**The effect of extender, method of thawing and duration of storage on in-vitro fertility
measures of frozen-thawed boar sperm**

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13 **ABSTRACT**

14 Frozen-thawed boar sperm (FTS) has reduced in-vitro and in-vivo lifespan compared to liquid
15 semen. Experiments tested whether extender, thawing procedures and storage temperatures
16 could extend the fertile lifespan of FTS. Experiment 1 tested six extenders on post thaw motility
17 (MOT) and viability (VIA). Straws from boars ($n = 6$) were thawed and diluted into each
18 extender and evaluated at 20, 60 and 120 min. There was a trend ($P = 0.08$) for an extender by
19 time interaction for MOT and effect of extender and time for MOT ($P < 0.0001$) and extender (P
20 $= 0.10$) and time ($P < 0.0001$) for VIA. Experiment 2 evaluated temperature and time of thawing
21 on in-vitro fertility at intervals after thawing. Straws (0.5 mL) from different boar ejaculates ($n =$
22 15) were thawed at 50 °C for 10, 20, or 30 s or at 70 °C for 5, 10, or 20 s and evaluated at 5, 30,
23 and 60 min. There was an effect of thawing treatment on MOT, VIA and ACR (viable sperm
24 with intact acrosomes, $P < 0.0001$) and an effect of time of evaluation ($P < 0.0001$) on MOT and
25 ACR. Thawing at 70 °C for 20 s reduced ($P < 0.05$) MOT, VIA and ACR compared to other
26 treatments. Experiment 3 tested the effects of storage temperature and time after thawing using
27 20 ejaculates. Samples were thawed, diluted and allotted to storage at 17, 26, or 37 °C with
28 evaluation at 2, 6, 12 and 24 h. There was a storage temperature and time effect and an
29 interaction for MOT and VIA ($P < 0.0001$). Storage at 17 and 26 °C increased ($P < 0.05$) MOT
30 over all times (38.5%) compared to 37° C (26%) while MOT was reduced at intervals. Viability
31 was also greatest with 17 and 26 °C compared to 37 °C and was also affected by time and
32 decreased with time. These results indicate that FTS can be held at 17 or 26 °C for up to 2 h
33 before use and would allow for preparation of multiple doses. These data suggest in-vitro fertility
34 of FTS is affected by extender, thawing, and storage.

35

36 **Keywords:** Extender; Fertility; Frozen boar sperm; Motility; Thawing; Viability

37

39 **1. Introduction**

40 In modern swine production there is limited use of frozen-thawed boar sperm for artificial
41 insemination due to the variable pregnancy rates and litter sizes when used for breeding [1, 2] .
42 The shortened lifespan of frozen-thawed boar sperm in comparison to liquid extended semen has
43 been documented in-vivo and in-vitro and is assumed to be the cause of lower fertility when used
44 in artificial insemination [3-5]. It has been hypothesized that post-thaw handling procedures may
45 have large implications for post-thaw boar sperm quality measures such as motility, viability and
46 acrosome status and could therefore impact in-vivo fertility.

47 The in-vitro motility and viability of boar sperm has been reported to be influenced by
48 various extenders and components for liquid [6, 7] and frozen sperm [2, 8-10]. Because of
49 considerable variation in freezing and thawing methodologies, there is no clear evidence on
50 whether specific post-thaw extenders could improve cryopreserved sperm fertility.
51 Cryopreserved boar sperm is packaged in plastic straws or plastic bags of 0.25 mL to 5 mL
52 volumes [1, 11-14]. In some research studies, improved post-thaw quality was observed in
53 lower volume straws and plastic bags (0.25 mL to 0.7 mL), due to the smaller surface area to
54 volume ratio, which allows for more uniform freezing and thawing [11, 15, 16]. Due to the
55 discrepancies in freezing volume, surface area, and surface area to volume ratio, different
56 thawing temperatures and durations have been explored for sperm packaged in different forms.
57 While thawing was initially performed at 37 °C [17], higher thawing temperatures of 50 °C [16,
58 18] and 70 °C [19] were chosen to minimize the exposure time of sperm cells to potential ice
59 crystal and osmotic damage. As a result, thawing duration differed with lower volume straws
60 (0.25-0.5 mL) which were thawed at 10 to 20 s at 50 °C [16, 20] or 5 to 10 s at 70 °C [19]
61 whereas greater volume straws (5-7 mL) were thawed for 40 to 50 s at 50 °C [18].

While there has been extensive research on the importance of pre-freeze semen handling [2, 21, 22], cooling rate [23, 24], freezing rate [19, 25] and on thawing rate [26, 27], there is limited information on many aspects of post-thaw semen processing. This includes information on diluents, temperature and time of thawing, temperature and duration of storage on the fertile lifespan of frozen-thawed boar sperm.

To determine the effects of post-thaw semen handling, three experiments were performed to evaluate the effect of different extenders, method of thawing and storage on measures of in vitro fertility for frozen-thawed boar sperm.

2. Materials and Methods

2.1. Boar sperm cryopreservation

2.2. Semen collection, freezing, and evaluation

The protocol for use of animals in these experiments was approved by the University of Illinois Institutional Animal Care and Use Committee. Semen was obtained from purebred boars that were in commercial boar studs and routinely collected for production of liquid semen. Ejaculates were frozen using a modified procedure [23] that has been previously described [28]. Briefly, ejaculates were collected and diluted 1:1 with 37 °C extender (Modena, Swine Genetics International, Cambridge, IA), and slowly cooled to 17 °C before overnight shipping to the University of Illinois, Urbana, IL. Upon arrival, samples were evaluated for motility and concentration and processed if motility was $\geq 80\%$. Samples were centrifuged for 12 min at 800 x g at 17 °C and the supernatant aspirated. The sperm pellet was re-suspended with Androhep CryoGuard Cooling Extender® (MOFA, Verona, WI) to a concentration of 2.8×10^9 sperm/mL.

Sperm were held at 5 °C for 2.5 h and diluted to a final concentration of 1.4×10^9 sperm/mL with Androhep CryoGuard Freezing Extender® (MOFA, Verona, WI). Straws (0.5 mL) were filled using a semi-automatic filling and sealing machine. Straws were placed into the Ice Cube® controlled rate freezer (MOFA, Verona, WI) at 2 °C and processed using the following freezing curve: Step 1: 2 to -4 °C in 3 min (2 °C/min), Step 2: -4 to -30 °C in 0.87 min (29.9 °C/min), Step 3: -30 to -25 °C in 1 min (5 °C/min), and Step 4: -25 to -140 °C in 11.5 min (10 °C/min). Following the completion of the freezing program, the straws were placed into containers for storage in liquid nitrogen at -196 °C.

2.1. Experiment 1: Effect of thawing extender on measures of sperm fertility at intervals after thawing

The aim of this study was to determine whether different extenders influenced the motility and viability of cryopreserved boar sperm after thawing. Frozen thawed samples were selected from cryopreserved ejaculates that had a 20 min post-thaw motility >40% in the extender designed for cryopreserved boar sperm (ACG, Androhep CryoGuard Extender). The other extenders were chosen based on their availability as short-term and long term extenders. The short term extenders chosen were Beltsville Thaw Solution (BTS, MOFA, Verona, WI USA) and Modena (MOD, Swine Genetics International, Cambridge, IA USA), while the long term extenders were Preserv® Xtra (PRX, Reproquest, Fitchburg, WI USA), Duragen (DUR, Magapor, Zaragoza, Spain) and Vitasem LD (VIT, Magapor). The extenders were diluted to the specifications of the manufacturer and stored at 5 °C. The experiment was performed using semen from 6 different boars and sperm frozen in 0.5 mL straws that had been stored in liquid nitrogen for 12 to 36 months. One boar was tested in each replicate. Within a replicate, straws

from a single ejaculate were thawed at 50 °C for 20 s and the contents expelled into a single glass tube in a dry heating block at 37 °C. Aliquots of the sample were diluted 1:40 into one of six different extenders in 1.5 mL polypropylene tubes. After 10 min, a vital stain (Viadent, Hamilton Thorne, Beverly, MA) containing the fluorescent dye, Hoechst 33258, that had been diluted in each of the six different extenders, was added to a sub-sample for a final dilution of 20 µg/mL of stain and then incubated for 8 min at 37 °C. The vital stain was added to the different extenders at 1 minute intervals to avoid different incubation times. To avoid bias, the sequenced order for extender evaluation was rotated equally among the different extenders over replicates. Following the incubation time, samples were analyzed using CASA (IVOS Hamilton Thorne, Beverly, MA) using 20 µm Leija slides (Leja Products B.V. the Netherlands) for motility in 5 fields followed by assessment for viability in the same 5 fields using excitation of the fluorescent stain with a blue LED and using the Viadent filter. Samples were evaluated at 20 min (T 0), 60 min and 120 min after thawing.

2.2 Experiment 2: Effect of thawing temperature and duration of thawing on sperm measures at intervals after thawing

2.2.1. Semen and experimental design

In experiment 2, cryopreserved sperm from the ejaculates (n = 15) of 13 boars were used. The sperm had been stored at -196 °C for approximately 6 to 12 months prior to use in this study. Straws from a single ejaculate were assigned to a thawing temperature and duration of thawing. A total of 90 straws was thawed using 6 straws from each ejaculate. Straws were individually thawed at 50 °C for 10, 20, or 30 s or at 70 °C for 5, 10, or 20 s and evaluated at 5,

30, and 60 min for post-thaw motility (MOT) viability (VIA) and viable sperm with intact acrosomes (ACR).

For evaluation of MOT, sub-samples were placed in a 37 °C warming block and diluted 1:40 with Androhep CryoGuard Extender. Samples were then placed onto a pre-warmed slide and a coverslip added and warmed for 2 minutes before evaluation. Post-thaw motility was performed manually using a phase-contrast microscope at 200 X magnification with a heated stage and external video monitor. Ten sperm were counted in each of 10 separate fields and motility expressed as the average of the fields. Viability was assessed using fluorescent staining with propidium iodide as previously described [28]. Samples were incubated with the vital fluorescent stain, propidium iodide (PI, Sigma–Aldrich, St. Louis, MO) and fluorescein isothiocyanate lectin from *Arachis hypogaea* for acrosome integrity (FITC-PNA, Sigma–Aldrich, St. Louis, MO) for 10 min and then fixed with 0.4% paraformaldehyde in PBS. The stained samples were observed for fluorescence on a Carl Zeiss AxioCam HRc microscope (Carl Zeiss Microscopy, LLC, Thornwood, NY) and sperm counted based on the bright field image, the PI fluorescence image, and the FITC-PNA fluorescence image. A total of 200 sperm cells were counted at 200 X magnification. Live sperm excluded PI and did not fluoresce red and those with intact acrosomes excluded FITC-PNA and did not show green fluorescence.

2.3. Experiment 3: Effect of storage temperature and storage time on measures of sperm fertility

2.3.1. Semen and experimental design

In experiment 3, cryopreserved sperm from ejaculates (n = 20) of 17 boars were selected. The ejaculates were initially extended 1:1 in Modena extender and shipped overnight and frozen in 0.5 mL straws and stored in liquid nitrogen for approximately 6 to 12 months prior to use. All

samples were assigned in a 3 x 4 factorial arrangement of treatments with samples stored at 17 °C, 26 °C and 37 °C for 2, 6, 12 and 24 h. Straws were thawed at 50 °C for 20 s and the contents of each straw diluted 1:20 in Androhep CryoGuard Thaw Extender® and an aliquot removed to evaluate pre-treatment MOT and VIA as described in experiment 2. Diluted samples were equally allotted into sealed plastic tubes and stored in digitally controlled thermal devices at 17 °C, 26 °C and 37 °C. Each treatment tube was sub-sampled at 2, 6, 12, and 24 h during storage and MOT and VIA assessed similarly to that described for experiment 2.

2.4. Statistical analysis

Data were analyzed using ANOVA procedures in SAS (SAS Institute Inc., Cary, NC). The continuous response measures of MOT, VIA and ACR were analyzed using the PROC MIXED procedures for significance of the main effects using the *F*-test and differences between least squares means identified using the *t* test with the Tukey-Kramer adjustment. The models for the dependent variables included the main effects and interactions for extender and time of evaluation after thawing for Experiment 1, thawing temperature combined with time of thawing (treatment) and evaluation time after thawing for Experiment 2, and storage temperature and time of storage for Experiment 3. The analyses were performed as repeated measures for data obtained from the same boar over time using the “repeated” statement with the ‘ar’ classification. The assumptions of ANOVA for normal distribution of data were evaluated using PROC UNIVARIATE and for homogeneity of variance using Levene’s test. Significant differences were identified at $P \leq 0.05$ and trends at $P > 0.05$ and ≤ 0.10 .

3. Results

3.1. Experiment 1

Data for MOT for extenders evaluated over time are shown in Figure 1a. There was a tendency ($P = 0.08$) for an interaction of extender and time and an effect of extender ($P < 0.0001$) and time of evaluation ($P < 0.0001$). Overall MOT was greatest with ACG ($P < 0.05$) compared to other extenders but did not differ from MOD ($P > 0.10$). The MOD extender showed greater MOT compared to VIT and PRX ($P < 0.05$) but did not differ from other extenders ($P > 0.10$). All MOT measures differed among the time intervals of 20, 60, and 120 min after thawing ($P \leq 0.05$). Data for VIA for extenders over time is shown in Figure 1b. There was no interaction of extender and time of evaluation for VIA ($P > 0.10$), but there was an effect of time ($P < 0.0001$) and a trend for extender ($P = 0.10$). The VIA at time intervals of 20, 60, and 120 min after thawing differed from one another ($P < 0.001$).

3.2. Experiment 2

There was no interaction among the main effects for any measures of sperm fertility ($P > 0.10$). Sperm MOT was affected by thawing method ($P < 0.0001$) and was similar among treatments except when thawing at 70 °C for 20 s (Figure 2a). Sperm MOT was also affected by time of evaluation ($P < 0.0001$) and differed among each time period. For VIA, there was an effect of thawing method ($P < 0.0001$) but no effect of time of evaluation ($P > 0.10$). Thawing at 70 °C for 20 s resulted in lower VIA compared to all other treatments (Figure 2b). Measures for ACR were affected by thawing method ($P < 0.0001$), which was reduced when thawing sperm at 70 °C for 20 s compared to other treatments (Figure 2c). Measures for ACR were also affected by time of evaluation ($P < 0.0001$) and differed among each of the periods.

3.3. Experiment 3

Sperm MOT and VIA were affected by storage temperature and duration of storage, with an interaction between the main effects ($P < 0.0001$). Pretreatment MOT averaged $48.6 \pm 2.5\%$ and VIA $52.5 \pm 2.6\%$. Samples stored at 17 and 26 °C maintained MOT for 2 h following thawing, while samples held at 37 °C showed a reduction compared to the pre-treatment mean (Figure 3a). At the 17 and 26 °C temperatures, there were duration of storage effects on MOT with a decline by 6 h but no change to 12 h, and then a further decline by 24 h. For samples stored at 37 °C, MOT was reduced at every time point with most sperm non-motile and non-viable at 24 h. Sperm VIA did not change from pre-treatment value with storage at 17 °C and 26 °C for 6 h of storage, but with noted declines at 24 h, while at 37 °C, VIA declined at each time point (Figure 3b).

4. Discussion.

The results of these studies indicate that for cryopreserved boar sperm, post-thaw extender, method of thawing, and storage temperature for diluted sperm after thawing can affect in-vitro fertility. Although not evaluated in these experiments, the in-vitro measures could be related to fertility when the sperm is used for insemination. Low motility samples have been reported to produce fewer offspring with the use of cryopreserved boar sperm [29] and with liquid semen [30]. Further, frozen-thawed boar sperm has been shown to have a reduced lifespan for in-vivo fertility compared to liquid semen with an optimal fertilization interval of only 4 hours prior to ovulation [5]. Methods to improve the fertile lifespan of cryopreserved boar sperm in-vitro, might also help extend the fertile lifespan of sperm in-vivo. The extender study revealed immediate effects of the diluent on motility and viability soon after thawing. These effects

became more evident with time after thawing with differences in motility and viability ranging from 8 to 35% at 2 hours after thawing. Although the extender ingredients or concentrations that would impact these measures are unknown, in-vitro motility and viability of boar sperm has been reported to be influenced by various extenders and components in liquid [6, 7] and frozen form [2, 8-10]. This would suggest that continued efforts in this area might yield improved fertility for cryopreserved boar sperm.

The optimal temperature and duration of thawing for cryopreserved boar sperm might logically depend upon the packaging material, form and volume [1, 11-14] in order to optimize the surface area to volume ratio to allow for uniform thawing [11, 15, 16]. Cryopreserved boar sperm is routinely thawed in the range of 37 to 70 °C for 5 to 40 s [16, 18, 19]. Small volume straws (0.25-0.5 mL) are typically thawed from 10 to 20 s at 50 °C [16, 20] or for 5 to 10 s at 70 °C [19] while larger volume straws (5-7 mL) are typically thawed for 40-50 s between 37 to 50 °C [15, 18]. A review on the effects of thawing indicate that rate of freezing dictates the rate of thawing, as rapid freezing requires rapid thawing to prevent cryo-injury from re-growth of ice crystals during the thawing process [1]. In the present study, we tested the thawing of 0.5 mL straws at 50 and 70 °C and our results indicated that both could result in similar motility and viability, but that at the higher temperature, there were potential problems with thawing times that were too long. Errors in time of thawing might be expected to occur in practical use and even in the lab as we detected changes in sperm fertility with 20 s thawing. In contrast, thawing at 50 °C resulted in similar measures for viability and motility within a range of thawing times and would provide a margin of safety with different thawing times.

Increasing the fertile lifespan of frozen-thawed boar sperm in storage could have practical application for insemination on farms. Thawing and processing cryopreserved boar sperm for AI

doses requires more time than for conventional AI with liquid semen and the preparation of multiple frozen thawed doses at one time would be more efficient. Our results indicated that frozen-thawed boar sperm can be extended and held at 17 or 26 °C for up to 2 h following thawing with no significant decrease in motility and viability. While not a very long period of time, two hours would provide a safe margin for the preparation of doses to be used that morning or in the afternoon. Unfortunately, storage up to 6 h and beyond showed significant reductions in sperm fertility and might be expected to reduce fertility when used for AI. However, the decline in fertility was not great at 17 and 26 °C and the motility measures that were observed at the 6 and 12 h intervals in this study, were shown to have no effect on the fertility when used for AI in another study [29]. Yet, as expected, storage at 37 °C showed a significant decrease in post-thaw quality by 2 h and thereafter as time progressed.

Conclusions:

The choice of diluent for cryopreserved boar sperm after thawing can affect in-vitro measures of sperm motility and viability within the 2 h period after thawing. Research into the modification of extenders could result in improved post-thaw boar sperm fertility. Thawing 0.5 mL straws of cryopreserved boar sperm at 50 °C allowed a safe range of 10 to 30 s while thawing at 70 °C limited thawing to a 10 s time period. Storage of thawed boar sperm at 17 or 26 °C in extender allowed fertility to be maintained for 2 h to allow for preparation or analysis of multiple doses before insemination.

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References

- [1] Johnson LA, Weitze KF, Fiser P, Maxwell WM. Storage of boar semen. *Anim Reprod Sci.* 2000;62:143-72.
- [2] Johnson LA. Fertility results when using frozen boar spermatozoa: 1970 to 1985. In: Johnson LA, Larsson K, editors. *Deep Freezing Boar Semen: Proc First Int Conf Deep Freezing of Boar Semen Uppsala, Sweden: Swedish University of Agricultural Sciences; 1985.* p. 192-222.
- [3] Pursel VG, Schulman LL, Johnson LA. Distribution and morphology of fresh and frozen-thawed sperm in the reproductive tract of gilts after artificial insemination. *Biol Reprod.* 1978;19:69-76.
- [4] Einarsson S. Deep freezing of boar spermatozoa. *World Review of Animal Production.* 1973;9:45-51.
- [5] Waberski D, Weitze KF, Gleumes T, Schwartz M, Willmen T, Petzoldt R. Effect of time of insemination relative to ovulation on fertility with liquid and frozen boar semen. *Theriogenology.* 1994;42:831-40.
- [6] Vyt P, Maes D, Dejonckheere E, Castryck F, Van Soom A. Comparative study on five different commercial extenders for boar semen. *Reprod Dom Anim.* 2004;39:8-12.
- [7] Estienne MJ, Harper AF, Day JL. Characteristics of sperm motility in boar semen diluted in different extenders and stored for seven days at 18 degrees c. *Reprod Biol.* 2007;7:221-31.
- [8] Larsson K, Einarsson S, Nicander L. Influence of thawing diluents on vitality, acrosome morphology, ultrastructure and enzyme release of deep frozen boar spermatozoa. *Acta Vet Scand.* 1976;17:83-100.
- [9] Großfeld R, Sieg B, Struckmann C, Frenzel A, Maxwell WMC, Rath D. New aspects of boar semen freezing strategies. *Theriogenology.* 2008;70:1225-33.
- [10] Garcia JC, Dominguez JC, Pena FJ, Alegre B, Gonzalez R, Castro MJ, Habing GG, Kirkwood RN. Thawing boar semen in the presence of seminal plasma: Effects on sperm quality and fertility. *Anim Reprod Sci.* 2010;119:160-5.
- [11] Bwanga CO, Einarsson S, Rodriguez-Martinez H. Deep freezing of boar semen packages in plastic bags and straws. *Reprod Dom Anim.* 1991;26:117-25.
- [12] Eriksson BM, Vazquez JM, Martinez EA, Roca J, Lucas X, Rodriguez-Martinez H. Effects of holding time during cooling and of type of package on plasma membrane integrity, motility and in vitro oocyte penetration ability of frozen-thawed boar spermatozoa. *Theriogenology.* 2001;55:1593-605.
- [13] Eriksson BM, Petersson H, Rodriguez-Martinez H. Field fertility with exported boar semen frozen in the new flatpack container. *Theriogenology.* 2002;58:1065-79.

305 [14] Eriksson BM, Rodriguez-Martinez H. Effect of freezing and thawing rates on the post-thaw
306 viability of boar spermatozoa frozen in flatpacks and maxi-straws. *Anim Reprod Sci.*
307 2000;63:205-20.

308 [15] Córdova A, Pérez-Gutiérrez JF, Lleó B, García-Artiga C, Alvarez A, Drobchak V, Martín-
309 Rillo S. In vitro fertilizing capacity and chromatin condensation of deep frozen boar semen
310 packaged in 0.5 and 5 ml straws. *Theriogenology.* 2002;57:2119-28.

311 [16] Pelaez J, Breininger E, Alegre B, Pena FJ, Dominquez JC. In vitro evaluation in the quality
312 of fertilizing capacity of boar spermatozoa in 0.25 ml straws. *Reprod Dom Anim.* 2006;41:153-
313 61.

314 [17] Salamon S, Visser D. Fertility test of frozen boar spermatozoa. *Aust J Biol Sci.*
315 1973;26:291-3.

316 [18] Cordova-Izquierdo A, Oliva JH, Lleo B, Garcia-Artiga C, Corcuera BD, Perez-Gutierrez JF.
317 Effect of different thawing temperatures on the viability, in vitro fertilizing capacity and
318 chromatin condensation of frozen boar semen packaged in 5 ml straws. *Anim Reprod Sci.*
319 2006;92:145-54.

320 [19] Hernández M, Roca J, Gil MA, Vázquez JM, Martínez EA. Adjustments on the
321 cryopreservation conditions reduce the incidence of boar ejaculates with poor sperm freezability.
322 *Theriogenology.* 2007;67:1436-45.

323 [20] Aamdal J, Andersen K. Fast thawing of semen frozen in straws. *Zuchthyg.* 1968;3:22-4.

324 [21] Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and
325 the assessment of their post-thawing function. *Reprod Fertil Dev.* 1995;7:871-91.

326 [22] Guthrie HD, Welch GR. Impact of storage prior to cryopreservation on plasma membrane
327 function and fertility of boar sperm. *Theriogenology.* 2005;63:396-410.

328 [23] Pursel VG, Park CS. Freezing and thawing procedures for spermatozoa. In: Johnson LA,
329 Larsson K, editors. *Deep Freezing of Boar Semen.* Uppsala, Sweden: Swedish University of
330 Agricultural Sciences; 1985. p. 147-66.

331 [24] Pursel VG, Schulman LL, Johnson LA. Effect of holding time on storage of boar
332 spermatozoa at 5°C. *J Anim Sci.* 1973;37:785-9.

333 [25] Almlid T, Stavne SE, Johnson LA. Fertility evaluation of the straw freezing technique for
334 boar semen under practical artificial insemination conditions. *Reprod Dom Anim.* 1987;22:193-
335 202.

336 [26] Fiser PS, Fairfull RW, Hansen C, Panich PL, Shrestha JN, Underhill L. The effect of
337 warming velocity on motility and acrosomal integrity of boar sperm as influenced by the rate of
338 freezing and glycerol level. *Mol Reprod Dev.* 1993;34:190-5.

339 [27] Thurston LM, Watson PF, Mileham AJ, Holt WV. Morphologically distinct sperm
340 subpopulations defined by fourier shape descriptors in fresh ejaculates correlate with variation in
341 boar semen quality following cryopreservation. J Androl. 2001;22:382-94.

342 [28] Ringwelski JM, Beever JE, Knox RV. Effect of interval between inseminations when using
343 frozen-thawed boar sperm on fertility and fetal paternity in mature gilts. Anim Reprod Sci.
344 2013;137:197-204.

345 [29] McNamara KA, Knox RV. Effect of using frozen-thawed boar sperm differing in post-thaw
346 motility in the first and second inseminations on pregnancy establishment, litter size, and fetal
347 paternity in relation to time of ovulation. J Anim Sci. 2013;91:5637-45.

348 [30] Flowers WL. Management of boars for efficient semen production. J Reprod Fertil Suppl.
349 1997;52:67-78.
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Figure 1. Least square means ($\pm$ SE) for the percentages of frozen thawed boar sperm that were motile (a) and viable (b) when extended in Androhep CryoGuard (ACG), Beltsville Thaw Solution (BTS), Modena (MOD), Preserv® Xtra (PRX), Duragen (DUR) and Vitasem (VIT). For motility there was a trend for an interaction ($P = 0.08$). Extender and time of evaluation affected motility and viability ($P < 0.0001$). For the main effects of extender and time, within an extender (^{a,b,c}) and among extenders (^{x,y,z}), means without a common superscript are different ($P < 0.05$). Straws ($n = 6$) were from different sires and were represented across all treatments.

Figure 2. Least squares means ($\pm$ SE) for percentages of frozen thawed boar sperm that were motile (a) viable (b) and live with intact acrosomes (c) in response to thawing temperature and duration of thawing (seconds) assessed at 5, 30 and 60 minutes after thawing. There was an effect of treatment and time after thawing ($P < 0.0001$) for all measures. For the main effects of extender and time, within a treatment ^(a,b,c), and among treatments ^(x,y,z), means without a common superscript are different ($P < 0.05$). Straws ($n = 15$) were from different sires and ejaculates were represented across all treatments.

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Figure 3. Least squares means ($\pm$ SE) for percentages of frozen thawed boar sperm motile (a) and viable (b) in response to the effect of holding temperature and duration of storage. There was an interaction and effect of treatment and time ($P < 0.0001$). Within a storage duration ^(x,y,z) and among durations ^(a,b,c,d), means without a common superscript are different ($P < 0.05$). Straws ($n = 20$) from ejaculates were thawed and aliquoted into sub-samples and were represented across all treatments.

a.

b.