Anim. Reprod., v.12, n.4, p.903-909, Oct./Dec. 2015

Impact of isothermic and bithermic dilution on quality of chilled boar sperm

M.C.S. Almeida[1], J.L. Moroni[1], M.L. Bernardi[2], I. Wentz[1], F.B. Bortolozzo[1,3]

[1]Setor de Suínos, Faculdade de Medicina Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.
[2]Departamento de Zootecnia, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Abstract

The use of bithermic dilution protocols is increasing in artificial insemination centres; therefore, it is necessary to guarantee that the quality of insemination doses remain the same when compared to isothermic dilution protocols. Four ejaculates from each of 19 crossbreed PIC® boars were collected and assigned, in a split sample design, in three treatments: two-step bithermic dilution, two-step isothermic dilution and one-step isothermic dilution. Temperature curves for the three treatments were recorded using a temperature sensor data logger. Semen doses were prepared with a BTS extender and stored at 16°C and then used to evaluate sperm parameters with the CASA system and sperm morphology for 120 h. The temperature in semen samples submitted to a two-step bithermic dilution reached 24.6ºC after 120 min, whereas one or two-step isothermic dilution samples reached 26.4ºC and 27.4ºC, respectively. Total motility, progressive motility and BCF were influenced ($P < 0.05$) by the storage time but not by the dilution procedure. Total and progressive motility decreased throughout the storage time (91.0 ± 0.91 to 81.5 ± 1.08% and 74.0 ± 2.48 to 60.4 ± 2.59% from 24 h to 120 h, for MOT and PROG, respectively) whereas BCF differed between 24 and 120 h (28.6 ± 0.76 and 27.3 ± 0.79 Hz). The following motility traits were not affected by the dilution procedure or by the time of storage: DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, and ALH. At 72 h of storage, sperm morphology was not different among treatments ($P > 0.05$), showing an overall mean of 9.2 ± 0.4 total defects. In conclusion, the bithermic dilution makes the production of artificial insemination doses faster by taking less time to reach a temperature close to that of storage, without impairing semen quality.

Keywords: boar, cold shock, dilution, semen preservation, temperature.

Introduction

Swine artificial insemination (AI) has seen a significant increase and improvement in its efficiency since 1990 (Roca *et al*., 2006). In the majority of intensive pig production systems worldwide, AI is routinely applied with semen extended in a liquid state, and usually stored at 16ºC up to five days (Johnson *et al*., 2000; Waberski, 2009).

The quality of semen doses assumes a greater importance considering the recent increase in the use of post-cervical semen deposition combined with sperm cell reduction (Garcia *et al*., 2007). However, despite all of the improvements concerning semen preservation, only a few attempts have focused on optimising the process of semen dilution.

Due to its lipid membrane composition, swine sperm is particularly sensitive to cold shock (De Leeuw *et al*., 1991). With the purpose of reducing the risks of cold shock, isothermic dilution protocols are usually performed in AI centres in one or two steps (López Rodriguez *et al*., 2011). However, semen dilution at 20ºC, compared to 32ºC, did not affect the motility of sperm stored at 16ºC (Petrunkina *et al*., 2005). Despite the fact that a bithermic protocol, using the extender in a lower temperature for the last dilution, is being routinely used in some artificial insemination centres (Schulze *et al*., 2013), there is a scarcity of studies evaluating the effect of a bithermic protocol on semen quality during storage (Petrunkina *et al*., 2005; López Rodriguez *et al*., 2011; Schulze *et al*., 2013). The use of bithermic dilution could accelerate semen processing due to the rapid cooling to reach storage temperature (Schulze *et al*., 2013), and decrease bacterial growth due to less time of exposure to higher temperatures (Althouse and Lu, 2005). However, it is necessary to assure that a bithermic protocol provides semen doses with a similar quality to those obtained with standard protocols.

The present study compared three different protocols of semen dilution, two using the extender at 34ºC, with one- or two-step isothermic dilution and one, the bithermic two-step dilution protocol, using the extender at 34ºC for the first dilution and at 23ºC for the final dilution. The aim of the study was to evaluate whether bithermic dilution provides semen doses with the same quality when compared to isothermic dilution with one or two steps.

Material and Methods

Animal and facilities

The study was performed in an AI station located in southern Brazil, in Santa Catarina State.

[3]Corresponding author: fpbortol@ufrgs.br
Received: July 23, 2014
Accepted: September 15, 2015

Nineteen crossbreed PIC® boars (Pietran x Duroc x Large White x Landrace - Agroceres PIC, Patos de Minas, MG, Brazil) aged 8 to 24 months were used for semen collection from February to May 2013. Boars were housed in individual crates in the same barn. The boars had *ad libitum* access to water and were fed daily with 2.5 kg of a corn-soybean diet (15% crude protein, 0.55% digestible lysine and 3300 kcal metabolisable energy) formulated to meet their specific nutritional needs (National Research Council - NRC, 2012). Room ventilation and temperature were controlled by an adiabatic ventilation system.

Boar sperm motility, analysed using the CASA system (Sperm Vision 3.7; Minitüb GmbH, Tiefenbach, Germany), was recorded from four to six weeks before the beginning of the trial. Boars that fulfilled the minimum requirements of 70% of total motility, after three days of storage, and at least 80% normal sperm morphology were included in this study.

Semen collection

Four ejaculates from each boar were collected once a week on a routine basis at the AI station. Boars were collected by glove hand method into a pre-warmed cup (38°C) coated with a disposable plastic bag (Minitüb GmbH, Tiefenbach, Germany) and covered with a filter that was used to remove the gel fraction. Immediately after collection, the raw semen was transferred to the laboratory.

Semen processing

Once in the laboratory with a controlled temperature (~24ºC), the weight of each ejaculate was recorded, temperature was measured and an aliquot of the ejaculate was collected for further morphological evaluation. Motility score and concentration of the ejaculates were obtained using the CASA system. All raw ejaculates used in the trial fulfilled the following requirements: a) minimum total motility of 80%, and b) a minimum of 80% normal sperm morphology. Each ejaculate was diluted in BTS (Beltsville Thawing Solution - BTS; Minitüb GmbH, Tiefenbach, Germany) and assigned, in a split sample design, to the following treatments:

Two-step bithermic dilution treatment: the ejaculate fraction was diluted in a 1:1 proportion, with one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. The second step for final dilution was performed 10 min later using the extender at 23 ± 1ºC.

Two-step isothermic dilution treatment: the ejaculate fraction was diluted in 1:1 proportion, with one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. The second step for final dilution was performed 10 min later using the extender at 34 ± 1ºC.

One-step isothermic dilution treatment: one dilution was performed using the ejaculate fraction at 34 ± 1ºC and the extender also at 34 ± 1ºC.

After dilution, semen doses were processed to contain 3 x 10^9 sperm cells in a final 80 ml volume, in sealed airtight plastic tubes (Minitüb GmbH, Tiefenbach, Germany). Ninety minutes after the dilutions described above, semen doses from each experimental treatment were stored at 16°C in a semen storage unit (Minitüb GmbH, Tiefenbach, Germany) for 120 h, and were used to evaluate the sperm motility parameters, in addition to sperm morphology.

Semen analysis

Motility traits were evaluated using the CASA system at 0 h and at 0.75, 24, 48, 72, and 120 h after final dilution. At 0 h, an aliquot of raw semen was diluted in a 2 ml microtube (Sarstedt®, Nümbrecht, Germany), using an electronic mixing pipette. A dilution rate of 1:9 was used, with 90 µl of raw semen and 810 µl of BTS extender being pre-warmed at 34ºC. Previously, the ejaculate in the collection bag was thoroughly homogenised by rotating it at least five times. This semen sample was stored on a warming plate at 38ºC and was homogenised five times. From that sample, an aliquot of 3 µl was used to fill, in a single step and by capillary flow, one of four chambers of standardised slide depth of 20 µm - Leja-4 chambers (Leja, 4 chamber counting slides; Leja Products B.V., Nieuw-Vennep, The Netherlands). Samples were individually analysed (one chamber filled, one sample analysed) by CASA, using eight fields per slide along the centre line, at 200X magnification. Both the microscope-warming stage and the Leja chamber were pre-warmed at 38°C. The analyses were accepted when the coefficient of variation per eight fields was lower than 15%. For the analyses after the final dilution, 1 ml of each stored semen dose was collected and kept for 5 min in a thermo block on the top of a warming plate at 38ºC. A 3 µl subsample was collected and placed on a pre-warmed glass slide, completely covered and spread with a pre-warmed cover slip (18 x 18 mm). To obtain CASA parameters, samples were analysed using a common starting position within each slide, at 200X magnification. To avoid incorrect estimation by CASA, particles such as droplets, spots and dirt, and sperm heads were identified and manually deleted by the operator. The mean of the four microscope fields was used for the analysis.

The following CASA semen parameters were assessed: DAP - Distance Average Path (µm); DCL - Distance Curved Line (µm); DSL - Distance Straight Line (µm), defined as the distance that the sperm cell

travels in a straight line (μm) from the first frame to the last frame of the analysis; VAP - Velocity Average Path (μm/sec), defined as the average velocity over the smoothed cell path; VCL - Velocity Curved Line (μm/sec), defined as the average velocity measured over the actual point-to-point track followed by the cell; VSL - Velocity Straight Line (μm/sec), defined as the average velocity measured in a straight line from the beginning to the end of the track; STR - Straightness (VSL/VAP); LIN - Linearity (VSL/VCL); WOB - Wobble (VAP/VCL); ALH - Amplitude of Lateral Head Displacement (μm), defined as the maximum measured width of the head oscillation as the sperm cells swam; BCF - Beat Cross Frequency (Hz), defined as the frequency in which the actual track crossed the smoothed track in either direction; Motility percentage of sperm >2.5 μm - average orientation change (AOC), where DSL is the distance that the sperm cell travels in a straight line (μm) from the first frame to the last frame of the analysis; and progressive forward motility (% sperm ≥4.5 μm AOC). Software settings recommended by Minitüb GmbH (Tiefenbach, Germany) for progressive motile cells were adjusted for the cut-off values of AOC. Settings were cell detection, with a minimum cell size of 20 μm and a maximum of 120 μm, and DSL <4.5 mm for local cells.

The morphological evaluation of raw and stored semen at 72 h was performed under phase-contrast microscopy at 1000X magnification. Two hundred sperm cells were counted and classified according to sperm morphology: normal, acrosome defects, abnormal head, neck defects, attached proximal cytoplasmatic droplets, attached distal cytoplasmatic droplets, middle piece defects, bent tail and coiled tail (Pursel *et al*., 1972).

The temperature curve for the three treatments was recorded using a temperature sensor data logger (myPCProbe, Novus Electronics, Brazil) that features a USB interface for computer communication. The temperature curve was performed three times for each treatment. A temperature probe (Pt100, myPCProbe, Novus Electronics, Brazil) was placed in a central position in the semen mixing cylinder. Temperature was recorded every minute for a total of 180 min, starting with the record of the temperature in raw semen, before dilution. The raw semen and AI doses were kept at room temperature (24ºC) until 120 min and then placed at 17ºC up to 180 min.

Statistical analyses were performed using the Statistical Analysis System software, version 9.2 (SAS Institute Inc., Cary, NC, USA, 2005). Variables concerning motility characteristics (DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, ALH, BCF, Total Motility, Progressive Motility) were analysed as repeated measures using the MIXED procedure. The treatment, storage time and its interaction were included in the model as fixed effects whereas boars and day of semen collection were used as random effects. Comparisons of treatments (LSMEANS) were performed with the Tukey-Kramer test with a 5% probability level for statistical significance. Percentages of sperm abnormalities were evaluated using the NPAR1WAY procedure and comparisons among treatments were performed with the Kruskal-Wallis test. A variance component analysis, using the VARCOMP procedure, was performed to estimate the proportion of variance in Total Motility and Progressive Motility explained by dilution treatment, storage time, individual boar and day of semen collection.

Results

Total Motility and Progressive Motility of raw semen were 93.2% ± 0.39 and 80.5% ± 0.81, respectively. At 45 min after the final dilution, there was no difference among treatments ($P > 0.05$) in any of the CASA parameters. The variables Total Motility, Progressive Motility and BCF were influenced ($P < 0.05$) by the storage time but not by the dilution procedure (Table 1). Total Motility and Progressive Motility decreased throughout the storage time, whereas BCF values were different between 24 and 120 h. The variation in Total Motility and Progressive Motility had a greater contribution, respectively, of individual boar (19.4 and 22.7%) and storage time (20.0 and 11.0%) than dilution treatment (0.6 and 0.07%).

Neither dilution procedure nor storage time affected the following motility traits: DAP, DCL, DSL, VAP, VCL, VSL, STR, LIN, WOB, and ALH. Overall means for these motility sperm traits are shown in Table 2. At 72 h of storage, sperm morphology was not different among treatments ($P > 0.05$), showing an overall mean of 9.2 ± 0.4 total defects, with the majority of these being acrosome defects (2.8 ± 0.2) and coiled tails (2.7 ± 0.2).

Within 20 min, temperature decreased to 25.3ºC in doses submitted to the bithermic dilution, whereas one and two-step isothermic curves reached 31.4 and 33.1ºC, respectively (Fig. 1). Within 120 min, the temperature reached 24.6ºC in two-step bithermic samples, whereas one and two-step isothermic samples reached 26.4 and 27.4ºC, respectively.

Table 1. Percentages of total motile and progressive motile sperm cells, and beat cross frequency (BCF) of boar AI semen doses stored for 120 h, according to the dilution procedure before being stored at 16ºC.

Variables	Two-step bithermic dilution	Two-step isothermic dilution	One-step isothermic dilution	Pooled SEM	Mean
Total motility, %					
24 h	91.3	91.1	90.8	0.99	91.0 ± 0.91[a]
48 h	89.6	88.9	88.0	1.07	88.8 ± 0.94[b]
72 h	87.0	85.3	85.4	1.20	85.9 ± 0.99[c]
120 h	83.0	81.5	80.1	1.42	81.5 ± 1.08[d]
Mean	87.7	86.7	86.1	1.03	
Progressive motility, %					
24 h	74.8	73.3	73.9	2.71	74.0 ± 2.48[a]
48 h	70.0	69.3	70.8	2.77	70.0 ± 2.50[b]
72 h	68.5	65.9	66.7	2.77	67.0 ± 2.50[c]
120 h	62.0	61.5	57.8	3.00	60.4 ± 2.59[d]
Mean	68.8	67.5	67.3	2.54	
BCF, Hz					
24 h	28.8	28.4	28.8	0.84	28.6 ± 0.76[a]
48 h	27.5	27.3	28.3	0.87	27.7 ± 0.78[ab]
72 h	27.9	27.7	28.0	0.84	27.9 ± 0.76[ab]
120 h	27.8	26.9	27.3	0.92	27.3 ± 0.79[b]
Mean	28.0	27.6	28.1	0.76	

Two-step bithermic dilution: The first step dilution consisted of one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. For the second step dilution, the extender was at 23 ± 1ºC; Two-step isothermic dilution: The first step dilution consisted of one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. For the second step dilution, the extender was at 34 ± 1ºC; One-step isothermic dilution: Only one dilution was performed using the ejaculate fraction at 34 ± 1ºC and the extender at 34 ± 1ºC. [a,b,c,d]Values followed by different letters indicate significant differences among the storage moments ($P < 0.05$).

Table 2. Motility sperm traits of boar AI semen doses submitted to different dilution procedures before storage at 16ºC.

Motility traits	Two-step bithermic dilution	Two-step isothermic dilution	One-step isothermic dilution	Mean
DAP	25.4	25.1	25.6	25.4 ± 0.20
DCL	45.5	44.4	45.5	45.1 ± 0.37
DSL	16.9	16.7	17.1	16.9 ± 0.14
VAP	54.8	53.8	54.9	54.5 ± 0.45
VCL	97.5	95.2	97.5	96.7 ± 0.81
VSL	36.5	36.0	36.9	36.5 ± 0.32
LIN	0.38	0.38	0.38	0.38 ± 0.002
STR	0.66	0.66	0.67	0.66 ± 0.002
ALH	2.62	2.56	2.60	2.60 ± 0.02
WOB	0.56	0.56	0.56	0.56 ± 0.002

Two-step bithermic dilution: The first step dilution consisted of one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. For the second step dilution, the extender was at 23 ± 1ºC; Two-step isothermic dilution: The first step dilution consisted of one part of the ejaculate at 34 ± 1ºC and one part of the extender at 34 ± 1ºC. For the second step dilution, the extender was at 34 ± 1ºC; One-step isothermic dilution: Only one dilution was performed using the ejaculate fraction at 34 ± 1ºC and the extender at 34 ± 1ºC. Abbreviations: DAP - Distance Average Path (μm); DCL - Distance Curved Line (μm); DSL - Distance Straight Line (μm); VAP - Velocity Average Path (μm/sec); VCL - Velocity Curved Line (μm/sec); VSL - Velocity Straight Line (μm/sec); LIN - Linearity (VSL/VCL); STR - Straightness (VSL/VAP); ALH - Amplitude of Lateral Head Displacement (μm); WOB - Wobble (VAP/VCL).

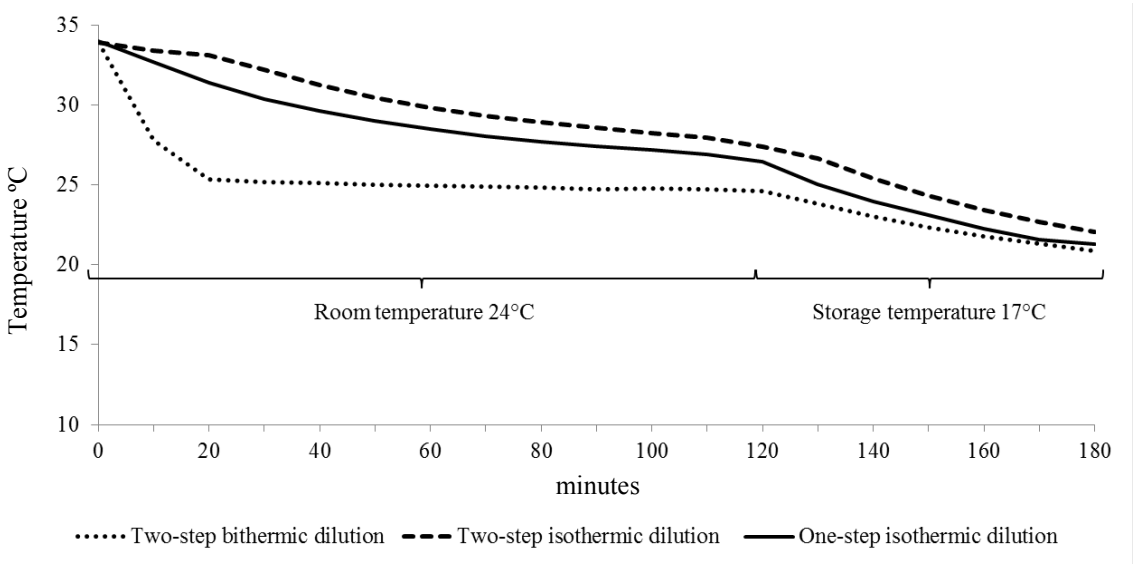

Figure 1. Temperature of semen samples during processing up to 180 min. Curves demonstrate mean temperature changes in semen samples submitted to three different dilution procedures (n = 3 each): Two-step bithermic dilution - first and second dilution with extender at 34ºC and 23ºC, respectively; Two-step isothermic dilution - both the first and second dilutions were performed with the extender at 34ºC; One-step isothermic dilution - single dilution with the extender at 34ºC. Minutes 0, 1 and 10 correspond to the temperature measurements in raw semen, final first dilution and final second dilution, respectively. After dilution, all samples were kept at room temperature (24ºC) until 120 min and then placed at 17ºC up to 180 min.

Discussion

As boar sperm is extremely sensitive to low temperatures compared to spermatozoa from other species (Althouse *et al*., 1998; Petrunkina *et al*., 2005), a two-step isothermic dilution protocol is usually performed to prevent the effect of cold shock (Weitze, 2012). Cold shock reduces the number of viable spermatozoa, declines motility and increases acrosomal defects, which affects spermatozoa function, reducing their fertilising potential (Althouse *et al*., 1998; Johnson *et al*., 2000). The 1:1 dilution with isothermic extender would provide a protective effect and adaptation of sperm cells; a slow and gradual temperature drop to the storage temperature at 15-17°C would result in less cell damage (Waberski, 2009; Weitze, 2012). However, in the present study, when comparing one-step isothermic dilution to two-step isothermic dilution, none of the motility traits were negatively affected, indicating that the temperature of the extender at the first dilution was more important than the volume of the extender to be added. Although a great volume of extender needs to be heated to 34ºC, this protocol has the advantage of the dilution being performed in only one step.

Although the adverse effects of lipid phase transition in boar sperm cells are concentrated between 5-15ºC (Watson, 2000), changes in membrane fluidity due to lipid phase transitions and protein reassembly may occur at temperatures above 20ºC (Canvin and Buhr, 1989; Parks and Lynch, 1992). The lipid phase transition can lead to the rupture of the plasmatic membrane, acrosome degeneration, and loss of ions and enzymes due to changes in membrane fluidity (De Leeuw *et al*., 1991). In a two-step procedure, bithermic dilution facilitates semen processing by reducing the time necessary to reach the desired storage temperature, when compared to isothermic dilution (Schulze *et al*., 2013). However, the lower temperature used in the last dilution can lead to a rapid cooling and result in altered membrane function and lower adaptation of the cells to temperature changes. The fact that none of the CASA semen parameters differed between the isothermic and bithermic two-step dilution is in agreement with the results of previous studies (Petrunkina *et al*., 2005; López Rodriguez *et al*., 2011). The results of VAP, VCL, VSL, ALH, BCF, STR and LIN also corroborate those of Schulze *et al*. (2013). Nevertheless, Schulze *et al*. (2013) showed better results for total motility, progressive motility and integrity of sperm plasma and acrosomal membranes, in isothermic rather than bithermic dilutions, in addition to a small significant difference in WOB, on day 6 of storage. The fact that differences between isothermic and bithermic dilutions were small or absent on the first three days of storage led these authors to suggest that the lower temperature of the extender for the final dilution compromised only a small portion of sperm cells, but this difference increased during the storage time up to six days.

It can be pointed out that not only the storage time, but also the extender used could influence the results obtained with different dilution procedures. When semen was preserved in BTS, and the evaluations

were performed for two days (López Rodriguez *et al*., 2011) or five days (current study), the dilution procedure did not affect CASA sperm parameters. On the other hand, the adverse effects of two-step hypothermic dilution were more pronounced in BTS than in Androstar Plus, mainly with a longer storage time (Schulze *et al*., 2013). This suggests that the choice of extender could minimise the possible negative effect of a faster temperature decline resulting from a two-step bithermic dilution. Nevertheless, the scarcity of information concerning different protective actions of semen extenders for bithermic dilution renders this aspect inconclusive, and further investigation is necessary for its elucidation.

The considerable individual variation among boars concerning the preservation capacity of semen quality during storage is well documented (Waberski *et al*., 1994; Reis *et al*., 2002). The boar variation in chilling sensitivity was confirmed in the present study since the individual boar explained more the variation in motility than dilution treatment. There is no concrete information about a possible difference among boars regarding the sensitivity to temperature variation during semen dilution but it may be possible that some boars are more sensitive to a two-step hypothermic dilution (Schulze *et al*., 2013). If this assumption is true, the group of boars used in each study could be a factor contributing to the discrepancy of results among studies. In the present study, although overall differences were observed among boars, they showed a similar response to all treatments (data not shown), suggesting that they were similarly tolerant to the dilution procedures studied. One of the positive aspects of this study is that it was performed with a relatively high number of boars. However, it is important to point out that before the beginning of the trial, all of the boars included in this study showed a satisfactory response in terms of motility during three days of semen storage. Thus, for the safe and widespread use of two-step bithermic or one-step isothermic dilutions, it would be interesting to validate these results with boars of unknown response to conventional semen preservation.

The use of bithermic instead of isothermic dilution has the advantage of facilitating semen processing, since it is necessary to heat only small volumes of extender used for the 1:1 dilution to 34°C (Schulze *et al*., 2013). We observed that bithermic dilution provides a faster drop in temperature to room temperature, making it possible to send semen doses in less time to commercial farms, as pointed out by Schulze *et al*. (2013). It must be also taken into account that spending less time at room temperature is beneficial because bacterial growth can be minimised (Althouse and Lu, 2005).

Semen quality during storage at 16ºC in BTS extender is not affected by dilution performed in one or two steps when the temperature of the extender is kept at 34ºC for both steps. When using a two-step dilution, the temperature of the extender can be reduced to 23ºC for the second dilution without impairing semen quality, which enables room temperature to be reached faster.

Acknowledgments

We would like to thank Master Genética Animal, especially all staff from Master Carijos (Papanduva, Santa Catarina, Brazil), for their partnership and Agroceres PIC and CNPq for their financial support of this project.

References

Althouse GC, Wilson ME, Kuster C, Pasrley M. 1998. Caracterization of lower temperature storage limitations of fresh-extended porcine semen. *Theriogenology*, 50:535-543.

Althouse GC, Lu KG. 2005. Bacteriospermia in extended porcine semen. *Theriogenology*, 63:573-584.

Canvin AT, Buhr MM. 1989. Effect of temperature on the fluidity of boar sperm membranes. *J Reprod Fertil*, 85:533-540.

De Leeuw FE, Colenbrander B, Verkleij AJ. 1991. The role membrane damage plays in cold shock and freezing injury. *Reprod Domest Anim*, 1:95-104.

Garcia JC, Abad M, Kirkwood RN. 2007. Effect of sperm numbers and time of insemination relative to ovulation on sow fertility. *Anim Reprod Sci*, 100:397-401.

Johnson LA, Weitze KF, Fiser P, Maxwell WMC. 2000. Storage of boar semen. *Anim Reprod Sci*, 62:143-172.

López Rodriguez A, Rijsselaere T, Vyt P, Van Soon A, Maes D. 2011. Effect of dilution temperature on boar semen quality. *Reprod Domest Anim*, 47:e63-e66.

National Research Council. 2012. *Nutrient Requirements of Swine*. 11th ed. Washington, DC: National Academy of Sciences. 400 pp.

Parks JE, Lynch DV. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology,* 29:255-266.

Petrunkina AM, Volker G, Weitze KF, Beyerbach M, Peterson ET, Waberski D. 2005. Detection of cooling-induced membrane changes in the response of boar sperm to capacitating conditions. *Theriogenology*, 63:2278-2299.

Pursel VG, Johnson LA, Rampacek GB. 1972. Acrosome morphology of boar spermatozoa incubated before cold shock. *J Anim Sci*, 34:278-283

Reis GR, Bernardi ML, Schwarz P, Bortolozzo FP, Wentz I. 2002. Diferença entre machos suínos na manutenção da viabilidade espermática a 17ºC. *Acta Sci Vet*, 30:159-166.

Roca J, Vázquez JM, Gil MA, Cuello C, Parrilla I, Martínez EA. 2006. Challenges in pig artificial insemination. *Reprod Domest Anim*, 41:43-53.

Schulze M, Henning H, Rüdiger K, Wallner U, Waberski D. 2013. Temperature management during semen processing: impact on boar sperm quality under

laboratory and field conditions. *Theriogenology*, 80:990-998.

Waberski D, Meding S, Dirksen G, Weitze KF, Leiding C, Hahn R. 1994. Fertility of long-term-stored boar semen: iInfluence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. *Anim Reprod Sci*, 36:145-151.

Waberski D. 2009. Critical steps from semen collection to insemination. *In:* Proceedings of the Annual Meeting of EU-AI-Vets, Ghent, Belgium. Ghent: University of Ghent. pp. 66-69.

Watson PF. 2000. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*, 60:481-492.

Weitze KF. 2012. Novos conceitos na diluição do ejaculado suíno: como as células espermáticas respondem aos desafios impostos? *In*: VII Simpósio Internacional de Suinocultura, Porto Alegre, RS. Porto Alegre, RS: SINSUI. pp. 17-30.