 9). It was concluded that the fertility assays evaluated are able to exclude most in- or subfertile males and eliminate wrong processing protocols of semen. Fertility assays are however, not able to point out males with superior fertility. Investment in philosophical thinking might develop our ability to take the total approach in order to get to the right questions. Finally with improvement of real knowledge therapeutic concepts for the in- or subfertile male might be developed as well as new ideas around processing and storage of semen.

## Samenvatting

In de huidige rundveefokkerij worden de meest waardevolle stieren geselecteerd op basis van melkproduktie en exterieur informatie van hun dochters. Op het moment dat de stieren zijn gekozen als fokstier is het doel zijn genen te introduceren en door te geven aan de populatie. Het is daarom belangrijk het aantal inseminaties per stier te maximaliseren zonder de kans op dracht in gevaar te brengen. In de runder-KI is er dus een continue noodzaak om het belang van de veehouder, namelijk zijn koe drachtig krijgen in balans te houden met haar eigen belangen, zoveel mogelijk inseminaties per stier te verrichten. Het doel van de runder-KI is een zo groot mogelijk aantal nakomelingen per genetisch superieure fokstier, daarbij wordt de genetische vooruitgang zo efficiënt mogelijk doorgegeven aan de rundvee populatie op de bedrijven. Dit doel wordt bereikt door het maximaliseren van het aantal verzamelde zaadcellen en het aantal inseminaties per zaadlozing, daarbij dient de kans op dracht na inseminatie behouden te worden. De motivering van de gevolgde aanpak in het onderzoek naar de dosis afhankelijkheid van inseminatie resultaten wordt in hoofdstuk 2 bediscussieerd tegen de achtergrond van onze huidige kennis van de biologie van de voortplanting. Om na kunstmatige inseminatie bevruchtingsresultaten te kunnen bestuderen wordt informatie uit de praktijk verzameld en worden non-return cijfers berekend. Non-return cijfers zijn gedefinieerd als het aantal koeien die binnen een bepaalde tijd na inseminatie, meestal 60 tot 90 dagen of in het geval van Nederland 28 en 56 dagen, niet opnieuw worden aangeboden voor inseminatie. In hoofdstuk 3 zijn deze non-return gegevens bestudeerd met als doel te komen tot een systeem ze te corrigeren voor systematische omgevingsinvloeden, met als doel een meer zuivere vergelijking van non-return resultaten tussen stieren en tussen inseminatoren mogelijk te maken. We concluderen dat onder Nederlandse omstandigheden non-return gegevens moeten worden gecorrigeerd voor bedrijf/seizoen, inseminatie seizoen, leeftijd van de koe en inseminator effecten om zuivere informatie te leveren voor management- en onderzoeksdoeleinden. Om de bekijken of het mogelijk was om meer elementaire informatie over vruchtbaarheidsresultaten te herleiden uit

de non-return gegevens wanneer daarbij kennis van de biologie van de conceptie en dracht werd betrokken hebben we de afname in non-return per dag bestudeerd. Een meerfase functie werd ontwikkeld om de afname in non-return te gebruiken om het niveau van conceptie en afkalven te schatten alsmede de karakteristieken van de eerste twee tochtigheidscycli na inseminatie.

In hoofdstuk 5 is het effect van het aantal geinsemineerde spermatozoa op de bevruchtingsresultaten bestudeerd. Sperma van 20 fokstieren werd (split sample) verdund, waarbij de dosis 2,1 tot 17,3 millioen zaadcellen bevatte. De exponentiele relatie tussen de geinsemineerde dosis en de non-return resultaten (hoofdstuk 3) en conceptie- en afkalfresultaten (hoofdstuk 4) werd voor elke stier bepaald. Voor 5 van de 20 stieren die werden getest werd er geen vermindering van inseminatie resultaten bij afnemend aantal geinsemineerde spermatozoa geconstateerd. De verwachte afname in non-return resultaten lag voor deze stieren beneden de geteste range. Het optimale aantal spermatozoa per inseminatie varieerde tussen stieren en ook de rangvolgorde, op basis van non-return, van stieren veranderde met het aantal geinsemineerde zaadcellen. Het is vergt veel tijd en het is kostbaar om dergelijke praktijktesten voor elke individuele stier uit te voeren. Bovendien is het noodzakelijk om betrouwbare en accurate praktijkgegevens beschikbaar te hebben om deze praktijstudies uit te kunnen voeren, daarom is het niet voor elke KI-vereniging mogelijk om haar stieren op deze wijze te testen. Het uiteindelijke doel is daarom om de dosis afhankelijke bevruchtingsresultaten te voorspellen om op deze wijze de praktijk testen overbodig te maken.

Rond 1985 werden verschillende beweeglijkheids analyse systemen geintroduceerd op basis van het volgen van zaadcellen op videobeelden. Deze systemen bleken objectief en herhaalbaar in tegenstelling tot de beweeglijkheidschatting met het oog die tot dan toe werd uitgevoerd. In hoofdstuk 6 zijn de dosis afhankelijke beweeglijkheidparameters bestudeerd na invriezen -ontdooien en incubatie stress. De verkregen informatie is vervolgens gebruikt om de relatie tussen inseminatie dosis en non-return-, conceptie- en afkalfresultaten te voorspellen. De beweeglijkheidsparameters hadden beperkte waarde om deze relatie te voorspellen. De hoogste correlaties die werden gevonde lagen in de orde van -0,5 tot -0.6.

De correlatie tussen maximale non-return resulaten en het percentage beweeglijke cellen direkt na ontdooien was 0.52. In hoofdstuk 7 is het vermogen tot capacitatie en acrosoom reactie van zaadcellen bestudeerd aan de hand van een simultane bepaling van vitaliteit en acrosoom status onder de microscoop. Bepaling van het percentage intacte cellen dat de acrosoom reactie kan ondergaan direkt na ontdooien en het percentage cellen dat acrosoom reactie vertoonde na toediening van lysophosphatidyl choline als trigger voor de acrosoom reactie na een incubatie van vier uur kan worden gebruikt om de optimale dosis zaadcellen per inseminatie te voorspellen wanneer geen informatie van praktijk data voorhanden is. In het geval dat er ook praktijk informatie aanwezig is, wordt deze test benut om aan te geven welke dosis range bestudeerd moet worden onder praktijk omstandigheden. Op deze manier zou de test voor elk KI-station van waarde kunnen zijn. In hoofdstuk 8 is de runder eicel penetratie bestudeerd voor 5 van de 20 stieren waarvan praktijk gegevens beschikbaar waren. Het was moeilijk om een herhaalbare test te ontwikkelen die alleen de eicel penetratie mat omdat er een effect aanwezig bleek wanneer de concentratie zaadcellen in de test veranderde, terwijl de zaadcel eicel ratio gelijk bleef. Ook het aantal eicellen per test had invloed op penetratie percentages. De variatie die in vitro werd gevonden had geen relatie met de bevruchtingsgegevens uit de praktijk.

In de eindbeschouwing is de onderzoeksstrategie gevolgd in dit proefschrift bediscussieerd tegen de achtergrond van het holistische en reductionistische concept in de wetenschapsfilosofie (hoofdstuk 9). Uiteindelijk is de conclusie dat de bestudeerde fertiliteitstesten het mogelijk maken om de slechtste stieren kwa bevruchting uit te selecteren danwel verkeerde behandeling van het sperma in het laboratorium op te sporen. Fertiliteitstesten kunnen echter niet dié stieren aanwijzen met een superieure vruchtbaarheid. Investering in onderzoeksfylosofie is nodig om ons vermogen te ontwikkelen om vanuit het totaal (holistische) redenerende te komen tot de juiste onderzoeksvraagstelling. Indien we deze vragen kunnen oplossen, dan wordt het mogelijk om een therapie voor de slecht bevruchtende stier te ontwikkelen en om nieuwe ideeen voor het bewaren en behandeling van sperma te gaan bestuderen.

## Dankwoord

Het is onmogelijk om iedereen die aan dit onderzoek heeft meegewerkt persoonlijk te bedanken. Dit proefschrift is het resultaat van de inspanning van velen over een periode van vele jaren. De eerste plannen zijn gemaakt met Hans Uwland, Jan Kruize, Chris Willems, Jan van Lieshout, Henk Jansen en Ebele Talstra. Bedankt voor jullie steun en enthousiasme, jullie hebben het project in de eerste fase gedragen en hebben bij de KI- besturen bepleit dat het kon worden uitgevoerd. In deze eerste fase was het ook vaak filosoferen 'geblazen' samen met Jan Aalbers, Hans Wilmink, Dick Koorn, Frank de Graaf, Albert Reurink en later ook Gerben de Jong. Ik herinner me sigaren op de vrijdag middag.... bedankt daarvoor. Daarna kwam het invriezen van het sperma op het laboratorium bij Mid-West samen met John Nieland, Dhr. P. Vos en medewerkers. Allemaal bedankt voor jullie werkspirit en enthousiasme. Helaas bleek de bestelde witte inkt niet te houden op de bruine rietjes... Erg onhandig voor onze inseminatoren, zelfs met het bijgeleverde leeslampje. Zonder jullie toewijding was het nooit gelukt, de registratie was bijna perfect! Dan is de veldproef af. Van elke concentratie van elke charge zijn rietjes achtergehouden.

Deze rietjes gaan dan naar het IVO waar Janneke (dan nog) de Leeuw net begonnen is, naar de Faculteit Diergeneeskunde en ook naar Embrytec. Samen met een aantal studenten en een groep die zich op voorstel van Tette van der Lende de 'Egg getters' noemt wordt bekeken of de testen iets opleveren. Henri Woelders, Ben Colenbrander, Theo Kruip, Tette van der Lende, Bas Kemp, Peter Ursem, Yvonne Wurth, Janneke van Wagtendonk, Richard de Jong, Hans Koole, Gerbrich Andringa, Bas Engel en Einco Topper zonder jullie vasthoudendheid en 'eindeloze' discussies was het nooit wat geworden. Vervolgens komt dan het schrijven. Het R&D team komt er min of meer alleen voor te staan, sorry maar ik heb voor jullie te weinig tijd gehad. Gelukkig kunnen we het samen prima vinden en hebben we inspirerende en flitsende discussies. Die heb ik nodig gehad om het uithoudingsvermogen te vinden... Rene Haring, Lucia Kaal, Janneke van Wagtendonk, Siebrand Merton en ook Joke Holland, jullie zijn zo vertrouwd dat het raar is om nu jullie achternamen te schrijven. Ik heb met name genoten van de diepgaande discussies over onderzoeksfilosofie en maatschappij

met Ronald van Giessen, Theo Kruip en anderen. Daardoor ben ik, tenminste zo voelt dat voor mezelf, in staat geweest het verhaal echt 'af' te maken. Ingrid Boerstoel, bedankt voor onze samenwerking en het leesbaar maken van het manuscript. Gelukkig houdt jij goede zin.

Beste Pim Brascamp en Tette van der Lende het moet voor jullie een gek idee zijn geweest de promotie van iemand te begeleiden zonder bij de opzet van het onderzoek te zijn betrokken. Bedankt voor jullie uithoudingsvermogen, de andere wijze van het kijken naar de gegevens en alle suggesties om mijn 'vreselijke' engels te verbeteren. In dit kader mag ik zeker Wiebe Koops en Peter de Boer niet vergeten, ook jullie bedankt voor de andere kijk op de dingen, discussie ...

En dan als laatste maar misschien wel het meeste moet ik het thuisfront bedanken. Mijn ouders, Diny en dit jaar ook Hilary die zoveel moeten opvangen omdat mijn drijfveren me in de weg zitten. Ook mijn vrienden die zich in de moeilijke dagen als een 'deken' om mij heen hebben gevouwen ben ik heel veel verschuldigd, ik hoop dat ik er nu ook wat meer voor hen kan zijn. Onder het motto van liever een inspirerende werkende moeder dan een sacherijnige thuis, heb ik (en doe ik) Wieteke en Lonneke dagelijks te kort gedaan. Meiden, nu komt er tijd voor zijdeverven, ontwerpen, toneel...en andere dingen. Peter, bedankt voor alle aandacht en begrip en ook voor alle ruimte die je mij en ons samen geeft, er zijn nu in ieder geval minder prioriteiten die in mij vechten.

Curriculum vitae

Johanna Hermina Geertruida den Daas was born on the 23rd of July 1955 in Bergh, one day after the 27th birthday of her father, a brick manufacturer. She was baptised Anneke, but over the years came to be known as Nanke. Nanke grew up in Klein Azewijn, in a house besides the factory. She attended Gemeentelijke Scholen Gemeenschap in Doetinchem for her secondary education and graduated in 1972. After spending a year in France and England she started a Bio-Chemistry degree at the University of Utrecht in 1973. After two years she decided that the study was too theoretical and missing the draw which would have enabled her to start studying veterinary science in 1976, she instead started work as a secretary for Prof. Dr. Hattinga Verschure at the Institute for Research on Hospitals and as veterinary assistant at the clinic of Dr. Kraan in Utrecht. In order to improve her chances in the draw for veterinary science she sat a second exam in secondary education 1977. Finally in 1978 the study of veterinary science could be started. In 1980 she left Utrecht to start a farm with her husband, Rick Zuure, along the river Rhine. On the first of June 1985, five days before graduating in veterinary science her career began as junior research assistant at the National Dutch Cattle Herdbook (NRS). Meanwhile her first daughter Wieteke was born in November 1984, followed by Lonneke in May 1987.

In January 1991 she started to work for Holland Genetics as manager of Research and Development. After her divorce in 1992 she has had the challenge of caring daily for her two children and furthering her career. With her children and Diny Rasing to act as second 'mum' for the girls, she spend a sabbatical of six months in New Zealand in 1996 with Livestock Improvement Cooperation. The field trials that form the basis of this thesis were performed in the period 1987/1988. Thereafter the laboratory tests were performed. Over the past years, besides her current job, the care of her children and the rather turbulent changes in her relationships, time has been found to write this thesis.

Curriculum Vitae

Johanna Hermina Geertruida den Daas werd op 23 juli 1955 geboren te Bergh, een dag na de 27ste verjaardag van haar vader, een baksteenfabrikant. Ze werd Anneke gedoopt, een naam die later is uitgegroeid tot Nanke. Nanke groeide op in Klein-Azewijn in een huis naast de fabriek. Ze bezocht de gemeentelijke scholengemeenschap te Doetinchem alwaar ze in 1972 het diploma HBS-b behaalde. Na een verblijf van (in totaal) een jaar in Frankrijk en Engeland begon ze in 1973 de studie (Bio-) Chemie aan de rijksuniversiteit te Utrecht. Na twee jaar studie ontdekte ze dat deze richting te theoretisch was. Wegens uitloting voor de studie diergeneeskunde werd in 1976 het diploma MoA-Schei- en Natuurkunde behaald. Hierna volgden anderhalf jaar waarin ze werkzaam was als secretaresse voor Prof.Dr. Hattinga Verschure van het Instituut voor Ziekenhuis-wetenschappen en als dierenarts-assistente op de praktijk van Dr. Kraan op de Biltstraat in Utrecht. Om de inlotingskansen te vergroten werd in 1977 een staatsexamen VWO afgelegd. Uiteindelijk kon Nanke in 1978 met de studie diergeneeskunde beginnen. In juli 1980 werd het appartement in Utrecht verruild om samen met Rick Zuure het akkerbouwbedrijf "de Roswaard" langs de Rijn in de Overbetuwe op te starten. Op 1 juni 1985, vijf dagen voor het behalen van het begeerde diergeneeskundige diploma, begon ze haar carrière als onderzoeksmedewerker bij het Nederlands Rundvee Syndicaat. Ondertussen was Wieteke, haar eerste dochter, in november 1984 geboren en in mei 1987 volgde Lonneke.

Per januari 1991 trad ze in dienst bij haar huidige werkgever Holland Genetics als manager Onderzoek en Ontwikkeling. Na de scheiding in 1992 draagt ze de dagelijkse zorg voor beide kinderen. Met haar kinderen en Diny Rasing, als steun en toeverlaat, werd in 1996 zes maanden in Nieuw-Zeeland bij Livestock Improvement Cooperative doorgebracht in het kader van een werkuitwisseling. Het veldonderzoek dat de basis vormt voor het bijgaande proefschrift is uitgevoerd in 1987/1988. Daarna zijn de laboratorium-bepalingen uitgevoerd. In de laatste jaren is naast de huidige baan, de opvoeding van de kinderen en de tumulteuze veranderingen in haar relaties, gewerkt aan de op schriftstelling van het onderzoek.

List of publications

Den Daas, J.H.G. and Aalbers, J.G., 1988. Effect of suboptimal number of spermatozoa per insemination on fertility of deepfrozen spermatozoa. 11th Int. Congr. on Animal Reprod. And Artif. Insem. Dublin, Ireland, 3: 236-238.

Den Daas, J.H.G., de Leeuw, A.M., and Woelders, H., 1992. Use of the Fix-Vital Stain method to Monitor Capacitation of Frozen Thawed Bovine Spermatozoa in Vitro. 12th Intern. Congr. on Animal Reprod., The Hague, The Netherlands, vol1: 432-434.

Den Daas, J.H.G., 1992. Laboratory assessment of semen characteristics. Anim. Reprod. Sci., 28: 87-94.

Den Daas, J.H.G. and Merton, S., 1994. In Vitro Embryo Production, Its Use. 10e Reunion Association Europeene de Transfert Embryonaire. Lyon, 9-10 sept., INRA, Nouzilly France: 117-124.

Den Daas, J.H.G. 1997. Genetic Gain Through Reproductive Technologies. In: Milk composition, Production and Biotechnology. Edt. R.A.S. Welsch, D.W.J. Burns, S.R. Davis, A.I. Popay and C.G. Prosser, CAB International USA: 357-362.

De Leeuw, A.M., den Daas, J.H.G., Kruip, Th.A.M. and Rall, W.F., 1992. The Relative Efficiency of Bovine Embryo Cryopreservation by Vitrification and Conventional Slow Freezing. 12th Intern. Congr. on Animal Reprod., The Hague, The Netherlands, vol 3: 1398-1400.

De Leeuw, A.M., den Daas, J.H.G. and Woelders, H., 1991. The fix vital stain method. Simultaneous determination of viability and acrosomal status of bovine spermatozoa. J. Androl., 12: 112-118.

De Leeuw, F.E., de Leeuw, A.M., den Daas, J.H.G., Colenbrander, B. and Verkleij, A.J. 1993. Effects of various enviromental conditions cryoprotective agents and membrane stabilizing compouns on Bull Sperm Membrane Integrity after Cooling and Freezing. Cryobiology, 30: 32-44.

Erkens, J.H.F. and den Daas, J.H.G., 1994. Seminal Plasma Acrosin Inhibitor BUSI-I Content is Correlated to Bull Fertility. Reprod. Dom. Anim., 29: 387-395.

Grossman, M., Koops, W.J. and den Daas, J.H.G., 1995. Multiphasic analysis of reproductive efficiency in dairy bulls. J. Dairy Sci. 78: 2871-2876.

Koops, W.J., Grossman, M. and den Daas, J.H.G., 1995. A model for reproductive effeciency in dairy bulls. J. Dairy Sci., 78: 921- 928.

Kruip, Th A.M. and den Daas, J.H.G., 1997. In Vitro Produced and Cloned embryos Effects on Pregnancy, Parturition and Offspring. Theriogenology, 47: 43-52.

Reurink, A., den Daas, J.H.G. and Wilmink, J.B.M., 1990. Effects of AI sires and technicians on non-return in the Netherlands. Livst. Prod. Sci., 26: 107- 118.

Te Meerman, G.J., Mullaart, E., van der Meulen, M.A., den Daas, J.H.G., Morolli B., Uitterlinden, A.G. and Vijg, J. 1993. Linkage Analysis by two dimentsionsal DNA Typing. Am. J. Hum. Genet. 53: 1289-1297.

Trommelen, G.J.J.M., den Daas, J.H.G., Vijg, J. and Uitterlinden, A.G., 1993. DNA profiling of cattle using micro- and minisatellite core probes. Animal Gen., 24: 235-241.

Trommelen, G.J.M., den Daas, J.H.G., Vijg, J. and Uitterlinden, A.G., 1993. Identity and Paternity Testing of Cattle: Application of a Deoxyribonucleic Acid Profiling Protocol. J. Dairy Sci., 76: 1403-1411.

Van der Schans, G.D., Timmerman, A.J. and den Daas, J.H.G., 1996. An Immunochemical assay for the assessment of bovine sperm quality with respect to fertility efficiency. The 13th Int Congr on Animal Reproduction, Sydney, Australia, 3: p24-15.

Van Os, J.L., de Vries, M.J., den Daas, J.H.G. and Lansbergen L.M.T.E. Trends in sperm count of dairy bulls. Submitted to J. of Andrology.

Van Wagtendonk-de Leeuw, A.M., den Daas, J.H.G., Kruip, Th.A.M. and Rall, W.F., 1995. Comparison of the Efficacy of Conventional Slow Freezing and Rapid Cryopreservation Methods for Bovine Embryos. Cryobiology, 32: 157-167.

Van Wagtendonk-de Leeuw, A.M., den Daas, J.H.G. and Rall, W.F., 1994. Pregnancy Rates in a Comparative Field Trial of Vitrification and One-Step Dilution or Conventional Slow Freezing and Three-Step Dilution of Bovine Embryos are Similar. Theriogenology 41: 326.

Van Wagtendonk-de Leeuw, A.M., den Daas, J.H.G. and Rall, W.F. Accepted for publication Theriologenology. Field Trial to Compare Preganancy Rates of Bovine Embryo Cryopreservation Methods: Vitrification and One-Step Dilution versus Slow Freezing and Three-Step Dilution.