

## Review Article

### Quality Control of Boar Sperm Processing: Implications from European AI Centres and Two Spermatology Reference Laboratories

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In recent years, increased automatization has resulted in a higher efficiency of boar semen processing in AI laboratories. Sophisticated laboratory management and efficient quality control programmes are needed for current tendencies in major pork-producing countries to reduce the sperm number per AI dose, to lengthen semen storage times and to adopt responsible methods for bacterial control and prevention of the development of multiresistant bacteria. The objective of the present review was to outline current trends in boar semen production and the critical steps in semen processing which affect sperm quality. In addition, integrated elements of a quality assurance programme in use by thirty European AI centres in association with the two German spermatology reference laboratories are described.

#### Introduction

Proper handling of semen during processing is critical for the optimal quality of semen used for AI, especially when semen doses are stored for several days before insemination. Temperature and hygiene management during semen collection, dilution, filling of tubes and during transport to farms are known factors that influence sperm quality in stored AI doses. In recent years, semen production efficiency in pig AI stations has increased enormously. At the same time, the expectations of clients to receive a high-quality product have also increased. In Germany, Austria and Switzerland, the challenges associated with modern boar semen production have resulted in a collaborative effort among 30 AI centres organized in the Association for Bioeconomy Research (FBF; [http://www.fbf-forschung.de/home\\_gb.html](http://www.fbf-forschung.de/home_gb.html)) and two German reference laboratories, IFN Schönnow, Bernau, and University of Veterinary Medicine Hannover. The aim of this review was first to outline recent trends in the production of boar semen doses at AI centres and second to report relevant factors during semen processing that affect the quality of extended semen. Implications for routine work in AI centre laboratories based on the long-term research cooperation between European AI organizations and the two reference laboratories are discussed.

#### Actual Challenges and Trends in Semen Production for Boar AI Centres

Pig reproduction efficiency has dramatically increased in the last decade in the major pork-producing countries and is now advancing rapidly in developing countries experiencing increased pork consumption. With respect to the boar, increased genetic indices, fertility and high efficiency in the production of AI doses are main factors contributing to the high performance of pig production (Knox 2014). Interestingly, an early goal of AI technology was to introduce frozen semen, but now this is no longer a priority. Instead, production efficiency of liquid semen doses and their efficient use for AI have become the preference. Currently, approximately 2000 AI doses can be produced per hour. With this high production intensity, a concomitant higher level of technology for semen collection (automatic collection systems), semen analysis (CASA systems) and semen processing (high-efficiency semen filling machines) has been incorporated into AI centres. Increased automatization requires well-trained laboratory personnel to ensure correct use of the sophisticated instruments. At the same time, customer demands for semen quality have become increasingly important. AI doses should be of high quality, have high biosecurity, should have high genetic value and should maintain high fertility over several days of *in vitro* storage. Due to a highly competitive market, AI centres seek to fulfil the high expectations of quality-conscious clients. Efforts to ensure high biosecurity have increased dramatically due to widespread prevalence of some pathogens (e.g. PRRS) which can be transmitted via semen. In addition, with the increased intensity of semen production in AI laboratories, hygienic demands have increased to avoid entrance of multiresistant bacteria into the AI doses during processing (Morrell and Wallgren 2014; Schulze et al. 2015b). Recently, there have been frequent discussions about the responsible use of antibiotics in semen extenders, especially about those classified as ‘critically important antibiotics for human medicine’ by the WHO (2011). Some countries have already designated prohibited drugs or have never allowed their use extralabel in food-producing animals, for example cephalosporins of the 3rd and 4th

generation and fluoroquinolones (US Government Electronic Code of Federal Regulations 2015, accessed online 2015; Downing, accessed online 2015). Together, these scenarios require modifications of semen processing procedures and strict quality assurance (QA) programmes. Nonetheless, AI centres need to be profitable.

In integrated production systems, where AI centres are part of large production companies, profitability calculations take into account sow population size and are based on the number of resulting market pigs. It has been demonstrated that insemination (conventional, intrauterine, deep intrauterine) and semen preparation (fresh, frozen) techniques have a non-additive effect on profit, return, total costs, fixed costs, variable costs and sow population size (Gonzalez-Peña et al. 2014). Boar utilization efficiency is currently being increased by lowering sperm numbers per insemination dose. This trend is most apparent in some European countries where total sperm numbers below 2 billion per dose are routinely used for conventional AI. In the Netherlands, for example, the total number of spermatozoa in an insemination dose has been decreased from 4.0 billion in 100 ml doses (1985) to 1.5 billion in 80 ml doses (2008), while further reductions are expected. A small, but significant decrease of  $-0.07$  piglet when decreasing the total number of sperm from 2.2 to 1.7 billion per dose was justified by the economic benefit due to the improvement of the average genetic index of boars (Feitsma 2009, 2013).

The challenges described above for modern boar semen production require sophisticated semen handling during processing to ensure optimal semen quality. From the perspective of AI centres, there are three main implications: first, support of research is important to expand understanding of boar sperm physiology under various preservation conditions. Second is the establishment of effective quality assurance programmes. And third, continuous education of laboratory personnel is vital. European boar AI stations organized in the FBF have followed these goals for many years and have established standards for semen processing, which are continuously being updated.

### Preparation of Semen Extenders

Schulze et al. (2015a) estimated that currently approximately 12.8 million litres of extender per year are used worldwide. Water quality and accuracy of extender preparation have a tremendous impact on sperm quality of stored semen. Modern water production equipment, for example reverse osmosis and water disinfection by UV lights, are common in many AI centres. Regular monitoring and periodic maintenance of water production system are mandatory.

Preparation of extenders requires precise measurement of purified water volume and accurate weighing systems for extender powders. At present, 15 of 30 AI boar studs participating in a periodic quality audit in Germany, Austria and Switzerland use simple canisters

or water-filling heights in vats to measure the amount of purified water for extender preparations (Schulze et al. 2015a). Thus, human and technical errors in extender preparation are potential sources for mistakes, especially in expanding studs with high employee turnover.

Poor results as measured by semen quality are often associated with bacterial contamination or osmotic imbalances due to weighing errors during extender preparation. Recently, refractometry was introduced in AI centres as a rapid and simple quality control tool for semen extender preparation prior to their use. Refractive indices are sensitive measures of deviations in osmolality caused by incorrect water/extender ratios (Schulze et al. 2015a). Boar spermatozoa are especially sensitive to hypotonic conditions resulting from by inaccurate [low] measurement by weight of extender powder. A significant loss of motility was observed when BTS extender was improperly prepared to 70% of its prescribed concentration. Additional parameters associated with sperm quality, such as thermoresistance and mitochondrial activity, and morphological status were demonstrated to be affected in a dose- and time-dependent manner (Schulze et al. 2015a). Refractive indices are now measured and documented as part of daily quality control steps in AI centres organized within the FBF.

### Dilution and Temperature Management

An important issue related to semen processing is holding time. There are several holding times during processing: first, directly after collection (raw semen); second, after pre-dilution prior to final dilution; and finally, holding time of AI doses prior to 16–18°C storage. From the physiological perspective, holding times may have a beneficial effect on sperm quality because they allow seminal plasma proteins to coat sperm membranes prior to dilution and to adapt to hypothermic storage temperatures. Holding times of undiluted semen of 30–60 min were, therefore, implemented in Swedish laboratory protocols (Rodríguez-Martínez et al. 2009). Ninety minute holding times of extended semen samples at room temperature are common before AI doses are transferred to 16–18°C cooling cabinets (Waberski 2009). From the practical perspective, holding times are unavoidable during any processing protocol, especially on labour-intensive days, or when pre-diluted samples are transported to a central AI laboratory from outlying boar semen collection facilities. Holding times under routine conditions may vary from a few minutes to several hours. Holding times of 1 : 1 pre-diluted semen at 21°C or 32°C for up to 6 h do not affect the quality and energy status of boar spermatozoa stored for several days (Wallner U., unpublished data). The length of holding times of pre-diluted semen appears to be of minor relevance, whereas the extender temperature at final dilution has a larger impact on

sperm quality. Several AI centres have recently introduced a two-step hypothermic procedure for extension of semen with the aim of saving energy costs related to extender pre-warming and faster cooling of AI doses to the desired storage temperature (Waberski 2009). Although, results of one study did not detect an influence of extender temperature at second dilution (22°C vs 29°C) on CASA parameters and membrane integrity (Lopez Rodriguez et al. 2012), subsequent studies under field and experimental condition clearly provided evidence of a negative effect on boar sperm quality of a hypothermic second dilution step (Schulze et al. 2013). However, this effect was extender dependent and was less apparent using Androstar Plus (Minitüb GmbH, Tiefenbach, Germany) compared to BTS (Minitüb GmbH). Holding of extended semen at lower temperature (17°C) is beneficial when semen is subsequently cooled to 5°C, due to a stabilizing effect on the plasma membrane architecture (Casas and Althouse 2013). As an overall result, the majority of European AI centres organized within the FBF has returned to the traditional one- or two-step isothermic dilution procedure.

Dilution *per se* presents a stress factor for spermatozoa due to any sudden change of the surrounding milieu. Most extenders possess a lower pH compared to the alkaline seminal plasma, as a pH of approximately 7.0 is commonly regarded as advantageous for *in vitro* storage of extended semen. Several studies have demonstrated a detrimental effect of elevated extender pH on sperm motility and other sperm kinematic parameters (Gatti et al. 1993; Cross 2007; Vyt et al. 2007). Storage experiments clearly have provided evidence that maintenance of pH of extended semen below 7.3 is beneficial for sperm quality. In extenders with the simple bicarbonate-citrate buffer system, for example the commonly used BTS extender, contact with environmental air must be minimized during extender preparation and semen storage to avoid alkalinization induced by loss of CO<sub>2</sub> from the liquid phase into the air (Vyt et al. 2007). A recent study indicates that rotation of semen doses during storage accelerates an increase in the extender pH and results in negative effects on boar sperm motility and membrane integrity during long-term storage (Schulze et al. 2015c). Interestingly, this study also indicated that other, yet unidentified, factors probably contributed to the loss of sperm quality in rotated semen samples.

### Quality Assurance in Semen Processing

Increasing demands on reliable semen processing procedures in modern boar semen production facilities require efficient QA programmes. At the onset, critical steps during these procedures need to be identified. Then, a HACCP (hazard analysis and critical control points) concept must be specifically designed for individual AI stations. HACCP analyses have been

followed since 1996 in FBF member organization in Germany, Austria and Switzerland with a science-based QA programme established by the two reference laboratories (reviewed in Waberski and Schulze 2015). The main focus of these analyses is boar semen production biosecurity. The aim is not only to avoid bacterial contamination of AI doses, but also to prevent the overuse of antibiotics in semen extenders and the development of multiresistant bacterial strains in the AI laboratories. There is an increasing recognition of the need for responsible semen production by the AI industry. During biennial audits by staff from the reference laboratory in Schönnow, hygienic critical control points have been identified in AI laboratories after multisampling of laboratory equipment for microbiological testing, analysis of work flows and detection of potential sources of cross-contamination and conditions that support the development of multiresistant bacteria. Laboratory sinks and drains have been identified as the highest risk sites for resulting cross-contamination of AI doses. Standard operating procedures and sanitary guidelines have been established accordingly. A 4-year review clearly provided support for efficacy of improved sanitary measures (Schulze et al. 2015b).

External quality control protocols for FBF members include periodic audits and evaluations of randomly selected semen samples by the two reference laboratories using an extended spectrum of diagnostic tools, including flow cytometry and screening for bacterial contamination. Special emphasis is placed on the verification of intended total sperm numbers per dose. Sperm numbers above the desired level have a high and negative impact on the profitability of AI centres, whereas doses with low sperm numbers may result in lower fertilization rates. Any deviations from intended values therefore require immediate action. HACCP during station visits include evaluation of photometer cleanliness and function using grey-scale standards, verification of correct dilution rates according to the manufacturer's instructions and evaluation of semen handling procedures (mixing of semen samples, pipetting, etc.). In 2013, in 87% of the 23 participating European AI stations, sperm concentration was measured by photometry. Schulze and Rüdiger (2014) concluded that incorrect sperm concentration in AI doses could be explained by dilution rate (approximately 13% of errors), photometer calibration (approximately 13% of errors), photometer cleanliness (approximately 13% of errors) and semen handling procedures (approximately 4% of errors).

A key QA tool is repeated training and education of AI personnel at all levels. Results of applied university and/or industry research projects should be transferred to production centres. In addition, hands-on training with adequate monitoring of success needs to be provided. For FBF members, this is accomplished by various seminars offered by the reference laboratories,



either at their institutions or on-site at AI stations and at annual member meetings.

## Conclusions

Increasing efficiency of the use of boar ejaculates and the need for responsible semen production require high-quality standards during semen processing. Strict quality assurance programmes based on recent scientific knowledge are mandatory. Overall, the goals of the AI centre are to produce a high-quality product offering maximal fertilization chances and to strengthen the customers' confidence in the production facility.

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## Author contributions

AR and DW prepared a draft of the manuscript. All authors contributed to the research underlying this review.

## Conflict of interest

The authors have no conflict of interest to declare.

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