FACULTY OF VETERINARY MEDICINE
Department of Reproduction, Obstetrics and Herd Health

Fresh boar semen: quality control and production

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“So one must not be childishly repelled by the examination of
the humbler animals. For in all things of nature there is something
wonderful… so one must approach the inquiry about each
animal without aversion, since in all of them there is something
natural and beautiful “

(Aristóteles In: De Partibus Animalium)
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<th>Description</th>
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<tr>
<td>ABN TAIL</td>
<td>Percentage of Spermatozoa with Tail Abnormalities</td>
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<tr>
<td>AI</td>
<td>Artificial Insemination</td>
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<td>ALH</td>
<td>Amplitude of Lateral Head Displacement</td>
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<td>ALP</td>
<td>Alkaline Phosphatase</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ASMA</td>
<td>Automated Sperm Morphology Analysis</td>
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<td>AST</td>
<td>Amino-Transferase</td>
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<td>BCF</td>
<td>Beat Cross Frequency</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>BTS</td>
<td>Beltsville Thaw Solution</td>
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<td>Ca</td>
<td>Calcium</td>
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<td>CASA</td>
<td>Computer Assisted semen Analysis</td>
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<td>CI</td>
<td>Confidence Interval</td>
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<td>Cl</td>
<td>Chloride</td>
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<td>CONC</td>
<td>Concentration</td>
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<td>CP</td>
<td>Crude Protein</td>
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<td>CV</td>
<td>Coefficient of Variation</td>
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<td>DIST</td>
<td>Percentage of Spermatozoa with Distal Cytoplasmic Droplet</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DFI</td>
<td>DNA Fragmentation index</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>FACS</td>
<td>Fluorescent Activated Cell Sorter</td>
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<td>FITC</td>
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<td>GA</td>
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<td>GGT</td>
<td>( \gamma )-glutamyl-transferase</td>
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<td>GPx</td>
<td>Glutathione peroxidase</td>
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<td>HEPES N-2-</td>
<td>Hydroxyethylpiperazine-N’-2-Ethanesulfonic Acid</td>
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<td>HOST</td>
<td>Hypo-osmotic swelling test</td>
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<td>HTR</td>
<td>Hamilton-Thorne Semen Analyzer</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>K</td>
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<td>LIN</td>
<td>Linearity</td>
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<td>Term</td>
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<td>MDA</td>
<td>Malondialdehyde</td>
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<td>MEDIUM%</td>
<td>Percentage of Spermatozoa with Medium Velocity</td>
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<td>P</td>
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<td>PM</td>
<td>Progressive motility</td>
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<td>PROGR%</td>
<td>Percentage of Progressively Moving Spermatozoa</td>
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<td>PSA</td>
<td>Pisum Sativum agglutinin</td>
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<td>PUFA</td>
<td>Polyunsaturated Fatty Acids</td>
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<td>r</td>
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<td>Percentage of Rapidly Moving Spermatozoa</td>
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<td>SD</td>
<td>Standard deviation</td>
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<td>Se</td>
<td>Selenium</td>
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<td>SEM</td>
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<td>SLOW%</td>
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<td>Sperm Motility Index</td>
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<td>Seminal Plasma</td>
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<td>SQA</td>
<td>Sperm Quality Analyzer</td>
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<td>STATIC%</td>
<td>Percentage of Static Spermatozoa</td>
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<td>Straightness</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>TBA</td>
<td>Thiobarbituric Acid</td>
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<td>TM</td>
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<td>VAP</td>
<td>Velocity Average Pathway</td>
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<td>VSL</td>
<td>Velocity Straight Line</td>
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<td>VCL</td>
<td>Velocity Curvilinear</td>
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<td>WHO</td>
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<td>Zn</td>
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<td>ZP</td>
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Chapter 1. General Introduction
Chapter 1.1. Boar semen quality analysis
1.1.1. Introduction

During the last decades, the use of porcine semen for artificial insemination (AI) by means of fresh diluted semen has increased considerably (Maes et al., 2011; Riesenbeck, 2011). Compared to natural mating, AI reduces the risk of disease transmission (Maes et al., 2008), it allows the introduction of superior genes into sow herds and additionally it leads to a better profitability of each boar ejaculate. Therefore, AI has become a very useful tool in countries with intensive pig production. In Western Europe, more than 90% of the sows have been bred by AI for more than two decades (Vyt et al., 2007a; Riesenbeck, 2011). Semen is obtained from boars either on farm or from specialised AI-centres. The latter offer a diversity of breeds and genetic lines and distribute ready-to-use semen doses of constant quality to different sow herds. In addition, modern production systems without weekly inseminations discourage the on-farm semen production.

The fertilizing potential of a semen dose is inherently linked to the quality of the spermatozoa (Vyt et al., 2008; Tsakmakidis et al., 2010). Examination of the ejaculates before AI is therefore absolutely required. Conventional methods for semen quality analysis such as visual examination under light microscope are cheap and easy to perform. However, conventional semen quality parameters only give a rough idea of the fertility potential of a given ejaculate. More sophisticated methods may be more suitable to elucidate subtle differences in semen quality between highly selected boars with high semen quality (Waberski et al., 2011a).

The role of seminal plasma (SP) in males has recently also received increasing interest. Several biomarkers such as peptides and proteins have been identified in the SP of boars and they may be related to fertility. Therefore, they could be of interest for semen quality analysis (Rodriguez-Martinez et al., 2008; Rodriguez-Martinez et al., 2010; Dyck et al., 2011).

The present chapter will review and critically discuss the state of the art on boar semen quality analysis. Traditional semen quality analysis as well as several more recently developed advanced techniques will be discussed.

1.1.2. Volume and concentration

The volume is routinely measured by weighing the ejaculate considering 1 gram equal to 1 mL.

Ready to use doses for AI are supplied in 80-100 ml packages containing
approximately 3 x10^9 spermatozoa (Martin-Rillo et al., 1996; Alm et al., 2006). The variation in concentration between breeds and individuals is evident (Johnson et al., 2000; Kommisrud et al., 2002) and it should be considered when preparing semen doses.

The number of spermatozoa should be adapted according to the morphological or motility characteristics and it is generally admitted that a fertile dose should contain at least 2-3 x 10^9 spermatozoa (Martin-Rillo et al., 1996; Alm et al., 2006). However, to maximize semen dose production, AI-centres tend to dilute the ejaculates as much as possible for evident economic purposes (Vyt et al., 2007a). Research during the last years has focussed on the reduction of the number of spermatozoa per dose without jeopardizing fertility results. Therefore, insemination strategies have been developed that require lower doses. Using intrauterine insemination, acceptable fertility results have been obtained with doses of 1 x 10^9 spermatozoa (Roca et al., 2003; Roca et al., 2011).

Visual evaluation of the opacity of the raw ejaculate only gives a subjective and rough idea of the sperm concentration. Microscopic evaluation using different reusable glass chambers (Figure 1) allows counting of immobilized spermatozoa in a grit with a known volume. Within the reusable glasses, haemocytometers are considered as the gold standard (Rijsselaere et al., 2003; Prathalingam et al., 2006; World Health Organization, 2010). However, many different haemocytometers such as Neubauer, Thoma or Bürker, are commercially available (Christensen et al., 2005). Variations ranging from 4% to 20% in haemocytometer counts have been observed. Consistent miscounts can partly be attributed to improper sub-sampling, improper pipetting and filling of counting chambers, together with under or over dilution of the sample (Hansen et al., 2002; Knox, 2004). Other reusable glass chambers such as the Mackler chamber (Figure 1) are used for assessing concentration as well as motility (Tomlinson et al., 2001). Both reusable chambers and several disposable low depth chambers (Figure 1) are used for computer assisted semen analysis to study both motility and concentration (Bjorndahl and Barratt, 2005).

Disposable chambers seem to have a lower coefficient of variation but they underestimate the sperm concentration compared to reusable chambers (Tomlinson et al., 2001; Bjorndahl and Barratt, 2005; Christensen et al., 2005). The debate on which is the most accurate type of chamber for assessing semen concentration is still open (Christensen et al., 2005; Maes et al., 2010). In any case, visual determination of semen concentration by either reusable and disposable chambers is rather time consuming since it requires the counting of a relatively high number of immobilized spermatozoa to achieve an acceptable level of precision (Christensen et al., 2005; World Health Organization, 2010). Therefore,
new techniques such as photometer, computer assisted semen analysis (CASA) or flow cytometry have been developed during the last years to achieve fast and accurate sperm counts (Vyt et al., 2008; Maes et al., 2010).

![Image of different semen concentration chambers]

Figure 1: Different chambers for assessment of semen concentration: A) reusable Makler chamber; B) reusable Bürker counting chamber (haemocytometer); C) disposable Leja chamber.

**Colorimeters and photometers**

During the last years, most AI-centres have introduced photometers (single wavelength) or spectrophotometers (multiple wavelengths) to assess semen concentration (Vyt et al., 2007a; Knox et al., 2008). These methods measure the optical density, *i.e.* the relative absorption and scattering of a light beam that is sent through a semen sample. The absorption and scattering is proportional to the sperm concentration. Photometry is commonly used in practice because it is fast and easy to perform but gel particles and debris can be confounded with spermatozoa resulting in an overestimation of the sperm count (Knox, 2004). Improper sampling, pipette error, the type of cuvette, incorrect dilution or the wavelength and many other factors may bias photometer counts (Knox, 2004). Currently there are several photometers on the market based on different optical systems and acquisition modes that partially explain differences between devices (Camus et al., 2011). The light sources (*i.e.* pre-adjusted halogen lamp or LED), the way in which light is dispersed (*i.e.* by a prism or optical fibre), the wavelengths or the number of readings per analysis are different between photometer models (Camus et al., 2011).

Very recently, Camus et al. (2011) compared different photometers with other methods for semen concentration. The photometers had a lower coefficient of variation (CV) and a higher repeatability compared to CASA, nucleocounter and haemocytometer and differences were explained by the extra dilution needed for the latter methods. Curiously, the gold standard for semen concentration (the haemocytometer) appeared to be the least repeatable and the least precise from all the evaluated methods. However, all
methods showed an acceptable agreement in the counts and can be used routinely in AI-centres. Accurate dilution and a correct calibration curve (adapted to each AI-centre) appear imperative to obtain reliable results (Knox, 2004; Camus et al., 2011).

**Computer assisted semen analysis (CASA)**

Besides detailed motility analysis, CASA systems additionally measure sperm concentration by means of image analysis of semen within a counting chamber (Verstegen et al., 2002; Prathalingam et al., 2006). CASA is considered to be an objective method, but many factors may influence the outcome regarding CASA concentration. The accuracy of these systems depends not only on the optical properties and the software settings but also on the technician and the type of chamber used for the analysis as well as the method for filling the chamber (Rijsselaere et al., 2003; Vyt et al., 2004b; Kuster, 2005). Thin, capillary-filled, disposable chambers are generally found to underestimate sperm concentration due to the Segre–Silberberg effect (Kuster, 2005). This is a theory based on the different flow dynamics of different depths of chambers. According to this theory, particles accumulate in the meniscus of the fluid entering a low depth chamber (Douglas-Hamilton et al., 2005; Kuster, 2005) (Figure 2). As a consequence, measuring concentration in the centre of a field may underestimate the actual sperm numbers. This explains why CASA systems generally underestimate the concentration compared to haemocytometers. The Segre–Silberberg effect is less pronounced in deeper (100-mm-depth) chambers such as for instance the improved Neubauer haemocytometer (Douglas-Hamilton et al., 2005). To compensate for this effect, compensation factors have been proposed (Douglas-Hamilton et al., 2005). In addition to the Segre–Silberberg effect, CASA-systems may also underestimate the concentration compared to the haemocytometer (Bürker chamber) because of clumping of the sperm cells (Maes et al., 2010). On the other hand Rijsselaere et al. (2003) and Vyt et al. (2004) reported that CASA may also overestimate concentration readouts due to debris and count of gel particles in the semen.

**Flow cytometry for semen concentration**

The use of flow cytometry to determine semen concentration has increased during the last years, mainly in research laboratories since it requires expensive equipment which is not economically profitable for AI-centres. A fluorescent activated cell sorted (FACS) flow cytometry has been tested in the last years and it seems to provide accurate
information (Hansen et al., 2002; Christensen et al., 2004). The main advantage of flow cytometry is that it allows counting of a large number of sperm in a short period of time (less than 1 minute) (Christensen et al., 2004). However, flow cytometry may interpret the actual concentration wrongly because of not being able to distinguish between debris, gel particles or unstained sperm (Petrunkina and Harrison, 2010).

Figure 2: A) Schematic representation of Segre Silberberg effect: velocity is higher in the centre of the chamber (blue arrows) and very low near the chamber walls (red arrows) and the sperm tends to accumulate in the meniscus; B) air bubble (arrow) in a slide for CASA analysis; C) sperm agglutination in a slide for CASA analysis

Other methods for assessment of semen concentration

Nucleocounters are counting chamber based instruments used for determining sperm concentration providing similar counts as those obtained with photometers (Camus et al., 2011). When using these devices, DNA is fluorescently labelled and counted by image analysis resulting in an accurate determination of sperm concentration.

A recent approach in human andrology has shown that it is possible to obtain
accurate measurements of semen concentration using a micro fluidic chip (Segerink et al., 2010). The system measures the impedance of sperm passing an electrode pair in a micro channel. Depending on the impedance, measurements semen concentration can be calculated.

1.1.3. Morphology and vitality

Semen of boars with poor sperm morphology will result in lower pregnancy rates and reduced litter size (Table 1) when used for AI insemination (Alm et al., 2006; Tsakmakidis et al., 2010) and morphology must therefore be analysed to identify subfertile boars. Sperm morphology aberrations are typically classified as primary, secondary or tertiary abnormalities (Donadeu, 2004). The first group comprises abnormalities in the shape of the head (Figure 3) which damage the genetic material or abnormalities of the mitochondrial sheet that would impair flagella function. Proximal and distal (Figure 3) cytoplasmic droplets are considered as secondary abnormalities. Morphological anomalies acquired by inappropriate handling of semen (e.g. coiled tails) are considered as tertiary abnormalities. Secondary and tertiary abnormalities but not primary anomalies can be compensated by increasing the number of sperm per dose (Donadeu, 2004). Even though the exact cut-off for a fertile ejaculate is still under discussion, some established criteria are accepted nowadays and at least 80% of normal morphology is considered necessary for a fertile dose of 2x10^9 spermatozoa per dose (Martin-Rillo et al., 1996; Shipley, 1999).

Membrane integrity is an indicator of sperm vitality and it is necessary to maintain sperm function. Many handling procedures such as dilution or storage at low temperatures, both for liquid storage and for cryopreservation, may damage the sperm membrane impairing fertility. It is therefore imperative to evaluate this parameter to assess boar fertility and storage effects (Leahy and Gadella, 2011; Waberski et al., 2011a).

Sperm staining

A first estimation of morphology can be obtained by looking at an unstained semen smear under contrast light microscopy. However, there are several staining methods such as Papanicolaou, Eosin-nigrosin (Figure 3), Trypan Blue, Giemsa, Diff-Quik or SpermBlue® that provide much more accurate information (Dott and Foster, 1972; Kruger et al., 1996; Shipley, 1999; van der Horst and Maree, 2009). Among these staining methods, the Papanicolaou® stain is considered as the standard by the World Health Organization (World Health Organization, 2010) for human semen analysis. It can
additionally be used for automated sperm morphology analysis (ASMA) (Coetzee et al., 2001). However the Papanicolau stain is very time consuming as it includes more than 20 steps and more than 12 different chemical solutions (van der Horst and Maree, 2009) which make it not suitable for use in porcine AI-centres. The eosin-nigrosin staining is widely used for boar semen analysis because it is easy to perform, it allows morphological and membrane integrity examination (Figure 3) and its outcome correlates with sow fertility (Bjorndahl et al., 2004; Tsakmakidis et al., 2010). Studies in human have shown that many factors such as the type of diluter used to prepare the staining and the time of exposure to it may affect the outcome of the eosin-nigrosin staining (Bjorndahl et al., 2004). The ideal staining should be simple (a single staining solution), osmotically adapted to semen, be able to stain different components of the sperm and discriminate artefacts. Furthermore it should be applicable to different species and be compatible with automated sperm morphology analysis (ASMA) (van der Horst and Maree, 2009).

Figure 3: Boar semen dose for artificial insemination and slides for eosin nigrosin examination (A); eosin staining of spermatozoa with abnormal head (B, arrow), eosin staining of spermatozoa with distal cytoplasmic droplet (C, arrow)

**CASA-morphometry (ASMA)**

Compared to staining techniques that only provide a general idea of normal vs. abnormal sperm, ASMA provides detailed morphological characteristics of the sperm head, midpiece and flagella of different species including porcine (Thurston et al., 1999; Verstegen et al., 2002; Peña et al., 2005; Rijsselaere et al., 2005). These systems provide
detailed information on the dimensions (length, width, and perimeter) of the different parts of the sperm that might help differentiate sperm subpopulations within an ejaculate (Thurston et al., 1999; Saravia et al., 2007; Gil et al., 2009). The outcome of morphometry analysis is associated with some semen quality parameters that are related to fertility. Morphometry of the head and midpiece of boar sperm by means of ASMA has been shown to be correlated with sperm motility but not with sperm chromatin integrity (Saravia et al., 2007; Gil et al., 2009). Whether morphometry analysis itself can be associated with fertility is under discussion.

During the last decade, several new CASA systems with software for ASMA analysis are commercially available (Verstegen et al., 2002; Rijsselaere et al., 2004; Hidalgo et al., 2006; Saravia et al., 2007). Computer assisted sperm morphometry analysis is normally performed on a slide with stained sperm. The staining technique as well as the background contrast is of high importance to obtain accurate results (Hidalgo et al., 2006). Therefore although objective information can be obtained with ASMA, standardization of the procedures is required in order to compare results. Moreover ASMA analysis can be time consuming being necessary up to 25 min per sample (Rijsselaere et al., 2004).

**Fluorescent dyes**

Several studies use fluorescent dyes that stain intact or damaged spermatozoa differently. These dyes can be measured in the sperm cell population by directly counting using a fluorescence microscope or by a flow cytometer (Ericsson et al., 1993; Althouse and Hopkins, 1995; Christensen et al., 2004). The SYBR14-PI stain is commonly used in porcine andrology research and it allows identifying three cell populations (*i.e.* live, dead, and moribund spermatozoa) compared to the conventional nigrosin/eosin stain that only discriminates between two groups (*i.e.* live and dead spermatozoa) (Garner and Johnson, 1995).

Fluorescent dyes allow not only studying membrane integrity but they make it possible to assess semen concentration simultaneously. When combining different probes, membrane and acrosome integrity together with mitochondrial function can be simultaneously assessed (de Andrade et al., 2007). To obtain an accurate count with a fluorescence microscope, a large number of spermatozoa must be counted which makes it very time consuming. Flow cytometry however allows counting thousands of sperm in a very short time. On the other hand, this device is sometimes not able to discriminate interference from gel particles resulting in overestimation of unstained sperm and
underestimation of stained sperm (Petrunkina and Harrison, 2010). Mathematical calculations have been proposed to identify non-sperm particles during flow cytometry analysis (Petrunkina et al., 2010).

The need for qualified personnel for using a fluorescence microscope or flow cytometry excludes the practical use of this technique in commercial AI-centres, although they are widely used in porcine research laboratories and universities (de Andrade et al., 2007; Petrunkina et al., 2010).

**Hypo-osmotic swelling test (HOST)**

The way in which sperm swell when they are submitted to hypo-osmotic stress (due to the influx of water) can be observed and measured to test the membrane (functional) integrity (Vazquez et al., 1995). This phenomenon is more easily observed in the sperm tail than in the head because the plasma membrane surrounding the tail appears to be more loosely attached (Jeyendran et al., 1984; Takahashi et al., 1990; Vazquez et al., 1997).

Although the correlation between the Hypo-osmotic swelling test (HOST) and other vital stains such as eosin-nigrosin, Trypan Blue, PI-CFDA, and SYBR-PI is weak, the osmotic resistance of the porcine sperm cells was correlated with fertility results (Vazquez et al., 1997; Perez-Llano et al., 2001; Foxcroft et al., 2008). This is logical because HOST measures activity of the membrane (ability to regulate flux of electrolytes and non-electrolytes) whereas a vitality staining only refers to the integrity of the membrane (Vazquez et al., 1997; Foxcroft et al., 2008).

Difference in sperm cell volume can be also measured by detecting voltage changes when cells pass a capillary pore in a CASY cell counter, a computerized method (Petrunkina et al., 2004).

### 1.1.4. Motility

**Subjective motility assessment**

Motility is known to be an important characteristic in predicting the fertilizing potential of an ejaculate (Vyt et al., 2008) (Table 1). Although inseminated spermatozoa are brought to the fertilization site (the oviduct) mainly by uterine contractions (Langendijk et al., 2002), a high motility is required for the sperm at the fertilization site to reach and penetrate the oocyte. Vyt et al. (2008) showed that a 1% increase in motility in the diluted semen was related to an increase of 0.14 piglets per litter. Motility rates higher than 60% are accepted to comply with the minimum requirements for a fertile ejaculate (Martin-Rillo
et al., 1996; Britt et al., 1999; Donadeu, 2004). Visual assessment of motility remains an acceptable method and is preferred by AI-centres over other methods mainly because of economic reasons. However, visual assessment, although consistent if performed by the same technician (Vyt et al., 2004b), requires special training and there is a large variability between technicians (Rijsselaere et al., 2003; Vyt et al., 2004b; Tejerina et al., 2008).

**Computer Assisted Sperm Analysis (CASA)**

Objective motility counts with CASA systems are based on the capture of multiple digital images from which individual sperm tracks can be reconstructed and different motility parameters can be calculated by the incorporated software (Verstegen et al., 2002; Rijsselaere et al., 2003; Vyt et al., 2004b). This way, different motility patterns can be observed, e.g. progressive movement, hyperactivity of spermatozoa and different subpopulations of spermatozoa within an ejaculate can be demonstrated (Verstegen et al., 2002; Vyt et al., 2004b; Peña et al., 2005; Rijsselaere et al., 2005). The detailed information on motility and velocity patterns of the sperm might be useful to identify slight differences between highly selected boars used for AI (Tejerina et al., 2008). Additionally, the capacitation status of the spermatozoa can be studied by means of CASA. It has been shown, for instance, that porcine sperm undergoing capacitation-like changes show high average path velocity (VAP) and low linearity (Garcia et al., 2005).

Several studies from our research group have shown that CASA systems provide consistent and reliable results for different species (dogs: Rijsselaere et al., 2002; pigs: Vyt et al., 2004; cattle: Hoflack et al., 2005; cats: Filliers et al., 2008; horses: Hoogewijs et al., 2011). Nevertheless the information obtained by CASA is still subjected to external factors such as sample preparation or type of chamber used for the analysis (Figure 2). Moreover trained personnel and standardized procedures are necessary for a reliable use of CASA (Verstegen et al., 2002; Rijsselaere et al., 2003; Feitsma et al., 2011). Although CASA provides reliable motility measurements, the relation between CASA outcome and fertility is still under discussion (Holt et al., 1997; Vyt et al., 2008; Broekhuijse et al., 2011a; Broekhuijse et al., 2011b) (Table 1). Holt et al. (1997) found an association between some velocity parameters and fertility. Similarly, Vyt et al. (2008) demonstrated a relation between the percentage of motile sperm as determined by CASA and litter size (Table 1). In an extensive field data analysis (Table 1), the percentage of motile sperm as determined by CASA was positively associated with the farrowing rate and litter size (Broekhuijse et al., 2011a; Broekhuijse et al., 2011b).
Nowadays many different CASA systems are commercially available. The suitability of CASA systems for use in AI-centres has been recently discussed (Feitsma et al., 2011). It was concluded that an economical evaluation for each AI-centre should be performed before implementing such systems. Furthermore properly trained personnel as well as standardized procedures are absolutely necessary to obtain reliable and consistent results (Feitsma et al., 2011).

**Sperm Quality analyzer (SQA)**

The SQA systems convert variations in optical density into electrical signals to determine sperm concentration and motility. These electronic signals are analysed by the SQA software algorithms and converted into sperm quality parameters. The effectiveness of different SQA systems for sperm analysis has been studied both in humans and animals, and different algorithms are needed for each species. A previous version of the SQA namely the SQA-IIC was consistent and suitable for the estimation of boar semen quality (Vyt et al., 2004b). There appeared to be a good correlation between the sperm motility index (SMI) obtained by SQA-IIC and several CASA parameters, especially with the percentage of motile sperm ($r=0.71; p<0.05$) and the straight line velocity (VSL) ($r=0.64; p<0.05$). However, the SQA-IIC is based on a rather old technology originally meant for use in human sperm analysis. Furthermore, SMI values provide global information of the quality of the semen, and do not discriminate between concentration, morphology and motility parameters.

**1.1.5. Other semen quality parameters**

**Membrane function**

During storage, reorganization of the sperm membrane lipids occurs leading to membrane destabilization that may cause sub-lethal changes in the sperm membrane. These changes could therefore affect the sperm function (Holt and Van Look, 2004; Petruhkina et al., 2007; Rodriguez-Martinez and Barth, 2007). Tests for the membrane function could detect these sub-lethal changes that may show up before membrane damage is detected by staining (Petrunkina et al., 2005; Hossain et al., 2011). For this technique, capacitation is induced by calcium ionophore and the amount of intracellular calcium is measured using a fluorescent probe which is able to stain intracellular calcium (Petrunkina et al., 2005). Membrane destabilization results in alteration of the Ca flow. Therefore, measuring intracellular calcium under capacitating conditions can be used to detect altered
membrane function (Petrunkina et al., 2005; Waberski et al., 2011a).

Table 1: Recent studies that showed none or positive association of sperm motility and/or morphology with *in vivo* fertility.

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of boars</th>
<th>Number of inseminations</th>
<th>Motility analysis</th>
<th>Morphology analysis</th>
<th>Association with fertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alm et al. (2006)</td>
<td>50</td>
<td>10773</td>
<td>-</td>
<td>Giemsa</td>
<td>NM</td>
</tr>
<tr>
<td>Vyt et al. (2008)</td>
<td>38</td>
<td>276</td>
<td>CASA</td>
<td>Eosin</td>
<td>None</td>
</tr>
<tr>
<td>Didion et al. (2008)</td>
<td>208</td>
<td>6266</td>
<td>CASA</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>Tsakmakidis et al. (2010)</td>
<td>7</td>
<td>1350</td>
<td>-</td>
<td>Eosin</td>
<td>None</td>
</tr>
<tr>
<td>Broekhuisje et al. (2011)</td>
<td>Unknown^a</td>
<td>Unknown^a</td>
<td>CASA</td>
<td>-</td>
<td>None</td>
</tr>
</tbody>
</table>

Normal morphology (NM); total motility (TM); progressive motility (PM); Straight line velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF), amplitude lateral head displacement (ALH).^a^study including 45532 ejaculates obtained from several AI-centres in the Netherlands over a period of 3 years.

**Acrosome integrity**

An intact acrosome is necessary for the penetration of the oocyte and therefore its integrity is considered vital for optimal fertilising capacity. Similar to membrane integrity, the acrosome integrity can be evaluated by means of fluorescent probes. Different probes based on lectins such as *Pisum Sativum agglutinin* (PSA) which are conjugated to a fluorescein isothiocyanate (FITC) are used in andrology research. These lectins bind specifically to the acrosomal content by interacting with the saccharide groups of the glycoprotein pro-acrosin (Peña et al., 1999). The acrosome integrity can be assessed by the staining pattern of sperm cells observed by fluorescence microscopy (Figure 4) or by means of flow cytometry (de Andrade et al., 2007). A triple labelling technique that allows assessing the membrane and acrosome integrity together with mitochondrial function has been tested in boar semen with good results (de Andrade et al., 2007). In combination with vitality staining, flow cytometry allows counting large numbers of particles but it is prone to miss-estimations (Petrunkina and Harrison, 2010).

**Sperm binding capacity**

Sperm binding to the zona pellucida (ZP) is necessary for the process of fertilization. Basically the sperm zona binding assay consists of incubation of spermatozoa
with oocytes followed by counting of the number of spermatozoa attached to the ZP. Although it has been shown that mainly boar sperm with intact acrosome initiate binding to ZP (Fazeli et al., 1997), the association of this test with \textit{in vivo} fertility is sometimes contradictory (Waberski et al., 2005).

Binding of spermatozoa to oviductal epithelium has also been studied in pigs. It can therefore be considered as an indicator of membrane integrity and of the ability of sperm to form a sperm reservoir in the caudal oviduct. Consequently it could be used as a marker of boar fertility (Waberski et al., 2005).

**Figure 4**: A) Fluorescent image of boar sperm cells stained with SYBRR-14/PI showing membrane intact (green), damaged membrane (red) or moribund cells (dual stained cells); B) PisumSativum agglutinin (PSA) conjugated to a fluorescein isothiocyanate staining (FITC-PSA) of intact and reacted (unstained, arrow) acrosome of boar spermatozoa.

\textit{Lipid peroxidation}

Boar spermatozoa are very sensitive to lipid peroxidation because of the high content of polyunsaturated fatty acids, the substrate of reactive oxygen species (ROS) (Bathgate, 2011). During storage, boar sperm undergoes lipid peroxidation that will result in destabilization of the sperm membrane that may affect sperm motility and acrosome integrity (Awda et al., 2009; Kumaresan et al., 2009; Radomil et al., 2011). Additionally, some sperm abnormalities like protoplasmic droplets are associated to higher lipid peroxidation as well as motility and acrosome integrity (Brouwers et al., 2005). Lipid peroxidation can be assessed with an indirect method by measuring the production of thiobarbituric acid reagent substances (TBARS) like for example malonaldehyde (Ohkawa et al., 1979) (Figure 5). During the last years, new methods based on fluorescence have
been developed and staining kits like for example the BODIPY are frequently used in andrology research to study sperm oxidative stress. The latter probe incorporates into biological membranes and responds to oxidation with a spectral emission shift from red to green. These changes can be measured by flow cytometry (Brouwers et al., 2005). Because lipid peroxidation seems to be one of the main causes of sperm damage during storage, recent interest has focussed on possible antioxidants that could be added to the feed or directly to semen extenders in order to protect sperm (Brouwers et al., 2005; Malo et al., 2011; Radomil et al., 2011).

Figure 5: Different steps for Thiobarbituric Acid Reactive Substances (TBARS) assay. Lipid peroxidation is induced by ferrous sulphate and sodium ascorbate (A) resulting on malonaldheide (MDA) production; MDA will react with Thiobarbituric Acid (TBA) originating TBARS with pink chromogen (B) that can be quantified with a spectrophotometer (C).

**DNA fragmentation**

Damage in the sperm DNA structure seems to be associated with low fertilization rates and reduced litter size (Evenson et al., 1994; Boe-Hansen et al., 2008). The sperm DNA can be damaged during spermatogenesis as well as during semen processing and storage (Boe-Hansen et al., 2005). There are many different tests to assess DNA integrity like the Comet assay, transferase mediated dUTP nick-end labeling (TUNEL), the sperm chromatin structure assay (SCSA) or the sperm chromatin dispersion test (SCD). The DNA fragmentation index (DFI) obtained by SCD seems to correlate with sperm motility, normal acrosomes, normal sperm morphology and cells positive to HOST (Perez-Llano et al., 2006). However, study results on the association of boar semen integrity and fertility are sometimes contradictory. Whereas some authors found that the DFI obtained by means of SCSA is related to fertility rate and litter size (Evenson et al., 1994; Boe-Hansen et al.,
2008), other studies on sperm from normospermic boars has shown that there is no relationship between DFI and sub fertility (Waberski et al., 2011b).

**Sperm proteome**

During the last years the interest for sperm proteomic profiles has increased aiming to identify sperm components related to capacitation and sperm function (Aitken and Baker, 2008). In human reproduction, several peptides have been identified and suggested to be involved in capacitation pathways as well as with sperm maturation during passing through the epididymis (Aitken and Baker, 2008). Additionally, the proteomic profile of sperm from fertile and infertile men seems to be different (Pixton et al., 2004; Zhao et al., 2007). The research on this area in porcine reproduction is also increasing and some attempts have been conducted to find sperm proteins associated with protection against oxidative stress or against heat shock during storage (Dube et al., 2004; Spinaci et al., 2005; Gonzalez-Fernandez et al., 2009; Belleeannee et al., 2011). Nevertheless, proteome profiling is a very complex process that is not suitable for routine analysis in AI-centres.

1.1.6. **Seminal plasma components**

The seminal plasma (SP) is a mixture of fluids from the cauda epididymidis and the accessory sexual glands (Davies et al., 1975). During many years, SP has been considered only as a medium to transport spermatozoa and it has been suggested that it can be substituted by an extender without exerting any detrimental effects. In some species, ejaculates are centrifuged and the SP discharged because it could have detrimental effects on the spermatozoa during storage (Rodriguez-Martinez et al., 2011). In boar semen, the SP is lost in the high dilution rate used to produce semen doses. However in the last years, several studies have shown that addition of SP may have beneficial effects on sperm maturation and sow fertility (Foxcroft et al., 2008; Rodriguez-Martinez et al., 2011).

Mixing the sperm from low fertility boars with SP from boars with high fertility resulted in improved *in vivo* fertility (Flowers, 1997). The use of pooled semen samples is a common practice in porcine AI and semen of good boars could mask the poor fertility of bad boars (Foxcroft et al., 2010). Whether the SP plays a role improving the fertility of bad boars when semen is pooled is not known. In all cases this practice should be avoided because it makes difficult to identify poor fertility boars and the full potential of a very fertile ejaculate is diluted when mixed with a poor fertility ejaculate (Foxcroft et al., 2010).

Moreover, addition of SP to boar semen seems to reduce detrimental effect of both
liquid and frozen storage over sperm cells. Dilution of semen in commercial extenders with 10% or 20% added SP from a pool, significantly improved motility and acrosome integrity after 5 days of liquid storage compared to semen diluted without SP. Semen was diluted to 1 or 0.5 x 10^9 sperm/80 ml and the effect was more expressed in the 0.5 x 10^9 compared to 1x10^9 group, suggesting a protective effect of SP against high dilution rates (Weitze et al., 2011). Furthermore, addition of seminal plasma reduced the dilution effect at different stages during cryopreservation and has a beneficial effect on sperm motility and vitality of frozen-thawed semen (Rodriguez-Martinez et al., 2008; Saravia et al., 2009). In a recent study, thawed semen incubated in 50% SP had increased percentages of live sperm and percentage of sperm motility compared to incubation in either 0% or 10% SP (Garcia et al., 2010).

Different roles have been attributed to SP (Foxcroft et al., 2008; Rodriguez-Martinez et al., 2011). It modulates sperm function and its interaction with epithelia and fluids of the female reproductive tract (Rodriguez-Martinez et al., 2008; Rodriguez-Martinez et al., 2011). Additionally, SP stimulates the female immune system to clear pathogens and to tolerate spermatozoa and embryos, and it could also influence female behaviour as it happens with other species. In insects, SP is responsible for physiological post-mating changes that, among others, seem to increase eggs production, modulate sperm storage or decrease receptivity to re-mating and modify feeding behaviour (Avila et al., 2011).

The research in the last years has shown that SP has a protective effect over sperm motility and vitality and plays an important role on the sperm interaction with the female tract (Foxcroft et al., 2008; Rodriguez-Martinez et al., 2011; Dyck et al., 2011). Therefore SP components could be indicators of boar fertility potential and the exact composition of the SP and the specific roles of each of its components need to be investigated. The mechanisms behind these beneficial effects of the SP are under study and recently there is an increasing number of research studies performed on SP proteomics (Rodriguez-Martinez et al., 2011). Some SP proteins seem to be related with sperm resistance to oxidative stress and temperature shock and others mediate the interaction of sperm with the female reproductive tract (Foxcroft et al., 2008; Rodriguez-Martinez et al., 2011). Spermadhesins like porcine seminal plasma proteins help maintain membrane stability and are involved in capacitation, sperm zona binding and sperm-oocyte interaction (Petrunkina et al., 2001; Liberda et al., 2006; Dyck et al., 2011). Other SP proteins regulate sperm motility or will serve as decapacitation factors.
Despite several proteomic studies, it is not yet known which SP components could be used as fertility predictors in the pig. In human semen, specific biochemical parameters are included in the routine semen quality analysis (World Health Organization, 2010). Enzymes like for example acid phosphatase and minerals such as zinc are believed to be indicators of prostatic health in human, and therefore the WHO manual warrants their investigation. In stallions, an association between SP enzymes and minerals and semen quality has been observed (Pesch et al., 2006). In boars, no basic studies are available on the presence or absence of specific minerals and enzymes in SP. One study has described low levels of alkaline phosphatase (ALP) in SP of one infertile boar, due to an obstruction of the ductuli efferentes, but no reference values are available on these biochemical parameters in boar semen (Clements et al., 2010). Moreover, minerals in the SP like for instance selenium have known antioxidant properties and zinc is known to help on the stabilization of sperm chromatin (Bjorndahl and Kvist, 2010). Although these biochemical parameters could be related to semen quality and fertility, research in this area is scarce for boar semen.

1.1.7. Concluding remarks

The practical relevance of many of these techniques is limited due to the fact that most of the research on porcine semen is based on the semen from good performing boars. Sub fertile or infertile boars are rapidly culled because of economic considerations, and therefore, there is a lack of information regarding sperm quality of infertile boars. Moreover fertility in sows depends on many factors and therefore in vivo trials to find associations between semen quality with fertility are scarce and the results are difficult to interpret. However, this should not stop us from doing research to find indicators of fertility. Additionally, many of these tests require trained personnel and expensive equipment and they are therefore not suitable for AI-centres. To overcome this problem, external quality controls have been proposed (Waberski et al., 2008). In this manner, AI-centres can routinely submit semen samples to external specialized laboratories to monitor in detail the reproductive health of their boars.
Chapter 1.2. Critical steps during fresh semen production
1.2.1. **Managing the boar: factors affecting semen production and quality**

*Boar selection*

Appropriate boar selection is crucial for an AI-centre. Breeding soundness examination should be performed before introduction of boars in production (Shipley, 1999). Clinical examination of the boar, inspection of the male genitalia, the display of a positive attitude towards the dummy and a proper investigation of semen quality should all be considered as crucial factors in boar selection (Shipley, 1999).

For obvious reasons, the customer demands semen from genetic lines that will give offspring with good potential for meat production thus with good growth efficiency, fast growth rate and lean carcass composition. Recently, it was suggested that selection of boars to improve growth rate may have a negative effect on semen quality as was described in other species such as chicken (Robinson and Buhr, 2005). Although there is no information available in this regard in boars, it has been observed that chickens selected for fast growth between 14 and 42 days, had a decreased fertility compared to non-selected chickens (Barbato, 1999). A negative correlation between exponential growth and reproductive phenotypes was described in the latter study.

In the last years it has also been suggested that, besides good attitudes for semen production and quality, some genetic markers should be investigated in boars before introducing them in production. Genetic selection of boars that will accomplish sexual maturity earlier or of boars with higher resistance to heat stress as well as selection of boars with semen more resistant to liquid or frozen storage may be possible (Flowers, 2008).

*Boar housing and climate conditions*

The pens where AI boars are housed must be animal welfare friendly and, according to the European legislation (Commission directive 2001/93/EC), they must have at least 6 m² of unobstructed floor area available and the construction must allow the boar to turn around and to hear, smell and see other boars. Although the type of housing for mature boars might not have a direct impact on semen quality, it may affect boar health. A non-slippery floor must be provided in order to avoid leg problems. When solid floors are used, bedding (straw or wood shavings) should be added in order to keep the boar comfortable and dry (Althouse, 2007). When bed is supplied, routine cleaning is necessary and remainders of bedding in the ventral abdomen of the boar should be cleaned before
semen collection to avoid bacterial contamination of the ejaculate (Althouse, 2008). Boar feed can be supplied by automated or manual drop feeders and the daily amount of feed is mainly determined by boar body condition and boar weight. Water is delivered by nipples (Knox et al., 2008). Moreover the type of boar housing must be safe for the personnel and should allow easy handling of the boars (Althouse, 2007).

Although adult boars are housed in individual pens, it has been shown that group housing of growing boars is beneficial for subsequent reproductive performance. Groups of 8 boars from 30 kg housed in pens of 4m x 4.3m until they successfully completed two mountings, had on average stronger legs for jumping, higher libido, earlier accomplishment of the first mating and higher sperm counts compared to boars housed individually (Hacker et al., 1994).

Besides an appropriate pen construction, the environment must also be adapted to the boars’ requirements. The ancestors of the current pigs were seasonal breeders with one litter/year. The European wild boar, which is already closer related to the current pig breeds has a decreased fertility when days become shorter and a reduced semen quality during summer months. Photoperiod seems therefore important for the regulation of boar reproduction. However, the role of photoperiod on semen quality is controversial. Berger et al. (1980) showed that boars kept under natural light plus artificial light supplementation (10-500 lux) to maintain constantly 15h of light/day from 11 weeks of age until puberty (24-26 weeks), had a faster sexual maturation and a higher libido than boars receiving only natural light during that period (15h at 11 weeks to 9h at the end of the trial). However, there was no effect on semen quality. A more recent study showed that submission of boars to either 24h of artificial light or 24h of complete darkness for a period of 3 months had a negative effect on semen volume and concentration, especially when boars were submitted to complete darkness (Sancho et al., 2006). In the latter study, there was a reduction in semen volume and concentration after one month of exposure to 24h of light or of complete darkness but, after 3 months, semen volume and concentration seemed to return to the values before treatment. The authors suggested that boars were able to adapt to these extreme photoperiods. However, similarly to other studies, photoperiod did not affect sperm motility or vitality (Berger et al., 1980; Trudeau and Sanford, 1986).

It is very likely that not only photoperiod but also heat stress during long summer days may influence semen quality. Boars exposed to 34.5°C for 8 h and 31.0°C for 16 h daily for 90 days had lower sperm motility and sperm morphology as well as reduced fertility compared to control boars maintained at 23.0°C (Wettemann et al., 1976).
stress might also have an indirect effect on semen quality via reduced feed and protein intake. A lower protein intake could lead to a decreased semen quality, although the effect on semen quality is not clear either as will be discussed later in this chapter. Moreover not only constant heat stress but also fluctuations in temperature between the day and the night may decrease fertility (Kunavongkrit et al., 2005). Such stress will increase corticosteroid levels which could have a detrimental effect on semen quality. To overcome heat problems, AI stables should be equipped with refrigeration systems that are normally based on evaporative or mechanical cooling (Kunavongkrit et al., 2005; Knox et al., 2008).

Until now there is no research performed on the influence of air quality, ammonia or other gas concentrations on semen quality but it is obvious that a good air quality is imperative for the comfort and welfare of the boar.

*Collection pen*

Similarly to the housing pen, the collection pen must be safe for boar and employees and should allow a fast processing of many boars (Levis and Reicks, 2005; Althouse, 2007). To avoid injuries, dummy sows should be solid in construction without sharp edges, and located in a quiet designated semen collection room with a non-slippery floor (Althouse, 2007). Automation of the collection line allowing almost hand free collection has been recently developed (Aneas et al., 2008). The system that includes, among other features, pneumatic opening of access doors and electronic identification of collector and boar, has been proven to increase the number of boars processed/collector/hour.

The design of the collection pen will also influence boar sexual behaviour (Levis and Reicks, 2005). Sexual behaviour traits that can be measured are for example duration of time between entering the collection pen and mounting the dummy, the number of mounts before the start of ejaculation and the duration of ejaculation time (Levis and Reicks, 2005). Although the sexual behaviour will influence the number of boars collected/hour, there is no relationship between sexual behaviour and semen quality (Levis and Reicks, 2005). Boars that have a sexual stimulus seem to complete collection faster thus resulting in more boars processed in a shorter period of time. Additionally these boars seem to have higher sperm counts (Hemsworth and Galloway, 1979; Levis and Reicks, 2005). Boars can be sexually stimulated just before collection by allowing them to see other boars in action with the dummy. This is possible when a so-called warm up area is available prior to the collection pen. Boar stimulation with prostaglandins (PGF2α) has
also been studied but, apart from a tendency to a reduced time to the start of ejaculation and a longer duration of the latter, no effect was observed on sperm counts or semen quality (Estienne and Harper, 2004).

Lack of hygiene during collection will result in bacterial contamination of the ejaculate and subsequently of the semen doses (Althouse et al., 2000; Althouse and Lu, 2005; Althouse, 2008). Bacterial contamination may cause a decrease in semen quality by direct effect of bacteria or by indirect action of bacterial by-products on sperm. This could result in sperm agglutination and loss of sperm motility (Althouse et al., 2000; Althouse and Lu, 2005; Althouse, 2008). Bacterial contamination may also decrease the longevity of the semen during storage. Therefore bacterial control strategies have been proposed which include regularly cleaning of the boar and the collection pen as well as good and strict hygiene practice by personnel (Althouse, 2008). Prior to collection, the ventral abdomen of the boar should be washed and dried if any dirt is present (Althouse, 2008). Additionally the hair surrounding the preputial orifice must be trimmed on a regular basis because it could result in bacterial contamination (Althouse, 2008).

Collection frequency

Generally semen collection from boars in AI-centres is performed approximately 2 times per week (Vyt et al., 2007a). It is known that a high frequency of collection has a negative effect on semen quality because sperm is forced to rapidly pass from caput to cauda of the epididymis thus having insufficient time for epididymal maturation (Strzezek et al., 1995). More recently, it was demonstrated that submitting boars to collection 4 days in a row affected the re-absorption/secretion pattern of fluids in the lumen of epididymis (Pruneda et al., 2005). This imbalance in the secretion of fluids which is necessary for sperm maturation resulted in an increase of abnormal sperm and a reduction in sperm motility.

Boar nutrition

Feed general composition

Studies of the influence of feeding on semen quality of boars are limited and the current knowledge is mainly based on old studies (for review, see Kemp and Soede, 2001). Besides the possible effect of diet on semen quality, boar nutrition will additionally affect the health and the performance of the boar at the time of mounting. Boars with an excessive weight for example, could have problems when mounting the dummy and are
more prone to leg problems. Excessive growth before puberty will result in excessive weight and incomplete skeletal development with consequent leg problems and difficulties to mount the dummy. Therefore feed restrictions are necessary (Kemp and Soede, 2001). It has been shown that boars fed from weaning to puberty with 70% and 50% of the National Research Council (NRC) requirements compared to boars fed 100%, had lower weight and delayed puberty. However, apart from a decrease of 30% in semen volume, no effect of feed restriction was observed on sperm concentration, motility and morphology or infertility of inseminated sows (Dutt and Barnhart, 1959). Once the boar is introduced in production at 7-8 months of age, feeding level only seems to have an effect on libido and sperm counts when severe restrictions are applied. When 13 week old boars were fed during 15 weeks at a low feed level (1.92 kg/day) a decrease of 69 and 46% was observed in the number of ejaculated sperm cells compared to boars fed at high level (5.74 kg/day) and at medium level (3.62 kg/day), respectively (Kemp et al., 1989). However, feed restriction did not have a negative effect on sperm motility or vitality nor on sow fertility. In this study, boars receiving the low feed level had lower sperm counts during restriction but they were able to recover after the amount of feed was increased to medium levels. Similarly, Louis et al. (1994) proved that reduced energy intake (25.5 MJ ME/day versus 32.2 MJ ME/day) results in a reduction in libido and sperm output. This negative effect could be compensated by increasing proteins level. In their review, Kemp and Soede (2001) concluded that feed levels below 1.4 times maintenance will have a negative effect on sperm output and/or libido.

Protein restriction of 12.0% crude protein (CP) compared to 18.0 or 23.0% CP during the growing period also resulted in a delayed puberty and a decrease of ejaculated sperm with 50% but it did not affect semen quality (Uzu, 1979). The adequate protein level in feed for boars already in production is controversial and based on old data and mainly from studies investigating amino acids supplementation. Whereas Poppe et al., (1974) found a positive effect of synthetic lysine (12g L-lysine/boar/day) and synthetic methionine (16g DL-methionine/boar/day) supplementation on sperm production of boars submitted to a high collection frequency compared to non supplemented boars, Kemp et al. (1988) could not find such an effect independently of semen collection frequency. It seems that only severe deficiencies in proteins in the diet will affect boar libido and sperm output but with no effect on semen quality as was described above for feed and energy intake (Kemp and Soede, 2001).
Chapter 1.2

**Antioxidants**

Although the current energy and protein requirements are based on old data, there has been an increasing amount of research being performed on different feed supplemetations in the last years. Special attention has been paid to antioxidants since it is believed that one of the main sperm damages during liquid storage is caused by lipid peroxidation of the membrane lipids (Leahy and Gadella, 2011; Radomil et al., 2011; Waberski et al., 2011a). The boar sperm membrane is rich in omega-3 polyunsaturated fatty acids (n-3 PUFA), especially docosahexaenoic acid (DHA) (Rooke et al., 2001; Castellano et al., 2010). Different studies have shown that sperm lipid composition can be modified by feed supplementation with n-3 PUFA (Rooke et al., 2001; Castellano et al., 2010). However whether this manipulation of the membrane lipid composition has an effect on sperm resistance to storage is under discussion. Rooke et al. (2001) found that tuna oil supplementation (30 g tuna oil/kg diet) during 6 weeks improved sperm motility, acrosome integrity and morphology. In contrast, Castellano et al. (2010) found no effect of supplementation during 6 months with tuna oil (60 g/boar/day) on semen quality or quantity compared to supplementation with hydrogenated animal fat (62 g/boar/day) or menhaden oil (60 g/boar/day).

The protective effect of other feed supplementations like rosemary or flaxseed against sperm lipid peroxidation during liquid and frozen semen storage has been studied recently with positive results (Malo et al., 2011; Radomil et al., 2011).

Minerals also play a role in the protection of sperm. Selenium (Se) is likely to be involved in the production and maturation of sperm for it can be found in high concentrations in the testes and epididymis of boars (Marin-Guzman et al., 1997; Marin-Guzman et al., 2000; Lasota et al., 2004). As it happens with feed intake and proteins, an adequate amount of this mineral is necessary during sexual development (Kolodziej and Jacyno, 2005). Additionally Se is a structural component of glutathione peroxidase (GPx), an enzyme present in boar sperm which protects cellular and sub-cellular membranes against peroxidation (Flohe et al., 1973; Rotruck et al., 1973; Lasota et al., 2004; Jelezarsky et al., 2008). Although GPx is present in the sperm and Se supplementation seems to increase its activity (Marin-Guzman et al., 1997), it is not clear whether sperm GPx actively protects against lipid peroxidation since it has been suggested that the enzyme loses activity when it incorporates in the sperm membrane (Ursini et al., 1999). In boars, supplementation with 0.5 ppm of a basal diet containing 0.63 ppm Se from weaning
to 9 months resulted in higher sperm motility and less abnormal sperm than the boars fed the non-supplemented basal diet (Marin-Guzman et al., 1997). In the latter study higher fertility rates were observed in gilts inseminated with semen from the boars fed the supplemented diet. Unfortunately, apart from Se, research on the effect of other minerals on boar semen quality is scarce. Typically, minerals in animal feed are included in the inorganic or organic form but very little is also known on the effects of different sources of minerals on male reproductive performance and sperm quality.

The association of some vitamins such as L-carnitine or Vit E with semen quality has also been investigated in the last years. Supplementation with L-carnitine (625 mg/boar/day) for instance, improved sperm morphology from Piétrain boars when photoperiod and temperature increased but this beneficial effect was not observed in Duroc and Large white boars (Yeste et al., 2010). Vitamin E works together with Se to protect sperm against lipid peroxidation and therefore an adequate combination of these two elements is necessary (Marin-Guzman et al., 1997; Marin-Guzman et al., 2000). In another recent study, supplementation of a mix of different fat and soluble vitamins did not protect sperm quality of boars submitted to high collection frequencies (Audet et al., 2009).

1.2.2. Managing the ejaculate: factors related to semen handling

Semen collection

Semen collection in AI-centre is normally performed by the gloved handed technique (Vyt et al., 2007a; Knox et al., 2008). Polyvinyl gloves can be used; latex gloves should be avoided as these are toxic for the semen (Ko et al., 1989). The end of the penis is grabbed firmly with a gloved hand and the collection process is initiated with firm pressure to the spiral end of the penis with the hand so that the penis cannot rotate. This process imitates the pressure applied by the corkscrew shape of the sow’s cervix. A pre-warmed (38°C) collection container is used to avoid rapid cooling of the ejaculate (Knox et al., 2008; Maes et al., 2011). The top of the container is covered with cheesecloth to filter out gel portion of the semen. The first part of the ejaculate (pre-sperm) should be discarded. It is a clear, watery fluid and does not contain sperm (~25 ml), but it may have a high bacterial count (Althouse, 2008). The sperm-rich fraction should be collected (40-100 ml). It is very chalky in appearance and contains 80-90% of all sperm cells in the ejaculate. Once the sperm-rich fraction is complete, the remainder of the ejaculate is again a clearer, watery fluid which should not be collected (70-300 ml). The ejaculation lasts up to 5 to 8
min, but may continue up to 15 min. About 100 to 300 ml of semen is routinely collected.

**Dilution procedures**

After collection, the filter with gel should be discarded, and the collection container should be placed in warm water. After ejaculation, sperm motility and vitality will only be retained for few hours (Johnson et al., 2000). To prolong sperm survival their metabolic activity must be inhibited by chemical inhibitors or by lowering the temperature and therefore the ejaculate needs to be extended shortly after collection (Johnson et al., 2000).

Compared to semen of other animal species, boar sperm is very susceptible to temperatures below 15ºC, due to a different composition of the phospholipids in their membrane (De Leeuw et al., 1990). The temperature of the ejaculate at the moment of collection is approximately 37ºC and of approximately 32-35ºC at arrival in the laboratory where it is processed (Waberski, 2009). Fast cooling of the ejaculate from body temperature to temperature below 15ºC will result in lipid phase separation that will alter the sperm membrane permeability with subsequent loss of sperm vitality (De Leeuw et al., 1990; Johnson et al., 2000). These changes in membrane permeability will result in calcium influx into the sperm cells that would stimulate capacitation like changes (Johnson et al., 2000; Petrunkina et al., 2005). Recent studies have shown that indeed capacitation like changes are the main reasons for the damage of sperm cells when submitted to cold shock (Petrunkina et al., 2005; Leahy and Gadella, 2011). Therefore, the temperature of the semen must be diminished gradually to avoid cold shock. Most of the AI-centres agree on a two-step dilution in which semen is first diluted (1:1) with preheated extender (~33°C) and subsequently diluted in either a preheated extender or an extender kept at room temperature (Vyt et al., 2007; Waberski, 2009). However, the dilution protocols and the temperature of extender for each dilution vary between AI-centres (Vyt et al., 2007a). Research on the effect of different dilution temperatures on semen quality is limited (Waberski, 2009) although this topic could have economic impact on AI semen production. It has been suggested that acclimation at 30ºC for several hours had a protective effect for samples to be stored at 17ºC (Pursel et al., 1972a; Pursel et al., 1972b). However, more recently a negative effect of acclimation at 32ºC was suggested arguing that, by keeping sperm closer to the physiological temperature, the sperm does not diminish its metabolism leading to changes impairing semen quality, based on in vitro response to capacitation assays (Petrunkina et al., 2005). Cold shock can also occur during long transports especially during winter and precautions are to be taken to avoid temperature fluctuations
Storage media

The media used for liquid storage are necessary in order to prolong sperm survival providing energy to the cells, buffering the pH of the suspension and avoiding the growth of bacteria (Johnson et al., 2000; Vyt et al., 2004b). There are many different semen extenders for boar semen claiming protection for short and long term storage (Vyt et al., 2004b). A detailed composition of frequently used extenders for boar semen can be found in the reviews of Johnson et al. (2000) and Gadea (2003).

Extenders should have a balanced composition of ions to maintain the osmotic pressure as well as energy sources (Johnson et al., 2000). The ions in the media for liquid boar semen are merely sodium bicarbonate and sodium citrate and are simultaneously used as buffer. Glucose is the main source of energy and it will also contribute to osmotic equilibrium. Increase in pH during storage has been observed and it was negatively correlated with sperm motility (Vyt et al., 2007b). Therefore buffering systems to stabilize the pH are requested.

EDTA is added for its chelating properties to delay the initiation of capacitation (Watson, 1995). Long-term extenders differ from short-term extenders mainly by the use of complex buffering systems (HEPES, Tris), mostly in addition to the bicarbonate buffering system, and by the presence of Bovine Serum Albumin (BSA) (Althouse et al., 1998; Murase et al., 2010). The latter has a positive influence on sperm survival due to the absorption of metabolic bacterial products from the extender (Waberski et al., 1994; Johnson et al., 2000). Cysteine is used as a membrane stabilizer (Johnson et al., 2000) inhibiting capacitation. Extender-concentrates are normally diluted in distilled or de-ionized water that is in most cases purchased by the AI-centre (Knox et al., 2008). Next to the microbiological quality of the water, the electrolyte content, especially the absence of calcium ions, is important.

Bacterial contamination proved to be detrimental for semen quality as it will cause sperm agglutination and reduced motility (Althouse and Lu, 2005). Antibiotics to prevent bacterial overgrowth are therefore commonly added to boar semen extenders to reduce bacterial concentration as well as to reduce the effect of bacterial toxins (Okazaki et al., 2010). In the context of careful use of antimicrobials, less use of antimicrobials in diluted semen may help to prevent antimicrobial resistance. To this respect single layer centrifugation of boar ejaculates can reduce bacteria concentration and consequently
reduce the use of antibiotics in semen extenders (Morrell and Wallgren, 2011). As it has been explained before in this chapter, good hygiene practices and water of high quality are imperative to avoid bacterial contamination (Althouse, 2008).

The effect of short versus long term extenders on semen quality and sow fertility has been investigated in different studies. According to in vitro and in vivo studies, most extenders in the market provide an acceptable sperm vitality protection during the first 72 hours of storage, although some differences were observed regarding fertility of semen stored during more than 4 days (Kuster and Althouse, 1999; Vyt et al., 2004b; De Ambrogi et al., 2006; Haugan et al., 2007).

Packing and storage

After dilution is completed, diluted semen is introduced in packages of 80-100 mL to be stored and distributed. This processing is made by automated systems in most AI-centres. Different containers such as plastic bottles, blisters, tubes or Gedis® can be used for storage (Figure 1), delivery and insemination of extended semen doses (Vyt et al., 2007a; Knox et al., 2008). A recent study of our group showed that packaging in blisters preserved boar semen better than Gedis® during liquid storage (de Jong et al., 2010).

Recent research has shown that sperm can be encapsulated in capsules of barium alginate that will protect them from damage during handling. The concentration of each capsule is ejaculate-dependent and they are inseminated in a conventional way giving good fertility results (Faustini, 2011).

Further storage of diluted semen is done at 17°C (Figure 1), at which temperature semen metabolism is reduced (Althouse et al., 1998), a condition necessary to extend the storage time. Different temperatures of storage have been studied in the past Althouse et al. (1998) established the critical lower temperature for sperm survival at 12°C whereas storage at 15-17°C showed no detrimental effect on sperm motility and vitality. The mechanism behind the aging of sperm during storage is studied in the last years by means of new semen quality assays and it seems related among others to lipid peroxidation and changes in the fluidity of the sperm membrane with entrance of calcium that will start capacitation like changes (Radomil et al., 2011; Leahy and Gadella, 2011; Waberski et al., 2011a).
1.2.3. Concluding remarks

Despite the new research on antioxidant and vitamins supplementation in the diet of AI boars, the current knowledge on boar nutrition is mainly based on old studies. Since then, new feed supplements including organic forms of minerals are available and might improve the longevity of fresh semen during storage. Therefore more research on this topic is warranted, also because major progress has been made in the sperm assessment techniques like for example CASA. It is possible that subtle changes in semen quality due to a different feed composition or feeding levels can now be detected whereas this was not the case with the conventional semen assessment techniques mainly used in the past.

Each step during semen processing needs to be controlled in detail, especially temperature fluctuations as cold shock destabilizes the sperm membrane. Although the critical temperature for sperm damage is established at 12°C, the effect of moderate changes in temperature must be also investigated. Dilution at room temperature could for instance simplify semen processing if it does not negatively affect semen quality.
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Chapter 2. Aims
Objective and accurate analysis of semen concentration and motility is absolutely required to prepare semen doses of controlled quality for porcine AI. The discussion on which are the best methods for semen concentration and motility is still open. Furthermore, validation of new devices coming to the market is necessary.

In combination with the traditionally assessed sperm quality parameters, other semen parameters such as biochemical components of the seminal plasma (SP) may be an extra tool to better identify boars of low fertility. Analysis of SP composition could therefore be a tool for routine quality analysis of boar semen.

Several factors can affect boar semen production and quality both before and after production of the ejaculate. Literature indicates that research on boar feed is very scarce. During processing, boar semen suffers oxidative stress and therefore feed supplementation with antioxidants like e.g. selenium could improve semen quality and subsequent preservation. Moreover, there is a lack of standardization in the protocols for semen handling in AI-centers especially regarding the dilution temperature.

The specific objectives of the present thesis were to:

- Compare different methods for semen concentration and motility analysis.
- Validate the semen quality analyzer for pigs (SQA-Vp).
- Provide reference values for enzymes and minerals of the seminal plasma in boars and to study their association with semen quality.
- Study the effect of different sources of selenium (organic vs. inorganic) in the diet of boars on semen quality.
- Study the effect of dilution temperature on porcine semen quality and subsequent storage.
Chapter 3. Boar Semen Quality Analysis
Chapter 3.1. Boar semen quality analysis: a comparison of methods

Adapted from:
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3.1.1. Contents

The Sperm Quality Analyzer (SQA-Vp) was evaluated for assessing concentration and motility of porcine semen. Both fresh and diluted semen from 50 different boars from a commercial AI-centre were investigated. For the fresh ejaculate, the concentration obtained with SQA-Vp was compared with a photometer and a haemocytometer. For the diluted samples, the concentration and motility were compared with computer assisted semen analysis (CASA) and visual sperm analysis. The agreement between methods was studied with Bland-Altman plots and the repeatability with coefficient of variation (CV) as well as Bland-Altman plots. The sperm concentration (x10^6/ml) obtained with SQA-Vp (379.3 ± 134.9) for fresh ejaculates agreed well with concentration by the photometer (447.2 ± 154.2; difference = -67.9 x 10^6/ml; difference + 2SD= 55.3 x 10^6/ml; difference – 2SD= -191.1 x 10^6/ml) and with the haemocytometer (332.8 ± 141.1; d= 92.8; d + 2SD= 448.6; d - 2SD= -263.0). For diluted semen, the agreement between the concentration (x10^6/ml) assessed with SQA-Vp (20.4 ± 4.3) was good with CASA (23.2 ± 5.8; d= -2.8; d + 2SD = 6.2; d - 2 SD = -11.8) but poor with the haemocytometer (18.8 ± 5.0; d = 1.6; d+ 2SD = 12.2; d - 2SD = -9.0). The % motile spermatozoa assessed by SQA-Vp (65.8 ± 10.0) in diluted semen agreed well with CASA (72.2 ± 13.7; d = -6.4; d+ 2SD = 20; d – 2SD = -32.8) and with visual assessment (64.1 ± 11.6; d = 1.7; d+ 2SD = 30.9; d - 2SD = -27.5). The SQA-Vp showed a good repeatability (CV; repeatability coefficient) for measuring the concentration of both fresh (3.9%; d= 10.7; d+2SD= 30.9; d - 2SD= -9.5) and diluted semen (2.6%; d= 1.0; d+2SD= 2.38; d - 2SD= -0.42) and for motility (3.2%; d= 0.9; d+2SD= 8.5; d-2SD= -6.7). The mean values SQA-Vp fell between the other methods’ results for both fresh and diluted semen. Moreover the repeatability was acceptable. Therefore SQA-Vp can be used as a valid device for sperm quality analysis in pigs.
3.1.2. **Introduction**

Although the real fertilizing capacity of sperm is difficult to assess and many parameters need to be studied (Holt, 2009), it is well-known that the concentration and the motility are two very important parameters of boar semen used for AI (Vyt et al., 2008). Consequently, these parameters are routinely evaluated in AI-centres (Vyt et al., 2007). Visual assessment of sperm quality, although consistent if performed by the same person, is time consuming, it requires special training and it lacks accuracy (Vyt et al., 2004). Therefore, new tools based on computerized instruments have been developed in the last decade to assess objectively and accurately the motility of porcine sperm used for AI (Vyt et al., 2004; Tejerina et al., 2008).

Two of these new instruments namely Computer Assisted Sperm Analysis (CASA) by means of the Hamilton-Thorne Ceros sperm analyzer (HTR) and the Sperm quality analyzer version pig (SQA-Vp) have been used in the present study. CASA provides different motility parameters from sperm tracks obtained from multiple digital images that are subsequently analyzed with software (Rijsselaere et al., 2003; Vyt et al., 2004). Several studies from our group have shown that CASA systems provide consistent and reliable results for different animal species (Rijsselaere et al., 2003; Vyt et al., 2004; Hoogewijs et al., 2011). Moreover, CASA provides detailed information on motility and velocity patterns of the sperm, which might be useful to identify slight differences between highly selected boars used for AI (Tejerina et al., 2008; Lopez et al., 2010).

The SQA systems convert variations in optical density into electrical signals to determine sperm concentration (Rijsselaere et al., 2002; Vyt et al., 2004; Hoogewijs et al., 2011) and motility is determined based on converting signals generated from light disturbances as the sperm cells transverse a light beam. These electronic signals are analyzed by the SQA software algorithms and translated into sperm quality parameters. The effectiveness of different SQA systems for sperm analysis has been studied both in humans and animals and different algorithms are needed for each species (Rijsselaere et al., 2002; Vyt et al., 2004; Hoogewijs et al., 2011). Vyt et al (2004) proved that a previous version of SQA namely the SQA-IIC was consistent and suitable for the estimation of boar semen quality. They showed a good correlation between the sperm motility index (SMI) by means of SQA-IIC and several CASA parameters, especially with percentage of motile sperm and with straight line velocity (VSL). However, the latter device is based on an old technology meant for human sperm analysis and the SMI values are based on overall
information of the quality of the sperm, and do not discriminate between concentration, morphology and motility parameters. Recently, the SQA-Vp was introduced as an SQA device specifically designed for boars in which the sperm movement can be visualized on a screen and motility is given as percentage of motile sperm.

The aim of this study was to compare the effectiveness of an upgraded version of the SQA device, the SQA-Vp, with other methods (1) Accucell photometer, 2) Bürker counting chamber, 3) CASA-HTR and 4) visual score for assessment of boar sperm concentration and motility, as well as to study its repeatability in the measurements.

3.1.3. Materials and methods

Semen samples

Fresh ejaculates and diluted semen from 50 ejaculates (10 samples per day during 5 days) of different boars (35 Piétrain, 8 Landrace, 7 Large White) in a commercial AI-centre (Hypor, Zulte, Belgium) were investigated. Immediately after collection and filtration of the ejaculate, this was diluted (1:1) using a commercially available semen extender (Beltsville Thawing Solution, BTS) and a sample was used for analysis of semen concentration. A subsample of the 1:1 dilution was kept for further analysis of the concentration with the Bürker chamber. Subsequently, the remainder of each ejaculate was diluted in BTS to a final concentration of $25 \times 10^6$ spermatozoa per millilitre as determined by the photometer. All diluted semen samples and the subsamples of the 1:1 dilution were transported at 17°C in isotherm boxes to the laboratory of the department of Reproduction, Obstetrics and Herd Health of the Faculty of Veterinary Medicine, Ghent University, Belgium, where they were further processed upon arrival at the laboratory. Prior to motility analysis, diluted samples were pre-heated at 37°C for 20 minutes in an incubator (IN, Memmert GmbH + Co.KG, Germany).

Analysis of the ejaculates (sperm concentration)

Concentration of the ejaculate was assessed using three different methods. First, each semen (1:1 diluted) sample was analyzed once with a photometer (Accucell, IMV technologies, L'Aigle, France) according to the manufacturer’s protocol and as routinely performed by the AI technicians. Subsequently, the SQA-Vp analysis was started. Briefly, 200 µl of the 1:1 dilution were diluted in containers especially designed for the SQA-Vp and filled with 2 ml of BTS extender based on SQA-Vp onscreen instructions. The extender and the capillaries used for the analysis were pre-heated at 37°C for 3-5 minutes.
in the incubator accompanying the SQA-Vp. Subsequently, the pre-heated capillary was filled with a drop of the aliquot taking care not to introduce air bubbles, inserted in the electro-optical chamber of the SQA-Vp and analyzed according to the SQA-Vp onscreen instructions. Data regarding sperm concentration were recorded and used for the statistical analysis. The test was run in duplicate in a 1 min interval using the same capillary. During the interval, the capillary remained in the SQA-Vp chamber. Once in the laboratory of the Faculty of Veterinary Medicine, 20 samples from 1:1 diluted ejaculates were gently mixed and used for determination of sperm concentration with a haemocytometer (Bürker counting chamber; VWR International, Haasrode, Belgium) following the manufacturer’s protocol. Haemocytometer count was also performed in duplicate in a 1 min interval.

**Analysis of diluted semen (sperm concentration and motility)**

**SQA-Vp**

Concentration (x10⁶/ml) and motility (% of motile spermatozoa) of the samples were assessed with the SQA-Vp. Pre-heated testing capillaries were filled with a pre-heated diluted semen sample and the test was run following onscreen instructions similar as performed and described for the fresh ejaculates. For repeatability, the test was performed twice in a 1 minute interval using the same capillary. The average of the data was used for the statistical analysis.

**CASA-HTR**

Concentration (x10⁶/ml) and motility (%) of the samples were assessed with CASA-HTR (HTR Ceros 12.3 semen analyzer, Hamilton-Thorne Research, Beverly, USA). Shortly, a 5 µl droplet of pre-heated diluted semen was placed in a 20 µm deep disposable counting chamber (Leja, Leja Products B.V., The Netherlands) that stayed in a minitherm stage warmer at 37°C during the analysis. Four randomly selected fields were measured 5 times each (Lopez et al., 2010; Hoogewijs et al., 2011), obtaining 20 scans from which the average was used for the statistical analysis. The analysis was repeated 1 minute later on the same counting chamber which stayed on the warming plate at 37° during that time.

**Manual analysis**

The concentration and motility of the sperm of the diluted samples were visually assessed. The sperm concentration (x10⁶/ml) was measured using the Bürker counting
Chapter 3.1

chamber as described above. The visual motility assessment was performed adapting the World Health Organization (WHO) protocol (WHO’99) (World Health Organization, 2010). In short, a drop 5 μl of diluted semen was deposited in a standard microscope slide and covered with a coverslip. Thereafter, the slides were observed using a light microscope with a warming plate at 37°C and up to 200 cells were counted to assess the motility (% motile spermatozoa). Both Bürker and visual motility assessments were done in duplicates within a 1 minute interval of each other.

Statistical analysis

Semen parameters obtained with the different methods were measured in duplicate and the average was used for the statistical analysis. The agreement between SQA-Vp and the other methods was studied using limits of agreement plots as described by Bland and Altman and calculating the limits of agreement (Bland and Altman, 1986). The repeatability on the measurements of the different methods was studied by calculating the coefficients of variation (CV) from the two measurements of a same sample (Microsoft Office Excel®, Excel 2007). Additionally Bland Altman plots were used for the repeatability study and the repeatability coefficient (Bland and Altman, 1986) was assessed.

3.1.4. Results and discussion

The effectiveness of a new SQA device, the SQA-Vp, for pig sperm concentration and motility analysis was compared with other methods of semen analysis. Misleading statistics are often used in method comparison analyses. Correlation coefficients and comparison of means are not appropriate as indicators of agreement (Bland and Altman, 1986). Therefore, and similar to previous studies (Vyt et al., 2004; Maes et al., 2010), plots of agreement were used to study how likely was SQA-Vp to agree with other methods for measuring sperm quality. The device performed well and the agreement with the other methods was in most cases good.
Figure 1: Limits of agreement plot showing the difference (d) in the measurements by Sperm quality analyzer version pig (SQA-Vp) and the different methods against the average of SQA-Vp with the corresponding method: A) concentration (x10^6/ml) of non diluted semen (n= 50 boars) by SQA-Vp and IMV Accucell photometer (d= -67.9 x 10^6/ml; d + 2SD= 55.3 x 10^6/ml; d – 2SD= -191.1 x 10^6/ml); B) concentration (x10^6/ml) of non diluted semen (n= 20 boars) by SQA-Vp and Bürker counting chamber (d= 92.8; d + 2SD= 448.6; d – 2SD= -263); C) concentration (x10^6/ml) of diluted semen (n = 50 boars) by SQA-Vp and Computer assisted sperm analysis by means of CEROS sperm analyzer (Hamilton Thorne Research CASA-HTR) (d= -2.8; d + 2SD= 6.2; d – 2 SD = -11.8); D) concentration (x10^6/ml) of diluted semen (n= 50 boars) by SQA-Vp and Bürker counting chamber (d = 1.6; d+ 2SD = 12.2; d – 2SD = -9); E) sperm motility (% motile) of diluted semen by SQA-Vp and CASA-HTR (d = -6.4; d+ 2SD = 20; d – 2SD = -32.8); F) sperm motility (% motile) of diluted semen by SQA-Vp and visual score (World Health Organization protocol, WHO’99) (d = 1.7; d+ 2SD = 30.9; d – 2SD = -27.5).
**Fresh semen concentration**

The plots of agreement showed that the concentration ($10^6$/ml ± SD) of fresh ejaculates measured with SQA-Vp (379.3 ± 134.9) corresponded well to those obtained with the photometer (447.2 ± 154.2) and with Bürker chamber (332.8 ± 141.1) (Figure 1). The photometer and the SQA-Vp devices both use changes in optical density to determine the sperm concentration which could explain why SQA-Vp results differed less from the photometer than from the haemocytometer. Both the SQA and Bürker had lower counts compared to the photometer. All the three methods need a different extra dilution prior to the analysis which could partially explain discrepancies between methods. Also photometers must be regularly calibrated for obtaining good accuracy (Vyt et al., 2004).

The same applies to the SQA-Vp. Although the haemocytometer is considered as the golden standard, its accuracy also depends on the type of chamber used and the operator’s skills (Christensen et al., 2005). Consequently, all methods have limitations (Mahmoud et al., 1997; Vyt et al., 2004; Christensen et al., 2005) and, taking into account how difficult it is to determine the most accurate method to study sperm concentration, the observed differences between SQA-Vp and the other methods can be considered as acceptable. SQA-Vp showed a good repeatability based on CV (2.6%) as well as repeatability coefficient (Figure 2). Therefore, the SQA-Vp seems to be suitable for estimation of the sperm concentration of fresh ejaculates in AI-centres.

![Figure 2: Bland-Altman difference plots for repeatability of two measurements (SQA-Vp 1 and SQA-Vp 2) of fresh semen concentration ($10^6$/ml) by SQA-Vp ($d= 10.7$; $d+2SD= 30.9$; $d-2SD= -9.5$)](image)
**Diluted semen**

**Sperm concentration**

For AI purposes determination of sperm concentration of the diluted semen is important. The capacity of SQA-Vp to analyze diluted semen was consequently studied.

The concentration (x10⁶/ml ± SD) assessed with the SQA-Vp (20.4 ± 4.3) seemed to agree well with both CASA (23.2 ± 5.8) and Bürker chamber (18.8 ± 5.0). The mean difference (x10⁶/ml) was 2.8 and 1.6, respectively (Figure 1). SQA-Vp however provided a lower count compared to CASA. It is known that CASA systems may recognize particles with a similar size as spermatozoa thereby overestimating the actual concentration (Vyt et al., 2004; Kuster, 2005). On the other hand CASA may underestimate the concentration compared to haemocytometer by the Segre–Silberberg effect (Kuster, 2005). Taking into account the variations in measurements that other methods can give, the differences between SQA-Vp and the other methods can be considered as acceptable. Moreover, repeatability was good both based on CV (3.9%) and repeatability coefficient (Figure 3).

![Figure 3: Bland-Altman difference plots for repeatability of two measurements (SQA-Vp 1 and SQA-Vp 2 of diluted semen concentration (x10⁶/ml) by SQA-Vp (d= 1.0; d+2SD= 2.38; d-2SD= -0.42)](image)

**Sperm motility**

The agreement between the motility (% ± SD) assessed with the SQA-Vp (65.8 ± 10.0) and CASA-HTR (72.2 ± 13.7) and visual assessment of motility (64.1 ± 11.6) was good with a mean difference of 6.4 (% motile) and 1.7, respectively (Figure 1). Again
SQA-Vp showed good repeatability (3.2% CV and Figure 4) for measuring motility.

The different techniques by which motility is assessed may explain the differences between the methods. CASA tracks and counts sperm while, the percentage of motile sperm with the SQA-Vp is obtained by mathematical algorithms, which analyze the signals caused by the mass of motile sperm traverse a light source. For visual assessment, motility is estimated from what the human eye is able to count. The subjectivity of the visual assessment of sperm and thus the variation in results is clear (Vyt et al., 2004; Tejerina et al., 2008). CASA only tracks a limited number (few hundred) of sperm and for a short period of time making biases possible (Tejerina et al., 2008). For SQA-Vp the measurement of sperm motility is still based on mathematical algorithms, assuming associations between parameters which may not always be present (Makler et al., 1999; Matson et al., 2007).

![Bland-Altman difference plots for repeatability of two measurements (SQA-Vp 1 and SQA-Vp 2) of diluted semen motility (%) by SQA-Vp (d= 0.9; d+2SD= 8.5; d-2SD= -6.7)](image)

Figure 4: Bland-Altman difference plots for repeatability of two measurements (SQA-Vp 1 and SQA-Vp 2) of diluted semen motility (%) by SQA-Vp (d= 0.9; d+2SD= 8.5; d-2SD= -6.7)

The SQA-Vp still has a disadvantage compared to CASA systems, which provides with different sperm motility and velocity parameters which are useful to detect slight differences between sperm of good performing boars (Tejerina et al., 2008; Lopez et al., 2010). Although SQA-Vp seems to give an appropriate estimation of the quality of a semen sample, which could be of value for quick screening both in AI-centres and farms, its usefulness for research proposes may be limited.

In conclusion, the SQA-Vp is suitable for estimation of boar sperm quality. The
device showed a good agreement with previous proven methods for boar sperm analysis such as photometer or CASA and proved to be consistent in the results. The device can therefore be useful to both prepare semen doses for AI and to estimate the sperm quality of these doses prior to their use.

Acknowledgements

The authors would also like to thank Marian Lezy and Wouter Deley (Hypor, Olsene, Belgium) for their excellent technical assistance.
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Chapter 3.2. Boar seminal plasma components and their relation with semen quality

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3.2.1. Contents

Selected boar seminal plasma (SP) components and their relation to semen quality were investigated. Thirty nine boars from 3 artificial insemination (AI) centres were divided in group A (GA: >80% normal sperm and >70% motility) and group B (GB: <80% normal sperm and <70% motility). One ejaculate/boar was collected and semen volume, concentration, sperm motility (CASA), morphology and vitality (both eosin nigrosin staining) were investigated. The SP was separated and analyzed for activities of aspartate-amino-transferase (AST), γ-glutamyl-transferase (GGT), alkaline phosphatase (ALP) and for concentrations of sodium (Na), potassium (K), chloride (Cl), calcium (Ca), phosphate (PO₄³⁻), magnesium (Mg), selenium (Se) and zinc (Zn). Repeated measures (2 months interval) were conducted in eight boars of GA from one AI-centre. The activity of GGT (r=-0.482) and ALP (r=-0.459) was moderately associated (p<0.05) with ejaculate volume and strongly associated with concentration (r=0.580 and r=0.618 respectively; p=0.000). Moderate associations (p<0.05) were found between ALP (r=0.439), GGT (r=0.387), Na (r=-0.428), K (r=0.354) and Se (r=0.354) with progressive motility. The SP concentration of Na (r=-0.401), Cl (r=-0.521) and K (r=0.350) was associated (p<0.05) with normal morphology. Only Mg was associated (p<0.05) with membrane damage (r=-0.335). Concentration of Na, Cl and Zn (1681.0 vs. 1701.0 μg/dL) was different between groups (p<0.05). Repeated measures showed significant differences in time for Na, Mg and Zn (P<0.05). In conclusion, several biochemical components of SP were related to semen quality. The analysis of biochemical parameters could therefore provide extra information about reproductive health of AI boars.
3.2.2. Introduction

The use of artificial insemination (AI) in commercial pig herds has increased significantly in the last decades (Maes et al., 2011; Riesenbeck, 2011). To provide high quality insemination doses, routine assessment of the semen quality of the ejaculates is necessary. Although some AI-centres start to implement computer aided semen analysis (CASA) in their routine semen assessment, semen quality analysis at AI-centre level is mainly based on conventional techniques i.e. concentration by photometer, and visual evaluation of the motility and morphology (Vyt et al., 2007; Knox et al., 2008). The relation of these parameters with fertility is still under discussion (Vyt et al., 2008; Tsakmakidis et al., 2010). Besides the routine semen quality analysis performed in AI-centres, more sophisticated methods such as flow cytometry or proteomic analysis, might be necessary to predict the potential fertility of boars and to assess minor differences in semen quality between highly selected boars (Foxcroft et al., 2008; Dyck et al., 2011; Waberski et al., 2011).

In human reproduction, assessment of seminal plasma (SP) including the analysis of various biochemical components is advised by the World Health Organization (WHO) for the routine seminogram (World Health Organization, 2010). In animal reproduction however, little is known about SP although there is currently an increase in research performed related to SP proteomics.

The SP is a mixture of fluids from the cauda epididymidis and the accessory sexual glands (Davies et al., 1975). In pigs, it has been proposed that the SP can be substituted by extender without remarkable detrimental effect, but recent studies have shown that SP has different functions for sperm metabolism (Rodriguez-Martinez et al., 2011). Additionally SP plays an important role during sperm capacitation and it stimulates the female immune system to remove pathogens and to tolerate spermatozoa and embryos (Rodriguez-Martinez et al., 2010; Rodriguez-Martinez et al., 2011). However, little is known on the biochemical composition of SP and its relation with semen quality and fertility in pigs. The activity of enzymes such as γ-glutamyl-transferase (GGT) or alkaline phosphatase (ALP) is related to semen quality and membrane function in stallions (Pesch et al., 2006; Kareskoski et al., 2010). In dogs and humans, low levels of ALP are associated to azoospermia mainly due to obstructions and are therefore routinely studied in cases of infertility. In vasectomized boars, this enzyme is not present and low ALP activity has been described in one azoospermic boar (King and Macpherson, 1966; Clements et al., 2010).
Minerals such as zinc (Zn) or selenium (Se) [structural component of glutathione peroxidase (GPx)], have been associated with semen quality in human due to their antioxidant properties (Bedwal and Bahuguna, 1994; Chia et al., 2000; Gavella et al., 2000; Powell, 2000; Bjorndahl and Kvist, 2010). Furthermore, Zn is included in the WHO manual as an indicator of prostatic function (World Health Organization, 2010) and low levels of Zn have been found in SP of infertile men (Chia et al., 2000; Colagar et al., 2009). Similarly several minerals in the SP such as chloride (Cl) or phosphate (PO$_4^{3-}$) are related to semen quality of stallions (Pesch et al., 2006; Kareskoski et al., 2010).

Based on the studies performed in other animal species and human, detection of several biochemical parameters could help to identify boars with infertility problems. Therefore, in the present study, we investigated several SP parameters in boars from 3 commercial AI-centres. Furthermore, the association of these components with various semen quality parameters was investigated.

3.2.3. Materials and Methods

Study population and semen samples

Forty eight boars from 3 different AI-centres were included in the study. In each AI-centre, the boars were housed within the same building in individual pens with straw bedding and received a commercial feed (2-3 kg feed/day, depending on age) and ad libitum drinking water from a deep pit. A feed sample was collected from each AI-centre to analyze the composition (Table 1).

One ejaculate of each boar was collected using the gloved hand technique (Shipley, 1999). Immediately after collection, volume (weight) and concentration of each ejaculate were measured, and the total number of sperm cells per ejaculate was calculated (volume x concentration). The technician performing the collection was recorded as well as the number of days to previous collection (Table 2). Subsequently semen quality parameters were investigated by personnel of the AI-centre and ejaculates were divided in two groups. Ejaculates with semen quality complying with the AI-centre’s cut-off values (minimum 80% normal spermatozoa morphology and sperm motility of at least 70%) were considered as good ejaculates and were included in group A (GA). The ejaculates with semen quality below these cut-off values were considered as poor ejaculates and were included in group B (GB). The numbers of boars per AI-centre, the different groups and the methods used in each AI-centre to determine semen quality are summarized in Table 2.
Prior to any dilution, 4-5 ml of raw ejaculated semen was collected into blood tubes to study the semen quality, and minerals and enzymes composition of the seminal plasma. The raw ejaculate samples were stored at room temperature until they were transported in isotherm boxes to the laboratory for sperm analysis at the Faculty of Veterinary Medicine (Ghent University, Belgium).

Table 1: Feed composition in the 3 artificial insemination (AI) centres and minimum requirements established by the National Research Council (NRC).

<table>
<thead>
<tr>
<th></th>
<th>AI centre</th>
<th>NRC recommendation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>B (ppm)</td>
<td>8.93</td>
<td>6.78</td>
</tr>
<tr>
<td>Ca (ppm)</td>
<td>7240</td>
<td>7000</td>
</tr>
<tr>
<td>Cu (ppm)</td>
<td>23.1</td>
<td>13.3</td>
</tr>
<tr>
<td>Fe (ppm)</td>
<td>366</td>
<td>244</td>
</tr>
<tr>
<td>K (ppm)</td>
<td>9440</td>
<td>7210</td>
</tr>
<tr>
<td>Mg (ppm)</td>
<td>2480</td>
<td>1880</td>
</tr>
<tr>
<td>Mn (ppm)</td>
<td>126</td>
<td>80</td>
</tr>
<tr>
<td>Na (ppm)</td>
<td>3030</td>
<td>2400</td>
</tr>
<tr>
<td>PO_4^{2-} (ppm)</td>
<td>5780</td>
<td>4720</td>
</tr>
<tr>
<td>Zn (ppm)</td>
<td>143</td>
<td>139</td>
</tr>
<tr>
<td>Se (ppb)</td>
<td>349</td>
<td>310</td>
</tr>
<tr>
<td>Vit E (mg/kg)</td>
<td>172.7</td>
<td>101.2</td>
</tr>
<tr>
<td>% Dry matter</td>
<td>88.5</td>
<td>88.5</td>
</tr>
<tr>
<td>% Ash</td>
<td>5.68</td>
<td>4.9</td>
</tr>
<tr>
<td>% N</td>
<td>2.661</td>
<td>2.686</td>
</tr>
<tr>
<td>% Crude protein</td>
<td>16.6</td>
<td>16.8</td>
</tr>
<tr>
<td>% Crude fat</td>
<td>4.6</td>
<td>3.39</td>
</tr>
</tbody>
</table>

**Sperm motility, morphology and vitality**

Upon arrival at the semen laboratory at the Department of Reproduction, Obstetrics and Herd Health (Ghent University, Belgium) (3-4h after collection), a subsample (1 mL) of the raw ejaculate was taken, placed in an eppendorf tube and used to study sperm motility and morphology.

Different sperm motility characteristics were measured objectively with a CASA system (HTR Ceros 12.3 semen analyzer, Hamilton-Thorne Research, Beverly, USA). Prior to the CASA analysis, a subsample of the raw ejaculate was extended in PBS (1:7) and warmed at 37°C in an incubator (IN, Memmert GmbH + Co.KG, Germany) for 30 min
Samples were subsequently submitted to CASA analysis in a random order and were consistently investigated by the same person. After gentle mixing, a 10 μl droplet of each sample was placed on a slide prepared according to WHO guidelines (World Health Organization, 2010) and analyzed by CASA. The slide was evaluated following the manufacturer’s protocol (Vyt et al., 2004). Up to 1000 tracks of sperm cells were obtained by analysing four randomly selected fields that were measured five times each (Rijsselaere et al., 2003; Filliers et al., 2008). The average of the measurements on these 4 fields was calculated and used for the statistical analysis. The software settings for the HTR Ceros 12.3 were those recommended by the manufacturer for analysis of boar sperm. Different motility parameters were determined but only MOTILE% (percentage of motile sperm) and PROGR% (percentage of progressively moving sperm: sperm cells with both VAP > 50 μm/s and STR > 70%) were used for the statistical analysis.

Morphology was assessed using eosin-nigrosin stained slides following standard procedures (Shipley, 1999). A total of 100 cells/sample were evaluated to determine the percentage of normal sperm and the percentage of sperm with abnormal heads, abnormal tails, and proximal and distal cytoplasmic droplets. In addition, up to 100 spermatozoa/sample were evaluated to establish the percentage of membrane damaged spermatozoa as evidenced by a pink-red color.

In order to clearly define good and poor ejaculates, doubtful ejaculates were excluded. Therefore, when an ejaculate was considered as good for the AI-centre (>80% normal morphology and >70% motility) or bad (<80% normal morphology and <70% motility) but the semen quality analysis at the sperm laboratory at Ghent University showed discordant results, the ejaculate was excluded from the study. From the initial 48 collected boars 39 boars were finally considered as valid and were included in the study (Table 2).

**Biochemical analysis of seminal plasma**

The raw ejaculates were centrifuged for 20 min at 1000×g. Subsequently, the SP was separated from the sperm layer and the remaining SP was sent to an external diagnostic laboratory for biochemical analysis. Absorbance photometry (Cobas 8000, Roche) was used to measure the activity of aspartate-amino-transferase (AST), GGT, ALP and lactate-dehydrogenase (LDH) as well as the concentrations of iron (Fe), calcium (Ca), phosphate (PO₄³⁻) and magnesium (Mg). The concentration of sodium (Na), potassium (K) and chloride (Cl) was measured with ion selective electrode system (Cobas 8000, Roche).
and copper (Cu), Se and Zn by atomic absorption spectrophotometry (AAS) (Perkin Elmer for Cu and Zn and Grafietoven SA600). Vitamin E was assessed by ultraviolet high performance liquid chromatography (Perkin Elmer Flexar UVHPLC).

Table 2: Descriptive data of the boars included in the present study and the semen quality analysis procedures used in the three artificial insemination (AI) centres to classify ejaculates in group A ( >80% normal sperm morphology and sperm motility >70%) and group B (<80% normal morphology and motility <70%).

<table>
<thead>
<tr>
<th></th>
<th>AI centre 1</th>
<th></th>
<th>AI centre 2</th>
<th></th>
<th>AI centre 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group A</td>
<td>Group B</td>
<td>Group A</td>
<td>Group B</td>
<td>Group A</td>
</tr>
<tr>
<td>Number of valid boars</td>
<td>7</td>
<td>3</td>
<td>8</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Boar age (months± SD)</td>
<td>49.8±21.6</td>
<td>26.6±7.2</td>
<td>33.0±15.0</td>
<td>32.5±24.2</td>
<td>41.9±19.6</td>
</tr>
<tr>
<td>Days to previous collection± SD</td>
<td>6.7±0.5 a</td>
<td>5.7±1.5 a</td>
<td>5.5±1.1 a</td>
<td>5.2±3.7 a</td>
<td>12.1±3.0 b</td>
</tr>
<tr>
<td>Sperm quality analysis</td>
<td>Concentration</td>
<td>Photometer</td>
<td>Photometer</td>
<td>CASA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Motility &amp; morphology</td>
<td>Visual</td>
<td>Visual</td>
<td>CASA</td>
<td></td>
</tr>
</tbody>
</table>

*a,b different superscripts within a row indicate significant differences.

**Repeatability**

To study the repeatability of the biochemical parameters in the SP, 8 boars with good ejaculates from AI-centre 2 were sampled a second time 2 months after the first sampling. The ejaculates were processed in exactly the same way as described above. The semen quality analysis and seminal plasma parameters were also measured in the same way as for the first analysis.

**Statistical analysis**

Data were close to the normally distributed as determined by Kolmogorov-Smirnov test although the test was positive for some parameters. Because of the presence of outliers, Spearman rank correlation was used to investigate the associations between the biochemical and the semen quality parameters. Differences between groups in semen quality and in SP biochemical parameters were studied with ANOVA. Group and AI-centre were included as fixed factor and their interaction was investigated. When the interaction was not significant, it was excluded from the model and only the main effects
were investigated. Differences were considered as significant if P-values were lower than 0.05 (2-sided test). Descriptive statistics of the biochemical parameters were additionally presented to establish baseline levels. The repeatability of the measurements of SP parameters was studied with repeated measures ANOVA. Statistical analyses were performed using the statistical software package SPSS version 19.00. Data are expressed as means ± SD, unless stated otherwise.

3.2.4. Results

Semen quality

There were no significant differences between the groups (GA vs. GB) for ejaculate volume, sperm concentration, sperm count and the percentage of sperm with intact membrane (Table 3). The percentage of motile sperm and the percentage of progressive motility were lower in GB (p<0.05; Table 3). The number of all morphological sperm abnormalities was higher in GB (p<0.05; Table 3). The interaction terms group x AI-centre were not significant for any of the semen quality parameters (p>0.05).

Table 3: Average values (mean ± SD) of semen quality parameters of boars with good (Group A, >80% normal sperm morphology and >70%, sperm motility; n=24) and poor (Group B, <80% normal morphology and <70%, motility; n=15) semen quality.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A Mean ± SD</th>
<th>Group B Mean ± SD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (mL)</td>
<td>247.3 ± 70.64</td>
<td>277.3 ± 136.4</td>
<td>0.622</td>
</tr>
<tr>
<td>Concentration (x10^6/mL)</td>
<td>414.2 ± 234.8</td>
<td>422.0 ± 225.6</td>
<td>0.548</td>
</tr>
<tr>
<td>Sperm count (x10^9/mL)</td>
<td>94.8 ± 37.5</td>
<td>105.1 ± 43.2</td>
<td>0.547</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>80.8± 7.0</td>
<td>61.1± 16.8</td>
<td>0.000</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>34.1± 13.8</td>
<td>18.7± 12.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Normal sperm (%)</td>
<td>90.7± 5.7</td>
<td>55.5± 21.4</td>
<td>0.000</td>
</tr>
<tr>
<td>Abnormal head (%)</td>
<td>1.7± 2.1</td>
<td>5.6± 7.0</td>
<td>0.001</td>
</tr>
<tr>
<td>Abnormal tail (%)</td>
<td>1.6± 1.7</td>
<td>13.5± 13.9</td>
<td>0.000</td>
</tr>
<tr>
<td>Proximal Droplet (%)</td>
<td>2.5± 2.7</td>
<td>8.3± 6.5</td>
<td>0.000</td>
</tr>
<tr>
<td>Distal Droplet (%)</td>
<td>3.5± 2.6</td>
<td>17.1± 12.3</td>
<td>0.000</td>
</tr>
<tr>
<td>Membrane intact (%)</td>
<td>75.5± 12.6</td>
<td>79.3± 7.4</td>
<td>0.374</td>
</tr>
</tbody>
</table>

Associations between SP parameters and semen quality

The associations between SP biochemical values and semen quality parameters are summarized in Table 4. The concentrations of GGT and ALP were negatively associated
with volume of the ejaculate and positively associated with concentration. There was a moderate positive association between GGT and ALP with progressive motility. There was no association between SP enzymes and sperm with normal morphology or membrane integrity. However, trends were observed on the association of GGT (r= 0.366; p= 0.024) and ALP (r= -0.359; p= 0.027) with abnormal heads and distal droplets, respectively.

The mineral concentration was associated with semen quantity and quality. Phosphate, K and Se were negatively correlated with semen volume and Se and P were correlated positively with semen concentration (Table 4). Furthermore, there was a moderate positive association of $\text{PO}_4^{3-}$, K and Se and a negative association of Na with progressive motility (Table 4).

Higher levels of Na and Cl were associated with a decrease in the number of spermatozoa with normal morphology. Whereas Na seemed to be positively associated with abnormal tails (r= 0.412; p= 0.010), high levels of Cl appeared to be associated with more abnormal heads (r= 0.449; p= 0.041) and abnormal tails (r= 0.431; p= 0.051). Higher levels of Mg and Se were associated with less membrane damage (r= -0.335; p= 0.040) and proximal droplets (r= -0.0352; p= 0.033), respectively. There was a trend for a negative association between Zn concentration and the number of abnormal tails (r= -0.327; p= 0.051). In addition, a moderate association was found between AST (r=0.481; p=0.002), ALP (r=0.353; p=0.030), K (r=0.421; p=0.009), Cl (-0.500; p= 0.003) and Zn (r= -0.398; p= 0.016) with days to previous collection. Selenium was strongly associated with days to previous collection (r= 0.628; p= 0.000).

**Seminal plasma reference values and intergroup comparison**

Mean, median and range (95% confidence interval) of the enzyme activity and mineral concentration in SP of GA and GB are summarized in Table 5. Most of the parameters except for GGT, Ca, $\text{PO}_4^{3-}$, Mg and Na were affected by AI-centre (p<0.05) but there was no group x AI-centre interaction.

From the selected parameters, LDH, Fe, Cu and Vit E were in most of the samples below the detection limit of the test, and therefore they were not included in the statistical analysis. The concentration of Na, Cl and Zn differed significantly between groups (Table 5). Sodium and chloride were higher in the GB whereas the concentration of zinc was lower in this group. No significant differences were observed between groups for all the other evaluated minerals.
Table 4: Spearman Rank correlation between SP enzymes and minerals concentration, and semen quality. Boars with good (Group A: >80% normal sperm morphology and >70% sperm motility, n=24) and poor (Group B: <80% normal morphology and <70% sperm motility, n=15) semen quality are included in the analysis.

<table>
<thead>
<tr>
<th></th>
<th>Volume (mL)</th>
<th>Concentration (x10^6/mL)</th>
<th>Motility (%)</th>
<th>Progressive (%)</th>
<th>Normal sperm (%)</th>
<th>Membrane intact (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>-0.050</td>
<td>0.343^a</td>
<td>-0.043</td>
<td>0.011</td>
<td>-0.134</td>
<td>0.197</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>-0.482^b</td>
<td>0.580^c</td>
<td>0.279</td>
<td>0.387^a</td>
<td>0.050</td>
<td>-0.158</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>-0.459^b</td>
<td>0.618^c</td>
<td>0.311</td>
<td>0.439^a</td>
<td>0.241</td>
<td>-0.178</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>-0.146</td>
<td>0.293</td>
<td>0.162</td>
<td>0.308</td>
<td>0.224</td>
<td>0.228</td>
</tr>
<tr>
<td>PO_4^3- (mg/dL)</td>
<td>-0.356^a</td>
<td>0.549^c</td>
<td>0.23</td>
<td>0.359^a</td>
<td>-0.048</td>
<td>-0.198</td>
</tr>
<tr>
<td>Mg (mg/dL)</td>
<td>-0.003</td>
<td>0.079</td>
<td>0.213</td>
<td>0.278</td>
<td>0.261</td>
<td>0.335^a</td>
</tr>
<tr>
<td>Na (meq/L)</td>
<td>0.197</td>
<td>-0.229</td>
<td>-0.396^a</td>
<td>-0.428^a</td>
<td>-0.401^a</td>
<td>-0.271</td>
</tr>
<tr>
<td>K (meq/L)</td>
<td>-0.338^a</td>
<td>0.201</td>
<td>0.278</td>
<td>0.354^a</td>
<td>0.350^a</td>
<td>0.162</td>
</tr>
<tr>
<td>Cl (meq/L)</td>
<td>-0.256</td>
<td>-0.077</td>
<td>-0.348</td>
<td>-0.394</td>
<td>-0.521^a</td>
<td>-0.390^a</td>
</tr>
<tr>
<td>Se (μg/l (ppb))</td>
<td>-0.324^a</td>
<td>0.436^c</td>
<td>0.302</td>
<td>0.354^a</td>
<td>0.246</td>
<td>0.137</td>
</tr>
<tr>
<td>Zn (μg/dL)</td>
<td>-0.005</td>
<td>0.062</td>
<td>0.140</td>
<td>0.194</td>
<td>0.094</td>
<td>-0.140</td>
</tr>
</tbody>
</table>

^a p<0.05; ^b p<0.005; ^c p<0.001

Repeatability of measurements of SP components

Values of SP parameters from 8 boars of GA from AI-centre 2 at two different time points are summarized in Table 6 together with the p-values for time effect of the repeated measures ANOVA. Most of the parameters varied numerically between samplings. Whereas AST, GGT, ALP, Na, K, Se were lower in the second sampling, Ca, Mg, Cl and Zn were higher. However, the time effect was only significant between time points for Mg, Na and Zn (Table 6).

3.2.5. Discussion

In the present study, selected biochemical components of boar SP and their association with various semen quality parameters were investigated.

When SP parameters from all boars of GA and GB were plotted with semen quality parameters, interesting associations were observed. The AST, ALP and GGT activity had a
positive association with semen concentration and a negative association with semen volume. Similar findings have been reported in stallion and the authors suggested a testicular/epididymal origin of these enzymes (Pesch et al., 2006). A testicular/epididymal origin seems obvious for ALP as no ALP was found in vasectomized boars (King and Macpherson, 1966). Furthermore, old studies based on immune histochemistry have shown the presence of ALP in testis of rats and low ALP in accessory glands of boars (Bern, 1949; Aitken, 1960). A low activity of ALP could therefore be indicative of insufficient ejaculate due to obstruction of the ductuli efferentes or ductus deferentes, but currently there is a lack of reference values in the literature (Clements et al., 2010). To our knowledge, there is only one report in which a single boar with low sperm counts and diagnosed of obstruction of the ductuli efferentes at slaughter had low ALP activity in SP (Clements et al., 2010). The observed ALP activity in that case report (8100 U/L) was markedly below the observed values in our study for both GA and GB. Because the ejaculate of this boar was only of 40 mL a dilution effect would not explain the low ALP activity.

Table 5: Reference values (mean, median, range) of seminal plasma parameters of boars with good (Group A: >80% normal sperm morphology and >70%, sperm motility n=24) compared to the values obtained for boars with poor (Group B: <80% normal morphology and <70% motility, n=15) semen quality.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A</th>
<th></th>
<th></th>
<th>Group B</th>
<th></th>
<th></th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Median</td>
<td>Range²</td>
<td>Mean</td>
<td>Median</td>
<td>Range²</td>
<td>(Group)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>15.6</td>
<td>12.0</td>
<td>3.0-26.3</td>
<td>25.4</td>
<td>14.0</td>
<td>11.6-39.2</td>
<td>0.075</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>6917.7</td>
<td>5715.0</td>
<td>5385.1-8772.4</td>
<td>5702.9</td>
<td>5361.0</td>
<td>3698.9-7706.8</td>
<td>0.586</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>45586.3</td>
<td>41363.0</td>
<td>37944.9-54869.7</td>
<td>33611.0</td>
<td>32569.0</td>
<td>23598.1-43623.9</td>
<td>0.183</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>3.1</td>
<td>2.9</td>
<td>2.6-3.4</td>
<td>2.7</td>
<td>2.4</td>
<td>2.3-3.2</td>
<td>0.250</td>
</tr>
<tr>
<td>PO₄³⁻(mg/dL)</td>
<td>1.4</td>
<td>1.3</td>
<td>1.1-1.5</td>
<td>1.3</td>
<td>1.1</td>
<td>1.0-1.5</td>
<td>0.767</td>
</tr>
<tr>
<td>Mg (mg/dL)</td>
<td>7.7</td>
<td>7.8</td>
<td>7.5-8.0</td>
<td>7.5</td>
<td>7.5</td>
<td>7.2-7.7</td>
<td>0.152</td>
</tr>
<tr>
<td>Na (meq/L)</td>
<td>105.8</td>
<td>106.0</td>
<td>100.7-111.6</td>
<td>116.7</td>
<td>120.0</td>
<td>110.3-123.1</td>
<td>0.015</td>
</tr>
<tr>
<td>K (meq/L)</td>
<td>15.7</td>
<td>15.2</td>
<td>14.7-17.0</td>
<td>14.5</td>
<td>13.9</td>
<td>13.1-15.8</td>
<td>0.228</td>
</tr>
<tr>
<td>Cl (meq/L)</td>
<td>107.2</td>
<td>97.4</td>
<td>93.8-120.5</td>
<td>136.4</td>
<td>138.8</td>
<td>112.9-159.9</td>
<td>0.022</td>
</tr>
<tr>
<td>Se (ppb)</td>
<td>22.6</td>
<td>20.0</td>
<td>18.0-28.6</td>
<td>18.7</td>
<td>13.0</td>
<td>12.4-24.9</td>
<td>0.871</td>
</tr>
<tr>
<td>Zn (μg/dL)</td>
<td>1927.5</td>
<td>1681.0</td>
<td>1690.4-2164.6</td>
<td>1718.8</td>
<td>1701.0</td>
<td>1438.3-1999.3</td>
<td>0.032</td>
</tr>
</tbody>
</table>

¹Range: 95% confidence interval
Table 6: Seminal plasma values of selected seminal plasma parameters of good boars (GA; n=8) from the same AI-centre at 2 different time points (approximately 2 months between samples).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time 1</th>
<th>Time 2</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>12.5</td>
<td>3.5</td>
<td>11.7</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>9749.9</td>
<td>9965.3</td>
<td>6083.6</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>57775.9</td>
<td>47730.0</td>
<td>34394.9</td>
</tr>
<tr>
<td>Ca (mg/dL)</td>
<td>2.7</td>
<td>1.1</td>
<td>3.1</td>
</tr>
<tr>
<td>PO₄³⁻ (mg/dL)</td>
<td>1.1</td>
<td>0.3</td>
<td>1.2</td>
</tr>
<tr>
<td>Mg (mg/dL)</td>
<td>6.6</td>
<td>1.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Na (meq/L)</td>
<td>121.6</td>
<td>12.1</td>
<td>107.8</td>
</tr>
<tr>
<td>K (meq/L)</td>
<td>16.5</td>
<td>1.7</td>
<td>15.5</td>
</tr>
<tr>
<td>Cl (meq/L)</td>
<td>102.7</td>
<td>13.8</td>
<td>115.7</td>
</tr>
<tr>
<td>Se (μg/l (ppb)</td>
<td>13.9</td>
<td>10.7</td>
<td>11.4</td>
</tr>
<tr>
<td>Zn (μg/dL)</td>
<td>1065.9</td>
<td>330.5</td>
<td>2262.0</td>
</tr>
</tbody>
</table>

We can only hypothesize why the studied components of the SP are related to semen quality as the literature on this topic in porcine andrology is very scarce, but some results are similar to those found in stallion and human. A positive association was found between ALP, GGT, K, PO₄³⁻ and Se with progressive motility. Similar positive associations of ALP and GGT with motility have been described in stallions (Pesch et al., 2006). The association of ALP with sperm motility has been attributed to the role which ALP plays in the synthesis of fructose, one of the main energy sources for sperm movement. In this process, ALP appears to be involved in the dephosphorylation of glucose-6-phosphate (King and Macpherson, 1966). However, fructose seems to be synthesized in the seminal vesicles (Mann, 1946). If indeed ALP is of testicular/epididymal origin, it should therefore not affect fructose production in the semen vesicles and the mechanism why it is related to motility remains therefore unclear. The relation between GGT and motility has been attributed to the protective effect of the enzyme against oxidative stress during sperm maturation in the epididymis (Kohdaira et al., 1986; Seligman et al., 2005).

For K, it has been shown that its addition to boar semen extenders helps to preserve motility and it is added to several commercial extenders (Johnson et al., 2000). The role of PO₄³⁻ on motility has been explained by phosphorylation required to activate proteins involved in the initiation of sperm motility (Arrata et al., 1978; Tash and Bracho, 1994).
Phosphate is also an important component of ATP and cAMP which are both necessary for motility. For Se, it has been shown that it is an important component of GPx, an enzyme that protects against lipid peroxidation resulting in improved sperm motility (Marin-Guzman et al., 1997).

In the present study a higher ALP was associated with a reduction of distal droplets. It has been shown that boar sperm loses cytoplasmic droplets during ejaculation and the number of sperm with these droplets increases with collection frequency (Pruneda et al., 2005). Distal droplets could therefore indicate insufficient sperm maturation and it could be possible that ALP plays a role in sperm maturation during ejaculation. The association with tail droplets could explain the observed positive association of the enzyme with motility. The exact mechanism behind this association is not clear but it has been suggested that fructose stimulates shedding of cytoplasmic droplets of boar spermatozoa (Harayama et al., 1996). As it was stated previously in this section, ALP has been associated with synthesis of fructose but we cannot confirm this association.

Na and Cl correlated negatively with normal morphology in the present study. It might be possible that abnormal sperm results in leakage of these elements to the SP and that Na/K flux needed for sperm metabolism is altered.

Although significant differences between groups were observed, we could not find any clear association of Zn concentration with semen quality. Only a trend of a negative association for Zn with abnormal tails was observed. Besides its antioxidant properties, Zn is involved in many aspects of spermatogenesis and sperm physiological function (Bedwal and Bahuguna, 1994). Therefore, Zn seems to act at different levels and it is possible that it affects semen quality in different ways which would make it difficult to associate it with one single parameter. Similarly to our results, a negative association of Zn with sperm abnormal morphology has been described in human semen (Colagar et al., 2009). It is possible that the concentration of Zn could be an indicator of boar fertility but the high variation in the measurements and the weak associations with semen quality that we observed limits its potential use for diagnostic purposes. Only Mg was associated with reduced membrane damage. Moreover, in agreement with this finding, addition of magnesium to boar extenders has been shown to improve semen vitality (Szczesniak-Fabianczyk et al., 2003).

Interestingly, many of the studied SP components were associated with days to previous collection. It has been shown that high collection frequency affects the excretion and reabsorption patterns of epididymal epithelium (Pruneda et al., 2005). Although the
differences in days to previous collection were not significant between groups, there seemed to be a numerical difference and there was a significant difference between AI-centre 3 and the other 2. This could partially also explain differences in SP composition between groups and AI-centres.

When comparing the 2 groups, only significant differences were found for Na, Cl, Zn although some numerical trends were also observed for the other parameters. Regarding Na and Cl, it seems that an appropriate osmolarity is needed for sperm motility and that a balanced combination of these elements is necessary for sperm metabolism (Quinn et al., 1965). With respect to Zn, it has been shown in human that low Zn concentration in seminal plasma is associated with low motility, low sperm vitality and infertility (Chia et al., 2000). In boars it has been shown that, when sperm cells are damaged, Zn accumulates in the sperm cells with a consequent reduction of Zn in SP (Westmoreland et al., 1967).

In the present study, reference values for SP parameters in boars were additionally provided. Many factors could explain the variations in the measurements. We did e.g. not study the different fractions of the ejaculate and their composition may be different. However all ejaculates were handled in the same way and they were homogenized before a sample was collected. Consequently, this should not have biased the comparison between groups. A previous version of the device used for most of the analysis (Cobas 8000) has been shown to have a good precision and coefficients of variation ranged between 0.6% and 4.4% for routine chemistry (van Gammeren et al., 2008). Therefore, the method of analysis could be responsible for only a minor part of the observed variation. Variation can also be attributed to differences between AI-centres as the statistical analysis showed differences between AI-centres. Differences in feed composition were observed that could result in different SP mineral composition. However in all three AI-centres feed composition complied with the NRC requirements. Therefore a deficiency that could have dramatically affected the results is very unlikely. Other sources of variation could be the water or a possible biting behaviour of the cages which are usually made of galvanized metal (Zn coated), but this possible sources of variation were not investigated. Interval of collection to analysis may affect Zn count as it has been shown in human, where samples analyzed 60 min after collection had respectively 29% and 17% lower Zn than samples analyzed 30 min or immediately after semen collection (Elzanaty and Malm, 2007). The authors hypothesized that after ejaculation zinc binds to spermatozoa but the assumption was not confirmed. Because boar semen diluters contain minerals (Johnson et al., 2000),
all samples remained undiluted to avoid interference of the diluters with the SP composition. Sperm motility can only be maintained for a few hours after collection without dilution (Johnson et al., 2000) and the composition of SP will likely also change with time after ejaculation. AI-centre 1 and 2 were sampled on the same date and batches of samples were processed in the same time interval, whereas AI-centre 3 was sampled on a different date. Because samples for mineral analysis were submitted to an external commercial laboratory we cannot ensure that all samples were analyzed in exactly the same time interval. However, the time from collection to submission of the samples was the same for both groups and similar between AI-centres (collection in the morning, processing at midday and submission to the laboratory within 12h) so this should have not biased the comparison between groups.

Interestingly repeatability measures analysis showed variability for all parameters in time although time effect was only significant for Na, Mg and Zn. Possible reasons for the variation in the measurements have already been explained above. In addition, we should also consider a possible seasonal effect as samples were collected at the beginning of the summer and at the end of the summer. Some seminal plasma components like proteins or citric acid seem to increase during the winter (Trudeau and Sanford, 1986). However, the same study did not show season effect on SP ALP activity.

In conclusion, reference values of selected SP components for boars are provided for the first time in the present study and could add valuable information to the increasing research performed on boar SP. A significantly different composition of Na, Cl and Zn of the SP of good and poor quality ejaculates was observed. Furthermore different associations of biochemical components of the SP with semen quantity and quality were found.
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Diergeneeskundig Tijdschrift 76 195-200.
Chapter 4.  Boar fresh semen production
Chapter 4.1. Effect of organic selenium in the diet on sperm quality of Boars

López Rodríguez A, Rijsselaere T, Van Soom A, Leroy JLMR, De Clercq JBP, Bols PEJ, Maes D

4.1.1. Contents

The effect of a diet supplemented with organic selenium (Se) on sperm production and quality of boars was investigated. Sixty mature boars from a commercial artificial insemination centre were randomly allocated at Day (D) into Group A and B. Group A received the regular ration supplemented with inorganic Se (0.4 mg/kg feed as Na$_2$SeO$_3$) whereas Group B was switched to the same diet but with organic Se (0.4 mg/kg fed as Se-yeast). The sperm was investigated during 4 months (D0, D30, D60, D75, D90, D105 and D120). Sperm concentration and motility were objectively measured using a photometer and Computer Assisted Semen Analysis (CASA), respectively. Morphology of the sperm was assessed using eosin-nigrosin staining and the resistance to induction of oxidative stress (production of malonaldehyde MDA) through thiobarbituric acid reagent substances (TBARS) analysis. Additionally, the Se concentration in sperm and blood plasma were measured. Repeated measures ANOVA from D60-120 (spermatogenesis of approximately 2 mo) or ANOVA at D120 (Se concentrations) were used for statistical analysis. The total number of ejaculated sperm was not significantly different between both groups, but boars of Group B had a significantly higher sperm concentration (434.6 vs 514.1 x10$^6$ sperm/ml; P<0.05). Small differences (P<0.05) were observed between both groups for some CASA parameters, namely straight line velocity (µm/s) (Group A: 48.3, Group B: 45.1), straightness (%) (Group A: 65.6, Group B: 62.2), and linearity (%) (Group A: 32.2, Group B: 29.3). The sperm of Group B showed more oxidative stress (4.1 vs. 4.9 µmol MDA/l; P<0.05) compared to those of Group A. No significant differences (P>0.05) were observed for the other parameters.

Under the present study conditions, changing from inorganic Se to organic Se in the diet of boars increased sperm concentration but reduced some motility parameters and resistance to oxidative stress.
4.1.2. **Introduction**

Trace minerals such as zinc, copper and selenium (Se) are usually present at very low concentrations in the diet of farm animals. They have, however, an essential role in many physiological processes (Hill and Spears, 2001). Selenium is widely recognized to have antioxidant properties and to stimulate immune function (Mahan, 2001). Apart from being a component of at least 25 selenoproteins (Kryukov et al., 2003), Se is additionally an integral component of glutathione peroxidase (GPx), an enzyme protecting cellular and sub cellular membranes against peroxidation (Flohe et al., 1973; Rotruck et al., 1973).

Selenium is also important in female and male reproduction (Bedwal and Bahuguna, 1994). In sows, Se deficiencies are related to a small litter size, weaker progeny, longer farrowing duration and/or low frequency of milk letdown (Van Vleet et al., 1973; Mahan et al., 1974). Regarding male reproduction, Se has been found in high concentrations in testes and epididymidis of boars, and is therefore likely important for the production and maturation of sperm (Marin-Guzman et al., 1997; Marin-Guzman et al., 2000; Lasota et al., 2004). For growing boars, it has been shown that higher levels of Se and vitamin E in the feed (0.5 mg Se + 60 mg vitamin E compared to 0.2 mg Se + 30 mg vitamin E/kg) improved semen quality (Kołodziej and Jacyno, 2005). Another study however reported that a basal diet for boars supplemented with Se as Na₂SeO₃ (0.10 and 0.25 ppm Se) decreased libido and testis size compared to a basal diet poor in Se (0.005 ppm) (Henson et al., 1983). GPx activity has been found in sperm of boars (Lasota et al., 2004; Jelezarsky et al., 2008) and therefore a higher resistance to oxidative stress should be expected when Se is provided. Nevertheless, the way GPx acts on sperm protection remains unclear. While some authors could demonstrate GPx activity in sperm of boars, others demonstrated that, in mammals, the enzyme loses activity once it is taken up in the sperm as a structural component (Ursini et al., 1999). Se supplementation has been shown to increase boars sperm GPx activity (Marin-Guzman et al., 1997) but, to our knowledge, no studies have demonstrated whether sperm resistance to oxidative stress in boars is enhanced by this GPx increase.

Typically, Se in animal feed is included in the inorganic (sodium selenite – Na₂SeO₃ or sodium selenate - Na₂SeO₄) or organic (Selenomethionine from Se-enriched yeast) form. Sodium selenite is most commonly used because of its lower cost. Several studies have been performed to elucidate the effect of these different sources of Se on the performance of pigs (Mahan, 2001). Higher toxicity of inorganic versus organic selenium
has been demonstrated for instance in finishing pigs (Kim and Mahan, 2001a). Mahan and Parrett (1996) demonstrated that the bioavailability of organic Se (Se-enriched yeast) was better than inorganic Se (\(\text{Na}_2\text{SeO}_3\) and \(\text{Na}_2\text{SeO}_4\)). Kim and Mahan (2001b) suggested that organic Se when supplied at high levels during prolonged periods of time also has more positive effects on gestating and lactating sows than inorganic Se.

Although semen production and quality are both very important for commercial artificial insemination (AI) centres, very little is known on the effects of different sources of Se on male reproductive performance and sperm quality. One study showed that feeding young boars organic Se (0.2 Se-enriched yeast and 60 g vitamin/E kg) from 70 until 180 d of age resulted in a better reproductive performance regarding sperm concentration and morphology of sperm compared to boars fed a diet containing inorganic Se (0.2 \(\text{Na}_2\text{SeO}_3\) and 30g vitamin E) (Jacyno et al., 2002). However, no differences in motility of sperm were observed. Dimitrov et al. (2007) recently found that organic Se enhanced sperm motility and the reproductive success in turkeys. In the latter studies, motility was assessed subjectively by visual scoring. Computer Assisted Semen Analysis (CASA) systems however provide more accurate information and have the advantage that a large number of different motility parameters can be assessed objectively (Holt et al., 1997; Vyt et al., 2004; Vyt et al., 2008). Although inorganic Se increases GPx activity in blood serum more than organic Se (Mahan and Parrett, 1996), there is almost no information whether different sources of selenium have an effect on GPx activity and resistance to oxidative stress in sperm.

The aim of this study was to assess the possible beneficial effects of a diet supplemented with organic Se in the feed of good performing mature boars of a commercial AI-centre on semen production and quality. Different semen parameters such as volume of the ejaculate, the concentration, motility and morphology of the sperm and the resistance to oxidative stress were investigated.

4.1.3. Materials and Methods

Study population and experimental design

Sixty Piétrain boars housed in a commercial AI-centre were included in this study. The age distribution (average ± SD) between the two groups was similar namely 21.8 ± 8.2 (minimum: 11; maximum: 37) for Group A and 21.8 ± 7.6 mo (minimum: 12; maximum: 35) for Group B. They were housed in individual pens within the same building and before
the start of the trial, they received a commercial feed containing a premix with inorganic Se (0.4 mg/kg feed as Na₂SeO₃/kg and 80 mg of vitamin E/kg). Upon entry into the AI-centre (8 mo of age) until D0, the boars were fed twice daily and depending on the age, they received each time 1 to 1.5 kg/feed plus supplementations. From 8 months of age onwards, the basal feed ration (2 kg) was increased at a ratio of 0.1 kg/day per month aging. Additionally, boars received an extra ration during winter months namely + 0.2 kg (April and October), + 0.4 kg (November and March), + 0.6 kg (December, January and February). The frequency of semen collection was 2 times/week with an interval of 3-4 days between two collections. The criteria of the AI-centre for boar selection were used namely a minimum 80% normal spermatozoa morphology and sperm motility of at least 70%.

The study lasted from January until May 2008. At the start of the study Day 0 (D0), the boars were randomly allocated into 2 groups of 30 boars. Boars of Group A received the same feed as before initiation of the study whereas boars from Group B were switched to a feeding program using the same feed but replacing inorganic Se by organic Se (Se-yeast, Sel-Plex®, Alltech, Inc., Nicholasville, KY, USA) at the same total concentration in feed. The general composition of the feed of both groups is given in Table 1. The initial feeding level (i.e. 2 times / day 1 to 1.5 kg / boar plus supplementations) and frequency of semen collection were maintained during the study.

**Semen samples**

Semen of the boars was collected according to standard procedures by means of the gloved-hand technique (Shipley, 1999). Immediately after collection, the ejaculates were extended using a commercially available semen extender (Beltsville Thawing Solution, BTS) to a final concentration of approximately 25x10⁶ sperm/ml, as determined by using a photometer (Accucell®, IMV technologies, L'Aigle, France). At Day 0 (D0) and Day 120 (D120), a subsample of the ejaculate of 10 boars from each group was selected, kept undiluted and further processed to determine Se concentration in sperm. The same 10 boars/group were used at D0 and D120. All semen samples were subsequently transported at 17°C in isotherm boxes to the laboratory of the Department of Reproduction, Obstetrics and Herd Health of the Faculty of Veterinary Medicine, Ghent University, Belgium. Upon arrival, approximately 4 hr post-collection, they were further processed and analysed for sperm quality. Sperm samples from the same ten boars/group used for determination of Se in sperm, were submitted to thiobarbituric acid reagent substances (TBARS) test at each
time point.

The semen was first investigated at D0 to obtain baseline levels of semen production and sperm quality in both groups. Thereafter, taking into account a spermatogenesis of 6 to 8 weeks (Swierstra, 1968), semen was further investigated at the following time points during the study: Day 30 (D30), Day 60 (D60), Day 75 (D75), Day 90 (D90), Day 105 (D105) and Day (D120).

Table 1: General nutrient composition of the feed supplied to the boars of Group A (n=30) and B (n=30) before the beginning of the trial and from Day 0 to Day 120.

<table>
<thead>
<tr>
<th>Item</th>
<th>Group A&amp;B Before D0</th>
<th>Group A D0 – D120</th>
<th>Group B D0 - D120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>17.1</td>
<td>17.1</td>
<td>17.1</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>2.8</td>
<td>2.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Fibre (%)</td>
<td>4.6</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Crude ash (%)</td>
<td>5.4</td>
<td>5.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Starch (%)</td>
<td>41.1</td>
<td>41.1</td>
<td>41.1</td>
</tr>
<tr>
<td>Sugar (%)</td>
<td>4.6</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>Net energy (MJ/kg)</td>
<td>9.5</td>
<td>9.5</td>
<td>9.5</td>
</tr>
<tr>
<td>Vit A (IU/kg)</td>
<td>12500</td>
<td>12500</td>
<td>12500</td>
</tr>
<tr>
<td>Ca (%)</td>
<td>0.7</td>
<td>0.7</td>
<td>0.7</td>
</tr>
<tr>
<td>P (%)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Na (%)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>K (%)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Cl (%)</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Cu (mg/kg)</td>
<td>5.9</td>
<td>5.9</td>
<td>5.9</td>
</tr>
<tr>
<td>Zn (mg/kg)</td>
<td>29.1</td>
<td>29.1</td>
<td>29.1</td>
</tr>
<tr>
<td>Vit D3 (IU/kg)</td>
<td>2000</td>
<td>2000</td>
<td>2000</td>
</tr>
<tr>
<td>Vitamin E (mg/kg)</td>
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<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Sodium selenite (mg Se/kg)</td>
<td>0.4</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Sel-Plex (mg Se/kg)</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
</tr>
</tbody>
</table>

**Blood and feed samples**

Blood samples from 10 randomly selected boars / group were collected at D0 and
from the same boars at D120. The blood samples were collected in heparin sterile tubes (Venoject®, Terumo Europe, Leuven, Belgium) and transported to the laboratory where they were centrifuged and the plasma was kept at -20°C (Lasota et al., 2004) until analysed.

Feed samples from both groups were collected every time a new batch of the experimental feed was supplied namely at D0, D30, D60 and D90 ± 5 days.

At the end of the study, the blood plasma was sent to the Institute of Animal Physiology (SASci, Soltesovej, Kosice, Slovakia) for vitamin E and Se determination and feed samples were submitted to Alltech (Alltech, Inc., Nicholasville, KY, USA) for the analysis of Se concentration in feed.

**Parameters of comparison**

**Volume and concentration of non-diluted semen**

Immediately after collection, the volume (weight) of each ejaculate was measured and the sperm concentration of the ejaculate from each of the boars was measured with a photometer (IMV Accucel®). The total number of sperm/ejaculate was estimated as the volume of the non-diluted semen multiplied by the sperm concentration of the non-diluted semen.

**Motility and morphology of the sperm**

Upon arrival at the laboratory in the Department of Reproduction, Obstetrics and Herd Health, Ghent University, different sperm motility characteristics were objectively measured with a CASA system (HTR Ceros 12.3 semen analyzer, Hamilton-Thorne Research, Beverly, USA). Prior to the CASA analysis, extended samples were introduced and warmed at 37°C in an incubator (IN, Memmert GmbH + Co.KG, Germany) for 20 min. Samples were then submitted to CASA analysis in a random order and by the same person. During the CASA analysis period and after the initial 20 min incubation, the samples waiting to be analyzed stayed in the same incubator at 37°C. Approximately 15-20 minutes transpired from the analysis of the first sample until the last one. For the CASA analysis and after gentle mixing a droplet of each sample was placed in a Leja counting chamber (Leja, Leja Products B.V., The Netherlands) and the test was run following the manufacturer’s protocol. Up to 1000 tracks of cells were obtained by analysing four randomly selected fields that were measured five times each to ensure enough number of tracked cells during sufficient time (Rijsselaere et al., 2003; Filliers et al., 2008). The
average of the measurements on these 4 fields was calculated and used for the statistical analysis.

The software settings for the HTR Ceros 12.3 were those recommended by the manufacturer for analysis of boar sperm. Different motility parameters were determined: MOTILE% (percentage of motile sperm); PROGR% (percentage of progressively moving sperm, VAP > 45 µm/s and STR > 45%); average path velocity (VAP, µm/s); straight-line velocity (VSL, µm/s); curvilinear velocity (VCL, µm/s); straightness (STR, %); linearity (LIN, %); percentage of rapid, medium, slow and static cells; amplitude of lateral head displacement (ALH, µm) and beat cross frequency (BCF, Hz).

Morphology was assessed using eosin-nigrosin staining following standard procedures (Shipley, 1999). A total of 100 cells/sample were counted to determine the percentage of normal sperm and the percentage of sperm with abnormal heads, abnormal tails and proximal and distal cytoplasmic droplets. In addition, up to 100 cells/sample were counted to establish the number of membrane damaged sperm (pink staining).

**TBARS analysis of the sperm**

The selected diluted sperm samples from each group (10 boars/group at D0 and the same 10 boars at each time point) were centrifuged in order to remove BTS extender. The remaining sperm was then re-diluted in PBS without Mg++ to a final concentration (sperm/ml ± SD) of approximately 25x10^6 as determined per CASA (Group A: 25.2 ± 2.7; Group B: 24.8 ± 1.6). Subsequently, samples were transported at 17°C in isotherm boxes to the Laboratory of Veterinary Physiology in Antwerp (Belgium) to determine the sperm resistance against peroxidation by performing TBARS assay. The measurements were made in accordance with a protocol first described by Ohkawa et al. (1979). Briefly, lipid peroxidation was induced by adding ferrous sulphate (250 ml, 4 mM) and sodium ascorbate (250 ml, 20 mM) to 1 ml of the sperm suspension in PBS. Subsequently, the mixture was incubated for 1.5 hr at 37°C. This process resulted in the production of lipid peroxidation residues, mainly malondialdehyde (MDA). These are capable to react, at high temperatures and low pH, with thiobarbituric acid (TBA) giving a TBARS with a pink chromogen that can be quantified with a spectrophotometer (Nichi et al., 2006). In order to get TBARS, after the 1.5 hr of the incubation period, 2 ml of trichloroacetic acid (TCA 10%) were added to 1 ml of the incubated sperm, mixed and centrifuged for 10 min at 2363 xg in order to precipitate protein. Subsequently, 500 ml of the supernatant was mixed with 500 ml of TBA 1% in a glass tube and placed into a boiling water bath (90 – 100°C).
for 20 min and immediately cooled in an ice bath (0°C) to stop the chemical reaction. TBARS were then quantified using a spectrophotometer (U-2001 spectrophotometer, Hitachi High Technologies America, Inc., San Jose, CA, USA) at a wavelength of 532 nm. Results were compared to a standard curve previously prepared with a standard solution of MDA. Spectrometry measurements for the TBARS analyses were done in duplicate. Each time, spectrometry was additionally done on one random subsample of each group for which lipid peroxidation was not induced, and this was used as a control to verify that the induction procedure was working (data not shown).

**Se concentration in sperm, blood plasma and feed, and vitamin E in blood plasma**

The 40 non diluted semen samples (10 boars/group at D0 and the same 10 boars at D120) were centrifuged and the resultant pellets were frozen (Lasota et al., 2004). The pellets were subsequently analyzed for Se concentration at the Clinic for cattle in the Faculty of Veterinary Medicine in Hannover, Germany. The Se level in the sperm (dry matter) was determined with inductively coupled plasma-mass spectrometry (ICP-MS, Varian 820-MS, Varian, Inc., Palo Alto, CA, USA).

Determination of Se in blood plasma was done by fluorometry (Rodriguez et al., 1994). Se was analyzed in the feed of Groups A and B by ICP-MS after a semi-open destruction with HNO$_3$ and H$_2$O$_2$.

Vitamin E levels in blood plasma were analyzed with high performance liquid chromatography (HPLC) (Tučková Mand Kašteľ, 1999).

**Statistical analysis**

Possible differences between both groups at D0 were analyzed using analysis of variance (ANOVA) to validate the randomization procedure and to ensure equality of groups for the different parameters.

The data of the different semen parameters obtained from D60 to D120 were analyzed using repeated measures ANOVA. In this model, the volume and concentration of the ejaculate, the number of sperm, the different CASA parameters, the MDA concentration (TBARS) and the percentage of sperm with specific morphology characteristics were considered each time as dependent variables. Group was included as independent factor and time was the within subjects variable. The boar was considered as the experimental unit. When significant differences were found for a parameter in the repeated measures analysis, one-way ANOVA was performed at the different time points.
of the study. Differences between groups in Se concentration in sperm and blood, and the vitamin E concentration in blood (at D120) were analysed with ANOVA and differences between D0 and D120 within groups were analysed with a paired t-test. The change in Se concentration in sperm (D120 vs. D0) between the two groups was also analyzed using ANOVA. The Se concentrations in feed of Group A and B were compared with an ANOVA. Boars that died during the trial or boars from which semen collection was not possible at one or more time points were excluded for either the repeated measures analysis and ANOVA at D0 or D120. Associations between motility parameters that were significantly different between groups (VSL, STR and LIN) and MDA concentration in sperm were investigated using Spearman rank correlations. All analyses were performed using SPSS 15.00 (SPSS Inc., Chicago, IL, USA). P-values (two-sided) were considered to be significant when P was lower than 0.05.

### 4.1.4. Results

One boar from Group A and 2 from Group B suddenly died during the study. One boar from Group A was reluctant to jump on one of the occasions (D120). Three blood samples from Group A and 4 from Group B could not be processed for Se and vitamin E analysis due to haemolysis in the samples at D0.

**Volume and concentration of the ejaculate**

At D0, following results (average ± SD) were obtained in Group A and B, respectively: volume (ml), 176 ± 63 and 156 ± 69 (P>0.05); concentration (x10⁶ sperm/ml), 435 ± 171 and 477 ± 139 (P>0.05) (Figure 1) and total number of sperm (x10⁹ sperm/ejaculate), 73 ± 32 and 70 ±23 (P>0.05). No overall significant difference between the two groups was observed regarding volume measurements. There was a significant overall difference in the repeated measures analysis between the sperm concentration of the 2 groups from Day 60 to Day 120 (P<0.05) but pairwise comparisons at different time points showed that the 2 groups were significantly different only at D105 for sperm concentration (P<0.05; Figure 1).

**Motility of the sperm**

The results of the different CASA parameters at D0, D60, D75, D90, D105, D120 and the average parameter values from D60 to D120 are presented in Table 2.

At D0, no significant differences (P>0.05) were observed between the groups for any of the CASA parameters. Repeated measures analysis from D60 to D120, showed an
overall significant difference for VSL, STR and LIN (P<0.05; Table 2). Pairwise comparison at each time point for these three parameters showed statistical differences on D105 and D120 for VSL, D60, D105 and D120 for STR and D60, D90, D105 and D120 for LIN (Table 2). No significant difference was observed for the other CASA parameters (P>0.05; Table 2). At D90, the sperm of one boar from Group A had very low motility values. This value was excluded as it was considered to be due to wrong handling of the sample.

Figure 1: Average (±SD) sperm concentration (x106/ml, IMV Accucel®) of ejaculated boar sperm in Group A (n=28) and Group B (n=28) at different time points. Group A received a diet based on a premix with inorganic Se (0.4 mg Na2SeO3/kg) whereas Group B received a diet based on a premix with organic Se (0.4 mg Se-yeast/kg). There was a significant overall difference in the repeated measures analysis between the 2 groups from Day 60 to Day 120 (P<0.05). Pairwise comparisons at different time points showed that the 2 groups were significantly different only at D105 (P<0.05).

Morphology of the sperm

The results of the morphology assessment at D0, D60, D120 and the average results for D60 to D120 are presented in Table 3. No significant differences (P>0.05) were observed between the groups at D0 nor along the study for any of the morphology parameters (P>0.05; Table 3).

TBARS analysis of the sperm
At D0, the concentration of MDA in sperm (average ± SD; µmol/l) for Group A and B were 6.78 ± 1.0 and 6.3 ± 1.1, respectively (P>0.05) (Figure 2). From D60 to D120, the concentration of MDA in sperm (average ± SD) for Group A (4.1 ± 0.8) and B (4.9 ± 0.9) were significantly different (P<0.05) (Figure 2). Pairwise comparison at each time point showed statistical differences between the groups on days 60 and 75 (P>0.05, Figure 2).

Figure 2: Average (±SD) malondialdehyde (MDA) concentration (µmol/l) in sperm from extended sperm samples after lipid peroxidation induction with ferrous sulphate in Group A (n=8) and Group B (n=10) from Day 0 to Day 120. Group A received a diet based on a premix with inorganic Se (0.4 mg Na₂SeO₃/kg) whereas Group B received a diet based on a premix with organic Se (0.4 mg Se-yeast/kg). There was a significant overall difference in the repeated measures analysis between the 2 groups from Day 60 to Day 120 (P<0.05).

*Timepoints with significant differences between the two groups (P<0.05).

Se concentration in sperm, blood plasma and feed, and vitamin E in blood plasma

The average (± SD) Se concentration in sperm (µg/g sperm dry matter) at D0 was significantly lower for Group A (24.1 ± 5.4) than for Group B (31.1 ± 3.5) (P<0.05; Figure 3). At D120, the average Se concentration in sperm (µg/g sperm dry matter) was 16.7 ± 5.5 and 23.5 ± 7.9 for Group A and B, respectively (P>0.05; Figure 3). The change in Se
concentration in sperm (D120 vs. D0) was not statistically significant between the two groups.

The average (± SD) Se (µmol/l) and vitamin E (µmol/l) concentrations in blood plasma at D0 were 2.3 ± 0.3 vs. 2.4 ± 0.2 (Se), and 3.3 ± 0.5 vs. 3.3 ± 0.5 (vitamin E) in Group A and B, respectively (P>0.05). At D120, the average (± SD) Se (µmol/l) and vitamin E (µmol/l) concentrations in blood plasma were 2.7 ± 0.5 vs. 2.4 ± 0.5 (Se), and 3.3 ± 0.2 vs. 3.5 ± 0.2 (vitamin E) in Group A and B, respectively (P>0.05).

The average Se concentration in the feed (4 samples/group) was 0.40 mg/kg for Group A and 0.41 mg/kg for Group B (P>0.05).

**Correlation between MDA and motility parameters**

There were no significant correlations between the MDA concentration in sperm cells and VSL (r= - 0.088; P > 0.05), STR (and r= - 0.038; P > 0.05) and LIN (r= 0.001; P > 0.05).

**4.1.5. Discussion**

The present study investigated the effect of a diet supplemented with organic Se on sperm production and quality of 60 mature Piétrain boars in a commercial AI-centre. To study the different parameters, proven tests which were performed in accordance to previous studies and in exactly same way for both groups, were used.

It appeared that changing from inorganic Se (Group A) to organic Se (Group B) in the diet only induced minor changes in a limited number of the measured parameters. Boars from Group B had a higher sperm concentration but some motility parameters and the resistance to oxidative stress were lower. Other parameters were not significantly influenced.
Table 2 Mean (± SD) motility parameters of the sperm of the boars in Group A (n=28, inorganic selenium) and B (n=28, organic selenium). The sperm parameters are from computer assisted semen analysis (CASA) using a Hamilton-Thorne at the start of the study (Day 0) and from Day 60 to Day 120.

<table>
<thead>
<tr>
<th>Group</th>
<th>D0</th>
<th>D60</th>
<th>D75</th>
<th>D90</th>
<th>D105</th>
<th>D120</th>
<th>Average (D60-D120)</th>
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<td>Motile %</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>A</td>
<td>65.3 ± 21.6</td>
<td>73.5 ± 16.8</td>
<td>76.8 ± 12.9</td>
<td>76.8 ± 16.9</td>
<td>78.2 ± 13.7</td>
<td>82.0 ± 11.5</td>
<td>77.4 ± 8.8</td>
</tr>
<tr>
<td>B</td>
<td>62.9 ± 20.0</td>
<td>74.3 ± 16.6</td>
<td>75.7 ± 14.9</td>
<td>76.3 ± 14.1</td>
<td>79.2 ± 12.7</td>
<td>80.4 ± 9.3</td>
<td>77.2 ± 10.4</td>
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<td>Progressive %</td>
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<td>A</td>
<td>37.3 ± 18.6</td>
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<td>57.2 ± 18.4</td>
<td>60.2 ± 18.0</td>
<td>66.1 ± 16.4</td>
<td>58.4 ± 11.7</td>
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<td>55.7 ± 14.5</td>
<td>55.6 ± 13.3</td>
<td>55.1 ± 11.3</td>
<td>57.2 ± 10.5</td>
<td>53.4 ± 9.5</td>
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<td>VAP (µm/s)</td>
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<td>A</td>
<td>61.8 ± 14.1</td>
<td>66.0 ± 9.9</td>
<td>73.5 ± 9.0</td>
<td>75.7 ± 10.6</td>
<td>75.4 ± 7.8</td>
<td>78.9 ± 9.3</td>
<td>73.9 ± 7.4</td>
</tr>
<tr>
<td>B</td>
<td>63.2 ± 17.3</td>
<td>66.9 ± 14.6</td>
<td>72.3 ± 8.0</td>
<td>78.0 ± 9.1</td>
<td>74.1 ± 9.0</td>
<td>76.5 ± 9.5</td>
<td>73.6 ± 7.9</td>
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<tr>
<td>VSL (µm/s)</td>
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<td>A</td>
<td>36.3 ± 7.5</td>
<td>43.1 ± 8.7</td>
<td>47.7 ± 6.0</td>
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<td>48.9 ± 6.7a</td>
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<td>44.4 ± 4.1b</td>
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<td>154.4 ± 18.1</td>
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<td>164.1 ± 18.5</td>
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<td>A</td>
<td>60.1 ± 4.7</td>
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<td>60.6 ± 4.3b</td>
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<td>61.2 ± 5.0b</td>
<td>62.9 ± 4.8b</td>
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<td>LIN (%)</td>
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<td>31.4 ± 4.7a</td>
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<td>32.2 ± 3.5a</td>
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<td>29.1 ± 2.2b</td>
<td>28.2 ± 2.4b</td>
<td>30.0 ± 3.1b</td>
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<td>30.4 ± 2.7</td>
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<td>33.0 ± 2.4</td>
<td>33.7 ± 2.7</td>
<td>35.6 ± 3.2</td>
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<tr>
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<td>32.7 ± 3.2a</td>
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<td>33.2 ± 2.2</td>
<td>34.4 ± 2.6</td>
<td>32.9 ± 1.7</td>
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</tbody>
</table>

At each time point or for the average values D60-D120, values of Group A and B with a different superscript, differ significantly (P<0.05). Motile% (percentage of motile sperm); Progressive% (percentage of progressively moving sperm); VAP (average path velocity); VSL (straight-line velocity); VCL (curvilinear velocity), STR (straightness); LIN (linearity) ALH (amplitude of lateral head displacement) BCF (beat cross frequency)
Table 3: Sperm morphology parameters (mean ± SD) of the sperm of the boars in Group A (n=28) and B (n=28). The sperm were investigated with an eosin-nigrosin staining at the start of the study (Day 0), Day 60 and Day 120. Group A received a diet based on a premix with inorganic Se (0.4 mg Na2SeO3/kg) whereas Group B received a diet based on a premix with organic Se (0.4 mg Se-yeast/kg). There were no significant differences between the groups (P>0.05).

<table>
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<tr>
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<th>Group</th>
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<th>D60</th>
<th>D120</th>
<th>Average (D60-D120)</th>
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</thead>
<tbody>
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<td>Normal morphology %</td>
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<td>74.8 ± 17.7</td>
<td>82.6 ± 11.4</td>
<td>80.3 ± 12.9</td>
<td>82.0 ± 8.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>72.1 ± 16.6</td>
<td>78.7 ± 15.8</td>
<td>79.2 ± 11.9</td>
<td>79.4 ± 10.9</td>
</tr>
<tr>
<td>Abnormal head %</td>
<td>A</td>
<td>8.0 ± 8.4</td>
<td>3.3 ± 2.8</td>
<td>5.4 ± 6.1</td>
<td>4.6 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>6.0 ± 3.2</td>
<td>1.9 ± 2.2</td>
<td>4.2 ± 3.9</td>
<td>3.3 ± 1.9</td>
</tr>
<tr>
<td>Abnormal tail %</td>
<td>A</td>
<td>5.5 ± 5.4</td>
<td>4.7 ± 4.1</td>
<td>4.4 ± 5.2</td>
<td>5.1 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>9.0 ± 10.5</td>
<td>8.7 ± 10.3</td>
<td>7.0 ± 6.2</td>
<td>7.8 ± 7.0</td>
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<tr>
<td>Proximal droplet %</td>
<td>A</td>
<td>5.5 ± 8.6</td>
<td>3.8 ± 4.9</td>
<td>5.1 ± 6.6</td>
<td>3.6 ± 4.6</td>
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<tr>
<td></td>
<td>B</td>
<td>6.9 ± 7.5</td>
<td>4.4 ± 5.9</td>
<td>5.1 ± 9.0</td>
<td>4.5 ± 6.6</td>
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<td>6.2 ± 8.7</td>
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<td>4.8 ± 5.7</td>
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<tr>
<td></td>
<td>B</td>
<td>5.4 ± 5.6</td>
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<td>4.6 ± 3.5</td>
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<td>Membrane damaged %</td>
<td>A</td>
<td>10.8 ± 5.4</td>
<td>17.3 ± 5.2</td>
<td>15.0 ± 6.1</td>
<td>17.2 ± 3.0</td>
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<tr>
<td></td>
<td>B</td>
<td>12.9 ± 4.7</td>
<td>16.5 ± 6.2</td>
<td>14.2 ± 5.5</td>
<td>16.8 ± 3.7</td>
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Boars of Group B performed better regarding the sperm concentration (434 versus 514 x10⁶ sperm/ml) (P<0.05), but there was no significant effect on the volume of the ejaculate or the total number of ejaculated sperm. Our results could only partially confirm the results of a previous study in which young boars fed an organic Se diet (Se-yeast), when compared to boars fed a diet with inorganic Se (Na₂SeO₃), had a significantly higher sperm concentration as determined by cytometry (Bürker chamber) but also a higher total number of sperm/ejaculate (Jacyno et al., 2002). In the latter study however, the boars received the experimental feed before puberty (from day 70 until day 180 of age) and the group receiving the organic Se also received higher concentrations of vitamin E (60 g versus 30 g/kg). The photometer was used in the present study as it is the method of choice for sperm concentration determination in 73% of Belgian AI-centres (Vyt et al., 2007a). Aberrations in the results given by the photometer can be related to the accidental confusion of particles of a size similar to that of sperm cells with actual sperm cells (Vyt et al., 2007a). Nevertheless, for practical reasons we preferred the photometer over more sophisticated systems and other researchers have also used this method successfully to study differences in concentrations between different groups of boars (Smital, 2009).
Some of the measured sperm motility parameters were slightly different in the boars of Group B compared to those of Group A namely VSL (-7%), STR (-5%) and LIN (-9%). Although the differences were small, the sperm of Group A boars had a more straightforward pattern of movement. Selenium has been proven to improve motility in the sperm from boars supplemented with dietary inorganic Se (sodium selenite) from weaning to sexual maturity (Marin-Guzman et al., 1997). Another study, however, based on subjective visual assessment, could not demonstrate differences in sperm motility when the same amount of different Se sources (sodium selenite and Se yeast) was supplied to boars (Jacyno et al., 2002). One study in turkeys, also based on visual semen assessment, showed that an organic source of Se enhanced the motility and the reproductive values. However, the Se yeast was supplied at a higher dose than the inorganic sodium selenite namely at 0.3 ppm compared with 0.1 ppm of sodium selenite (Dimitrov et al., 2007). It is therefore difficult to verify whether the beneficial effects of Se in the latter study could be ascribed to the effect of Se source, dose or both. Assessing motility parameters is delicate as parameters are easily influenced by external factors such as extender pH, air contact, temperature and incubation time and this could explain differences within groups along the study (Vyt et al., 2007b; Vyt et al., 2008). The samples in the present study however were processed in random order and analysed following the same procedures by the same person in both groups. Therefore, it is unlikely that environmental conditions have biased the results and results by chance, although always possible, are very unlikely. In contrast with previous studies, CASA was used in the present study to assess the effect of Se on the motility of the sperm. CASA analyses have the advantage, compared to visual evaluation, that a large number of motility parameters can be assessed, and that the assessment can be made accurately and objectively (Holt et al., 1997; Vyt et al., 2004).

There were no significant differences regarding the different morphology parameters, in contrast with the study of Jacyno et al. (2002) where significant differences were found. As mentioned earlier, in the latter study, the experimental set-up was different. It is known that both Se and vitamin E can enhance boar sperm quality by increasing sperm motility and reducing the number of sperm with abnormal morphology, resulting in higher fertilization rates (Marin-Guzman et al., 1997). However, it is not clear how they exactly interact and which factor has a higher effect on semen quality and production. In a recent study (Audet et al., 2009), addition of extra vitamin complexes, including vitamin E, to the diet of the boars affected the vitamin levels in blood and plasma but had no influence on the production or in the quality of boar sperm.
Chapter 4.1

Figure 3: Average (±SD) Selenium (Se) concentration in ejaculated boar sperm dry matter at the beginning of the trial (Day 0) and at the end (Day 120) for Group A (n=7) and B (n=9). Group A received a diet based on a premix with inorganic Se (0.4 mg Na$_2$SeO$_3$/kg) whereas Group B received a diet based on a premix with organic Se (0.4 mg Se-yeast/kg).

$x,y$ Values between Day 0 and Day 120 within each group with a different superscript differ significantly ($P<0.05$). $a,b$ Values between groups A and B with a different superscript differ significantly at a certain day of study ($P<0.05$).

Semen quality and especially concentration is known to be affected by several factors such as seasonal variation, collection frequency or the age of the boar (Audet et al., 2009; Kemp et al., 1988; Smital, 2009). However samples from both groups were processed in the exact same way and compared simultaneously. In this regard, time or season could not have biased the results. Also, the semen collection frequency was the same for both groups and has therefore not biased the results. For this experiment, only mature boars were used which were already selected for their semen quality to be used in AI. With the use of selected boars with proven fertility, factors leading to reproductive failure, such as infectious diseases or genetics, can be excluded. On the other hand, the fact that the semen production, motility and morphology of the boars in the present study were already very good and left little possibility for improvement, might explain the absence of significant differences between both groups (Gadea et al., 2004). The concentrations of inorganic Se (0.4 mg of Na$_2$SeO$_3$/kg) and vitamin E (80 mg/kg) in the feed before the
study and in Group A exceeded the National Research Council (NCR 1998) recommendations. It is also possible that, given these high concentrations, the source of Se has only a little beneficial effect on sperm production and quality.

A higher concentration of MDA was observed in sperm of Group B, indicating less resistance to induced peroxidation thus suggesting a possible effect of dietary Se source on the protective effect of GPx. Previous studies found that supplementation with inorganic Se increased GPx in blood and liver of rats and blood of pigs (Goehring et al., 1984; Mahan et al., 1999; Mahan and Parrett, 1996; Sunde and Hoekstra, 1980) more than organic Se, suggesting that the form Na₂SeO₃ is more easily metabolized and incorporated into GPx. Selenomethionine is the form in which Se is mainly found in Se yeast (Beilstein and Whanger, 1986; Kelly and Power, 1995) and selenocysteine the form in which Se is incorporated into glutathione peroxidase (Forstrom et al., 1978). A study done in rats suggested that selenomethionine competes with methionine for incorporation in non-Se-requiring proteins leading to selenomethionine to be less available for the synthesis of GPx (Beilstein and Whanger, 1986).

Breininger et al. (2005) suggested a negative association between TBARS and sperm motility of boars. A decrease in the reproductive potential of male mice has been associated with oxidative damage in spermatozoa (Sanchez-Gutierrez et al., 2008). Similarly, Payne et al. (2005) suggested that a higher number of cracked eggs from hens fed selenium yeast compared to a sodium selenite group could be due to higher oxidative stress caused by a lower GPx activity in the selenium yeast group. According to the results of our study, one could think that the better protection against lipid peroxidation obtained with inorganic selenium would result in better sperm motility. However, such correlations could not be proven in the present trial likely due to the limited number of samples.

In the present study, there were no significant differences between the two groups in the concentration of Se and vitamin E in the blood plasma. Previous studies showed that the Se source may determine the Se distribution in the body, but also no or marginal differences were found in blood Se concentrations (Mahan and Parrett, 1996). The Se concentration in blood plasma in the present study remained at similar levels throughout the study in both groups, whereas the Se concentration in the sperm decreased in both groups. According to the literature, many different factors, apart from the Se source, may determine the distribution of Se in the body. A recent study in humans, in which high dietary Se-yeast was supplemented, showed that Se in sperm declined along the study both in the control and treatment groups (Hawkes et al., 2009). In the latter group, also a
decrease in serum concentrations of luteinizing hormone and testosterone was observed. The authors raised the hypothesis that these hormones may act on the Se distribution towards sperm, but this remains to be confirmed. A study in rats showed an increase in testis Se content at the beginning of spermatogenesis (Behne et al., 1986) which could also suggest a hormonal effect on Se distribution. Lasota et al. (2004) suggested that the mechanisms controlling the Se content and GPx activity in blood and semen are independent, and that the age of the boars may influence the Se content and GPx activity in blood and semen. As the present study lasted for only 4 months, it is unlikely that the age of the boars explains the higher Se concentrations found in sperm at D0 compared to D120. Additionally, there was no correlation between the age of the boars and the Se concentration in the sperm (data not shown). Wahlstrom et al. (1984) and Kim and Mahan (2001b) suggested that the Se distribution in the body may also depend on the breed as symptoms of selenosis occur earlier in white haired breeds than in dark hair ones. As only Piétrain boars were included and both groups were randomized, a breed or age effect may not have biased the results and the differences between the groups. The reason for the reduced Se concentration in semen of both groups in our study is not clear. It might be due to the lower feed intake at the end of the study, as the feeding level diminished from January to May, and therefore there was also less intake of selenium. Previous studies demonstrated that a reduced feed intake (from 5.74 kg/day to 1.92 kg/day) resulted in lower sperm production but there was no effect on motility or morphology of the sperm (Kemp et al., 1989).

In conclusion, the present study documented that changing from inorganic to organic Se in the diet of good performing boars in a commercial AI-centre only induced minor changes in a limited number of semen parameters. Feeding organic instead of inorganic Se resulted in a higher sperm concentration. On the other hand, sperm from inorganic Se group showed a more straightforward movement. A higher incorporation of inorganic Se into sperm GPx resulting in higher resistance to oxidative stress is suggested.

**Acknowledgements:**

The artificial insemination centre (Hypor) is acknowledged for the collaboration with this study. The authors thank Martin Hoeltershinken (Clinic for Cattle, University of Veterinary Medicine, Bischofsholer Damm, Hannover, Germany) and the Institute of Animal Physiology (SASci, Soltesovej, Kosice, Slovakia) for their cooperation and technical assistance.
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Chapter 4.2. Effect of dilution temperature on boar semen quality

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Since boar semen is very sensitive to cold shock and changes in temperature during semen processing can have a profound impact on semen quality, the effect of the extender temperature at the time of dilution was investigated in a 2-step dilution protocol for boar semen being processed for liquid storage. Fifteen boars of different breeds and ages from a commercial artificial insemination centre were included. One ejaculate per boar was collected and processed with Beltsville Thawing Solution semen extender. Each ejaculate was diluted (1:1) at 30°C and subsequently the samples were diluted (30 x 10⁶ sperm/mL) with either pre-heated extender [29.3°C ± 0.2°C, group A (GA)] or extender at room temperature [22.7°C ± 0.6°C, group B (GB)]. Samples were transported to the Faculty of Veterinary Medicine (University of Ghent, Belgium) in 2 isotherm boxes (1 per group), stored at 17°C and investigated during 3 consecutive days (D0 to D2). At D0, D1 and D2, motility parameters [Computer Assisted Semen Analysis (CASA)] and the % of sperm with intact membrane (% IM) by eosin nigrosin staining were evaluated. At D0 and D2, the % of sperm with intact acrosome (% IA) was studied by Pisum Sativum Agglutinin staining. The average temperature of the 1:1 dilution was 29.4°C ± 1.1°C immediately after extender addition. No significant differences were found between groups for % motility [79.3 ± 9.0 for GA and 81.1 ± 9.2 for GB (p=0.372)], % progressive motility [56.5 ± 13.3 for GA and 58.4 ± 13.8 for GB (p=0.737)] or any CASA parameter. No differences were found for % IM [85.1 ± 10.7 and 84.5 ± 3.8 for GA and GB respectively (p=0.761)] and % IA [72.2 ± 9.4 for GA and 68.3 ± 16.6 for GB (p=0.792)]. In conclusion, when a 2-step dilution is performed, preheating the extender for the second dilution to match the semen temperature, did not result in better semen quality compared to a dilution at a moderate room temperature.
Chapter 4.2

4.2.2. Introduction

Artificial insemination (AI) is widely used nowadays in porcine production. Boar sperm is very susceptible to cold temperatures, due to a different composition of the phospholipids in the membrane of boar sperm compared to other animal species (De Leeuw et al., 1991). When boar semen is cooled too fast, lipid phase separation occurs. This results in an alteration of the membrane proteins and the membrane permeability with a subsequent leakage of cations and enzymes (Robertson et al., 1988, De Leeuw et al., 1991). When diluting boar semen, the temperature of the semen is diminished gradually to avoid cold shock. Two different dilution protocols are commonly used in AI-centres: 1) one-step dilution with either preheated diluter (~33°C) or diluter at room temperature or 2) a two-steps dilution with first a 1:1 dilution with preheated diluter (~33°C), followed by a second dilution in either a preheated diluter or a diluter kept at room temperature (Waberski, 2009). Most AI-centres use a first dilution with a preheated diluter, but the temperatures used for the 1:1 dilution and for the second final dilution depend on the AI-centre (Vyt et al., 2007). Different AI practices may cause differences on semen quality between AI-centres (Waberski et al., 2008).

Althouse et al. (1998) established the critical cold shock temperature for boar semen at 12°C. This temperature is normally not reached during actual semen processing where the temperature is controlled and semen is not cooled below 16-17°C. Research on how moderate temperature changes may affect semen quality is however limited and might be of economic interest for AI-centres. Preheating the diluter increases the costs and labour in the AI-centres but it is not clear yet whether this practice has any beneficial effect on semen quality (Petrunkina et al., 2005; Waberski 2009). Pursel et al. (1972) showed that acclimation at 30°C for several hours had a protective effect for samples to be stored at 17°C. On the contrary, Petrunkina et al. (2005) suggested a negative effect of acclimation at 32°C arguing that, by keeping sperm closer to the physiological temperature, the sperm does not diminish its metabolism leading to changes impairing semen quality. In this study, sperm diluted at 32°C seemed less capable to undergo capacitation changes induced in vitro. This suggestion was made based on how fast or how slow the intracellular calcium increased when in vitro capacitation was induced by calcium ionophore. This test is however complex and not yet fully understood (Petrunkina et al., 2005). An alternative to detect minor changes due to moderate temperature fluctuations could be computer assisted semen analysis (CASA). Some motion parameters provided by CASA have been related to
capacitation changes and fertility (García-Herreros et al., 2005; Vyt et al., 2008). However, the effect of moderate temperature changes during dilution on CASA parameters has not yet been discussed. In the present study, we therefore investigated the effect of 2 different temperatures (30°C and room temperature) for the second dilution in a 2-steps dilution protocol for boar semen, on semen quality parameters (CASA parameters, membrane damage and acrosome integrity) during liquid storage for 3 days.

4.2.3. Materials and methods

Experimental animals

Fifteen boars (12 Piétrain, 2 Large white, 1 Landrace) housed in a commercial AI-centre were included in this study. The age distribution (average ± SD) of the boars was 27.1 ±13.2 months (range: 9 to 54). They were housed in individual pens within the same building and they received a commercial feed (pellets) for AI boars. From 8 months of age onwards, the basal feed ration (2 kg) was increased at a ratio of 0.1 kg/day/month ageing. Boars were dewormed three times per year and were vaccinated against E.rhusiopathiae. The AI-centre was free of mange and atrophic rhinitis. The frequency of semen collection was 2 times/week with an interval of 3–4 days between two collections. The criteria of the AI-centre for boar selection were used namely a minimum 80% normal spermatozoa morphology and sperm motility of at least 70% (Martín Rillo et al., 1996).

Experimental design

The experiment was conducted during three consecutive weeks including ejaculates from 5 boars each week. Semen of the boars was collected by the gloved handed technique (Shipley et al., 1999) and the sperm rich fraction was processed. A first dilution (1:1) with diluter at 30°C was followed by a final dilution to 30 x10⁶ sperm/mL (commercial dose concentration) with either diluter at 30°C [Group A (GA)] or diluter kept at room temperature [Group B (GB)]. The temperature of 1:1 dilution prior to the final dilution was measured. For the final dilution, 1 mL of the 1:1 diluted semen was further diluted either in 2 mL of preheated diluter at 30°C in a water bath or in diluter that was stored at room temperature of 22°-23°C. The diluter at the corresponding temperature was added to obtain the final concentration and the final dilution ratio was recorded. For each group, the temperature of diluter was measured at the moment of dilutions. For all dilutions, a commercially available and widely used semen extender (Beltsville Thawing Solution, BTS, Minitüb, Tiefenbach, Germany) was used, and the semen concentrations were
determined by photometer (Accucell®, IMV Technologies, L'Aigle, France) as described by Maes et al. (2010). Shortly after dilution, semen samples were placed in 2 different but identical isotherm boxes (one box per group) and were subsequently transported (approximately 40 min.) to the laboratory of the Faculty of Veterinary Medicine, Ghent University, Belgium. The temperature inside the car and inside each isotherm box upon arrival at the laboratory was recorded. Upon arrival at the laboratory, samples were allowed to cool down at room temperature for 1 hour. A subsample was taken to perform a first analysis on semen quality. The remaining of the sample was stored at 17°C to be further analysed on D1 and D2. At D2, the temperature of the stored diluted semen was measured.

**Semen quality analysis**

Sperm motility was studied with CASA by means of a Hamilton Thorne system (HTR Ceros 12.3 semen analyzer, Hamilton-Thorne Research, Beverly, USA) at D0, D1 and D2 as previously described (López et al., 2010).

The percentage of sperm with damaged membrane was studied with eosin nigrosin staining at D0, D1 and D2 (World Health Organization, 2010).

At D0 and D2, the percentage of sperm with intact acrosome was investigated by means of fluorescent staining namely Pisum Sativum Agglutinin (PSA) as described by Filliers et al. (2008).

**Statistical analysis**

The number of boars sampled (15) permitted to investigate a difference in most CASA parameters up to 10% with 95% certainty and a power of 80% considering mean values and SD from previous studies (López et al., 2010). The number of boars sampled permitted to investigate a difference in intact membrane sperm up to 5% (for example 85-80) with 95% certainty and a power of 80% considering mean values and SD from previous studies (López et al., 2010). The number of boars sampled permitted to investigate a difference in intact acrosome sperm of 10% (for example 75-67.5) with 95% certainty and a power of 80% considering a standard deviation of 10. Power calculations were made in Win Episcope (Win Episcope 2.0, CLIVE, Edinburgh, UK).

Data were normally distributed as determined by Kolmogorov-Smirnov test of normality. For all parameters of comparison, a repeated measures analysis of variance was performed to identify possible differences between the groups during storage. Group was
included as fixed factor and time as within subjects’ factor and their interaction was also investigated. Spearman rank correlation between dilution ratio and semen quality parameters was calculated. Differences were considered as significant if p-values were lower than 0.05 (2-sided test). Statistical analyses were performed using the statistical software package SPSS version 18.00. Data are expressed as means ± SD, unless stated otherwise.

4.2.4. Results

The average temperature of the 1:1 dilution was 29.4±1.1°C, with fluctuations ranging from 27.7°C to 31.6°C. The mean transport time from the AI-centre to the laboratory during the 3 weeks was 43 minutes. The temperature inside the car during transport was 26.3°C, 22.8°C and 24.2°C for week 1, 2 and 3, respectively. The temperature in the isotherm box during transport was: 27.5°C (GA) and 26.0°C (GB) in week 1; 26.0°C (GA) and 24.7°C (GB) in week 2 and 27.2°C (GA) and 24.0°C (GB) in week 3. The average temperature of the semen after 3 days of storage was 16.5±0.3°C (GA) and 16.6±0.4°C (GB) (p>0.05).
Table 1: Semen quality parameters of boar semen doses (n=15). Semen was first diluted (1:1) with diluter at 30°C. Semen was subsequently diluted with pre-heated extender (29.3°C ± 0.2°C; GA) and at room temperature 22.7°C ± 0.6°C; GB). From D0 to D2, samples were stored in a refrigerator set at 17°C.

<table>
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<th>Day 0</th>
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<th>Day 2</th>
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<td>72.8 ± 6.8a</td>
<td>65.5 ± 7.6b</td>
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<td><strong>LIN (%)</strong></td>
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</tr>
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<td>40.6 ± 8.3a</td>
<td>33.5 ± 7.1b</td>
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<tr>
<td>GA</td>
<td>62.3 ± 13.3a</td>
<td>65.1 ± 12.9a</td>
<td>50.8 ± 19.1b</td>
<td>51.7 ± 19.4b</td>
<td>0.579</td>
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<tr>
<td>GB</td>
<td>65.1 ± 12.9a</td>
<td>50.8 ± 19.1b</td>
<td>51.7 ± 19.4b</td>
<td>54.4 ± 19.8b</td>
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<td><strong>Static (%)</strong></td>
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<td>GA</td>
<td>10.4 ± 6.1a</td>
<td>18.6 ± 19.9b</td>
<td>15.6 ± 10.5b</td>
<td>17.0 ± 15.0b</td>
<td>0.552</td>
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<td>GB</td>
<td>8.8 ± 6.1a</td>
<td>15.6 ± 10.5b</td>
<td>17.0 ± 15.0b</td>
<td>17.3 ± 15.2b</td>
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<td><strong>Membrane intact (%)</strong></td>
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<tr>
<td>GA</td>
<td>85.1 ± 10.7a</td>
<td>85.8 ± 8.0a</td>
<td>86.9 ± 8.1a</td>
<td>85.6 ± 6.6a</td>
<td>0.761</td>
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<tr>
<td>GB</td>
<td>84.4 ± 3.8a</td>
<td>85.8 ± 6.2a</td>
<td>85.6 ± 6.6a</td>
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<td><strong>Acrosome intact (%)</strong></td>
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<tr>
<td>GA</td>
<td>72.2 ± 9.5a</td>
<td>68.3 ± 16.6a</td>
<td>69.1 ± 18.0a</td>
<td>70.7 ± 12.8a</td>
<td>0.792</td>
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<tr>
<td>GB</td>
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Motile% (percentage of motile sperm); Progressive% (percentage of progressively moving sperm); VAP (average path velocity); VSL (straight-line velocity); VCL (curvilinear velocity); ALH (amplitude of lateral head displacement); BCF (beat cross frequency); STR (straightness); LIN (linearity). ^ab^ Different superscripts within the same row indicate significant differences (p < 0.05).
Chapter 4.2

The CASA parameters and the percentage of sperm with intact membrane and acrosome are summarised in Table 1. All studied semen quality parameters were affected by boar and study week (p<0.05). No interaction of group with boar, week or day was observed (p>0.05). No significant differences were found between groups for any of the CASA parameters. Total and progressive motility numerically decreased in both groups during storage but this decrease was only significant from D0 to D1 (Table 1). Some CASA parameters varied similarly in both groups during storage but variation was only significant from D0 to D1 (Table 1).

The percentage of sperm with intact membrane was similar both between days of storage and groups (p>0.05). The percentage of sperm with intact acrosome was not different between groups (p>0.05) and did not seem to change in any of the groups during storage (p>0.05).

The final dilution ratio ranged from 1:4 to 1:8.4 and this ratio was not associated with any of the studied semen quality parameters (p>0.05).

4.2.5. **Discussion**

In the present study, the effect of different temperatures of dilution on boar semen quality was investigated.

Although temperature was carefully controlled, fluctuations were still observed. Different dilution ratios could explain different temperatures of the 1:1 dilution. This pre-dilution has been identified as a critical step during semen processing and, if not performed properly, may render spermatozoa more sensitive for any damage during the final dilution (Waberski 2009). This pre-dilution is not consistently performed at a 1:1 ratio and a higher or lower dilution ratio may be applied depending on the volume of the ejaculate. It is likely that a lower dilution ratio was applied to those ejaculates with a higher volume which would result in a higher temperature of the 1:1 dilution of these ejaculates. In boar spermatozoa, the cold shock appears immediately after ejaculation and therefore the 1:1 dilution is very important for the acclimation to the subsequent final dilution (Pursel et al., 1972). The mechanisms behind this acclimation are not completely known but they seem to be related to reorganization of the membrane’s lipids and proteins (Johnson et al., 2000). Waberski (2009) identified that a low 1:1 dilution ratio reduced the stabilizing effect of sperm. However, because of biosecurity reasons, the 1:1 dilution was done by the staff at the AI-centre as routinely performed and therefore, the effect of this dilution was not investigated in the present study.
Similarly to other studies, no major changes in semen quality characteristics were observed in diluted semen stored during less than 3 days which is the conventional storage time of semen in most farms (Althouse et al., 1998; De Ambrogi et al., 2006). This is most likely due to the protective effect of BTS extender which keeps sperm viable for a period up to 3 or 4 days (Vyt et al., 2004).

Cold shock can reduce motility as well as sperm vitality (Pursel et al., 1972; Althouse et al., 1998). Nevertheless, the critical cold shock temperature established at 12°C by Althouse et al. (1998) was not reached during the study. It is therefore likely that working at a temperature above 12°C does not affect sperm motility nor membrane or acrosome integrity. However, gross motility or morphology assessed using eosin nigrosin staining, seem not sufficient to elucidate minor / subtle changes in semen quality and more sensitive tests are needed (Waberski et al., 2009). Petrunkina et al. (2005) did not find any effect of dilution temperature on conventional semen quality parameters. However, measuring calcium influx in sperm submitted to induced capacitation, they found that sperm diluted at 32°C was more rapidly saturated by intracellular calcium compared to sperm diluted at 20°C. This effect was observed when semen was stored at 10°C but not when stored at 16°C. The authors therefore suggest that when sperm is kept at a temperature close to the physiological one, the membrane starts suffering from capacitating-like changes which involve reorganization of membrane’s lipids and proteins. It is possible that these changes in the structure of the membrane render sperm diluted at 32°C more sensitive to cold shock. It is also possible that the negative effect of dilution at 32°C only becomes evident when severe cold shock is applied and that it is masked at temperatures above 12°C since these temperatures do not cause severe cold shock. In contrast, we did not find any effect of dilution temperature on semen quality based on CASA parameters but we did not test different temperatures of storage and we did not submit semen to temperatures below 16°C. Testing the calcium influx in sperm under capacitation conditions might be a sensitive tool to detect minor changes in semen quality (Petrunkina et al., 2005). However, this technique is not yet fully standardized and the outcomes are difficult to interpret (Petrunkina et al., 2005). Moreover, it requires trained staff and expensive equipment for flow cytometry. On the other hand, CASA could be a more easy method to monitor semen processing. As cold shock induces capacitation-like changes, it might be possible to identify these changes by evaluating specific CASA motion patterns. It has been shown that sperm undergoing capacitation-like changes show high average path velocity (VAP) and low linearity (García-Herreros et al., 2005). None of
these parameters was affected by dilution temperature. It is less clear which of the CASA parameters are related to in vivo fertility. It has been demonstrated that the percentage of total motility as determined by CASA has a positive effect on litter size and number of live born piglets, but no major predictive values were obtained for the other CASA parameters (Vyt et al., 2008). However, Holt et al. (1997) found a positive association between CASA velocity parameters like VAP and straight line velocity (VSL) and fertility. Anyway, a sensitive method which is able to detect minor changes during semen processing would be very suitable for routine and practical use in AI-centres.

The semen quality parameters for both groups were above the recommended cut-off values for boar semen quality for AI (Waberski et al., 2008; Martin Rillo et al., 1996). Moreover, motion parameters (CASA) were not affected and therefore there is no clear indication that sow fertility would have been affected by different dilution temperatures. However, all in vitro studies on boar semen should ideally be confirmed by in vivo assays.

In conclusion, no significant differences were found in semen quality between boar semen diluted at 30°C and at a room temperature of 22-23°C after a 1:1 pre-dilution (30°C). From a practical point of view, this implies that, when a 2-step dilution is performed, preheating the diluter for the second dilution step is not required for maintaining good semen quality. The study also showed that, even when diluted semen samples are processed in a controlled manner, fluctuations in temperature are commonly observed.
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Chapter 5. General Discussion
This thesis investigated several practical aspects of liquid semen quality analysis and production in pigs. During the last years, research in porcine semen has mainly focused on the optimization of the cryopreservation process. However, artificial insemination (AI) with liquid stored semen is the preferred and for practical reasons still the most frequently used method in commercial swine farms (Johnson et al., 2000; Riesenbeck, 2011). Research in this area is therefore imperative in order to improve semen productivity and fertility results. Semen quality analysis is a necessary step during semen production; not only to identify boars with poor semen quality but additionally to detect possible semen damage during processing and storage (Shipley, 1999; Foxcroft et al., 2008; Waberski et al., 2011).

In the first study of this thesis (Chapter 3.1) we investigated different methods to assess boar semen concentration and motility. A new device for boar semen quality analysis was validated: the sperm quality analyser (SQA-Vp). Besides evaluating the spermatozoa themselves, the seminal plasma (SP) is also an important player in determining quality of a given ejaculate. Research on SP components is increasing in the last years because some of these components could be associated with fertility (Foxcroft et al., 2008; Dyck et al., 2011). Biochemical analysis of SP has been reported in several other animal species and in human, but it is poorly documented in boars (Pesch et al., 2006; Kareskoski et al., 2010; World Health Organization, 2010). Therefore, in Chapter 3.2 the association of selected biochemical components with semen quality was investigated. Enzymes like ALP and GGT as well as several minerals like Na, Cl, Se and Zn could be associated with semen quality.

Furthermore, the thesis showed that proper management of the boars and attention for specific details during semen handling may improve the semen production. Boar productivity and the quality of the semen might be improved by feeding strategies (Kemp and Soede, 2001). However, research on boar nutrition is very scarce and mainly based on old studies (for review see Kemp and Soede, 2001). We have shown in Chapter 4.1 that different sources of selenium (Se) in feed could affect semen quality parameters.

Next to boar management, semen handling is important to avoid sperm damage and bacterial contamination (Vyt et al., 2007; Althouse, 2008; Waberski et al., 2008). We have shown in Chapter 4.2 that some practices that were believed to be necessary like e.g. matching the semen temperature for final dilution could be replaced by more simple dilution protocols without decreasing semen quality. Dilution at room temperature did not have a negative effect on semen quality and it could therefore simplify the production...
process.

In this final chapter, the results of the different studies are discussed. We will successively focus on (1) semen quality analysis (2) analysis of the seminal plasma (3) boar feed supplementation, (4) effect of dilution temperature on semen quality and (5) future perspectives.

**Semen quality analysis**

Although semen quality is directly related to *in vivo* fertility (Vyt et al., 2008; Tsakmakidis et al., 2010; Broekhuijse et al., 2011a), there is currently no consensus on which method is the most precise, accurate and objective. Various methods are used in different AI-centres (Vyt et al., 2007; Knox et al., 2008; Waberski et al., 2008). New devices claiming better precision, accuracy and objectivity are constantly coming to the market. The increase in the availability of new devices is very positive as it could improve semen quality analysis. On the other hand, the abundance of methods for semen quality analysis makes it difficult to compare results between AI-centres and andrology laboratories. Additionally, each new method needs to be validated properly before used in practice.

Validation studies of methods to measure biological parameters, including semen analysis, are often based on correlations and p-values between the method to be validated and a gold standard (Bland and Altman, 1999). However it has been clearly shown that this is not the adequate statistical approach for validation studies (Bland and Altman, 1999). Correlations give only a rough indication on whether the measurements of two different devices are related in a linear way but give no overview of their agreement. Two methods will agree if, when plotted against each other, the points lie along the line of equality, but they will correlate if the points lie along any straight line (Bland and Altman, 1986). In this respect, we used the method proposed by Bland and Altman in which the mean difference is plotted against the mean measurements of the 2 methods (Bland and Altman, 1986; Bland and Altman, 1999). This statistical analysis gives a good estimation of the agreement between methods and the precision of each method over the entire range of values. Following this statistical approach, we observed that the SQA-Vp agrees with the measurements of traditional and computer assisted semen analysis (CASA) for semen concentration and motility.

The validation study was also useful to make a comparison between the outcomes provided by different current and frequently used methods for semen quality analysis. The
question of which method is the best for assessing semen concentration and motility parameters has been raised many times in the last years, and still, based on the studies in this thesis and in the literature, it is not yet possible to provide a straightforward answer.

For semen concentration, it was interesting to observe that the haemocytometer (Bürker chamber in this case) showed higher variations in the counts than all the other evaluated methods. The same finding has been reported recently in a study comparing photometers with different methods for semen concentration (Camus et al., 2011). The possible reasons for these findings have been stated in the general introduction. One should therefore question whether the haemocytometers are the adequate gold standard for boar semen analysis since they seem to lack accuracy and precision. In an attempt to standardize semen quality analysis, the WHO has proposed the improved Neubauer haemocytometer as the gold standard for human semen concentration. This haemocytometer was not tested in this thesis but a recent study in boars has shown that the outcome of the improved Neubauer can be easily biased by the technician (Hansen et al., 2006). Therefore the discussion on the best method for boar semen concentration remains open and there is a call for standardization and for the establishment of a gold standard for boar semen. The haemocytometers are time consuming and show higher coefficients of variation. Photometers provide accurate and precise counts in a short period of time and that makes it the preferred method by AI-centres. However, photometers can confound particles with similar size as sperm and they need regular calibration. Taking into account how difficult it is to determine the most accurate method to study sperm concentration, the observed differences between SQA-Vp and the other methods can be considered as acceptable. The agreement plots showed that Bürker, photometer, CASA and SQA-Vp can all be used to measure boar semen concentration.

When evaluating motility, both CASA and SQA-Vp had lower variability in the measurements than visual motility. Visual motility is fast and consistent when performed by the same person but it is subjective and only provides a rough idea of the sperm motility. Both automated methods therefore seem to be more objective than visual motility (Rijsselaere et al., 2002; Vyt et al., 2004a). Our results showed that SQA-Vp is suitable for boar semen motility estimation. However, the SQA-Vp presents a disadvantage compared to CASA. When working with highly selected boars, the motility will consistently be high for all boars. Therefore, other motion parameters besides gross motility should be studied. However, the SQA-Vp is not able to discriminate progressive from total motility, and does not provide any information on velocity and patterns of straight forward movement while
all these different motion patterns can be obtained with CASA systems. The information on motion patterns obtained by CASA allows for the detection of subtle differences between highly selected boars. Even different sperm subpopulations within the same ejaculate can be identified (Garcia et al., 2005). Furthermore, the relation of the detailed motion parameters as measured by CASA with in vivo fertility, although not always consistent, seems to become more and more clear (Vyt et al., 2008; Broekhuijse et al., 2011b). Recent studies have shown that CASA parameters such as total motility, velocity average path, velocity straight line and amplitude of lateral head displacement are associated with litter size. Moreover, progressive motility, velocity curvilinear, and beat cross frequency on farrowing rate are associated with farrowing rate (see Table 1 in Chapter 1.1). This makes CASA the preferred tool for porcine research. However, its use for AI-centres remains limited mainly because of the rather high cost (Vyt et al., 2007; Feitsma et al., 2011).

The question of whether CASA systems are 100% objective is probably not properly formulated. One should rather ask whether CASA is more objective than visual assessment. Based on our results and on previous studies (Vyt et al., 2004a) we are inclined to answer “yes”. CASA appears to be more accurate than visual assessment, more precise and provides more detailed information which may be related to in vivo fertility. However, many factors which have been discussed in Chapter 1.1 can bias CASA results. The Segre Silberberg effect in low depth chambers used for CASA, sample preparation (i.e. introduction of air in the slide) and sperm agglutination can result in miscounts of concentration and/or motility (see Figure 2 in Chapter 1.1). Therefore proper and regular training of technicians and standardization of the CASA protocols are imperative (Vyt et al., 2004c; Feitsma et al., 2011).

**Lipid peroxidation**

In addition to the assessment of concentration and motility, other assays may be useful to study sperm integrity and function. Most of the methods currently available have been discussed in the general introduction. In experiment 3, we used an assay to assess resistance to lipid peroxidation, namely analysis of Thiobarbituric Acid Reactive Substances (TBARS). Our results suggest that TBARS analysis could be a valid method to study differences in protection against lipid peroxidation. Boar sperm is very sensitive to lipid peroxidation because of the high content of polyunsaturated fatty acids, the substrate of reactive oxygen species (ROS) (Bathgate, 2011). Therefore, sperm resistance against
lipid peroxidation seems to be a useful tool for future research.

**Biochemical components of the seminal plasma**

In a second part of this thesis, we focused on the SP composition. The seminal plasma is composed of inorganic compounds, amino acids, peptides and low- and high-molecular-weight proteins (Rodger, 1975; Maxwell and Johnson, 1999; Rodriguez-Martinez et al., 2011). This composition has been ignored in boar semen research as extenders seem to be able to substitute SP and provide a good protection for the spermatozoa during storage (Maxwell and Johnson, 1999; Johnson et al., 2000). However, assessment of SP composition is currently included in human semen analysis as some of its components are indicators of prostatic (dys)function and/or are related to infertility in men (Chia et al., 2000; World Health Organization, 2010).

In boars there is an increasing interest on biomarkers in the SP for fertility (Dyck et al., 2011; Rodriguez-Martinez et al., 2011) but research has mainly focused on the role of specific proteins and little is known about the biochemical composition of the SP.

In our study we found several interesting associations of SP biochemical parameters with both quantitative and qualitative semen parameters. Our findings regarding ALP and GGT activity in SP were to a large extend similar to those reported in stallions. Their positive association with semen concentration and negative association with semen volume could suggest a testicular/epididymal origin of these enzymes (Pesch et al., 2006). This is in agreement with old studies that showed the presence of ALP in testis of rats and low ALP in accessory glands of boars by IHC (Bern, 1949; Aitken, 1960). Low levels of these enzymes could therefore be used as indicators of insufficient ejaculate for example due to obstruction in ducti efferentes (Clements et al., 2010).

In addition, a positive association was found between ALP, GGT, K, P and Se with progressive motility. Similar positive associations of ALP and GGT with motility have been described in stallions (Pesch et al., 2006). However, the exact mechanism for this association is not elucidated yet. The activity of ALP seems to be related with the synthesis of fructose which is one of the energy sources for sperm metabolism (King and Macpherson, 1966). However, fructose is synthesized in the seminal vesicles (Mann, 1946). If indeed ALP is of testicular origin, it should not affect fructose production in the semen vesicles and the mechanism for its relation to motility remains unclear and further research is therefore warranted. We can only hypothesize about the possible mechanisms. It could be possible that ALP interacts with fructose precursors released in the accessory
glands. The activation of fructose precursors by ALP could be one of the triggers for spermatozoa activation when they come in contact with SP. This assumption could not be demonstrated in this thesis. The relation between GGT and motility has been attributed to a protective effect of the enzyme against oxidative stress during sperm maturation in the epididymis (Kohdaira et al., 1986; Seligman et al., 2005). This enzyme mediates in the supply of cysteine to cells and helps maintaining intracellular glutathione level (Chikhi et al., 1999).

The association of minerals with semen quality can be explained by their role on sperm cell metabolism and by the antioxidant properties of some of them. Initiation of sperm motility is a complex physiological process involving the exchange of ions like Na\(^+\), K\(^+\) and Ca\(^{2+}\) (Darszon et al., 1999; Luconi et al., 2006; Torres-Flores et al., 2011). Entrance of Ca is essential to initiate motility and this entrance is regulated by Na and K dependent ionic channels. Furthermore, the Na-K pump regulates the entrance of Na in sperm cells and the release of intracellular K, and this mechanism plays an important role in initiation of motility (Wong and Lee, 1983). Addition of low concentrations of potassium chloride to boar extenders facilitates the preservation of sperm motility by maintaining the sperm Na/K pump, avoiding excessive loss of intracellular K (Harrison et al., 1978; Alvarez and Storey, 1982).

Other minerals are also important for sperm motility and vitality. For example, P is involved in the phosphorylation required to activate proteins that assist in the initiation of sperm motility (Arrata et al., 1978; Tash and Bracho, 1994). As we will discuss further in this section, selenium is a main component of glutathione peroxidase (GPx) which has antioxidant properties and deficiencies in this mineral are reported to negatively affect sperm production and motility (Marin-Guzman et al., 1997; Marin-Guzman et al., 2000). Additionally, antioxidant properties are attributed to Zn and this mineral is subject of study in human medicine in the last years. Deficiency of this mineral for instance has been reported in smokers and it seems to be associated with male infertility (Chia et al., 2000). The prostate seems the main source of Zn in SP and Zn levels in SP are indicators of prostate (dys)function in men (World Health Organization, 2010). In boars it seems to originate in the vesicular glands (Boursnell et al., 1972), but no studies have shown the relation of Zn with infertility in pigs.

Interestingly, many of the studied SP components were associated with days to previous collection. It is clear that high collection frequency affects semen quality (Pruneda et al., 2005). However, the exact mechanisms for this phenomenon are not
completely clear. It might be possible that sperm is forced to pass from the head to the tail of the epididymis with insufficient time for maturation. Furthermore, it has been shown that high collection frequency affects the excretion and reabsorption behaviour of epididymal epithelium (Pruneda et al., 2005). If the excretion and reabsorption process is altered, the SP composition will be influenced which could partially explain our findings. The SP is a mixture of fluids from the epididymis and the accessory sexual glands (vesicular, prostate and bulbo-urethral gland) (Davies et al., 1975) but the exact origin of each component is not clearly known.

Furthermore, we have provided some reference values for the SP biochemical parameters as these are very scarce in the literature. Although variations were observed in the measurements and many factors can affect them, the proposed reference values seem valid as the repeatability in healthy boars was acceptable. The analysis of SP components is not suitable to be included in the daily semen analysis in AI-centres because the necessary equipment and the analyses are expensive. However, in cases of boars with fertility problems it might help in the diagnosis of obstruction in the ductuli efferentes/ductus deferente. Furthermore it could be a tool to monitor antioxidant capacity of boar semen by measuring Se or Zn. Further research to establish the potential of SP analysis as predictor of boar fertility is warranted.

Boar feed supplementation with selenium

Feed supplementation with antioxidants receives increasing interest although studies focusing on boar nutrition are scarce. Semen handling and storage induce lipid peroxidation which affects sperm motility (Parrilla et al., 2011). Therefore, it could be useful to add antioxidants which improve or maintain semen quality. Selenium is a known antioxidant and supplementation with this mineral may be important for boar semen quality (Marin-Guzman et al., 1997; Marin-Guzman et al., 2000). Moreover, treatment of infertility in men by supplementation of Se was recently reported to be effective (Agarwal and Sekhon, 2010; Moslemi and Tavanbakhsh, 2011). Selenium is routinely supplemented in boar feed in higher levels than the NRC recommendations. However, it can be supplemented in inorganic or organic form. Which source has the most beneficial effect on boar semen quality was not known when starting this study (Jacyno et al., 2002; Speight et al., 2011).

We therefore studied different sources of Se supplementation in feed and we found that the form in which the mineral is supplemented, may be important even when boars are
fed the recommended levels of Se. Boars receiving the inorganic form performed better which could be explained by a protective effect of glutathione peroxidase (GPx). It seems that the inorganic form (Na₂SeO₃) is more easily metabolized and incorporated into GPx (Sunde and Hoekstra, 1980; Mahan et al., 1999). On the other hand, selenomethionine, the form in which organic Se is administered (Beilstein and Whanger, 1986), competes with methionine for incorporation in non-Se-requiring proteins leading to selenomethionine to be less available for the synthesis of GPx (Beilstein and Whanger, 1986).

The presence of GPx in spermatozoa is controversial and it is not clear whether sperm GPx actively protects against lipid peroxidation since it has been suggested that the enzyme loses activity when it incorporates in the sperm membrane (Ursini et al., 1999). In this respect, our study suggests that there is GPx activity in boar sperm as antioxidant capacity was affected by Se source. This is in agreement with an older study in which GPx was detected in boar sperm and Se supplementation seemed to increase its activity (Marin-Guzman et al., 1997).

Our results were however surprising as a previous similar study had shown a better semen quality when organic Se was supplied (Jacyno et al., 2002). However, differences in the experimental design could partially explain these different results. First, in the latter study, the group of boars receiving the organic Se also received extra supplementation of Vit E which could have biased the results as it may also affect semen quality (Marin-Guzman et al., 2000). Furthermore, the study of Jacyno et al. (2002) was performed with prepubertal boars whereas in our study, only mature boars were used. Whether differences on semen quality where already present at the moment of randomization in this study is difficult to say because semen quality was first analyzed when boars were already in production. Many factors can affect the sexual development during puberty. Moreover, a very recent study has also shown more beneficial effect of organic compared to inorganic Se on sperm motility (Speight et al., 2011). In the latter study however, boars were fed different diets from weaning age onwards, and moreover no oxidative stress parameters were measured.

Although only minor changes in semen quality were observed in our study, these findings in combination with our results on SP analysis, suggest an important role of Se on semen quality in boars.

**Effect of dilution temperature on semen quality**

As stated earlier, research on dilution temperature of liquid semen in pigs is scarce.
Most of the studies performed during the last years of the temperature effect on semen quality have focused on optimization of the cryopreservation process. Fast cooling of the ejaculate from body temperature to a temperature below 15°C results in capacitation like changes due to lipid phase separation and alteration of the sperm membrane permeability (De Leeuw et al., 1990; Johnson et al., 2000; Petrunkina et al., 2005; Leahy and Gadella, 2011). Therefore, the temperature of the semen at dilution must be controlled. Surprisingly little attention is paid to the temperature of dilution. This is likely due to the fact that the critical cold shock temperature for sperm survival, established at 12°C by Althouse et al. (1998), is normally not reached during actual semen processing.

A few years ago, a questionnaire performed in Belgium showed that, in Belgian porcine AI-centres, there is a difference between the semen temperature and the temperature of the extender added to the ejaculate. In 14% of the cases, the extender temperature was below 22°C. This study additionally showed a large variation in the protocols between different AI-centres (Vyt et al., 2007). However, it was not investigated whether these different protocols affected semen quality. In AI-centres where dilution was performed below 22°C, no negative effect on semen quality was reported by the staff. Our study on dilution temperature (Chapter 4.2) was carried out in an AI-centre in which the temperature of dilution was matched to the temperature of the semen (i.e. 30°C). The personnel in this AI-centre confirmed that, when the diluter heater failed, they experienced problems with semen quality. Consequently the temperature of the semen diluter is of direct importance to practice.

Despite the different opinions of the effect of dilution temperature on semen quality and its possible economic impact, few studies are published on this topic (Waberski, 2009). Many years ago, it was stated that storage of sperm at 30°C for several hours had a protective effect for samples which should subsequently be stored at 17°C (Pursel et al., 1972). A more recent study however indicated different results (Petrunkina et al., 2005). The latter authors suggested a negative effect of acclimation at 32°C arguing that, by keeping sperm closer to the physiological temperature, the sperm does not diminish its metabolism leading to changes impairing semen quality. External quality control in Germany has shown that the different protocols used in each AI-centre for semen dilution may result in differences in semen quality among AI-centres (Waberski et al., 2008). Therefore, their protocols should be studied and standardized. We designed a protocol in which dilution at room temperature (approximately 23°C) was compared with dilution matching semen temperature at the moment of dilution (approximately 30°C).
We expected worse semen quality in the samples diluted at room temperature because cold shock has been widely reported to have a negative effect on semen quality and fertility (Martin-Rillo et al., 1996; Althouse et al., 1998; Johnson et al., 2000), especially when semen was cooled rapidly below 15°C. The absence of symptoms of cold shock in our study compared with older studies may be explained by the fact that previous experiments used very low temperatures (below 17°C) and moderate temperatures (e.g. 22-23°C) were ignored. In contrast to Petrunkina et al. (2005) who found that dilution at room temperature was better for semen quality compared to dilution at 32°C, we did not find any differences between groups. However, this effect was only evident when semen was stored at 10°C but not when stored at 16°C and we did not further investigate different storage temperatures in our study.

We can only speculate on the reasons why problems with semen quality occurred in this AI-centre when the extender heater failed. If the heater is placed in a room where the temperature is not controlled, it is possible that room temperature can decrease below 12°C resulting in too cold extender and consequently damage of the semen. This could be the case in the AI-centre of our study as the tank was placed outside the laboratory in a room without any temperature control. Therefore, it is recommended that these tanks should be kept in places where the room temperature is more or less stable. Additionally it is necessary to check routinely whether the incubator is working properly and temperature of the diluter should be measured daily before processing starts. Furthermore these incubators have been identified as a source of bacterial contamination and consequently, they should be cleaned routinely (Althouse, 2008).

Our study additionally confirmed that no major changes in semen quality characteristics occurred in diluted semen stored during less than 3 days which is the conventional storage time of semen in most farms (De Ambrogi et al. 2006; Althouse et al.1998). The extender used (BTS) apparently provided effective protection up to 3 days as described in the literature (Vyt et al., 2004b).

There is however a clear need for more in vivo studies as all in vitro studies on porcine semen should be confirmed by in vivo assays to determine the relevance of the in vitro findings. Unfortunately, research on boar semen faces a problem when progressing from in vitro to in vivo studies. Many management practices may influence pregnancy rates in sows. Furthermore for economical reasons, it frequently becomes difficult to perform in vivo studies with manipulated semen since it might be a risk for the involved farm for possible detrimental fertility results.
Conclusions

In conclusion, different practical aspects concerning the production of boar semen for AI have been studied in this thesis. We have evaluated different methods for boar semen concentration and motility analysis and discussed the differences in their outcomes. Furthermore, analysis of biochemical parameters in seminal plasma could add additional information to the currently performed semen analysis methods. Finally, it was shown that feed can affect boar semen quality and that the dilution process can be simplified by dilution at room temperature.

The specific conclusions of this thesis are:

- The SQA-Vp is suitable for estimation of boar sperm quality. The device showed a good agreement with previous proven methods for boar sperm analysis such as photometer and CASA. Moreover, the SQA-Vp proved to produce consistent results.
- Reference values of selected SP components are proposed and could add
information to the increasing research performed on boar SP. A significant different composition of Na, Cl and Zn of the SP of good and poor quality ejaculates was observed. Furthermore, different associations of biochemical components of the SP with semen quantity and quality were found and several parameters were associated with collection frequency.

- Feeding organic instead of inorganic Se resulted in a higher sperm concentration. On the other hand, sperm from boars fed inorganic Se showed a more straightforward movement. A higher incorporation of inorganic Se into sperm GPx resulting in higher resistance to oxidative stress is suggested.

- After a 1:1 pre-dilution (30°C), no significant differences were found in semen quality between boar semen diluted at 30°C versus 22-23°C. From a practical point of view, this implies that, when a 2-step-dilution is performed in an AI-centre, preheating the diluter for the second dilution step is not required for maintaining good semen quality.

**Future perspectives**

Liquid semen is a forgotten topic in porcine research as it is believed that there are few possibilities for any further improvement. However, we have shown in this thesis that semen analysis, boar management and semen handling can be improved.

Concentration and motility are clear indicators of boar fertility. Different outcomes in the measurements of these parameters are observed depending on the method used for the analysis. Therefore there is still a need for standardization in order to be able to compare semen quality parameters between andrology laboratories. The gold standards for boar semen analysis should be established as it has been proposed in human andrology (WHO manual). Furthermore, new devices coming in the market need proper validation. The relation of semen quality parameters with fertility is however still difficult to demonstrate due to the practical difficulties to perform in vivo trials.

In addition to the assessment of concentration and motility parameters, biochemical components of the seminal plasma seem to play an important role on semen quality. Therefore, research on SP should be further developed. The mechanisms why some of the biochemical parameters of the SP affect semen quality need to be further elucidated. To better understand which changes occur in the environment of the sperm from spermiogenesis to ejaculation. We additionally need further knowledge on where in the genital tract the different components are produced. This knowledge could help us to better
understand the processes of sperm maturation before, during and after ejaculation.

We have also shown that feeding strategies could improve semen quality. An interesting finding in our studies was that feed composition was different between the AI-centres and the mineral supplements were always at higher levels than those recommended by NRC. The specific nutritional requirements for boars, not only mineral supplements but also proteins and energy intake, need to be further clarified, as they are mainly based on old studies. If deficiencies may be hazardous for semen it is possible that too high quantities may also be detrimental. Furthermore there is very little research available on the effect of different ingredients used for boar feed on semen quality. For instance, soybean (a very commonly used ingredient in porcine feed) has recently been shown in human to be related with reduced sperm concentration due to its estrogens content (Chavarro et al., 2008) rendering it an important topic for further investigation in boars. Finally, detailed studies on temperature effects at each step of semen handling may help to improve and eventually simplify the currently used semen production systems. Not only final dilution but also the effect of the first dilution as well as moderate temperatures of storage need to be further investigated.
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Summary
Artificial insemination (AI) is the preferred method for sow mating in most European pig herds. To this end, fresh extended semen is produced in AI-centres and delivered to the farms in ready-to-use doses. Production efficiency will depend on proper identification of boars with potential fertility and identification of usable semen. Therefore semen quality analysis is a necessary step during semen production not only to identify boars with poor semen quality that need to be culled, but also to detect possible semen damage during processing. Next to semen analysis, a second essential step for a profitable semen production is to obtain the maximum productivity out of each boar. Since all physiological processes in the body are somehow related to feeding, this obviously is also the case for sperm function. The addition of antioxidants in the diet could for example improve semen quality as sperm suffers lipid peroxidation during processing and storage. Last but not least, ejaculates need to be handled in a controlled manner to avoid semen damage. Little attention has been paid in the literature to dilution temperature and dilution at room temperature could simplify the processing of semen doses.

In Chapter 1.1 the current knowledge on boar semen quality analysis is discussed. Concentration assessment can be performed using counting chambers, photometry, flow cytometry or image analysis (CASA). Morphology examination is done by staining or by computer assisted morphology analysis (ASMA). Different fluorescent staining procedures can be used to assess membrane integrity and acrosome integrity, sometimes in combination with flow cytometry. Motility is mostly assessed visually although computerized methods are available. New advanced methods to study semen quality such as assessment of the sperm function or lipid peroxidation as well as the potential use of seminal plasma (SP) analysis are presented. The advantages and limitations of these methods are discussed.

In Chapter 1.2 the current knowledge on boar management and semen processing is presented. The boars and consequently the semen production are sensitive to environmental factors. Different housing conditions and differences in temperature, humidity and light may have an impact on semen quantity and quality. The current literature on nutritional factors that could affect semen production and quality focused on antioxidant supplements is summarised. Furthermore, during processing, semen undergoes a series of changes that may be detrimental for its quality. In a second part of Chapter 1.2, handling of the semen and its effect on semen quality are presented with special attention to the current knowledge of temperature effect.

The experimental part of this thesis investigated new tools for semen quality
analysis and possible factors before and after ejaculation that could optimize semen production and quality in boars.

Validation studies for new devices for semen quality analysis are necessary. In Chapter 3.1, we validated a new device for boar semen quality analysis: namely the sperm quality analyzer (SQA-Vp). Both fresh and extended semen from 50 ejaculates of different boars were investigated. The concentration of fresh samples obtained with SQA-Vp was compared with IMV Accucel photometer and Bürker chamber. For extended samples, results were compared with CASA (Hamilton Thorne) and visual semen analysis. The agreement between methods was studied with Bland-Altman plots and the repeatability with coefficient of variation (CV) as well as with Bland-Altman plots. The agreement between SQA-Vp and the other methods was good for fresh ejaculates concentration. In diluted semen, the agreement between SQA-Vp and CASA for concentration was good, but poor with the haemocytometer. For diluted semen, the % motile spermatozoa assessed by SQA-Vp agreed well with CASA and with visual assessment. Moreover, the SQA-Vp showed a good repeatability for measuring the concentration of both fresh and diluted semen and for motility. The SQA-Vp seems therefore suitable for semen quality estimation as compared with Bürker chamber, photometer, CASA and visual assessment. Among the limitations of SQA-Vp, we discuss the fact that it is not able to distinguish progressive motility. In addition to the validation of the SQA-Vp, practical applications and pitfalls of the different methods have been discussed.

Although motility and concentration are clearly associated with fertility, they are not always sufficient to discriminate good from bad boars. Not only sperm but also the SP could help to differentiate boars with lower fertility. In Chapter 3.2, we hypothesized that analysis of SP enzymes and minerals could help to identify boars with low fertility potential, as it happens in human and stallion. Therefore, we tested the association of selected biochemical components with semen quality. The ejaculates of 39 boars from 3 different AI-centres with good (>80% normal sperm and >70% motility) and poor semen quality (<80% normal sperm and <70% motility) were investigated. The association of aspartate-amino-transferase (AST), γ-glutamyl-transferase (GGT), alkaline phosphatase (ALP) as well as for concentrations of sodium (Na), potassium (K), chloride (Cl), calcium (Ca), phosphate (P), magnesium (Mg), selenium (Se) and zinc (Zn) with semen volume, concentration, sperm motility (CASA), morphology and vitality (both eosin nigrosin staining) was investigated. The concentrations of GGT and ALP were negatively associated with volume of the ejaculate and positively associated with concentration. There
was a moderate positive association between GGT and ALP with progressive motility and trends were observed for the association of GGT and ALP with abnormal heads and distal droplets, respectively. The mineral concentration was associated with semen quantity and quality. Phosphate, K and Se were negatively correlated with semen volume and Se and P were correlated positively with semen concentration. Furthermore, there was a moderate positive association of P, K and Se and a negative association of Na with progressive motility. Higher levels of Na and Cl were associated with a decrease in the number of spermatozoa with normal morphology. Whereas Na seemed to be positively associated with abnormal tails, high levels of Cl appeared to be associated with more abnormal heads and abnormal tails. Higher levels of Mg and Se were associated with less membrane damage and proximal droplets, respectively. There was a trend for a negative association between Zn concentration and the number of abnormal tails. In addition, the concentration of some parameters e.g. selenium was associated with days to previous collection. These results showed associations of several biochemical components of SP with semen quantity and quality parameters. The analysis of biochemical parameters could therefore provide extra information about reproductive health of AI-boars. The possible mechanisms why these parameters could be associated with semen quality are discussed in this chapter. Furthermore, reference values of selected biochemical parameters of the SP are provided.

Feed supplementation with antioxidants is a raising topic in human and veterinary andrology. One of the supplements with known antioxidant properties is Se as a component of the glutathione peroxidase (GPx) and it is commonly used in boar feed. However, Se can be supplemented in organic and inorganic form. In Chapter 4.1 the effect of different forms of Se supplementation in feed (organic vs. inorganic) on semen production and quality of boars was investigated. Sixty mature boars from a commercial AI-centre were randomly allocated into two groups and fed either premix with inorganic Se (0.4 mg Na₂SeO₃/kg) and Vit E (80 mg/kg) or a commercial premix with organic Se (0.4 mg/kg, Se-yeast) and Vit E (80 mg/kg). The effect of different sources of Se on sperm concentration (photometer), motility (CASA), morphology (eosin-nigrosin staining), oxidative stress [production of malonaldehyde (µg MDA/l) measured with thiobarbituric acid reagent substance (TBARS) assay] was investigated during 4 months (D0, D30, D60, D90, D105 and D120). Differences were observed in sperm concentration which was higher for the organic group although the diet did not affect the total sperm count. The resistance to oxidative stress was higher in the inorganic group. This difference was explained by the fact the selenomethionine (organic Se) competes with methionine to
incorporate in proteins other than GPx and it is therefore less available for the synthesis of the latter enzyme. Some motility parameters (CASA-HTR straight-line velocity, straightness and linearity) seemed higher in the inorganic group. No differences (P>0.05) were shown for the other parameters. Under the presented study conditions, changing from inorganic to organic Se in the diet of boars increased sperm concentration but reduced some motility parameters and resistance to oxidative stress. We have therefore shown that different sources of Se in feed could slightly affect semen quality.

The critical temperature for sperm survival seems clearly defined at 12ºC. Although working at room temperature could simplify processing of semen doses for AI, there is little research on the effect of different dilution temperatures on semen quality. In Chapter 4.2 we investigated the effect of the temperature of the extender for the final dilution on semen quality in a 2-step dilution protocol. One ejaculate of each of 15 boars housed in a commercial AI-centre was included in the study. Each ejaculate was first diluted (1:1) in a commercially available semen extender (Beltsville Thawing Solution, BTS) at 30ºC and the temperature of the dilution was measured. A final dilution to 30 x 10⁶ sperm/ml (concentration of commercial dose) in BTS, with either pre-heated extender [29.3ºC ± 0.2ºC, Group (G) A] or extender kept at room temperature (22.7ºC ± 0.6ºC, GB), was performed. Samples were stored at 17ºC and investigated during 3 consecutive days. The following parameters were studied at D0, D1 and D2: % of motile sperm (% M), % of progressive sperm (% P) [both by Computer Assisted Semen Analysis (CASA)] and the % of sperm with intact membrane (% IM) by eosin nigrosin staining. At D0 and D2, the % of sperm with intact acrosome (% IA) was studied by PSA staining. No significant differences were found between groups for motility parameters or membrane integrity and the acrosome integrity was neither different between groups. In conclusion, we have shown in this study that dilution at a moderate room temperature (22-23ºC) has no detrimental effect on semen quality. When a 2-step dilution is performed, preheating the extender for the second dilution to match the semen temperature, did not improve sperm quality compared to a dilution at a moderate room temperature.

Finally, results obtained in these experimental trials are discussed in the light of current scientific knowledge (Chapter 5). Our results and the current scientific knowledge allowed us to draw the following conclusions:

- The SQA-Vp is suitable for estimation of boar sperm quality. The device showed a good agreement with previous proven methods for boar sperm analysis such as photometer and CASA. Moreover, the SQA-Vp proved to produce consistent results.
• Analysis of the SP composition should be included in the semen quality analysis of boars with fertility problems. Reference values of selected SP components are proposed and could add information to the increasing research performed on boar SP. A significant different composition of Na, Cl and Zn of the SP of good and poor quality ejaculates was observed. Furthermore, different associations of biochemical components of the SP with semen quantity and quality were found and several parameters were associated with collection frequency.

• Feed supplementation with Se has an impact on semen quality. The form in which supplements are supplied may affect semen quality differently. Feeding organic instead of inorganic Se resulted in a higher sperm concentration. On the other hand, sperm from boars fed inorganic Se showed a more straightforward movement. A higher incorporation of inorganic Se into sperm GPx resulting in higher resistance to oxidative stress is suggested.

• The processing of semen doses could be simplified by diluting at room temperature (22-23°C). After a 1:1 pre-dilution (30°C), no significant differences were found in semen quality between boar semen diluted at 30°C versus 22-23°C. From a practical point of view, this implies that, when a 2-step-dilution is performed in an AI-centre, preheating the diluter for the second dilution step is not required for maintaining good semen quality.
Samenvatting
Kunstmatige inseminatie wordt in de meeste varkensbedrijven in Europa toegepast om zeugen drachtig te krijgen. In de KI-centra wordt vers sperma verdund en geleverd aan de varkensboerderijen in gebruiksklare dosissen. De efficiëntie van de productie hangt af van een goede identificatie van de vruchtbare beren en van de spermakwaliteit. Daarom is kwaliteitscontrole van sperma een noodzakelijke stap tijdens de productie van de spermadosissen; niet alleen om beren te identificeren met een slechte spermakwaliteit, maar ook om minderwaardig sperma te detecteren tijdens de verwerking. Een tweede essentiële stap is het optimaliseren van de spermadoosproductie bij zo veel mogelijk beren. Alle fysiologische processen in het lichaam zijn op een of andere manier gerelateerd aan de voeding. Dit is ook het geval voor de spermafunctie. De toevoeging van antioxidanten in de voeding zou bijvoorbeeld de spermakwaliteit kunnen verbeteren omdat het sperma te lijden heeft onder peroxidatieve processen tijdens de verwerking en bewaring. Tenslotte, moeten ejaculaten op een gecontroleerde manier behandeld worden om spermaschade te voorkomen. In de literatuur wordt weinig aandacht besteed aan de temperatuur bij de verdunning: indien het verdunnen bij kamertemperatuur kan gebeuren, zal dit de behandeling van sperma vereenvoudigen.

In Hoofdstuk 1.1 wordt de huidige stand van zaken besproken wat het onderzoek van varkens sperma betreft. Beoordeling van de concentratie kan worden gedaan met behulp van telkamers, fotometrie, flowcytometrie of computer geassisteerde sperma analyse (CASA). Morfologisch onderzoek wordt gedaan door kleuring of door computer geassisteerde morfologische analyse (ASMA). Verschillende fluorescente kleuringen kunnen gebruikt worden om de membraanintegriteit en de integriteit van het acrosoom te beoordelen, soms in combinatie met flowcytometrie. Beweeglijkheid wordt meestal visueel beoordeeld alhoewel nu ook geautomatiseerde methoden beschikbaar zijn. Nieuwe geavanceerde methoden om de kwaliteit van het sperma te bestuderen, worden besproken, zoals de beoordeling van het bevuchtend vermogen door middel van in vitro fertilisatie, het nagaan van de graad van lipidenperoxidatie (wat negatief kan zijn voor spermakwaliteit) of de mogelijke toepassing van bepaalde markers in het seminaal plasma (SP) als indicatoren voor spermakwaliteit. Tevens worden de voordelen en beperkingen van deze methoden toegelicht.

In Hoofdstuk 1.2 wordt de huidige stand van zaken overlopen wat de huisvesting en behandeling van de beren en de verwerking van het sperma betreft. De invloed van omgevingsfactoren op de beren en bijgevolg ook op hun spermadoosproductie staat buiten kijf. Verschillen in huisvesting en bijkomende veranderingen in temperatuur, vochtigheid en
licht kunnen een invloed hebben op spermaproductie en kwaliteit. Tevens kan ook de voeding van de beer een invloed uitoefenen op de spermaproduktie. De literatuur betreffende nutritionele factoren specifiek gericht op het supplementeren met antioxidantant wordt samengevat. Bovendien ondergaat sperma tijdens de verwerking een reeks veranderingen die nadelig kunnen zijn voor de kwaliteit ervan. In het tweede deel van Hoofdstuk 1.2 wordt de behandeling van het sperma en het effect ervan op de kwaliteit van het sperma besproken met speciale aandacht voor eventuele temperatuur invloeden.

Het experimentele deel van dit proefschrift bestaat uit onderzoek naar mogelijkheden voor nieuwe spermakwaliteitsanalyse en naar mogelijke factoren die de spermaproductie en kwaliteit kunnen optimaliseren.

Bijkomende validatiestudies van nieuwe apparaten die gebruikt kunnen worden voor sperma-analyse zijn noodzakelijk. In Hoofdstuk 3.1 wordt de validatie van een nieuw apparaat voor analyse van varkenssperma beschreven: de Sperm Quality Analyser (SQA-Vp). Zowel vers als verdun sperma van 50 ejaculaten van verschillende beren werd onderzocht. De concentratie van verse stalen verkregen met SQA-Vp werd vergeleken met de IMV Accucel fotometer en de Bürkerse telkamer. Voor verdunde stalen werden de resultaten vergeleken met CASA (Hamilton Thorne) en visuele sperma-analyse. De overeenkomst tussen de methoden werd bestudeerd aan de hand van Bland-Altman grafieken en de herhaalbaarheid d.m.v. de variatiecoëfficiënt (CV) en Bland-Altman grafieken. De overeenkomst tussen de SQA-Vp en de andere methoden was goed voor wat de concentratiebepaling van verse ejaculaten betrof. Voor de concentratiebepaling van verdunde sparmastalen was de overeenkomst tussen de SQA-Vp en de CASA goed, en slecht met betrekking tot de hemocytometer. Voor verdun sperma kwam het percentage beweeglijke spermatozoa beoordeeld door de SQA-Vp goed overeen met CASA en met de visuele beoordeling. Bovendien vertoonde de SQA-Vp een goede herhaalbaarheid voor het meten van de concentratie van zowel vers als verdun sperma en voor de beweeglijkheid ervan. De SQA-Vp lijkt dus geschikt voor de beoordeling van de spermakwaliteit in vergelijking met de Bürkerse telkamer, fotometer, CASA en de visuele beoordeling. Een van de beperkingen van de SQA-Vp is dat dit toestel niet in staat blijkt te zijn om de progressieve beweeglijkheid te beoordelen. Tenslotte werden verschillende praktische beperkingen en mogelijke valkuilen van de verschillende methoden besproken.

Hoewel de beweeglijkheid en concentratie duidelijk geassocieerd zijn met de vruchtbaarheid van beren zijn deze parameters niet altijd voldoende om goede en slechte beren te onderscheiden. Niet enkel de spermacellen maar ook het SP zou kunnen helpen
een onderscheid te maken tussen beren met een goed en een lagere vruchtbaarheid. In Hoofdstuk 3.2 was de hypothese dat de analyse van bepaalde enzymen en mineralen in het SP kan helpen om beren met een lage vruchtbaarheid te identificeren, zoals dit reeds gebeurt bij de mens en de hengst. Daarom werd het verband tussen bepaalde biochemische componenten en de spermakwaliteit getest. Het ejaculaat van 39 beren uit 3 verschillende KI centra met een goede (>80% normale raadcellen en >70% beweeglijkheid) en een slechte spermakwaliteit (<80% normale zaadcellen en <70% motilititeit) werd onderzocht. Het verband tussen aspartaat-amino-transferase (AST), γ-glutamyl-transferase (GGT), alkalische fosfatase (ALP), de concentraties natrium (Na), kalium (K), chloride (Cl), calcium (Ca), fosfaat (PO₄³⁻), magnesium (Mg), selenium (Se) en zink (Zn) en spermavolume en -concentratie, beweeglijkheid van de zaadcellen (CASA), morfologie en vitaliteit (beiden eosine nigrosinekleuring) werd onderzocht. De concentraties van GGT en ALP waren negatief gecorreleerd met het volume van het ejaculaat en positief gecorreleerd met de concentratie. Er was een matig positieve correlatie tussen GGT en ALP met de progressieve beweeglijkheid. Een positieve trend werd ook waargenomen tussen GGT en ALP met respectievelijk het percentage spermacellen met een abnormale kop en distale protoplasmadruppels. De mineraalconcentratie was gecorreleerd met de spermakwantiteit en -kwaliteit. Fosfaat, K en Se waren negatief gecorreleerd met spermavolume en Se en P waren positief gecorreleerd met spermaconcentratie. Verder was er een matig positieve correlatie van P, K en Se en een negatieve correlatie van Na met de progressieve beweeglijkheid. Hogere concentraties van Na en Cl waren gecorreleerd met een afname van het aantal spermatozoa met normale morfologie. Natrium bleek positief gecorreleerd te zijn met het percentage spermacellen met een abnormale staart. Een hoge concentratie Cl bleek samen te hangen met meer abnormale koppen en abnormale staarten. Hogere concentraties van Mg en Se waren gecorreleerd met respectievelijk minder membraanschade en met proximale druppels. Er was een trend voor een negatieve correlatie tussen de Zn concentratie en het percentage spermacellen met abnormale staart. Bovendien waren enkele parameters (bv. selenium) gecorreleerd met het aantal dagen tot de vorige collectie. De resultaten van deze studie toonden een verband aan tussen verschillende biochemische componenten van SP met kwantitatieve en kwalitatieve spermaparameters. De analyse van biochemische parameters zou bijgevolg extra informatie kunnen geven over de reproductieve gezondheid van KI beren. De mogelijke verklaringen waarom deze parameters gecorreleerd kunnen worden met de spermakwaliteit werden in dit hoofdstuk besproken. Bovendien worden de referentiewaarden van
specifieke biochemische parameters van het SP gegeven.

Voederssupplementatie met antioxidanten is momenteel een belangrijk onderwerp in de humane en veterinaire andrologie. Een van de supplementen met bekende antioxiderende eigenschappen is selenium (Se) wat een onderdeel is van het enzyme glutathionperoxidase (GPX). Bovendien wordt Se, zowel in organische als anorganische vorm, vaak gebruikt als voederadditief bij beren. In Hoofdstuk 4.1 werd het effect van deze verschillende vormen van Se supplementatie in het voeder (organisch vs. anorganisch) onderzocht op de spermaproductie en -kwaliteit van beren. Zestig volwassen beren uit een commercieel KI centrum werden willekeurig verdeeld in twee groepen en gevoederd met een premix met anorganisch Se (0,4 mg Na2SeO3/kg) en vitamine E (80 mg/kg) of met een commerciële premix met organisch Se (0,4 mg/kg, Se-gist) en vitamine E (80 mg/kg). Het effect van de verschillende vormen van Se werd gedurende 4 maanden (D0, D30, D60, D90, D105 en D120) onderzocht op de spermaconcentratie (fotometer), beweeglijkheid (CASA), morfologie (eosine-nigrosine kleuring), oxidatieve stress [productie van malondialdehyde (µg MDA/l) gemeten met TBARS assay].

De spermaconcentratie was hoger bij beren die de organische vorm van Se kregen, maar er was geen invloed op het aantal zaadcellen. De weerstand tegen oxidatieve stress bleek hoger te zijn in de anorganische groep. Dit is mogelijk te verklaren door het feit dat de selenomethionine (organisch Se) in competitie gaat met methionine om opgenomen te worden in andere eiwitten dan de GPX waardoor het minder beschikbaar is voor de synthese van dit laatste enzym. Sommige motiliteitsparameters zoals de VCL, de rechtlijnig en lineariteit bleken hoger te zijn in de anorganische groep. Er werden geen verschillen gevonden voor de andere parameters. In deze studie, wanneer anorganisch Se werd vervangen door biologisch/organisch Se in de voeding van beren, was de spermaconcentratie hoger, maar verminderden bepaalde motiliteitsparameters en de weerstand van spermacellen tegen oxidatieve stress. Uit deze studie blijkt dus dat verschillende bronnen van Se in het voeder de spermakwaliteit kunnen beïnvloeden.

De kritische temperatuur voor overleven van berensperma is 12ºC. Hoewel het werken bij kamertemperatuur de routinebehandeling van spermadosissen voor KI kan vereenvoudigen, is er weinig onderzoek uitgevoerd naar het effect van een verschillende verdunningstemperatuur op de spermakwaliteit. In Hoofdstuk 4.2 werd onderzocht, wanneer een 2-staps verdunningsprotocol wordt toegepast, in welke mate de temperatuur van de verdunner die gebruikt wordt voor de uiteindelijke verdunning, een effect heeft op de spermakwaliteit. Een ejaculaat van 15 beren die gehuisvest werden in een commercieel KI-centrum werd willekeurig verdeeld in twee groepen en gevoederd met een premix met anorganisch Se (0,4 mg Na2SeO3/kg) en vitamine E (80 mg/kg) of met een commerciële premix met organisch Se (0,4 mg/kg, Se-gist) en vitamine E (80 mg/kg). Het effect van de verschillende vormen van Se werd gedurende 4 maanden (D0, D30, D60, D90, D105 en D120) onderzocht op de spermaconcentratie (fotometer), beweeglijkheid (CASA), morfologie (eosine-nigrosine kleuring), oxidatieve stress [productie van malondialdehyde (µg MDA/l) gemeten met TBARS assay].

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KI centrum, werd gebruikt. Elk ejaculaat werd eerst verdund (1:1) bij 30°C met een commercieel verkrijgbare spermaverdunner (Beltsville Thawing Solution, BTS) en de temperatuur van de verdunning werd vervolgens gemeten. Een uiteindelijke verdunning van 30x10^6 spermacellen/ml (concentratie van een commerciële dosis) in BTS met ofwel voorverwarmde verdunner (29,3°C ± 0,2°C, Groep A) ofwel verdunner bewaard bij kamertemperatuur (22,7°C±0,6°C, Groep B), werd verkregen. De verdonde spermastalen werden bewaard bij 17°C en onderzocht gedurende 3 opeenvolgende dagen. De volgende parameters werden beoordeeld op D0, D1 en D2: % beweeglijke spermacellen (% M), % progressief beweeglijke spermacellen (% P) (beiden d.m.v. CASA) en het % spermacellen met een intacte membraan (% IM) (d.m.v. een eosine nigrosine kleuring). Op D0 en D2 werd het % spermacellen met een intact acrosoom (% IA) beoordeeld d.m.v. een PSA kleuring. Er werden geen significante verschillen gevonden tussen de groepen wat betreft de motiliteitsparameters, de membraanintegriteit en de acrosoomintegriteit. Tenslotte kon worden aangetoond dat verdunning bij kamertemperatuur (22-23°C) geen nadelig effect heeft op de kwaliteit van het sperma. Wanneer er een 2-stapsverdunning wordt uitgevoerd, verbetert het voorverwarmen van de verdunner voor de tweede verdunning tot dezelfde temperatuur als het sperma de spermakwaliteit niet.

Tenslotte worden de resultaten verkregen in de verschillende studies besproken in het licht van de huidige wetenschappelijke kennis (Hoofdstuk 5). Uit de resultaten van de verschillende studies kunnen de volgende conclusies worden getrokken:

- De SQA-Vp is geschikt om de spermakwaliteit van beren te beoordelen. Dit toestel bleek een goede overeenkomst te hebben met vroeger gevalideerde sperma-analysesystemen bij beren zoals de fotometer en CASA. Bovendien konden met de SQA-Vp consistente resultaten verkregen worden.

- Analyse van de samenstelling van SP zou opgenomen moeten worden in de kwalitatieve sperma-analyse van beren met vruchtbaarheidsproblemen. Referentiewaarden van bepaalde specifieke componenten van SP zijn beschikbaar en kunnen extra informatie geven bij verder onderzoek van SP bij varkens. Een significant verschillende concentratie van Na, Cl en Zn in het SP werd waargenomen in ejaculaten van goede en slechte kwaliteit. Verder werden verschillende correlaties tussen biochemische componenten van SP en de spermahoeveelheid en -kwaliteit gevonden. Bovendien waren een aantal parameters gecorreleerd met de frequentie van spermacollectie.

- Het toevoegen van voedersupplementen heeft invloed op de spermakwaliteit. Bovendien kan de vorm waarin deze supplementen worden toegediend van belang zijn.
Toevoegen van biologisch/organisch in plaats van anorganisch selenium resulteerde in een hogere spermaconcentratie maar in een minder rechtlijnige beweging. Mogelijk resulteert een hogere opname van anorganisch selenium in sperma GPX in een hogere weerstand tegen oxidatieve stress.

- Het aanmaken van spermadosissen in KI-centra kan vereenvoudigd worden door verdunning bij kamertemperatuur (22-23°C). Na een 1:1 voorverdunning (30°C) werden geen significante verschillen gevonden in kwaliteit tussen varkenssperma verdund bij 30°C ten opzichte van sperma verdund bij 22-23°C. Vanuit praktisch oogpunt betekent dit dat, wanneer een twee stapsverdunning wordt uitgevoerd in een KI-centrum, voorverwarmen van de spermaverdunner niet vereist is voor het behoud van een goede spermakwaliteit.
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Curriculum Vitae
Alfonso López Rodríguez was born on June 23rd 1980, in Sevilla, Spain. He finalized his secondary studies in Biomedical Sciences with special distinction in 1998 in Spain. He continued his academic formation in the Faculty of Veterinary Medicine of Córdoba (Spain) where he obtained his DVM diploma in 2006.

After a short experience in the meat industry in United Kingdom, he enrolled in an internship program for the European College of Porcine Health Management in October 2007 at the Department of Faculty of Veterinary Medicine in Ghent University. This internship period was followed one year later for a 3 years residency program for the same college and at the same department. His interest in research led him to combine this residency with different research projects that resulted in a PhD project under the title “Fresh boar semen: quality control and production“.

Alfonso is first author and co-author of several articles published in international peer reviewed journals. His experimental work has been presented in different European and international congresses.
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Empty spaces –
what are we living for?
Abandoned places –
I guess we know the score..
On and on!
Does anybody know what we are looking for?
…
The show must go on!
(Freddie Mercury)