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Original article

Freezability and fertility of frozen-thawed boar semen supplemented with ostrich egg yolk lipoproteins

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Abstract

Lipoproteins, isolated from ostrich egg yolk (LPFo), provide excellent protection for boar spermatozoa against cryo-induced damage. The present study was performed to investigate the effects of LPFo on the freezability and fertilizing capacity of frozen-thawed (FT) boar semen after post-cervical artificial inseminations (post-CAIs). Semen, collected from 7 Polish Large White (PLW) and 4 Polish Landrace (PLR), was frozen in an extender containing LPFo. Post-CAIs were performed in 38 multiparous sows, using a catheter-cannula kit. Sows were inseminated 2× within one oestrus, and fertility parameters were recorded after farrowing. Neither boar (within breed) nor breed affected the quality of the pre-freeze (PF) semen, such as total motility (TMOT), mitochondria membrane potential (MMP), plasma membrane integrity (PMI), osmotic resistance test (ORT) and DNA fragmentation. Differences in the freezability of boar semen were observed among the boars, whereas there were no marked breed effects. Post-thaw TMOT markedly declined over storage time in most of the boars, particularly at 60 min after thawing. Inseminations of post-weaned oestrus sows resulted in pregnancy and farrowing rates of 84.2% and 81.6%, respectively. Neither the mean number of piglets born (NB) nor the mean number of piglets born alive (NBA) was affected by boar or breed. The total number of piglets born was 365, resulting in 11.8 NB piglets, whereas the total number of piglets born alive was 353, with 11.4 NBA piglets per litter. The findings of this study reaffirm the variations in the freezability of boar semen. In this study the supplementation of ostrich egg yolk lipoproteins to the freezing extender of boar semen produced high proportions of functionally viable FT spermatozoa that were capable of providing acceptable fertility results after post-CAIs in multiparous sows.

Key words: boar, egg yolk lipoproteins, cryopreservation, artificial insemination

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Introduction

Cryopreservation of semen allows the prolonged storage of genetically important reproductive traits through the use of assisted reproductive techniques (ARs), such as a artificial insemination, (AI) (Didion et al. 2013, Knox 2015, Gliozzi et al. 2017). Despite many years of research on the cryopreservation of boar semen, the fertility results are not satisfactory sufficient for commercial use compared with other domestic animals (Roca et al. 2011, Knox, 2015, Yeste 2016, Yeste et al. 2017). Freezing of boar semen requires more spermatozoa, and the litter size and the farrowing rate are markedly reduced compared with liquid-stored semen (Didion et al. 2013, Knox, 2015). Moreover, the production of a high number of functionally viable frozen-thawed (FT) boar spermatozoa is required to minimize economic loss and increase the worldwide application of FT in AI technologies (Almiñana et al. 2010, Spencer et al. 2010, Didion et al. 2013, Knox 2015). In recent years much effort has been directed towards optimizing the freezing protocol for boar semen (Alkmin et al. 2014, Estrada et al. 2014, Wasilewska and Fraser 2017, Yeste 2016, Yeste et al. 2017).

Hen egg yolk (HEY) is commonly used as a cryo-protective agent in the freezing extender, even though it represents a potential risk of contamination of semen and can adversely affect sperm respiration, microscopic analyses and biochemical assays (Manjunath 2002, Bergeron et al. 2004, Pillet et al. 2011). There has been increasing interest in replacing the HEY in the freezing extender, and in our laboratory we have shown that the lyophilized form of lipoprotein fractions, isolated from ostrich egg yolk (LPFo), is a suitable alternative to the HEY for the cryopreservation of boar semen, based on analysis of post-thaw sperm quality (Strzeżek et al. 2005, Fraser et al. 2010a, 2011, 2014a,b, Wasilewska et al. 2016, Wasilewska and Fraser 2017). Furthermore, in a preliminary study, we reported that post-cervical artificial insemination (post-CAI) of semen frozen in LPFo-supplemented extender provided acceptable fertility results (Fraser et al. 2007). Moreover, post-CAI allows the deposition of a low number of sperm cells in the uterine body, and does not negatively influence the fertility outcome compared with traditional AIs, using liquid-stored and FT semen (Casas et al. 2010, Roca et al. 2011, Hernández-Caravaca et al. 2012).

The main objective of this study was to assess the effects of LPFo-supplemented extender on the freezability and fertilizing capacity of FT boar semen after post-CAIs in multiparous sows. In addition to the assessment of the fertility results, the sperm parameters analyzed in the pre-freeze (PF) and FT semen included total motility (TMOT), mitochondria membrane potential (MMP), plasma membrane integrity (PMI), osmotic resistance test (ORT) and DNA fragmentation.

Materials and Methods

Animals and semen collections

Ejaculates were collected from seven Polish large white (PLW) and four Polish landrace (PLR) boars (average age 2 years). All the animals were stationed at the Artificial Insemination (AI) centers in Poland (Olecko, Pętkowice and Sławęcinek), except for two PLW boars, which were held at the Cryopreservation Laboratory of the Faculty of Animal Bioengineering, University of Warmia and Mazury in Olsztyn (Poland). A total of three to four ejaculates were collected from each boar, using the gloved-hand technique. To facilitate transport to the Cryopreservation Laboratory, semen (120 ml) collected from the boars at the AI centers, was dialyzed, against Kortowo 3 (K 3) extender (69.3 mM fructose, 64.6 mM sodium citrate, 8.0 mM Na_2-EDTA, 14.2 mM potassium acetate, pH 6.8 (Fraser et al. 2014b). Only semen samples that had more than 70% TMOT and less than 15% abnormal spermatozoa were used in this study. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

Semen processing and cryopreservation

At the laboratory semen samples were processed according to a standard cryopreservation protocol (Fraser et al. 2010a). Briefly, the semen samples were centrifuged (800 x *g*, 10 min at room temperature) and the recovered sperm pellets were re-suspended in an extender (Extender I) containing 11% lactose and 5% lipoprotein fractions of LPFo (Strzeżek et al. 2005). After mixing, the semen samples were cooled at 5°C for a 2-h period, and were further diluted (2:1) with a second extender (Extender II) consisting of 89.5 ml Extender I (lactose-LPFo), 9 ml glycerol and 1.5 ml Orvus Es Paste (Nova Chemical Sales, Inc. (Scituate MA, USA). The cooled semen samples were packaged into sterilized aluminum tubes and loaded onto a programmable computer freezing machine (Ice Cube 1810, SY-LAB, Austria). For post-thaw analysis, frozen semen samples were thawed in a water bath at 50°C for 60 sec. The thawed samples were diluted (1:10) with K 3 extender (50 x 10^6 spermatozoa/ml) and incubated for 15 min at 37°C (0 min post-thaw), prior to semen quality assessments.

Semen quality assessments

Sperm TMOT

For the assessments of sperm TMOT, aliquots of diluted semen samples were placed on a pre-warmed slide, covered with a glass cover slide (20 x 20 mm), and examined under a light microscope at 200x magnification (Olympus BX 40, Tokyo, Japan), equipped with an attached heated stage (38°C). TMOT was assessed randomly in at least five fields at 15 (0 min), 60, 120 and 180 min incubation after thawing.

Sperm MMP

The percentage of spermatozoa with intact MMP was assessed with the dual fluorescent probes, JC-1 and propidium iodide, PI (Molecular Probes (Eugene, OR, USA), as previously described (Fraser et al. 2014b, Dziekońska et al. 2017). Aliquots of the stained sperm samples (JC-1/PI) were examined at 600× magnification under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). Two slides were evaluated per sample and 100 spermatozoa were counted per slide.

Sperm PMI

The percentage of spermatozoa with intact plasma membrane (PMI) was assessed with dual fluorescent probes, SYBR-14 and PI, using the Live/Dead Sperm Viability Kit (Molecular Probes (Eugene, OR, USA), as described by Garner and Johnson (1995). A minimum of 100 cells per slide were examined at 600× magnification under an epifluorescence microscope (Olympus CH 30). Each slide was analyzed in duplicate.

Osmotic resistance test (ORT)

The percentages of spermatozoa with osmotically tolerant acrosome membranes (ORT) were assessed according to a previously described method (Fraser et al. 2014b). A minimum of 100 sperm cells per slide were examined at 1000× magnification under a bright light microscope (Olympus CH 30), and ORT was calculated by the mean of the percentages of spermatozoa with intact acrosomes after incubation in iso-osmotic and hypo-osmotic media. Each slide was analyzed in duplicate.

DNA fragmentation

The percentage of spermatozoa with fragmented DNA was assessed according to a previously described method (Fraser et al. 2010a). Aliquots of agarose-embedded sperm samples on microscopic slides were stained with ethidium bromide and examined at 400× magnification under an epifluorescence microscope (Olympus BX 41, Tokyo, Japan). A minimum of 100 cells per slide was examined in random fields, and sperm cells were classified as spermatozoa with non-fragmented DNA (undamaged) and with fragmented DNA (damaged). Each slide was analyzed in duplicate.

Post- CAI procedure

Post-CAIs were performed under field conditions at two commercial pig farms in Olsztyn (Poland), using a total of thirty-eight multiparous sows (two to six pregnancies). Oestrus detection in the post-weaned sows (twenty five PLW and thirteen PLR sows) was performed twice daily by experienced personnel. The occurrence of oestrus was defined by the standing reflex in front of a boar (back pressure test), and reddening and swelling of the vulva. Sows were individually located to gestation crates and fed daily with a commercial ration. All post-CAIs were performed at 12-15 h after the detection of oestrus in the sows. The animals were inseminated twice per day (AM/PM), with an interval of about 8-10 h within one oestrus, using a combined catheter-cannula kit (Soft & Quick®, Import-Vet S.A., Barcelona, Spain), according to a previously described procedure (Fraser et al. 2007). Briefly, the catheter was inserted through the vagina into the uterine cervix followed by the application of a small volume of citrate-caffeine solution. The cannula device was then inserted through the AI catheter to the uterine body, and the semen dose, comprising 2×10^9 motile FT spermatozoa, suspended in K 3 extender (60 ml, 38°C), was slowly infused into the cannula. Possible returns to oestrus were determined by exposing the sows once daily to a boar from day 21 to 28 after insemination. Pregnancy was diagnosed between 28 to 35 d after insemination by ultrasonography (Ultrasound Scanner Animal Profi, Dramiński S.A., Olsztyn, Poland). All pregnant sows were allowed to carry litters to term, and the pregnancy and farrowing rates (PR and FR, respectively) were recorded. The total number of piglets born (live and stillborn) (TB) and the total number of piglets born alive (TBA) were recorded immediately after birth. The mean number of piglets born (NB) per litter and the number of piglets born alive (NBA) per litter were also recorded.

Statistical analysis

Data were subjected to ANOVA, using the General Linear Model (GLM) procedure from the Statistica software package, version 10 (StatSoft Incorporation, Tulsa OK., USA). The statistical model used to analyze

the parameters of the PF and FT semen quality, and the mean number of the NB and NBA piglets included the random effect of boar nested within breed, and breed (boar × breed). Post-thaw TMOT was analyzed using a factorial design comprising the random effect of boar nested within breed, breed and incubation time period (boar × breed × incubation time). Significant main effects and the interaction means were compared using the Neuman-Keuls *post hoc* test. Values were considered significant at $p<0.05$.

Results

PF and FT semen quality

Table 1 summarizes the quality of the PF boar semen, along with the minimum and maximum values of the analyzed sperm variables. ANOVA results showed that the PF semen quality was not significantly ($p>0.05$) affected either by boar (within breed) or breed.

It was found that boar significantly affected the analyzed sperm parameters after freezing-thawing (Table 2). However, breed was not a significant ($p>0.05$) source of variation in any of the analyzed FT sperm parameters (Table 2). Neither boar (within breed) nor breed was a significant source in the variation in the mean number of NB and NBA piglets (Table 2). Post-thaw sperm TMOT was significantly affected by boar variability ($p<0.001$) and incubation time period ($p<0.001$). No breed effects on post-thaw sperm TMOT were observed in the analyzed samples. Wide variations were observed in post-thaw sperm TMOT at different incubation time periods, being less marked in Boars 6, 7 and 9, particularly at 60 min after thawing (Fig. 1A). A marked reduction in the percentages of motile spermatozoa was observed following prolonged post-thaw incubation, particularly for Boar 2 at 180 min after thawing (Fig. 1A). Within breed, a significant reduction ($p<0.05$) in post-thaw sperm TMOT was observed at 60 min after thawing, and no significant differences ($p>0.05$) were observed between the PLW and PLR boars in either treatment (Fig. 1B).

Post-thaw sperm MMP averaged 48.2 ± 0.9 %, PMI was 49.5 ± 0.8 %, ORT was 61.5 ± 1.2 % and DNA fragmentation was 7.6 ± 0.9 %, regardless of breed. It was found that the percentage of spermatozoa with intact MMP varied markedly among the boars (Fig. 2A). Boars 6 to 9 showed significantly higher ($p<0.05$) MMP than Boars 2 and 5 after freezing-thawing (Fig. 3A). Similarly, significantly higher ($p<0.05$) sperm PMI was observed in the FT semen of Boars 6 and 7 (Fig. 2B), whereas the FT semen of Boars 4 and 6 exhibited markedly higher ($p<0.05$) ORT values when compared with most of the boars (Fig. 2C). Compared with the other boars, cryo-induced DNA damage was significantly lower ($p<0.05$) in the FT spermatozoa of Boar 7, whereas the incidence of spermatozoa with DNA fragmentation was more prevalent in the FT semen of Boar 10 (Fig. 2D).

Fertility results of FT semen

Besides Boars 2 and 5, post-CAIs of sows with FT semen from the nine boars (Boar 1, Boars 3 and 4, and Boars 6 to 11) resulted in a TB of 33 to 45 piglets (Table 3). A similar trend was observed for the TBA piglets (Table 3).

Inseminations of the post-weaned oestrus sows at doses of 2×10^9 motile FT spermatozoa resulted in a PR of 84.2% (Table 4). The FR was 81.6%, whereas the TB and TBA piglets from a total of 31 farrowed animals were 365 and 353, respectively (Table 4). Irrespective of the animal breed, the mean number of NB piglets sired per litter was 11.8 (range 11.0 to 12.7), whereas the mean number of NBA piglets sired per litter was 11.4 (range 11.0 to 12.3) (Table 4).

Discussion

In a preliminary study we demonstrated that the supplementation of LPFo to the freezing extender provided satisfactory fertility results after post-CAI of eight sows (Fraser et al. 2007). In the present study a larger animal population was used to provide more compelling evidence indicating that, besides minimizing the cryo-induced sperm damage, the LPFo could give acceptable fertility results after post-CAIs of FT boar semen. It should be emphasized that studies in our laboratory have confirmed that LPFo, due to its complex biochemical composition, with respect to the protein, cholesterol, phospholipid and fatty acid content (Strzeżek et al. 2005), provides excellent protection for boar spermatozoa during liquid storage (Fraser et al. 2002, 2010b, Dziekońska et al. 2017) and cryopreservation (Fraser et al. 2010a, 2011, 2014a,b, Wasilewska et al. 2016, Wasilewska and Fraser 2017). We have confirmed that the protective action of LPFo on sperm function during semen preservation is mainly due to the interactions of various components of the extender and seminal plasma with the ostrich egg yolk lipoproteins (Dziekońska et al. 2017, Wasilewska et al. 2016, Wasilewska and Fraser 2017). A similar mechanism of action for low-density lipoproteins (LDL), isolated from hen egg yolk, has been demonstrated for bull spermatozoa during liquid semen storage (Manjunath 2002, Bergeron et al. 2004).

Evidence has shown that the prediction of male fertility is very important in the swine AI industry, and that

Table 1. Quality of pre-freeze (PF) boar semen.

Sperm parameters (%)	Mean ± SEM (n = 38)	Range (min-max)
Total motility (0 min, TMOT)	74.1 ± 0.5	72.5 - 79.5
Mitochondria membrane potential (MMP)	87.4 ± 0.8	84.4 - 90.4
Plasma membrane integrity (PMI)	86.4 ± 0.6	83.1 - 89.3
Osmotic resistance test (ORT)	86.2 ± 1.0	81.8 - 93.7
DNA fragmentation	2.6 ± 0.3	1.3 - 3.5

Table 2. ANOVA sources of variations in boar semen quality following freezing-thawing.

Source	MMP	PMI	ORT	DNA fragmentation	NB	NBA
	p-value	p-value	p-value	p-value	p-value	p-value
Boar*	<0.014	<0.035	<0.006	<0.015	>0.354	>0.188
Breed	>0.342	>0. 929	>0.237	>0.343	>0.971	>0.752

A nested ANOVA model was used to analyze the interactions of the main effects: boar nested within breed and breed;
* random effect of boar nested within breed; MMP: mitochondria membrane potential; PMI: plasma membrane integrity; ORT: osmotic resistance test; NB: mean number of number of piglets born/litter; NBA: mean number of number of piglets born alive/litter

Table 3. Individual fertility results of frozen-thawed (FT) semen from the Polish large white (PLW) and Polish landrace (PLR) boars.

Treatment	Boars										
	1	2	3	4	5	6	7	8	9	10	11
Number of sows inseminated*	3	3	4	4	3	4	4	3	3	4	3
Number of pregnant sows	3	1	3	4	3	3	3	3	3	3	3
Farrowed (n)	3	1	3	4	2	3	3	3	3	3	3
Number of piglets born	37	11	36	45	23	36	34	38	33	36	36
Number of piglets born alive	36	11	35	44	23	33	30	37	33	36	35

* indicates the number of FT semen/boar; 1-7 represented PLW boars; 8-11 represented PLR boars

Table 4. Fertility results of frozen-thawed (FT) semen from eleven boars.

Treatment	Total
Sows inseminated (n)	38
Total volume of AI doses/sow (ml)	60 (2×)
Sperm concentration/AI (× 10^9 motile spermatozoa)	2
Pregnant sows (n)	32
Pregnant rate, PR (%)	84.2
Total number of farrowed sows (n)	31
Farrowing rate, FR (%)	81.6
Total number of piglets born (TB)	365
Mean number of piglets born (NB)/litter (± SEM)	11.8 ± 0.3
Total number of piglets born alive (TBA)	353
Mean number of piglets born alive (NBA)/litter (± SEM)	11.4 ± 0.2
Mean number of litters/boar (SEM)	2.8 ± 0.2

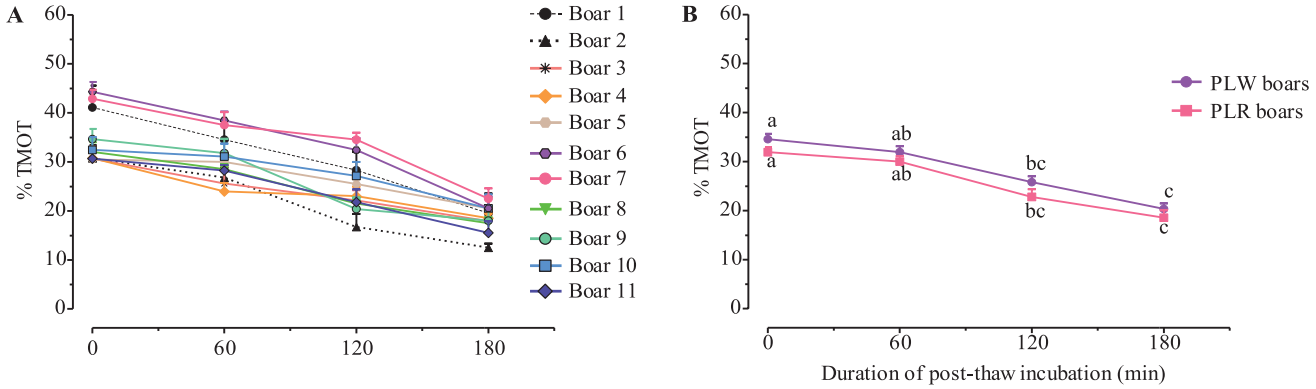

Fig. 1. Post-thaw total motility (TMOT) of spermatozoa from individual boars (A), and boar of different breeds (B) at different incubation time periods. Values are expressed as the mean (± SEM) of three or four ejaculates each from the boars. Values with different letters (a, b, and c) within the same line are significant at $p<0.05$. Boars 1-7: Polish large white (PLW); Boars 8-11: Polish landrace (PLR).

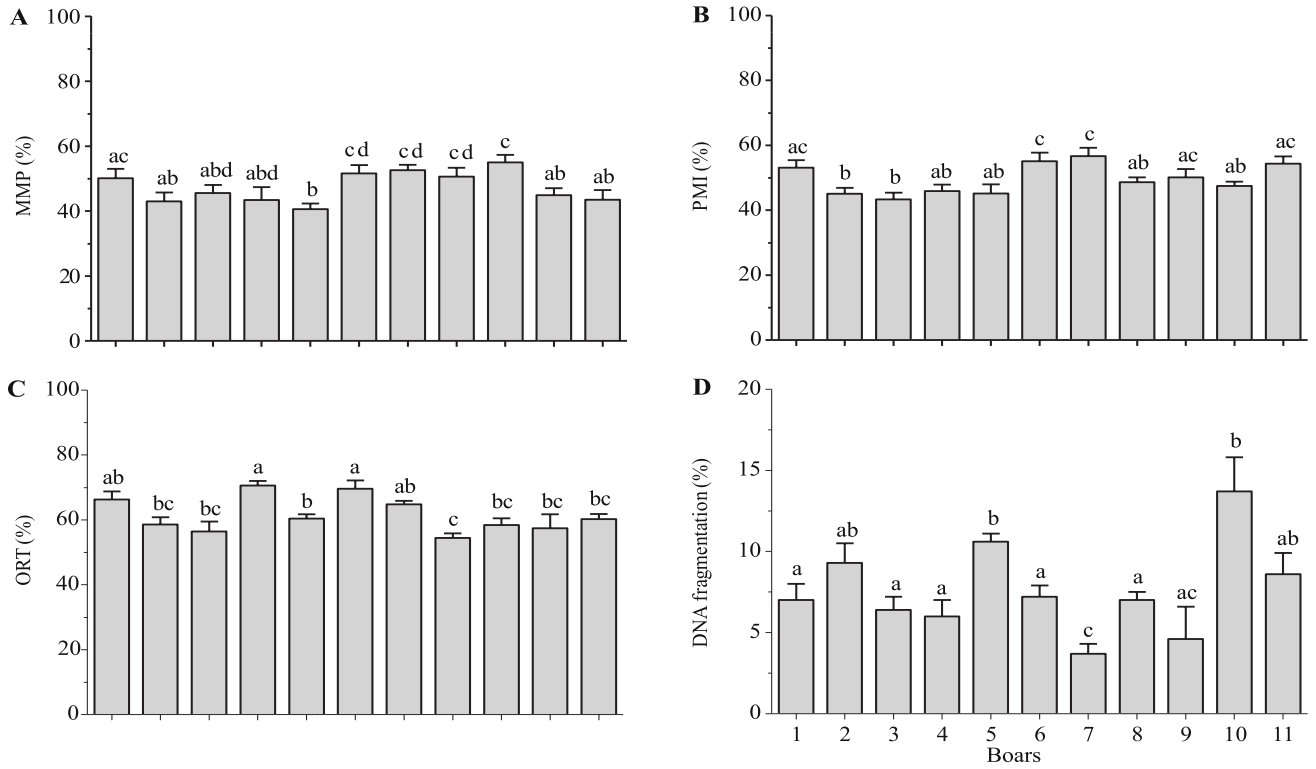

Fig. 2. Quality of frozen-thawed (FT) boar semen. Values are expressed as the mean (± SEM) of three or four ejaculates from 11 boars. Values with different letters (a, b, c, and d) are significant at $p<0.05$. MMP – mitochondria membrane potential; PMI – plasma membrane integrity; ORT– osmotic resistance test

multiple trait comparisons rather than a single trait analysis provide a better fertility prediction for the FT semen (Gadea 2005, Daigneault et al. 2015). Moreover, the assessment of several sperm attributes have been used to monitor the quality of FT boar semen (Casas et al. 2010, Fraser et al. 2011, 2014b, Wasilewska et al. 2016, Yeste 2016, Wasilewska and Fraser 2017), and some of these attributes have been shown to correlate with fertility outcome (Gadea 2005, Spencer et al. 2010, Broekhuijse et al. 2012, Daigneault et al. 2015, Knox 2015). Among these sperm attributes, motility is undoubtedly an important trait that is routinely used, under commercial conditions, to assess the semen quality prior to AI procedure, since it gives a qualitative estimate of fertility (Gadea 2005, Gliozzi et al. 2017). More importantly, sperm motility characteristics have been shown to be a reliable predictor of high- and low-fertility bulls following cryopreservation (Gliozzi et al. 2017).

The viability of FT boar spermatozoa is generally thought to be 6 to 8 h *in vivo* (Waberski et al. 1994, Didion et al. 2013). Even though the detection of oestrus is done with precision, the ovulation time of individual sows varies between 10 and 85 h after the onset of oestrus, and is therefore too imprecise to be predictable

(Bolarín et al. 2006). Moreover, inseminated FT spermatozoa must remain intact within the female reproductive tract for a considerable period of time, and a certain number of viable spermatozoa are needed to form the sperm reservoir to maximize fertility (Rodríguez-Martínez et al. 2005, Bolarín et al. 2006, Almiñana et al. 2010). In our study, the analyzed sperm parameters (motility, mitochondrial function, PMI, ORT and DNA fragmentation) showed wide variations among the boars within breed after freezing-thawing, and there were consistent variations in sperm TMOT among the boars at the different post-thaw incubation time periods. Several authors have confirmed that variations among boars have a significant effect on post-thaw semen quality, and might affect the fertility outcome (Casas et al. 2010, Daigneault et al. 2015, Yeste 2017, Yeste et al. 2017). The results of the current study reaffirm that semen freezability differs among boars within breed, and might have a genetic origin (Thurston et al. 2002), which probably influences the response of the sperm populations to the cryopreservation procedure (Holt et al. 2005, Fraser et al. 2014b, Wasilewska and Fraser 2017). Furthermore, the quality of pre-freeze semen differs among boar breeds at AI stations (Kondracki 2003, Kondracki et al. 2015), but in the current study breed was not a significant factor affecting either the quality of the PF semen or FT semen. Similar findings were reported in a previous study (Waterhouse et al. 2006).

It is worth nothing that litter size is one of the most important economic factors in pig production, and is affected by several factors, such as the quality of AI doses, which could compromise the reproductive performance of FT semen (Didion et al. 2013, Yeste 2016, Yeste et al. 2017). The genetic profiles of FT spermatozoa, in relation to fertility, have been of recent interest, and several studies have sought to characterize the mRNA (messenger ribonucleic acid) expression profiles in bull and boar spermatozoa following cryopreservation (Card et al. 2013, Zeng et al. 2014a,b, Zhang et al. 2015, Card et al. 2017, Zhang et al. 2017). Although the functions of sperm-borne RNAs are not fully understood, it has been reported that spermatozoa retain mRNA that can be translated into proteins facilitating male fertility (Gur and Breitbart 2006, Card et al. 2017) and semen freezability (Card et al. 2013, Yeste 2016). Using Illumina RNA-Sequencing (RNA-Seq), Card et al. (2013) identified several transcript profiles in FT bull spermatozoa, which varied among individuals with differed fertility. In other studies it has been demonstrated that the cryopreservation procedure induces significant changes in the mRNA expression and protein levels of epigenetic-related genes in boar spermatozoa (Zeng et al. 2014a,b). It is likely that differences in the sperm mRNA profiles, induced by the cryopreservation procedure, could also explain the reduction in the reproductive performance of FT semen. Interestingly, Zhang et al. (2015, 2017) have demonstrated that cryo-induced changes in the expression levels in the populations of freeze-related microRNAs (miRNAs), known as CryomiRs, compromise the anti-freeze mechanisms of boar spermatozoa, resulting in reduced fertility. Even though CryomiRs are rapidly emerging as potential modulators of cryo-tolerance of boar spermatozoa, further studies are still needed to elucidate the mechanisms responsible for cryo-injury to spermatozoa, which affects the PR rates and litter size after insemination. However, we suggest that analysis of the transcript profile of spermatozoa from boars differing in freezability might be a step towards overcoming the different problems associated with poor reproductive performance of FT semen following AIs. Moreover, the results of the present study provide evidence that the LPFo-supplemented extender gives satisfactory fertility results in most of the boars after post-CAIs of FT semen. Our findings are in accordance with those of other studies, indicating that the reproductive performance of FT semen varies among boars (Bolarín et al. 2006, Almiñana et al. 2010, Casas et al. 2010). It has been suggested that the sow reproductive tract probably has a mechanism which prolongs the viability of FT spermatozoa until fertilization (Didion et al. 2013). However, it is not known whether this hypothesis was associated with the acceptable fertility results observed in the current study. According to some authors, to precisely evaluate the reproductive performance of FT semen from each boar, it is necessary to inseminate a large number of sows to obtain significant levels (Gadea 2005, Spencer et al. 2010). In the current study it is most likely that the small number of sows that was inseminated with the FT semen from each boar, as indicated in Table 3, does not provide enough information to precisely assess the individual reproductive performance of the FT semen, with respect to the fertility measures.

It should be emphasized that the FR and mean number of NBA piglets are commonly used as estimates of the reproductive performance of either the liquid-stored semen or FT semen (Gadea 2005, Broekhuijse et al. 2012, Didion et al. 2013). We suggest that the fertility results (for examples, FR > 80% and NBA > 10 piglets per litter), obtained with the LPFo-supplemented extender after post-CAIs of FT semen, are comparable to those with HEY-based extender, using either post-CAIs (Roca et al. 2011), or traditional AI (Bolarín et al. 2006, Didion et al. 2013, Knox 2015). Collectively, studies have shown that the reproductive performance of FT boar semen is com-

promised, and several strategies have been developed to improve the freezing protocol and AI procedure (Almiñana et al. 2010, Casas et al. 2010, Spencer et al. 2010, Estrada et al. 2014, Yeste 2016, Wasilewska and Fraser 2017, Yeste et al. 2017). Also, our results show that the post-CAI procedure, which is not commonly used in the insemination of FT boar semen, was very efficient in our study. However, drawbacks of the post-CAI include its time-consuming procedure and its limited application in gilts or primiparous sows, mainly due to the increased risk of injuring the cervix and uterine body during insemination (Hernández-Caravaca et al. 2012).

The findings of this study reaffirm that supplementation of the freezing extender with LPFo provides protection for spermatozoa against cryo-induced injury, which is reflected in the satisfactory fertility results obtained after post-CAIs in multiparous sows. We suggest that such findings in these field trials confirm that LPFo can be used as an efficient cryoprotective additive to boar semen extender on the basis of fertility measures. However, in spite of these promising findings, further studies on commercial pig farms that will allow AIs of FT semen on a large number of sows are required to improve the overall acceptability of ostrich egg yolk lipoproteins as an efficient cryoprotective additive in the freezing extender used for cryopreservation of boar semen. With the increasing relationship of reduced fertility with sperm genetic and epigenetic factors, profiling of gene transcripts of FT spermatozoa from boars with high and low litter sizes would identify potential molecular markers, at transcriptome level, that are associated with semen freezability, and would contribute to genetic improvement in the cryopreservation of boar semen.

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