Prediction of porcine male fertility

Maria Leonarda Wilhelmina Johanna Broekhuijse

M.L.W.J. Broekhuijse
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Maria Leonarda Wilhelmina Johanna Broekhuijse

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Science is sizzling, slimey, strange, and successful!

The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.
(Albert Einstein)

Voor mijn ouders

Gaat het niet zoals het moet, dan moet het maar zoals het gaat

Voor Jef

Het zijn niet alleen mooie veren die mooie vogels maken

Contents

General Introduction

Outline

Efficient artificial insemination (AI) is essential for future challenges in the pig industry. Knowledge on the exact relation between semen quality characteristics and fertility can have a major impact on both the genetic merit of future animals and efficiency of AI. In this general introduction the history of pig AI is briefly discussed, followed by the status of pig AI in the Netherlands. Porcine semen production is introduced with the different methods to assess semen quality, resulting in the scope and the outline of this thesis.

History of artificial insemination

Artificial insemination (AI) has been first mentioned around 1400 in undocumented tales about an Arabic chief inseminating a mare. A cotton clough was drenched in the semen of a stallion belonging from rival groups and was placed in the vagina of their own mares. Leeuwenhoek (1678) was the first to report sperm cells. Another century passed before an Italian priest, Spallanzani (1784), inseminated a bitch which whelped three pups (Siebenga, 1937). The insemination in human was first attempted around 1800 by Hunter, followed by Sims in 1866. Heape *et al.* (1897) reported successful AI in studies with rabbits, dogs and horses. The Russian professor Elie Ivanov started working with sperm cells from testis, epididymis or vas deferens by the end of the 19th century and published in 1907 his first report (Ivanov, 1907). An English paper is written on studies in domestic farm animals, dogs, foxes, rabbits and poultry (Ivanov, 1922). Both studies included the use of porcine sperm for AI purposes. All over the world research on AI was stimulated. Milovanov (1964) established major projects for sheep and cattle breeding and designed artificial vaginas, similar to those used today. In 1912, Ishikawa started a similar program in horses (Nishikawa, 1962; 1964) and this developed in AI being applied in Japan in cattle, swine, goats, sheep and poultry. Using AI became widespread in the world with the book on AI by Watson (1933). Growth of AI occurred in the 1940s in the United States and procedures developed became established worldwide (Salisbury *et al.*, 1978).

More studies on pig AI were conducted in the United States (McKenzie, 1931), Japan (Niwa, 1958) and in Western Europe (Polge, 1956). Boars are easily trained on mounting dummies (Polge, 1956). All artificial vaginas developed for semen collection provided a means of applying pressure to the glans (McKenzie, 1931; Polge, 1956) or a gloved hand (Hancock and Hovel, 1959) could be used directly. The gloved hand method was beneficial for AI as it minimised the amount of bacterial contamination in collected semen (Althouse and Lu, 2005). The

development of a method to store semen long enough for shipment and use in the field was initiated in the United States where a yolk-phosphate semen extender was developed (Philips and Lardy, 1940). When extenders were developed for bull semen, they were used or modified for boar semen as well. Ito *et al.* (1948) was the first to recommend a storage temperature of 15-20°C. With AI expanding rapidly, demands for semen increased. The simplest solution was to dilute each ejaculate further by using less number of sperm cells per insemination. The Beltsville Thawing Solution (BTS) was developed by the USDA laboratory in Beltsville and made it possible to dilute the semen and increase the storage time up to 48 hours.

Pig AI became available in the Netherlands at the end of 1950s (Strikwerda, 2007). The use of pig AI was stimulated by the Dutch Ministry of Agriculture, the Animal Health Service and was adopted by the regional herd books. The main reason was to prevent the spread of contagious diseases. Before, breeding boars were shared among different breeding farms to mate the sows. The first pig AI stations were independent cooperative boar stations. The breeding companies were working close together with the AI companies to make the boars with the highest genetic index available for AI and the exclusive use of those boars for the nucleus breeders. Pregnancy rates of 60% and litter sizes of six piglets were common in the beginning of the use of pig AI. More research and development was funded since 1977 and in the years 1980-1990 the results improved. There was more knowledge on oestrus cycle of the sow, timing of the insemination and treatment and dilution of boar semen. Once the results were similar to natural mating, commercial farms started to use AI and the use of pig AI increased rapidly. Where technicians played an important role in the good fertility results at that moment, the demand for self-service AI increased in order to save labour (Strikwerda, 2007).

Current status of pig artificial insemination in the Netherlands

Today, most sows (98%) in the Netherlands are bred through AI, with one of the highest fertility results worldwide. In Europe, the pig AI rate is between 25% and 98% (Feitsma, 2009). Table 1 illustrates some characteristics of current sow fertility results in the Netherlands. In 1992, the Netherlands introduced the pooling of semen for commercial herds. Since the outbreak of classical swine fever in 1997, the use of pooled semen was prohibited by the Dutch government.

Pooled semen increases the impact of infected semen on farms and complicates effective tracing of potentially infected boars (Stegeman *et al.*, 1999; Stegeman *et al.*, 2000). Nowadays only single sire insemination doses are used.

Table 1. Characteristics of sow production in the Netherlands.

Number of sows	1,000,000
Number of AI doses sold	3,500,000
Number of cycles per sow per year	2.35
Number of doses per sow per cycle	1.6
Farrowing rate	86%
Total number of piglets born per litter	13.9
Live number of piglets born per litter	12.9
Number of piglets weaned per sow per year	26.2

Results are based on 2010 records (Dutch Association of Cooperative Pig AI centres year report, 2010; Agricultural Economic Institute Wageningen University, 2010; Agrovision, 2011).

Monitoring field fertility

Economic losses due to reproductive inefficiency in male animals can be substantial (Roberts, 1986). Common problems in sub fertile and infertile human patients and animals include low number of sperm cells or low semen quality. It is generally accepted that lower motility semen samples have lower or limited potential to fertilise oocytes (Flowers, 1997; Donadeu, 2004). The question remains how differences in sperm properties from high fertile AI boars can explain variety in field fertility results, which is also the main question in current thesis. Minimal differences in pig fertility may already lead in lower efficiency of producing piglets and thus to a loss of economic prospects. Although efficiency of piglet production is dependent on many factors one of them that we can measure is the quality of semen. Indeed sub- or infertility is a significant problem in humans and domestic animals (Lunenfeld and Insler, 1993). Ultimately one would like to design a semen quality test that could predict the fertilising potential of the semen of individuals.

The most critical aspect of predicting the fertilisation potential of semen samples is to have specific, precise and accurate fertility tests and precise and accurate fertility data. It is very difficult to obtain reliable field fertility data. The problems that can occur are 1) boars or sows are not representative for the population, and are too few in number, 2) insufficient sows are inseminated with sperm cells from each semen sample, 3) too few semen samples are assessed per boar, 4)

inappropriate number of cells is used for each insemination, 5) fertility outcome is not reported properly. Predicting the fertilising potential of semen samples on basis of semen quality characteristics and analysing relationships with fertility needs high quality fertility data to compare with standardised laboratory test results.

For successful implementation of AI, field fertility results of semen used are critical. In the Netherlands, data on both pig AI level and on farm level are recorded, which is the basis data in current thesis. Large data sets of fertility data and ejaculate data are more suitable to analyse effects of semen quality characteristics on field fertility. It solves the problems mentioned 1) boars or sows represent the actual population in the Netherlands, 2) insemination doses produced from each semen sample are transported to sow farms, 3) all routine ejaculate production per boar is used for AI, 4) each insemination is performed with a relatively low concentration, 5) fertility outcome is reported in sow management systems, which are recorded properly. Variation in fertility in sows is large. The effect of semen factors is relatively small and therefore impossible to find in smaller data sets. Large data sets allow for statistical corrections on both sow- and boar-related parameters. Remaining sow fertility variation can then be assigned to semen quality parameters.

A standard calculation of technical results and data exchange is available. Linking breeding and AI databases makes it possible to analyse the relation between semen quality characteristics and fertility. New techniques and remaining research questions can be tested in this dataset. Field trials can be designed with accurate data retrieval. Differences in litter sizes as far as 0.1 piglet can be detected (Feitsma, 2009). Institute for Pig Genetics B.V (Beuningen, the Netherlands) maintains the database of both breeding company TOPIGS B.V. (Vught, the Netherlands) and the cooperative pig AI centres Varkens KI Nederland B.V. (Deventer, the Netherlands) and Varkens KI Twenthe B.V. (Fleringen, the Netherlands). Results of over 8 million litters are recorded and semen quality information of over 1 million ejaculates is known (status in 2011). With this infrastructure semen quality characteristics can be validated for their relation with field fertility.

Semen quality assessment

Boars are introduced at Dutch AI centres via quarantine and strict health controls. The semen production is performed under strict hygienic conditions. Boars are collected in separate collection pens on dummy sows with the gloved hand technique (Hancock and Hovel, 1959) or in an automated semen collection system (Collectis®, IMV, L'Aigle, France). The semen is pre diluted within 10 minutes after collection with Solusem® (Varkens KI Nederland, Deventer, the Netherlands) with similar temperature as the semen. Each semen quality assessment starts with the macroscopic evaluation: colour, smell, contamination with dirt, blood or urine and viscosity. After volume determination, the concentration and the motility are measured. Until 2006, the concentration was measured using a colorimeter or spectrophotometer and the motility was microscopically estimated by experienced laboratory technicians. Currently, a computer assisted semen analysis (CASA) system is used for the evaluation of sperm motility characteristics. The results of assessing semen motility microscopically or with a CASA system and the merging of these motility data with field fertility results of the same semen samples, is part of this thesis.

After quality assessment the semen is diluted (Solusem®, 20°C) to a current minimum level of 1.5 billion motile sperm cells in 80 ml (NEN-ISO 9001, Varkens KI Nederland, 2011). The insemination doses are transported to the distribution area. The transport temperature is 17°C ± 2°C and the transport boxes are temperature controlled. Also all farmers do have temperature controlled cabinets to store the doses until use on the farm. The motivation of the Dutch AI company is to produce these insemination doses as efficiently as possible, without loss in fertility results. This continuous search for improved efficiency triggered the question if variation in semen motility could explain variation in field fertility and if, by objective measurements, this variation due to motility could be reduced.

For the current AI companies it is important to achieve the highest reproductive efficiency per sold insemination dose obtained and diluted from boars. Reproductive performance has a genetic basis, however is highly affected by environment. For optimal genetic expression the environmental effects should be reduced. One of the variables in the environment is the semen quality. Hence reducing the variation in fertility results caused by variation in semen quality will enhance genetic expression in fertility results. Once the selection criteria for semen approval are validated, the semen dose production per ejaculate will increase and become more efficient. Therefore, the constant achieving of the fertilising efficiency in the field with

insemination doses produced will result in a faster dissemination of desired genes by which the difference with the nucleus breeding top decreases (personal communication EHAT Hanenberg, TOPIGS). High quality semen can be defined as having an optimal fertilising capacity. Rejecting or approving an ejaculate is based on semen quality assessments. But what is the value of assessing semen quality if the relation with field fertility is unknown?

Semen assessment starts with semen quantity assessment (macroscopic evaluation: semen volume and sperm density), followed by semen quality assessment (microscopic evaluation; sperm motility, morphology and integrity). From the initial stages of AI development until the present time, the assessment of the percentage moving (motile) semen is the most widely used test of semen quality (Salisbury *et al.*, 1978). To improve quantifying the semen motility bright field microscopy, differential interference contrast microscopes, CASA, multiple stains and flow cytometry have contributed. The relevancy of assessing semen quality parameters is answered in this thesis by relating these results to actual field fertility.

Microscopic semen motility analysis

Anthony van Leeuwenhoek (1632-1723) was a Dutch tradesman and is known to have made over 500 microscopes. Fewer than ten have survived to the present day. His discoveries on the presence and movement of "animalculi", which were sperm cells, were reported in 1677 in a letter to the *Philosophical Transactions* and published as a Latin translation (Leeuwenhoek, 1678).

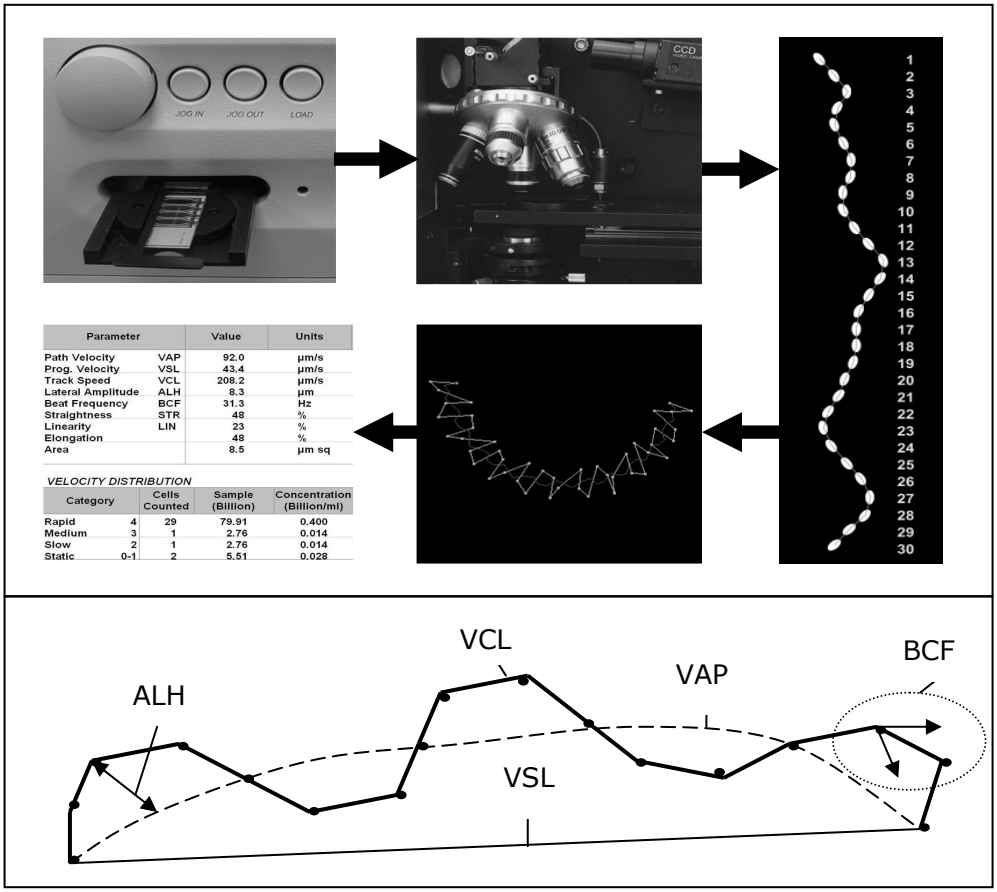
Nowadays the movement of semen is manually measured with phase contrast microscopy by trained technicians. Usually there are two parameters for motility: quantity and quality of motile sperm cells. The cut-off values are set arbitrarily. Visual estimation of semen motility is a common laboratory test, but is subject of discussion regarding the predictive value of fertility, the subjective nature and the human bias. The World Health Organisation (WHO) recommends assessing by categorising semen as immotile (no movement), non-progressive motility and progressive motility (WHO, 2010). The laboratory technicians of Varkens KI Nederland use a more detailed scoring system grading per 10% quality increase. It is difficult for a technician to grade the velocity of moving semen. Essential for this thesis is the question whether there is a relation between this microscopically assessed semen motility score and field fertility results. Such an eventual relation will become extremely relevant for an AI company and for a breeding company in the process of improving efficiency in both AI and breeding.

Computer assisted semen analysis

In the 1940s, scientists started recognising the need for objective data on the movement of sperm cells (Amann and Katz, 2004). Dott and Foster (1979) first proposed a computer assisted semen analysis (CASA) approach to obtain an overall objective semen motility analysis. The first validated CASA system was presented at the Third International Conference on the Spermatozoon in 1978 (Amann, 1979). Commercially the first CASA system developed was the CellSoft system (Cryo Resources Ltd), starting in 1985. Hamilton Thorne Research developed an other commercial system: the HTM-2000w, which was a system in a box, introduced in 1986. Davis and Katz (1993) report an overview of over 120 papers that have been published, verifying CASA technology for semen analysis.

CASA is a computerised system, which visualises and digitises the image of sperm cell movement. By means of a stroboscopic principle with a frequency of 60 Hz pictures are taken from which the exact trajectory of each individual cell in the observed microscopic field is followed and recorded, as is shown in Figure 1. Information on the kinematics of individual cells and ejaculate summary statistics can be calculated from CASA data. Next to motility and progressive motility (which are calculation results, based on settings of basic parameters), more movement parameters are assessed. Some standard terminology for variables measured by CASA is illustrated by Figure 1. Different CASA systems use different mathematical algorithms, so results are not automatically comparable among systems. But the results do give a perfect tool to link the CASA motility assessment scores with field fertility.

Despite the possibilities for objective and multiparametric motility analysis that are allowed by CASA it is still of concern that different CASA systems intrinsically give different results. Another concern is the intensive need for training of the users and evaluation and calibration of the settings of the equipment. CASA is currently one of the most popular methods to evaluate semen motility (Verstegen *et al.*, 2002). In 2003, there were systems in use at approximately 1,200 sites worldwide (Amann and Katz, 2004), primarily in human andrology laboratories. Since then the highest market growth occurred in large human clinics and in large animal companies (e.g. pigs). Using a CASA system in a high productive pig AI laboratory can have additional effects over microscopic semen motility assessments, which are pointed out in this thesis. Furthermore, implementing a CASA system means the possibility to establish the relation between semen motility and field fertility because more detailed semen motility parameters are studied.



Parameter		Value	Units
Path Velocity	VAP	92.0	µm/s
Prog. Velocity	VSL	43.4	µm/s
Track Speed	VCL	208.2	µm/s
Lateral Amplitude	ALH	8.3	µm
Beat Frequency	BCF	31.3	Hz
Straightness	STR	48	%
Linearity	LIN	23	%
Elongation		48	%
Area		8.5	µm sq

VELOCITY DISTRIBUTION

Category		Cells Counted	Sample (Billion)	Concentration (Billion/ml)
Rapid	4	29	79.91	0.400
Medium	3	1	2.76	0.014
Slow	2	1	2.76	0.014
Static	0-1	2	5.51	0.028

VAP	Velocity Average Path	µm/s	Time averaged velocity of a sperm head along its average path.
VSL	Velocity Straight Line	µm/s	Time averaged velocity of a sperm head along the straight line.
VCL	Velocity Curvi Linear	µm/s	Time averaged velocity of a sperm head along its actual curvilinear path.
ALH	Amplitude of Lateral Head	µm	Magnitude of lateral displacement of a sperm head about its average path. Expressed as maximum or average.
BCF	Beat Cross Frequency	Hz	Frequency of the sperm head crossing the sperm head average path

Figure 1. Schematic presentation of motility parameters recorded in a single sperm trajectory as recorded by Computer assisted semen analysis (CASA) systems.

Flow cytometry

Another way for objective assessments of sperm samples using a computer interface is flow cytometry. The principle of this technique is that a cell suspense is introduced in fluid stream through a laser excitation source. Cells can be fluorescently labelled for certain cell characteristics. The cell size morphology is determined by scattered excitation light detection while the fluorescent properties per cell can be determined by fluorescence detectors. This technique allows rapid (up 5000 cells/second) analysis of multiparametric properties (for a review about flow cytometry see Nunez, 2001). Semen motility only quantifies the movement of the cell, but does not give an indication about any other sperm cell characteristics. Other sperm quality characteristics could for instance thus be assessed by flow cytometric and may relate to field fertility results. Different parameters are assessed for thousands of sperm cells per second (for principle see Nunez, 2001 and Figure 2). Flow cytometry is not only used for semen assessment but also routinely in the diagnosis of any given cell suspense for instance in human health for assessing blood cancer. The first flow cytometer was issued in 1953, to Wallace H. Coulter. The first fluorescent flow cytometer was developed in 1968 by Wolfgang Göhde and commercialised in 1969.

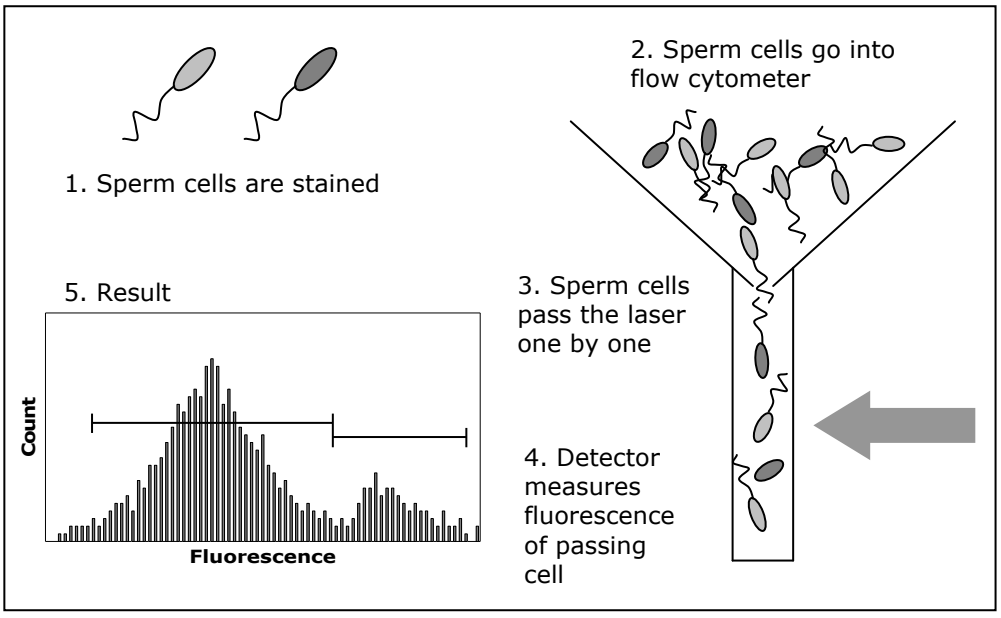


Figure 2. Schematic presentation of the semen quality assessment using flow cytometry.

The strength of the flow cytometry technique is that measurements on large numbers of sperm cells can be made within a short time frame. Different subsets of cells can be identified and quantified. Flow cytometry allows assessment of different semen quality characteristics like plasma membrane integrity, acrosome intactness and responsiveness, mitochondrial and chromatin structure. The system is relative expensive and trained technicians to operate the system are necessary. Therefore, flow cytometry is not often used as routine semen quality assessment method for pig AI. However, detailed flow cytometric data analysis of sperm cell and sperm cell subpopulation properties may add predictive value to a certain insemination dose and its fertilisation competence.

In vitro fertilisation

In vitro fertilisation (IVF) is a commonly known concept in the human industry. Infertility affects a lot of people and the IVF technique, among others, helps to get a woman pregnant. In 1978, IVF was with the birth of baby, successful for the first time. Robert G. Edwards developed the treatment and was awarded with the Nobel Prize in Physiology or Medicine in 2010.

There is always an interest in improving technologies in pig reproduction. IVF of pig oocytes has developed in the last years. Transferable *in vitro* produced embryos can be produced with acceptable farrowing rates and litter sizes after transfer to recipient animals (Gil *et al.*, 2008). Although both farrowing rate and litter size are still too low to compare with current field results. Moreover, a specific problem for pig IVF is the relative high occurrence of polyspermic fertilisation lowering the IVF efficiency. Despite of these drawbacks IVF can be used as a diagnostic for the fertilising capacity of semen. IVF is often mentioned as an indicator for *in vivo* fertility (Gadea, 2005). Relevant for this thesis is whether or not differences between ejaculates and/or boars can be screened by their capacity to fertilise oocytes in an IVF test. Therefore, in current thesis an IVF test is validated as predictor for fertilising capacity of semen from high fertile boars in a commercial AI industry.

Scope of the thesis

The tests performed in pig AI labs to assess semen quality are aimed to exclude sub or non-fertile boars and their ejaculates. Most AI boars are fertile but nevertheless there is variation in their field fertility performance. Once the relation between semen quality characteristics and field fertility is known, AI companies will be able to distinguish between ejaculates for fertility potential and adapt dilution rates of ejaculates accordingly. Therefore questions as: "Can we predict the fertility outcome based on assessing semen quality in a laboratory? How large is this effect and should we implement new assessment techniques in routine AI laboratories in order to improve the effect of semen quality assessment?

Outline of the thesis and experimental approaches

Farmers efficiently use semen to maximise the number of sows farrowing and the number of piglets produced per litter. There is variation in fertility results and multiple variables cause this variation. These variables are for the largest part related to farm and sow but are related to boar and semen as well. Finding causes of this variation makes it possible to reduce variation. Therefore research is necessary. Research was initiated by the pig AI company and Utrecht University participates in this research because of the possibility to validate semen quality assessment tests.

This study consists of 6 parts, presented as Chapter 1 – 6 in this thesis. In Chapter 1 an overview of studying boar fertility is given. What is generally known about field fertility analysis of boar semen quality? How the use of large datasets, merging breeding and AI datasets, make it possible to retrospectively analyse the relation between semen quality characteristics and fertility and learn about factors significantly affecting fertility. In Chapter 2, results from microscopic semen motility analysis, performed between 1998 and 2006 by AI laboratories in practice, were related to field fertility, by which an overview is given of all boar and semen parameters influencing this relation. This knowledge is valuable for the AI company on efficient dose production, but also for the breeding company on knowledge of effects on technical field fertility results. Chapter 3 continues on the microscopic semen motility assessment and compares this method with assessing semen motility with the use of a CASA system. The additional value of using a CASA system for the pig AI industry is described. In 2006 the laboratories implemented a CASA system and Chapter 4 describes the relation between motility parameters measured with CASA and field fertility.

Implementing a CASA system involves important adaptations and therefore, analysing the relation between CASA motility parameters and field fertility is of great importance to an AI company. Motility is not the only parameter of interest, which is shown in Chapter 5, describing a detailed flow cytometry study. It is known that there are differences in semen quality which can be established by flow cytometry tests, but often the relation with field fertility is missing in literature. Before such a test can be implemented in an AI routine practice, this relation has to be established first. Multiple flow cytometry tests are evaluated in this chapter. Selecting ejaculates based on their quality and fertility potential resulted in comparing IVF results of different ejaculates, which is described in Chapter 6, again as a possible tool to predict field fertility by semen quality assessment. Finally, the benefits and progress of analysing all these different semen quality characteristics in relation to fertility are discussed in the concluding discussion with future perspectives.

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Chapter 1

Field data analysis of boar semen quality

Broekhuijse MLWJ, Feitsma H, Gadella BM.
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Abstract
This contribution provides an overview of approaches to correlate sow fertility data with boar semen quality characteristics. Large datasets of fertility data and ejaculate data are more suitable to analyse effects of semen quality characteristics on field fertility. Variation in fertility in sows is large. The effect of semen factors is relatively small and therefore impossible to find in smaller datasets. Large datasets allow for statistical corrections on both sow and boar related parameters. Remaining sow fertility variation can then be assigned to semen quality parameters which are of huge interest to AI companies. Previous studies of Varkens KI Nederland to find the contribution to field fertility of (1) the number of sperm cells in an insemination dose, (2) the sperm motility and morphological defects and (3) the age of semen at the moment of insemination, are discussed in context of the possibility to apply such knowledge to select boars on basis of their sperm parameters for AI purposes.

Introduction
The most important question for a pig AI company is how the semen quality characteristics can be related to fertility results. By knowing the boar and semen factors which explain the variation in fertility, the value of such a factor can be estimated and hence the criteria for release or rejection of an ejaculate for distribution. This strategy is underlined in literature (Foxcroft *et al.*, 2008; Gadea, 2005; Amann, 1989). However, the analysis of a good field fertility dataset with sufficient data is complicated and has not been performed yet. We here provide an overview of approaches in the past and discuss them in order to comment on current emerging approaches to show the value of field data analysis of boar semen quality as well as the applications in future AI industry.

Semen collection, processing and data recording
The working methods at the AI stations of Varkens KI Nederland are standardised and controlled during the whole production procedure (ISO 9001-2008, Varkens KI Nederland, Deventer, the Netherlands). Boar ejaculates are collected on a routine base at the AI stations, collecting the sperm-rich fraction using the gloved hand technique (Hancock and Hovel, 1959). The semen is diluted via a two-step dilution method. The volume of the ejaculate (ml) is diluted with approximately the same volume (ml) of Solusem® short term (≤ 72 hours) extender (Varkens KI Nederland, Deventer, the Netherlands) at 32°C within 15 minutes after collection (step 1). After this first dilution, semen quality is assessed by

measuring semen motility (fresh, 24 and 72 hours after production) and semen concentration with the Computer Assisted Semen Analysis (CASA) system UltiMate™ (UltiMate™ Sperm Analyzer, Hamilton Thorne Inc., Beverly, MA, USA) with standardised Leja 4-chamber counting slides (Leja Products B.V., Nieuw-Vennep, the Netherlands). Each fourth ejaculate of a boar is manually assessed on morphological abnormalities (fixed, stained samples) using phase contrast microscopy. After semen quality assessment, the final dilution (step 2) is performed using Solusem® 20°C. AI doses are produced (1.5 billion motile homospermic cells per 80 ml) and then stored in a temperature conditioned room at 17°C. Varkens KI Nederland records boar and ejaculate related information such as boar identification (tattoo number and name), genetic line and age of the boar (current mean: 25 ± 12 months), days between ejaculation (current mean: 4.5 ± 2.5 days), AI station, location and date of ejaculate production, semen collector, laboratory where ejaculate is processed, laboratory technician who assessed semen quality, volume and concentration of ejaculate (current mean: 84×10^9 ± 11×10^9 cells per ejaculate), motility (fresh, 24 and 72 hours after production), morphological abnormalities, concentration of the insemination dose and number of doses produced (current mean: 35 ± 15) in the AI database: AI Information System (KIS) under a unique ejaculate number.

Data recording in the field

The farmer uses the ordered insemination doses to inseminate the sows. Ejaculate number of the insemination dose used, date of return to oestrus, date and ejaculate number used for following inseminations, date of farrowing and number of piglets born (total, live, dead, and mummified) are recorded. At the farms in the Netherlands, the number of inseminations per cycle is 1.6 with a range of 1 – 3. This insemination protocol is based on the results of Steverink *et al.* (1997, 1999). Heat detection is performed every 12 hours, with recommended boar in front of the sow for positive effect on heat detection (Langendijk *et al.,* 2005). When there is no heat detected, no insemination is performed. If a sow is still in heat after 24 hours, a second insemination is performed. The majority of inseminated sows in the database are purebred sows, producing either purebred offspring or more frequently crossbred offspring (F1 production). Sows that are inseminated twice within the same cycle using insemination doses from two different boars are excluded from this analysis. There are two possible sow management systems (Pigmanager and Farm) which are available in the Netherlands containing the standardised fertility results from each farmer. Data of

both systems can be exchanged electronically with the breeding database. These data are 1) to estimate the breeding value and 2) to advise the farmer on reproduction management (on request) because fertility related technical results can be calculated as well. Merging these results with ejaculate information leads to the establishment of a large dataset to be analysed.

Description of the dataset

In the Netherlands 98% of the annual 4,275,000 sow matings are performed by AI. At Varkens KI Nederland approximately 1,800 boars produce ± 3.7 million insemination doses per year. For successful implementation of AI the field fertility results of the semen utilised are critical. The breeding database (Pigbase) of the Institute for Pig Genetics (IPG, Beuningen, the Netherlands) contains fertility records from purebred and crossbred sows. These data are used for genetic evaluation and breeding value estimation. From this dataset two fertility parameters of the inseminations, namely (1) the farrowing rate (FR), indicating the percentage of sows that produced offspring from the initial insemination, and (2) the litter size indicating the total number of piglets born (TNB) per litter, are recorded and related to the semen quality of the underlying insemination doses used. Currently, the dataset of Varkens KI Nederland contains over 1 million ejaculates (1998-2010) which are merged to the Pigbase breeding dataset of IPG which contains 8.6 million farrowing records from 750 farms (< 2010). With this merge a unique dataset is built, which is used to analyse the relation between boar or semen quality parameters and field fertility.

Statistical approach

From each ejaculate 35 ± 15 (mean ± SD) insemination doses are produced. Approximately 10-15 sows are inseminated with semen from 1 ejaculate. Therefore per ejaculate a maximum of 10-15 sow records are available. However, as not all farms exchange the information with IPG only 5-6 sow fertility records are available per ejaculate. The fertility results vary because of sow related and herd related factors. Therefore, the fertility results per ejaculate are first corrected for sow related factors calculating the least square means (LSM) per ejaculate for FR and TNB. This method of calculating a LSM is a statistical approach to find a solution for the multiple fertility results of one ejaculate (SAS 9.1 Institute Inc., Cary, NC, USA). LSM is calculated dealing with correction factors by adding a weight statement. Sow and herd related correction factors are: parity and genetic line of the sow, the effect of the farm and the season, whether it is a first or remating, the effect of a purebred or

crossbred litter, the weaning to oestrus interval, the number of inseminations per oestrus, the effect of the year, month and day of the week of insemination and the age of the semen (days after production) at insemination.

Next step in the analysis is to focus on the parameter of interest and to correct for other boar and semen factors which affect the fertility results. In order to analyse the relation between e.g. sperm motility and fertility, the effect of all other boar and semen related factors must be identified and analysed to determine if these factors significantly affect the fertility. Significant factors which are included in the final statistical model are: genetic line of the boar and the effect of the individual boar, the effect of age of the boar and days between ejaculation, the effect of AI station and laboratory technician, the effect of the number of sperm cells in an ejaculate and the number of sperm cells in an insemination dose. Remaining variation could be assigned to the semen quality characteristics of interest. Next step is to analyse if the specific quality characteristics such as percentage motile sperm and percentage progressive motile sperm have a significant effect on fertility and the magnitude of the effect is calculated. For an AI company this is relevant, since with this method the *in vitro* tested semen quality can be related to field fertility and test results can be used to effectively select good performing ejaculates from poor performing ejaculates.

Pros and cons of field data analysis

The use of field data is disputable since they might contain multiple errors, due to incorrect data collection and recording at farm level. The field breeding database contains results from nucleus farms (purebred and crossbred) where farmers do focus on their data recording and therefore, results are of high reliability. All relevant data are first checked for correctness because this is essential for the purpose of these data: breeding value estimation. Hence the field dataset used for this kind of analysis is unique with regard to the number and the quality of the data.

In a dataset from numerous farms with several factors affecting the fertility results, it is difficult to find significant differences between ejaculates and fertility records. In order to be able to correct for these factors, a large dataset is necessary for showing statistically significant differences. However, if significant differences appear from this analysis, the power of the analysis result is huge, since it represents actual differences found in the field.

Table 1. Literature on boar semen quality AI related research. Results represented are without field fertility results (A), with *in vivo* field fertility results based on small field fertility trials (<100 boars/ejaculates) (B) or with *in vivo* fertility results based on field fertility under (commercial) conditions with large number of results (>100 boars/ejaculates and/or >1000 inseminations) (C).

A Author	Semen quality characteristic	# of boars	# of ejaculates
Boe-Hansen *et al.*, 2005	DNA integrity	20	60 (3 per boar)
Gil *et al.*, 2009	Morphometry, motility (CASA)	5	65 (13 per boar)
Paulenz *et al.,* 2000	Long term storage	16	16 (1 per boar)
Smital, 2009	Year-season, age of boar and days between ejaculation	2,712	230,705 (± 85 per boar)
Vyt *et al.*, 2004	Motility (CASA)	30	900 (30 per boar)

B Author	Semen quality characteristic	# of boars	# of ejaculates	# of insem.
Mircu *et al.*, 2008	Motility (CASA)	10	270 (± 27 per boar)	125
Popwell and Flowers, 2004	Motility, morphology	3	120 (40 per boar)	664
Sutkeviciene *et al.*, 2009	Fluorescence properties of sperm nucleus	19	57 (3 per boar)	2,296
Sutkeviciene *et al.*, 2005	Methanol-stressed motility	36	288 (8 per boar)	13,993
Tardif *et al.*, 1999	Motility	9	9 (1 per boar)	74

C Author	Semen quality characteristic	# of boars	# of ejaculates	# of insem.
Anil *et al.*, 2004	Age of semen	Unknown		35,300
Boe-Hansen *et al.*, 2008	DNA integrity	145	435 (3 per boar)	3,276
Gadea *et al.*, 2004	Motility, morphology, membrane integrity	57	273 (± 5 per boar)	1,818
Didion, 2008	Motility (CASA)	208	3,077 (± 15 per boar)	6,266
Holt *et al.*, 1997	Motility (CASA)	53	170 (± 3 per boar)	2,200
Vyt *et al.*, 2008	Motility, morphology	38	100 (± 3 per boar)	276

Why are field data analyses of boar semen quality so relevant?
Most research on the effect of semen quality on fertility is performed *in vitro*, but hardly in the field. Table 1A shows some examples of research performed on semen quality characteristics but without *in vivo* fertility results (varying from 16 to 230,705 ejaculates assessed). For example, Smital (2009) performed a very detailed research on 2,712 boars analysing 230,705 ejaculates, assessing quality and quantity of boar semen. Unfortunately, the results from such study do not consider relations with field fertility. And although of interest, the results do not guarantee their use in the field. Overall, publications show differences between methods, protocols, different tests or systems, but no author can describe what this means for the actual fertility. A test can be of interest when it relates to fertility effects, but without knowing field results, we cannot establish cut off values for processing semen. Therefore, it is hard to implement such a new test/parameter only based on *in vitro* trials.

Other researchers base their results on *in vivo* trials, but the field data are often minimal. In their trials, semen samples of only a small number of boars (often from one genetic line) are used and doses are inseminated at a conditioned experimental farm with standardised sows and insemination management or after oestrus and ovulation induction. Examples of small (<100 ejaculates) *in vivo* fertility trials, in experimental setups, are shown in Table 1B. The advantage of small *in vivo* trials is that the experimental groups are kept and managed under the same conditions and the results of the semen quality parameters can be easily correlated with the actual *in vivo* results, possibly leading to a conclusion that a significant relation is present. However, the differences must be quite large to find significant variations in an *in vivo* trial. An important practical question is if these experimental results can be extrapolated to other genetic sow lines and to other farms or to other ejaculates from different genetic boar lines. The results of boars/ejaculates used from experimental trials in literature could differ significantly, but be within the normal fertility range of the overall boar/ejaculate population and therefore have no meaning for practice. The unique field dataset used at Varkens KI Nederland combines all these criteria. Since the variation in FR and TNB is quite large and affected by many factors, a considerable number of fertility results are required to find significant differences. Table 1C shows research where more than 100 ejaculates and/or more than 1,000 inseminations are used under more commercial/practical conditions, with more variation in parameters like boar, breeding line, etc. Gadea *et al.* (2004) conclude that semen analysis conducted under commercial conditions leads to

detection of ejaculates with very poor quality, but a high number of sperm cells per dose and a high quality of the semen used reduce the variability. Didion (2008) states that despite his large dataset (6,266 inseminated sows) the low number of sows mated per boar and the number of inseminations per oestrus or the number of sperm cells in an AI dose could cause the lack of correlation between semen parameters and fertility. Our built up dataset now contains a number of ejaculates, boars and matings per boar (1 million ejaculates and 8.6 million farrowings) that exponentially outnumber the results in literature, which enables to find even the smallest difference at a very significant level, which is the most important value of this dataset.

Results from field data analysis

As mentioned above the large field fertility dataset gives us the possibility to relate semen quality characteristics with field fertility. Overall, it is concluded that there is a significant effect ($P<0.05$) of both breeding line of the boar and of individual boar on FR and TNB (Feitsma, 2009; Chapters 2 and 4). An important conclusion for AI companies, dealing with different individual boars from different breeding lines. Results from previous statistical analyses are summarised below.

The relation between morphological defects and fertility is analysed based on the results of 132,345 ejaculate records with morphology results. There is a significant relation ($P<0.05$) between the number of morphological defects and fertility (Bergsma and Feitsma, 2005). Decreasing the threshold for percentage of abnormal cells from 30% to 20% results in a positive effect on fertility of +0.07% FR and +0.08 TNB (Feitsma 2009). Based on the analysis results, new cut-off values are calculated and implemented in the semen processing procedures of our AI company.

Also interesting for AI practice is how the age of the semen at the moment of insemination affects fertility. Varkens KI Nederland guarantees the quality of the insemination doses for 72 hours after production. In the field however, farmers use the insemination doses up to 7 days after production. Since these results are recorded, the relation between age of semen and fertility is analysed. There is no significant effect ($P=0.09$) between age of semen and FR and TNB from day of production up to 5 days after production (personal communication JI Leenhouwers, Institute for Pig Genetics B.V.). This result can be used to reorganise distribution of semen in the AI company and equalise workload over days avoiding peak production at the start of the week.

The laboratory technicians of Varkens KI Nederland have been using a microscope for semen motility assessment until 2006. In the period of 1998-2006 these motility scores were recorded and a dataset of >110,000 ejaculates with known fertility results has been built up using these data. The analysis of the relation between microscopic assessed semen motility and field fertility shows a significant but only small positive effect ($P<0.0001$), although highly affected by the influence of AI laboratory and technician (Chapter 2).

In order to objectivise and standardise semen motility assessment, Varkens KI Nederland implemented a CASA system (UltiMate^{TM}) in all AI laboratories in 2006. From the results of the first 3 years (2006-2009, >45,000 ejaculates with known fertility) we conclude that there is a significant relation ($P<0.05$) between basic CASA motility parameters and FR and TNB (Chapter 4). Furthermore, the method is concluded to be objective, since there is no longer an effect of laboratory technician and AI centre on FR ($P=0.91$) and TNB ($P=0.40$).

Number of sperm cells in an AI dose is partly depending on the percentage of motile sperm cells in an ejaculate. We have compared the effect of number of motile sperm cells for two time periods. The time period 1998-2006 shows a significant effect of number of motile sperm cells in an AI dose on TNB ($P=0.01$). For the last four years (2006-2010), the number of motile sperm cells in an insemination dose no longer shows an effect on FR ($P=0.75$) and TNB ($P=0.52$). This conclusion is confirmed in an overall statistical analysis in 2010 (personal communication JI Leenhouwers, Institute for Pig Genetics B.V.).

Although not routinely produced, there is a certain demand for cryopreserved semen in the pig AI industry especially for genetic linking of nucleus herd of internationally operating breeding companies. In principle, cryosurvival of frozen-thawed sperm and fertilisation results of those insemination doses can be monitored as mentioned in this review in the same way as for fresh diluted semen. Moreover, the characteristics of fresh diluted sperm appear to correlate to certain extent to their post-thaw properties (personal communication H Feitsma and JI Leenhouwers, Institute for Pig Genetics BV, Beuningen, the Netherlands). As mentioned, frozen boar semen is currently only a small part of the AI market. Therefore, the actual available number of frozen ejaculates used for insemination is too small to be able to get reliable relations between cryopreservation results and fertility. In principle with large datasets such correlations can be made in the future and results may be used to select boars suitable for freezing.

Monitoring results in practice

From a commercial perspective it is important for an AI company to monitor their results in practice. Knowing the performance of semen in the field and keeping track records, enables efficient analysis of the causes of poor performance at individual farm level. Determining the proper number of motile cells in an insemination dose, the maximum allowed morphological defects, the optimal value for CASA motility parameters and the shelf life of an insemination dose permits the AI company working as efficient as possible at lowest possible cost price and with maximum field fertility. An example is decreasing the number of cells in an insemination dose. Lowering the number of cells in an insemination dose is not possible without knowing the optional risk of decrease in field fertility. Varkens KI Nederland knows this effect and we are able to calculate the lower limits for our AI company and for our clients. The effect of performed adaptation in cut-off levels is guaranteed by continuing statistical analyses on these parameters.

Finally, the field fertility dataset is and will be used to test other and new semen quality characteristics such as membrane integrity, acrosome intactness, acrosome responsiveness, chromatin structure and potential of the inner mitochondrial membrane using flow cytometry and recorded field fertility results. In this way we could evaluate the practical relevance of these flow cytometry tests (Chapter 5). The ultimate goal is to find a sperm quality index resulting from the combination of relevant results from *in vitro* tests that will allow deciding which ejaculates should be used, how many sperm cells should be used per insemination dose, what the guaranteed shelf life is and what the expected fertility is. On the other side, this index can be used to identify sub fertile boars. The use of such a system gives enormous progress in the quality and efficiency management at AI stations

Conclusive remarks

In order to monitor field results and to analyse the relation between semen quality characteristics and fertility, collecting field fertility data and merging these with ejaculate records is a very strong tool. It is of higher value for AI centres than collecting data in an experimental set up. Using field data has the advantage over experimental data that all factors affecting fertility can be quantified and corrected, which leaves the variation in fertility due to semen factors. This variation can be assigned to semen quality characteristics as morphological abnormal cells, age of semen, semen motility, and number of sperm cells in a dose. Small experimental data can reveal the effect of a specific semen quality parameter in the experimental setting and/or conditions.

However, extrapolation for other lines, farms, boars and ejaculates cannot be done. Results from small experimental trials are impossible for pig AI centres to use. Changing their control policy for the processing and production of insemination doses should be based on data analysis representing the practical relation between semen quality and field fertility.

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Chapter 2

The value of microscopic semen motility assessment at collection for a commercial artificial insemination centre, a retrospective study on factors explaining variation in pig fertility

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Broekhuijse MLWJ, Soštarić E, Feitsma H, Gadella BM.
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Abstract
This study was conducted to evaluate the relationship between boar and semen related parameters and the variation in field fertility results. In eight years' time semen insemination doses from 110.186 ejaculates of 7,429 boars were merged to fertility parameters of inseminations of 165,000 sows and these records were used for analysis. From all ejaculates boar and semen related data were recorded at the artificial insemination (AI) centres. Fertility parameters such as farrowing rate (FR), ranging between 80.0 and 84.0%, and the total number of piglets born (TNB), ranging between 12.7 and 13.1, were recorded and from these the least square means per ejaculate were calculated. Only 5.9 % of the total variation in FR was due to boar and semen variability of which 21% (P=0.0001) was explained by genetic line of the boar, 11% (P=0.047) was explained by laboratory technician and 7% (P=0.037) was explained by the AI centre. For TNB the total variation was 6.6% boar and semen related of which 28% (P<0.0001) was explained by genetic line of the boar and 7% (P=0.011) was explained by the AI centre. Only 4% of the boar and semen related variation was caused by sperm motility (microscopically assessed at collection, ranging from 60% to 90%). Other variation in FR and TNB was explained by management and semen related parameters (age of boar (3% (P=0.009) and 8% (P=0.031) respectively), days between ejaculations (1% (P<0.0001) of FR), number of cells in ejaculate (1% (P=0.042) of TNB), year (9% (P=0.032) and 13% (P=0.0001) respectively), month (11% (P=0.0001) and 5% (P=0.0001) respectively)). Although semen motility is considered to be an important parameter to validate the quality of the ejaculate processed, it only minimally relates to fertility results under the current Dutch AI practice. Other boar and semen related parameters, like genetic line of the boar, are more relevant factors to select boars for AI purposes.

Keywords
sperm, semen motility, porcine, fertility, genetic line

Introduction

In commercial pig breeding great emphasis is put on improving field fertility results. In the Netherlands, at farm level a farrowing rate (FR) of > 83% and a total number of piglets born (TNB) of > 13 is considered to be sufficient. It is generally accepted that there is large variation in these fertility results, mainly caused by farm and sow related parameters (Hanenberg *et al.*, 2001). Factors that affect this variation in fertility can be minimised, by breeding, management and by artificial insemination (AI). Examples of these effects are seasonality, shown by differences in heat stress tolerance between sow lines (Bloemhof *et al.*, 2008) or minimising the effect of farm by training and supervision on standardising insemination protocols. Remaining variation in pig fertility is explained by boar and semen related parameters. Nowadays, artificial insemination (AI) is a tool for efficient distribution of high quality genetics and efficient running of a genetic program. AI centres should always aim to minimise their effect on variation in pig fertility and at the same time know which role they play in the pig breeding program by, among others, semen quality assessment. It is essential for AI centres to guarantee that only the high quality ejaculates will be processed further. Sperm motility is commonly believed to be one of the most important semen quality characteristics (Ruiz-Sanchez *et al.*, 2006). In this retrospective study, fresh sperm motility is used as an example parameter which possibly affects variation in pig fertility. The usefulness of semen motility assessments to judge semen quality has been debated (Baker *et al.*, 1957) although it is commonly used and has been shown to correlate with the fertilising capacity of the semen (Flowers, 1997; Holt *et al.*, 1997; Tardif *et al.*, 1999; Flowers, 2002; Donadeu, 2004; Gadea *et al.*, 2004; Gadea, 2005; Foxcroft *et al.*, 2008). Besides sperm motility, there are several other boar and semen related parameters affecting pig field fertility, such as: the number of spermatozoa inseminated (Holt *et al.*, 1997; Xu *et al.*, 1998; Tardif *et al.*, 1999) and the existing variation between different laboratories and technicians (Baker and Clarke, 1987; Gadea *et al.*, 2004). Also other parameters that could be related to the boar (individual boar, genetic line and age of the boar (Xu *et al.*, 1998)) and non-boar related factors (season (Janett *et al.*, 2005)) can affect the fertilising capacity of a given semen sample.

Optimising the fertilising capacity of the semen is critical for a successful AI program. One aim of this study was to investigate how useful and reliable fresh microscopic semen motility assessments are for predicting the fertilising capacity of boar semen. Other factors were simultaneously evaluated retrospectively from a very large dataset, which was obtained from one commercial breeding company and was

used to establish predictive values accurately. Based on the results described in this manuscript several boar and semen related parameters and their effect on pig field fertility are discussed.

Material and methods

Animals and semen

Data records of ejaculates collected at four AI centres, belonging to Varkens KI Nederland (Deventer, the Netherlands) from January 1998 until April 2006 were analysed retrospectively. These four AI centres in total had 7 AI laboratories and 11 semen production locations. The ejaculate data recorded (n=110,186) originated from 7,429 boars (15 different genetic lines).

Boar ejaculates were collected on a routine basis at the AI centres with a standardised protocol which was constant over the years (on average 1.6 collections per boar per week); the sperm rich fraction was collected using the gloved hand technique (Hancock and Hovel, 1959). The ejaculate was collected in a pre-warmed (40 ± 2°C) plastic container (370 ml, Graham Packaging Company Inc., York, England) without insulation cover cup. At the moment of collection, the ejaculate was filtered with a milk filter (nonwoven disc, 200 mm, Universal Filters Inc., New Jersey, USA) to remove the gel fraction. The volume of the ejaculate (ml) was diluted with approximately the same volume (ml) of Solusem® extender (Varkens KI Nederland, Deventer, the Netherlands) at 32 ± 2°C within 15 minutes after collection. This was the first step in the two step dilution method, used and standardised by Varkens KI Nederland (personal communication). The first dilution was not too long after collection, to prevent the risk of forming agglutination. Furthermore, first dilution with a small temperature decrease prevented temperature shock during later dilution procedures which was tested before establishing this standard procedure (personal communication). Semen concentration was assessed using a colorimeter (Ciba-Corning Colorimeter 252; Ciba Corning Diagnostics Ltd., Suffolk, United Kingdom, used by AI centres 1 and 3) or a spectrophotometer (Spectronic®, Thermo Scientific, Waltham, USA used by AI centres 2 and 4). The systems have a CV of 10.4% (Hansen *et al.*, 2006) or less and were calibrated every three months with the same NucleoCounter (NucleoCounter® YC-100™, ChemoMetec A/S**,** Allerød, Denmark) as a standard (started in 2003). Results should be within a window of 5%, otherwise the equation was adapted. Before 2003, the calibration was performed every three months by the Animal Health Service (Deventer, the Netherlands) with the use of a CoulterCounter (CoulterCounter®,

Beckman Coulter, Inc., Brea CA, USA). The CoulterCounter was calibrated with World Health Organisation standards for counting total number of sperm cells.

The weight from the approximately 1:1 diluted ejaculate was retrieved. The weight was used as the measure for volume. The number of cells in billions was calculated by volume (ml) multiplied by concentration (million/ml) and divided by 1000 ((volume × concentration) / 1000). The AI centres used a minimal percentage of motile cells in an insemination dose to calculate the number of doses that could be produced. After fresh sperm motility assessment at collection (described in paragraph 2.2), the final dilution in order to reach an overall average over the years of 2.7 ± 1.5 billion total sperm cells per dose, was performed using Solusem® (20 ± 1°C). This was the second step in the two step dilution method, used and standardised by Varkens KI Nederland (personal communication). The protocol of dilution remained constant during the years, although the number of cells per insemination dose decreased over the years. After final dilution, polyethylene insemination tubes (Minitüb GmbH, Tiefenbach, Germany) were filled (80 ml). The tubes were airtight sealed and stored in an acclimatised area (17 ± 2°C). Within four hours after production, the insemination doses went on transport. The transport of the insemination doses was temperature controlled in a transport box (17 ± 2°C). At the farm, the farmer had an acclimatised box as well (17 ± 2°C, digital recorder with a min and max thermometer) and the insemination doses were stored until use for AI. Varkens KI Nederland provided semen with longevity of 72 hours after production although unpublished data revealed that farmers use the semen for more days, but that age of semen (at least until day 5) did not have an effect on fertility results.

Assessment of the sperm motility

The motility score of semen was routinely obtained by experienced laboratory technicians, working in a laboratory at room temperature (20°C ± 2°C). The assessments were performed at fresh ejaculates directly after collection and the same criteria were used over the years. The AI centres in the Netherlands have strict standards for ejaculate approval, partly based on the motility characteristics of an ejaculate (percentage motility and quality of movement). For the fresh sperm motility assessment a 10 µl drop of the initially diluted ejaculate was placed on a pre-warmed (38± 2°C) glass slide and covered with a cover slip (microscope slides, Mediware®, Wesel, Germany). The evaluation was performed at 200× magnification by a phase contrast microscope and a thermal plate, (BH-2, Olympus, Tokyo, Japan). The experienced

laboratory technicians estimated the percentage of non-moving sperm in 3-4 fields. The classification system for the 10 categories of fresh sperm motility is represented in Supplementary table 1. Based on this estimation, the laboratory technicians gave a percentage of motile cells in classes of increasing steps of 10% motility. Only ejaculates with sperm motility of minimal 70% at day of collection were initially accepted. The class of 60% motility at collection was accepted in combination with an increased number of sperm cells in the insemination dose (±33% increase of the initial number of cells in a dose).

Fertility results, breeding database

The breeding database (Pigbase) of the Institute for Pig Genetics (IPG, Beuningen, the Netherlands) registers fertility records from purebred and crossbred sows. This breeding database includes the records of ejaculate identification, the boar and day of insemination. At farm level in the Netherlands a strict timing of the insemination was advised, the average number of inseminations per cycle was 1.6 with a range of 1 – 3. Short weaning to oestrus intervals (WEI) in sows (5-6 days) in general gave longer oestrus duration. Therefore the first insemination for this group of animals was later after onset of oestrus than in the group with a long WEI (>7 days). Timing was farm dependent but aimed for the first insemination used within a window of 24 hours pre-ovulation. When 24 hours after the first insemination the sow was still in heat, a second insemination was performed. This insemination protocol was based on the results of Steverink *et al.* (1997, 1999). Heat detection was performed every 12 hours. During insemination it was advised to have a boar in front of the sow, which induced uterine contractions. These standards were set by Langendijk *et al.* (2005).

The fertility records extracted from the database could be correlated to the ejaculate parameters recorded at the AI centres. In this case the relation between semen motility characteristics for individual boar ejaculates and the fertility results obtained from the insemination doses derived from these ejaculates was analysed. The majority of inseminated sows were purebred sows, producing either purebred off-spring or more frequently crossbred off-spring (F1 production). Sows that were inseminated twice within the same cycle using insemination doses from two different boars were excluded from this analysis. In Dutch practice only two different sow management programs were used by farmers. The definitions for technical results were standardised in the Netherlands. The farmer recorded first and if performed a second and third insemination per sow. Therefore always

the ejaculate number or boar used, date of returning to heat (non-pregnancy), date of following insemination, date of farrowing and number of piglets born (total, live, dead, mummified) were recorded on a per sow basis. These data were exchanged electronically via an electronic data interchange (EDI) module with the breeding database (Pigbase). Data were loaded in this database following several procedures which checked for faults. Once all data were considered valid, the data were accepted in Pigbase. From this database we extracted a dataset which was used in order to analyse the per ejaculate fertility. Two fertility traits of the inseminations, namely: (1) the farrowing rate (FR) indicating the number of sows that produced offspring, and (2) the litter size indicating the total number of piglets born (TNB) per litter, were recorded and related to the semen motility assessment scores of the insemination doses used.

Statistical analyses

Data collection resulted in 1) a dataset with sow fertility records and parameters affecting these results and 2) a dataset with ejaculate records and parameters affecting these results. The two datasets with sow fertility and ejaculate records were merged. With the insemination doses of one ejaculate more than one sow were inseminated and thus resulted in more than one fertility result per ejaculate. For the statistical approach the results were first corrected on the sow related parameters, calculating the least square means (LSM) for the two fertility traits (FR and TNB) using the statistical package SAS (SAS 9.1 Institute Inc., Cary, NC, USA). The method for LSM is an approach to find a solution for a system in which there are more results for the unknown. In this study, there were ejaculates with multiple fertility results per ejaculate. In order to calculate the LSM, the data were corrected for factors significantly affecting sow fertility results, not being boar or semen effects. We calculated a LSM in which we deal with all those factors by adding a weight statement. The observations were weighted according the inverse of the standard error of the estimates for the ejaculate. Unit of analysis was ejaculate, because all observations were measured per ejaculate. Testing against ejaculates with inseminations as unit of observation, took care of the insemination related fertility traits. The best possible LSM was estimated for ejaculate effect and then these LSM were analysed. The boar and semen related effects were corrected for in another model (model 2), which is described in the results paragraph.

The following model was used to describe the sow related parameters:

$Y = \mu + parity + line♀ + farm \times quarter + 1^{st}/remating + purebred / crossbred\ litter + \#\ inseminations + weekday + age\ semen + error$ [1]

Where Y was the value of FR or TNB; μ is the mean value of FR and TNB; parity was the effect of actual parity of the sow; line♀ was the effect of the line of the sow, farm × quarter was the effect of the herd and the season (first quarter was January – March, second quarter was April – June, third quarter was July – September and fourth quarter was October – December); 1^{st}/remating was the effect of a first or a remating of the insemination outcome (rematings were known to have better results); purebred/crossbred litter was the effect of having a purebred or crossbred litter and assumed heterosis effect of crossbred animals; # inseminations was the effect of the number of inseminations per heat; weekday was the effect of the day of the week of insemination which indicates the longitude of the interval weaning to insemination; age semen was the effect of the age of the semen (days after production) at insemination (this is a farm effect, since the farmer decides when to use the semen); and error was the random residual effect. The parameters in this model were tested for interaction, but no more interactions, other than farm × quarter, were significant and were therefore not taken into model 1.

Data were checked for normality and the Pearson correlation test (SAS Inst. Inc., Cary, NC, USA) was used to study the correlations among the semen motility and fertility parameters. Boar related parameters were put into a general linear model (SAS Inst. Inc., Cary, NC, USA) to study the relation between the sperm motility and fertility, taking information from the boar (line, age, ejaculate interval), the AI centre (laboratory technician, centre) and the ejaculate (volume, concentration) into account. The variance component analysis (SAS Inst. Inc., Cart, NC, USA) was used to determine the sources of variation in the fertility results. Results are presented as means and standard deviations. Differences were considered to be statistically significant when $P \leq 0.05$.

Results

Descriptive statistics

The dataset was limited to ejaculates used to inseminate sows that produced fertility records. The descriptive results from this dataset are combined in Table 1 for farm and sow (Table 1A) and boar and semen (Table 1B) related parameters. The number of ejaculates at each semen motility assessment score from the contributing AI centres that were inseminated and generated fertility data are listed in Table 2. Table 3 shows the variation in fertility results per semen motility class (60-90%).

Relation between sperm motility and sow fertility

Sperm motility at the day of collection was correlated with the field fertility traits farrowing rate (FR) and total number of piglets born (TNB). The correlations were significant, but weak (n=110,186 ejaculates, 165,000 inseminated sows; FR: $r=-0.03$, $P<0.0001$; and TNB: $r=0.06$, $P<0.0001$). These correlations, uncorrected for boar and related parameters, demonstrated only a minimal relationship of fertility with fresh sperm motility. Corrections for boar and semen related parameters are made in next sections.

The combined 60-70% semen motility classes (n=50,907 ejaculates) had a significantly, and unexpectedly higher FR ($P=0.0034$; Table 5) than that of the 80% motility class (n=55,533 ejaculates), but lower than the 90% motility class (n=3,746 ejaculates). As expected TNB increased ($P=0.001$) from the 60% (n=8,649 ejaculates) to the 70% (n=42,258 ejaculates) to the 80% (n=55,533 ejaculates) motility classes; the 90% class (n=3,746 ejaculates) did not differ from 80%.

Table 1. Descriptive results of sow and boar related parameters. A. Sow related parameters, B. Boar related parameters. Data registered from January 1998 until April 2006. Results are based on ejaculates from Varkens KI Nederland with known fertility results in breeding database IPG.

Parameter	Number of records or mean ± SD
A.	
Number of farms	350
Number of inseminated sows	165,000
Number of genetic sow lines / crossings	22
Sows per farm	475 ± 51
Weaning to oestrus interval, d	6.22 ± 2.92
Number of inseminations per cycle	1.6 (1-3)
Parity	3.3 ± 2.5
Gestation length, d	115.1 ± 1.5
Farrowing rate, %	81.4 ± 30.1
Number of total born piglets	12.9 ± 2.1
Number of live born piglets	11.7 ± 3.1
Number of still born piglets	1.0 ± 1.2
Number of mummified born piglets	0.2 ± 0.7
Number of piglets weaned	10.7 ± 2.1
B.	
Number of ejaculates	110,186 ejaculates (with known fertility)
Number of boars	7,429
Number of genetic boar lines	15
Age of boars, mos	25 ± 12
Number of days between ejaculations, d	4.5 ± 2.5
Number of cells in ejaculate	$84 \times 10^9 \pm 11 \times 10^9$
Number of cells in dose (80 ml)	$2.7 \times 10^9 \pm 1.5 \times 10^9$
Number of doses produced per ejaculate	35 ± 15

Table 2. Variability in sperm motility and number of ejaculates used for the relation studied in total and per AI centre.

Mot(%)	Total (7)	AI centre 1 (2)	AI centre 2 (3)	AI centre 3 (1)	AI centre 4 (1)
60 %	8,649 (7.8%)	416 (0.7%)	7,991 (16.7%)	1 (0.03%)	241 (8.5%)
70 %	42,258 (38.4%)	11,760 (21.0%)	28,815 (60.1%)	183 (5.4%)	1,500 (53.0%)
80 %	55,533 (50.4%)	40,304 (72.0%)	11,078 (23.1%)	3,149 (93.1%)	1,002 (35.4%)
90 %	3,746 (3.4%)	3,532 (6.3%)	80 (0.2%)	49 (1.4%)	85 (3.0%)
Ejac.	110,186	56,012	47,964	3,382	2,828

Number between brackets is number of laboratories, Ejac is the number of ejaculates
For results per motility class, see Table 5.

Table 3. Descriptive results of semen motility assessment and results of farrowing rate (FR), and total number of piglets born (TNB). Mean and standard deviation (SD). In total 110,186 ejaculates were assessed.

Motility, %	Number of ejaculates	FR, % mean ± SD	TNB mean ± SD
60 %	8,649	83.1 ± 29.9	12.7 ± 2.2
70 %	42,258	82.7 ± 29.2	12.8 ± 2.1
80 %	55,533	80.0 ± 31.8	13.1 ± 2.0
90 %	3,746	84.0 ± 27.6	13.0 ± 2.0
Mean		81.4 ± 30.1	12.93 ± 2.1

Boar related parameters as source of variation in fertility

Sources of variation in fertility can be divided in farm and sow related parameters and boar and semen related parameters, the so called direct boar effect (DBE). We statistically corrected for sow related parameters already, calculating the LSM of fertility traits. The remaining variation therefore is the DBE on fertility. Part of this variation is due to non-semen factors, others are related to semen factors. In order to analyse the relation between sperm motility and fertility, we have to quantify the effect of all boar related parameters which affect fertility. Therefore, we included boar related parameters which had a significant effect on fertility, in the statistical model for calculating the relation between sperm motility and fertility:

$Y^* = \mu^* + line♂ + age + days\text{-}ejac + AI\ centre + lab\text{-}ID + year + month + sperm\text{-}ejac + sperm\text{-}dose + motility + error^*$ [2]

Where Y* was the LSM value of FR or TNB; μ* is the mean value of FR and TNB; line♂ was the effect of the genetic line of the boar (boar line); age was the effect of the age of the boar at ejaculation; days-ejac was the effect of the number of days between current and previous ejaculation; AI centre was the effect of all the different AI centres of Varkens KI Nederland; lab-ID was the effect of the laboratory technician performing the semen assessment; year was the effect of the year (1998-2006); month was the effect of the month during the year (Jan-Dec); sperm-ejac was the effect of the number of cells in the ejaculate (volume × concentration); sperm-dose was the effect of number of cells in a commercial dose; motility is the effect of semen motility assessment results; and error* was a random residual term. While using GLM, the weight statement was used. The observations were weighted according to the inverse of the standard error of the estimates for the ejaculate. The parameters in this model are tested for interaction, but no interactions were significant and were therefore not taken into model 2.

Table 4. Boar related sources of variation (direct boar effect) and their effect on farrowing rate (FR) and total number of piglets born (TNB).

Sources of variation	FR		TNB	
	%	P-value	%	P-value
Direct boar effect	5.9		6.6	
Line of boar	21	0.0001	28	<0.0001
Age of boar	3	0.009	8	0.031
Days between ejac	1	<0.0001	ns	0.371
No cells in ejaculate	ns	0.127	1	0.042
No cells in dose	ns	0.315	ns	0.127
Year	9	0.032	13	0.0001
Month	11	0.0001	5	0.0001
AI centre	7	0.037	7	0.011
Laboratory technician	11	0.047	ns	0.087
Semen motility	4	0.0001	4	<0.0001
Residual	33	<0.0001	34	<0.0001

ns = non significant. % = percentage of explained variation by boar related parameters. P-value = significance value, differences were considered to be statistically significant when P≤0.05.

Of the variation in total fertility, the DBE was small: for FR 5.9% and for TNB 6.6% of the variation was explained by the model. As part of the DBE, sperm motility explained 3.8% of the variation in FR and 4.3% of the variation in TNB, as indicated in Table 4. Most of the variation was explained by the line of the boar, the AI centre, the laboratory technician, the year and the month of production. These mentioned boar and semen related parameters are described in more detail below.

Table 5. Results of farrowing rate (FR) and total number of piglets born (TNB) per semen motility assessment score, for 4 AI centres.

Mot, %	All AI centres	AI centre 1	AI centre 2	AI centre 3	AI centre 4
# labs	7 mean ± SD	2 mean ± SD	3 mean ± SD	1 mean ± SD	1 mean ± SD
Farrowing rate (%)					
60 %	83.12[a] ± 30.1	82.74[a] ± 28.7	83.08[a] ± 30.1	74.87[a] ± 31.9	85.02[a] ± 29.4
70 %	82.69[a] ± 29.1	84.38[b] ± 29.1	81.83[b] ± 27.1	70.24[a] ± 32.5	87.46[a] ± 30.1
80 %	79.90[b] ± 27.7	80.65[c] ± 26.5	79.62[c] ± 30.2	68.99[a] ± 29.4	87.38[a] ± 31.7
90 %	84.02[c] ± 28.9	84.00[b] ± 27.1	87.27[d] ± 29.8	68.93[a] ± 28.5	91.32[a] ± 30.2
Total number born (#)					
60 %	12.66[a] ± 2.4	12.47[a] ± 1.8	12.66[a] ± 2.1	11.60[a] ± 2.1	12.79[a] ± 1.6
70 %	12.83[b] ± 1.9	12.73[b] ± 2.3	12.88[b] ± 2.0	13.04[b] ± 2.0	12.78[a] ± 1.9
80 %	13.05[c] ± 1.8	13.04[c] ± 2.6	12.99[b] ± 1.7	13.59[c] ± 2.1	12.54[a] ± 2.2
90 %	12.98[c] ± 2.8	12.99[c] ± 2.1	13.10[b] ± 2.5	13.56[c] ± 1.8	12.24[a] ± 1.9

Superscripts a,b,c,d represent whether column results are significantly different at P≤0.05.
For number of ejaculates, see Table 2.

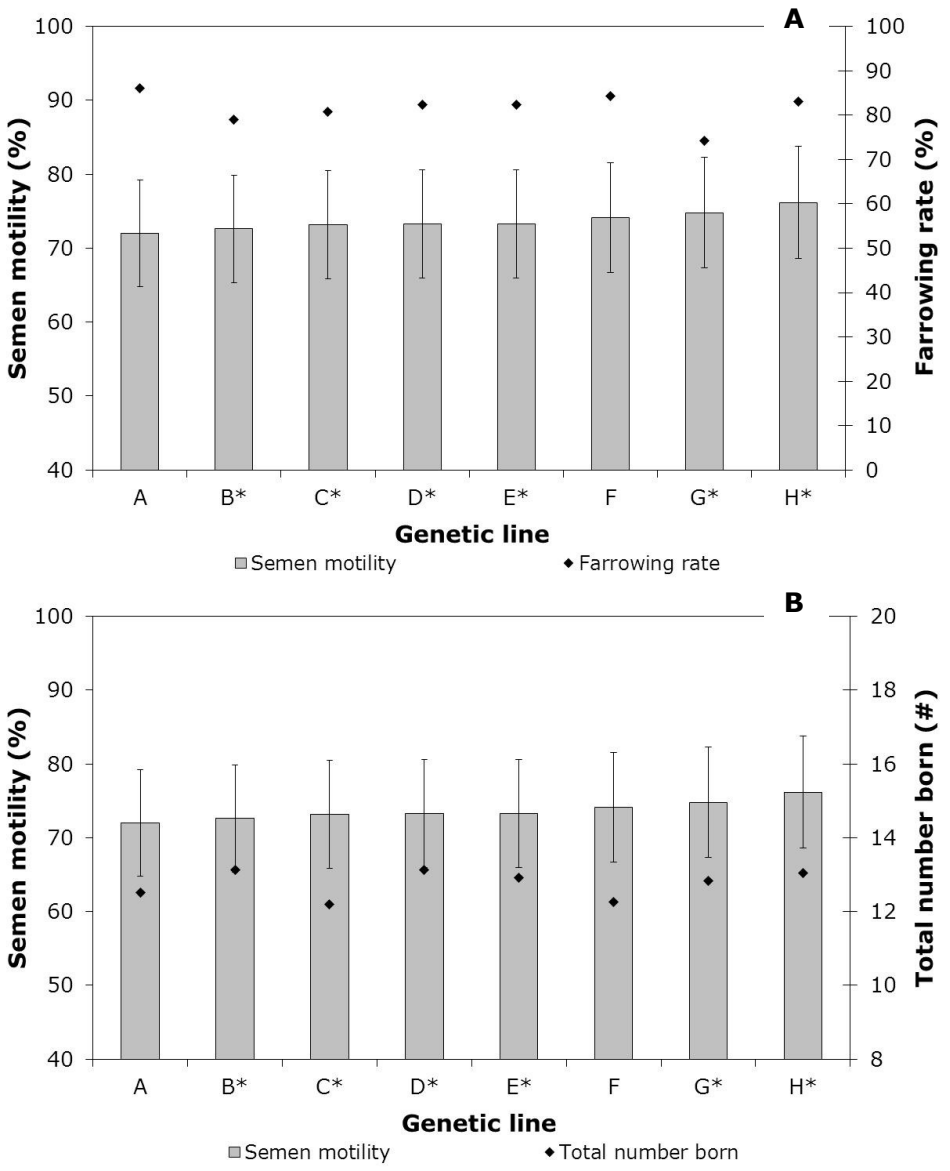

Figure 1. Effect of genetic line of the boar (boar line) on the relation between sperm motility (mean ± SD) and (A) farrowing rate (FR) and (B) total number of piglets born (TNB) in 8 boar lines. Boar lines with a * show a significant relation between semen motility and fertility. The descriptive results per genetic line are shown in Supplementary table 2.

Genetic line of the boar as source of variation in fertility

In general, genetic line of the boar explained 21% of the boar related variation in FR and 28% of the variation in TNB, as indicated in Table 4. When analysing in detail 8 out of the total of 15 genetic lines, 6 out of these different genetic lines significantly (P<0.0001) affected the relation between semen motility assessment and FR (Figure 1A), but the fresh sperm motility score varied between positive and negative relationships with FR. Similar patterns were seen for TNB (Figure 1B). Descriptive results per genetic line are shown in Supplementary table 2. When results from individual boars were isolated within the different genetic lines, they showed that some boars affected the relation between sperm motility and FR and/or TNB, and others did not show this relation. Within the genetic lines, individual boars significantly affected the nature of relationship between sperm motility and FR and/or TNB (P=0.001).

Year and month of semen production as source of variation in fertility

In the years 1998 and 1999 lower sperm motility was reported (P<0.001) when compared to the other years (2000-2005), as illustrated in Figure 2. Also a significant lower TNB in 1998 compared to 1999 and following years (Figure 2) is seen. The mean TNB increased over the years (1.2 piglets increase between 1998 and 2006). For FR no clear pattern is seen over the years, although differences are found (Figure 2). Descriptive results per year are shown in Supplementary table 3.

The month of semen collection explained 11% of the boar related variation in FR and 5% of the variation in TNB. Sperm motility itself was not directly affected by month: no clear patterns were seen in the semen motility results (Figure 3) and corresponding FR and TNB. The highest motility results were seen in March-May and decreased in July and August, but the difference was not significant (P=0.1154). Descriptive results per month are shown in Supplementary table 4.

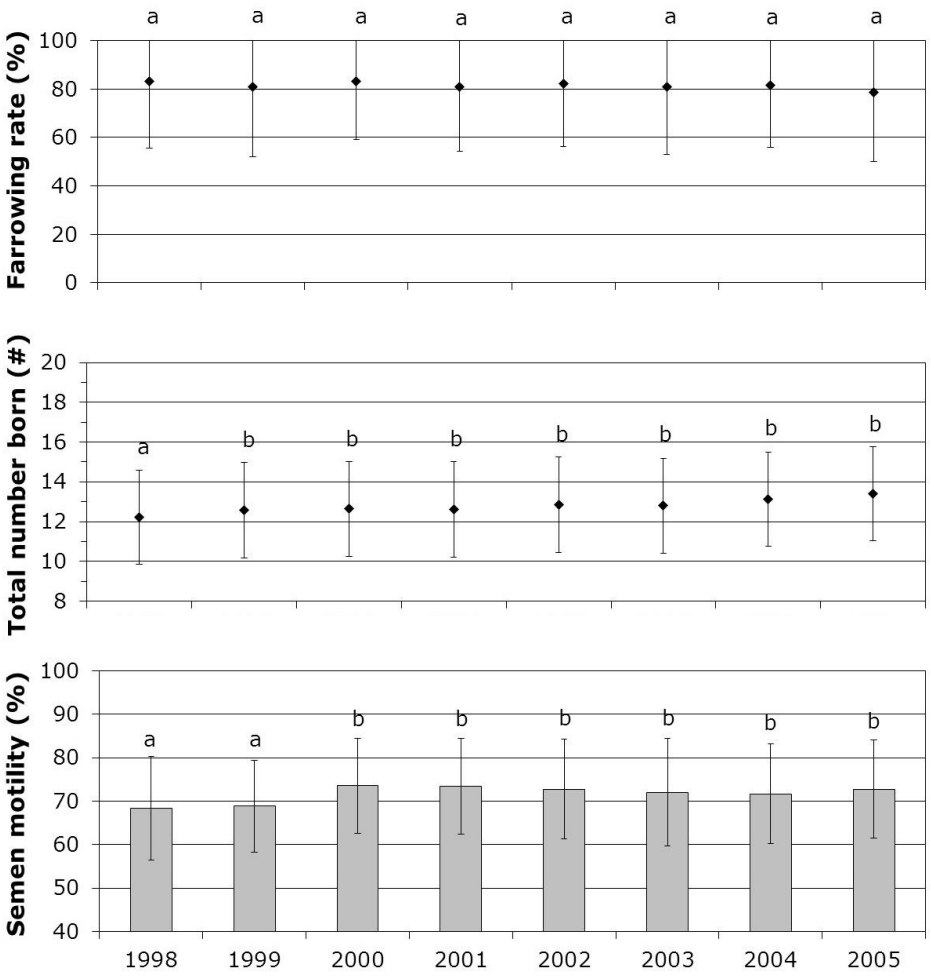

Figure 2. Results of sperm motility, farrowing rate (FR) and total number of piglets born (TNB) over the years (mean ± SD). Results with different superscripts differ significantly (P≤0.05). The descriptive results per year are shown in Supplementary table 3.

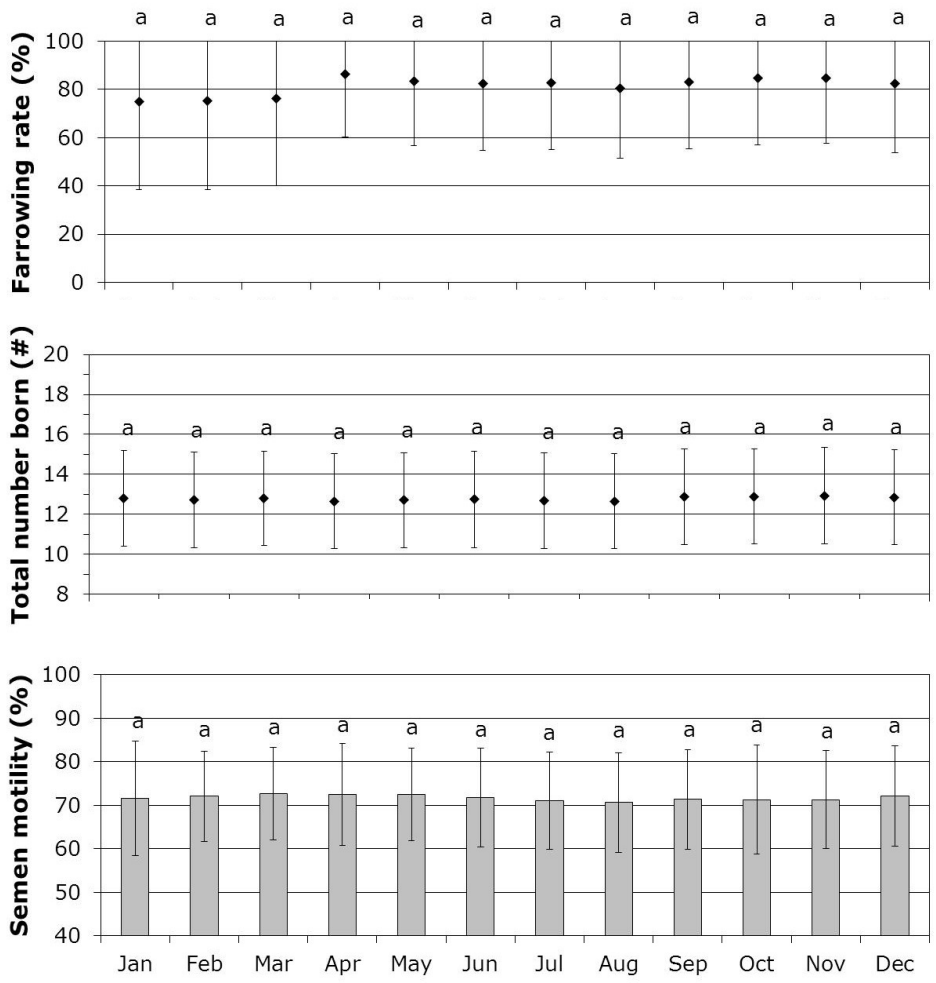

Figure 3. Results of sperm motility, farrowing rate (FR) and total number of piglets born (TNB) over the months (mean ± SD). Results with different superscripts differ significantly (P≤0.05). The descriptive results per month are shown in Supplementary table 4.

AI centre and laboratory technician as source of variation in fertility
Statistical analysis showed that the AI centre explained 7% of the variation in FR (P=0.037) and 7% of the variation in TNB (P=0.011). The effect of the AI centre is indicated in Table 5, where results for the four AI centres are presented. Overall FR declined as the motility classes increased from 60% to 80%. AI centre 1 showed a significant increase (P=0.0001) in FR between the 60% and the 70% and 90% motility

classes. The decrease in FR at the 80% motility class was also significant (P=0.0001). AI centre 2 showed decreasing FR from the 60% motility class to the 70% and 80% motility class (P=0.021). The 90% motility class was significantly higher. The smaller AI centres 3 and 4 did not show a significant relation between sperm motility and FR (P=0.076 and P=0.128 respectively). All AI centres, except for AI centre 4, showed a significant relation between sperm motility and TNB (P=0.0001). However for the second largest AI centre (AI centre 2) only between semen motility class 60% versus the classes 70%, 80% and 90% a significant increasing TNB was found (P=0.0001). AI centres 1 and 3 showed a significant increase in TNB from the motility class 60% to the class 70% (P=0.021) and the classes 80-90% (P=0.001).

Individual laboratory technicians had a significant effect on FR: 11% of the variation due to boar related parameters was explained (P=0.047) by laboratory technician, for TNB no significant effect of laboratory technician was shown (P=0.087). When isolating results from individual laboratory technicians, this did not result in a higher significant relation between semen motility assessment and fertilising capacity, but it did show differences between laboratory technicians.

Boar age as source of variation in fertility

Statistical analysis showed that age of the boar at the time of ejaculation explained 3% of the variation in FR (P=0.009) and 8% of the variation in TNB (P=0.031). These percentages were small but significant, and the variation for boar age (in months) is shown in Figure 4, focusing on the period from the start (age of 10 months) till the age of five years (60 months).

Other boar related parameters as source of variation in fertility

Part of the variation in FR was explained by the days between ejaculations (1%, P<0.0001). The number of sperm cells in an ejaculate (P=0.217) and the number of sperm cells in a dose (P=0.315) were both not significant. The variation in TNB was explained by the number of sperm cells in an ejaculate (1%, P=0.042). No effect was found for number of days between ejaculations (P=0.371) and number of sperm cells in a dose (P=0.127). Part of the variation could not be explained: 33% for FR and 34% for TNB.

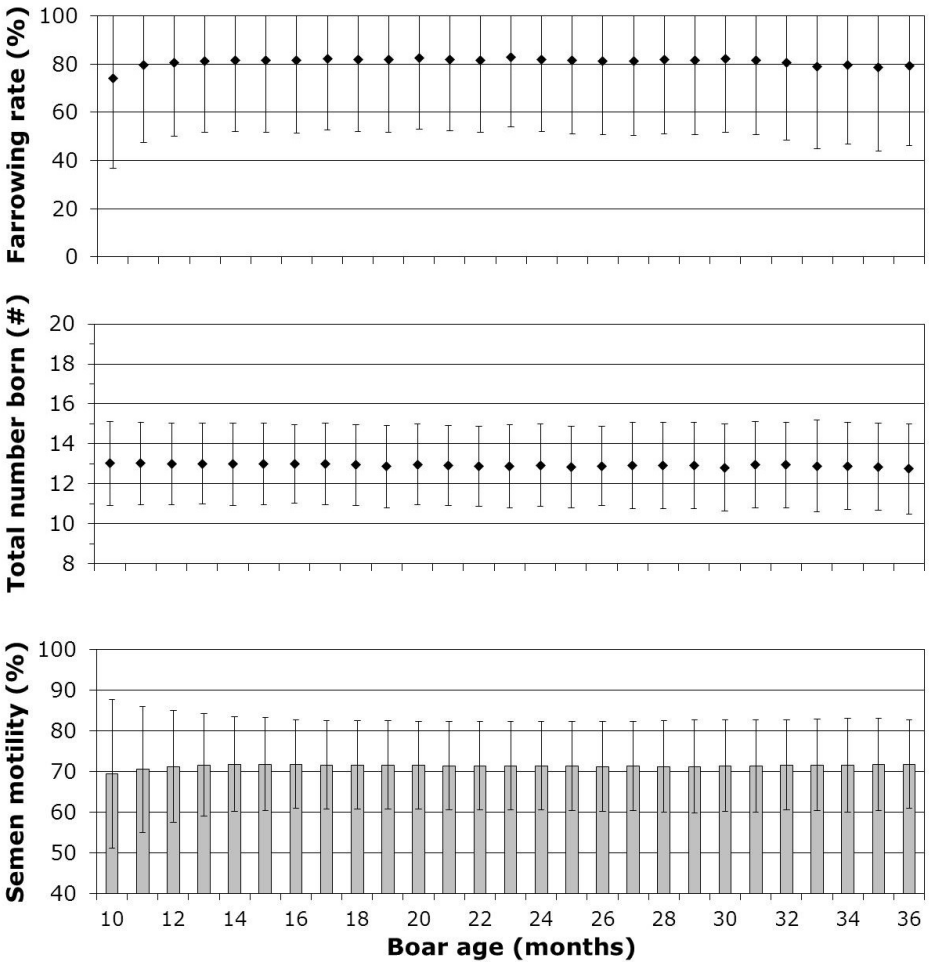

Figure 4. Results of sperm motility, farrowing rate (FR) and total number of piglets born (TNB) per boar age (in months) (mean ± SD). For the number of ejaculates per boar age see Supplementary table 5.

Discussion

In this retrospective review from a very large commercial Dutch AI database boar and semen related parameters were correlated with the sow field fertility parameters farrowing rate (FR) and total number of piglets born (TNB). The variation in FR was for 5.9% explained by boar and semen related parameters (the so called direct boar effect (DBE)), of which genetic line of the boar explained the largest part of this variation (21%). The results for TNB indicated a DBE of 6.6%, of which 28% was explained by genetic line of the boar.

As mentioned before, the reliability of the study depended on the number of data used for analysis (see Amann, 1989) who illustrated this principle by comparing confidence intervals). Studies (Gadea *et al.*, 1998; Juonala *et al.*, 1998; Xu *et al.*, 1998; Tardif *et al.*, 1999; Flowers, 2002) often reported fertility data of limited practical value because numbers of animals involved are far too few in number, which is also reviewed in Chapter 1. From the present study it can be concluded that the number of data (110,186 ejaculates) was not limiting to represent the relation between boar and semen related parameters and fertility. Obviously there was a large SD for FR which is a 0 or 1 parameter as either the sow farrowed or she did not. Consequently, the LSM for all ejaculates results in a high SD for the parameter FR.

The variation in FR and TNB is only minimally explained by fresh sperm motility assessed at collection (4% of the DBE). So under the Dutch standard protocol for artificial insemination (AI), the motility parameter of sperm cannot be used to select boars, as has been described before in studies using much smaller datasets (Johnson *et al.*, 2000; Flowers, 2002; Rodriguez-Martinez, 2003). This suggests that other factors involved in the fertilisation process (Foote, 2003) are more relevant. Indeed we detected large variation between boars (age, genetic lines) but also variable relations were found between the four AI centres and between laboratory technicians involved in this comparison.

The genetic line of the boars explained the largest part of the variation in FR and TNB (21% and 28% of the DBE respectively). This is in accordance to several studies (Kommisrud *et al.*, 2002; Kondracki, 2003; Gadea *et al.*, 2004; Janett *et al.*, 2005; Sondermann and Luebbe, 2008). Smital (2009) stated that the boar's impact on the herd fertility can be high, particularly if the male is mated to many females, but most authors agree that no breed excels in all semen characteristics (Kennedy and Wilkins, 1984; Oh *et al.*, 2003). With sub-fertile boars, the effect on field fertility is much larger (unpublished data from 1-3 sub-fertile boars annually) and therefore, it is important to identify sub-fertile boars. This supports the study from Popwell and Flowers (2004) who concluded that

fresh sperm motility can be a good predictor for one boar, but not automatically the same good predictor for another boar. In general, the choice for a genetic line is not based on fertility results but on market perspective of the offspring. AI centres need to include breed differences into their decision-making processes to ensure adequate use of the (genetic line of the) boar (Sondermann and Luebbe, 2008).

Next to the boar line effect, there was an effect of age of the boar on FR (3%) and TNB (8%). There will obviously come a time in a boar's reproductive life when fertility is diminished by advanced age. Both in fresh motility and FR there was an increase seen in the first months at the AI centre, this increase was not seen in TNB. When boars were over 3 years of age, the results of both FR and TNB started to fluctuate and decrease, although the sperm motility at collection increased. This is similar to Knox (2006) who observed older boars showing lower pregnancy rates and litter sizes and higher incidences of sperm abnormalities. We could not establish the age where reduced fertility starts, probably due to the low number of boars at a higher age. Replacement rate of boars at the AI centres was ± 50% per year, of which 60% was by semen quality reasons (unpublished data). This is the result of aimed genetic improvement in the field.

The minimal percentage of explained variation in field fertility by fresh sperm motility was overruled by the differences between AI centres (explaining 7% over the variation in fertility). This is in accordance with several studies (Baker *et al.*, 1957; Jorgensen *et al.*, 1997), showing intra and inter laboratory variation. The smaller AI centres did not show a relation between sperm motility assessed at collection and FR and/or TNB registered in the field. This could be due the fact that the number of data was too small for that particular AI centre and/or the number of ejaculates per motility class differed between larger and smaller AI centres. The parameter AI centre includes several parameters which are not clearly indicated separately in this retrospective study. Parameters like climate conditions in the barn, effect of semen collector, effect of minor diseases or moment of vaccination within one barn or differences between feed over the years are confounded within the parameter AI centre and should be considered to study separately. This is only possible when this kind of data is recorded in a standardised way.

Fresh semen motility assessment by eye is consistently an area of subjective estimations with an effect of the individual laboratory technician. In fact the laboratory technician affected the fertilising capacity of boar ejaculates (11% of the variation in FR) despite the fact that all technicians were trained to estimate the motility of a semen

sample as uniform as possible. In practice this seems hard to accomplish. Variations in evaluations of 30 to 60% have been reported in the estimation of sperm motility between technicians (Gadea *et al.*, 2004; Jorgensen *et al.*, 1997; Yeung *et al.*, 1997; Verstegen *et al.*, 2002; Vyt *et al.*, 2004; Tejerina *et al.*, 2008) and within technicians (Baker and Clarke, 1987; Amann, 1989; Knuth *et al.*, 1989; Jorgensen *et al.*, 1997; Yeung *et al.*, 1997; Verstegen *et al.*, 2002; Foote, 2003; Gadea *et al.*, 2004; Vyt *et al.*, 2004; Tejerina *et al.*, 2008), which emphasises the need for standardisation (Yeung *et al.*, 1997). In the current study steps of 10% motility were used, which could be too indiscriminative for research and were based on the practical setup of the data collected. Methods with continuous and objective results (computer assisted systems) could be valuable for estimating a more detailed relation between sperm motility and fertilising capacity of boar semen.

It is well known that semen quality in mammals can be affected by the time of the year (Colenbrander and Kemp, 1990; Buhr, 2001; Janett *et al.*, 2005). Several studies confirm that temperature, heat stress and/or hot weather (Cameron and Blackshaw, 1980; Rivera *et al.*, 2005), next to photoperiod (Bartness and Golman, 1989) and humidity (Rivera *et al.*, 2005) have an effect on semen production and semen quality. In this study results were corrected for the influence of month, representing both day length as well as temperature variation. Fresh motility of spermatozoa varies only moderately during the year (Murase *et al.*, 2007; Smital, 2009) with a tendency to increase in autumn and winter months and the lowest percentages in August-September, which is in accordance to our current study. The percentages of explained variation by month on FR (11%) and on TNB (5%) were larger than the effect of sperm motility assessed at collection.

The year of analysis did show an explained variation of 11% for FR and 5% for TNB as part of the DBE. The results from this retrospective study showed the lowest TNB in 1998 and increasing over years, which was amongst improved reproduction management a positive effect of genetic selection. The lowest fresh motility scores were also given in 1998 and 1999. This could be a result of the classical swine fever outbreak in 1997 (February 1997 till June 1998), and the higher demand for semen afterwards, because of the repopulation of over 1,000 farms. Since this study only involves AI centres in the Netherlands, this could have had a large effect on the outcome of both fresh motility score and field fertility. Because of increased demand for semen and the fact that new boars could enter AI centres for the first time since months, it can be speculated that the average age of the

population of boars in the AI centres was lower than normal and this could have affected sperm motility as well as fertility. Other explanations include that for both boars for AI and the need for semen at the breeding and commercial farms (for replacing pigs), unconsciously, laboratory technicians were more reluctant rejecting ejaculates not meeting the standard criteria because of shortages. Differences in years is not an unknown effect, it is also seen in other studies (Smital *et al.*, 2004; Janett *et al.*, 2005; Smital, 2009).

From the analyses we conclude that the number of cells in a commercial dose did not explain the variation in FR and TNB, which is similar to Xu *et al.* (1998). Furthermore, lower percentage of motile sperm could not be compensated simply by adjusting the number of sperm in an insemination dose. Both probably imply that the number of sperm cells in the doses used was well above the minimal number of sperm to obtain maximal FR and TNB. In the 60% motility class, the lower percentage motile cells were in practice compensated by increasing the number of sperm cells in an insemination dose. Whether such compensations have a positive effect on fertility rates could not be concluded since the higher number of sperm cells was confounded with the lower motility and there was no comparative class without compensation at the motility level of 60%. However we did not find a significant difference between the 60% and 70% motility class, although this can also be due to the fact that the number of ejaculates with 60% fresh motility was quite small. Furthermore, this means that the number of cells per dose is confounded with the fresh motility assessment score, which makes the motility parameter biased. Several other studies stated that the number of sperm cells per insemination used, exceeded the threshold value of sperm necessary to observe a fertility effect (Tardif *et al.*, 1999; Johnson *et al.*, 2000; Flowers, 2002; Watson and Behan, 2002; Alm *et al.*, 2006; Ruiz-Sanchez *et al.*, 2006; Foxcroft *et al.*, 2008). The total number of sperm cells per dose for the Dutch pig AI decreased over the years from 3.44 billion/80 ml in 1998 to 2.19 billion/80 ml in 2006. Also remarkable was the small, but significant relation between number of sperm cells in an ejaculate and TNB. Since the number of sperm cells in an ejaculate is corrected to a well-defined number of cells in an insemination dose, there is no explanation for the 1% variation of the DBE explaining the TNB.

Days between ejaculations had a significant but minimal effect on FR (1%). Several studies (Rutten *et al.*, 2000; Smital, 2009; Wolf and Smital, 2009) suggest that a time interval of 7 to 10 days between ejaculations is a good choice for optimising all semen traits from the biological point of view. This interval is much larger than the interval in

this study and in common AI practice. Unpublished data showed that too low ejaculation frequency (< 1.4 / week) resulted in increased morphological abnormalities. In our data the ejaculation frequency almost never exceeded 2 times per week, however, it was shown that when the inter ejaculation interval becomes >7 days, the fresh sperm motility was decreasing, and at the same time the FR in the field decreased (data not shown). So optimising the interval between ejaculations can improve the sperm motility of the ejaculates at collection and the FR can increase. The effect was, however, rather small and not seen in TNB (data not shown).

Although the relation between fresh sperm motility and field fertility is weak, it is generally accepted that immotile or poorly motile sperm cells are incapable of fertilisation (Clark *et al.*, 1989; Flowers, 1997; Tardif *et al.*, 1999; Turner, 2003; Popwell and Flowers, 2004; Gadea, 2005; Alm *et al.*, 2006; Ruiz-Sanchez *et al.*, 2006; Turner, 2006; Tejerina *et al.*, 2008). We should note that in the Dutch AI situation ejaculates with poor motile sperm (< 60% motility) were scarce and were routinely rejected for AI purpose. The effects of 60%, 70%, 80% and 90% motility classes on field fertility only explained 4% of the DBE of FR and TNB. Within the marginal differences we noted an unexpected decrease in FR for the fresh sperm motility class 80% compared to 70% and 90%. This decrease was similar for all AI centres used in this study and we do not have a proper biological explanation for this result. Another reason for limited explained variation in fertility is the bulk ejaculates at 70-80 % motile cells at the moment of production. Objective and multi parametric scores are expected to show more variation in results and therefore more reliable relation with fertility, which was already concluded in 1982 (Saacke, 1982). Computer assisted semen analysis (CASA) systems are potentially the best equipments for analysing sperm motility (Rozeboom, 2001; Chapter 3). Didion (2008) showed no significant relation for any unique motion parameter with fertility data, which is in contrast to the studies of Holt *et al.* [6] and Vyt *et al.* (2008). The routine use of CASA systems in order to assess sperm motility has been initiated in the Netherlands in October 2006 and the relation between CASA retrieved semen motility results and fertility is analysed (Chapter 4).

In conclusion, this study showed the unreliability of fresh semen motility assessment by eye. It explained only a relatively small percentage of the total variation in field fertility. By standardisation of other boar and semen related factors affecting fertility, the AI organisation could diminish their effect on field fertility, and at the same time play a more important role in the selection of semen. They need to

focus on the effect of genetic line and age of the boar, year and month, and AI centre and laboratory technician, which were all parameters explaining more variation in both FR and TNB compared to sperm motility. For AI laboratory practice it is important to conclude, that microscopic semen motility assessment is recommended just for discriminating between good ejaculates (≥ 60%) and bad ejaculates (< 60%). By doing so we showed that differences within good ejaculates (60% - 90%) on fresh sperm motility scores did result in minimal differences in FR and TNB.

The ultimate goal of working in pig reproduction is to critically consider that there are parameters one has to take in for granted (just because there are e.g. different boar lines, and there is an effect of parity of the sow), and there are other parameters that can be optimised to get an optimal fertility result. Displaying the subjectivity of microscopic semen motility assessment suppresses the recommendation to use CASA systems for more standardised semen assessment. More accurate and objective semen motility assessments may become critically relevant for lower dose insemination approaches. By retrospectively analysing an eight year dataset of 110,186 boar ejaculates and inseminations of 165,000 sows, we showed that the percentages of variation which you can explain by several boar and semen related parameters were significant and should be studied in detail to use as a criterion to select boars for AI purposes. Other parameters that turned out to be more relevant could be considered for such purpose.

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Supplementary table 1. Classification system of categories for sperm motility.

Motility	Movement pattern
10%	almost non-moving, weak non-progressive movement
20%	almost non-moving, weak non-progressive movement
30%	active, but non-progressive movement
40%	very active, but non-progressive movement
50%	weak and irregular movement
60%	weak, continuous progressive movement
70%	average up to good, continuous progressive movement
80%	good up to very good, continuous, progressive movement
90%	good up to very good, continuous, progressive movement
100%	good up to very good, continuous, progressive movement

Supplementary table 2. Descriptive results per genetic line (mean ± SD). The parameters represented per genetic line (line) are number of ejaculates (N), motility (%), farrowing rate (FR, %), total number of piglets born (TNB), boar age (months), number of cells per dose (#cells/dose, $\times 10^9$ spermatozoa/80 ml) and number of cells per ejaculate (#cells/ejac, $\times 10^9$ spermatozoa).

Line	N	Motility	FR	TNB	Boar age	#cells /ejac	#cells /dose
A	9,317	72.0 ± 10.9	86.1 ± 24.4	12.5 ± 2.3	28 ± 13	87 ± 16	2.9 ± 1.9
B	5,383	72.6 ± 11.5	79.1 ± 26.3	13.1 ± 2.4	25 ± 10	86 ± 13	2.4 ± 1.7
C	13,458	73.2 ± 10.2	80.8 ± 25.5	12.2 ± 1.9	27 ± 10	80 ± 13	2.7 ± 1.8
D	7,162	73.3 ± 11.1	82.3 ± 26.4	13.1 ± 2.0	24 ± 10	81 ± 11	2.7 ± 1.4
E	9,758	73.3 ± 10.5	82.4 ± 25.4	12.9 ± 2.5	23 ± 9	85 ± 13	3.1 ± 1.1
F	3,296	74.1 ± 11.4	84.3 ± 24.9	12.3 ± 2.3	23 ± 11	80 ± 10	2.4 ± 1.6
G	5,507	74.8 ± 10.9	74.2 ± 24.3	12.8 ± 2.7	25 ± 9	84 ± 14	2.3 ± 1.5
H	24,115	76.2 ± 11.4	83.0 ± 23.5	13.1 ± 2.6	26 ± 12	86 ± 15	2.3 ± 1.7

Supplementary table 3. Descriptive results per year (mean ± SD). The parameters represented per year are number of ejaculates (N), motility (%), farrowing rate (FR, %), total number of piglets born (TNB), boar age (months), number of cells per dose (#cells/dose, $\times 10^9$ spermatozoa/80 ml) and number of cells per ejaculate (#cells/ejac, $\times 10^9$ spermatozoa).

Year	N	Motility	FR	TNB	Boar age	#cells /ejac	#cells /dose
1998	11,459	68.4 ± 11.9	83.1 ±27.6	12.2 ± 2.4	30 ± 13	66 ± 19	3.4 ± 0.9
1999	12,468	68.9 ± 10.5	81.0 ± 29.1	12.6 ± 2.4	29 ± 14	72 ± 12	3.0 ± 1.0
2000	9,957	73.6 ± 10.9	83.3 ±24.1	12.6 ± 2.3	28 ± 14	76 ± 11	3.0 ± 1.0
2001	9,448	73.4 ± 11.1	80.7 ± 26.3	12.6 ± 2.3	28 ± 12	77 ± 12	2.7 ± 1.0
2002	11,246	72.8 ± 11.4	82.1 ±25.9	12.9 ± 2.4	27 ± 13	75 ± 12	2.6 ± 0.9
2003	16,314	72.1 ± 12.3	80.8 ± 27.8	12.8 ± 2.3	25 ± 11	75 ± 10	2.5 ± 0.8
2004	19,432	71.7 ± 11.5	81.4 ± 25.4	13.1 ± 2.3	25 ± 12	83 ± 16	2.5 ± 0.9
2005	19,862	72.8 ± 11.3	78.6 ± 28.7	13.4 ± 2.3	25 ± 12	87 ± 16	2.4 ± 0.8

Supplementary table 4. Descriptive results per month (mean ± SD). The parameters represented per month are number of ejaculates (N), motility (%), farrowing rate (FR, %), total number of piglets born (TNB), boar age (months), number of cells per dose (#cells/dose, × 10^9 spermatozoa/80 ml) and number of cells per ejaculate (#cells/ejac, × 10^9 spermatozoa).

Month	N	Motility	FR	TNB	Boar age	#cells /ejac	#cells /dose
Jan	10,074	71.6 ± 13.1	75.0 ± 36.6	12.8 ± 2.3	24 ± 11	88 ± 10	2.7 ± 1.4
Feb	9,289	72.1 ± 10.3	75.3 ± 36.8	12.7 ± 2.4	25 ± 10	85 ± 10	2.7 ± 1.5
Mar	10,414	72.7 ± 10.5	76.2 ± 36.2	12.8 ± 2.3	25 ± 12	83 ± 10	2.7 ± 1.3
Apr	8,000	72.4 ± 11.7	86.4 ± 25.9	12.7 ± 2.4	25 ± 11	82 ± 12	2.6 ± 1.4
May	8,519	72.5 ± 10.6	83.5 ± 26.6	12.7 ± 2.4	25 ± 10	81 ± 12	2.6 ± 1.5
Jun	8,921	71.8 ± 11.3	82.5 ± 27.7	12.7 ± 2.4	26 ± 11	81 ± 13	2.6 ± 1.7
Jul	9,176	71.0 ± 11.1	82.7 ± 27.4	12.7 ± 2.3	26 ± 10	81 ± 13	2.6 ± 1.5
Aug	9,305	70.6 ± 11.3	80.5 ± 29.0	12.7 ± 2.3	26 ± 11	82 ± 13	2.6 ± 1.4
Sep	9,151	71.3 ± 11.3	83.2 ± 27.7	12.9 ± 2.3	25 ± 10	83 ± 12	2.7 ± 1.6
Oct	9,247	71.3 ± 12.5	84.7 ± 27.5	12.9 ± 2.4	25 ± 12	88 ± 11	2.7 ± 1.5
Nov	9,165	71.3 ± 11.3	84.6 ± 27.1	12.9 ± 2.4	25 ± 11	89 ± 12	2.7 ± 1.7
Dec	8,925	72.1 ± 11.5	82.4 ± 28.5	12.9 ± 2.4	24 ± 10	88 ± 11	2.7 ± 1.5

Supplementary table 5. The number of ejaculates per boar age.

Boar age (months)	Number of ejaculates	Boar age (months)	Number of ejaculates
10	766	36	1090
11	3426	37	1030
12	5881	38	947
13	7590	39	938
14	7525	40	820
15	7080	41	768
16	6540	42	676
17	6327	43	658
18	5350	44	575
19	5184	45	538
20	4604	46	462
21	4399	47	449
22	3989	48	357
23	3638	49	312
24	3273	50	291
25	3269	51	259
26	2817	52	216
27	2703	53	197
28	2320	54	181
29	2248	55	153
30	2044	56	143
31	1898	57	153
32	1617	58	143
33	1552	59	120
34	1319	60	91
35	1260		

Chapter 3

Additional value of computer assisted semen analysis (CASA) compared to conventional motility assessments in pig artificial insemination

Broekhuijse MLWJ, Soštarić E, Feitsma H, Gadella BM.
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Abstract
In order to obtain a more standardised semen motility evaluation, Varkens KI Nederland has introduced a computer assisted semen analysis (CASA) system in all their pig AI laboratories. The repeatability of CASA was enhanced by standardising for (1) an optimal sample temperature (39°C), (2) an optimal dilution factor, (3) optimal mixing of semen and dilution buffer by using mechanical mixing, (4) the slide chamber depth, and together with the previous points (5) the optimal training of technicians working with the CASA system and (6) the use of a standard operating procedure (SOP). Once laboratory technicians were trained in using this SOP, they achieved a coefficient of variation of < 5 % which was superior to the variation found when the SOP was not strictly used. Microscopic semen motility assessments by eye were subjective and not comparable to the data obtained by standardised CASA. CASA results are preferable as accurate continuous motility dates are generated rather than discrimination motility percentage increments of 10% motility as with motility estimation by laboratory technicians. The higher variability of sperm motility found with CASA and the continuous motility values allow better analysis of the relationship between semen motility characteristics and fertilising capacity. The benefits of standardised CASA for AI is discussed both with respect to estimate the correct dilution factor of the ejaculate for the production of artificial insemination (AI) doses (critical for reducing the number of sperm per AI doses) and thus to get more reliable fertility data from these AI doses in return.

Keywords
sperm motility, computer assisted semen analysis, standardisation procedure, fertility, boar, artificial insemination

Introduction

One of the important parts of semen quality evaluation in human and different animal species is microscopic semen motility assessment. Traditionally semen motility is assessed by a technician who scores by eye when looking through a phase contrast microscope. However, this practice gives rise to subjective interpretation of individual technicians (which in part also depends on the level of skills and training of the technician). This technician-based variation in results makes it difficult to estimate the effect of sperm motility on fertility traits. The method is not standardised and subjective. Moreover, it only allows discrete motility scores (increments of 10%) only estimating the number of motile sperm (Chapter 2). The limitations and subjectivity of the microscopic motility evaluation by eye has also been reported elsewhere (Deibel *et al.*, 1976; Chong *et al.*, 1983; Jenq and Ukombe, 1983; Mortimer *et al.*, 1986; Amann, 1989; Dunphy *et al.*, 1989; Knuth *et al.*, 1989; Rozeboom, 2000; Verstegen *et al.*, 2002).

To overcome this variability in technician based semen motility assessment results, systems such as turbidimetry, laser-Doppler spectroscopy and photometric methods have been proposed (Verstegen *et al.*, 2002). Dott and Foster (1979) first proposed computer assisted semen analysis (CASA) to obtain an overall objective semen analysis. The main advantage of CASA is that it allows an objective analysis of sperm motility in samples, and that identical parameters can be used in serial sample analysis. Moreover, the technique provides a continuous value of the exact number of sperm cells that were considered to be motile or immotile. The main reservations for using these CASA systems are the high investment costs, the extreme need for standardisation, and the validation of the system, that should be implemented before any practical use is possible (Comhaire *et al.*, 1992; Holt *et al.*, 1994; Lenzi, 1997; Smith and England, 2001; Verstegen *et al.*, 2002; Rijsselaere *et al.*, 2003; Rijsselaere *et al.*, 2005; Tejerina *et al.*, 2008). For example, CASA systems cannot always discriminate between immotile spermatozoa and other similar sized static cells and particles in the semen, which can lead to incorrect motility estimation (Larsen *et al.*, 2000). Moreover, errors of measurements in too dense sperm suspensions are due to the fact that many sperm cells in the sample collide to other sperm cells or cross each other's trajectories, which frustrates CASA measurements (Iguer0ouada and Verstegen, 2001). On the other hand, there are several reports that claim CASA to be objective and more standardised when compared to sperm motility analysed microscopically by eye (Farrell *et al.*, 1995; Krause, 1995; Farrell *et al.*, 1996; Abaigar *et al.*, 1999; Hoflack *et al.*, 2007). For an

overview about CASA technology for semen analysis see (Davis and Katz, 1993).

The rationale for performing the current studies is that in the Netherlands over 98 % of approximately 2.4 million annual sow matings are performed by artificial insemination (AI) and, therefore, the sow farms essentially rely on AI. For successful implementation of AI, the fertility results of the semen utilised are critical: semen parameters relate to farrowing rate and total number of piglets born (Holt *et al.*, 1997; Juonala *et al.*, 1998; Tardif *et al.*, 1999; Gadea *et al.*, 2004; Popwell and Flowers, 2004; Gadea, 2005; Chapters 2 and 4) and thus are of great economic importance. Since sperm motility is considered as one of the most important semen quality aspects, the AI laboratories of Varkens KI Nederland (Deventer, the Netherlands) were willing to optimise semen motility evaluation in a way that it was more objective and standardised. At all 7 laboratories a CASA system was already introduced with the primary goal to implement CASA as a more precise and thus reliable semen concentration measurement (Hansen *et al.*, 2006). Moreover, the use of CASA also allows getting more objective, detailed and repeatable determination of semen motility characteristics of sperm cells in boar ejaculates. Thus, CASA will provide more semen motility parameters and should result in a lower degree of variation in motility characteristics of a given sample when compared to technician variation (performing semen motility assessments by eye) as well as lower variation between AI stations.

Positive correlations between microscopic and CASA semen motility assessment were reported previously (Schönlärl and Krause, 1991; Klimowicz *et al.*, 2008). The fact that these correlations were found under laboratory conditions does not automatically mean that similarly a CASA system can be implemented in an AI laboratory practice. In the first part of this study we report on factors that were found to significantly improve the repeatability of the CASA measurements. Furthermore, the need to identify and quantify factors that may influence CASA assessments and to adjust laboratory procedures for using a CASA system is implemented in this manuscript. The aim of this study is to identify the factors that cause variation in CASA results. Bringing those factors together in a standard operating procedure (SOP) which minimises this variation, is expected to be instrumental for high throughput applications of CASA systems in pig AI.

This study also deals with the acceptance of such a new system by the laboratory technicians, the microscopic evaluation method by eye was compared with the standardised CASA system. Results from both semen motility assessment methods and fertility competence of these

ejaculates as measured in total number of piglets born (TNB), were used as being indicative for the value of both semen assessment methods.

Material and Methods

Material
The boar ejaculates were retrieved from Varkens KI Nederland (Deventer, the Netherlands), that produces insemination doses for pig artificial insemination (AI) for sow herds commercially. Ejaculates were diluted approximately 1:1 by adding the same volume (ml) of Solusem® extender (Varkens KI Nederland, Deventer, the Netherlands) at 32°C within 15 min after collection. Semen samples in the following experiment were samples from these 1:1 diluted ejaculates.

CASA motility assessment
CASA semen motility assessment was performed using the UltiMate™ (UltiMate™ Sperm Analyzer, Hamilton Thorne, Inc., Beverly, MA, USA) with Leja-4 standardised counting chambers (Leja, 4 chamber counting slides, Leja Products B.V., Nieuw-Vennep, the Netherlands), according to the method described in the accompanying manual (HTB manual) resulting in sperm motility (%) and the progressive motility (%). Both UltiMate™ and Leja-4 slides were pre-warmed at 39°C and samples were single analysed (one chamber filled, one sample analysed). The Leja counting slides were filled with a pipette set at 3 µl, no pressure used (capillary flow of chamber) and filling was stopped when chamber was completely filled. The factory settings of Hamilton Thorne were adapted to standard settings of Varkens KI Nederland, where the progressive cells average path velocity (VAP) cut-off was altered from 45 µm/s to 25 µm/s and the progressive cells straightness (STR) cut-off from 45% to 30%.

Effect of sample temperature on CASA motility assessment results
In order to determine the effect of sample temperature on CASA motility assessment results, 10 ejaculates of each of 10 different boars were collected. Semen samples were split in two aliquots and diluted (200 µl semen added to 3.0 ml extender) in Solusem® (Varkens KI Nederland, Deventer, the Netherlands) of either room temperature (20°C) or pre-warmed at 39°C in 5 ml tubes (Sarstedt AG & Co, Nümbrecht, Germany). Samples were incubated for minimal 2 min (with a maximum of 5 min). From each sample a chamber of a Leja-4 slide was filled and analysed continuously for 5 min (31 measurements). From the same ejaculate a duplicate was run.

Effect of dilution factor on CASA motility assessment results

According to the accompanying manual of the CASA system used, the number of sperm cells for an optimal measurement should be between 30 and 80 sperm cells per analysis field. Measuring 8 fields results in a cell count between 240 and 640 cells per measurement. Therefore, the right dilution factor for the sample was determined in order to fulfil these requirements. Starting with a dilution factor of 1:15 (200 µl semen added to 3.0 ml Solusem® (pre-warmed at 39°C), in 5 ml Sarstedt tubes), 1,630 motility assessments (279 boars, 2-10 ejaculates per boar) were performed. The number of samples within this window was compared with the number of samples in the window diluted with a factor 1:12. Therefore 1,731 samples (283 boars, 2-10 ejaculates per boar) using a dilution factor of 1:12 (250µl semen added to 3.0 ml Solusem® (pre-warmed at 39°C), in 5 ml Sarstedt tubes) were analysed for sperm motility with the CASA equipment, with adapted settings.

Effect of frequency and duration of mixing on CASA motility assessment results

Improper mixing of cell suspensions could result in incorrect motility scores. Five approximately 1:1 diluted semen samples of 5 different boars were split in 3 equal aliquots, diluted (1:15) in Solusem® (pre-warmed at 39°C) in 5 ml Sarstedt tubes (incubated for 2 min). Samples were mixed manually by turning the tube approximately 5 times, 2-3 s or mechanically mixed with a vortex mechanical mixer (Reax top, Heidolph Instruments GmbH & Co. KG, Kelheim, Germany) either at 80 % speed for 3 s or at 100 % speed for 5 s. The CASA with adapted settings was used. Checking the homogeneity of the suspension after mixing and the effect of the sample spot, either at the top (1) or in the middle (2) of the test tube, was investigated by comparing the concentration and semen motility results of 60 samples in total, derived from 4 different boars, per boar 5 ejaculates and per ejaculate 3 samples.

Effect of variation in chamber depth on CASA motility assessment results

Depending on chamber depth and volume wherein sperm cells could move, one could expect effects of variation in chamber depth on motility assessment results. From 25 Leja-4 slides with an indicated chamber depth of 20 µm and an indicated variation from 19 – 21 µm (Leja certificate of analysis), the chamber depth was measured using an AvaSpec-USB2 spectrometer (AVASOFT ThinFilm version 7.0 for AvaSpec-USB2, Avantes BV, Eerbeek, the Netherlands), an infrared

equipment for precision measurement of depths. Duplicate semen samples of 50 different boars were analysed for sperm motility in the CASA equipment with adapted settings.

Establishing a SOP to reduce sample variation and laboratory technician variation

In order to minimise the variation of CASA motility assessment results, above mentioned standardisation factors (section 2.2.1-2.2.4) were implemented and transformed into a standard operating procedure (SOP). Results of technicians using CASA that were not trained with the established SOP were compared with the results during their training en eventually compared with the results after training and by working on routine basis with the CASA using the established SOP. Variation in repeat measurements of CASA motility was tested by performing duplicate measurements from raw ejaculates. In this way remaining variation in sample preparation including the human factor could be analysed. The progress of technician skills through training was followed by testing 600 samples in duplicate at the start-up of the training sessions, 614 duplicate samples during training and 635 duplicate samples at the end of the training period. All samples were retrieved from boar ejaculates of varying quality and concentration, and were analysed with the CASA equipment, with adapted settings. The repeatability of the CASA motility assessment results was established by calculating the R^2 and CV; which represents the reproducibility of the test results.

Microscopic evaluation of semen motility by eye

Laboratory technicians estimated sperm motility using phase contrast microscopy. A 10 µl drop of approximately 1:1 diluted semen was placed on a pre-warmed glass slide and covered with a pre warmed cover slip (microscope slides, Mediware®, Wesel, Germany). The evaluation was performed using a microscope (BH-2, Olympus, Tokyo, Japan) equipped with phase contrast optics and a thermal plate (39°C*)*, at 200X magnification. The microscopic semen motility assessment score consisted of two parameters, namely the percentage of motile cells and the percentage of quality of movement, both resulting in a score between 10 % and 100 %. The quality of movement was classified subjectively into 10 categories as follows: 10% and 20% (almost non-moving, weak non-progressive movement), 30% (active, but non-progressive movement), 40% (very active, but non-progressive movement), 50% (weak and irregular movement), 60% (weak, continuous progressive movement), 70% (average up to good,

continuous progressive movement), 80%, 90% and 100% (good up to very good, continuous, progressive movement).

Analysing agreement between microscopic and CASA semen motility assessment methods

From split samples of a total number of 1,499 diluted boar ejaculates (approximately 1:1) the motility was evaluated using both CASA with the original settings and by microscopically assessment by the eye of trained laboratory technicians.

The breeding database (Pigbase) of the Institute for Pig Genetics (IPG, Beuningen, the Netherlands) contains fertility records from nucleus, sub nucleus and (limited) commercial sows. Fertility records were extracted from this database and were merged to the semen motility assessment results of the ejaculates of which the insemination doses used, originated from. Of the 1,499 ejaculates analysed both microscopically and with CASA, for 341 ejaculates fertility results (farrowing rate and total number piglets born) could be retrieved from Pigbase. Analysing the relation between sperm motility and fertility involves taking both sow related and boar related parameters into account. Since the number of inseminations with known fertility results is too low to show significant differences, this publication uses the fertility results just for indication of differences between the two methods of semen motility assessment.

Statistics

Data are presented as means and standard deviations. Data were distributed normal with homogene variances and therefore could be used without transformation. Results were compared and analysed with one-way analysis of variance (ANOVA) using statistical package SAS 9.1 (SAS Inst. Inc., Cart, NC, USA). Coefficient of variation (CV, %) was calculated based on (SD/mean)×100. Differences were considered to be statistically significant when $P \leq 0.05$.

Results

Risk factors using CASA for semen assessment in AI laboratory practice
Sample temperature, dilution factor, preparation of the sample and counting chamber depth variation affecting the precision and accuracy of CASA motility assessment results are presented in the next sections.

Effect of sample temperature on CASA motility assessment results
The results of motility assessment of semen samples are shown, when the start temperature was 20°C, compared to 39°C (Figure 1). No effect between start and after two min was shown on percentage motile cells. The results for progressive motility showed that the samples with a start temperature of 20°C had 25% lower progressive motile cells at the start of the measurement than after 2 min in the CASA. The difference in progressive motility of samples at a temperature of 20°C and 39°C at the start of the measurement was significant (P=0.001) (Table 1). There was no significant difference in motility during the 5 min in the counting chamber (P>0.05). For both motility and progressive motility there was no significant difference after 2 min in the counting chamber on the heated stage (P>0.05).

Table 1. Effect of sample temperature on CASA motility assessment results. Results for (progressive) motility by using CASA equipment for semen samples analysed continuously after filling the standardised counting chamber with a semen sample diluted with 20°C extender vs. diluted with 39°C extender.

Pre-incubation temperature	Motility, %		Progressive motility, %	
	39°C	20°C	39°C	20°C
N	20	20	20	20
t=0: start	94 ± 3[a]	93 ± 2[a]	70 ± 4[a]	47 ± 6[b]
t=2: after 2 min	94 ± 4[a]	93 ± 4[a]	70 ± 4[a]	71 ± 3[a]

Results represent mean ± SD. Rows with different superscripts differ significantly (P≤0.05)

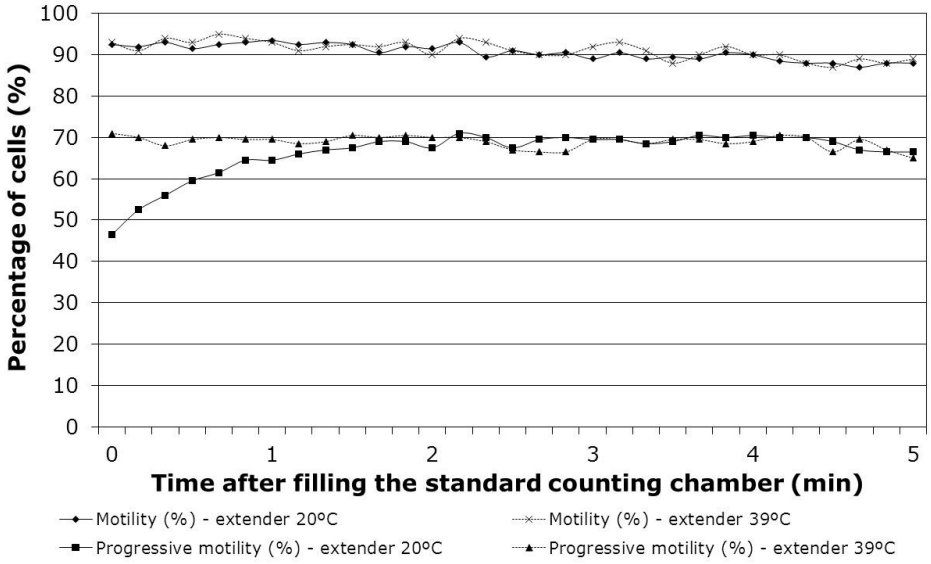

Figure 1. Effect of sample temperature on CASA motility assessment results. Results (n = 20) for (progressive) motility by using CASA for semen samples analysed continuously after filling the standardised counting chamber with a semen sample diluted with 20°C extender vs. diluted with 39°C extender. For SD values, see Table 1.

Effect of dilution factor on CASA motility assessment results

The effect of dilution factor of the sample analysed is shown in Table 2. The results with a dilution factor of 1:15 showed 5% of the measurements with a total number of counted cells being <30 cells per field and 40% of the measurements with a total number of counted cells >80 cells per field. The results with a dilution factor of 1:12 showed 10% of the measurements with a total number of counted cells <30 cells per field cells and 15% of the measurements with a total number of counted cells >80 cells per field cells. Comparing dilution factor 1:15 with 1:12 resulted in respectively 55% and 75% of the measurements being in the optimum range of 30-80 cells per field. Therefore the 1:12 dilution rate was chosen for this AI station for that moment in order to have as many ejaculates in the recommended range without the necessity for additional dilution which raises extra labour.

Table 2. Ejaculates fulfilling required sample density for proper analysis. Distribution of ejaculates over 3 categories of number of cells per field. The number of cells per field between 30 and 80 cells is optimum. Comparing results of two dilution factors (before (1:15) and after (1:12) adjustment) using CASA equipment with standardised counting chambers.

	Before adjustment		After adjustment	
	# ejaculates	%	# ejaculates	%
<30 cells per field (too low)	72	5	169	10
30-80 cells per field (required range)	900	55	1296	75
>80 cells per field (too dense)	658	40	266	15
Total	1630		1731	

Effect of frequency and duration of mixing on CASA motility assessment results

Using a mechanical mixing method, mixing for 3 s at 80% speed, showed significantly higher results, when compared to manual mixing for: concentration (+ 17.7 million/ml, $P<0.0001$), motility (+ 4%, $P<0.0001$) and progressive motility (+ 3%, $P<0.0001$), which is shown in Table 3. Mixing mechanically for 5 s at 100% speed also showed higher results compared to a manual mixing method, however these differences were not significant. Both mechanical methods did not differ significantly although 5 s at 100% speed showed a larger variation in results, especially in the concentration measurement result (Table 3). The homogeneity of the sample was checked and no significant differences were found at different sample spots in the tube ($P>0.05$).

Effect of variation in chamber depth on CASA motility assessment results

The average chamber depth measured for the counting slides was 19.93 ± 0.16 µm. Samples analysed showed on average an absolute difference for motility of 4.3 ± 4.7% and for progressive motility 3.4 ± 3.5%. The difference in motility and progressive motility showed low correlations (0.090 and 0.096 respectively, n = 100) with differences in chamber depth, as indicated in Figure 2.

Table 3. Comparison of manual and mechanically mixing methods. Mixing methods in relation to semen concentration and motility assessment results using CASA with standardised counting chambers.

	A	B	C
Number of ejaculates	50	50	50
Concentration, M/ml	210.7 ± 15.1[a]	228.4 ± 7.1[b]	225.3 ± 11.3[ab]
Motility, %	75.1 ± 3.3[a]	79.3 ± 2.4[b]	78.9 ± 4.9[ab]
Progr motility, %	71.3 ± 5.1[a]	74.3 ± 1.9[b]	73.7 ± 5.0[ab]

Results represent mean ± SD. Rows with different superscripts differ significantly (P≤0.05)

A: manual mixing = ± 5 times tube upside down for 2-3 s

B: mechanical mixing = using mechanical mixing 80% speed, for 3 s,

C: mechanical mixing = using mechanical mixing at 100% speed, for 5 s

Establishing a SOP to reduce sample variation and laboratory technician variation

The effect of training of laboratory technicians and the remaining variation in CASA results is shown in Table 4. The precision is dependent on the number of cells counted. The more cells counted, the more precise the counting will be. Based on the numbers of cells counted for the motility assessment, between 400 and 450 cells, the theoretical obtainable coefficient of variation is about 4.7 % for the CASA. Before training the coefficient of variation was 4.7%, but by practicing and repeating measurements the performance of the laboratory technicians improved considerably to a coefficient of variation of 1.9% in the current situation (Figure 3).

Table 4. Effect of training on repeatability. The repeatability and coefficient of variation (CV) of the CASA motility measurements based on duplicate measurements, before, during and after training using CASA with standardised counting chambers.

	Before training	During training	Current situation
Number of ejaculates	600	614	635
Number of cells per analysis	413 ± 35	456 ± 43	387 ± 61
Repeatability	71%	85%	96%
CV in motility scores	4.7%	3.3%	1.9%

Results represent mean ± SD

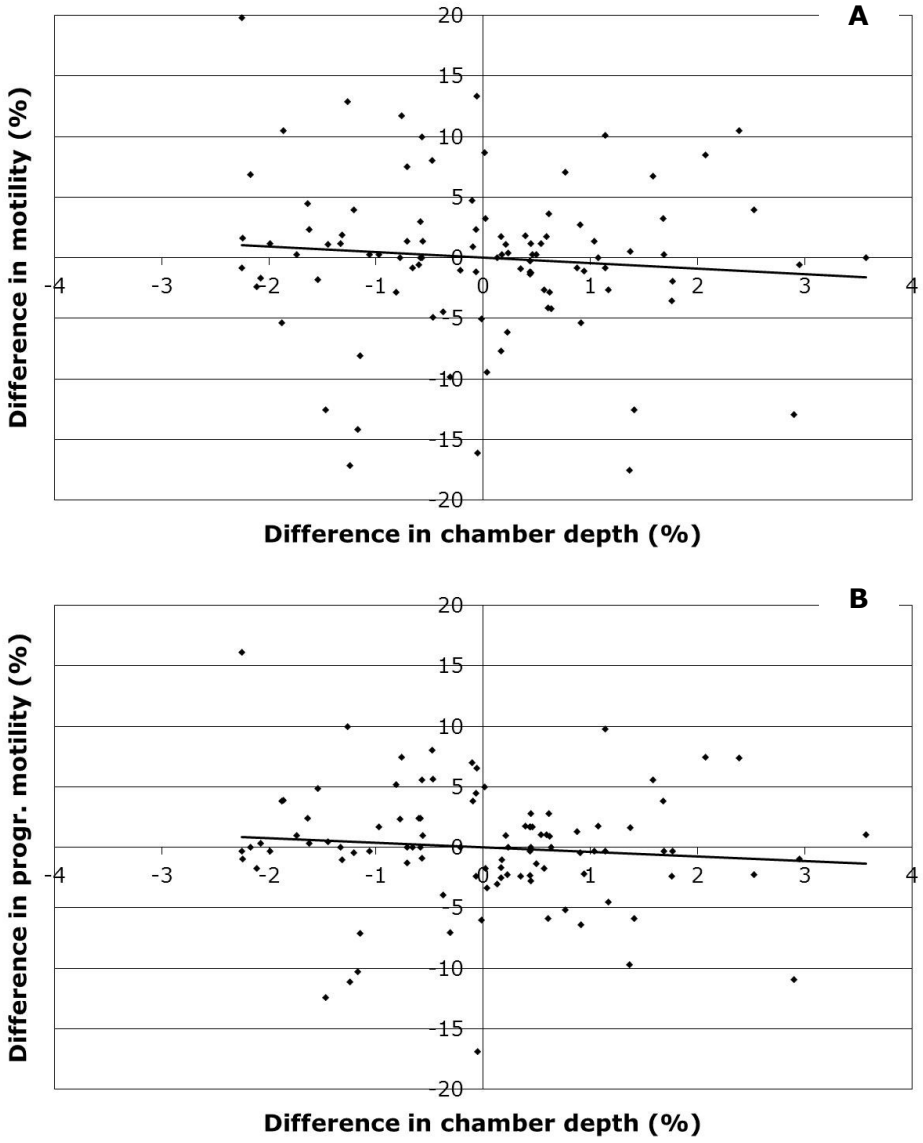

Figure 2. Effect of variation in chamber depth on CASA motility assessment results. Relation between differences in chamber depth and differences in motility (A) and progressive motility (B). The measurements (n = 100) were performed using the CASA equipment with standardised counting chambers.

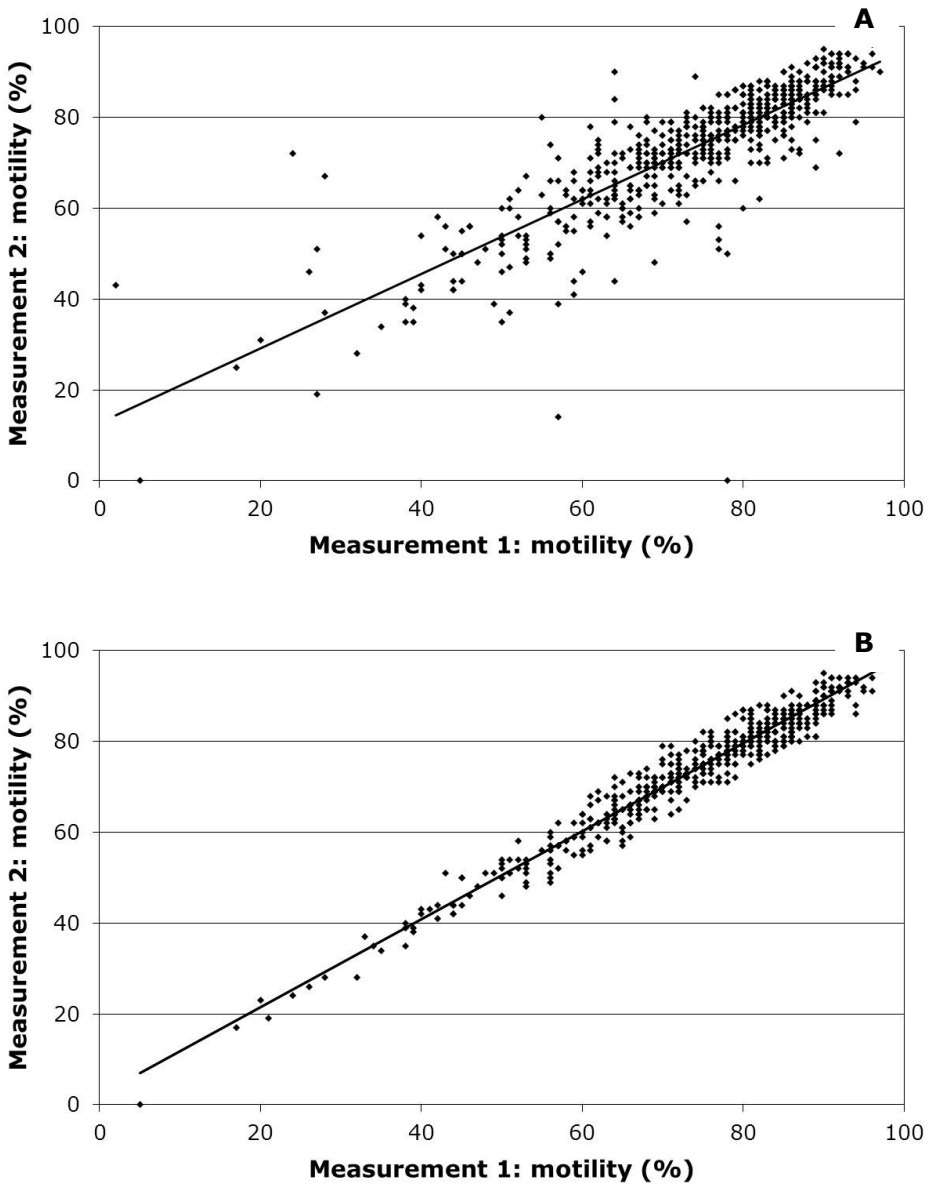

Figure 3. Effect of training on repeatability. The repeatability of the CASA motility measurements with CASA and with standardised counting chambers based on duplicate measurements, before, (A) and after (B) training.

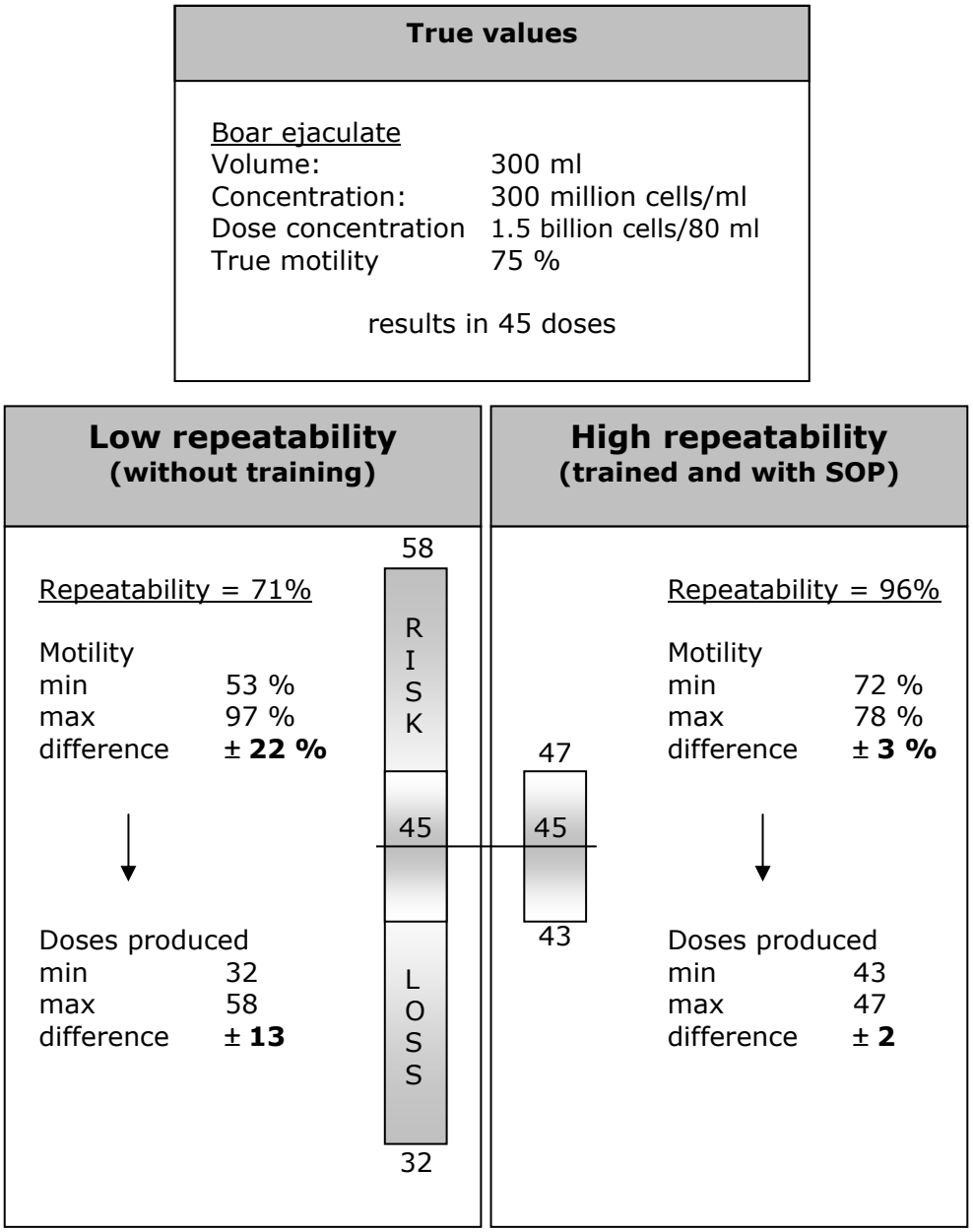

Figure 4. Example of beneficial effect of precise, standardised and objective motility assessment. Shows effect on number of doses produced and semen motility quality of the dose. Low repeatability (without training) vs. high repeatability (trained and with SOP). Varkens KI Nederland.

Improved efficiency in semen dose production after CASA standardisation

Following high precision enables producing more doses per ejaculate and this is shown in a schematic illustration. Figure 4 shows an example of a boar ejaculate of 300 ml with a concentration of 300 million sperm cells/ml. The dose concentration used at Varkens KI Nederland is currently 1.5 billion motile cells in an 80 ml volume. With a true motility of 75 %, dose calculation results in a production of 45 doses of this example ejaculate. When the repeatability achieved in practice is only 71% (untrained) instead of the current 96%, CASA motility assessment can give scores between 53% and 97% motility, resulting in 32-58 insemination doses produced. With a repeatability of 96% (after training personnel conducting according to SOP), scores are between 72% and 78% motility, resulting in 43-47 insemination doses produced.

Comparison between microscopic and CASA semen motility assessments and their relationship with fertility potential of insemination doses

Results from semen motility assessment using phase contrast microscopy (percentage motile cells and quality of movement) were compared with results from semen motility assessment with the use of a CASA system with its original settings (% motile and progressive motile cells), as shown in Table 5. The results of 1,499 samples are randomly grouped per 100 measurements, which results in averages with SD values. For the microscopic results, we found a clear peak in results at 80% motile cells (83% of the results); less than 1% of the ejaculates scored < 60% motile cells. Due to the settings and principle of the CASA measurements, it showed a broader range with a standard deviation of 14% for percentage motile cells measured (Figure 5). Furthermore the CASA equipment showed continuous semen motility results (Figure 6) with 10.7% of the ejaculates scoring < 60% motile cells. For the statistical approach on estimated limits of agreement, see Supplementary figure 1. The results of microscopic semen motility assessment were statistically different from the CASA results ($P<0.0001$). CASA measurement of percentage progressive motile cells leaded to an average score of 39 ± 16%, where phase contrast microscopy gives a result of 77 ± 5%. The correlation between the two methods was low (0.24 and 0.21 for percentage motile cells and percentage progressive motile cells (quality) resp.)

For 341 ejaculates, results of farrowing rate and total number piglets born could be traced back. The number of fertility records was too limited to show statistically significant differences in fertility using microscopy or CASA results as basis. However we could calculate raw

means and Figure 7 shows the variation in total number born which was more indicative with broader ranges in using CASA compared to microscopy.

Table 5. Comparing microscopic with CASA semen motility assessment. Results for 1,499 ejaculates, using phase contrast microscopy compared with CASA with standardised counting chambers. Correlation between percentage motile cells estimated with microscopy and CASA.

	Microscope		CASA	
	Motile cells	Quality of movement	Motility	Progressive motility
N	1499	1499	1499	1499
Mean ± SD	80.2 ± 5.3	76.5 ± 5.4	74.5 ± 13.8	39.5 ± 16.2
< 60 % mot	0.3%	0.1%	12.7%	87.5%

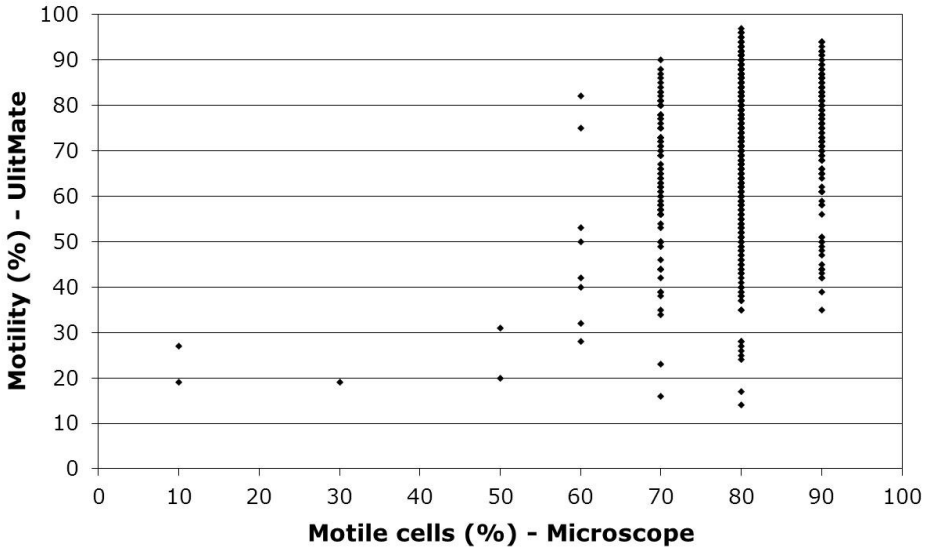

Figure 5. Comparing microscopic with CASA semen motility assessment. Results for 1,499 ejaculates, using phase contrast microscopy compared with CASA with standardised counting chambers. Differences in percentage motile cells assessed by microscopy vs. motility CASA.

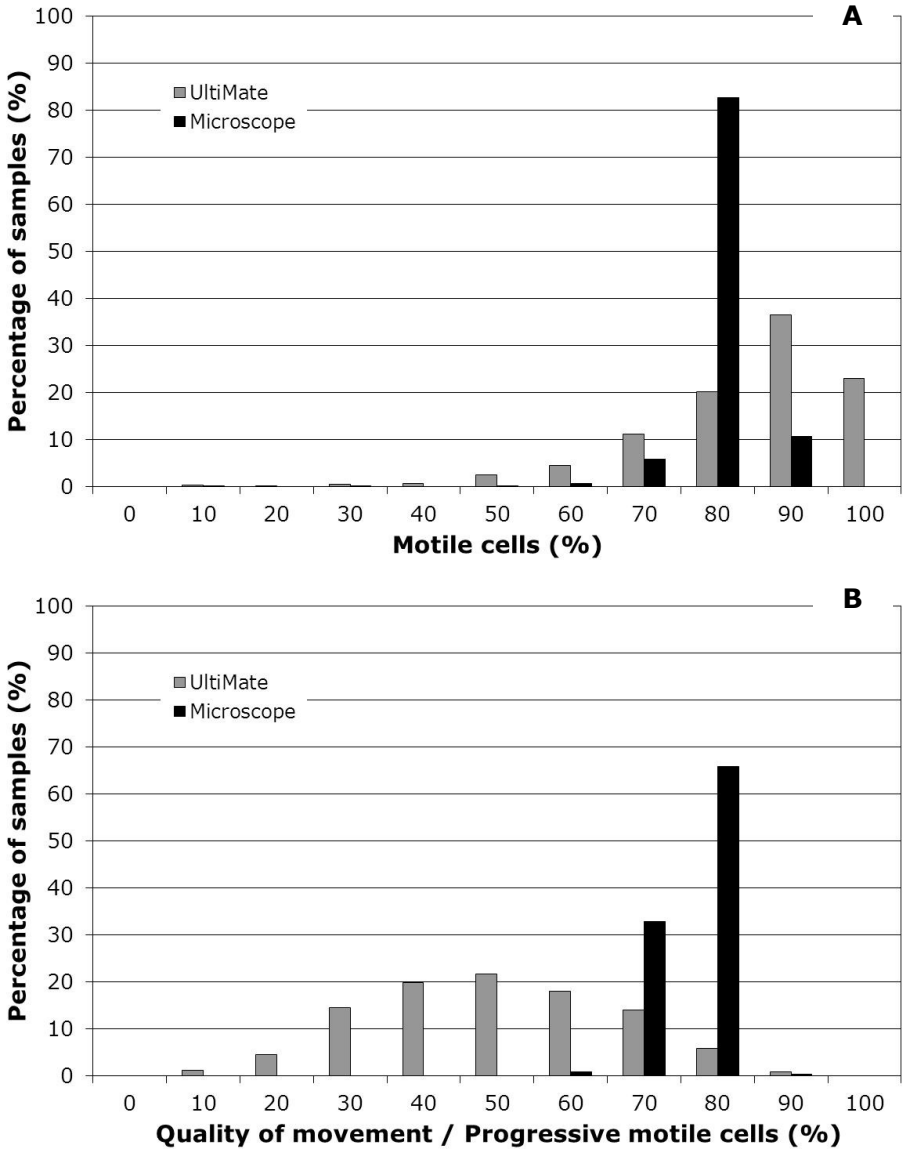

Figure 6. Comparing microscopic with CASA semen motility assessment. Results for 1,499 ejaculates, using phase contrast microscopy compared with CASA with standardised counting chambers expressed in motility categories of 10%. A. Motile cells (microscopy) vs. motility (CASA). B. Quality of movement (microscopy) vs. progressive motility (CASA). For the statistical approach on estimated limits of agreement, see Supplementary figure 1.

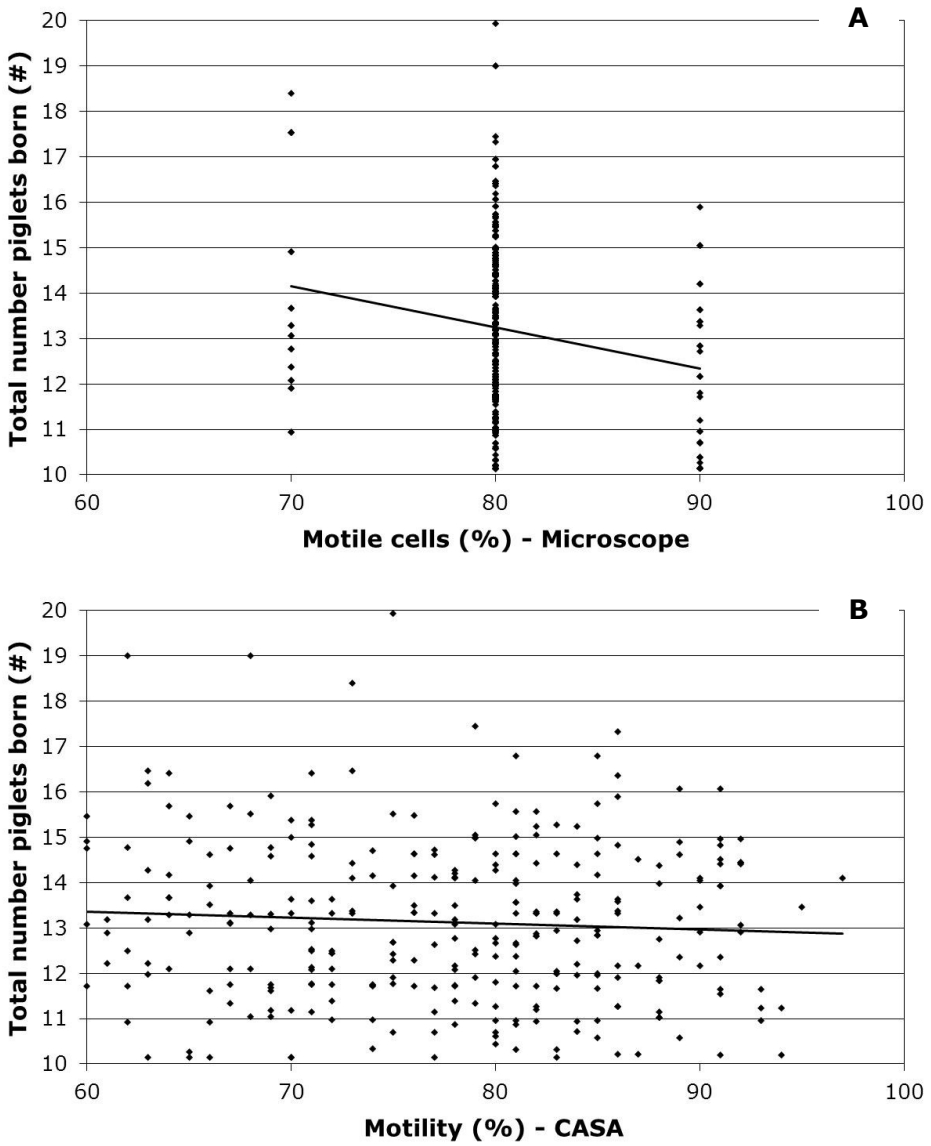

Figure 7. Comparing microscopic with CASA semen motility assessment in relation to total number of piglets born. Results for 341 ejaculates. A. Motile cells (microscopy) vs. total number of piglets born. B. Motility (CASA) with standardised counting chambers vs. total number of piglets born.

Discussion

This study shows that after optimising sample preparation (temperature, dilution factor and mixing method), standardisation (chamber depth) and training with computer assisted semen analysis (CASA) the repeatability of the method is highly increased. As a result the variation in motility assessment score decreased from ± 22% (untrained) to ± 3% (trained).

CASA equipment is claimed to be more precise and accurate and therefore objective (Farell *et al.*, 1995; Krause, 1995; Farell *et al.*, 1996; Abaigar *et al.*, 1999; Iguer-ouada and Verstegen, 2001; Hoflack *et al.*, 2007). However to live up to that expectation repeatability needs to be at an acceptable level (>95%). The number of cells counted should be over 400, which gives a theoretical precision of 95.0% and higher. The establishment of a SOP and the training of laboratory technicians are critical for accuracy and precision of CASA measurements, as is also expressed by Mortimer *et al.* (1986). We analysed the effect of external factors to establish a SOP: sample temperature (Birks *et al.*, 1994; Kraemer *et al.*, 1998; Iguer-ouada and Verstegen, 2001; Klimowicz *et al.*, 2008), sample dilution factor (Varner *et al.*, 1991; Davis and Katz, 1993; Farell *et al.*, 1996; Verstegen *et al.*, 2002; Rijsselaere *et al.*, 2003; Contri *et al.*, 2010), mixing (Collins and Donoghue, 1999), pipeting and the use of the standard counting chamber (Mortimer *et al.*, 1986; Ginsburg and Armant, 1990; Le Lannou *et al.*, 1992; Bailey *et al.*, 2007; Contri *et al.*, 2010).

Ejaculate sample temperature causes variation in CASA results. The optimal dilution and assessment temperature (39°C) has been tested and the results were in accordance with those reported previously (Mortimer *et al.*, 1986; Birks *et al.*, 1994; Kraemer *et al.*, 1998; Iguer-ouada and Verstegen, 2001). It is shown that too low sample temperature will lead to underestimation of the progressive motility. This will lead to either rejection of the sample or less production of insemination doses from the ejaculate. Hence the economic efficiency is lower than might be expected with good sample temperature.

Based on the sperm cell density in the counting chamber, a CASA system needs to correct for the Segre-Silberberg effect (Segre and Silberberg, 1961), because this can affect the estimate of the percentage (progressive) motile sperm cells in a sample. The algorithm for the CASA system of the current study is described in detail (Douglas-Hamilton *et al.*, 2005a; Douglas-Hamilton *et al.*, 2005b) and it is based on a density between 30 and 80 cells per field [D. Douglas-Hamilton, personal communication]. Higher density samples showed more crossing of trajectories, or collisions of spermatozoa which affect the analysis

outcome by overestimating the percentage motile cells (Davis and Katz, 1992; Iguer-ouada and Verstegen, 2001; Contri *et al.*, 2010). When using CASA, the dilution factor for preferred concentration for different species or systems is advised on (Nieuwinger *et al.*, 1990; Varner *et al.*, 1991; Davis and Katz, 1993; Farell *et al.*, 1995; Farell *et al.*, 1996; Verstegen *et al*, 2002; Rijsselaere *et al.*, 2003; Contri *et al.*, 2010). However, density ranges of ejaculates differ between boars and/or AI stations due to different collection procedures, different post collection dilution methods and seasonal variation (Le Lannou *et al.*, 1992) based on environmental temperature (Cameron and Blackshaw, 1980; Stone, 1982; Malmgren, 1989; Rivera *et al.*, 2005), photoperiod (Bartness and Goldman, 1989) or humidity (Rivera *et al.*, 2005). We showed that individualised and frequent adjustments of the dilution factor at an AI station resulted in an increased number of samples being in the optimal density range for proper analysis. This improves the analysis speed in the line of production since no extra dilution steps of the ejaculate have to be performed, which is quite important for running a CASA system in the field. We recommend using a dilution factor which gives the highest number of samples in the preferred density range. Furthermore, we recommend monitoring the total number of cells counted per field on a monthly base and adjust the dilution factor when the percentage of samples in the optimal density range is less than 70 %.

Since sperm cells tend to settle on the bottom of the tube, homogeneity of the sample can change. Evaluating various mixing techniques is essential to optimise the survival of sperm cells (Collins and Donoghue, 1999). Mechanical mixing was preferred, showing significantly higher results for concentration and percentage (progressive) motile cells compared to manual mixing. We recommend choosing the method which results in the lowest variation (80%, 5 s) and is easy to standardise. Regularly check the effect of the mixing on homogeneity by sampling from the same sample tube at different heights and analyse the concentration and motility.

Chamber depth of a counting slide is of high importance because it affects cell detection ability of a CASA system (Douglas-Hamilton *et al.*, 2005a; Douglas-Hamilton *et al.*, 2005b) and is indicated as a cause of variation in motility (Klimowicz *et al.*, 2008; Contri *et al.*, 2010). The volume variation in routinely used counting slides can dramatically influence the results (Mortimer *et al.*, 1986; Ginsburg and Armant, 1990; Le Lannou *et al.*, 1992; Contri *et al.*, 2010). Our study shows just small variation in the sperm counting chamber depth and moreover the variation seen in motility assessment results was not related to this variation in chamber depth. For concentration the results

were similar (unpublished data). This conclusion could be different for other counting chambers (Bailey *et al.*, 2007) and/or other extenders by for example ruling out the sticking of sperm in case of surfactant coating of the counting chamber used or different movement patterns of the sperm cells in other extenders.

Distance from measurement position in the counting chamber to the edge of the chamber is affecting the assessment results (Douglas-Hamilton *et al.*, 2005a; Douglas-Hamilton *et al.*, 2005b). Motility assessment due to effects of glue on the motility, concentration assessment due to uneven distribution of cells in the chamber. Therefore, we recommend using a starting position with a minimum of 2 fields distance from the chamber edge. This recommendation can differ for other types of counting chambers (Baily *et al.*, 2007; Contri *et al.*, 2010), so validation against a standard is necessary.

Samper (2000) claims that pressure on the pipette during chamber filling and the duration of the analysis are the most important technical factors to consider, but the effect on the results is not quantified in literature. We reached a repeatability of over 95% in duplicate measurements. The question is how much emphasis one should put on an issue that only causes a minor part of the total variation. Furthermore, under field conditions a standardised filling routine is probably hard to reach when not automated.

Time of sample analysis after chamber loading significantly affects the motility results, although differences were only seen after 5-10 min in the counting chamber (Contri *et al.*, 2010), which is in accordance with our results. It is therefore recommended to analyse the sample directly after loading the chamber.

The importance of the use of a SOP and the training of laboratory technicians when working with a CASA system is obvious from our study (see also Farrell *et al.*, 1995; Krause and Viethen, 1999). The repeatability reached the required level of 95%. CASA semen evaluation should not be implemented before reaching this sufficient level of repeatability and this repeatability level should be monitored at least 4 times a year by analysis of duplicate samples.

High precision is essential for reaching guaranteed levels of insemination dose quality, efficient semen dose production and field fertility results. Moreover, objective, precise and accurate semen motility assessments enables to investigate the relation between sperm motility and fertilising capacity of the semen in future, not only using mean values for (progressive) motility, but also parameters analysed per individual cell.

The (progressive) motility assessment results with CASA were structural lower than microscopic assessment results, which is related to the settings of CASA measurements (Davis and Katz, 1992; Kolibianakis *et al.*, 1992; Krause, 1995; Verstegen *et al.*, 2002; Rijsselaere *et al.*, 2003; Klimowicz *et al.*, 2008). The evaluation of sperm motility by eye does not allow precise discrimination of motility differences between samples. With the settings of our CASA system a broader, continuous range of results was measured. Ejaculates for production at Varkens KI Nederland were rejected when motility was under 60% as assessed by eye. When using the cut-off value of 60%, CASA comparing to microscope, a higher number of ejaculates would be rejected which is a problem accepting a system with standard settings. To overcome this we adjusted the software settings. Another possibility is to adapt the cut-off levels for rejection, which was never recommended before and probably would give acceptance problems in practice. Studies recommend proper programming of species-specific settings, but no action in adjustment was taken (Knuth *et al.*, 1987; Klimowicz *et al.*, 2008). Laboratory technicians would like to agree on results with each other and with the CASA system, which was stressed in literature (Knuth *et al.*, 1987; Morris *et al.*, 1996; Verstegen *et al.*, 2002; Rijsselaere *et al.*, 2003) and in accordance with our own experience. When no action is taken, the number of boars needed for production will increase and the acceptance of CASA is limited.

Visual estimation of percentage motile cells is more basic (Deibel *et al.*, 1976; Chong *et al.*, 1983; Jenq and Ukombe, 1983; Mortimer *et al.*, 1986; Amann, 1989; Dunphy *et al.*, 1989; Knuth *et al.*, 1989; Rozeboom, 2000; Verstegen *et al.*, 2002) than a CASA system analysing each individual cell on its movement pattern. It is not possible to expect a detailed insight of individual sperm cell motility results by a (trained) laboratory technician; the human eye is not able to do so. The laboratory technician makes a difference in steps of 10% motile cells, as compared to a CASA system which calculates the estimated percentage motile cells in a continuous range of results. Furthermore, a CASA system analyses over 400 cells per sample, which is not possible for a laboratory technician analysing 2-3 microscopic fields. It must be added that the CASA system is still dependent on a trained laboratory technician, who is responsible for the sample preparation and loading of the counting chamber.

Production of insemination doses by using a CASA system with a SOP and with trained laboratory technicians improves efficiency and reliability in the production of insemination doses. It has an additional value to conventional motility assessment in pig AI, which is beneficial

for both farmer and AI companies. The ejaculate quality data recorded, when available over a longer period, can be used to analyse the relation with fertility.

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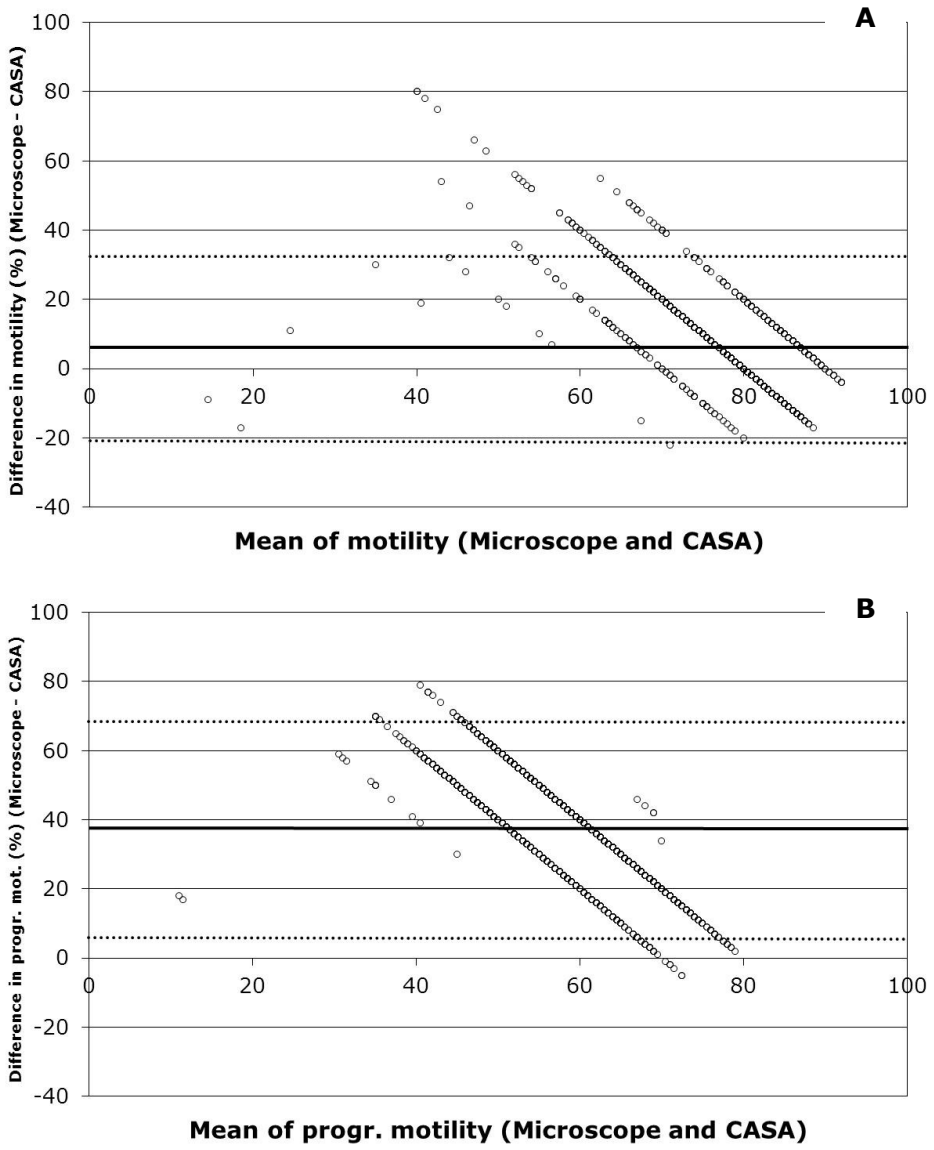

Supplementary figure 1. Precision of estimated limits of agreement in differences in (progressive) motility measured by microscope and by CASA against their means. A. Results for motility. B. Results for progressive motility. Solid line represents mean, upper dashed line shows the mean + 1.96 SD and lower dashed line the mean – 1.96 SD.

Chapter 4

Application of computer assisted semen analysis to explain variations in pig fertility

Broekhuijse MLWJ, Soštarić E, Feitsma H, Gadella BM.
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Abstract
Sperm quality is often evaluated through computer assisted semen analysis (CASA) and is an indicator of boar fertility. The aim of this research was to study the relationship between CASA motility parameters and the fertility results in pigs. Insemination records and semen parameters from a total of 45,532 ejaculates collected over a 3 year period were used. The statistical model for analysis of fertility data from these inseminations included factors related to sow productivity. The boar and semen related variance (direct boar effect) was corrected for effects of individual boar, genetic line of the boar, age of the boar, days between ejaculations, number of sperm cells in an ejaculate, number of sperm cells in an insemination dose and AI station. For remaining variance it was analysed if semen motility parameters had a significant effect. This analysis revealed ~~a~~ significant ($P<0.05$) effects of progressive motility, velocity curvilinear (VCL) and beat cross frequency (BCF) on farrowing rate (FR). Total motility, velocity average path (VAP), velocity straight line (VSL) and amplitude of lateral head displacement (ALH) significantly affected total number of piglets born (TNB). Boar and semen related parameters explained 5.3% of the variation in FR and 5.9% of the variation in TNB. Motility parameters, measured by CASA, explained 9% of the boar and semen related variation in FR and 10% of the boar and semen related variation in TNB. Individual boar and genetic line of the boar significantly ($P<0.0001$) affected the variation in FR and TNB. There were no significant differences between effects of AI stations on fertility outcome, underscoring the objectivity of the CASA system used. Measuring motility parameters with CASA can be used to assess sperm motility in an objective manner and it enables to discriminate the fertilising capacity of ejaculates based on the motility pattern depending on genetic line of the boar in AI stations.

Keywords
CASA system, fertilising capacity, porcine, semen motility, sperm

Introduction

In current AI practices, an important element is accurate and precise quality assessment of the motility characteristics of spermatozoa (e.g. Tardif *et al.*, 1999; Gil *et al.*, 2009). Although common practice, microscopic semen motility scores have a small effect on fertilising capacity whereas the evaluator's skills have a large influence (Amann, 1989; Gadea *et al.*, 2004; Foxcroft *et al.*, 2008). Hence more objective and standardised semen motility assessment methods are needed (Rijsselaere *et al.*, 2003).

One possibility of enhancing accuracy and standardisation of semen motility assessment is through the use of computer assisted semen analysis (CASA) systems (Amann and Katz, 2004). CASA is currently the most popular method used to evaluate sperm motility (Verstegen *et al.*, 2002; Gil *et al.*, 2009). A wide variety of CASA systems are available, however, the relation between parameters measured and fertilising capacity has not been analysed extensively. Besides human studies, there are surprisingly few comparable studies on the value of CASA measurements. Significant correlations between motility and fertility have been described for bovine (Budworth *et al.*, 1988), equine (Samper *et al.*, 1991), human (Hirano *et al.*, 2001), rabbit (Lavara *et al.*, 2005) and pig (Vyt *et al.*, 2008). Other studies did not demonstrate an association between sperm motility and fertility (Bataille *et al.*, 1990; Liu *et al.*, 1991; Didion, 2008).

The aim of the study was to determine the relation between CASA semen motility parameters and the fertilising capacity of boar ejaculates. Furthermore, the CASA system was evaluated as an objective measure of motility in a commercial AI.

Material and Methods

Animals and semen

Data from CASA records of boar ejaculates collected from October 2006 until October 2009 were analysed. The CASA was conducted by seven AI laboratories that process commercial AI doses for Varkens KI Nederland (Deventer, the Netherlands) according to International Standards Organisation (ISO 9001:2008) certified protocols. Boar ejaculates were collected on a routine basis at the AI stations, using the gloved hand technique (Hancock and Hovel, 1959). Each ejaculate was collected in a pre-warmed (40°C) plastic container (370 ml, Graham Packaging Company Inc., York, England) without an insulated cover cup. At the time of collection the ejaculate was filtered with a milk filter (nonwoven disc, 200 mm, Universal Filters Inc., New Jersey, USA) to remove the

gel fraction. The ejaculate was diluted 1:1 with the same volume of Solusem extender (Varkens KI Nederland, Deventer, the Netherlands) at 32°C within 15 mins after collection. The weight of this diluted ejaculate was recorded.

Assessment of sperm motility

The motility scores of semen were obtained on a routine basis using the UltiMate™ CASA system (Hamilton Thorne Inc., Beverly, MA, USA) in a laboratory at room temperature (± 20°C). The 1:1 diluted semen was further diluted (1:10 ~ 1:15 (varies between laboratories) in Solusem extender at 39°C) and a 2.6 μl aliquot was placed into a standardised Leja 4-chamber counting slide (Leja Products B.V., Nieuw-Vennep, the Netherlands). With an automated stage 8 microscope fields were analysed using a common starting position within each chamber. The mean of the microscope fields was used for statistical analysis.

The recorded CASA parameters include: velocity average path (VAP, μm/s), defined as the average velocity over the smoothed cell path; velocity straight line (VSL, μm/s), defined as the average velocity measured in a straight line from the beginning to the end of the track; velocity curvilinear (VCL, μm/s), defined as the average velocity measured over the actual point to point track followed by the cell; amplitude of lateral head displacement (ALH, μm), defined as the maximum of the measured width of the head oscillation as the sperm cells swam; beat cross frequency (BCF, Hz), defined as the frequency with which the actual track crossed the smoothed track in either direction; motility (%), defined as the percentage motile cells of total; and progressive motility (%), defined as the percentage progressive motile cells of total (VAP ≥25 μm/s and straightness (STR) ≥30 %).

Software settings recommended by Hamilton Thorne Inc. for progressive motile cells were adjusted for the cut-off values of VAP and STR. The settings were: image capture by 60 frames per second, total of 45 frames captured; cell detection with minimum contrast of 4 and minimum cell size of 7 pixels; the default for cell size was 7 pixels and the cell intensity ~~is~~ was 50; the cut-off value for progressive cells was for VAP 25 μm/s and for STR 30.0%; slow cells were recorded as static and had a VAP cut-off of 20.0 μm/s and a VSL cut-off of 5.0 μm/s.

Semen processing

After sperm motility assessment, to produce the insemination dose, the final dilution was prepared using Solusem at 20°C. The calculations for this dilution were based on the minimal percentage of motile cells in an insemination dose. After final dilution, polyethylene conic tubes (Minitüb

GmbH, Tiefenbach, Germany) were filled (80 ml) and airtight sealed. The tubes were stored in an acclimatised area (17 ± 2°C). The insemination doses were transported in a transport box maintained at 17 ± 2°C. At the farm, farmers had an acclimatised box (17 ± 2°C, digital recorder with a min and max thermometer). The insemination doses were stored until use for AI.

Fertility records

The Institute for Pig Genetics (Beuningen, the Netherlands) owns and manages a breeding database (Pigbase), which contains fertility records from purebred and crossbred sows. These records include the data of the ejaculate identification, the boar that produced the ejaculate and the day of insemination.

In Dutch sow farming practice, two different sow management systems (Pigmanager and Farm) are used by farmers for recording and analysing farm results. The definitions for technical results are standardised in the Netherlands. The farmers recorded the date of the first, and if performed, the second and third insemination per sow. The boar ejaculate used, date of return to oestrus (non-pregnancy), date of following insemination, date of farrowing and the number of piglets born (total, live, dead, mummified) were recorded for each sow. These data were exchanged electronically via an Electronic Data Interchange (EDI) module with Pigbase. Data were uploaded in the database after several procedures which check for errors according to criteria of the Institute for Pig Genetics, such as missing boar name or incorrect sow identification. Once all data were validated, the data were accepted in Pigbase. A dataset extracted from this database was used to analyse the fertility traits associated with the ejaculates. Two fertility parameters of the inseminations, namely (1) the farrowing rate (FR) indicating the percentage of sows that produced offspring, and (2) the litter size indicating the total number of piglets born (TNB) per litter, were recorded and related to the CASA semen motility assessment scores of the insemination doses used.

Statistical analyses

The fertility results were first analysed for effects of farm and sow related factors and the least square means for the two fertility traits (FR and TNB) were calculated using a statistical package (SAS 9.2 Institute Inc., Cary, NC, USA).

The method for least square means is used because per ejaculate an average of 37 ± 16 insemination doses were produced, which resulted in multiple fertility results. Hence the corrected average fertility result per ejaculate was calculated using this statistical method. The method of least square means was first described by Carl Friedrich Gauss around 1794. The method is widely used in agricultural scientific journals, one of the earliest occasions being Damon *et al.* (1959), more recently being Xu *et al.* (1998) or Bhakat *et al.* (2011).

The following model was used to calculate the least square means for fertility results, where farm and sow factors with significant effect on fertility were used as correction factors:

$Y = \mu + parity + line♀ + farm + 1^{st}/remating + purebred / crossbred\ litter + number\ of\ inseminations + int\text{-}wean\text{-}1 + gest\_length + year*season + weekday + age\ semen + error$ [1]

Where Y was the value of FR or TNB; μ was the mean value of FR and TNB; parity was the effect of the actual parity of the sow; line♀ was the effect of the genetic line of the sow, farm was the effect the farm; 1^{st}/remating_was the effect of a first or a remating of the sow (rematings are known to have better results); purebred/crossbred litter was the effect of having a purebred of crossbred litter; number of inseminations was the effect of the number of inseminations per heat; int-wean-1 was the effect of the interval between weaning and the first insemination; gest_length was the effect of the gestation length; year*season was the effect of the combination between year and season (first season is January – March, second season is April – June, third season is July – September and fourth season is October – December); weekday was the effect of the day of the week of insemination; age semen was the effect of the age of the semen (days after production) at insemination (the farmer decided when to use an insemination dose); and error was the random residual effect that could not be explained by the variables in the model. The observations were weighted according the inverse of the standard error of the estimates for the ejaculate. The variables in this model were tested for possible interactions. However, no interactions other than year*season, were significant. Therefore, no other interactions were included in model 1.

Data were first tested for normality (SAS Inst. Inc., Cary, NC). The residuals for FR and TNB were calculated for each ejaculate by correcting with effects estimated with model 1 to study the relation between sperm motility and fertility.

The boar and semen related effects on these residuals were then analysed using the following model:

$$Y^* = \mu^* + line♂ + boar(line♂) + age + days\text{-}ejac + AI\ station + vol\text{-}conc + dose\text{-}conc + CASA + error^* \quad [2]$$

Where Y* was the residual value of FR or TNB ; μ*_was the mean value of Y*; line♂ was the effect of the genetic line of the boar; boar(line♂) was the effect of the individual boar within the genetic line of the boar; age was the effect of the age of the boar at ejaculation; days-ejac was the effect of the number of days between current and previous ejaculation; AI station was the effect of the AI station; vol-conc was the effect of the number of cells in an ejaculate, dose-conc was the effect of the number of cells in the insemination dose used; CASA was the effect of measured CASA parameters; and error* was the random residual effect that could not be explained by the variables in the model. When using general linear model (SAS Inst. Inc., Cary, NC), the observations were weighted according the inverse of the standard error of the estimates for the ejaculate. The variables in this model were tested for possible interactions. However no interactions were significant and were, therefore, not included in model 2.

The CASA parameters were analysed separately. In a first analysis (analysis 1), both motility and progressive motility were included in the model as CASA parameters. In a second analysis (analysis 2), these were replaced by the basic parameters VAP, VSL, VCL, ALH and BCF. Thereafter, a variance component analysis (SAS Inst. Inc., Cart, NC, USA) was used to estimate the proportion of variance explained by the different sources of variation in fertility traits. Statistically significant boar and semen related parameters were modelled with a proc mixed procedure which shows the percentage of variation explained by each parameter. Differences were considered to be statistically significant when $P \leq 0.05$.

Results

The effect of boar and semen related parameters on fertility
A dataset of 45,532 boar ejaculates could be linked to sow fertility records from 364 farms of which the results are listed in Table 1. The sperm motility assessment results of the ejaculates directly after collection are reported in Table 2.

Table 1. Descriptive statistics of farm and sow characteristics.

Farm and sow characteristics	Number of records or mean ± SD
Insemination records	389,960
Number of farms	364
Number of sows	33,765
Number of genetic sow lines / crossings	22
Sows per farm	483
Weaning to oestrus interval, d	6.22 ± 6.73
Number of inseminations per cycle (min-max)	1.6 (1-3)
Parity	3.5 ± 2.2
Gestation length, d	115.1 ± 1.5
Farrowing rate, %	87.2 ± 33.4
Number of total born piglets	13.5 ± 3.2
Number of live born piglets	12.4 ± 3.0
Number of still born piglets	1.0 ± 1.3
Number of mummified born piglets	0.1 ± 0.6
Number of piglets weaned	11.7 ± 2.2

Table 2. Descriptive statistics of boar and semen characteristics.

Boar and semen characteristics	Number of records or mean ± SD
Number of boars	2,367
Number of ejaculates	45,532
Number of genetic boar lines	15
Number of AI laboratories	7
Number of AI production locations	9
Age of boars, mos	24 ± 11
Number of days between ejaculation	4.34 ± 2.51
Number of sperm cells per ejaculate	$84 \times 10^9 \pm 11 \times 10^9$
Number of sperm cells in a dose (80 ml)	$1.87 \times 10^9 \pm 0.42 \times 10^9$
Number of doses produced per ejaculate	37 ± 16
General	
Motility, %	87.4 ± 6.4
Progressive motility, %	78.2 ± 8.6
Direction and movement	
VAP, μm/s	95.1 ± 20.5
VSL, μm/s	68.5 ± 18.4
VCL, μm/s	175.2 ± 37.3
ALH, μm	7.3 ± 1.3
BCF, Hz	39.3 ± 2.8

There were significant ($P \leq 0.05$) effects of individual boar (within boar line), genetic line of the boar and boar age (on FR only) on FR and TNB and were therefore included in model 2 as correction factors (Table 3). Number of sperm cells in an ejaculate and number of sperm cells in an insemination dose both did not have an effect on FR and TNB ($P > 0.05$). Also, the effect of AI station was not significant for FR and TNB ($P > 0.05$).

Variation due to the boar and semen related parameters is allocated to the direct boar effect. This direct boar effect explained 5.3% of the total variation in FR and 5.9% of the total variation in TNB. Most of the variation of the direct boar effect was explained by the individual boar (29% and 31% for FR and TNB respectively, $P < 0.0001$) and the genetic line of the boar (22% and 18% for FR and TNB respectively, $P = 0.0012$ and $P < 0.0001$ respectively). Boar age explained 0.3% of the variation in FR ($P = 0.0008$). Respectively 40% and 41% of the variation in FR and TNB could not be explained (residual).

Results of the variance component analyses are shown in Figure 1A and Figure 1B for FR and TNB, respectively. The CASA parameters explained 9% of the variation in FR and 10% of the variation in TNB. According to Analysis 1, 9% of the variation in FR was explained by progressive motility (P=0.0134) while for TNB, 10% of the variation was explained by motility (P=0.0041). The results of analysis 2 suggest that VCL (2%, P=0.0151) and BCF (7%, P=0.0062) together explained 9% of the variation in FR. In case of TNB, VAP (3%, P=0.0034), VSL (1%, P=0.0012) and ALH (6%, P=0.0045) together explained 10% of the variation.

Table 3. Boar related sources of variation (direct boar effect) and their effect on farrowing rate (FR) and total number of piglets born (TNB). P-value per variable for variation in FR[1] and variation in TNB[2].

P-values	Analysis 1		Analysis 2	
Parameter	FR	TNB	FR	TNB
Ind. boar (within line)	<0.0001	<0.0001	<0.0001	<0.0001
Line of the boar	0.0012	<0.0001	0.0003	<0.0001
Age of the boar	0.0008	0.3746	0.0011	0.4724
No of days between ejac	0.6370	0.4086	0.2054	0.2978
No of cells in ejaculate	0.7523	0.5212	0.5063	0.7555
No of cells in insem. dose	0.2703	0.4393	0.6625	0.2424
AI station (11 locations)	0.9143	0.4013	0.8397	0.3762
Motility	0.5259	0.0041		
Progressive motility	0.0134	0.3795		
VAP			0.1059	0.0034
VSL			0.3553	0.0012
VCL			0.0151	0.5634
ALH			0.4328	0.0045
BCF			0.0062	0.7451

[1] See Figure 1A for the percentage explained variation in FR; [2] See Figure 1B for the percentage explained variation in TNB. P≤0.05 means that the variable had a significant effect on FR or TNB

The effect of CASA parameters on fertility

Table 4 shows the effects for different CASA parameters included in model 2. It includes the estimate which gives the direction and magnitude of change in FR and TNB per increase of 1 SD in each of the CASA parameters, to give an impression of the effect. The results of analysis 1 show that FR was significantly affected by progressive motility (+ 1 SD: + 1.058%, P=0.0134) and TNB was significantly affected by

motility (+ 1 SD: + 0.128, P=0.0041). The results of analysis 2 show that FR was significantly affected by VCL (+ 1 SD: -0.373%, P=0.0151) and BCF (+ 1 SD: -0.728%, P=0.0062). The TNB was significantly affected by VAP (+ 1 SD: + 0.0246, P=0.0034), VSL (+ 1 SD:-0.0092, P=0.0012) and ALH (+ 1 SD: -0.027, P=0.0045). The actual values of FR and TNB are represented as an example in Table 5, which represents the classified values of progressive motility and VCL in relation to FR and the classified values of motility and VAP in relation to TNB as most significant motility factors as an example. Figures of these examples are shown in Supplementary figures 1 and 2.

Table 4. Effects of CASA semen quality parameters in relation to farrowing rate (FR) and total number of piglets born (TNB). Effects are expressed as change in FR or TNB by + 1 SD value of the CASA variable.

Effect of + 1 SD on	FR, %	TNB
Motility	ns	0.128
Progressive motility	1.058	ns
VAP	ns	0.246
VSL	ns	-0.092
VCL	-0.373	ns
ALH	ns	-0.027
BCF	-0.728	ns

ns means that the CASA variable had no significant effect on FR or TNB (for P-values see Table 3)

Concluding these results, split by the two analysis methods, the models for explaining the variation in FR (model 3) and in TNB (model 4) due to the direct boar effect are:

For analysis 1:

$FR^* = \mu^* + line♂ + boar(line♂) + age + progressive\ motility + error^*$ [3a]

$TNB^* = \mu^* + line♂ + boar(line♂) + motility + error^*$ [4a]

For analysis 2:

$FR^* = \mu^* + line♂ + boar(line♂) + age + VCL + BCF + error^*$ [3b]

$TNB^* = \mu^* + line♂ + boar(line♂) + VAP + VSL + ALH + error^*$ [4b]

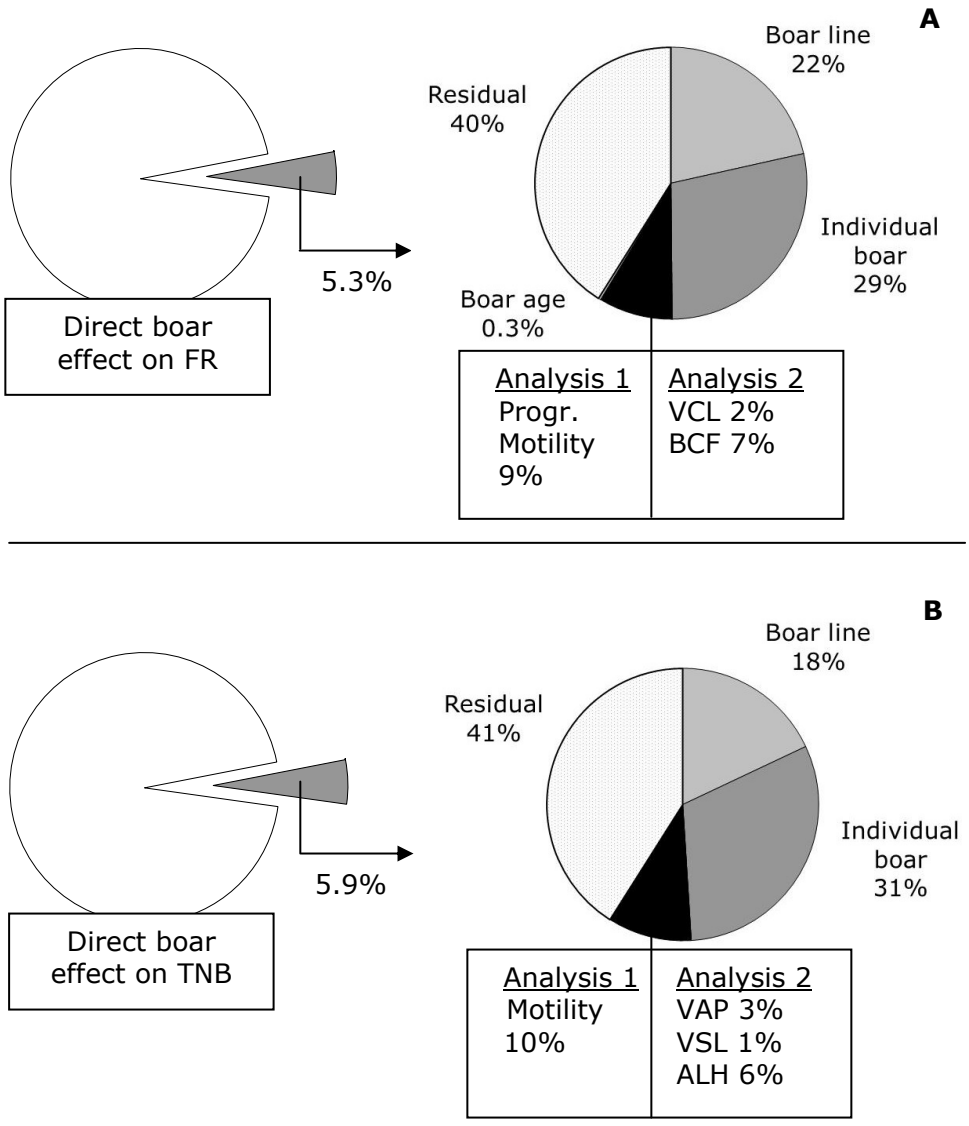

Figure 1. Boar and semen related sources of variation (direct boar effect) and their effects on farrowing rate (A) and total number of piglets born (B). The left pie shows the total variation in fertility. Part of this variation is due to the direct boar effect. This direct boar effect is explained by the parameters in the right pie. For significance levels, see Table 3. VAP = velocity average path (μm/s), VSL = velocity straight line (μm/s), VCL = velocity curvilinear (μm/s), ALH = amplitude lateral head displacement (μm) and BCF = beat cross frequency (Hz).

Table 5. Number of records for different CASA parameters.

Motility[1], %									
	65	70	75	80	85	90	95	100	
N	16	120	754	2554	8006	17450	15586	1046	
TNB	12.6	13.4	13.9	14.1	14.0	14.0	14.1	14.5	
Progressive motility[1], %									
	55	60	65	70	75	80	85	90	95
N	52	395	1672	3113	6166	10788	13191	9240	902
FR	84.6	83.2	86.3	84.0	85.6	86.6	86.7	87.6	87.0
Velocity average path[2], μm/s									
	50	60	70	80	90	100	110	120	130	140
N	838	1784	2758	3844	5536	7671	9559	8473	3983	982
TNB	13.7	13.8	13.9	13.9	14.0	14.1	14.1	14.1	14.2	14.0
Velocity curvilinear[3], μm/s									
	100	125	150	175	200	225	250	275	300	
N	982	3430	6352	9486	12022	8752	3498	802	160	
FR	88.2	86.3	86.4	85.5	87.1	86.5	86.6	86.8	80.4	

[1] Parameters were classified as e.g. 80% = 75.1 – 80%, 85% = 80.1 - 85%, etc.

[2] Parameters were classified as e.g. 80 μm/s = 70.1 - 80μm/s, 90μm/s = 80.1 - 90μm/s, etc.

[3] Parameters were classified as e.g. 175μm/s = 150.1 - 175μm/s, 200μm/s = 175.1 - 200μm/s, etc.

The effect of genetic line of the boar on fertility

The genetic line of the boar explained 22% of the boar related variation in FR (P=0.0012) and 18% of the boar related variation in TNB (P<0.0001), as illustrated in Figure 1. The differences between genetic lines in their relation between VCL and FR and in their relation between VAP and TNB as examples are illustrated in Figure 2A and Figure 2B respectively. The genetic lines in the figures are ranked based on the value of VCL or VAP. The same genetic lines in both figures are indicated by the letters A – G. Boar lines showing the greatest VCL or VAP did not per definition result in the greatest FR or TNB. Supplementary table 1 shows the P-values, representing the relation between CASA parameters and FR and TNB for the different genetic lines of the boars.

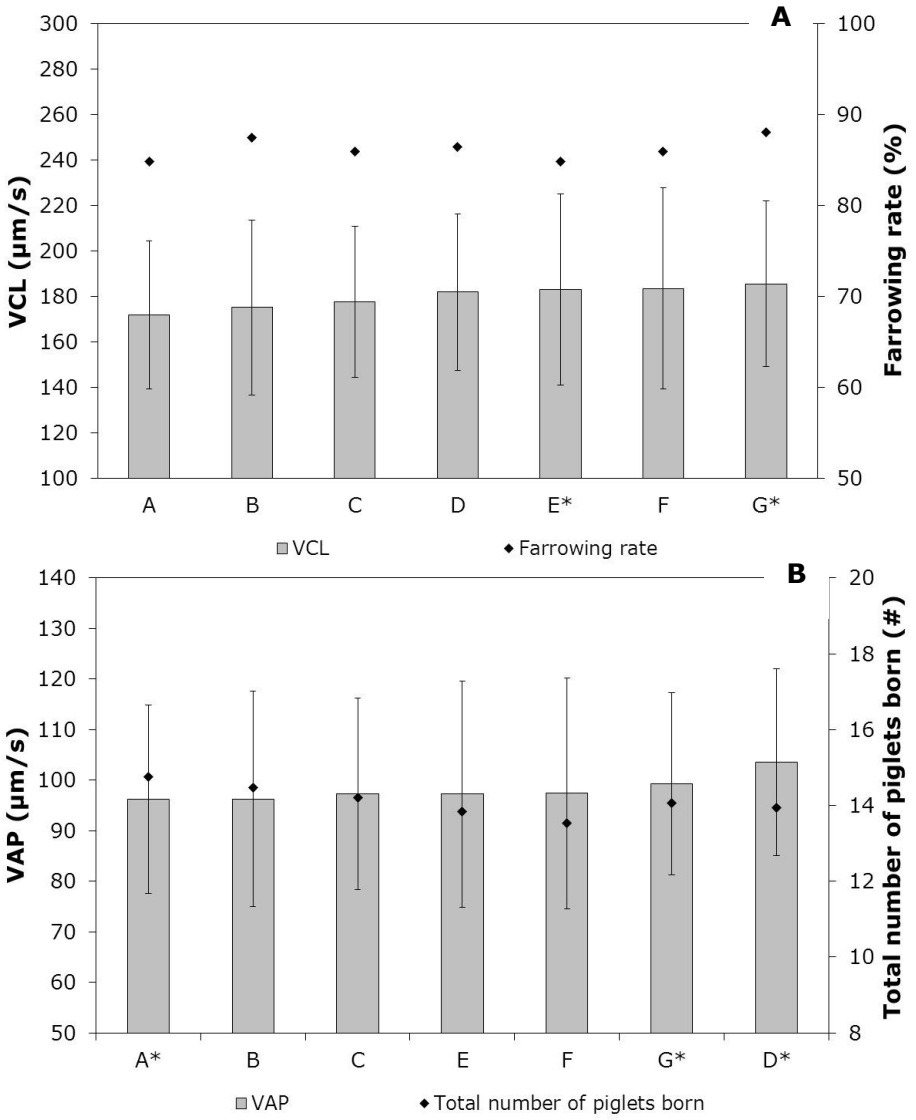

Figure 2. Effect of genetic line of the boar (boar line) in 7 genetic boar lines (A-G). Figure A: The relation between velocity curvilinear (VCL) and farrowing rate. Boar lines are sorted by increasing VCL. Figure 2B: The relation between velocity average path (VAP) and total number of piglets born. Boar lines are sorted by increasing VAP. The letters for the genetic boar lines in Figure A are the same as in Figure B. Genetic boar lines with a * show a significant relation (P≤0.05) between the sperm motility variable and fertility. The number of ejaculates per boar line is: A: 7,108, B: 13,312, C: 3,173, D: 2,908, E: 4,852, F: 5,450, G: 5,803. For P-values see Supplementary table 1.

Discussion

Several attempts to evaluate the relation between CASA motility parameters and fertility have been reported with variable results (Holt *et al.*, 1985; Aumüller and Willeke, 1988; Budworth *et al.*, 1988; Bataille *et al.*, 1990; Liu *et al.*, 1991; Samper *et al.*, 1991; Barrat *et al.*, 1993; Hirano *et al.*, 2001; Lavara *et al.*, 2005; Sutkeviciene *et al.*, 2005; Didion, 2008; Vyt *et al.*, 2008). As a model species for investigating this relationship, the pig offers several advantages over for example the human species where, in general, sub fertile couples are tested on sperm motility parameters and the only parameter tested is the pregnancy of the mother (Giltay *et al.*, 1999; Jacobsen *et al.*, 2000; Hirano *et al.*, 2001; Kolettis, 2003; Kamel, 2010; Awadalla *et al.*, 2011). Whereas pigs are polyestrous mammals producing > 20 oocytes per cycle and from the male side multiple ejaculations can be split into > 10 insemination portions per ejaculate and used to inseminate multiple sows. The boars and sows used are both of high fertility in contrast to the human species where sperm CASA parameters are normally not monitored in couples without fertility problems. In pigs, two types of fertility traits were obtained: 1) farrowing rate (FR) and 2) the number of piglets born (TNB).

To produce fattening pigs to accommodate the need for pork consumers, large numbers of inseminations are performed and management practices are aimed at maximising success rates. This provides an opportunity to analyse large datasets from optimised and standardised field conditions to determine whether the fertilising capacity of boar semen could be predicted by CASA parameters. The statistical approach of fitting fertility data from inseminations used in the present study was similar to Didion (2008). However, in contrast to our present results, Didion (2008) did not find significant correlations for any unique CASA parameter. The number of sperm cells per dose used in the period studied in the Netherlands (Table 2) was lower by a factor of 2 which could explain this discrepancy. Another discrepancy in Didion's study (2008) was the low number of females that were inseminated per boar. In a similar study, although with a different statistical approach Holt *et al.* (1997) revealed relations between the basic parameters (VAP, VSL, VCL, ALH and BCF) and fertility. The results in our study represent the actual situation in the pig industry, and thus enable full corrections for sow and boar related parameters. The percentage explained variation in this study was smaller when compared to the results of Holt *et al.* (1997). The study of Vyt *et al.* (2008), which was a smaller trial, supported our findings and found small predictive values of sperm motility on TNB.

Part of the variation in fertility is explained by the direct boar effect. Progressive motility has not been mentioned before in other reports relating this CASA parameter to fertility. This can be due to the fact that progressive motility depends on cut-off values for basic parameters, which differ for different CASA systems. From the current study we concluded that part of the variation in FR is caused by differences in progressive motility.

A positive correlation was found between VAP and TNB which is in contrast to Hirai *et al.* (2001) who did not find any effect. The VAP is a sperm motility parameter that apparently is required in boar semen processed for AI purposes. It was the only CASA parameter that showed a positive relation with boar fertility. This study showed a negative relation between VSL and TNB. A relation between VSL and TNB was shown before in boar (Holt *et al.*, 1997), rooster (McLean *et al.*, 1997), turkey (King *et al.*, 2000) and human (Liu *et al.*, 1991); although in our approach a negative instead of a positive effect was found. Despite one may conclude that increase of VSL enable sperm better to fertilise the oocyte (Liu *et al.*, 1991) it is possible that semen produced for AI purposes should not have this motility parameter apparent. It is more likely that semen processed for delayed use as in fresh semen AI with high VSL properties have preliminary signs of capacitation-like responses, making it vulnerable for deterioration (for review see Leahy and Gadella, 2011). This phenomenon could explain the negative correlation between VSL and TNB. In future studies the correlation between VSL in *in vitro* capacitated boar sperm and TNB should be considered, but this is out of the scope of the current study.

The ALH is considered to be an important motility parameter, acquired during sperm capacitation, and required to accomplish penetration of fertilisation barriers surrounding the oocyte such as the cumulus cell layers and the zona-pellucida (Gadea, 2005). This acquired ALH, which is needed during penetration, implies a positive relation with fertility, which was shown in our study with TNB. Again, a negative effect was found which may be attributed to the fact that we observed semen processed for pig AI rather than semen processed (and thus capacitated) for pig IVF. Probably, the ALH parameter needs to be low in semen for AI as is discussed for VSL (Leahy and Gadella, 2011). BCF is a useful semen cell characteristic in the estimation of gross changes in the flagella beat pattern (Selles *et al.*, 2003), but it may be hampered by the number of observations per second that can be performed by a CASA system. The image frame rate of the CASA system is 60 Hz (UltiMate system specifications) whereas the BCF is 39.3 ± 2.8 Hz (data presented in this study). The results in our study demonstrated a

significant but negative relation between BCF and FR. This indicates that at the moment of motility assessment (fresh semen), it is preferred that sperm cells do not use their beating function yet. In agreement, Gil *et al.* (2009) suggested that the beating must be saved till the moment of penetration of the zona pellucida. ALH and BCF were not significantly correlated with fertility in other studies (Budworth *et al.*, 1988; Liu *et al.*, 1991). In multiple other human studies, the value of ALH and BCF is confirmed (Holt *et al.*, 1985, 1989; Davis *et al.*, 1991; Barratt *et al.*, 1993; Macload and Irvine, 1995; Hirano *et al.*, 2001), although showing opposite effects. This probably again relates to the fact that in those studies semen was processed for IVF purposes rather than AI.

Implications for AI practice

The results in our study showed both negative and positive relations between CASA parameters and fertility and these relations deserve further attention. Typically, one may consider treatments resulting in the greatest velocity outcome to be the best (Amann and Katz, 2004). There is no biological logic to support this assumption. The results in our study clearly indicated negative relationships between 4 out of 5 basic CASA parameters in relation with fertility. An optimal value with a cut-off value to use in daily AI practice still has to be determined. Different parameters have an opposite effect on FR and TNB. Progressive motility or VCL and BCF explained variation in FR and motility or VAP, VSL and ALH explained variation in TNB.

This study is valuable for the AI practice. In routine AI centres, subjective estimation of spermatozoa motility is the main parameter used to select ejaculates. Amann (1989) highlights the most critical aspects of the problem of predicting fertility. It is essential to have 1) specific, precise and accurate laboratory tests and 2) precise and accurate fertility data. In our study all criteria were fulfilled. In a previous study (Chapter 2) it was stated that assessing sperm motility microscopically was less accurate and standardised and that there was a significant effect of AI station and technician on fertility. Own experience showed that working with CASA parameters resulted in precise measurements of concentration and motility parameters (Chapter 3). The current study revealed that there was neither a significant effect of AI station nor technician (included in AI station). Therefore, the method can be considered to be highly objective and effective in selecting ejaculates for AI. CASA can become quite useful for AI practice for optimising semen dose production.

Another important aspect that remains relevant in the procedure of assessing semen quality is the effect of individual boar and genetic line of the boar. Boars have been reported to differ in various semen characteristics (Sondermann and Luebbe, 2008). Both differences among individual boars and between genetic lines of the boars are independent of the semen assessment method, concluded from current (CASA) and previous (microscope, Chapter 2) studies. Sondermann and Luebbe (2008) emphasised that breeds differed in the sustainability of motility, and in fertility. In support we found these differences in the main level of CASA parameters per genetic line of the boar and in the related level of FR and TNB. This does not mean that boar lines with lower sperm motility automatically have lower fertility. AI centres need to factor genetic line differences into their decision-making processes to ensure adequate use of boars and customer satisfaction.

The precision of CASA systems increases the probability of detecting changes in sperm motility in relation to success to fertilise an oocyte (Amann and Katz, 2004). Without a direct solution, the discussion of ”how much is enough” is appropriate in this context. The relatively high number of sperm cells in a dose used in commercial AI practice likely masks reduced fertility (Foxcroft *et al.*, 2008). Previous study (Chapter 2) already underlined that lower number of sperm per insemination dose may cause more variation in fertility. At the same time, the precise and objective method, such as CASA, enables a more efficient use of the ejaculates by increasing the number of insemination doses per ejaculate (Chapter 3). It ensures improved reliability of the fertilising capability of the insemination doses produced. In this study, it has been concluded that FR and TNB are independent of the total number of cells in an insemination dose. This does not hold true automatically for all AI stations. Many other factors, such as hygiene, extender, transport conditions and health status of boars, might affect the threshold for the number of sperm cells per insemination dose. More accurate methods using CASA can be critically relevant for low dose insemination approaches.

The statistical approach of our study focused on the mean values of the CASA motility parameters. Only a small part of the total number of sperm cells is able to fertilise an oocyte (Holt and Van Look, 2004). Sperm quality assessment methods in the laboratory would improve if they could incorporate aspects of these selective processes. Little is known about subpopulations of sperm cells within a semen ejaculate and if unique motility patterns and specific abnormalities influence boar fertility (Didion, 2008). Braundmeier and Miller (2001) suggested that the sperm cells that fertilise the oocytes *in vivo* may be a subpopulation

that is small and highly selected but not representative of the average sperm evaluated in the ejaculate. In the current study, we could only store average motility parameter results; so not all individual cell results were collected. This leaves the research question on subpopulations and their relation with fertility unanswered.

Conclusion

In conclusion this study showed that the use of a CASA system in AI laboratory practice is a valuable tool. The dataset of 45,000 records revealed that there is a significant relation between CASA parameters and FR and TNB, representing the value of CASA motility parameters in relation to the fertilising capacity of boar ejaculates. Furthermore, the study confirmed that the method is highly objective. The effect of individual boar and the effect of genetic line of the boar are continuous effects which have to be taken into account for ejaculate rejection policy. In overall conclusion, our large dataset proves that CASA is valuable for selecting boar ejaculates. In this study we report on the pig which suited as the model species of choice to validate CASA parameters. Fertility parameters are obtained from fertile boars and sows and this validation is applicable for the pig AI industries. The fact that CASA parameters can be related to female fertility in other species can be especially useful in the human reproductive centres where the sperm output can be much more variable.

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Supplementary table 1A. Effect of genetic line of the boar (boar line). P-values for CASA parameters for variation in farrowing rate for 7 boar lines (A-G). P-values <0.10 are in bold. P≤0.05 means that the variable had a significant effect on farrowing rate.

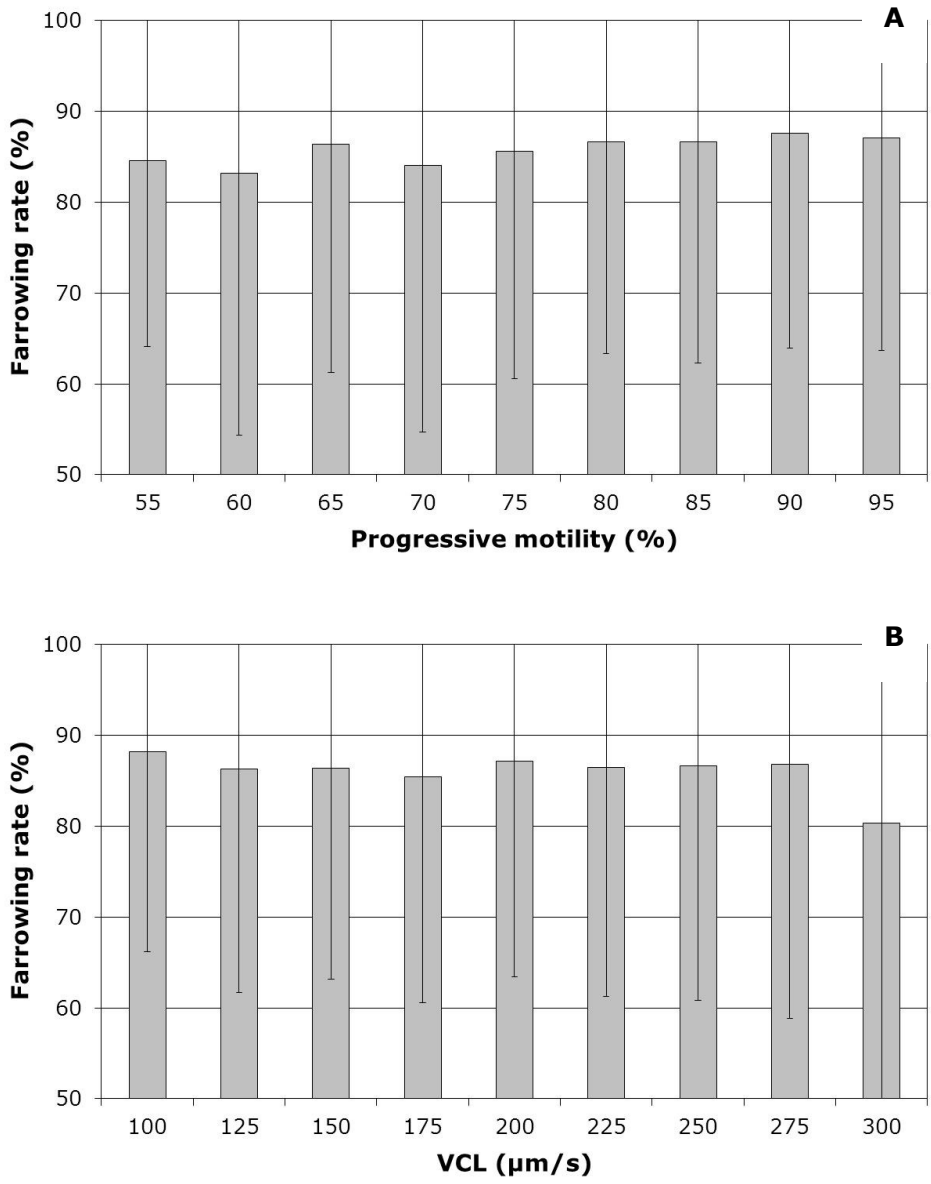
	A	B	C	D	E	F	G
N	7,108	13,312	3,173	2,908	4,852	5,450	5,803
Mot	0.529	0.563	0.768	0.798	0.316	**0.002**	0.192
PM	0.634	0.403	0.666	0.236	0.900	**0.085**	**0.093**
VAP	0.717	0.993	0.780	**0.054**	0.266	0.909	0.221
VSL	0.869	0.660	0.876	0.235	0.974	0.279	0.280
VCL	0.496	0.482	0.337	0.148	**0.031**	0.938	**0.030**
ALH	0.878	0.300	0.141	0.772	**0.048**	0.227	**0.002**
BCF	0.715	**0.082**	0.755	0.374	0.321	0.917	0.516

Mot = motility, %; PM = progressive motility, %; VAP = velocity average path, μm/s; VSL = velocity straight line, μm/s; VCL = velocity curvilinear, μm/s; ALH = amplitude lateral head displacement, μm; and BCF = beat cross frequency, Hz.

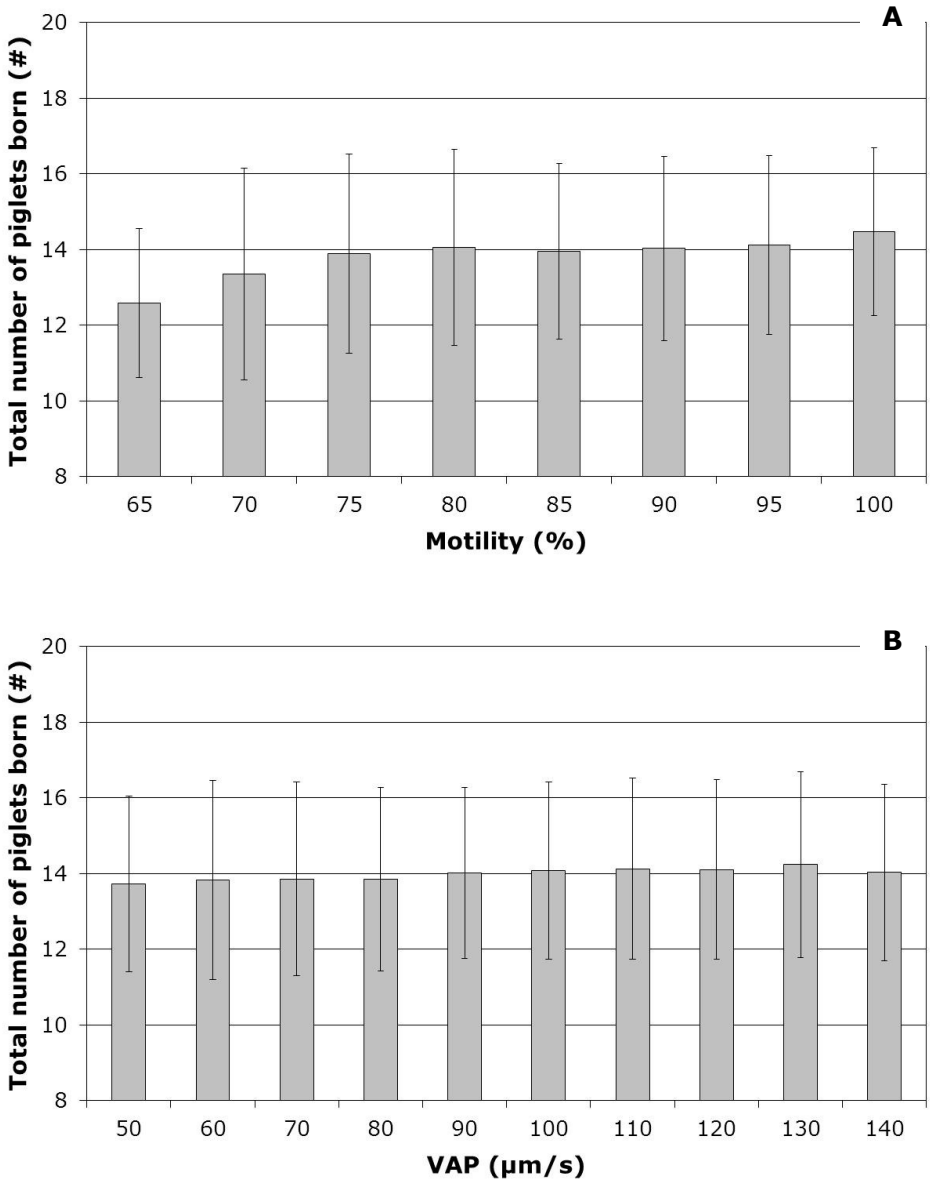
Supplementary table 1B. Effect of genetic line of the boar (boar line). P-values for CASA parameters for variation in total number of piglets born for 7 boar lines (A-G). P-values <0.10 are in bold. P≤0.05 means that the variable had a significant effect on total number of piglets born.

	A	B	C	D	E	F	G
N	7,108	13,312	3,173	2,908	4,852	5,450	5,803
Mot	0.879	0.200	0.449	**0.090**	0.112	**0.013**	0.180
PM	0.578	**0.070**	0.385	**0.044**	**0.057**	0.908	**0.076**
VAP	**0.010**	0.459	0.808	**0.025**	0.639	0.106	**0.008**
VSL	**0.074**	0.604	0.866	**0.018**	0.982	0.677	**0.062**
VCL	**0.004**	0.852	0.869	**0.071**	0.906	**0.021**	**0.001**
ALH	0.218	0.487	**0.063**	0.869	0.154	**0.091**	**0.001**
BCF	0.219	0.714	**0.006**	0.799	0.384	0.250	0.119

Mot = motility, %; PM = progressive motility, %; VAP = velocity average path, μm/s; VSL = velocity straight line, μm/s; VCL = velocity curvilinear, μm/s; ALH = amplitude lateral head displacement, μm; and BCF = beat cross frequency, Hz.



Supplementary figure 1. Farrowing rate in relation to progressive motility (A) and velocity curvilinear (VCL) (B). The numbers of records are described in Table 5. Parameters were classified as e.g. progressive motility = 80% = 75.1 – 80%, 85% = 80.1 - 85%, etc or VCL = 175µm/s = 150.1 - 175µm/s, 200µm/s =175.1 - 200µm/s, etc. Results represent mean ± SD.



Supplementary figure 2. Total number of piglets born in relation to motility (A) and velocity average path (VAP) (B). The numbers of records are described in Table 5. Parameters were classified as e.g. motility = 80% = 75.1 – 80%, 85% = 80.1 - 85%, etc or e.g. VAP = 80 µm/s = 70.1 - 80µm/s, 90µm/s = 80.1 - 90µm/s, etc. Results represent mean ± SD.

Chapter 5

Relation of flow cytometric sperm integrity assessments with boar fertility performance under optimised field conditions

Broekhuijse MLWJ, Soštarić E, Feitsma H, Gadella BM.
J Anim Sci 2012; in press.

Abstract
The number of intact and functional spermatozoa can be assessed with flow cytometry and is believed to relate with male fertility. The aim of this study was to examine whether or not currently used sperm integrity assessments with flow cytometry correlated with field fertility data obtained with boar semen. For this purpose 20 boars were followed for a 20 week period (with a total average production of 33 ejaculates per boar) and the obtained fertility results (farrowing rate and number of piglets born) of commercial artificial insemination doses made from these ejaculates were recorded and the fertility results were corrected for farm, sow, boar and semen related parameters. From the same semen samples sperm cell integrity was assessed with respect to DNA and to membrane integrity, acrosome intactness and responsiveness and mitochondrial potential using established flow cytometric assays. This was done on freshly produced semen and on semen stored for up to 15 days. Remarkably none of the individual membrane integrity parameters were significantly related to fertility results. In contrast the amount of DNA damage as assessed at 7-10 days and at 14-15 days of semen storage significantly related to farrowing rate (P=0.0400) and total number piglets born (P=0.0310) respectively. Thus the detection of the degree of DNA damage in stored boar semen samples can be used to predict the fertilising capacity of boar ejaculates.

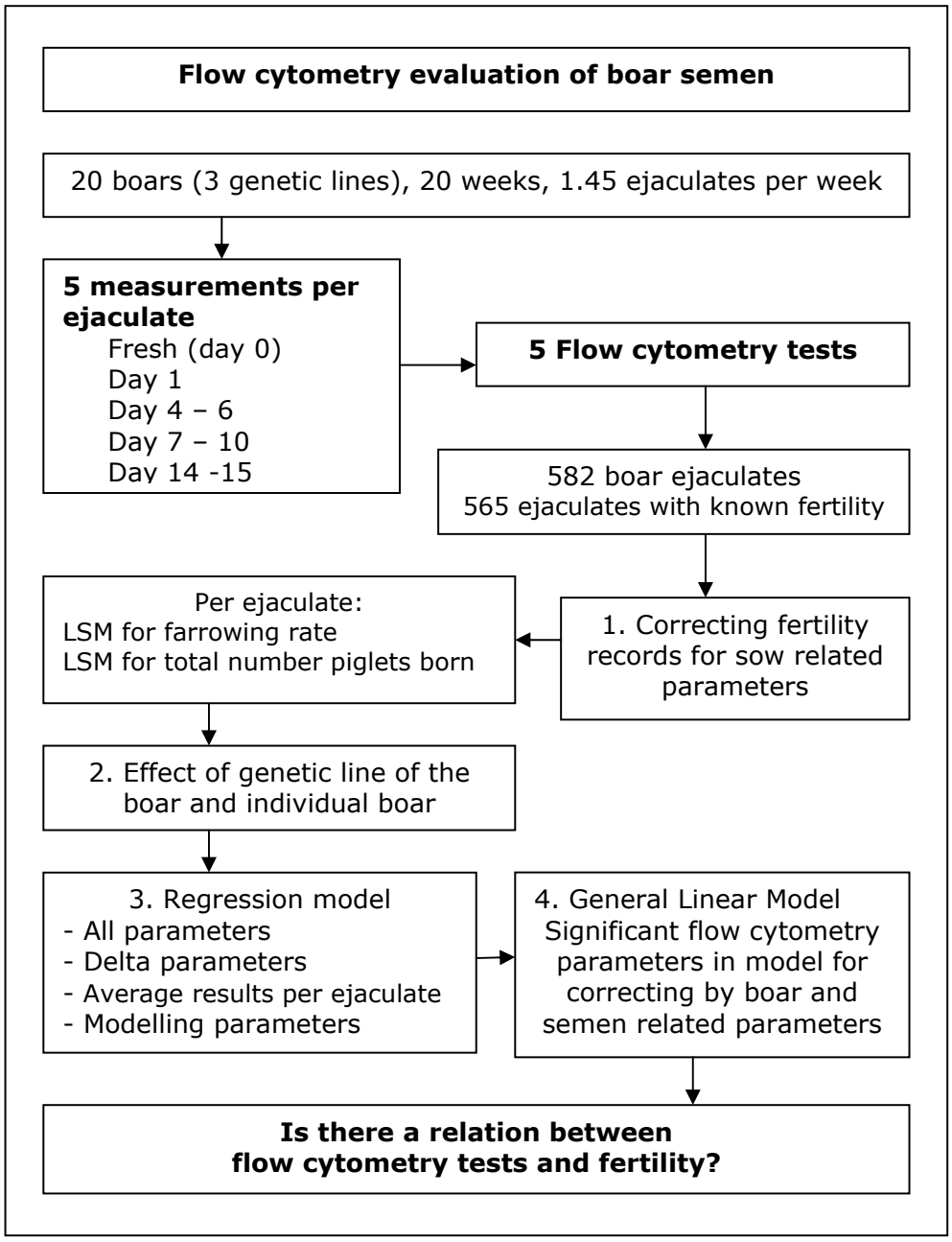
Keywords
DNA fragmentation, membrane integrity, flow cytometry, boar, fertility

Introduction

When a pig breeder is using an insemination dose of boar sperm for artificial insemination (AI), he expects the highest quality of semen in order to ensure maximum litter sizes and farrowing rates. To this end semen ejaculates are analysed at the AI centre where sperm concentration, morphology and motility are assessed and an optimal sperm insemination dose is calculated following the station's criteria. However, the predictive value of these classical sperm assessments is quite low; semen quality is only explaining a small part of the total variation in fertility (Chapters 2 and 4). It is likely that other semen characteristics are important to explain remaining variation, which is an important research question for pig AI centres. For instance a number of flow cytometry tests can be employed to assess the functional integrity of sperm and a naive assumption is that a higher degree of sperm deterioration is related to lower fertility competence of a given sperm sample. However, results obtained in different studies are often controversial (Sutkeviviene *et al.*, 2005).

The integrity of mammalian sperm DNA is of importance for the male genetic contribution to normal offspring. Damaged DNA can lead to early embryonic or foetal death and can have a dramatic impact on health of the offspring (Evenson and Jost, 2000). Membrane integrity assays are also suggested as key parameters in assessing fertilising capacity (Pintado *et al.*, 2000; Frazer *et al.*, 2002). On the other hand, plasma membrane integrity is a requirement for fertilisation (Flesch and Gadella, 2000; Andrade *et al.*, 2007) as is the presence of an intact acrosome and the induction of the acrosome reaction in intact sperm with proper stimulation (Õura and Toshimori, 1990). Finally, the mitochondria play a key role in the generation of sperm movement (Gąxzarzewicz *et al.*, 2003), because they are the major generator of energy (Flesch and Gadella, 2000).

These sperm integrity assessments are currently not taken into account by the classic spermiogram, but may provide added value to predict the fertilising capacity of boar ejaculates. Therefore, the aim of this study was to analyse the relation of flow cytometric assessments of sperm integrity with detailed field fertility results. The potential added value is discussed.



Scheme 1. Experimental and statistical design of flow cytometry analysis. The design represents the experiment, the moments of assessment and the statistical approach for the results of the flow cytometry analysis.

Material and Methods

Trial setup

During a period of 20 weeks, from July 2009 until December 2009, ejaculates from 20 boars, from three genetic lines (A, B, C) were processed (on average 1.45 ejaculates per boar per week). The ejaculates were collected at Varkens KI Nederland (Deventer, the Netherlands) for routine use at sow farms in the Netherlands, according to the method described previously in Chapters 2 and 4. In short, the volume of the ejaculate (mL) was diluted with approximately the same volume (mL) of Solusem® extender (Varkens KI Nederland, Deventer, the Netherlands) at 32°C ± 2°C within 15 minutes after collection. This was the first step in the two-step dilution method. Semen quality (sperm cell concentration and motility) was measured using the computer assisted semen analysis (CASA) system UltiMate (UltiMate^{TM}, Hamilton Thorne Inc., Beverly, MA, USA) with standardised Leja 4-chamber counting slides (Leja Products B.V., Nieuw-Vennep, the Netherlands). The final dilution to reach an average of 1.67×10^9 motile sperm cells per insemination dose was performed using Solusem® (20°C ± 1°C). This was the second step in the two-step dilution method. After final dilution, polyethylene insemination tubes (Minitüb, GmbH, Tiefenbach, Germany) were filled (80 ml). The tubes were airtight sealed and stored in an acclimatized area (17°C ± 2°C). From each ejaculate, one insemination dose was transported within 4 hours after production to the research laboratory of the Faculty of Veterinary Medicine (Utrecht University, Utrecht, the Netherlands), where the remaining doses were transported to the sow farms. Flow cytometric sperm integrity assessments were performed on samples, immediately after the samples arrived at the research laboratory (day 0) and after 1 day, 4-6 days, 7-10 days and 14-15 days of storage. All tests were performed at the same time, for the same samples. For each sample all tests were performed within 1 hour, resulting in a combination of different flow cytometry test results per sample. The test results were included in the fertility records retrieved from the farms. Detailed methods are described below. Scheme 1 shows the trial setup and statistical approach of this experiment.

Flow cytometry

Insemination doses arrived at the research laboratory at the same day of collection of the original ejaculate. Of each insemination dose, five tubes (1.5 ml tube, Eppendorf AG, Hamburg, Germany) were prepared containing 1 ml of semen from the insemination dose. Four tubes were

stored in an acclimatised box (17°C) for repeated measurements and one sample was analysed at the day of arrival. Samples were reactivated at 38°C for 30 minutes in a warming cabinet preceding measurement. Samples were analysed at room temperature in the Guava® EasyCyte™ microcappillary flow cytometer with Cytosoft™ software (Guava Technologies Inc., Hayward, CA USA). The flow cytometer contained one solid phase blue laser (488 nm) and two photodiodes (FSC, SSC). It measured particle (in our case only those that are sperm specific) emission properties with three photo multiplayer tubes (PMTs; green 525 nm, yellow 583 nm and red 655 nm) and accommodating optical filters and splitters. For each analysis, the recording of scatter and fluorescence properties was stopped when 10,000 sperm specific events were obtained. The performance of the flow cytometer was tested daily with the Guava Check™ (Guava Technologies, Inc., Millipore, Billerica, USA, for details see Guava® EasyCyte™ system user's guide). The semen samples were analysed in a 96 well micro plate (round bottom, sterile 96-well assay plate with lid, BD Falcon™, NJ, USA) and loaded in the sample tray. A maximum of 20 samples were assessed per session. With this number there was no time effect on assessments. This was checked in pre-trial tests (which is in line with personal communicated observations, Christophe Staub, IMV Technologies, L'Aigle, France).

Sperm chromatin structure assay

The integrity of sperm was assessed using the sperm chromatin structure assay (SCSA; Evenson and Jost 2000). Briefly, 5 µl semen sample was diluted with 200 µl TNE buffer (0.01 M Tris-Cl, 0.15 M NaCl, 1 mM disodium EDTA, pH 7.4) and 400 µl of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 1.2) was immediately added to the tube for 30 s. Then 1.2 ml of 48 µg/ml acridine orange (AO) in 0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM disodium EDTA, 0.15 M NaCl, pH 6.0 was added and the samples were immediately run through the flow cytometer. Analysis of the data was performed using FCS Express (DeNovo Software, Ontario, Canada).

Membrane integrity, acrosome intactness and responsiveness

The membrane integrity and acrosome intactness were determined for each sample simultaneously. Briefly, 2 µl semen sample was diluted with 196.5 µl Solusem® extender (Varkens KI Nederland, Deventer, the Netherlands) were stained for 5 min in the dark after addition of, respectively, 0.5 µl peanut agglutinin (fluorescein isothiocyanate conjugated; FITC-PNA) (1 mg/ml stock solution, Sigma Chemical Co.,

St. Louis, Mo.), and 1 µl propidium iodide (PI) (1 mg/ml stock solution, Sigma Chemical Co., St. Louis, Mo.) and run through the flow cytometer. Freshly collected and diluted sperm samples (at day 0) were also pre-treated for 30 min with 1 µM of ionophore (calcium ionophore A23187, calcimycin, 10 mg, Sigma Chemical Co., St. Louis, Mo.) to induce the acrosome reaction (Watson *et al.*, 1991). The difference with the corresponding measurement without induction of ionophore was indicated as acrosome responsiveness.

Mitochondrial membrane potential
The Guava® EasyCyte™ MitoPotential™ kit (Guava Technologies, Inc., Millipore, Billerica, USA) was used to calculate the aerobic functionality of the mitochondria (Garner *et al.*, 1997). This kit uses a combination of the 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye to evaluate the aerobic functionality of mitochondria (Smiley *et al.*, 1991). Per sample a mix was prepared of 2 µl JC-1, 2 µl 7-AAD and 46 µl of Solusem® (20°C). This mix was added to 148 µl Solusem® (20°C) and 2 µl semen sample and sperm was stained for 30 min at 38°C in the dark. After staining, the membrane depolarisation was assessed with the EasyCyte mitopotential setup (Guava® EasyCyte™ MitoPotential™ kit).

Data retrieval
In order to analyse the relation between flow cytometry tests and fertility we had to retrieve data from 3 sources: the ejaculate records from the AI centres, the fertility records from the farms who used insemination doses from the ejaculates involved in this trial and the results from the flow cytometry measurement as described above. Fertility records were retrieved according to the method described previously in Chapters 2 and 4. At the Institute for Pig Genetics B.V. (Beuningen, the Netherlands) a breeding database (Pigbase) was available containing fertility records from purebred and crossbred sow farms which were recording these data. From this database we extracted a dataset, complemented it with ejaculate data and test results from flow cytometry and used this as the dataset for statistical analysis.

Statistical analysis
In order to analyse the relation between flow cytometry results and fertility results, the effect of all parameters which significantly affect fertility were quantified. In this study two fertility parameters of the inseminations, namely: (1) the farrowing rate (FR) indicating the

percentage of sows that produced offspring, and (2) the litter size indicating the total number of piglets born (TNB) per litter, were recorded and related to the flow cytometry assessment scores of the insemination doses used. Sources of variation in fertility were on one side farm and sow related factors, on the other side boar and semen related factors (the so called direct boar effect). In order to prepare a dataset for analysis, sow fertility data were corrected for farm and sow related factors and the remaining variation was the direct boar effect on fertility. Differences were considered to be statistically significant when $P \leq 0.05$.

Modelling sow related parameters
One ejaculate resulted in multiple fertility results. For the statistical approach the results were first corrected for the farm and sow related parameters. The model used is described previously (Chapters 2 and 4) and is added in the supplementary information 1.

Statistical approach
Approaching this dataset was started with univariate analyses, where the individual measurements were linked to the fertility records. This resulted in a few significant relations, but without a clear pattern (data not shown). Therefore, the data were approached differently. First of all, the results were grouped and analysed in a regression model. The significant parameters resulted from this analysis were corrected for boar and semen related parameters in a general linear model. The methods are described below.

Regression model
For each ejaculate flow cytometry assessments were performed at 5 moments after production. The dataset contained results from day 0 (fresh), day 1, between day 4 and 6, between day 7 and 10 and at day 14 or day 15. We intended to have the results spread over different times after production in order to analyse the optimal evaluation moment. For the final statistical approach however, the results were first grouped in five classes of measurement moment, resulting in a dataset where all measuring moments were equal for each ejaculate. Data were first checked for normality (SAS Inst. Inc., Cary, NC). The residuals after correcting for farm and sow related parameters from each ejaculate were analysed with a regression model (SAS Inst. Inc., Cary, NC), including the flow cytometry results in relation to FR and TNB.

Next step in the regression model was to include the difference (Δ = delta) between the results for each measurement moment class. In the original dataset, for each ejaculate with five measurement results, the delta was calculated. This resulted in a dataset with records for the delta parameters: Δ[1-0], Δ[(4-6)-0], Δ[(4-6)-1], Δ[(7-10)-0], Δ[(7-10)-1], Δ[(7-10)-(4-6)], Δ[(14-15)-0], Δ[(14-15)-1], Δ[(14-15)-(4-6)], Δ[(14-15)-(7-10)]. The last parameter per ejaculate analysed was the mean over time. This means the mean result from the 5 measurement moments per ejaculate. For the complete overview of the analyses see Supplementary table 1.

Modelling flow cytometry results and boar and semen related parameters

The significant parameters resulting from the regression models were included in a general linear model (SAS 9.2 Inst. Inc., Cary, NC), taking information from the boar, the AI centre and the ejaculate into account. Boar and semen related parameters (the direct boar effect) were put in a second model, which is described previously (Chapters 2 and 4) and is added in the Supplementary information 2.

Results

Flow cytometric analysis of sperm integrity

A total number of 582 ejaculates from the 20 boars were analysed on the day of collection (day 0) and at four consecutive moments: day [1], day [4-6], day [7-10] and day [14-15]. From each boar on average, 1.45 ejaculates were analysed per week (in total 29 ± 6 ejaculates per boar). All the ejaculates analysed, were used for insemination; the boar semen used for flow cytometry analysis was fully conform to approved quality. The average number of sperm cells per ejaculate was 84×10^9 ± 12×10^9 and the average motility and progressive motility at day 0 were 88.0 ± 5.0 % and 78.2 ± 7.1 % respectively. The boar and semen quality results of the fresh ejaculates are reported in Table 1A. From the total of 582 ejaculates, 565 ejaculates could be linked to the fertility records of inseminated sows of which the results are listed in Table 1B.

Table 1. Descriptive results of boar and semen related parameters and farm and sow related parameters. A. Boar and semen related parameters, B. Farm and sow related parameters.

Parameter	Number of records or mean ± SD
A.	
Number of ejaculates	582 (565 with known fertility)
Number of boars	20
Number of genetic boar lines	3
Age of boars, mos	20.6 ± 9.7
Number of days between ejaculation	4.2 ± 2.1
Number of sperm cells in ejaculate	$84 \times 10^9 \pm 12 \times 10^9$
Motility, %	88.0 ± 5.0
Progressive motility, %	78.2 ± 7.1
DNA fragmentation index (day 0), %	3.15 ± 1.75
Membrane integrity (day 0), %	87.67 ± 8.66
Acrosome intactness (day 0), %	90.34 ± 5.68
Acrosome responsiveness (day 0), %	75.34 ± 6.21
Depolarized mitochondria (day 0), %	23.61 ± 4.68
Number of motile cells in dose (80 ml)	$1.67 \times 10^9 \pm 0.43 \times 10^9$
Number of doses produced per ejaculate	33 ± 11
B.	
Number of farms	51
Number of inseminated sows	2,371
Weaning to oestrus interval, d	5.6 ± 4.6
Number of inseminations per cycle (min-max)	1.6 (1-3)
Parity	3.6 ± 2.2
Gestation length, d	115.5 ± 1.5
Farrowing rate, %	87.9 ± 32.7
Number of total piglets born	14.5 ± 3.3
Number of live piglets born	13.2 ± 3.0
Number of still born piglets	1.0 ± 1.4
Number of mummified piglets born	0.3 ± 0.7
Number of piglets weaned per litter	11.5 ± 1.9

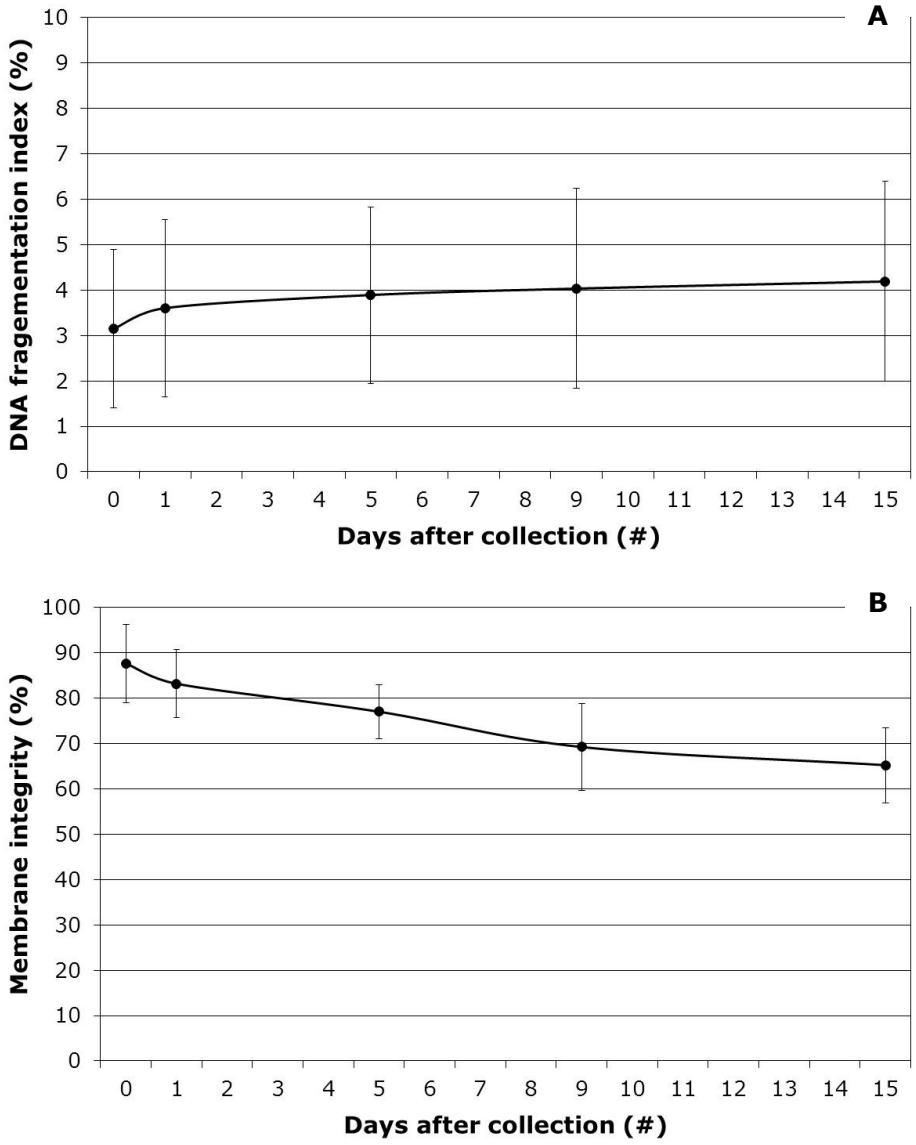


Figure 1. Results for membrane integrity, acrosome integrity, DNA defragmentation and depolarised mitochondria. Figures represent DNA fragmentation index (A) and membrane integrity (B). Results represent mean ± SD.

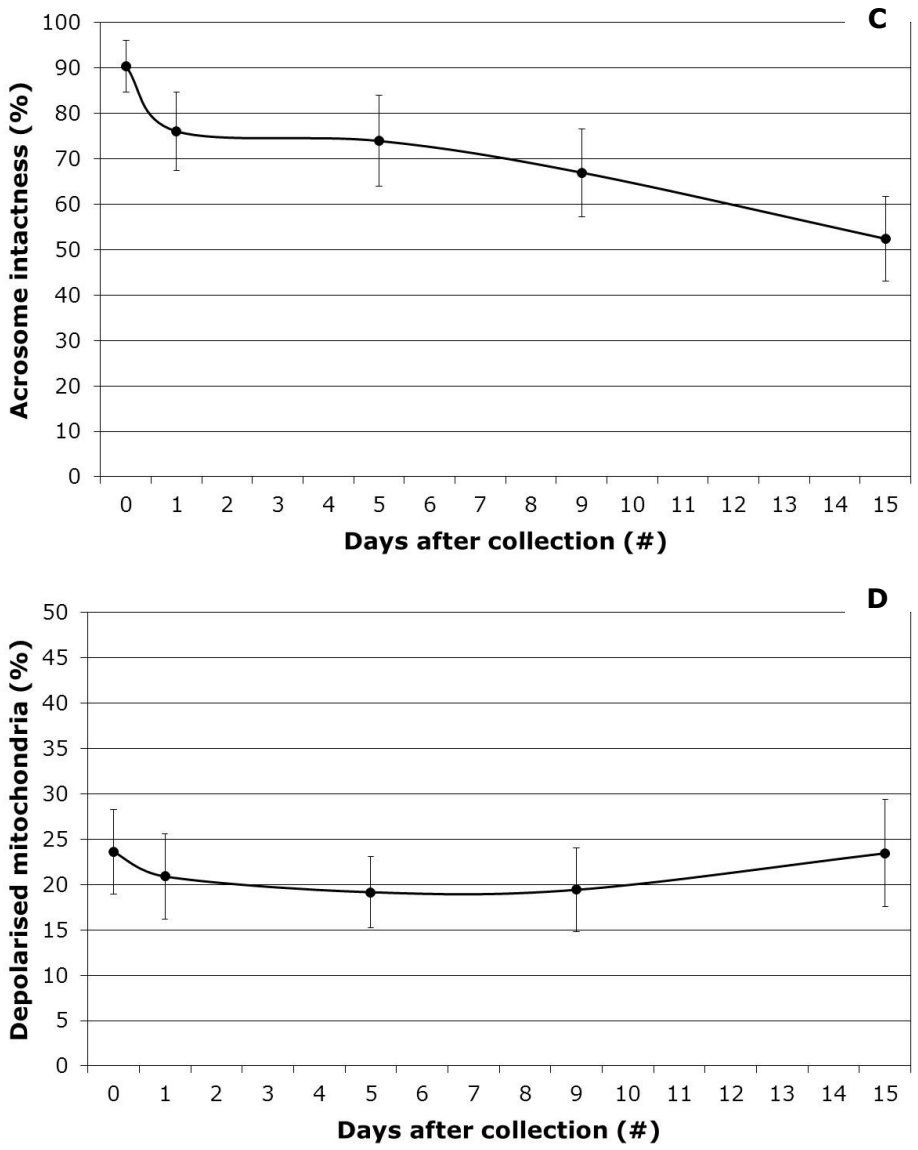


Figure 1. Results for membrane integrity, acrosome integrity, DNA defragmentation and depolarised mitochondria. Figures represent acrosome intactness (C) and depolarised mitochondria (D). Results represent mean ± SD.

The average DNA fragmentation index (DFI) (Figure 1A) was 3.15 ± 1.75% at day 0. DFI increased during the period of 15 days after production up to an average of 4.19 ± 2.20%. Between day 0 and day 1 this increase was highest (+0.46%). The membrane integrity (Figure 1B) and acrosome intactness (Figure 1C) before calcium ionophore induction were both at high level at day 0 (87.67 ± 8.66% and 90.34 ± 5.68% respectively). In the presence of the ionophore, the percentage of spermatozoa with a reacted acrosome was 75.34 ± 6.21% after a period of 30 min (resulting in a relative rate of potential responding cells of 83%). The membrane-intact cells and the acrosome-intact cells before calcium ionophore induction decreased in percentage over the time period of 15 days. On average the membrane integrity was 65.23 ± 8.30% and the acrosome intactness was 52.40 ± 9.38% at day [14-15]. However, the decrease in membrane integrity and acrosome intactness varied between boars. The decrease was highest between day 0 and day 1 (-4.48% and -14.28% for membrane integrity and acrosome intactness respectively), compared to the other measurement moments. The mitochondrial membrane potential (Figure 1D) showed a different pattern compared to the other flow cytometry tests. The percentage of spermatozoa with depolarised (inactive) mitochondria at day 0 was on average 23.61 ± 4.68%. After a small decrease to day 1 (-2.71%) the percentage increased to a percentage of 23.45 ± 5.90% at day [14-15] after collection, which was a similar level compared to day 0.

Regression model

Linear regression resulted in significant flow cytometry variables showing a relation with farrowing rate (FR) or total number piglets born (TNB) (Table 2). Flow cytometry variables that significantly influenced FR were the delta acrosome intactness between day [4-6] and day [7-10] (P=0.0210), the DFI at day [7-10] (P=0.0428) and the delta DFI between day [1] and day [4-6] (P=0.0072). Flow cytometry variables that significantly influenced TNB were the delta membrane integrity between day [0] and day [1] (P=0.0065), the delta DFI between day [0] and day [7-10] (P=0.0006), the DFI at day [14-15] (P=0.0020), and the mean DFI (P=0.0132).

Table 2. Estimates for flow cytometry semen quality variables in relation to farrowing rate (FR) and total number of piglets born (TNB). Effects are expressed as change in farrowing rate (%) or total number piglets born by +1 value of the flow cytometry variable.

Parameter	P-value in regression analysis	P-value in GLM analysis	Effect of 1 value of parameter
Farrowing rate			
AI Δ[(7-10)-(4-6)]	0.0210	0.0899	0.16
DFI [7-10]	0.0428	**0.0400**	**-0.14**
DFI Δ [(4-6)-(1)]	0.0072	0.2531	-1.23
Total number piglets born			
MI Δ[(1)-(0)]	0.0065	0.4899	-0.0199
DFI Δ [(7-10)-(0)]	0.0006	0.3493	-0.083
DFI [14-15]	0.0020	**0.0310**	**-0.124**
DFI_mean	0.0132	0.9238	-0.099

MI = membrane integrity, AI = acrosome intactness, Mito = depolarised mitochondria, DFI = DNA fragmentation, Δ = the delta of the variable between 2 measurement moments. P≤0.05 means that the variable had a significant effect on FR or TNB and are expressed in bold.

Modelling flow cytometry results and boar and semen related parameters

When modelling the flow cytometry variables with other boar and semen related parameters in model 2, it showed that genetic line of the boar had a significant effect on FR and TNB (P=0.0235 and P=0.0222 respectively). This represents that there were differences between the three genetic lines used in this trial. Within the genetic line there was no significant difference between boars in the relation with FR and TNB (P>0.05). Extrinsic and intrinsic factors like AI centre, number of cells in an insemination dose, age of boar and days between ejaculations did not have a significant effect on FR and TNB (P>0.05) and were therefore not considered in the final model 2. The number of cells in an ejaculate did not have a significant effect on FR (P>0.05) but did show a significant relation with TNB (P<0.0001). A high number of cells in an ejaculate correlated to a higher TNB. The *P*-values of these boar and semen related parameters are shown in Table 3.

Table 3. *P*-values per boar and semen related parameters for variation in farrowing rate (FR) and total number of piglets born (TNB).

Parameter	FR, %	TNB
Individual boar (within line)	0.1251	0.1843
Genetic line of the boar	**0.0235**	**0.0222**
Age of the boar at ejaculation	0.5623	0.7742
Number of days between ejaculations	0.7211	0.8645
Number of cells in ejaculate	0.5614	**<0.0001**
Number of cells in insemination dose	0.6231	0.4586
AI center	0.4523	0.7234

P ≤ 0.05 means that the variable had a significant effect on FR or TNB

The flow cytometry variables that significantly showed a relation in the regression model with FR and TNB were analysed in model 2 with the significant boar and semen related parameters. The results are shown in Table 2. The DFI at day [7-10] showed a significant relation with FR (P=0.0400). When increasing by 1% DFI, the FR decreased with 0.14%. The DFI at day [14-15] showed a significant relation with TNB (P=0.0310). When increasing by 1% DFI the TNB decreased with 0.124 piglets.

With significant relations between DFI at day [7-10] and FR, and between DFI at day [14-15] and TNB, there was a significant effect of genetic line of the boar, but within this line, there was no effect of individual boar (P>0.05). The results from the boars are shown in Table 4 and Figure 2, representing the DFI at day [7-10] and FR (A) and representing the DFI at day [14-15] and TNB (B).

Table 4. Impact of genetic line. Results per genetic line (A, B, C) for DNA defragmentation index at day [7-10] and at day [14-15] and farrowing rate (FR) and total number of piglets born (TNB).

Genetic line	DFI [7-10], %	DFI [14-15], %	FR, %	TNB
A	3.40 ± 1.89	3.50 ± 2.09	89.7 ± 32.6	15.2 ± 3.2
B	3.70 ± 1.91	3.70 ± 1.61	88.8 ± 30.2	14.8 ± 2.9
C	5.19 ± 1.61	5.31 ± 1.59	87.3 ± 31.4	14.4 ± 3.4

Results represent mean ± SD.

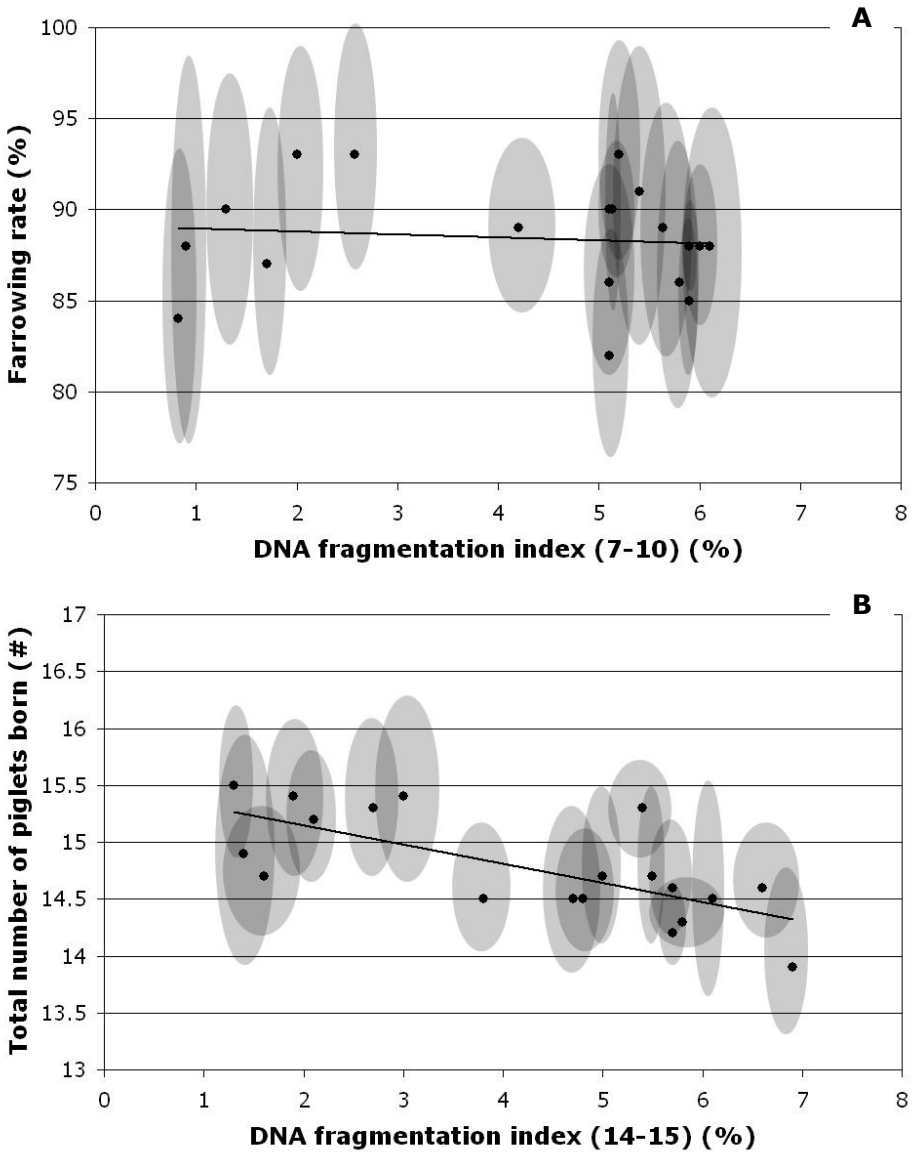


Figure 2. Average results per boar for DNA fragmentation. A. Relation between DNA defragmentation index at day [7-10] and farrowing rate; B. Relation between DNA defragmentation index at day [14-15] and total number of piglets born. Results represent mean (dot) ± SD (circle).

Discussion

This is the first study in which a combination of sperm integrity assessments with flow cytometry is related to the fertilising capacity of the corresponding semen in pig artificial insemination practice. We showed a significant relation between the DNA fragmentation index (DFI) measured between day 7 and day 10 after semen production and farrowing rate (FR) (P=0.04) and between the DFI measured at day 14 or day 15 after production and total number piglets born (TNB) (P=0.03). This indicates that the assessment of DNA integrity gives an additive predictive value for sperm quality with regard to field fertility. In contrast, none of the individual membrane integrity parameters did show such a relation with field fertility.

Damage in the DNA is considered to affect the first cleavage divisions and therefore reduce embryo development (Larson-Cook *et al.*, 2003; Fatehi *et al.*, 2006). It has also been shown that sperm cells with compromised chromatin organisation show a reduced capability to bind to oviductal epithelium (Ardón *et al.*, 2008) while spermatozoa that bind to oviduct cells have superior DNA integrity. This suggests that females can select DNA intact sperm to fertilise the oocyte (Ellington *et al.*, 1999). This selection makes sense as it has been demonstrated that oocytes that are fertilised with sperm that carried damaged DNA do not develop (Larson-Cook *et al.*, 2003; Fatehi *et al.*, 2006) and thus affect TNB and possibly even FR. Indeed this effect also was prominent in our results and thus can be used to predict field fertility results. Despite the fact that the DNA deterioration is only significantly affecting fertility at more than 7 days after production of the ejaculate, it still can be used as a predictor for boar fertility. Of course we realise that the semen of that particular ejaculate is already inseminated, but the semen quality results are reproducible. Therefore, determining the DNA fragmentation can be a valuable test for the routine monitoring of boars fertility level in AI practice.

In general, a relatively low level of DFI has been reported in both fresh (Evenson *et al.*, 1994; Rybar *et al.*, 2004) and liquid stored (Waberski *et al.*, 2002; Boe-Hansen *et al.*, 2005; De Ambrogi *et al.*, 2006; Waberski *et al.*, 2011) boar semen. DNA fragmentation of the boars used in the current study was below 10% which is representing high fertility potential. This is demonstrated in other studies (Evenson *et al.*, 1999; Waberski *et al.*, 2011). Literature proposes cut-off levels for boar sperm cells between 2 % and 18 % for DFI (Rybar *et al.*, 2004; Boe-Hansen *et al.*, 2008; Didion *et al.*, 2009; Waberski *et al.*, 2011). From the results from our study we do not recommend a cut-off level, since the results are dependent on multiple factors and therefore an AI

company has to determine this cut-off value for their own boar population and production conditions.

A number of studies have indicated the potential of assessing the DFI in the assessment of semen from bulls (Ballachey *et al.,* 1987; 1988; Evenson, 1999), stallions (Evenson *et al.*, 2000) and boars (Evenson *et al.*, 1994; Didion *et al.*, 2009; Rybar *et al.*, 2004; Boe-Hansen *et al.*, 2005; 2008; Waberski *et al.*, 2011). The boars used in the current study were of high fertile quality. All ejaculates used were of high quality, according to AI standards. This could be one of the reasons why there was not a large effect of DNA fragmentation in relation to fertility results. Human studies showed that the effect of DFI level is dependent on the standard sperm parameters (Erenpreiss *et al.*, 2006; Nicopoullos *et al.*, 2008; Giwerman *et al.*, 2010). If motility and morphology were normal, there was no effect on fertility for DFI < 20%. In contrast, if one of the standard parameters was abnormal, fertility was reduced at DFI above 10% (Giwercman *et al.*, 2010). In our study we did not find a correlation between the DFI results and the standard semen parameters (results are not published), indicating that assessing DNA fragmentation has an additive value on the standard semen assessment in relation to the fertilising capacity of the sperm. The fact that only the results of the repeated measurements in time were related to field fertility can be a valuable indication for human industry as well.

Fertility can be categorized as compensable or uncompensable (Saacke *et al.*, 2000, DeJarnette, 2005). Defective chromatin structure (increased DFI) is an uncompensable trait that affects the function of sperm cells during later stages of fertilization and in embryonic development (Saacke *et al*., 2000). The failing of membrane integrity parameters to relate to fertility is therefore categorized as a compensable trait. Defects in compensable traits can be overcome by a large number of sperm cells per insemination. Apparently, the number of motile cells per insemination dose (1.67×10^9 per 80 mL) is still compensating for deficiencies in membrane, acrosome or mitochondria.

The membrane integrity exerts a vital role on sperm survival inside the female reproductive tract (Õura and Toshimori, 1990; Flesh and Gadella, 2000; Andrade *et al.*, 2007). In our study we did not find a relation between membrane integrity assays and field fertility, which is in contrast to other studies (Pintado *et al.*, 2000; Frazer *et al.*, 2002; Andrade *et al.*, 2007). We believe that this is due to the fact that the number of intact sperm cells in all insemination experiments was still more than sufficient to obtain maximal FR and TNB results. This implies that the remaining number of normal cells in an insemination dose is not critically low to discriminate between sub fertile and fertile quantities of

membrane intact sperm. It is expected that the membrane damaged sperm do not fertilise.

Likewise, sperm mitochondria play a key role in the generation of energy of sperm movement. Decreased motility during storage may result from functional abnormalities of sperm mitochondria (Gączarzewicz *et al.*, 2003). In the current study, the aerobic functionality of mitochondria did not show a significant relation with fertility. Probably the population of remaining cells with sufficient energy generation and thus with normal motile sperm is sufficient to achieve maximum FR and TNB.

In contrast, sperm with intact membranes and functional mitochondria but carrying DNA damage, do fertilise the oocyte (Larson-Cook *et al.*, 2003; Fatehi *et al.*, 2006). Therefore, although in the Netherlands we are using critically low commercial AI doses (i.e. at a level where there is still no significant numeric sperm effect on fertility) this insemination strategy does not allow to discriminate how large the proportion of membrane intact and motile sperm is required for optimal fertility results (the quantity of functional sperm). The stability and integrity of the DNA in such sperm samples is of predictive value (the quality of functional sperm).

Assessing sperm DNA fragmentation is currently not used in the commercial practice of Varkens KI Nederland, but can have added value to the knowledge on the next ejaculates produced by that particular boar. In the current study, all boars had a high fertilizing potential. But assessing DNA fragmentation could especially have an added value if the boar is sub-fertile, but not identified by the daily spermiogram. The relation between DNA fragmentation and fertility is established, but the AI company has to validate these results before implementation is possible. In the current study, semen is used within 3 days after production. Further research is recommended on using semen after a longer storage time and to study the effect on fertility parameters. The practicality of the DNA fragmentation assay in a routine AI setting is questionable. In a high producing laboratory (e.g. > 50 ejaculates per hour) it is not possible to assess the DFI in line. A possibility is to assess the DFI during quarantine period before routine production at an AI station. If the results are repeatable within a boar, you could decide to repeat this assessment every month or 2 months, depending on the differences. Therefore, current DNA fragmentation assay is not practical for routine assessment on fresh semen in the daily production routine, but can be used as a screening test.

Similar to previous studies (Chapters 2 and 4) the genetic line of the boar significantly affected the fertility outcome in this study, which is similar to the study of Boe-Hansen *et al.* (2005). The boars within the genetic line, used in our study, did not have a significant affect, which is in contrast to Sutkeviciene *et al.* (2009) but similar to Boe-Hansen *et al.* (2005). This study concerns only a minimal number of boars (20) and we do know from previous studies that in the overall population, the individual boar does explain variation in the fertility outcome (Chapters 2 and 4). For implementation such a flow cytometry test in AI practice, the company has to know the variation within the whole boar population, taking the differences between genetic lines into account. There was a significant positive effect of the concentration of sperm cells in an ejaculate on farrowing rate. The number of sperm in an insemination dose is due to a further dilution of such ejaculates normalised and therefore cannot attributing to this positive effect. However, seminal plasma components are further diluted from high sperm concentrations in ejaculates compared to lower sperm concentrations. Seminal plasma has a stabilising but also inhibiting role on sperm capacitation and thus could the variation in seminal plasma (and perhaps variation of composition thereof causes the noted differences (for review see Leahy and Gadella 2011). In the current study, there was no effect of days between ejaculation and age of boars on fertility, but this can be due to the selection of boars in advance. In human, age of men and long periods of abstinence appear to have influence on flow cytometry data (Spanò *et al.*, 1998). So especially, if one uses flow cytometry tests in the quarantine period of the boars career, one has to take into account that there needs to be progress in ejaculation before qualifying the flow cytometry results.

Conclusion

In the current study the DNA fragmentation, in contrast to membrane integrity, acrosome intactness and responsiveness and mitochondrial potential, did show a relation with fertility. Probably compromised membrane and mitochondrial integrity of sperm prevented the sperm population to fertilise the oocyte. The insemination doses as well as the sperm quality were so optimal that variation in the percentage of functionally intact sperm was not correlated with the fertility outcome. Damaged or instable DNA on the other hand may exist in the deteriorated but also in the functional intact sperm population and thus can fertilise the oocyte and exert negative effects on embryonic or foetal development and health of the offspring. We may stress here that the stability of sperm chromatin showed a relation with fertility only days

after ejaculation. The initial defragmentation did not show this relation. This may be due to the selection of high fertile boars for the Dutch AI centres. Breeding management wants to create additional tools for predicting boar fertility under those settings. For this purpose the sperm longevity experiments and the impact of DNA deterioration in that period are an important addition for the assessment of semen quality. When implementing a DNA fragmentation test in the routine pig AI practice, the differences between genetic lines and individual boars always have to be taken into account, as well as other boar and semen related parameters, the costs of the test and the fact that the test is time consuming. Therefore it is recommended not to assess DNA defragmentation on routine scale, but at a set number of times over the year or as an entrance criterion for boars to an AI centre. Furthermore we anticipate that the value of assessing DNA fragmentation especially when using sperm longevity experiments is a possible addition to the assessment of semen quality and can be relevant for predicting fertility results not only for pigs but also for other mammalian species.

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Supplementary table 1. Flow cytometry parameters in statistical modeling. Flow cytometry parameters which were tested for significant effect on FR or TNB.

Statistical approach	Parameters
Each individual parameters MI, AI, AR, Mito, DFI	0 – 15
Grouped parameters	[0], [1], [4-6], [7-10], [14-15]
Delta (Δ) parameters	Δ[1-0], Δ[(4-6)-0], Δ[(4-6)-1], Δ[(7-10)-0], Δ[(7-10)-1], Δ[(7-10)-(4-6)], Δ[(14-15)-0], Δ[(14-15)-1], Δ[(14-15)-(4-6)], Δ[(14-15)-(7-10)]
Mean over time	Mean ([0], [1], [4-6], [7-10], [14-15])

MI = membrane integrity, AI = acrosome intactness, AR = acrosome responsiveness, Mito = depolarised mitochondria, DFI = DNA fragmentation, Δ = the delta of the variable between 2 measurement moments.

Supplementary information 1. Calculating the least square means per ejaculate for the two fertility traits farrowing rate (FR) and total number piglets born (TNB). The following model was used to describe the farm and sow related parameters.

$$Y = \mu + \text{parity} + \text{line}\varphi + \text{farm} + 1^{st}/\text{remating} + \text{purebred/crossbred litter} + \#\ \text{inseminations} + \text{int-wean-1} + \text{gest\_length} + \text{month} + \text{weekday} + \text{age semen} + \text{error} \quad [1]$$

Where Y was the value of FR or TNB; μ was the mean value of FR and TNB; parity was the effect of the actual parity of the sow; line♀ was the effect of the line of the sow, farm was the effect the farm; 1^{st}/remating was the effect of a first or a remating of the sow; purebred/crossbred litter was the effect of having a purebred of crossbred litter; #_inseminations was the effect of the number of inseminations per heat; int-wean-1 was the effect of the interval between weaning and the first insemination; gest_length was the effect of the gestation length; month was the effect of the six months of trial; weekday was the effect of the day of the week of insemination; age semen was the effect of the age of the semen (days after production) at insemination; and error was the random residual effect that could not be explained by the variables in the model.

Supplementary information 2. Corrected observation (Y*****) for FR and TNB were calculated for each insemination with the direct boar effects on flow cytometry results in the model. The following model was used to describe the boar and semen related parameters.

$$Y^* = \mu^* + line♂ + boar(line♂) + age + days\text{-}ejac + AI\ centre + vol\text{-}conc + dose\text{-}conc + FLOW + error^* \quad [2]$$

Where Y* was the value of FR or TNB ; μ* was the mean value of FR and TNB; line♂ was the effect of the line of the boar; boar(line♂) was the effect of the individual boar within the boar line; age was the effect of the age of the boar at ejaculation; days-ejac was the effect of the number of days between current and previous ejaculation; AI centre was the effect of the AI centre; vol-conc was the effect of the number of cells in an ejaculate, dose-conc was the effect of the number of cells in the insemination dose used; FLOW was the effect of measured flow cytometry parameters; and error* was the random residual effect that could not be explained by the variables in the model.

Chapter 6

In vitro fertilisation competence of semen from AI boars has no predictive value for its fertilising capacity under commercial insemination conditions

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Broekhuijse MLWJ, Soštarić E, Schoevers EJ, Feitsma H, Gadella BM. 2012; submitted.

Abstract
The aim of this study was to validate whether *in vitro* fertilisation (IVF) results can be used for predicting the fertilising potential of boars using commercial artificial insemination (AI) conditions. Twelve boars from one genetic line (Pietrain) were selected and ranked based on their fertilising potential using track records. The relatively higher and lower half of the boars with respect to fertilising potential were compared in a standardised IVF protocol, which was repeated three times per boar. Cumulus oocyte complexes were cultured and subjected to *in vitro* fertilisation. One portion of these oocytes was fixed and stained to determine normally fertilised oocytes; another portion was incubated further to determine embryo development. After IVF, fertilisation rates for the high and low ranked fertile boars were 52 ± 8 and 51 ± 10% respectively (P≥0.05). The degree of maturation in the unfertilised oocytes was also not significantly different (P≥0.05). Early embryonic development 16 ± 9% and 17 ± 7% (blastocyst rates) was not significantly different between high and low ranked fertile boars (P ≥ 0.05). Non significant differences between individual boars were observed. Additionally, the IVF and embryo development results did not correlate with sperm motility parameters, or with *in vivo* farrowing rate and *in vivo* total number piglets born. Thus it appears that IVF results are unsuitable for explaining the differences between fertility levels of boars in a commercial AI practice and therefore lack potential as a discriminating test.

Keywords
Boar semen, *in vitro* fertilisation (IVF), artificial insemination (AI), sperm motility, farrowing rate, litter size

Introduction

Development and validation of new tests for predicting the fertilising capacity of semen is of great value for selecting boars for artificial insemination (AI) centres. The most valid method for boar semen quality is to analyse results *in vivo*. However, an easier and quicker test to validate the fertilising capacity of boar semen is desirable. *In vitro* fertilisation (IVF) has been implemented to be potentially informative for selecting boar semen and determining fertilising ability (Bavister, 1990).

Technologies like cryopreservation and embryo transfer, but also IVF have become part of commercially applied breeding techniques (Faber *et al.*, 2003). Several attempts have been made to relate semen quality characteristics to fertility *in vitro* with either none or low significance (Flowers and Turner, 1997; Marchal *et al.*, 2002; Rodriguez-Martinez, 2003). Motility parameters are indicated to be important for predicting human IVF results (Hirano *et al.*, 2001) and this predictive value can become useful for couples before they make the decision to proceed with IVF. However, there are not much studies relating semen motility to the IVF outcome (Xu *et al.*, 1998; Oehninger *et al.*, 2000; Popwell and Flowers, 2004; Gadea, 2005; Ruiz-Sánchez *et al.*, 2006). By the use of motility assessments (Chapters 3 and 4) and an IVF protocol (Schoevers *et al.*, 2003), an AI station could predict the fertilising potential of a boar with possible benefits on its future career in the AI industry. Currently the IVF test results lack evidence for effectiveness in predicting the fertility of boar ejaculates that meet laboratory criteria for dilution and use for AI. Therefore, in the current study we have studied whether motility and IVF results can be used to predict male fertility in pigs.

In this study, the 6 highest and 6 lowest fertile boars within a genetic line from a commercial AI centre were selected, based on their fertility results in the field. Differences in IVF results were analysed for these boars. The semen quality of boar ejaculates and the field and IVF fertility results were assessed. The aim of this study was to validate whether the IVF test can be used as a predictor for field fertility potential of AI boars.

Material and Methods

Trial setup

Boars of different fertilising potential were compared for *in vitro* fertilisation (IVF) results. Semen of six lower and six higher end fertilising capacity (see section boar and semen selection) were compared with each other. Boars were compared in IVF trials in pairs,

resulting in six trials. This set of experiments was repeated three times, resulting in eighteen IVF experiments comparing three ejaculates from each of the twelve boars. IVF trails were performed at the research laboratory of the Faculty of Veterinary Medicine (Utrecht University, Utrecht, the Netherlands).

Culture media
All chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA) unless indicated otherwise. The basic medium for *in vitro* maturation (IVM) is the oocyte maturation medium (OMM) which was NCSU-23 stock (Petter and Well, 1993) supplemented with 0.1 mg/ml cysteine, 1mM L-glutamine, 0.4% ß-mercaptoethanol (BME) and 25.05 mM $NaHCO_3$. The *in vitro* fertilisation (IVF) medium was modified Tris-buffered medium (Abeydeera *et al.*, 1997), containing 1 mM caffeine and 0.1% (w/v) BSA (Faction V, fatty acid free). The *in vitro* culture (IVC) medium NCSU-23 was combined with 0.4% BSA.

Selection and culture of cumulus oocyte complexes
Ovaries were collected from adult sows at a local slaughterhouse and were stored in a thermo flask and transported to the laboratory within 2 h. Isolation and selection of cumulus oocyte complexes (COCs) was as described previously (Schoevers *et al.*, 2003). Briefly, the ovaries were rinsed under running tap water after dissection of the surrounding tissue. They were kept at 30°C in physiological saline solution supplemented with 0.1% (v/v) penicillin and streptomycin (Gibco BRL, Paisley, UK). By means of a suction pump under pressure, COCs were aspirated from 3 to 6 mm follicles, using a 19-gauge needle attached to a 50 ml polystyrene conical tube (BD Falcon™, BD Biosciences, Franklin Lakes, NJ, USA). The follicular contents were allowed to sediment and washed three times with a Tyrode's lactate-HEPES medium (Bavister *et al.*, 1983) supplemented with 0.1% (w/v) PVP (TL-HEPES-PVP).

In vitro maturation
Oocytes with a compact cumulus cell mass were selected washed three times in IMV-I medium, containing IVM medium supplemented with 500 µL PFF, 125 µL ECG and 125 µL HCG. The COCs were transferred to four-well culture dishes (Nunc A/S, Roskilde, Denmark) and per well 40-50 COCs were cultured under warm mineral oil for 20-22 h in 500 µL IVM-I medium. After culture, the COCs were washed three times in IVM-II. This was the IVM medium supplemented with 1 ml PFF. The COCs were further cultured for 20-22 h under oil in 500 µl IVM-II medium. All media were equilibrated at 38.5°C and 5% CO_2 in an incubator for at

least 2 h before use. Also COCs were cultured in a humidified incubator at 38.5°C and 5% CO_2.

Boar and semen selection

Twelve boars were selected for IVF. This selection was based on the field fertility results of previous inseminations (at least > 1000 inseminations) recorded in the breeding database Pigbase of the Institute for Pig Genetics B.V. (Beuningen, the Netherlands). From one genetic line (Pietrain) six boars with highest fertility were compared to six boars with lowest fertility. This ranking was based on the total number of piglets born per insemination, after correction for farm and sow related parameters, previously described (Chapters 2 and 4). Doses from these boars, used for artificial insemination (AI), were obtained from Varkens KI Nederland (Deventer, the Netherlands) which is processing commercial AI doses. From each ejaculate, one insemination doses was transported within 4 h after production to the research laboratory in a temperature controlled box at 17°C. The other insemination doses were transported to farms for AI use.

Of each insemination dose eight tubes (1.5 ml tube, Eppendorf AG, Hamburg, Germany) were prepared containing 1 ml of extended semen and a 50 ml conical ml tube was filled with extended semen. Tubes were stored in an acclimatised box (17°C ± 2°C). The small tubes, containing 1 ml extended semen, were reactivated at 39°C for 30 min. Semen motility and concentration were assessed at day 0, 1, 2, 5, 7, 9, 12 and 14 after production, using the computer assisted semen analysis (CASA) system UltiMate™ (Hamilton Thorne Inc., Beverly, MA, USA) with standardised Leja 4-chamber counting slides (Leja Products B.V., Nieuw-Vennep, the Netherlands). The semen used for IVF was used at day 1.

In vitro fertilisation

Quality of the oocytes was controlled by induction of parthenogenesis, using the protocol previously described (Lee *et al.*, 2004) (results not shown). *In vitro* fertilisation is previously described by (Schoevers *et al.*, 2003). Briefly, prior to scheduled fertilisation in a 10 ml conical tube (BD Falcon™, BD Biosciences, Franklin Lakes, NJ, USA) 4 ml of extended semen was added to 4 ml of IVF medium. The sample was centrifuged at 700 x g for 5 min at 20°C and the supernatant was removed. The pellet was resuspended in 2 ml IVF medium and centrifuged under the same conditions. After removing of the supernatant, the pellet was resuspended again in the IVF medium and was reactivated to 39°C before concentration and motility were determined, using the UltiMate.

The concentration was adjusted to 10^6 spermatozoa/ml before added to the oocytes.

After culture in the IVM-II medium, cumulus cells were removed from oocytes by repeated pipetting. The oocytes were washed three times and placed in a four well plate containing 500 µL IVF medium per well, previously covered with warm mineral oil and equilibrated (38.5°C with 5% CO_2 in air). For fertilisation 40-50 µL of 1×10^6 spermatozoa/ml was added to achieve a ratio of 1000 spermatozoa per oocyte. Oocytes with spermatozoa were incubated for 24 h at 38.5°C with 5% CO_2 in air.

In vitro culture

After fertilisation and 20-22 h of incubation, presumptive zygotes were washed three times and groups of 40-50 presumptive zygotes were transferred to 500 µl synthetic oviductal fluid (SOF) medium supplemented with essential and nonessential amino acids and 0.1% BSA (w/v) (Tol *et al.*, 2008) at 39°C in a humidified atmosphere of 5% CO2 and 7% O2 in air for 5 days.

Assessing results of IVF

After fertilisation and 24 h after incubation, adherent sperm cells were removed from the oocytes by repeated pipetting. Similar procedure was followed after five days of incubation in the IVC medium. The status of the oocytes and zygotes was determined by DAPI staining. The cells were fixed with 4% (w/v) formaldehyde in phosphate buffered saline (PBS), washed with PBS, stained for 5 min with 2.5% (w/v) 4,6-diamino-2-phenyl-indole (DAPI) (Molecular Probes, Eugene, OR, USA), and mounted on slides in a drop of antifade solution (Vectashield, Vectorlab, Burlingame, CA, USA). The state of the stained oocytes was assessed by fluorescence microscopy.

In vivo results

Fertility records from the insemination doses also used for IVF were retrieved according to the method described previously (Chapters 2 and 4). Briefly, a breeding database (Pigbase) was available at the Institute for Pig Genetics B.V. (Beuningen, the Netherlands) containing fertility records from purebred and crossbred sows. From this database a dataset was extracted, merging IVF fertility results with *in vivo* fertility results. Fertility results presented are farrowing rate (FR) and total number piglets born (TNB). These results were corrected for farm and sow related parameters, which was described previously (Chapters 2 and 4).

Results

Descriptive results

Twelve boars were ranked based on their fertilising potential as being high or low ranked fertile boars. This ranking was based on the corrected total number of piglets born (TNB), which was corrected for farm and sow related parameter (the so called direct boar effect on TNB) of >1,000 inseminations per boar (Table 1). The TNB represents the corrected difference from 0 (average) for the whole boar population of Varkens KI Nederland. The maximal difference between high and low ranked fertile boars was 0.8 piglets; the minimal difference was 0.4 piglets. Furthermore, Table 1 shows the number of COCs, on average assessed per boar, which was 48 ± 3 and 50 ± 4 for the high and low ranked fertile boars respectively.

Table 1. Selection of high and low ranked fertile boars based on corrected values for total number of piglets born (TNB). The number of inseminations represents the number of inseminations on which the TNB result is based. The number of oocytes assessed represents the mean ± SD and is based on three replicates.

Fertility	Boar	Number of insemination records	Corrected TNB	Number of oocytes
High ranked	1	1897	0.282	47 ± 2
	3	2580	0.281	48 ± 3
	5	3877	0.281	43 ± 1
	7	2647	0.272	51 ± 6
	9	2530	0.259	54 ± 7
	11	1897	0.282	47 ± 3
	Mean	2571	0.276	48 ± 3
Low ranked	2	1660	-0.552	51 ± 5
	4	1769	-0.412	54 ± 3
	6	1893	-0.246	47 ± 5
	8	2324	-0.220	44 ± 3
	10	2047	-0.181	47 ± 6
	12	1168	-0.162	57 ± 4
	Mean	1810	-0.296	50 ± 4

In vitro fertilisation results

Semen collected from high versus low ranked boars gave a similar fertilisation rate (52 ± 8% versus 51 ± 10%, respectively) which was not a significant difference (P>0.05). During the IVF incubations the remaining non-fertilised oocytes showed no aberrant signs of maturation or degeneration for low versus high ranked boar semen (Table 2; all mean data are not significantly different between both groups P>0.05). The fertilised oocytes in both groups showed similar embryo developmental competence as the percentages of oocytes that developed into blastula stage at day 6 of culture were 16 ± 9% and 17 ± 7% of the total for high and low ranked fertile boars respectively and not significantly different (P>0.05; Table 3). For fertilisation and embryo development percentages we noted variations between boars but these were not dependent on their field fertility ranking (Table 2 and Table 3).

Table 2. Results of maturation rates of porcine oocytes 24 h after *in vitro* fertilisation. Results represent mean ± SD of the three experiments.

Fertility	Boar	Stage of non-fertilised oocytes, % (Mean ± SD)				Fertilisation, %
		GV	MI	MII	Degenerated	
High ranked	1	3 ± 2	19 ± 3	68 ± 5	10 ± 3	57 ± 4
	3	2 ± 3	13 ± 2	73 ± 6	12 ± 3	44 ± 8
	5	5 ± 3	4 ± 2	78 ± 4	13 ± 4	49 ± 5
	7	4 ± 3	17 ± 1	71 ± 5	8 ± 2	58 ± 15
	9	2 ± 3	7 ± 2	80 ± 6	11 ± 4	51 ± 6
	11	5 ± 2	9 ± 2	72 ± 5	14 ± 2	56 ± 9
	Mean	4 ± 3 [a]	12 ± 2 [a]	74 ± 6 [a]	11 ± 3 [a]	52 ± 8 [a]
Low ranked	2	4 ± 2	13 ± 4	71 ± 5	12 ± 3	42 ± 6
	4	6 ± 3	7 ± 3	72 ± 6	15 ± 2	51 ± 5
	6	3 ± 2	20 ± 2	68 ± 7	9 ± 2	59 ± 10
	8	2 ± 2	14 ± 2	72 ± 4	12 ± 3	59 ± 4
	10	5 ± 2	2 ± 1	74 ± 4	19 ± 3	57 ± 15
	12	1 ± 3	9 ± 3	81 ± 5	9 ± 2	39 ± 12
	Mean	4 ± 2 [a]	9 ± 3 [a]	75 ± 4 [a]	13 ± 3 [a]	51 ± 10 [a]

GV = germinal vesicle stage; MI = metaphase I stage; MII = metaphase II stage

Values with similar superscripts (a) are not significantly different (P>0.05)

Table 3. Results of fertilised oocytes after six days of maturation after *in vitro* fertilisation. Results represent mean ± SD of the three experiments.

Fertility	Boar	Embryo development, %
High ranked	1	19 ± 7
	3	10 ± 5
	5	12 ± 11
	7	24 ± 9
	9	12 ± 7
	11	20 ± 11
	Mean	16 ± 9 [a]
Low ranked	2	14 ± 11
	4	25 ± 8
	6	18 ± 7
	8	24 ± 6
	10	13 ± 8
	12	12 ± 2
	Mean	17 ± 7 [a]

Values with similar superscripts (a) are not significantly different (P>0.05)

Semen motility

Semen motility was assessed immediately after collection and processing as well as during storage up till 14 days. Figure 1 shows the pattern of semen (progressive) motility over time. The semen motility percentage at the day of *in vitro* fertilisation (day 1 after production of the insemination dose) was in the range of 59-83% motility for both groups of boars (with no significant difference between groups P>0.05 see Figure 2). The percentage of motile sperm did not significantly correlate with *in vitro* fertilisation rates or with the embryo developmental competence of oocytes (Figure 2A and B, respectively P>0.05). The figures show a decrease in IVF results by increasing motility, although correlation coefficient (R^2) values <0.10 indicate that no relation was seen. Semen motility results in time show similar patterns (data not shown), also concluding that there is no significant relation (P>0.05) between semen motility and IVF results.

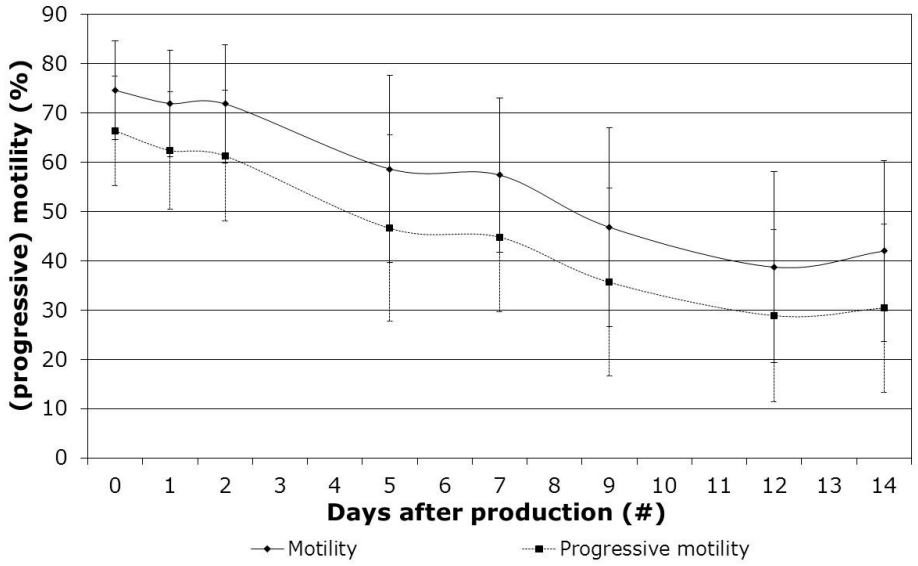

Figure 1. Results for motility and progressive motility in longevity. Results during storage up till 14 days. Results represent mean ± SD.

In vitro versus in vivo fertility

Per ejaculate on average 47 ± 16 and 32 ± 9 insemination doses were produced for the low and high ranked boars respectively. Of these produced doses on average 15 ± 7 (low ranked) and 11 ± 4 (high ranked) insemination records were retrieved from the breeding database. Although correcting for farm and sow related parameters, the number of results for the 38 ejaculates (18 high ranked versus 18 low ranked) is too low. The results indicate no relation between *in vitro* and *in vivo* fertilisation. Both *in vivo* farrowing rate (Figure 3A) and *in vivo* total number piglets born (Figure 4A) do not show a relation with *in vitro* fertilisation rate and *in vitro* embryo development, as indicated with low correlation coefficient values. The correlation coefficients are represented in Table 4. Also relating *in vivo* farrowing rate to *in vitro* embryo development, and *in vivo* total number piglets born to *in vitro* fertilisation rate results in low correlation coefficient values, and no relation (data not shown in figures, but correlation coefficients are shown in Table 4). Averaging the results per boar does show a similar pattern compared to the individual results for fertilisation (Figure 3B) and embryo development (Figure 4B).

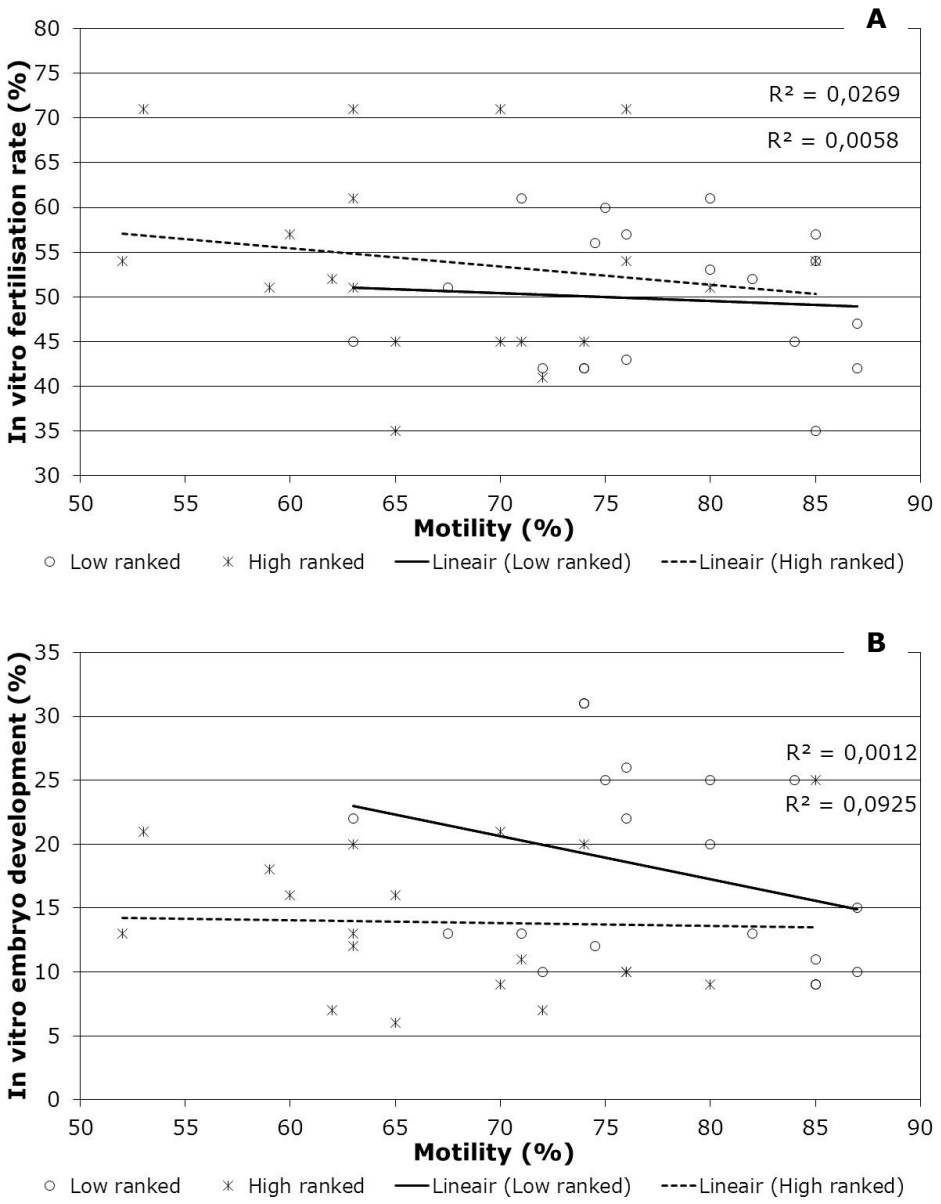

Figure 2. Relation between semen motility (measured at the moment of *in vitro* fertilisation) and fertilisation rate (A) and embryo development (B). Representing results of each ejaculate analysed and used for *in vitro* fertilisation.

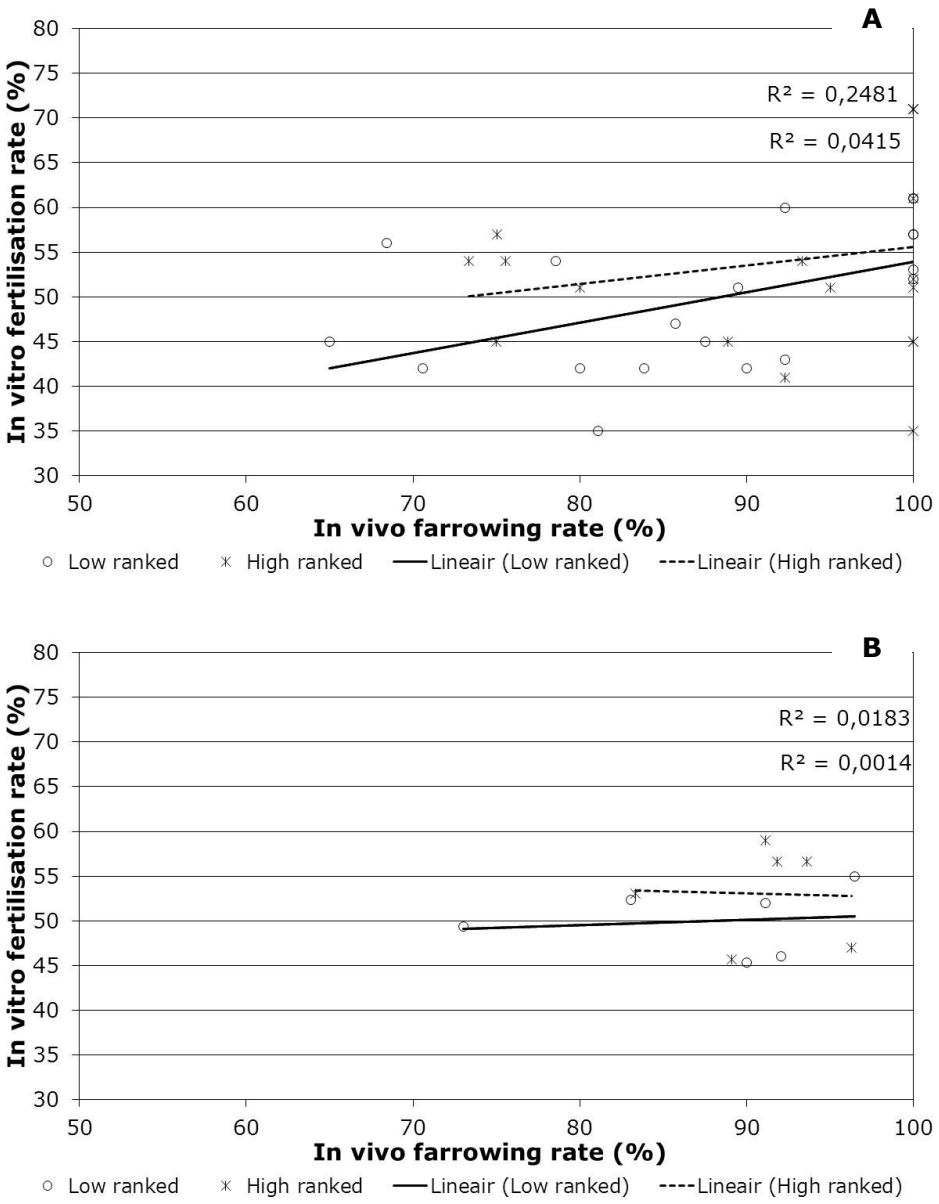

Figure 3. Relation between *in vivo* farrowing rate and *in vitro* fertilisation rate. *In vivo* farrowing rate vs. *in vitro* fertilisation rate per ejaculate (A) and per boar (B).

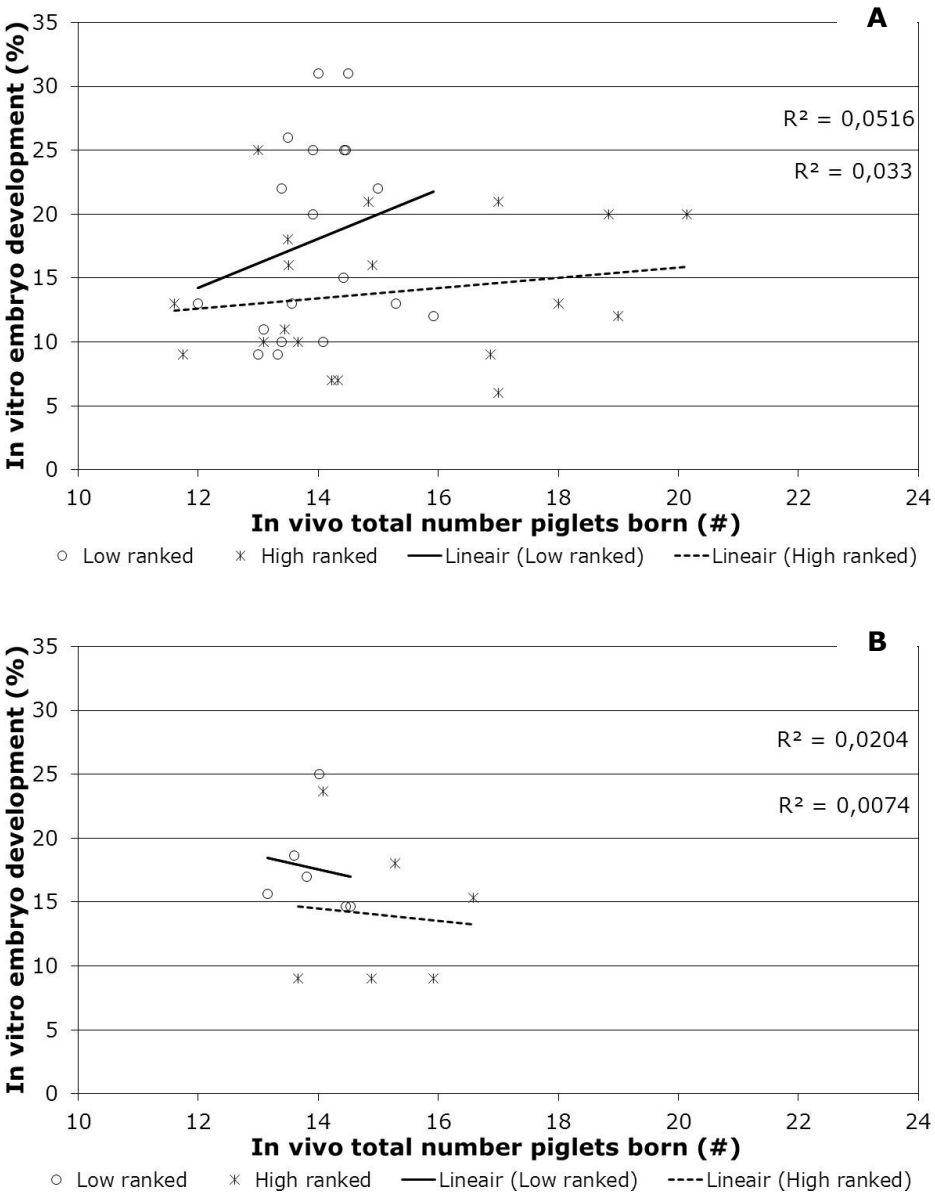

Figure 4. Relation between *in vivo* total number piglets born and *in vitro* embryo development. *In vivo* total number piglets born vs. *in vitro* embryo development per ejaculate (A) and per boar (B).

Table 4. Correlation coefficients between *in vivo* fertility and *in vitro* fertility results. Correlation coefficients for *in vivo* fertilisation (farrowing rate (FR) and total number piglets born (TNB)) vs. *in vitro* fertilisation (fertilisation rate and embryo development).
L = low ranked, H = high ranked

Correlation coefficients			*In vitro* fertilisation					
			Fertilisation rate			Embryo development		
			L	H	Mean	L	H	Mean
In vivo fertilisation	FR	L	0.498			0.103		
		H		0.204			0.062	
		Mean			0.356			0.020
	TNB	L	0.068			0.227		
		H		0.062			0.182	
		Mean			0.127			0.053

Discussion

This study was performed to evaluate whether IVF is a suitable tool for predicting the potential field fertility of boars and/or of boar ejaculates after commercial AI. Clearly is shown that there were no differences found between two groups of boars which were ranked according to their fertilising capacity. Furthermore, *in vitro* fertility results were not related to *in vivo* fertility results. This implies that IVF is not a suitable screening method for selecting boars on high versus low field fertility (as obtained after commercial AI).

Other studies showed no relation or low significant relation between IVF and field fertility (Flowers and Turner, 1997; Marchal *et al.*, 2002; Rodriguez-Martinez, 2003; Popwell and Flowers, 2004; Gadea, 2005; Ruiz-Sánchez *et al.*, 2006). Hirano *et al.* (2001) mentioned that although IVF is the best means of investigating sperm-oocyte interaction, it cannot be used as a test to select on field fertility. Obviously, the processing of sperm to achieve IVF is different from the processes sperm undergo during *in vivo* fertilisation (Popwell and Flowers, 2004) and beyond this IVF itself is a technically complex process making it difficult if not in fact impossible to standardise between laboratories. Many IVF conditions have been described to influence fertilisation results (Gil *et al.*, 2005). IVF protocols initially were developed (Schoevers *et al.*, 2003) to enable comparisons between boars and their ejaculates. Still, if the differences in input (like in current study, minimal differences between boars) are small, the IVF is not a suitable tool for predicting fertilising capacity.

There are not much studies relating semen motility to the IVF outcome (Xu *et al.*, 1998; Oehninger *et al.*, 2000; Popwell and Flowers, 2004; Gadea, 2005). In human, sperm motility parameters, measured with computer assisted semen analysis (CASA) systems are indicated being important for predicting IVF results (Hirano *et al.*, 2001). We did not find such relation in our study but should note that all samples used had relatively good motility (> 55% remained motile at day 1) when compared to human semen (Menkveld *et al.*, 2001; WHO, 2010): Men with low motile semen (cut-off is 20% motile spermatozoa are still used for IVF, which is different for commercial AI practice in which only ejaculates with semen motility >70% are used for production of insemination doses). It is therefore important to emphasise that in this study only ejaculates approved for commercial practice were used, which resulted in semen motility at day of production >80%. This is in contrast to other studies (such as (Gadea *et al.*, 1998), where semen with < 60% motility were used for an *in vitro* fertilisation assay.

Large variation within and between boars have been reported in the success of IVF (Sirard *et al.*, 1993; Matas *et al.*, 1996; Xu *et al.*, 1996; Long *et al.*, 1999; Suzuki *et al.*, 2003), which is partly similar to our study. Indeed we also did see differences between boars, although not significant in this study. For a whole boar population, it can therefore, be hypothesised that the IVF results will be affected by individual boar. Previous studies confirmed the effect of individual boar on field fertility (Chapters 2 and 4) but it remains to be established whether the individual differences in IVF results obtained with the boars really correlate with field fertility or was based on coincidence. The current analysis did not show such a relation between *in vitro* and *in vivo* fertility results, both on ejaculate level and on boar level. Our study only included one genetic Pietrain boar line. Suzuki *et al.* (2003) mentioned that fertilisation rates are more variable among genetic lines than among boars within the genetic line. Therefore, if a pig AI organisation would like to implement the IVF technique, it should consider whether it is used to discriminate fertility between genetic lines or individual boars.

Fertilising capacity of semen is normally measured *in vivo* by farrowing rate and total number of piglets born of the sows inseminated. It is not only time consuming, because one has to wait till the end of the gestation period. Furthermore, it is also expensive to use *in vivo* trials to assess the fertilising potential of a boar. To get a reliable prediction, one needs a lot of fertility results before one can discriminate between higher and lower fertilising capacity of individual boars. Xu *et al.* (1998) speculated that because changes in boar fertility measured *in vivo*

appear more slowly than changes seen in the IVF system, the use of IVF variables can predict the onset of sub fertility in boars more precisely and at an earlier stage than routine semen assessment. Previous results indicated that the routine semen quality assessments (motility and morphology) significantly related with field fertility (Feitsma, 2009; Chapters 2 and 4). Datasets of > 100,000 ejaculates were needed to validate both farm and sow related parameters and boar and semen related parameters. An evaluation period of at least 9 months would be necessary to obtain adequate records if individual farrowing rates and litter size were to be used as an index of fertility (Flowers, 1997). Using the IVF test as a predictor for boar fertility could have minimised this, but current study did not show a relation and therefore, it is an unsuitable tool.

Gadea *et al* (1998) used for an *in vitro* penetration assay test semen of boars with more variation in fertility: Semen of low fertile boars (with a farrowing rate of only 8% with on average 8.25 piglets born) was compared with semen of high fertile boars (with 89% farrowing rate and 9.37 piglets born). It is difficult to compare our study with other studies, since the fertility *in vivo* is at a higher level (87% farrowing rate and 13.5 total number piglets born on average). From the current study we learned that for a commercial practice where semen was used of boars with much higher fertility standards it was not possible to show discrepancy between the boars. Differences between higher and lower fertile boars (on basis of commercial AI) was not recovered in the IVF results probably because the differences in fact are too small to have an effect on IVF results. Despite, this IVF screening can possibly be of interest for AI companies when for instance they are involved in producing insemination doses in a commercial setting with lower fertility results. Furthermore, IVF screening can be considered when inseminating frozen thawed semen as cryopreservation and thawing causes a reduction in the fertilising capacity of processed semen and causes more variation in the fertility results as some boars produce semen that is affected more by cryopreservation then semen from other boars (the so called good and poor freezers). Gil *et al.* (2005) indicated that post thaw semen parameters were good indicators of *in vitro* fertility results.

In summary, we conclude that the high fertility level of the boars used in this current study did not show enough discrepancy to properly validate the IVF technique. It was concluded that the IVF test is not a suitable tool for predicting field fertility in commercial AI practice with high fertile boars. Our results indicate that both on ejaculate level and on boar level there was no relation between *in vitro* fertility and *in vivo* fertility.

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Concluding Discussion

Predicting porcine male fertility: what is the added value for the pig industry?

The work in this thesis has shown details on different semen quality assessments and their relation with field fertility. This can be profitable for AI companies (e.g. improved efficiency in insemination dose production), for breeding companies (e.g. genetic merit in the breeding pyramid) and for sow farmers (e.g. improved field fertility). In the Netherlands pig meat production is characterised through high cost prices (Agricultural Economic Institute Wageningen University, 2011) These cost prices must be compensated with technical results to enhance the sustainability of pig production. Fertile boars produce insemination doses, resulting in field fertility results, which are a prerequisite for efficient production. The pig industry continuously wants to improve the field fertility to reach efficient meat production.

Approximately 40% of the red meat consumed worldwide is pig meat (Gerrits *et al.*, 2005), which makes it the most widely consumed meat in the world. Animal production is increased and genetic quality has improved (Waberski *et al.*, 2008). Over the last decades knowledge on artificial insemination (AI) in pigs has developed rapidly. Results improved where production of insemination doses has become more efficient. For instance, in the Netherland the number of sperm cells in an insemination dose (80 ml) has decreased to a current level of 1.5×10^9 cells (NEN-ISO 9001, Varkens KI Nederland, 2011), which doubled the efficiency of the ejaculates used when compared to other European countries.

An AI company can use the results from the analysis on the relation between semen quality characteristics and field fertility as a tool for efficient semen production, the criteria for release or rejection of an ejaculate for distribution can be adapted to produce insemination doses more optimally. Using semen more efficiently, means using boars more efficiently, which is beneficial for the breeding company using more strict selection based on genetic merit. This efficiency is economically beneficial and in the gilts and finishers production this means faster dissemination of genes in the breeding pyramid, which results in less distance to the nucleus breeding population. In conclusion the farmer can benefit on the results from this thesis in an overall way: improvement of field fertility compensates the cost price of meat production. Finally, this thesis is representing Dutch data, but the results can be implemented and become beneficial for pig industries worldwide. Every pig producing industry can validate the results presented in this thesis against their own system.

The different chapters in this thesis provide data and conclusions on the effects (relative contribution to the variation in fertility) of the different semen quality characteristics which turned out to be small (boar and semen related parameters explained 5-6% of the variation in

fertility). Although, the effects may be little they can be of significant economic value in the high productive pig industry in order to diminish variation between farms. Semen quality characteristics can be related to field fertility and this concluding discussion points out the opportunities and the challenges the results of this thesis give. With small examples the economic perspectives of semen quality assessments are described, finishing with the conclusion on how the results from this thesis are beneficial for the pig industry. I will discuss these items with answers that I can formulate on basis of the studies described in this thesis to five questions relevant for predicting porcine male fertility.

Can we relate semen quality with field fertility?

Fertilisation is a complex process involving a large number of events. Not only should fertility research be concerned about testing new assessment methods *in vitro*, but it should also validate the value of such new assessment methods for predicting field fertility *in vivo*. The pig industry requires increased efficiency in piglet production in order to remain competitive; however there remains a wide variation in field fertility results. Pig farms with sows from breeding company TOPIGS B.V. (Vught, the Netherlands) weaned an average of 28.1 piglets per sow per year in 2010. The top 10% of farms achieved 30.9 weaned piglets per sow per year. These results are taken from the technical results of 748 Dutch farms with a total of almost 337,000 sows (TOPIGS year report, 2010). In comparison other countries had even higher variation: North-America uses 20-25 billion sperm cells per sow per year to wean 22-25 piglets per year while in the Netherlands only 7.5 billion cells are used to wean 27-29 piglets (personal communication H Feitsma, IPG; CE Kuster, Kuster Research and Consulting). Compared with the United Kingdom (21 piglets), Czech Republic (18 piglets) or with China, the biggest pig producing country of the world, where only 13 piglets per sow per year are produced (Agricultural Economic Institute Wageningen University, 2011), this clearly points out the opportunities for improvement. The results in this thesis contribute to the possibilities on improving the number of piglets weaned per sow per year combined with efficient insemination doses production (e.g. by the reduction of semen cells used per cycle).

Merging ejaculate records with field fertility records can be used to predict porcine male fertility

The fertility parameters mainly focused on in this thesis are farrowing rate (FR), indicating the percentage of sows that produce offspring from the initial insemination and litter size, indicating the total number of piglets born (TNB) per litter. These fertility parameters measure retrospectively the boar's fertility and can highly be influenced by breeding management and quality of the females (Colenbrander *et al.*,

2003). Collecting field fertility data and merging them with ejaculate records can be used to predict porcine male fertility in order to monitor field results and to analyse the relation between semen quality characteristics and fertility, as is described in Chapter 1. Merging over 1 million ejaculate records (Varkens KI Nederland, Deventer, the Netherlands) with over 8.6 million farrowing records (TOPIGS, Vught, the Netherlands) resulted in a unique dataset (Pigbase, Institute for Pig Genetics, Beuningen, the Netherlands). This dataset was used to estimate breeding values as described in this thesis.

Field fertility was affected by farm and sow related parameters, showing wide variations between farms. Although this thesis did not focusing on farm and breeding effects, these parameters could not be neglected and were used in all statistical approaches in this thesis in order to standardise the data. Part of the variation in fertility was due to genetics of the sow. In farms with better management this genetic effect becomes even larger, because the effect of the sow is larger. Boar and semen related parameters only explained a relatively small part of the variation in fertility (5-6%, Chapters 2 and 4). However, the effect of a sub fertile boar in an optimal functioning farm will have large economic impact, as is described in a later paragraph of this discussion.

Motility and morphology are sperm cell parameters that have traditionally been used to assess semen quality (World Health Organisation, 2010). Despite of this the clinical value of these parameters has been questioned (Alvarez *et al.*, 2003; Nallella *et al.*, 2006; Lewis, 2007). Semen motility is the most widely used test of semen quality, from the initial stage of AI development (Salisbury *et al.*, 1978) until present. Also in pig AI, it is a parameter generally used, as shown in Chapter 2 on microscopic semen motility assessment and in Chapter 4 on computer assisted semen analysis (CASA). Morphology assessment was analysed before (Feitsma, 2009) and is therefore not one of the parameters studied in this thesis. Chapter 5 of this thesis focused on semen quality parameters that are assessed with flow cytometry. The last chapter of this thesis, the *in vitro* fertilisation (IVF) test was analysed as possible predictor for boar fertility. All semen parameters tested in this thesis linked to fertility data sets belonging to the semen in order to monitor their predictive value for male porcine fertility in the field.

Which opportunities does it give us?

The sperm cell population within an ejaculate is heterogeneous and it seems that only a small subpopulation of the sperm cells have the potential to fertilise oocytes (Waberski *et al.*, 2008; Holt, 2009). A sperm cell that achieves successful *in vivo* fertilisation has to perform perfectly in many functions and had to overcome a series of obstacles in the female reproductive tract (transport through male and female

genital tract, binding and penetration through the cumulus and zona pellucida with concomitant acrosome reaction, binding and fusion with the oocyte (fertilisation) and subsequent inhibition of polyspermic fertilisation, for review see Flesch and Gadella, 2000)) The rationale behind the research described in this thesis is that within an ejaculate of 100% sperm cells, there are cells lacking motility or having abnormal morphology. It is questionable whether the motility and morphology tests applied are powerful enough to indicate the potential fertility of a semen sample. Every cellular component of a sperm cell must not only be intact but must also respond to intracellular and extracellular signals (Holt, 2009). Other semen quality assessments analyse for instance membrane integrity, acrosome intactness and responsiveness. Or maybe there are possibilities in mitochondrial activity or DNA fragmentation. In the end, a percentage of the original 100% sperm cells have all semen quality characteristics that are needed to optimise the odds that the sperm will meet the egg (title used from Holt, 2009). Using field data, as was done in this thesis, has an advantage over experimental data: Namely, all factors affecting fertility data can be quantified and the data can be corrected for such influences (Chapter 2). Small experimental datasets can reveal a significant difference of a specific semen parameter between boars. However, no extrapolation for other boars, other ejaculates, other genetic lines or other farms can be performed on small data sets (Chapter 1).

Microscopic semen motility assessment only minimally relates to field fertility

Since decades, laboratory technicians use microscopic semen motility as their basic semen parameter to assess the quality of the boar ejaculate before processing and producing insemination doses. Generally it is believed that semen motility assessment can identify sub fertile boars, but cannot identify the relative fertility of boars that already met the accepted industry standards for ejaculate quality (Ruiz-Sanchez *et al.*, 2006). Up to 2006, the laboratories of Varkens KI Nederland assessed ejaculates microscopically as described in Chapter 2. But does this microscopical test provide any predictive value for average farrowing rates and/or litter sizes which are so important for the breeding companies and thus for a commercial AI centre? The study presented in Chapter 2 showed that microscopic semen motility assessment only minimally relates to field fertility. Only 5-6% of the total variation in fertility was boar and semen related. This result seems very low, but in fact is similar to the effects found by Christensen *et al.* (2004) who accounted 5.5% of the total variation in litter size to boar and semen effects. We found only 4% of the boar and semen related variation to be caused by semen motility. Additionally, the control of semen quality by microscopic motility assessment is biased because the method is difficult

to standardise and differs between laboratories and technicians. An AI laboratory using microscopic assessments should understand that it is basically a yes-no parameter, without continuous results that could be used in a valid analysis in relation with fertility. In summary Chapter 2 showed that, despite the fact that almost all AI centres asses semen motility by eye under a microscope and consider this an important parameter for the fertilising capacity of the ejaculate, it relates to field fertility only minimally.

CASA has an additional value for the pig AI

The lack of standardisation of the method and the subjective interpretation of the laboratory technicians give rise to discussion about the microscopic semen motility assessment. This is a known phenomenon: variations between and within technicians emphasise the need for standardisation. The absence of variation in results makes it difficult to estimate the effect of semen motility on fertility. To overcome this variability, computer assisted semen analysis (CASA) has been introduced. Chapter 3 of this thesis describes the added value of CASA compared to conventional motility assessment. The first advantage using CASA was the more precise calculation of number of sperm cells in an ejaculate; therefore, a reduction in sperm cells per dose was possible which will make the semen production more efficient. Hence less boars will be necessary to produce the necessary doses and therefore a more strict selection based on genetic merit can be executed. This can lead to more efficient dissemination of superior genes in the breeding pyramid. Overall, this will lead to an advantage for the whole pig industry: improvement of field fertility and increased levels in finisher traits.

There was no additional value of CASA when the laboratory technicians were not skilled by training. Proper introduction to the system was needed, including the risks involved and working procedure (Chapter 3). The repeatability of CASA was enhanced by using a standardised operating procedure (SOP) combined with optimal training of technicians. A false perception from AI managers might be that a CASA system runs itself. The human factor remains a point of attention and can be of significant impact when not controlled. Chapter 3 clearly pointed out the additive value of using CASA in efficient insemination dose production. The AI company has to realise that if a repeatability level of 95% is not reached, the impact of using CASA is less although it still can be beneficial over microscopic semen motility assessments by eye.

Of course there will be some points missing in describing the implementation of CASA. For instance in a personal communication with RP Amann, it was pointed out that the value of quality control over the years which was not described in detail in Chapter 3. It is important to keep results consistent over time. Over the years there are technician

changes and possible technical improvements in the instrumental set up. The risk is that procedures that were validated years ago are assumed to still work great, while in fact it is agreed upon that even a standardised procedure must routinely be checked. It is important to monitor how the procedures are followed and if the variation of results is acceptable or out or range. Training and updating of laboratory personnel in the use of CASA is implemented in the quality control program of Varkens KI Nederland. The laboratory technicians are trained in using the SOP and monitored on a regular base, but at least 4 times per year. By that achieving a coefficient of variation of <5% is achieved. Without training and using the SOP this was 25% for motility.

CASA is a tool to objectively discriminate between fertilising capacity of ejaculates

The use of CASA resulted in a higher variability of semen motility with continuous results. This allowed better analysis of the relationship between semen motility characteristics and fertilising capacity of ejaculates. Chapter 4 concluded on a 5-6% of the variation in fertility explained by boar and semen related parameters. Motility parameters, measured by CASA, explained 9-10% of this boar and semen related variation. The significant CASA parameter affecting FR were opposite to the parameters affecting TNB. FR and TNB were apparently fertility parameters that should be validated independently. This is in agreement with other studies (Juonala *et al.*, 1998; Tsakmakidis *et al.*, 2010) showing a low relation between FR and TNB. Tsakmakidis *et al.* (2010) showed that FR differed among boars used for AI, but litter size did not. Chapter 4 clearly pointed out that CASA is a tool for the AI company to predict the fertilising capacity of ejaculates. Ejaculates and/or boars can be approved or rejected by using cut-off values based on CASA motility parameters. The prediction was more precise compared to microscopic semen motility assessment, because the variation in boar and semen related parameters explained by semen motility had increased. Furthermore, the relation with fertility was more profound, because there was no longer an effect of AI laboratory on fertility, which states the objectivity of CASA. These results give the AI company a strong tool to discriminate on the fertilising capacity of ejaculates based on motility pattern.

Similar to the retrospective study shown in Chapter 2 on microscopic semen motility, also the statistical analysis of CASA semen motility revealed a relatively large effect of individual boar and genetic line of the boar affecting the variation in fertility (Chapter 4). Identifying differences in genetic lines can be practical and interesting for the AI company and for the breeding company. All boar and semen related parameters that have or have not an effect on field fertility can be used in an index. Therefore, the relation between factor and fertility should be

calculated and weighed against other factors. This index can be used for an AI company to establish standards for approval or rejection based on objective data. An AI company can discriminate the results in detail, e.g. specifically for each genetic line or even for each individual boar. Depending on the level of details used to calculate the index, an AI company needs to build in a larger or smaller safety margin in the insemination dose depending if such "risks" are compensative yes or no. The possibilities resulting from our study described in Chapter 4 on using boar and semen related parameters in an index will be described in the paragraph on opportunities.

Flow cytometric semen quality assessment gives possibilities for an AI company

Can CASA motility parameters predict enough to explain the variation in fertility or do we add any value by assessing semen quality via the use of flow cytometry? This question resulted from the fact that part of the variation due to boar and semen related parameters is not explained. Despite that, non motile does not automatically mean that these cells are dead and incapable of fertilisation. Their motility can be activated in the oviduct (Suarez *et al.*, 1992). Therefore, assessing more detailed semen functionalities can be an additive value for the semen quality assessment in the AI laboratory.

General laboratory practice involves measuring semen motility and possibly morphology, although, flow cytometry is being used more and more for semen assessment. The methodology improves the objectivity, accuracy and reproducibility of membrane assessments (Flesch *et al.*, 1998). Despite the large number of publications about semen assessments with flow cytometry, a proper study assessing multiple flow cytometric parameters and relating these results with field fertility was missing until now. Therefore, in Chapter 5 of this thesis we studied the relation between flow cytometric sperm assessments and field fertility. Sperm cell integrity was assessed with respect to DNA and membrane integrity, acrosome intactness and responsiveness and mitochondrial potential using established flow cytometric assays. The DNA fragmentation assessed at 7-10 days and 14-15 days of storage relates to FR and TNB respectively. This indicated that an AI laboratory can detect DNA damage in boar semen samples after storing it 1 to 2 weeks and use this information to predict the fertilising capacity of future produced ejaculates from boars. Surprisingly, the membrane integrity assessments did not relate to FR or TNB. The initial quality of the semen doses assessed was at such a high level that these assessments did not add any value to the semen quality in relation with fertility. These assessments were not suitable for current Dutch AI practice, but can have an additive value for other AI companies. In sub-optimal pig producing systems, the discrimination between ejaculates based on

membrane integrity, acrosome intactness and responsiveness or mitochondrial potential can relate to field fertility in another way.

The flow cytometric assessment of sperm DNA fragmentation is currently not used in the commercial practice of Varkens KI Nederland, but can have an added value to the knowledge on the ejaculate produced by that particular boar. A number of studies indicated the potential of assessing the DNA fragmentation index (DFI) in the assessment of semen from boars (Evenson and Jost, 2000; Rybar *et al.*, 2004; Boe-Hansen *et al.*, 2005; 2008; Didion *et al.*, 2009; Waberski *et al.*, 2011). Whether one can use the test in this sense depends on the variation in DFI within the boar population of an AI company. What happens if there is a sub-fertile boar in the population? How soon can such a boar be identified? What is the prevalence of such a boar? The DFI shown from the research described in Chapter 5 was below 10% and representing high fertility potential. Other studies concluded that only a small portion of boars had semen with DFI > 5 % (Evenson and Jost, 2000; Waberski *et al.*, 2011), which was similar to the results from our study. Literature proposed cut-off levels for boar sperm cells between 2% and 18% for DFI (Rybar *et al.*, 2004; Boe-Hansen *et al.*, 2008; Didion *et al.*, 2009; Waberski *et al.*, 2011). From the results described in Chapter 5 such a cut-off level cannot be recommended. Each AI company has to determine this cut-off value for their own boar population. This research was based on 20 boars, representing only part of the boar population, so it is yet unknown if the result for those boars is representative for the entire stud population.

Sub fertile boars demonstrate the value of assessing DNA fragmentation

Using low quality ejaculates and sub fertile boars reduces production efficiency and lowers profit margins for the producer (Foxcroft *et al.*, 2008). Testing threshold of DFI > 10% was not achieved in the current study, but the value of DFI assessment was demonstrated before in two sub fertile boars from Varkens KI Nederland (unpublished data). These boars were identified as being sub-fertile, since the fertility was determined significantly lower at 3 farms. The non return rate at 28 days after insemination for the two boars was 30% (n = 44 inseminations) and 65% (n = 26 inseminations), respectively, compared to 84% (n = 1,075 inseminations) normal. The TNB for the two boars was 10.6 (n = 9 farrowings) and 9.0 (n = 16 farrowings), respectively, compared to 12.4 (n = 625 farrowings) normal. In the daily semen quality assessment, no abnormalities were shown. More detailed study revealed an abnormal head shape which was missed during routine assessment and which was consistent in all cells in the ejaculate for one of the boars. For both boars a DFI of on average 30% and 60% in fresh semen respectively (n = 10 ejaculates) was detected. This is an indication that assessing DNA fragmentation could be a helpful assay for

screening on sub fertility which would remain undetected using conventional semen quality assessment methods. With 30% abnormal cells it can be assumed that the other 70% can contribute to normal fertilisation. Evenson and Jost (2000) indicated that when 30% of the semen is indicated as a discrete population, likely the same kind of damage exists throughout the whole semen population in sufficient amount to cause sub fertility.

The performance of the boar is recorded and monitored. Since the outbreak of classical swine fever in 1997, the use of pooled semen was prohibited by the Dutch government. Therefore it is relative easy to calculate the fertility on a boar level and we can indicate those boars where fertility is on the lower side. Since the use of single boar semen for AI, a few times per year boars with low fertility results are detected (sub fertile boars). In the history of Varkens KI Nederland a total of 46 sub fertile boars were identified within a population of approximately 20,000 boars. With the current routine semen quality assessment, not all abnormalities will be detected, as was illustrated by the two sub fertile boars identified with DFI assessment. But this assay is currently not applied in practice. Therefore, it should be evaluated whether adding this test is worthwhile.

Are the costs of performing an extra assessment an even match with the benefits in preventing the lower fertility, by which costs for failure compensation are saved? The conclusion for this study shows that assessing DNA fragmentation is valid 1-2 weeks after production, showing a relation with field fertility. Furthermore, in a high producing laboratory (e.g. > 50 ejaculates per hour) it is not possible to assess the DFI in line. This means that assessing DNA fragmentation is not practical for routine assessment on fresh semen in the daily production routine, but can be used as a screening test although we have not yet validated this test. It is recommended to test the whole boar population, to check how many times the tests should be performed in a boar's career and by that to evaluate the cost benefit calculation of the test for the company. It can be carried out during the quarantine and training period of potential boars. Compare the total costs with the total benefits of identifying these boars and an AI company can decide whether the DNA fragmentation should be implemented in their routine assessment. Depending on the repeatability of DFI between ejaculates in time, the DFI-assessment must be repeated with a certain frequency if necessary. Similar validation has to be performed for each new test that is implemented in a commercial AI laboratory.

In vitro fertilisation has no predictive value for fertilising capacity of semen

Both microscopic and CASA assessments, but also flow cytometric assessments, can analyse semen quality from a boar's ejaculate at the moment after production. The predictive value of *in vitro* fertilisation (IVF) for field fertility of individual sperm samples was studied in Chapter 6. The imitation of *in vivo* fertilisation, although standardised laboratory conditions may provide a discrimination between high and lower fertilisation capacity of a given insemination dose. The advantage could be that an IVF test could predict the field fertility, without waiting for actual piglets to be born. By that, the IVF test could be used to validate *in vitro* tests, without *in vivo* fertility needed.

Despite this theoretical possibility, the results from Chapter 6 clearly pointed out that the IVF test protocol was not suitable to discriminate between ejaculates/boars. Ranking boars on their fertilising potential did not result in significant differences in both fertilisation rate and embryo development *in vitro*. The differences in fertilising potential were too small to discriminate between ejaculates. There were no relations between IVF results and semen motility parameters or with field fertility. I did expect such outcome because semen characteristics which are successful in IVF, do not necessarily give optimal fertility when used for AI (Ruiz-Sanchez *et al.*, 2006; Novak *et al.*, 2010). Extensive interactions between components of the ejaculate and the female reproductive tract *in vivo* are very different to the conditions in which sperm cell maturation is carried out *in vitro* (Novak *et al.*, 2010).

Unfortunately, this study is based on a limited number of boars, lacking a large number of performed IVF tests. Also the total number of oocytes per boar is not very high. The IVF trials have been done while only using one concentration of sperm with relatively high number of sperm cells per oocyte. This can also cause lack of revealing differences in fertilising ability between individual boars and/or ejaculates. To scientifically validate current conclusions you need larger number of IVF trials, different doses of sperm cells per oocyte, larger contrast between low and high fertile boars or lower concentrated sperm cell insemination doses in the field. The IVF technique is expensive and time consuming and thus is unlikely to become of practical use for routine semen assessment. Based on the results of Chapter 6, an AI company cannot use the current IVF technique as a tool for discriminating between boars nor as a valid test system for new quality assessment tests.

Are there future opportunities resulting from this thesis?
AI companies could use the conclusion from the analyses in this thesis to improve their efficiency and quality in the production of insemination doses. An index can be made to calculate and weight all boar and semen related parameters that have or have not an effect on field fertility. There are differences between genetic lines and between individual boars in both semen quality and field fertility, as was clearly pointed out in all chapters. Missing in current thesis is how to interpret the results from different genetic lines. It is recommended to subdivide between genetic lines and determine individual cut-off levels for semen quality. This will result in a more optimal way of producing high semen quality doses with genetic impact.

The conclusions on CASA motility assessment (Chapter 4) are a very good first step on the exact relation between semen motility and fertility, but can only be based on mean results. Little is known about subpopulations of sperm cells within a given ejaculate and if unique motility patterns in those subpopulations have specific abnormalities influencing boar fertility. Braundmeier and Miller (2001) suggested that the sperm cells that fertilise the oocytes *in vivo* may be a small and highly selected subpopulation that is not representative of the average semen evaluated in the sample. Current understanding of CASA motility assessments can be expanded with the results of the minimal and maximal value, of the standard deviation of the results or to subdivide differences classes within an ejaculate. Identifying different semen populations by means of CASA (Holt and Van Look, 2004; Satake *et al.*, 2006) is still at a research level and waits for further development. More detailed studies could be added to current results, being additive for a final use in semen quality assessment.

This thesis has information on the semen motility results fresh at the day of production. This is an important time point as at that moment the decision has to be made whether the ejaculate can be processed and insemination doses can be produced. Despite that, the decay rate of semen quality in time can be relevant factor as well. Semen quality in extended semen at day 7 has been correlated with *in vitro* fertility (Xu *et al.*, 1996) and with *in vivo* fertility (Juonala *et al.*, 1998; Xu *et al.*, 1998; Sutkeviciene *et al.*, 2005) although with variable results. Motility at different days of storage may offer a practical and inexpensive approach to identify boar fertility. Varkens KI Nederland assesses semen quality in time (24 and 72 hours after production). Adding this information to the results of the fresh ejaculates can be beneficial for assessing the following ejaculates of that particular boar.

Bergsma and Feitsma (2005) concluded a significant relation between morphological abnormalities and field fertility, as is confirmed in more repeated studies by the Institute for Pig Genetics (personal communication H Feitsma and JI Leenhouwers). Ideally, if there is a

correlation between CASA parameters and morphological abnormalities, the assessed CASA parameter could be enough indication for the determined relation between morphology and fertility. With this knowledge an AI company can decide whether they want to assess morphology in detail (time consuming), or to use the motility results as enough information on the ejaculate assessed. First results show that assessing semen motility with CASA is not automatically an indication for other semen quality parameters like morphology (data not shown).

The number of sperm cells in an insemination dose is currently at a relatively low level in the Netherlands (1.5×10^9 cells per 80 ml dose); especially compared to other AI companies ($\geq 3.0 \times 10^9$ cells). Continuous statistical analysis of the Dutch database (similar to current research) revealed that there is no significant relation between number of cells in an insemination dose and field fertility, except for one genetic boar line (Leenhouwers and Feitsma, 2011). The use of large numbers of cells can mean that semen traits that prevent sperm cells to enter the oviduct may be compensated for. Intrinsic differences in semen quality between individual ejaculates/boars are masked (Tardif *et al.*, 1999; Saacke *et al.*, 2000). In current study the relation between semen quality and fertility at an even lower number of sperm cells per insemination dose (e.g. $< 1 \times 10^9$ cells per 80 ml dose) was not analysed. This could possibly even show a better discrimination between ejaculates and/or boars, but was not possible in current research approach.

With the conclusions of the current research on semen motility, combined with previous research on morphology and number of cells in an insemination dose, an index can be developed. The relation between DNA fragmentation and fertility is established as well, but the AI company has to validate these results before application. The practicality of that assay in a routine AI setting is questionable. The AI company can choose whether they want an overall semen quality index, or they would like to split up per genetic line. Whatever the preference of the AI company is, the results of such detailed studies between semen quality parameters and field fertility are a convenient tool for the AI in their quality control routine. Better fertility related tests will increase efficiency in insemination dose production which will at the end be responsible for a more efficient dissemination of genes from nucleus to production level. Therefore the field can benefit a great deal from this work in achieving less distance between genetic level at the nucleus and at production farms.

What are the economic perspectives of semen quality assessment?

AI contributes positively to worldwide swine production. The goal of semen analysis in the pig industry is to estimate fertilisation in a large number of females. Thousands of sows are inseminated with semen from a given boar, with an obvious impact of the male fertility. The efficiency in spreading superior genetics in the production pyramid is positively affected by increase of production of high fertile insemination doses. Commercially it is important for an AI company to monitor their results in practice. Knowing the performance of semen in the field enables efficient analyses of the causes of poor performance. It was emphasised before that the percentage of the variation that is explained by boar and semen related parameters is very small (± 5-6%). More important is the conclusion that there are relations found between CASA motility parameters and fertility. With this thesis knowledge is obtained on how much the fertility results will change when the CASA parameter varies. The exact cut-off values are not discussed in this thesis, since this depends on the goals of the AI company. But a calculation model is given as an example of what these small statistical effects mean in economic perspective at field/farm level.

With a sow population of 1 million sows in the Netherlands and 2.35 cycles per sow per year (average per farm including empty days), it means that calculations can be based on 2.35 million cycles per year. In this example motility is used with a significant effect of 0.128 piglets per 1 SD (based on the results of Chapter 4). This means that per percentage of motility, the total number of piglets born increases with 0.02 piglets. Ejaculates with 10 percentage difference in motility are to be expected to give a 0.2 piglet difference in the field. This means a 1.4% difference in production (0.2 / 13.9), which still seems like a small part. However, with 2.35 million cycles per year and a farrowing rate of 86%, we have 2,021,000 farrowings per year. If the effect of 10% motility is 0.2 piglets for each farrowing, this means that the effect is 404,200 piglets. With a current price of €37.00 (December 2011), this means an economic equivalent of almost 15 million euros (€1.50 per sow). Combined with economic losses because of poor morphology (0.1 piglets per 10% abnormal cells) of approximately €4.00 per sow, reciprocal translocation losses of €1.60 (both based on personal communication H. Feitsma, IPG) per sow, at the end it will add up to between €7.50 and €10.00. For a farm with 500 sows this is €5,000.00 at least. These results are all corrected for other farm and sow related parameters and for other boar and semen related parameters. The effects on individual farms of a 10% difference in motility can be much larger.

There are not only benefits in the direct effect on field fertility. As mentioned before the spreading of superior genetics can be more efficient and as a result less boars are required to produce the needed number of doses. A more strict selection can be executed, based on genetic merit. Current genetic progress in the last year is €1.75 per pig sold (personal communication EHAT Hanenberg, TOPIGS). This is due to the increasing number of live born piglets per litter, with decreasing pre weaning mortality. Furthermore, the feed conversion ratio decreases and the daily gain increase, with focus on loin and fat depth. This means that efficiency in high quality semen dose production, which can be achieved with knowledge from current research, helps decreasing the distance between nucleus breeding and sub nucleus (gilts and finishers) pig production.

Can the worldwide population use less number of pigs?

Fertilisation in mammals is a complex process, involving multiple interactions between the semen and the seminal plasma components of the ejaculate (Novak *et al.*, 2010). This thesis has described multiple semen quality assessment methods, with the focus on semen motility, and flow cytometry and IVF tests. Assessing semen motility with the use of a CASA system turned out to be of added value for the AI company, because it gives a laboratory an additional tool for the process and production of high quality insemination doses. Besides that it gives the breeding company a tool to use superior genes more efficiently. And adding this up it this improves the whole pig production.

Animal farming plays an important role in European society. The Sustainable Farm Animal Breeding and Reproduction Technology Platform (FABRE-TP) is supported by 116 organisations across Europe to enhance the sustainability of animal breeding and reproduction in (and outside) Europe. In the research agenda, FABRE-TP highlights the challenges and opportunities of animal breeding and reproduction. The technology platform shows that optimised animal production systems contribute to a safe and healthy diet help to maintain human communities and offer opportunities to reduce the environmental footprint (Strategic research agenda, FARBRE-TP, 2011).

It is to be expected that in 2050 there are over 9 billion people in the world. It is expected that over the period 2010-2019 the meat consumption will increase with 19% (OECD and FAO, 2010). Pork is the most widely consumed meat in the world and will increase over the next decades. At the same time, the raw materials for the feed will become scarce. Efficient pig meat production will be an additive tool for the problem. In 2009 the worldwide production of pig meat was 106 million tons carcass weight (FAOstat, 2010). With Dutch standards of 92 kg slaughter weight per finishing pig this means that on a yearly basis you need 1.15 billion pigs. With a Dutch sow producing 26 finishing pigs per

year, you need 44 million sows to produce the 106 million tons of carcass weight. To calculate the number of sows worldwide it is assumed that on every sow there are 12 pigs (sow + weaned piglets + finishing pigs). This results in a worldwide population of 528 million pigs (LEI, 2011). In 2009, the records show that there are 941 pigs worldwide (FAOstat, 2010). This shows a less efficient pig production system, compared to the Netherlands. Producing in an efficient way, as is already reached in the Netherlands, will result in a decrease of 40% pigs to be housed. Clearly a future challenge to keep in mind.

Within the region of production the pig must adapt to the local environment, in terms of climate, housing system, health and availability of nutrients. A limited number of purebred animals of sire and dam lines in nucleus herds are the basis for cross breeding for the production level. Mentioned before is the benefit of current research on decreasing the distance between nucleus herds and production level. The available genetic variation is large, but with efficient insemination dose production, this will mean that the genetics can be used more efficiently. Breeding goals have evolved from heritable traits, like growth and feed efficiency, to sustainability related traits like litter size and piglet vitality. Breeding is becoming more and more technology intensive, with sequencing the porcine genome and genomic selection as a good example. Increasing research efforts, as this thesis is part of, devotes to improved management of genetic diversity. To reach high efficiency in semen and piglet production, semen quality must be guaranteed and controlled. Furthermore, efficient male and female reproduction must be supported.

Presenting new reproduction techniques is an on-going process, but not all techniques are helpful for improved fertility and use in AI. Current research adds value to improved fertility by knowing the effect of CASA motility parameters on field fertility. For progress in pig production with future research, only a few research subjects are of interest. Mentioned before are the possibilities to extend the current knowledge on semen motility to be used as a tool to select ejaculates. Other areas are e.g. seminal plasma, post cervical insemination, incapsulation of semen, boar feed additives or new ideas for sex sorting of semen. Results from current thesis are a good starting position by which research groups can collaborate and develop new research proposals.

Conclusion

Assessing fertilising capacity of the highest genetically indexed boars is one of the main goals of both AI companies and breeding companies. The economic impact of these boars is realised throughout the breeding pyramid and the number of sows bred per boar must be maximised. The use of proven high fertile boars must be optimised. At nucleus level it

allows increased selection pressure by increasing number of offspring bred. At the finisher production level it allows improvements in production efficiency. This results in decreasing the distance between nucleus and finisher production breeding. Identifying boars with relatively low fertility becomes more and more critical. With the results from current thesis, the AI companies can make a step forward in efficient and guaranteed high semen quality production of insemination doses.

Collecting field fertility data and merging this with ejaculate records is a very strong tool for an AI company and breeding company. The data analyses performed in the research described in this thesis are based on actual data, representing the practical relation between semen quality characteristics and field fertility. The AI company can use the results as a tool for efficient production and processing of insemination doses. This thesis already enables AI companies to make a step forward, the results from current tests used (semen motility) show a clear relation with fertility, and with standardised objective methods such as CASA even more than with microscopic methods. Optional other tests (flow cytometry) are suggested. DNA fragmentation is indicated as a predictive test at a high producing boar population, but also other membrane integrity tests are optional to be relevant in sub optimal boar populations. Finding a minimum set of tests with maximal functional coverage is an on-going process, along with the understanding of the quality a sperm cell needs for fertilisation in large population of females. The value of an accurate and reproducible semen analysis is clearly addressed in this thesis.

CASA motility parameters and DNA fragmentation are semen quality characteristics that are shown to affect field fertility. In the Netherlands, we are currently using high fertile boars. This is the result of a long period of indirect selection, where nucleus boars with lower fertility results were not used for producing the next generation. Despite this high fertility, the DNA fragmentation and the semen motility (especially assessed with CASA) are indicated as very significant parameters affecting field fertility. In a less selected population for boar fertility, the value of these tests might be even higher and tests that did not show a relation with fertility in our data, might prove to be related with sub fertility in less selected populations. The optimal value for e.g. CASA motility parameters permits the AI company working as efficient as possible at lowest possible cost price and with maximum field fertility. The pig industry will benefit from the larger genetic progress in the breeding program. The knowledge gained from this thesis can be extremely helpful in stepwise development of producing semen with the quality for improvement in field fertility.

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Summary

From semen cell to meat production: it all starts with fertilisation!

Fertility is estimated by several factors and therefore, achieving high fertility is not obvious. Predominantly, artificial insemination (AI) is used for breeding pigs. The advantage of AI is that you can dilute semen from high fertile breeding boars and in this way inseminate many sows. The last years, the pig industry is increased in scale and evaluated in specialisation. This means that also AI organisations value semen quality and guarantee of fertility. Fertile semen, inseminated at optimal timing in a healthy uterus results almost all times in pregnancy. Despite that, good semen is a broad concept (a healthy uterus is out of the scope of this thesis). A good ejaculate contains live, motile sperm cells. Unfortunately there is no golden standard concerning the requirements for qualitative good semen. This thesis handles different semen quality characteristics in relation to fertility.

How to estimate field fertility of breeding boars?

It is very important to assess fertility of breeding boars in a correct way. Fertility results are the foundation for the relation between semen quality and fertility. Chapter 1 shows worldwide research with questionable results. For example, only a minimal number of animals are used, from a single genetic line or the inseminated sows originate from one farm. Extrapolation of results for the whole population is often impossible. Data in this thesis is based on semen quality (results from the laboratory) and fertility (results from Dutch farms). Currently, the dataset contains > 1 million ejaculate records and > 8.5 million farrowings. In this thesis, two parameters are used to express field fertility: farrowing rate (FR) and total number piglets born (TNB).

An ejaculate is collected in the barn at an AI location. At the laboratory semen quality is assessed and based on the results the ejaculate is diluted to an average of 35 insemination doses per ejaculate. These doses are transported to different farms where they are used to inseminate sows. Insemination doses of one ejaculate are used at different farms which causes variation in fertility results. To get one fertility record per ejaculate statistical analyses corrects for these factors. Farm and sow related parameters like management, genetic line of the sow, parity, number of inseminations, interval weaning first insemination, gestation length, year and season, and age of semen at moment of insemination are corrected for to retrieve one fertility result per ejaculate. The influence of these farm and sow related parameters is out of the scope of this thesis. Boar and semen related parameters also affect variation in field fertility. These factors are corrected for,

concerning parameters like genetic line of the boar, individual boar, age of the boar, days between ejaculations, AI station and technician, number of cells in an ejaculate and number of cells in an insemination dose. Correcting for all these parameters results in the analysis between semen quality characteristics and field fertility, which is analysed in current thesis.

What is the value of microscopic semen motility assessment?
In Chapter 2 the most widely used and easiest way of assessing semen quality is shown: analysing semen motility. Trained laboratory technicians assess a semen sample using a phase contrast microscope and scoring for motility. The cut-off levels for this assessment are depending on the experience of the technician and are always subject of discussion. The subjectivity of this method is shown in the analysis on the relation between semen motility and fertility. Extensive data collection resulted in 110,186 ejaculates (1998-2005) with known fertility. Analysing these results showed the percentage of variation explained by boar and semen related parameters. This turned out to be only 6% of the total variation in field fertility. Within this variation, only 4% is explained by semen motility. Concluding, semen motility only explains a small percentage of the variation in fertility. Analysis showed that AI station (location and laboratory technician) explained a larger part of the variation, demonstrating the subjectivity of microscopic motility assessment. Furthermore it confirms that the largest part of the boar and semen related variation in fertility is explained by genetic line of the boar and individual boar within this line.

What is the advantage of automatic semen motility assessment?
Assessing semen motility microscopically turned out to be very subjective. Using a CASA (computer assisted semen assessment) system gives the possibility to automate semen motility assessment. CASA is applied in both human and veterinary industry. In 2006, CASA systems were implemented in the Dutch pig AI laboratories, after a very intensive implementation period, in which laboratory technicians were guided in the determination of a standard operating procedure and in increasing repeatability of the measurements. Do not forget that a CASA system is an automated system which is affected by the laboratory technician operating the system. Therefore, at first a standard operating procedure had to be determined, validating the effect of temperature, homogenising of the sample, dilution factor, standard counting chamber and the effect of training of technicians. With training on repeatability a coefficient of variation smaller than 5% was achieved. Improving the

repeatability showed the additional value of a CASA system, which is shown in Chapter 3. Accurate results make that insemination doses can be produced more efficiently, which is one of the goals of an AI company.

Is there a relation between CASA motility and fertility?
By using a CASA system an AI company can accurately assess semen motility and dilute the ejaculate in an efficient way. This raises the question on whether there is a relation between semen motility (assessed with CASA) and fertility. Using CASA means not only assessing motility and progressive motility, but even more detailed basic motility parameters. These parameters exceed the possibilities of the microscopic semen assessment, but at the same time the value of these parameters in relation with fertility of breeding boars was not known in detail. Chapter 4 describes the analysis of 45,532 ejaculates with known fertility during a 3 year period (2006-2009). Of the total variation in fertility only 5-6% could be explained by boar and semen related parameters. Of this variation, 9-10% was explained by detailed semen motility parameters. Objectivity using a CASA system was shown by the absence of an AI station effect (location and technician) on fertility. Furthermore, it was clearly shown that there are differences between genetic lines of the boars. With this knowledge, cut-off levels for semen quality could be established which can lead to an even more accurate and precise product.

What are the possibilities of assessing semen quality characteristics with flow cytometry for predicting fertility?
Only semen motility assessment is maybe not enough. Flow cytometry is a method in which you can assess more detailed characteristics of the sperm cell. This could be membranes, acrosomes or possible DNA damage. Chapter 5 describes the sperm cell characteristics that are analysed in relation with fertility. In this study, 20 boars were selected from three genetic lines. These boars were followed for a 20 week period, at which the semen quality of the ejaculate was assessed fresh and at 4 measurement moments (until 14 days after production). After correcting for both farm and sow related parameters, and boar and semen related parameters it was concluded that only DNA damage measured at more than 7 days after production of the ejaculate could be related to fertility. However, measuring DNA damage is a time consuming test, which is more difficult to implement in the daily routine of semen assessment. In the Discussion of this thesis it is underlined that before implementation of this test, a detailed study must be

performed on the repeatability of the measurements and on the variation within the whole boar population. Furthermore, a cost-benefit analysis must be performed: what are the costs of performing this test and what are the benefits if a sub fertile boar is identified. This study clearly points out that there is no relation between membrane integrity tests (membranes, acrosomes, mitochondria) and fertility. Possible cause is the boars, and with that the ejaculates of these boars, are high fertile, by selection by the AI organisation. Differences in semen quality did not result in an effect on fertility. There are enough sperm cells in the insemination dose to compensate for these semen quality characteristics. The effect of sperm cells with DNA damage is not compensable which shows an effect on fertility.

Is IVF a suitable test to predict fertility?

IVF (= *in vitro* fertilisation) is a technique which is known from the human industry. However, IVF is commonly used in the veterinary industry as well, and also in Chapter 6 of this thesis. Not to retrieve embryos, but to determine whether an IVF test can be a predictor for fertility. When measuring fertility *in vivo* (in the field), you have to wait for farrowing and count the total number piglets born. Gestation length of pigs is about three months, three weeks and three days, which means that only after this gestation period it can be established whether the quality of the semen (and/or other parameters) have an effect on fertility. By inseminating oocytes with semen from breeding boars (performing an IVF test) it was hypothesised that in an earlier stage you could determine the effect of semen quality of that particular ejaculate on fertility. This was studied with 3 ejaculates from 12 boars. These boars were selected based on fertility, comparing 6 high fertile boars with 6 low fertile boars from one genetic line. The study did not show any significant differences. Possible causes were the minimal difference in fertility between boars, the complicated IVF technique not relating variations in results to semen quality, or by the large number of inseminated sperm cells, or by the low number of performed IVF tests per boar. Despite that, this study concluded that the IVF test as a predictor for fertility of breeding boars is not suitable to implement as a semen quality control.

Conclusion

The Discussion of this thesis continues on the relevancy of the performed research. Results show that the effect of semen quality characteristics on the variation in fertility of breeding boars is relatively small. Despite that these effects seem small, the economic value in a

high producing pig industry is large, with the objective to minimise the variation between farms. An interesting worldwide goal is the calculation that if the production worldwide would be as efficient as it is in the Netherlands, you need 40% less pigs. The concluded relations between semen quality and fertility provide challenges and possibilities to extend this efficiency position. In the Discussion, the possibility to develop a semen quality index are described, in which different semen parameters, but also other boar related parameters could be weighted in. This results in a tool for semen quality control for an AI organisation. Tests related to fertility result in an efficient production of insemination doses, which results in an efficient spreading of the genes.

CASA motility and DNA damage are semen quality parameters which show a relation with fertility, in high fertile breeding boars. The value of these tests is possibly even higher in a population in which less selection is performed, added with other tested parameters from this thesis, but only if standardised and objective assessment methods are used. The conclusions in this thesis contribute to the development of semen quality assessments which improve the prediction of porcine male fertility.

Samenvatting

Van zaadje tot karbonaadje: het begint bij de bevruchting!
Goede resultaten bereiken is niet vanzelfsprekend aangezien de vruchtbaarheid door verschillende factoren beïnvloed wordt. Voor het fokken van varkens wordt bijna uitsluitend gebruik gemaakt van kunstmatige inseminatie (KI). Het voordeel van KI is dat je sperma van goede fokberen kunt doorverdunnen en op deze manier kun je er vele zeugen mee bedienen. De varkensindustrie is in de laatste jaren door schaalvergroting en specialisatie sterk geëvolueerd. Dit betekent ook dat KI organisaties veel waarde hechten aan spermakwaliteit- en vruchtbaarheidgarantie. Goed sperma, op het ideale tijdstip ingebracht in een gezonde baarmoeder leidt bijna altijd tot dracht. Echter geeft het begrip goed sperma ruimte tot onderzoek (een gezonde baarmoeder gaat buiten het aandachtsveld van dit proefschrift). Een goed ejaculaat bevat levende, bewegende sperma cellen. Helaas is er geen gouden standaard voor de eisen waar kwalitatief goed sperma aan moet voldoen. Dit proefschrift behandelt verschillende spermakwaliteit parameters in relatie met vruchtbaarheid.

Hoe bepaal je de vruchtbaarheid bij fokberen?
Het is van belang dat de vruchtbaarheid van fokberen op een correcte manier wordt vastgelegd. Alleen hiermee kun je de relatie tussen spermakwaliteit en vruchtbaarheid goed onderbouwen. Hoofdstuk 1 laat zien dat er wereldwijd veel onderzoek wordt uitgevoerd, maar dat de kwaliteit van de data vaak discutabel is. Er worden bijvoorbeeld slechts enkele dieren gebruikt, van een enkele genetische lijn of de geïnsemineerde zeugen komen maar van één bedrijf. Dit maakt extrapolatie van deze resultaten naar de gehele populatie vaak onmogelijk. In dit proefschrift is data geanalyseerd van zowel spermakwaliteit (resultaten uit het laboratorium) als vruchtbaarheid (resultaten van zeugenbedrijven in Nederland). Momenteel bevat de data > 1 miljoen ejaculaat gegevens en > 8.5 miljoen worpen. In dit proefschrift zijn een tweetal parameters gebruikt om de vruchtbaarheid weer te geven. Afbigpercentage (FR = farrowing rate) geeft het percentage zeugen weer dat biggen geworpen heeft. Toomgrootte (TNB = total number piglets born) geeft het totaal aantal geboren biggen weer.

Een ejaculaat wordt gevangen in de berenstal op een KI locatie. Op het laboratorium wordt de spermakwaliteit beoordeeld en aan de hand van deze metingen wordt het ejaculaat verdund tot gemiddeld 35 inseminatiedoses per ejaculaat. Deze doses worden verspreid naar verschillende bedrijven waar ze worden ingezet om zeugen te insemineren. Doordat de doses van één ejaculaat op verschillende

bedrijven geïnsemineerd worden, ontstaat er veel variatie in vruchtbaarheid resultaten. Om per ejaculaat een vruchtbaarheid resultaat te verkrijgen moet er voor deze factoren gecorrigeerd worden. Bedrijf- en zeuggerelateerde factoren als bijvoorbeeld management, genetische lijn van de zeug, pariteit, het aantal inseminaties, de tijd tussen het spenen van de vorige worp en de eerste inseminatie, de draagtijd, het jaar en het seizoen en de leeftijd van het sperma op moment van insemineren worden meegenomen in de statistische correctie naar één vruchtbaarheid resultaat per ejaculaat. De invloed van deze bedrijf- en zeuggerelateerde factoren valt buiten de doelstelling van dit proefschrift. Maar ook beer- en spermagerelateerde factoren beïnvloeden de variatie in vruchtbaarheid resultaten. Ook voor deze factoren wordt gecorrigeerd, waarbij factoren als genetische lijn van de beer, individuele beer, leeftijd van de beer, dagen tussen ejaculaties, KI station en laborant, aantal cellen in een ejaculaat en aantal cellen in een inseminatie dosis worden meegenomen. Al deze correcties resulteren in de analyses tussen spermakwaliteit kenmerken en vruchtbaarheid van fokberen, die onderzocht zijn in dit proefschrift.

Wat is de waarde van microscopische spermamotiliteit beoordeling?

In Hoofdstuk 2 wordt de eenvoudigste en meest voorkomende manier van spermakwaliteit beoordelen toegelicht: het analyseren van beweeglijkheid van spermacellen. Door getrainde laboranten wordt een spermamonster beoordeeld onder een fase contrast microscoop en daarbij geeft de laborant een score voor motiliteit (mate van beweeglijkheid). De afkapwaardes voor deze scores zijn zeer arbitrair en de microscopische beoordeling is afhankelijk van de ervaring van de laborant en blijft altijd een onderwerp van discussie. De subjectiviteit van deze beoordeling kwam duidelijk tot uiting in de analyse naar de relatie tussen spermamotiliteit en vruchtbaarheid. Uitgebreide dataverzameling resulteerde in 110.186 ejaculaten (1998-2005) met bekende vruchtbaarheid resultaten. Met deze gegevens kon geanalyseerd worden welk percentage van de variatie in vruchtbaarheid wordt veroorzaakt door beer en sperma gerelateerde kenmerken. Dit bleek slechts 6% van de totale variatie in vruchtbaarheid te zijn. Binnen deze variatie werd slechts 4% verklaard door motiliteit. Kortom, spermamotiliteit verklaart slechts een heel klein percentage van de variatie in vruchtbaarheid. Daarbij liet de analyse zien dat KI station (locatie en laborant) een groter deel van de variatie verklaart, waarmee subjectiviteit van de methode werd aangetoond. Bovendien werd nogmaals bevestigd dat het grootste deel van de beer- en

spermagerelateerde variatie in vruchtbaarheid werd verklaard door de genetische lijn van de beer en de invloed van individuele beer binnen deze lijn.

Wat is het voordeel van automatische spermamotiliteit analyse?
Het beoordelen van spermamotiliteit met de microscoop is gebleken zeer subjectief te zijn. Het beoordelen van spermamotiliteit kun je automatiseren met behulp van een CASA systeem. CASA staat voor computer geassisteerde sperma analyse en wordt veel toegepast in zowel de humane als de veterinaire industrie. In 2006 werden deze systemen geïmplementeerd in de KI laboratoria. Hieraan vooraf ging een intensieve implementatieperiode, waarbij een standaard werkwijze is vastgesteld en de laboranten zijn begeleid in het verhogen van de herhaalbaarheid van de metingen. Er moet niet worden vergeten dat CASA een geautomatiseerd systeem is dat beïnvloed kan worden door de laborant die het systeem bedient. Er is daarom eerst een standaard werkwijze vastgesteld, waarbij is gekeken is naar de effecten van temperatuur, homogeniseren, verdunningsfactor, telkamer en daarnaast is het effect van trainingen van laboranten beoordeeld. Door deze training werd bereikt dat de variatie coëfficiënt van de metingen op een niveau kleiner dan 5% kwam. Door verbetering van deze herhaalbaarheid kon ook de toegevoegde waarde van het gebruik van een CASA systeem worden aangetoond, wat naar voren komt in Hoofdstuk 3. Door nauwkeurige resultaten kunnen inseminatiedoses efficiënter geproduceerd worden. De efficiënte en betrouwbare productie van inseminatiedoses is een van de doelstellingen van een KI organisatie.

Is er dan ook een relatie tussen CASA motiliteit en vruchtbaarheid?
Nu dat een KI organisatie met behulp van een CASA systeem op een betrouwbare manier de spermamotiliteit van de ejaculaten kan beoordelen en het vervolgens efficiënt kan doorverdunnen, komt de vraag naar voren welke waarde deze beoordelingen hebben voor het voorspellen van vruchtbaarheid bij fokberen. Met een CASA systeem worden veel gedetailleerde spermamotiliteit parameters gemeten. Veel meer dan het menselijk oog kan, maar tegelijkertijd was de waarde van deze motiliteit parameters in relatie tot vruchtbaarheid nog niet in detail bepaald. Hoofdstuk 4 beschrijft de analyse van 45.532 ejaculaten met bekende vruchtbaarheid gedurende een 3 jarige periode (2006-2009). Van de totale variatie in vruchtbaarheid kon 5-6% worden verklaard door beer- en spermagerelateerde kenmerken. Gedetailleerde

spermamotiliteit kenmerken verklaarde 9-10% van de beer- en spermagerelateerde variatie in vruchtbaarheid. De objectiviteit van het gebruik van een CASA systeem kon worden geconcludeerd uit de afwezigheid van een effect van KI station (locatie en laborant) op vruchtbaarheid. Daarbij kwam opnieuw duidelijk naar voren dat er verschillen zijn tussen genetische beerlijnen. Met deze kennis kun je afkapgrenzen (wel/niet goedkeuren) voor spermakwaliteit beoordelingen nauwkeuriger bepalen en daarmee een nog betrouwbaarder product afleveren.

Wat voegen spermakwaliteit kenmerken geanalyseerd met flowcytometrie toe aan het voorspellen van vruchtbaarheid?
Met het meten van motiliteit worden wellicht andere spermakwaliteit kenmerken over het hoofd gezien. Flowcytometrie is een methode waarbij meer gedetailleerde kenmerken van een spermacel beoordeeld kunnen worden. Denk hierbij aan membranen, acrosomen of mogelijke DNA schade. In Hoofdstuk 5 wordt beschreven hoe deze spermakwaliteit kenmerken zijn geanalyseerd, opnieuw in relatie met vruchtbaarheid. Voor deze studie zijn 20 beren geselecteerd uit 3 genetische lijnen en deze beren zijn gedurende een periode van 20 weken gevolgd, waarbij zowel vers als op 4 meetmomenten (tot 14 dagen na productie) de spermakwaliteit is beoordeeld. Na correctie op zowel bedrijf en zeug, als beer en andere sperma factoren kon geconcludeerd worden dat alleen de DNA schade gemeten na dag 7 na productie van het ejaculaat een relatie vertoond met vruchtbaarheid. Echter is het meten van deze DNA schade een tijdrovende test die moeilijk geïmplementeerd kan worden in de dagelijkse sperma beoordelingsroutine. In de Discussie van dit proefschrift komt dan ook naar voren dat voorafgaand aan implementatie van deze test, eerst een vervolgstudie moet worden gedaan naar de herhaalbaarheid van de metingen en naar de variatie binnen de gehele beerpopulatie. Daarnaast moet een kostenbaten analyse worden uitgevoerd: wat kost het uitvoeren van deze test en wat levert het op als een subfertiele beer wordt geïdentificeerd. Ook kwam uit dit onderzoek heel duidelijk naar voren dat andere spermakwaliteit metingen gericht op de membranen van een spermacel (membraan, acrosoom, mitochondriën) geen relatie met vruchtbaarheid lieten zien. Dit kwam mogelijk omdat de beren en daarmee de ejaculaten die gebruikt worden door de KI organisatie al van een hoog fertiele kwaliteit zijn, waardoor de verschillen in spermakwaliteit geen effect op vruchtbaarheid hebben. Een oorzaak hiervan kan zijn dat deze spermakwaliteit kenmerken compenseerbaar zijn. Er zitten genoeg cellen in een inseminatie dosis die de functie van de slechter

bevruchtende spermacellen kunnen overnemen. Cellen met DNA schade kunnen wel een eicel bevruchten en het effect of vruchtbaarheid is dan niet meer compenseerbaar door andere cellen. Dan zie je dus wel een effect op vruchtbaarheid.

Is IVF een geschikte test als voorspeller voor vruchtbaarheid?
IVF (= *in vitro* fertilisatie) is een techniek die vooral bekend is uit de humane wereld. Echter wordt deze techniek ook in de veterinaire wereld veelvuldig toegepast, zo ook in Hoofdstuk 6 van dit proefschrift. Niet voor het verkrijgen van embryo's, maar voor het bepalen of deze IVF test een geschikte voorspeller voor vruchtbaarheid bij fokberen zou kunnen zijn. Het bepalen van de vruchtbaarheid *in vivo* (in het veld) is afhankelijk van het feit of er wel/niet biggen geboren worden en hoeveel dit er dan zijn. Gezien het feit dat de dracht bij varkens ongeveer drie maanden, drie weken en drie dagen duurt, betekent het dat pas na de drachtperiode bepaald kan worden of de kwaliteit van het sperma en/of andere factoren van invloed zijn geweest op de vruchtbaarheid. Door eicellen te insemineren met spermacellen van fokberen (IVF test) kan al in een eerder stadium bepaald worden wat het effect is van de spermakwaliteit van het betreffende ejaculaat op vruchtbaarheid. Dit is getoetst aan de hand van 3 ejaculaten van 12 beren. Deze beren waren geselecteerd op vruchtbaarheid waarbij de 6 hoogst en de 6 laagst fertiele beren van één genetische lijn zijn vergeleken. Echter kwamen uit deze studie geen verschillen. Dit werd mogelijk veroorzaakt door of het minimale verschil in vruchtbaarheid tussen de beren of door de gecompliceerde IVF techniek waarbij verschillen in resultaten niet direct aan spermakwaliteit konden worden gerelateerd of door het grote aantal geïnsemineerde spermacellen of door een te laag aantal uitgevoerde IVF testen per beer. Echter toonde de studie wel aan dat het gebruiken van IVF als voorspeller voor vruchtbaarheid van fokberen niet geschikt bleek om te implementeren als spermakwaliteit controle.

Conclusie
De Discussie van dit proefschrift gaat verder in op de relevantie van het uitgevoerde onderzoek. De verschillende onderzoeken laten zien dat het effect van spermakwaliteit kenmerken op vruchtbaarheid bij fokberen relatief klein is. Ondanks dat deze effecten klein lijken, is de economische waarde in een hoog producerende varkenshouderij groot, met daarbij de doelstelling om ook de variatie tussen bedrijven te minimaliseren. De wereldwijde varkensindustrie kan veel efficiënter: een berekening van het LEI (2010) laat zien dat als de productie binnen de

varkensindustrie wereldwijd net zo efficiënt zou zijn als in Nederland, dit resulteert in 40% minder varkens. De gevonden relaties tussen spermakwaliteit en vruchtbaarheid bieden uitdagingen en mogelijkheden om deze efficiëntie slag nog verder uit te breiden. In de Discussie wordt ingegaan op de mogelijkheid tot het ontwikkelen van een zogenaamde spermakwaliteit index, waar de verschillende spermakwaliteit parameters, maar ook andere beergerelateerde kenmerken kunnen worden ingewogen. Dit geeft een tool voor spermakwaliteit controle voor een KI organisatie. Testen die gerelateerd zijn aan vruchtbaarheid zorgen voor een efficiëntere productie van inseminatiedoses. Deze efficiënte productie betekent dat je snellere genetische vooruitgang kunt boeken.

CASA motiliteit en DNA schade zijn spermakwaliteit kenmerken die een effect laten zien op vruchtbaarheid bij hoog fertiele fokberen. De waarde van deze testen is wellicht nog veel hoger in een populatie waar minder selectie is toegepast, mogelijk aangevuld met andere getoetste parameters uit dit proefschrift, vooropgesteld dat men gebruik maakt van gestandaardiseerde en objectieve beoordelingsmethoden. De conclusies uit dit proefschrift dragen bij aan de ontwikkeling van spermakwaliteit controles ter verbetering van het voorspellen van vruchtbaarheid bij fokberen.

Dankwoord

"Als je doet wat je leuk vindt, hoef je nooit te werken"
maar ook……
"we zullen dat varkentje wel eens even wassen".

Daar sta ik dan aan het einde van mijn promotie onderzoek. Door de wisselwerking tussen het bedrijfsleven en de universiteit kijk ik met veel plezier terug op het bijzondere traject dat we hebben doorlopen. Ik ben nu het *feestvarken*, maar dit proefschrift kon niet gerealiseerd worden zonder de hulp van veel mensen!

Ik vond het fascinerend om te merken dat mijn inspirerende promotor Arjan Stegeman zo snel het onderwerp kon oppakken en heel direct de knelpunten en aandachtspunten opmerkte. Daar kon ik wat mee. Dit heeft mij zeer geholpen in de voortgang van het onderzoek.

Ik heb de prachtige kans gekregen om in dit unieke promotie traject te stappen, wat is opgezet door Bart Gadella en Hanneke Feitsma. Veel dank wil ik uiten aan mijn co-promotor Bart. Je kennis over spermafysiologie gaat ontzettend ver in detail, maar je kunt er boeiend en vol overgave over vertellen. Vooral toen het zo druk was tijdens het schrijven van wetenschappelijke publicaties ben je me tot grote steun geweest! Op welk tijdstip ik ook back-up nodig had, altijd kreeg ik een antwoord waar ik mee vooruit kon!

Een weg vinden tussen wetenschap en commercie was misschien nog wel de grootste uitdaging in dit traject. Het maakte werk besprekingen interessant en het was een uitdaging een goede middenweg te vinden. Hanneke, je bent een grote steun geweest. Vanaf het begin dat ik bij de KI kwam, heb ik heel prettig met je samengewerkt. Ik bewonder je inzet en passie voor het vak. Heel hartelijk bedankt voor al die jaren hulp en kennis overdracht. Ik heb veel van je geleerd.

Er werken veel meer mensen bij IPG en TOPIGS, die ik in de laatste jaren als goede collega heb ervaren! Vooral met statische hersenbreuk-gevallen kon ik altijd bij het R&D team terecht! Daar weten ze hoe je grote datasets moet aanpakken. Bedankt voor jullie hulp!

Een dag na m'n afstudeerfeestje begon ik in 2005 bij de KI in Vught aan een parttime baan voor een experiment van een paar maanden. Ik ging de uitdaging aan en met dit proefschrift is het tot een mooi einde gekomen. In Vught is er altijd tijd voor een ruim Brabants kwartiertje om een bakkie te doen en bij te praten. Bedankt voor al die gezelligheid! Vervolgens werd ik het CASA meisje, en heb ik bijgedragen aan de implementatie van deze systemen. Nog net niet letterlijk met de machine onder de arm heb ik verschillende malen de KI stations bezocht. Hoewel de implementatie een hele klus was, heb ik het altijd

als uitdaging gezien. Uiteindelijk is het gelukt, maar niet zonder de onuitputtelijke inzet van Jan Koks, alle laboratorium- en ook stalmedewerkers die er bij betrokken waren. Dank voor jullie geduld en hulp.

A special -thank you- for the financial partners in this project: Varkens KI Nederland, Hamilton Thorne Inc., Leja Products B.V., and GFS (in the first years). Thank you for realising the value of this PhD project by supporting my work, I absolutely appreciated your efforts.

Toen ik echt officieel met m'n promotie traject begon, kwam ik in 2007 in Bunnik terecht. Een ideale locatie, dicht bij de Uithof. Een kleine KI locatie, waardoor we erg van elkaar afhankelijk waren. Een hok vol mannen, maar kletsen dat ze konden! Ik kon het ook niet laten, om (soms iets te vaak) een helpende hand uit te steken in het lab. Ik heb het met plezier gedaan, het was een welkome afleiding. Vaak vroeg beginnen (half 5!!), maar gelukkig ben ik een typisch geval van een ochtendmens. Inmiddels bestaat Bunnik niet meer. *Als hadden komt, is hebben te laat*. Ik kijk vooral terug op de fijne tijd!

Veel plezier heb ik ook beleefd op mijn werkplek op de Uithof. Hiemke Knijn, de eerste jaren van mijn promotie traject zat je in de begeleidingscommissie. Praktisch gezien kon ik je altijd om raad vragen. Jammer dat je deze taak niet kon volbrengen vanwege je nieuwe baan bij CRV! (leuk dat we nu weer naast elkaar werken!). Edita Soštarić, je nam deze functie met liefde over, dat straal je ook uit. Je bent zorgzaam en hebt altijd tijd voor een praatje. Elke bespreking discussieerde je trouw mee over de voortgang van het project. Je was werkelijk betrokken, het was voelbaar dat het gemeend was.

In de wondere IVF wereld werd ik wijzer gemaakt door Eric Schoevers. Je gedachtegang over de actualiteiten in politiek en muziek maakte je als kamergenoot een welkome afleiding van het vele laptopwerk. Bovendien zijn jouw drumsessie (of ze nou op de drums of op de rand van de tafel zijn) van grootse waarde! En dan het sperma lab! Waar een variatie aan apparatuur te vinden is, met een doorlopende vraag om hulp waar je altijd op antwoord van Mabel Beitsma en Arend Rijneveld kunnen rekenen. Wie wil er nu niet in zo'n inspirerend lab maandenlang haar metingen uitvoeren? Bedankt dat ik zoveel ruimte in beslag mocht nemen, maar vooral ook hartelijk dank voor jullie hulp! Op de 2^{e} verdieping van het JDV lopen zoveel mensen rond. Waar moet ik nou beginnen? Vooral de vaste mensen wil ik niet vergeten, maar ik kan ze haast niet allemaal bij naam noemen. Dus ik hou het algemeen: dank voor jullie gastvrijheid. Het is fijn te merken dat er nog zoveel behulpzaamheid bestaat: daar kan de rest van de wereld nog van leren! Tijdens m'n IVF experimenten kon ik elke week rekenen op jullie hulp bij

het puncteren van eierstokken. Ook de rest van de BRC onderzoeksgroep: hartstikke leuk om met jullie samengewerkt te hebben in de afgelopen jaren. Deze contacten zijn me zeer dierbaar.

Cecile Veiga, I certainly do not want to forget you. When I almost drowned in all the flow cytometry measurements I had to perform per day, you were there! I really enjoyed the time we have spent together at the laboratory. Thanks again for all your efforts!

Lezers van dit dankwoord zijn toch op zoek naar de mens achter de auteur. Daarbij realiseer ik me ook dat dit ongetwijfeld het meest gelezen stuk van mijn proefschrift zal zijn, met name door familie en vrienden die niet altijd iets (konden) begrijpen van mijn "sperma proeven".

"Ik leef om te werken of ik werk om te leven"

Soms was er bijna geen verschil, maar dan is het fijn te merken dat er zoveel mensen om je heen staan, die je af en toe eens uit deze werksfeer trekken. *Een echte vriend(in) is degene die altijd raadt wanneer je hem nodig hebt*. M'n familie en vrienden zijn me allemaal heel dierbaar, ik ben hen veel dank schuldig en daarom wil ik er een aantal in het bijzonder noemen.

De dames van Ve"Nu"S, nog steeds sinds onze studententijd houden we contact, ondanks dat we naar alle windstreken zijn getrokken. Ik dank jullie hartelijk voor onze bijklets momenten. Daniëlle, Dorine en Marijn: wat moeten we toch zonder elkaar. We hebben verdrietige, maar vooral ook hele mooie momenten met elkaar gedeeld. *Goede vrienden zie je niet, zij staan achter je.* Geniet met jullie gezinnen van het leven! Zo ook de vriendschap met Karin: vele baantjes gezwommen, creabea geknutseld, kilometers geskeelerd of gewandeld (wie weet worden we ooit nog wel eens ingeloot voor de Vierdaagse): het kletsen maakte dat het nog meer energie kostte. We overlappen in onze interesses en hebben dezelfde kijk op het leven: dat geeft die hele goede band!

Een schoonfamilie krijg je vanzelf. Maar ik heb niks te klagen, want ik werd liefdevol ontvangen door de familie Willemsen. Piet, Roelie, Rob en Nathalie, Pieter, Albert: ik kwam met veel plezier bij jullie thuis, nog steeds natuurlijk! Bedankt voor de gezellige tijd, de vele maaltijden die ik bij jullie heb genuttigd en voor de spekjes die ook bij jullie altijd klaar staan. Mélanie, je gaf veel afleiding in het weekend! Ik heb geleerd van een steeds wijzer wordende kleine meid, met soms wat streken, maar vooral heel veel lieve gebaren. Zoals het spreekwoord zegt: *ze bevindt zich tussen big en zwijn*!

Ik ben in een fantastisch gezin opgegroeid. (Jan-)Willem, je bent m'n kleine, grote broer. Wij zijn zo verschillend dat we toch weer hetzelfde zijn. Ik kan altijd om je lachen, jouw humor brengt gezelligheid als we elkaar weer zien. Ik hoop dat je samen met Desiree net zo gelukkig blijft als dat je nu bent. M'n ouders, Wim en Maria, hebben me opgevoed volgens hun geweldige normen en waarden. Ik ben trots op wat ik bereikt heb, maar dit had ik niet zonder jullie kunnen waarmaken. Jullie hebben me de kans gegeven om te studeren en jullie hebben me laten zien dat je van het leven, samen met de mensen om je heen, iets bijzonders moet maken. Pa en ma, bedankt! Dit is de perfecte plek om dat nog weer eens een keer aan jullie te uiten. Jullie basis heeft me gemaakt tot wie ik nu ben. Ook daarom heb ik jullie gevraagd om mijn paranimfen te zijn: jullie hebben me groot gebracht, en ik voel me groots om voor de commissie te staan, met jullie aan m'n zijde.

En dan tot slot… *Eigenwijs zijn en een eigen wijsheid hebben*. Twee mooie karaktereigenschappen. Ik zie het terug in mezelf, maar heb het ook gevonden in de liefde. Jef, ik kijk terug op onze goede vriendschap in Bunnik, vooral de bakkies op de vrijdagochtend (met de wijze les: thee hoeft niet op koffie te lijken…). Dat het uiteindelijk, nadat je al lang weg was uit de KI, uitgroeide tot meer, maakte het niet minder gecompliceerd. Maar wij zouden dat *varkentje wel eens wassen*, ook toen we samen aan onze Boekvink-klus begonnen. Inmiddels is het een echte beestenboel, waar ik me zeer thuis voel! Jef, je zet me af en toe weer met beide benen op de grond, dat heb ik nodig. Je relativeert en je geeft mij niet altijd gelijk. Een weerwoord heb ik nodig en krijg ik van jou! Zowel privé als werk, onze passies overlappen. Ik dank je dan ook heel erg voor je geduld, de afleiding en je liefde die nodig was voor mij om dit proefschrift succesvol af te ronden.

Samen een toekomst in, *tomorrow is a mystery*!

Tot slot wil ik iedereen meegeven…..

Herinner je gisteren
Droom van morgen
maar Leef vandaag

Curriculum Vitae

About the author

Maria Leonarda Wilhelmina Johanna (Marleen) Broekhuijse was born on September 13th, 1982 in Utrecht and raised in De Meern. After finishing high school at St. Gregorius College in Utrecht in 2000 she started her study on Animal Sciences at Wageningen University. During her studies she did a minor on vigilance behaviour in rabbits (Resource Ecology) and a major on Para tuberculosis in cattle (Qualitative Veterinary Epidemiology in cooperation with Animal Health Service). In her last year she went to New-Zealand, studying sub-clinical parasitism in deer (Massey University). After her graduation in 2005, she started working for Institute for Pig Genetics B.V. on developing new extenders and other small trials (under supervision of Hanneke Feitsma) and she was involved in the implementation of Computer Assisted Semen Analysis (CASA) systems at the laboratories of Varkens KI Nederland and Varkens KI Twenthe. This work lead to starting a PhD in cooperation with the department Biology of Reproductive Cells of the faculty of Veterinary Medicine of Utrecht University and at the same time working for Varkens KI Nederland (under supervision of Bart Gadella en Hanneke Feitsma). After finishing her work, she started working for CRV Holding B.V., again on semen quality characteristics assessed with CASA and flow cytometry systems, although this time with bull semen.

Over de auteur

Maria Leonarda Wilhelmina Johanna (Marleen) Broekhuijse werd geboren op 13 september 1982 in Utrecht en groeide op in De Meern. Na het behalen van haar VWO diploma aan het St. Gregorius College in Utrecht, startte ze in 2000 met haar studie Dierwetenschappen aan de Wageningen Universiteit. Tijdens haar studie deed ze een afstudeervak naar gedrag van konijnen (Resource Ecology) en ze deed een afstudeervak naar paratuberculose bij rundvee (Qualitative Veterinary Epidemiology in samenwerking met de Gezondheidsdienst voor Dieren). In het laatste jaar ging ze naar Niew-Zeeland voor een studie naar subklinisch parasitisme in herten (Massey University). Na haar afstuderen in 2005 begon ze bij Institute for Pig Genetics B.V. met onderzoek naar de ontwikkeling van nieuwe verdunners en andere kleine onderzoeken (onder begeleiding van Hanneke Feitsma) en ze was betrokken bij de implementatie van Computer Assisted Semen Analysis (CASA) systemen op de laboratoria van Varkens KI Nederland en Varkens KI Twenthe. Dit werk leidde tot het starten van het promotie onderzoek in samenwerking met de vakgroep Biology of Reproductive Cells van de faculteit Diergeneeskunde van de Universiteit Utrecht, waarbij ze tegelijkertijd werkte voor Varkens KI Nederland (onder hoofdbegeleiding van Bart Gadella en Hanneke Feitsma). Na beëindiging van haar werk, is ze begonnen bij CRV Holding B.V., opnieuw in onderzoek naar sperma kwaliteit kenmerken beoordeeld met CASA en flowcytometrie systemen, al betreft het nu sperma van stieren.

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