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Estrategias para mejorar la fertilidad y prolificidad del semen criopreservado en la especie porcina utilizando la inseminación intrauterina profunda

Strategies for enhancing the fertility and prolificacy of cryopreserved boar spermatozoa by using deep intrauterine insemination

Alfonso Bolarín Guillén

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Tesis Doctoral como compendio de publicaciones

1 Validation of trans-rectal ultrasonography for counting preovulatory follicles in weaned sows. Bolarín A, Vázquez JM, Parrilla I, Vázquez JL, Martínez EA, Roca J. Anim Reprod Sci 2009;113:137-142.

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Jordi Roca Aleu y Emilio A. Martínez García, Profesores del Departamento de Medicina y Cirugía Animal de la Universidad de Murcia,

AUTORIZAN

La presentación de la Tesis Doctoral titulada "**Estrategias para mejorar la fertilidad y prolificidad del semen criopreservado en la especie porcina utilizando la inseminación intrauterina profunda**" realizada por D. Alfonso Bolarín Guillén, bajo nuestra inmediata dirección y supervisión, en el Departamento de Medicina y Cirugía Animal. Dicha Tesis reúne las condiciones legales para optar a la obtención del grado de Doctor en Veterinaria por la Universidad de Murcia. La Tesis consta de tres artículos publicados en revistas internacionales, todos ellos con fecha posterior a la aprobación del proyecto de Tesis Doctoral, y presentan objetivos comunes y complementarios en el campo de la inseminación artificial con espermatozoides criopreservados en la especie porcina, configurando una unidad científica que justifica la presentación de la Tesis en el formato de compendio de publicaciones.

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Que una vez evaluado, de conformidad con el procedimiento establecido en el artículo 21 del Reglamento de doctorado de la Universidad de Murcia, el expediente completo de la tesis doctoral titulada "**Estrategias para mejorar la fertilidad y prolificidad del semen criopreservado en la especie porcina utilizando la inseminación intrauterina profunda**", realizada por D. Alfonso Bolarín Guillén, bajo la inmediata dirección y supervisión de D. Jordi Roca Aleu, este Consejo de Departamento, en sesión celebrada en fecha 30 de octubre de 2012, ha dado su autorización para su presentación ante la Comisión General de Doctorado.

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Contenidos

RESUMEN

Resumen

La utilización eficiente de los espermatozoides criopreservados en los programas de inseminación artificial (IA) sigue siendo una asignatura pendiente para la industria porcina. Entre los factores limitantes para dicha aplicación eficiente destacan el elevado número de espermatozoides congelados-descongelados necesarios por dosis de inseminación y la elevada variabilidad observada entre lotes de cerdas y/o entre granjas en los resultados de fertilidad y prolificidad. Con el objetivo final de mejorar la eficiencia reproductiva de los espermatozoides criopreservados en los programas comerciales de IA de porcino, esta Tesis Doctoral propone una serie de experimentos orientados a (1) reducir el número de espermatozoides criopreservados por dosis de inseminación, sin perjudicar fertilidad ni prolificidad de las cerdas inseminadas; y a (2) profundizar en el conocimiento de factores que pueden influir de manera directa y relevante en la variabilidad tradicionalmente observada en los resultados de fertilidad y prolificidad de las cerdas inseminadas con espermatozoides congelados-descongelados. Como estudio preliminar, se evaluó la eficacia de la ecografía trans-rectal como herramienta de diagnostico para evaluar la funcionalidad ovárica, la cual resultaría determinante para la realización de los posteriores experimentos. Los resultados del estudio ecográfico seriado de los ovarios de 63 cerdas, cuyas imágenes fueron contrastadas con aquellas obtenidas tras laparoscopia, demostraron que la ecografía trans-rectal es un procedimiento inocuo, útil y preciso para contar los folículos pre-ovulatorios presentes en el ovario de las cerdas, además de ser también eficaz para detectar el momento de ovulación. En un primer grupo de experimentos, realizados en dos granjas comerciales donde se inseminaron 407 cerdas con espermatozoides congelados-descongelados empleando el procedimiento DUI, y destinado a evaluar la influencia del número de espermatozoides congelados-descongelados por dosis de IA, se observó que los resultados de fertilidad y prolificidad de los espermatozoides criopreservados de porcino depende más del intervalo entre la inseminación y el momento de ovulación (visualizado mediante ecografía trans-rectal) que del número de espermatozoides empleado por dosis de IA. Así, observamos que podemos alcanzar excelentes resultados de fertilidad y prolificidad, comparables a los obtenidos en cerdas inseminadas con semen fresco y/o refrigerado, inseminando con dosis de IA de tan solo 1.000 millones de espermatozoides criopreservados, siempre y cuando la inseminación se realice dentro de las 4-8 h previas a la ovulación. A su vez, estos experimentos pusieron de relieve que la variabilidad entre granjas en los resultados de fertilidad y prolificidad estaría relacionada con diferencias entre granjas en la eficacia de la detección del estro y, consecuentemente, con diferencias en las pautas de inseminación las cuales repercutirían en diferencias entre granjas en los intervalos entre las inseminaciones y el momento de la ovulación. Finalmente, en otro grupo de experimentos de inseminación, también realizados en una granja comercial, y con el objetivo de profundizar en los factores de variación en la respuesta reproductiva de las cerdas inseminadas con espermatozoides congelados-descongelados, se evaluó si la época

del año en la que se realizan las IA podría ser también una fuente de variabilidad en los resultados de fertilidad y prolificidad de las cerdas inseminadas con espermatozoides criopreservados. Para ello se realizaron tres experimentos. En el primero y tras la inseminación de 100 cerdas durante el verano-otoño (VO) y de 116 durante el invierno-primavera (IP) mediante DUI con espermatozoides congelados-descongelados, se comprobó que la fertilidad y prolificidad en VO era significativamente menor que durante IP; y aunque dicho patrón era similar al observado en cerdas inseminadas con semen fresco o refrigerado, la magnitud de la diferencia entre VO e IP era mucho mayor en las cerdas inseminadas con espermatozoides criopreservados. En el segundo experimento, en el que se realizaron ecografías seriadas cada 6 h a los ovarios de 31 y 30 cerdas durante en VO y IP, respectivamente; se demostró que el intervalo entre el inicio del estro y el momento de la ovulación en las cerdas difería sustancialmente entre los dos periodos del año. Mientras que durante IP las cerdas mostraban un menor intervalo y más homogéneo entre ellas, en las de VO dicho intervalo era más largo y mucho más heterogéneo entre ellas. Dichas diferencias claramente explicarían las significativas diferencias en los resultados de fertilidad y prolificidad entre los dos periodos del año en las cerdas inseminadas con espermatozoides congelados-descongelados. Por último, en un tercer experimento, en el que se inseminaron con espermatozoides congelados-descongelados durante el periodo VO un total de 55 cerdas tratadas con eCG (24 h después del destete) y hCG (72 h después de la eCG) para sincronizar el momento de la ovulación, se comprobó que dicho tratamiento hormonal no era eficiente para minimizar la menor fertilidad y prolificidad que muestran las cerdas inseminadas con espermatozoides criopreservados durante el periodo VO.

PALABRAS CLAVE: Porcino; cerda; espermatozoides criopreservados; inseminación intrauterina profunda; ecografía trans-rectal; ovarios; folículos pre-ovulatorios; ovulación; estacionalidad.

SUMMARY

Summary

Efficient use of cryopreserved spermatozoa in AI strategies is still a pending task in swine industry. Among the limiting factors, it is especially relevant the high numbers of frozen-thawed spermatozoa necessary per AI dose, and the high variability observed among different lots of sows and/or farms regarding fertility and total litter born (TLB). With the aim of enhancing the reproductive efficiency of cryopreserved spermatozoa in commercial swine AI strategies, this research proposes a series of experiments aimed at (1) the reduction of the number of cryopreserved spermatozoa per insemination dose, with no reduction of fertility and TLB; and (2) the study of those factors that can influence directly and relevantly the variability traditionally observed in fertility and TLB when using cryopreserved spermatozoa. As a preliminary study, transrectal ultrasonography was evaluated as a diagnostic tool for ovary function; this was determinant for the design of the following studies. Results of serial ovary scanning in 63 sow where compared to direct visualization of the pre-ovulatory ovary by laparoscopy; it was demonstrated that trans-rectal ultrasonography is an accurate, useful and safe procedure for pre-ovulatory follicle counting in weaned sows, and it is also effective for the detection of the ovulation time. In the first group of experiments, performed in two commercial farms, a total of 407 weaned sows were inseminated with frozen-thawed spermatozoa using the deep uterine insemination (DUI). It was intended to evaluate the influence of the numbers of frozen-thawed sperm cells per AI dose. It was observed that fertility and TLB results when using frozen-thawed spermatozoa depends on the interval between insemination and ovulation (assessed by trans-rectal ultrasonography), rather than on the numbers of spermatozoa used in each AI dose. Thus, we observed that we can achieve excellent fertility and TLB results, similar to those obtained by fresh/chilled semen, when using AI doses of only 1 x 10^9 cryopreserved spermatozoa, when AI is performed within 4-8 h before ovulation. At the same time, these experiments highlighted the fact that the variability among farms in fertility and TLB would be related to differences in estrus detection and, consequently, differences in AI moments related to ovulation. Finally, in another series of experiments, also performed in a commercial sow farm, and with the aim of deeper study those factors that may cause these variations in the reproductive behavior of weaned sows inseminated with frozen-thawed spermatozoa, it was evaluated whether the season of the AI could influence or not. Three experiments were designed. The first one, after 100 sows inseminated during summer-autumn (SA) and 116 sows inseminated in winter-spring (WS) using DUI and frozen-thawed spermatozoa, it was assessed that fertility and TLB in SA was significantly lower than in WS. The same trend was observed in sows inseminated with fresh-chilled semen; nevertheless, the difference between SA and WS was much higher in sows inseminated with cryopreserved spermatozoa. In the second experiment, transrectal ultrasonography was performed every 6 h to ovaries of 31 and 30 sows during SA and WS respectively. It was demonstrated that the interval between the onset of oestrus and the ovulation time was substantially different between both periods. Whereas during WS the sows

exhibited shorter and homogeneous intervals, during SA these intervals were longer and much more heterogeneous. Those differences clearly explain the significant differences obtained in fertility and TLB for both periods of the year in sows inseminated with frozen-thawed spermatozoa. Finally, in a third experiment, 55 sows were inseminated with frozen-thawed spermatozoa during SA, and injected with eCG (24 h post-weaning) and hCG (72 h after the injection of eCG), to synchronize the ovulation time. It was assessed that this hormonal treatment was not efficient to minimize the lower fertility and TLB observed in sows AI with cryopreserved spermatozoa during SA.

KEY WORDS: Swine; sow; cryopreserved spermatozoa; deep uterine insemination; trans-rectal ultrasonography; ovaries; pre-ovulatory follicles; ovulation; seasonality.

INTRODUCCIÓN

Introducción

Importancia de la inseminación artificial en la especie porcina

La inseminación artificial (IA) es la biotecnología reproductiva más ampliamente utilizada en la especie porcina. Desde mitad de la década de los 70 del siglo XX, la IA ha ido incrementando su presencia en la producción porcina hasta superar ampliamente el 90% de implantación en Europa y América del Norte en la actualidad [Weitze, 2000]. La mayor parte de las inseminaciones en el mundo se realiza con semen fresco-refrigerado, en dosis con caducidad de entre 1 y 3 días, y con cantidades totales de espermatozoides por dosis de inseminación variables entre 1'5 y 3'5 x 10^9 espermatozoides [Wagner y Thibier, 2002; Feitsma, 2009; Reicks y Lewis, 2008]. Los resultados de fertilidad y prolificidad obtenidos mediante IA con semen fresco-refrigerado son, por lo general, mejores que aquellos alcanzados mediante monta natural, siendo normales tasas de fertilidad superiores al 80-85 % y con 12 o más lechones nacidos por parto. Por otra parte, la optimización del verraco mediante IA es un hecho, y permite inseminar a un gran número de cerdas con las dosis seminales procedentes de aquellos verracos genéticamente superiores, justificando así las grandes inversiones y avances que las empresas dedicadas a la mejora genética porcina vienen realizando durante las últimas décadas.

La gran implantación de la IA en la especie porcina y el enorme avance ocurrido a lo largo de los últimos años en otras tecnología reproductivas y en la mejora genética, junto a la situación de alta competitividad empresarial existente en el sector porcino, incrementan la necesidad de implementación de nuevas tecnologías tales como la criopreservación espermática o la inseminación intrauterina profunda (DUI), y nos estimulan a profundizar en nuestro conocimiento sobre la fisiología reproductiva porcina, en la búsqueda de procesos reproductivos que ofrezcan mayor eficiencia y rentabilidad a la industria, mediante la optimización de los resultados productivos, incluyendo los de fertilidad y prolificidad.

Relevancia del semen criopreservado en los programas de inseminación artificial en la especie porcina y factores de variación en los resultados de fertilidad y prolificidad

Solo un pequeño porcentaje de cerdas, entre el 1-2 % del total de inseminadas, lo son con semen criopreservado [Weitze, 2000] y son normalmente cerdas que se encuentran en programas de mejora genética. Entre otras razones, el escaso empleo del semen criopreservado queda justificado por el elevado número de espermatozoides empleado por dosis de inseminación, entre 5-6 x 10^9, que minimiza la difusión genética de los mejores verracos, y los resultados de fertilidad y prolificidad alcanzados, sensiblemente menores que con el semen fresco o refrigerado, situándose en tasas de partos aproximadas al 70-75 % y obteniéndose de media un

lechón menos por parto [revisado por Roca y cols., 2006]. Junto a estos peores resultados productivos, la fertilidad y prolificidad de las IAs con semen criopreservado evidencian una mayor variabilidad entre lotes de cerdas y entre granjas respecto al semen fresco o refrigerado, lo que complica todavía más la aceptación del semen criopreservado en los programas comerciales de inseminación en la especie porcina. Buscar explicaciones a dicha variabilidad será objetivo prioritario de la presente Tesis Doctoral.

No obstante a lo expuesto anteriormente, el semen criopreservado tiene evidentes ventajas sobre el fresco y refrigerado, todas ellas relacionadas con el hecho de poder almacenar congeladas las dosis de inseminación por tiempo indefinido sin que ello deteriore la capacidad fecundante de los espermatozoides así conservados. Esta cualidad permite separar en el tiempo y en el espacio los centros de recogida de los eyaculados de las granjas donde se realizan las inseminaciones. Tal circunstancia a su vez permite maximizar el uso de reproductores de alto valor genético, tanto en granjas de producción como en los programas de mejora genética. También facilita el intercambio comercial de semen, no sólo a nivel nacional sino a nivel internacional, sustituyendo incluso al propio intercambio comercial de animales vivos, con el consiguiente incremento en bioseguridad y disminución de costes en las exportaciones e importaciones. Así mismo, favorece crear bancos de semen congelado que permitan almacenar indefinidamente líneas genéticas, incluso aquellas obsoletas o en peligro de extinción. Además, posibilita abastecer de forma ordenada y planificada las demandas de las explotaciones, y también eliminar el factor estacional en la fertilidad y prolificidad atribuible al verraco a través del eyaculado, con lo que cualquier factor estacional en la respuesta reproductiva quedaría exclusivo de la cerda. Por otra parte, el semen criopreservado está destinado a desempeñar un papel fundamental en el desarrollo de las biotecnologías emergentes tales como el sexaje espermático o la transgénesis [Gerrits y cols., 2005].

La alta variabilidad observada entre lotes de inseminación y entre granjas en la fertilidad y prolificidad obtenida tras inseminaciones con semen criopreservado es atribuible a diversos factores. Entre los más relevantes estarían la enorme variabilidad entre verracos e incluso entre eyaculados de un mismo verraco en la congelabilidad espermática [revisado por Holt y cols., 2005], lo cual podría directamente influir en su capacidad fecundante tras la inseminación. También la vida funcional de los espermatozoides congelados-descongelados, considerada como mucho más limitada que la de los espermatozoides frescos o refrigerados [Watson, 2000]. En conexión directa con estos factores, estaría el número total de espermatozoides empleados por dosis de IA y el número de IAs por cerda y estro [revisado Roca y cols., 2006], además del estado funcional del ovario en el momento de la IA [Waberski y cols., 1994]. Todo ello sin olvidar la propia cerda como individuo, su alimentación, salud y bienestar, y sin descartar los factores medioambientales, sobretodo los cambios en el fotoperiodo y la temperatura ambiental que definen las diferentes estaciones del año [revisado por Flowers, 2001]. Evaluar la influencia de algunos de estos factores sobre los resultados de fertilidad y prolificidad del semen criopreservado será el principal objetivo de esta Tesis Doctoral.

La ecografía trans-rectal como herramienta para evaluar la funcionalidad de los ovarios en las cerdas

El estado funcional del ovario en el momento de la IA es fundamental para la consecución de óptimos resultados tanto de fertilidad como de prolificidad en las explotaciones porcinas, especialmente cuando empleamos espermatozoides criopreservados [Waberski y cols., 1994; Roca y cols., 2006]. El desarrollo de nuevas tecnologías y herramientas aplicables a la reproducción porcina nos permite la optimización de la IA con espermatozoides congelados-descongelados, alcanzando en ocasiones valores muy próximos a aquellos obtenidos mediante IA tradicional con semen fresco-refrigerado [Roca y cols., 2008]. Entre estas nuevas tecnologías y herramientas, destacaría la ecografía, tanto trans-rectal como trans-abdominal [Kauffold y Althouse, 2007]. Con ella podemos obtener una nítida visualización del aparato reproductivo de la cerda, incluyendo textura y tamaño, y consecuentemente nos permite realizar un efectivo examen funcional tanto de los ovarios como del útero [Kauffold y cols., 2004]. En el ovario, la ecografía permite monitorizar la actividad funcional, incluyendo la dinámica folicular [Lucy y cols., 1999; Lucy y cols., 2001] y la ovulación [Soede y cols., 1994; Knox y Rodriguez Zas, 2001]. También permite detectar desórdenes reproductivos como quistes ováricos, ovarios inactivos u ovarios poliquísticos [Martínez y cols., 1992; Waberski y cols., 1999]. En cuanto al útero, la ecografía es de gran utilidad para la detección precoz de la gestación [Inaba y cols., 1983; Maes y cols., 2006], además de para visualizar alteraciones funcionales [Kauffold y cols., 2005].

La ecografía trans-rectal ha demostrado, además, ser una herramienta efectiva para identificar los folículos maduros presentes en el ovario [Soede y cols., 1998]. Un correcto contaje de los folículos pre-ovulatorios presentes en los ovarios sería importante para predecir el número previsible de ovulaciones y, consecuentemente, para predeterminar el potencial tamaño de camada. Con ello, se podría indirectamente también evaluar si camadas de bajo número pudieran ser ajenas a problemas de ovulación y por lo tanto estar relacionadas con otras cuestiones como por ejemplo una insuficiente capacidad fecundante de los espermatozoides empleados en las dosis de inseminación. Al respecto, es conocida la mayor variabilidad en el tamaño de las camadas cuando las cerdas son inseminadas con espermatozoides congelados-descongelados respecto a cuando lo son con espermatozoides frescos-refrigerados [Roca y cols., 2006], variabilidad atribuida precisamente a una menor capacidad fecundante de los espermatozoides criopreservados. Sin embargo, no existen todavía estudios que hayan demostrado la eficiencia de la ecografía para identificar con cierta exactitud el número de folículos pre-ovulatorios presentes en el ovario de las cerdas. Entre los objetivos de esta Tesis Doctoral se contempla evaluar la eficiencia de la ecografía trans-rectal para identificar el número de folículos pre-ovulatorios presentes en los ovarios de cerdas, además de verificar su utilidad para el diagnóstico de ovulación. Conocer ambos aspectos funcionales de los ovarios de las cerdas podría ayudar a explicar situaciones de variabilidad en los resultados de fertilidad y principalmente de prolificidad observados en cerdas inseminadas con espermatozoides criopreservados.

La inseminación intrauterina profunda como procedimiento para mejorar la rentabilidad productiva de los espermatozoides criopreservados en los programas de inseminación artificial de porcino

La criopreservación espermática es una tecnología todavía en fase de implantación en la industria porcina, y los protocolos de congelación y/o descongelación siguen bajo investigación, estando por lo tanto en continuo desarrollo [Carvajal y cols., 2004; Devireddy y cols., 2004; Grossfeld y cols., 2008; Rath y cols., 2009] bien sea para eliminar la variabilidad observada entre eyaculados y, sobretodo, entre verracos en la calidad espermática a la descongelación, como para aumentar la supervivencia en el tiempo de los espermatozoides tras la descongelación y así prolongar su vida funcional en el tracto genital de la cerda [Roca y cols., 2006; Hernández y cols., 2007a,b]. Sin embargo, todas estas fructíferas investigaciones deben ir acompañadas de una reducción efectiva del número necesario de espermatozoides por dosis de inseminación y por añadidura por cerda y ciclo, y todo ello mejorando los resultados tanto de fertilidad como de prolificidad actuales del semen criopreservado, para resultar en su implantación rutinaria en la industria porcina.

El número total de espermatozoides empleados para inseminar a una cerda es un factor limitante para la rentabilidad de los verracos genéticamente superiores. Un excesivo número de espermatozoides por dosis reduce la difusión genética del verraco. Para garantizar buenos resultados reproductivos, la IA tradicional utiliza una cantidad demasiado alta de espermatozoides por dosis de inseminación, y los programas convencionales de inseminación programan varias inseminaciones por cerda y ciclo, habitualmente entre 2 y 3. Además, al inseminar con espermatozoides criopreservados, el número de espermatozoides utilizados por cerda y ciclo se duplica o incluso triplica al emplear dosis de inseminación con el doble de espermatozoides respecto al semen fresco o refrigerado, y al realizar incluso 4 inseminaciones por cerda y ciclo. Evidentemente, estas propuestas de inseminación no son las más adecuadas para rentabilizar productivamente a los verracos genéticamente superiores. Entonces y desde una doble perspectiva tanto de rentabilidad como de productividad, es evidente la necesidad de buscar alternativas de inseminación que permitan minimizar el número de espermatozoides usados por dosis de IA y por cerda y ciclo, sin comprometer ni la fertilidad ni la prolificidad [Krüger y Rath, 2000; Flowers, 2001]. La DUI es una técnica de inseminación incipiente que ha demostrado ser útil para reducir el número de espermatozoides por dosis de IA, tanto cuando empleamos semen fresco-refrigerado como espermatozoides criopreservados, sin que dicha reducción en el número de espermatozoides comprometa la fertilidad y la prolificidad [Martínez y cols., 2002], por lo cual la DUI permite mejorar la rentabilidad de los verracos, especialmente de aquellos cuyos eyaculados están destinados a la criopreservación. Respecto a los espermatozoides criopreservados, si bien se han conseguido buenos resultados de fertilidad inseminando con tan solo 1.000 $x10^6$ espermatozoides por dosis de IA [Roca y cols., 2003], el número mínimo requerido para alcanzar las máximas tasas de fertilidad y la más alta prolificidad está todavía por definir. Será también objetivo de esta Tesis Doctoral evaluar la influencia del número de espermatozoides criopreservados por dosis de IA sobre los resultados de fertilidad y prolificidad en cerdas inseminadas mediante el procedimiento DUI.

Importancia del intervalo entre la inseminación y la ovulación en los resultados de fertilidad y prolificidad de los espermatozoides criopreservados de porcino

Actualmente la vida funcional de los espermatozoides congelados-descongelados de porcino se considera que es extremadamente corta si la comparamos con la que muestran los espermatozoides frescos o refrigerados. Mientras que los espermatozoides frescos y refrigerados parecen ser capaces de mantener su capacidad fecundante durante 18-20 h una vez depositados en el tracto genital de la cerda [Soede y cols., 1995ª], los espermatozoides descongelados parece que a penas la mantienen más allá de 4-6 h [Waberski y cols., 1994; Wongtawan y cols., 2006]. Por lo tanto, actualmente el éxito reproductivo de la IA con espermatozoides criopreservados pasa inexorablemente por su deposición en el tracto genital de la cerda dentro de las 4-6 h previas a la ovulación. Entonces, es obvio que predecir o aproximar el momento de la ovulación es primordial para mejorar la fertilidad de los espermatozoides criopreservados, a la vez que también podría ayudar para reducir el número de ellos necesario por cerda inseminada.

La ovulación es un proceso íntimamente relacionado con la duración del estro, siendo éste bastante estable dentro de una misma granja cuando se mantienen unas mismas condiciones de manejo, pero es altamente variable entre granjas [Steverink y cols., 1999]. La ovulación sucede transcurrida cerca del 70% de la duración del estro. En la mayoría de cerdas con un intervalo destete-estro de 4 d, suele suceder entre las 32 y 48 h tras el inicio del estro [revisado por Soede y Kemp, 1997]. Sin embargo, la duración del estro es un factor imposible de predecir. Ante la ausencia de un procedimiento mejor, las mejores herramientas para predecir el momento óptimo de ovulación son una correcta detección del estro y una monitorización de la duración del mismo [Beltra y cols., 2004]. Los intervalos destete-inicio del estro, inicio del estro-ovulación y su relación con la expresión del estro son factores determinantes en la decisión del momento de realizar las IAs y, obviamente, condicionan los resultados de fertilidad y prolificidad [Mburu y cols., 1995; Nissen y cols., 1997]. Desgraciadamente, a pesar de la importancia de la necesidad de controlar correctamente el inicio del estro y su duración, éstos parámetros reproductivos no suelen estar totalmente bajo control por los operarios de las granjas, al no realizarse las detecciones correctamente, normalmente debido a falta de personal o de la adecuada especialización del mismo. En cualquier caso, el momento de la ovulación es siempre difícil predicción. Como norma general, las cerdas con menor intervalo destete-inicio del estro presentan mayor duración del estro y por lo tanto un mayor intervalo inicio del estro-ovulación, y viceversa para aquellas con un largo intervalo destete-inicio del estro [Weitze y cols., 1994]. Conocer correctamente estos patrones fisiológicos es fundamental para el establecimiento de protocolos eficientes de IA en cualquier granja, y pueden ser determinantes en los resultados de fertilidad y prolificidad obtenidos con espermatozoides criopreservados, dada su corta vida funcional en el aparato reproductor de la cerda. Evaluar la importancia que tiene el intervalo inseminación-ovulación como factor de variación en la fertilidad y prolificidad de los espermatozoides criopreservados de porcino, será uno de los objetivos de la presente Tesis Doctoral.

La infertilidad estacional en la cerda y su magnitud en aquellas inseminadas con espermatozoides criopreservados

El descenso en los parámetros reproductivos, tanto de fertilidad como de prolificidad, característico de ciertos períodos del año en las granjas de porcino se denomina comúnmente infertilidad estacional. Este fenómeno, que sucede en todo el mundo, ha sido profundamente estudiado en los últimos años, sobre todo en zonas productivas donde los factores predisponentes son extremos o de muy marcada variabilidad a lo largo del año. Entre dichos factores, el fotoperiodo y la temperatura ambiental parecen ser los más determinantes [revisado por Peltoniemi y Virolainen, 2006]. El descenso de los valores reproductivos corresponde a la época del año en que el jabalí europeo (*Sus Scrofa*), antecesor del cerdo domestico, presenta a su vez anoestro estacional [Mauget, 1982] y se corresponde con el periodo del año en el que el fotoperiodo es más largo y las temperaturas ambientales son más elevadas (Love y cols., 1993).

La infertilidad estacional afecta tanto a la cerda como al verraco. En la cerda se caracteriza por un síndrome que incluye retrasos en la entrada en pubertad [Paterson y cols., 1991], reducción de la tasa de partos [Love, 1978] y aumento del intervalo destete-inicio del estro [Prunier y cols., 1996], pudiendo coexistir una reducción en el tamaño medio de la camada [Claus y Weiler, 1985; Reilly y Roberts, 1991; Peltoniemi y cols., 1999a]. También se ha evidenciado que el estrés por calor puede retardar la función ovárica, particularmente el desarrollo folicular [Guzeloglu y cols., 2001; Roth y cols., 2001], lo cual podría explicar un incremento en el intervalo inicio del estro-ovulación en períodos calurosos. En el verraco dichos factores medioambientales inducen cambios hormonales que repercuten sobre su capacidad reproductiva reflejándose en el eyaculado el cual se caracteriza por un menor volumen y concentración y por una pérdida de la calidad espermática [Trudeau y cols., 1986]. Los análisis anuales de los resultados productivos de granjas de porcino en las que las cerdas son inseminadas con semen fresco o refrigerado demuestran que en épocas ambientales desfavorables su eficiencia reproductiva se ve reducida significativamente [Martinat-Botte y cols., 1984; Peña y cols., 1998; Gaustad-Aas y cols., 2004; Peltoniemi y cols., 2006; Suriyasomboon y cols., 2006]. Entre las razones de dicha infertilidad estacional estarían las propias de la cerda antes mencionadas y también aquellas relacionadas con la calidad seminal de los eyaculados recogidos durante las épocas ambientales desfavorables. Al contrario de lo que ocurre con el semen fresco-refrigerado, no existen estudios en cerdas inseminadas con espermatozoides criopreservados, por lo que desconocemos si la magnitud de la estacionalidad sería la misma o podría ser menor en la medida que la calidad espermática post-descongelación puede mantenerse constante a lo largo de todo el año debido a que los espermatozoides congelados-descongelados utilizados para inseminar las cerdas en las épocas ambientales desfavorables no necesariamente deben proceder de eyaculados recogidos en las citadas épocas desfavorables. Estudiar la magnitud de la infertilidad estacional en cerdas inseminadas con espermatozoides criopreservados será otro de los objetivos de la presente Tesis Doctoral.

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OBJETIVOS

Objetivos

1 Evaluar la idoneidad de la técnica de ecografía trans-rectal para contar el número de folículos pre-ovulatorios presentes en los ovarios de las cerdas, así como para determinar el momento de ovulación, además de identificar posibles trastornos en dichos procesos fisiológicos.

2 Evaluar la adecuación del procedimiento de inseminación intrauterina profunda para la aplicación eficiente de los espermatozoides criopreservados en los programas comerciales de inseminación artificial en la especie porcina, con especial consideración al número de espermatozoides necesario por dosis de inseminación y a la importancia del intervalo entre la inseminación y el momento de ovulación.

3 Estimar la influencia de la estación del año sobre el intervalo estro-ovulación y la relación de éste con los resultados de fertilidad y prolificidad obtenidos en cerdas inseminadas con espermatozoides criopreservados.

Artículo 1

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Validation of trans-rectal ultrasonography for counting preovulatory follicles in weaned sows

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Follicles

ABSTRACT

The present study investigated the accuracy of trans-rectal ultrasonography (TRU) for assessing the exact number of preovulatory follicles (POFs, with a diameter from 6 to 10 mm) present in the ovaries of weaned sows. The ovaries of 63 hormonally treated (1500 IU of eCG) weaned sows were checked with TRU (7.5-MHz multiple scan angle transducer) in two successive scanning sessions performed at 26–27 and 30–31 h after the beginning of oestrus signs, and the maximum number of POFs were counted. Sows were subjected to laparoscopy (LAP) immediately after the last TRU scan to confirm the number of POFs. The differences (mean ± S.D.) in the number of POFs counted with TRU and LAP on each ovary were analyzed as a whole and after sorting the ovaries into three classes according to the number of POFs visualized by LAP: (1) less than 7; (2) from 7 to 13; and (3) more than 13. A significant correlation ($P < 0.01$) was found between TRU and LAP for both the whole data set (126 ovaries) and in each of the three ovarian classes. Despite the significant correlation, TRU underestimated the number of POFs by 1.40 ± 1.67 compared with LAP ($P < 0.001$). However, the underestimation varied among the ovarian classes. This difference was not significant ($P > 0.05$) in class 1 and was significant ($P < 0.001$) in classes 2 (1.11 ± 1.30 less POFs than counted by TRU) and 3 (3.19 ± 1.54 less POFs than counted by TRU). In conclusion, TRU is a valuable tool to count the number of POFs present in the ovaries of weaned sows, but a certain degree of underestimation should be expected when the number of POFs is large.

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1. Introduction

Real-time (B-mode) ultrasonography is an effective diagnostic tool for reproductive examinations in female pigs (see the recent review by Kauffold and Althouse, 2007). It reliably diagnosis pregnancy (Inaba et al., 1983; Maes et al., 2006), detects reproductive disorders (Martinez et al., 1992; Waberski et al., 1999; Kauffold et al., 2005) and monitors ovarian activity (Kauffold et al., 2004a,b), including follicular dynamics (Lucy et al., 2001) and ovulation (Soede et al., 1994; Knox and Rodriguez Zas, 2001). However, there have been no studies focused on the effectiveness of ultrasonography in determining the exact number of preovulatory follicles (POFs) in sows. The identification of the exact number of POFs in sows by non-invasive procedures, such as ultrasonography, could be interesting for determining both the ovulation rate and the duration of the ovulation process.

Small litter size is a major economic concern for commercial swine producers, and ovulation rate is considered one of the most important influencing factors (Geisert et al., 2007). In previous studies that focused on this topic, laparoscopy (Christenson, 1993) or post-mortem (Geisert et al., 2007) examination of ovarian structures were used to determine the ovulation rate. These methods are invasive, expensive and time-consuming.

In pigs, embryo diversity within a litter has been suggested to contribute to early embryonic mortality and subsequent small litter size at farrow. One of the causes of embryo diversity is a long ovulation duration (Geisert and Schmitt, 2002). Accurate determination of the number of POFs in both ovaries is essential for assessing the beginning and duration of the ovulation process (Soede et al., 1998). In addition, determining the beginning of ovulation allows for the establishment of efficient artificial insemination (AI) strategies (Soede et al., 1995; Roca et al., 2006). An inadequate synchronization of semen deposition and ovulation time is one of the most frequent causes of fecundation failure in commercial pig AI programs, mainly when frozen-thawed semen (Waberski et al., 1994; Bolarin et al., 2006) or sex-sorted spermatozoa (Vazquez et al., 2006) are used.

Trans-rectal ultrasonography (TRU) has been proven to be a feasible and reliable method for determining the number of POFs in conscious and unstressed sows (Soede et al., 1998; Ryan et al., 1994; Nissen et al., 1995). Therefore, the aim of the present study was to investigate the accuracy of TRU for counting the exact number of POFs in weaned sows. To achieve this, the data obtained by TRU were validated by laparoscopy (LAP).

2. Materials and methods

2.1. Animals

The experimental study was carried out on a commercial pig breeding farm (Grupo Agropor SL) located in the Region of Murcia, Spain. Multiparous (2–7 parity) crossbred (Landrace × Large White) sows were randomly selected at weaning (18–25 days after farrowing) and placed into individual crates in large rooms with windows exposed to natural daylength. Sows were fed a commercial diet twice daily and had *ad libitum* access to water. Detection of oestrus was performed twice a day (07:00 a.m. and 07:00 p.m.) beginning 3 days after weaning, using fence line contact with a mature boar. Only sows exhibiting a standing heat reflex in the presence of a boar were considered to be in oestrus. The University of Murcia Care and Use Committee approved the experimental procedures.

2.2. Trans-rectal ultrasonography for ovarian examination

All TRU scanning sessions were performed by the same person (A. Bolarin) under farm conditions using a Pie Medical SC100 scanner (Pie Medical, Maastricht, The Netherlands) equipped with cine-loop and fitted with a trans-rectal 7.5-MHz multi-angle transducer. Ovaries were scanned according to the procedure described by Soede et al. (1992) and modified by Bolarin et al. (2006). After removal of feces, the transducer was manually inserted into the rectum and held face down to maintain constant contact with the rectal mucosa. The hand was introduced up to a depth of 35–45 cm beyond the anal sphincter, where the ovaries could be detected. All ovaries were scanned in different cross-sections, and the cross-sections were fixed as images for subsequent follicle evaluation. Spherical anechogenic

structures with thin borders and occasional irregular outlines were counted as follicles. The sizes of the follicles were measured using the calibrated measurement functions of the ultrasound machine software.

2.3. Laparoscopy technique

To laparoscopically examine the ovaries, a laparoscopy technique (LAP) that was previously described (Brüssow and Ratky, 1994) and recently modified (Garcia et al., 2007) was used. The sows were sedated (Azaperone, Stresnil [Dr. Esteve, Barcelona, Spain]), then anesthetized (sodium thiopenthal [Abbot, Madrid, Spain]) and maintained in general anesthesia with halothane [Fluothane®, AstraZeneca, Madrid, Spain]. The anesthetized animals were placed in the Trendelenburg position. The laparoscope was inserted at the umbilical level, through a trocar-canula, after insufflation of the abdominal cavity with CO_2. Subsequently, two accessory trocars were inserted laterality between the second or third pair of nipples to manipulate abdominal structures and visualize the ovaries. For all sows, images of both ovaries were recorded on a videotape, and the number of POF was counted. The POFs were identified by size, using the size of the grasping forceps for comparison, and particular appearance, which was a light pink color due to extensive vascularization of the wall.

2.4. Experimental design

Seventy weaned sows were treated with 1250 IU eCG (Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning to stimulate follicular development (Roca et al., 2003). Only sows that exhibited first standing reflex on the morning of the fourth day after weaning were used in this experiment. Over a 14-week period, five sows were examined per week. The ovaries were scanned by TRU at 22–23, 26–27 and 30–31 h after the beginning of oestrus. The first scan was performed to check the feasibility of performing the trans-rectal ultrasound and assess the ovarian health. The number of POFs per ovary was defined as the maximum number of follicles with a diameter from 6 to 10 mm counted per ovary in the two last TRU scanning sessions. LAP was performed immediately after the last scanning session.

2.5. Statistical analysis

In seven animals, it was not possible to visualize both ovaries in one or more of the scanning sessions. Accordingly, data obtained from 126 ovaries of 63 animals were included in the statistical analysis. Statistical analyses were performed using SPSS version 14 (SPSS Inc., Chicago, IL, USA). Because the data were not normally distributed, the nonparametric Wilcoxon signed-rank *t*-test was used to test for differences in the POF number counted by TRU and visualized by LAP on the same ovary. The differences were analyzed both as a whole and according to the number of POFs per ovary. For this last assessment, ovaries were sorted into three different specific classes, according to the number of POFs visualized by LAP on each ovary: class 1, less than 7 POFs; class 2, from 7 to 13 POFs; and class 3, more than 13 POFs. Finally, a Spearman nonparametric correlation test was used to calculate the relationships between the number of POF counted on the same ovary in the two successive TRU scans and between TRU and LAP. Results are expressed as mean ± S.D. A $P < 0.05$ was considered statistically significant.

3. Results

The number of POFs showed a great variation among ovaries, ranging from 1 to 27. The distribution of the 126 ovaries into the three specific classes according to the number of POFs visualized by LAP on each ovary, is shown in Table 1. Most ovaries had POFs in a range of 7–13 POFs (55.6%), followed by ovaries with more than 13 POFs (24.6%).

The preovulatory follicle counts per ovary obtained with TRU at both 26–27 and 30–31 h after the beginning of oestrus correlated at $r = 0.98$ ($P < 0.001$). The numbers of POFs as determined by TRU and LAP also correlated significantly when analyzed in total ($r = 0.98$, $P < 0.001$) and separately for each

Table 1
The mean ± S.D. and range ( ) of the difference in the maximum number of preovulatory follicles (POFs) visualized by laparoscopy (LAP) and counted by trans-rectal ultrasonography (TRU) on the same ovaries

Ovarian class[a]	Ovaries no. (%)	LAP	TRU	Differences	*P*-value
1 (<7 POFs)	25 (19.8)	4.24 ± 0.34 (1–6)	4.24 ± 0.36 (1–7)	0.0 ± 0.58	NS
2 (7–13 POFs)	70 (55.6)	10.23 ± 0.24 (7–13)	9.09 ± 0.19 (5–13)	1.14 ± 1.30	0.001
3 (>13 POFs)	31 (24.6)	18.65 ± 0.69 (14–27)	15.45 ± 0.51 (11–22)	3.19 ± 1.54	0.001
Total	126 (100)	11.11 ± 0.49 (1–27)	9.71 ± 0.38 (1–22)	1.40 ± 1.67	0.001

[a] Ovaries sorted according to the number of POFs visualized by laparoscopy.

POF class (correlation coefficient ranging from 0.87 to 0.96, $P < 0.01$). However, the number of POFs per ovary was underestimated using TRU (Table 1). Significant ($P < 0.001$) discrepancies in the POF counts between TRU and LAP were observed for ovary classes 2 (7 to 13 POFs) and 3 (>13 POFs). Moreover, a higher number of POFs per ovary corresponded to more frequent underestimation (Fig. 1).

4. Discussion

Trans-rectal ultrasonography (TRU) is a feasible and reliable procedure to examine the anatomical structures of ovaries of weaned sows and can be completed without stress to most sows. In the present study, it was not possible to introduce the transducer into the rectum of only 2 sows (2/70, 2.9%). Although this percentage is low, it may have been even lower if the transducer had been introduced using a PVC handle instead of a hand as a guide. In relation to the scan successes, both ovaries were successful visualized in 63 of the 68 scanned sows (94.3%). This percentage was slightly higher than the 87.1% achieved by Kauffold et al. (2004a) using transcutaneous ultrasonography, suggesting that TRU could be a complementary procedure to transcutaneous scanning to visualize ovaries in weaned sows.

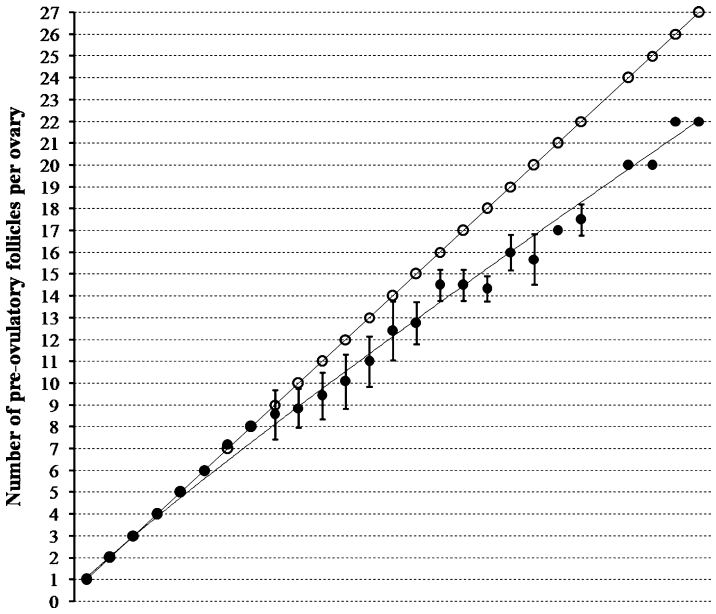

Fig. 1. Differences (mean ± S.D.) in the number of preovulatory follicles (POFs) per ovary counted using trans-rectal ultrasonography (●) in relation to those visualized using laparoscopy (○) on the same ovary ($n = 126$) of weaned sows. The lines represent the statistical trend.

To the best of our knowledge, this is the first study that validates the efficiency of ultrasonography to identify the exact number of POFs present in the ovaries of domestically farmed animals in real time. Previously, the potential efficiency of TRU for counting POFs was validated retrospectively by visualizing the corpora lutea (CL) in the ovaries of slaughtered females. This was the procedure used by Soede et al. (1992) in the only previous study carried out to validate the effectiveness of TRU to identify the exact number of POFs in sows. Despite the limited number of ovaries checked ($n = 26$) and the small average number of POF counted per ovary (slightly above 10), Soede et al. (1992) recognized the occurrence of unclear counts in those ovaries with a large number of POFs, always visualizing more CL than POF counted. This observation was clearly confirmed in our study, where the most evident differences between TRU and LAP were in ovaries with a large number of POFs. In this regard, it is important to emphasize that the number of POF counted in our experiment was >20 in 11 of the 126 ovaries (8.7%) evaluated. The existence of ovaries with a high number of POFs was likely related to the relatively high dose of eCG (1250 IU) used to stimulate uniform follicle development. The reason that TRU underestimated the exact number of POFs present on the ovaries, particularly in those with a large number of POFs, is likely related to the ultrasonography procedure. We suggest that the underestimation is due to the inability to view all POFs in a single image, making it difficult to estimate their exact number with certainty. Moreover, the POFs often appeared as clusters, depending upon the position of transducer, which could potentially lead to an erroneous count.

In the present experiment, three successive TRU scanning sessions were carried out in each sow, with the POF measurements recorded in the two last scans. A high and significant correlation between the POF measurements in the two scans was achieved, indicating that one single scan just before ovulation would be sufficient to count the exact number of POFs present in the ovaries of weaned sows. However, carrying out a single scan could give erroneous data based on the probability of scanning ovaries during the ovulation period, thus not counting the maximum number of POFs. Therefore, to count the exact number of POFs, we suggest carrying out at least two successive scans before the expected ovulation time.

In conclusion, TRU is a valuable tool to count the number of POFs present on the ovaries of weaned sows, but a certain degree of underestimation should be expected when a large number of POFs is present. Despite this, continuous TRU scanning might be helpful to determine ovulation time, as the disappearance of a number of POFs has been used to indicate the beginning of ovulation.

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Artículo 2

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Dissimilarities in sows' ovarian status at the insemination time could explain differences in fertility between farms when frozen-thawed semen is used

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Abstract

Deep intrauterine insemination (DUI) offers a suitable alternative for the commercial use of frozen-thawed boar semen. The present study evaluated how the ovarian status at DUIs of frozen-thawed spermatozoa (1×10^9 spz/dose, two DUIs, 30–31 and 36–37 h after detection of oestrus) in 179 sows would explain differences in fertility between two farms with similar, but not equal, reproductive management (experiment 1). A further experiment investigated whether an increase in sperm number per AI-dose (1 versus 2×10^9 spz/dose, two DUIs, 30–31 and 36–37 h after detection of oestrus, on 228 sows) could minimize this effect (experiment 2). Ovaries were checked by transrectal ultrasonography at the time of DUI and sows were classified into three categories: F-: ovarian pre-ovulatory follicles were visible during two examinations; O-: ovulation visible during one examination; and C-sows: corpora hemorragica visible during the two examinations. Overall farrowing rates differed ($P < 0.01$) between farms (70.1 versus 51.2%, farms A and B, respectively). Distribution of sows among ultrasonography categories also differed ($P < 0.05$) between farms (17.5, 72.2 and 10.3% were classified as F-, O- and C-sows in farm A, versus 40.2, 29.3 and 30.5% in farm B). Nevertheless, farrowing rates and litter sizes within categories did not vary between farms ($P > 0.05$). In addition, a two-fold increase in the number of spermatozoa per DUI improved ($P < 0.05$) fertility in F- and C-sows, but not in O-sows. In

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conclusion, the interval DUI-to-ovulation provides a major explanation for fertility differences between farms when frozen-thawed spermatozoa are used.

Keywords: Frozen-thawed spermatozoa; DUI; Fertility; Ovulation; Transrectal ultrasonography; Sow

1. Introduction

Artificial insemination (AI) has become the most common breeding tool on European and American pig farms. There is a parallel trend for introducing new genetics in the porcine breeding population based on the use of cryopreserved boar semen. Compared with fresh or chilled semen, frozen-thawed semen is a useful alternative to replace transportation of live animals and is an excellent way to store valuable genetic material.

Recent improvements in cryopreservation protocols, such as new package systems [1] or the optimization of centrifugation during processing [2] have contributed to better post-thaw sperm viability, one of the drawbacks of cryopreservation in this species. In consequence, fertility with frozen-thawed spermatozoa has increased to more than 70% and nine piglets born alive per sow [1]. Moreover, the development of new procedures for AI, such as the deep intrauterine AI (DUI) [3], have decreased the number of frozen-thawed spermatozoa required for acceptable fertility down to 1×10^9 [4], thus improving the output of this methodology.

However, fertility of frozen-thawed spermatozoa is still very variable. This variability is attributed to several factors, such as post-thaw sperm quality, number and handling of AIs, total number of inseminated spermatozoa, breeding management of the sows and the sow itself. Health, nutrition and ovarian status at the time of AI are also relevant intrinsic issues that affect fertility in the sow [5].

The interval between AI and ovulation is a major factor affecting fertility when frozen-thawed boar spermatozoa are used. Insemination must be done close to ovulation to achieve acceptable fertility, due to the limited life span of the frozen-thawed spermatozoa. For these spermatozoa, the interval between AI and ovulation has been considered to be optimal when deposition has been done between 4 and 0 h before spontaneous ovulation [6]. In contrast, such interval rises up to 24 h before ovulation when fresh or chilled boar liquid semen is employed [7].

The objective of the present study was to evaluate whether the interval between DUIs and spontaneous ovulation could explain differences in fertility between farms with similar, but now equal, reproductive management, when frozen-thawed semen was used. Furthermore, we aimed to determine whether an increase in sperm number per AI-dose could minimize such an effect.

2. Materials and methods

2.1. Farms reproductive management

The DUIs were carried under field conditions, during a period of 7 months (from November to May) in two consecutive years (2003 and 2004), on two commercial pig

farms (hereby named A and B) located about 20 km from each other, in the Region of Murcia, Spain (37°59′N, 1°08′W). The farms, with a similar sow inventory (more that 3000 breeding sows per farm), were selected for their similar, but not equal, standard of reproductive management. This standard was maintained throughout the period of the study. Routinely, sows were weaned at 18–25 days post-farrowing, followed by manual testing of standing oestrus twice daily. Traditional AI was performed with chilled liquid semen (3×10^9 total spermatozoa) by the staff of the farms on the morning standing oestrus was detected followed by a second AI 24 h later. Farrowing rate and litter size after AI with chilled liquid semen did not differ between farms, ranging from 70 to 75% and 9 to 10 piglets during summer and early autumn, and from 85 to 90% and 10 to 11 piglets during months when the experiments were carried out (data obtained from the farm databases).

To carry out the experiments, healthy, weaned, hybrid sows (landrace × large white) with a weaning-to-oestrus interval (WOI) of 4 days were selected. Oestrus detection was performed twice a day (07:00 a.m. and 07:00 p.m.) by the farm staff, beginning 3 days after weaning. The procedure of oestrus detection varied between the two farms. On farm A, the sows were individually introduced into the male pen and those that allowed the (vasectomized) boar to mount them were considered to be in oestrus. On farm B, sows were housed in pens containing 8–10 each, and were continuously allowed snout-to-snout contact with a mature boar, and back-pressure was applied by an experienced farm staff member. Sows exhibiting a standing reflex were considered to be in oestrus. At both farms, sows showing oestrous signs were relocated into individual crates into large rooms with windows exposed to the natural day length, which varies from 14 h 54 min of light at the summer solstice to 9 h 30 min of light at the winter solstice. Sows were fed once (usually) or twice (during weaning-to-oestrus interval) daily with a commercial diet and water was provided ad libitum.

2.2. Semen processing, sperm cryopreservation and post-thaw sperm evaluation

Three mature and fertile Pietrain boars from a commercial boar station were used as semen donors. The sperm-rich fraction was collected once a week by the gloved-hand method, into a 500 mL plastic container whose top was covered by a double layer of cheesecloth gauze to avoid eventual mixture of the gelatinized fraction with the collected semen. The sperm-rich fraction was extended (1:1, v/v) in Beltsville Thawing Solution (BTS) [8] and sperm characteristics were microscopically evaluated by standard laboratory techniques [9]. Only ejaculates with more than 75% motile spermatozoa and more than 80% normal acrosomal ridges were used.

Spermatozoa were cryopreserved using a modification of the straw-freezing procedure described by Westendorf et al. [10] adapted to 0.5 mL straws by Thurston et al. [11] and modified by Carvajal et al. [2]. Briefly, extended sperm-rich fractions from the three boars were pooled and slowly cooled to 17 °C within 4 h, and thereafter centrifuged ($2400 \times g$) for 3 min. The supernatant was discarded and the sperm pellet re-extended with lactose–egg yolk extender (LEY) to a concentration of 1.5×10^9 sperm/mL. After further cooling to 5 °C for 2 h, the extended spermatozoa were resuspended with LEY–glycerol–Orvus–ES–Paste (LEYGO) extender to a final concentration of 1×10^9 sperm/mL, and dispensed into 0.5 mL straws. Spermatozoa packaged in this manner (0.5×10^9 spermatozoa/straw) were further cooled from 5 to −5 °C at a rate of 6 °C/min, from −5 to −80 °C at a rate of 40 °C/min, held

for 30 s at −80 °C, and then cooled to −150 °C using a programmable cell freezer (IceCube 1810, Minitüb, Tiefenbach Germany), before plunging the straws into liquid nitrogen.

The frozen semen was evaluated after thawing. Two straws were randomly chosen on the day of AI, and were thawed in a water-bath with circulating water at 37 °C for 20 s. Sperm motility was evaluated using a computer-assisted semen motility analysis system (Sperm Class Analyzer; Microptic, Barcelona, Spain). Sperm viability was evaluated using the triple fluorescent procedure described by Graham et al. [12] and modified by Carvajal et al. [2]. The percentages of motile spermatozoa ranged from 46 to 62% and those of sperm viability between 53 and 68%. For the AI-trial, semen with more than 45% of motile spermatozoa and more than 50% of viable spermatozoa were used.

All frozen sperm samples were thawed before DUI, as described above, and immediately re-extended at 37 °C in BTS (1:5, v/v).

2.3. Ovarian status determined by transrectal ultrasonography

Ovarian status of the sows exhibiting a standing reflex was examined by transrectal ultrasonography (Pie Medical SC100 Scanner, Maastrich, The Netherlands), using a 7.5 MHz multiple scan angle transducer as described by Soede et al. [13]. Briefly, one arm wrapped with a transrectal glove, covered with lubricating vaseline, was carefully introduced into the sow's rectum. Faeces were removed, and then the transducer, held facing down on the hand was introduced, taking care to maintain good contact between the transducer and the rectal tissue. The hand was introduced to a depth of 35–45 cm beyond the anal sphincter, where the ovaries normally are located. The urinary bladder was used as a reference location during scanning. The ovaries often appeared adjacent and laterally to the bladder. The location of both ovaries, however, varied among sows.

Both ovaries were checked simultaneously for functional structures such as pre-ovulatory follicles (POFs) (diameter of antrum >4 mm), corpora hemorrhagica (CH) or corpora lutea (CL). The POF appeared as an echogenic or black spherical structure with thin borders, and sometimes irregular outlines due to follicles pressing each other. The CH and CL could be identified as circular, homogeneous and hypo-echoic relative to the ovarian stromal structures.

The ovaries were examined three times: at 12 h after onset of oestrus and just before each of the two DUIs. The first ultrasonography (12 h after onset of oestrus) was performed as a control to check the feasibility of the transrectal examination and to assess the ovarian healthy. Based on the scanning performed just before each of the two DUIs, the sows were classified into three groups: F-sows (POFs visible in both examinations), O-sows (ovulation had obviously occurred during or between the two examinations, either because the number of POFs at the time of first DUI was 50% lower than at the first ultrasonography 12 h after onset of oestrus, or because they disappeared during the second DUI) and C-sows (CH were visible in both examinations).

2.4. Deep intrauterine insemination (DUI)

Sows showing the standing reflex (8–10 sows/week per farm) were double DUI inseminated at 30–31 and 36–37 h after detection of oestrus according to the results

obtained by Roca et al. [14]. The DUI took place for each sow in gestation crates and was performed following the procedure described by Martínez et al. [15]. A commercial AI catheter was inserted through the vagina into the cervix. Afterwards, a Firflex® device (length 180 cm, outer diameter 4 mm and working inner channel 1.8 mm) was inserted through the AI catheter, handled through the cervix and propelled forward along one uterine horn. Thereafter, a syringe containing frozen-thawed spermatozoa suspended in BTS (5 mL dose) was connected to the device. The extended spermatozoa were slowly infused into the uterine horn through the device. Finally, before removing the device, an extra 2 mL of sperm-free BTS was flushed through to force any remaining spermatozoa out of the device. DUI inseminations were performed at the two farms by the same two operators who had experience in the DUI procedure.

2.5. Pregnancy diagnosis

Possible returns to oestrus were determined by exposing females once daily to teaser boars, beginning on day 17 after the last DUI until the day of pregnancy diagnosis by ultrasonography (day 28 after the last DUI). All pregnant sows were allowed to go to term and farrowing rates and litter sizes were recorded.

2.6. Experimental design

2.6.1. Experiment 1: fertility of frozen-thawed spermatozoa after DUI in relation to the occurrence of spontaneous ovulation

The DUIs were performed on two commercial farms, A and B. A total of 186 commercial hybrid sows were randomly selected (101 on farm A and 85 on farm B). The mean ± S.E.M. parity was 3.85 ± 0.15 and 3.68 ± 0.16 on farms A and B, respectively, ranging from 2 to 7 on both farms. Four and three sows on farms A and B, respectively, had a small rectal orifice, too narrow to insert the transrectal ultrasound device, and were removed from the experiments. The other 179 sows fulfilled the following two requirements: were weaned at 21 days (range from 18 to 25 days) and exhibited the standing heat reflex 90 ± 6 h post-weaning.

Sows were inseminated twice, 30–31 and 36–37 h after detection of standing oestrus. All DUIs were performed with 1×10^9 frozen-thawed spermatozoa (approximately between 500 and 650 $\times$ 10^6 live spermatozoa per dose) re-extended with BTS (1:5, v/v) to a 5 mL volume, and each inseminated sow was categorized as F, O or C, according to its ovarian ultrasound scan. Fertility was recorded in relation to the ovulatory status.

2.7. Experiment 2: influence of frozen-thawed sperm number per AI dose on fertility in F, O and C sows

A total of 236 commercial hybrid sows (mean ± S.E.M. parity 3.88 ± 0.11, range 2–7) were randomly selected on farm B, as described in experiment 1. Eight of them were too thin to allow transrectal ultrasonography, and were discarded from the study.

A total of 100 weaned sows were subjected to DUI twice with 2×10^9 frozen-thawed spermatozoa (approximately 1–1.3 $\times$ 10^9 live spermatozoa per dose) re-extended with

BTS (1:5, v/v) to 10 mL volume. The other 128 weaned sows were inseminated twice with 1×10^9 frozen-thawed spermatozoa (approximately 500–650 $\times$ 10^6 live spermatozoa per dose) re-extended with BTS (1:5, v/v) to 5 mL volume. Inseminations were conducted 30–31 and 36–37 h after detection of oestrus. As in experiment 1, every sow was catalogued as F, O or C, according to its ovarian ultrasound scan, and fertility was recorded for each ovulation status.

2.8. Statistical analysis

Data were analyzed using the SPSS 12.0/PC statistics package (SPSS Inc., Chicago, IL, US). Data for ovarian status (F, O C) and both pregnancy and farrowing rate (%) as well as litter size (numbers) were analyzed by ANOVA using a mixed model. The statistical models included the fixed effects of farm (experiment 1) or number of spermatozoa per AI dose (experiment 2), as well as ovarian status and the random effect of the day of DUI. Data in percentage were modelled according to the binomial model of parameters [16]. When the ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $P < 0.05$. Results are presented as mean $\pm$ S.E.M.

3. Results

3.1. Experiment 1

The overall fertility results after DUIs with frozen-thawed boar spermatozoa on both farms are shown in Fig. 1. The overall pregnancy and farrowing rates achieved on farm A were higher ($P < 0.05$) than those obtained on farm B. No differences were found, however, between farms for litter size ($P > 0.05$).

The pattern of distribution of sows according to ovarian status at the time of DUI (F-, O- and C-sows) varied between the farms ($P < 0.01$). As shown in Table 1, on farm A, there were more peri-ovulatory sows (O-sows) at the time of AI than F- or C-sows ($P < 0.01$), whereas on farm B there were no differences within the distribution ($P > 0.01$).

Fertility results obtained in F-, O- and C-sows in farms A and B are compiled in Table 2. Fertility within each sow group did not vary between farms ($P > 0.05$). Overall, pregnancy and farrowing rates were significantly ($P < 0.05$) higher for O-sows than for F- or C-sows, and litter size was significantly ($P < 0.05$) higher for O-sows as compared to C-sows.

3.2. Experiment 2

The percentage of sows in each respective ovarian status was similar ($P > 0.05$) for sows inseminated with 1×10^9 ($n = 100$ sows) or 2×10^9 ($n = 128$ sows) frozen-thawed spermatozoa. Moreover, the distribution of F-, O- and C-sows was also similar ($P > 0.05$) to that found on farm B in the first experiment.

Fertility results are presented in Table 3. Pregnancy and farrowing rates of weaned sows inseminated with 1×10^9 frozen-thawed spermatozoa were significantly ($P < 0.01$) higher

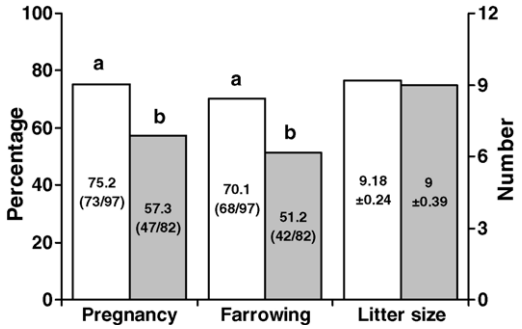

Fig. 1. Pregnancy (%, number of pregnant sows/total sows between brackets), farrowing rates (%, number of farrowed sows/total number of sows between brackets) and litter size (mean ± S.E.M.) in weaned sows at farm A (white) and farm B (grey) after double DUI with 1×10^9 frozen-thawed spermatozoa per AI dose. Different superscripts above bars mark significant differences between farms ($P < 0.05$).

in O-sows than in F- or C-sows, similar to the results observed in experiment 1. Litter size did not differ significantly ($P > 0.05$) among the three ovarian statuses at DUI. However, differences in pregnancy/farrowing rates were absent when sows were inseminated with 2×10^9 frozen-thawed spermatozoa ($P > 0.05$). Therefore, doubling the number of

Table 1
Distribution of weaned sows according to ovarian status at the moment of DUIs

		Ovarian status, n^o (%)		
	Sows (n)	Pre-ovulatory (F)	Peri-ovulatory (O)	Post-ovulatory (C)
Farm A	97	17 (17.52)^{a,1}	70 (72.16)^{b,1}	10 (10.31)^{a,1}
Farm B	82	33 (40.24)^{2}	24 (29.27)^{2}	25 (30.49)^{2}

a,b: values with different superscript in the same row are significantly different ($P < 0.05$); 1,2: values with different superscript in the same column are significantly different ($P < 0.05$).

Table 2
Pregnancy rates, farrowing rates and litter size of weaned sows according to their ovarian status at DUI on farms A and B

	Farm	Ovarian status		
		Pre-ovulatory (F)	Peri-ovulatory (O)	Post-ovulatory (C)
Pregnancy n^o/total (%)	A	9/17 (52.8)^{b}	59/70 (84.3)^{a}	5/10 (50)^{b}
	B	15/33 (45.4)^{b}	20/24 (83.3)^{a}	12/25 (48)^{b}
	Mean	24/50 (48)^{b}	79/94 (84)^{a}	17/35 (48.6)^{b}
Farrowing n^o/total (%)	A	7/17 (41.2)^{b}	58/70 (82.9)^{a}	3/10 (30)^{b}
	B	14/33 (42.4)^{b}	20/24 (83.3)^{a}	8/25 (32)^{b}
	Mean	21/50 (42)^{b}	78/94 (83)^{a}	11/35 (31.4)^{b}
Litter size (mean ± S.E.M.)	A	8.29 ± 0.68	9.38 ± 0.26	7.33 ± 0.67
	B	8.86 ± 0.75	9.65 ± 0.51	7.62 ± 0.84
	Mean	8.67 ± 0.54^{a,b}	9.45 ± 0.23^{a}	7.54 ± 0.62^{b}

Inseminated twice with 1×10^9 frozen-thawed spermatozoa at 30–31 and 36–37 h after oestrous detection. a,b: values with different superscript in the same row are significantly different ($P < 0.05$).

Table 3
Pregnancy rates, farrowing rates and litter size of weaned sows according to their ovarian status at DUI on farm B

	Sperm doses ($\times 10^9$)	Ovarian status			Total
		Pre-ovulatory (F)	Peri-ovulatory (O)	Post-ovulatory (C)	
Pregnancy n^o/total (%)	2	30/41 (73.2)[1]	22/26 (84.6)	19/33 (60.6)	71/100 (71)[1]
	1	29/56 (52.8)[b,2]	30/37 (81.1)[a]	15/35 (43.9)[b]	74/128 (57.8)[2]
Farrowing n^o/total (%)	2	30/41 (73.2)[1]	21/26 (80.8)	19/33 (60.6)[1]	70/100 (70)[1]
	1	27/56 (48.2)[b,2]	30/37 (81.1)[a]	11/35 (31.4)[b,2]	68/128 (53.1)[2]
Litter size (mean ± S.E.M.)	2	9.07 ± 0.53	10.14 ± 0.58	9.05 ± 0.60	9.38 ± 0.33
	1	8.70 ± 0.59	9.93 ± 0.46	7.82 ± 0.72	9.10 ± 0.34

Inseminated twice with either 1 or 2×10^9 frozen-thawed spermatozoa at 30–31 and 36–37 h after oestrous detection. a,b: values with different superscript in the same row are significantly different ($P < 0.05$); 1,2: values with different superscript in the same column are significantly different ($P < 0.05$).

spermatozoa per insemination dose improved pregnancy and farrowing rates in F- and C-sows ($P < 0.05$), but not in O-sows ($P > 0.05$). Litter size did not differ significantly ($P > 0.05$) in any case.

4. Discussion

The present study showed that the ovarian status of sows at the time of DUI significantly influenced fertility when sows were inseminated with frozen-thawed spermatozoa. Moreover, differences in fertility between the two farms could be associated with dissimilarities in ovarian status at the time of DUI.

In the first experiment, there were overall differences in farrowing rates between farms (75.26 versus 57.31% on farms A and B, respectively). Some authors have found a significant farm effect on the fertility of frozen-thawed spermatozoa [1,17,18]. Inseminations were carried out in the same season of the year, using the same ejaculates which were frozen and thawed following a standard protocol, and similar standard operating procedures. Therefore, differences in the interval DUI-to-ovulation between farms could explain the differences in farrowing rates.

Transcutaneous [19] or transrectal [13] ultrasonography are widely used in experimental studies of pig reproduction. Both procedures have been successfully used as diagnostic tools to determine the occurrence of ovulation without adverse effects associated with repeat scanning [20]. In the present experiments, transrectal ultrasonography was used a few minutes before or at the time of DUI to classify the sows into three groups, according to their ovarian status: pre-ovulatory (F), peri-ovulatory (O) and post-ovulatory (C). Significant differences in fertility related to the ovarian status were observed. Peri-ovulatory inseminations resulted in the highest pregnancy and farrowing rates and litter size. However, it is interesting that fertility results were similar in

F-, O- or C-sows on both farms. It can be concluded that the higher farrowing rates obtained on farm A, compared with those obtained on farm B, relate to differences between the farms in the distribution of sows according to their ovarian status at the time of DUI. Whereas on farm A more than 70% of the sows were inseminated at peri-ovulation, less of 30% of the sows inseminated on farm B were at this stage. The optimal time for AI with frozen-thawed boar semen has been reported between 4 and 0 h before ovulation [6]. This short interval relates to the well-documented reduction in the functional lifespan of thawed boar spermatozoa in the female genital tract, which is probably due to the damage caused to the spermatozoa during the cryopreservation process [21].

The disparate distribution between farms of sows according to their ovarian status at the time of DUI was notable. Nevertheless, it is well documented that the average duration of oestrus varies between farms, a fact that is closely related to the WOI, but is highly consistent within a farm [22]. Inseminations and ultrasonography were carried out at 30–31 and 36–37 h after oestrus detection on both farms. The interval between two oestrus detections was 12 h. Hence, on farm A, most of the sows ovulated between 30–31 and 48–49 h after the theoretical onset of oestrus. Nevertheless, only a few sows ovulated within this time range on farm B. Soede and Kemp [23] showed that the interval from the onset of oestrus to ovulation varies considerably (between 10 and 58 h), although the majority of sows with a WOI of 4 days ovulated between 32 and 48 h after the onset of oestrus. With the two DUIs performed in the present experiments, we intended to include those hours in which ovulation was most likely to occur, taking into account the lifespan of frozen-thawed spermatozoa in the uterus. The distribution of ovulation described above occurred on farm A, but not on farm B, within the present experimental conditions. Thus, it is clear that there were marked differences between these farms in the interval between oestrus detection and ovulation, that could be associated with an incorrect detection or with an intrinsic variability in the duration of oestrus, and therefore in time of ovulation among farms.

Ultrasonography can be used as a diagnostic tool to monitor the occurrence of ovulation in herds, but it is not a useful predictor for spontaneous ovulation [20]. In the absence of a better predictive tool, the correct detection of the beginning of oestrus as well as monitoring the duration of oestrus at the farm is, to date, probably the best strategy to forecast the time of ovulation and hence, a possible way to achieve high fertility rates after AI with frozen-thawed spermatozoa. Possible reasons for the different timing of oestrus detection and ovulation between the two farms are nutrition, stress, housing conditions, season of the year, weather conditions (e.g. temperature), genetic background, diseases and an insufficient oestrus detection [24]. In the present field trial none of these factors were significantly different between farms.

The presence of the boar is fundamental when attempting the detection of oestrus on-farm. Boars can, however, also negatively influence the expression of oestrus by means of the phenomenon called ‘‘habituation’’ [25,26]. In our experiments, sows at farm A were guided one by one to a pen where a young, mature and fertile boar, with no continuous fence-line contact with sows, was used for oestrus detection. At farm B, an old boar was in continuous fence-line contact with the sows, while an experienced operator manually checked the sow’s standing reflex response to back pressure. To perform a correct detection of oestrus, important signs of the sow in heat must be considered. Among these, mucous vulva discharge, interest in the boar and other

females, mounting activity, voluntary immobility in close proximity to a boar or induction of the standing reflex when back pressure is applied, are known to indicate standing heat. It is possible, on farm B, that not all signs of heat were registered completely. Instead, only the standing reflex was used to indicate the onset of heat, and animals were probably predisposed to habituation. Mounting by a boar, a more accurate method to detect standing oestrus (albeit more time consuming) compared to human-detected oestrus, might explain the differences in the timing of insemination to spontaneous ovulation. Unfortunately, incorrect oestrus detection strategies are not unusual on farms, due to a lack of time and skilled labour. In addition, as AI is usually performed with fresh or chilled liquid semen, its long functional lifespan in the female genital tract masks incorrect or inconsistent oestrus detection procedures.

In the second field experiment, we evaluated whether an increase in the number of frozen-thawed spermatozoa in the DUI dose could improve fertility on farm B, where a very heterogeneous ovulation time occurred. Although 1×10^9 frozen-thawed spermatozoa are sufficient to achieve high fertility rates when DUI is used [4], it is clear that the number of spermatozoa per AI dose influences fertility [27], especially when using frozen-thawed spermatozoa [21]. Accordingly, we doubled the number of frozen-thawed spermatozoa per AI dose and obtained a significant overall increase in farrowing rates. Nevertheless, when we evaluated how this increase in the number of spermatozoa influenced the fertility of F-, O- or C-sows, we found that farrowing rate was improved for F- and C-sows, but not for O-sows. Thus, doubling the number of frozen-thawed spermatozoa increases the probability of fertilization within a certain range. The increased farrowing rate in F- and C-sows when 2×10^9 frozen-thawed spermatozoa were used, suggests that the more functional spermatozoa that are inseminated, the higher the chance of competent spermatozoa reaching the isthmus to await the oocyte [28]. Nevertheless, the farrowing rate achieved in O-sows also demonstrated that 1×10^9 frozen-thawed spermatozoa were sufficient to achieve acceptable farrowing rates and litter size when DUI was performed around ovulation time. Thus, insemination close to the time of ovulation is important, irrespective of the number of spermatozoa being inseminated.

No change in litter size was found when the number of spermatozoa used for AI was doubled. This agrees with results previously obtained using either DUI [15] or uterine surgical sperm deposition [29] with different low doses of fresh boar spermatozoa. A possible explanation is that the increased number of spermatozoa per dose had a positive influence on sperm transport through the uterus and ensured sufficient functional spermatozoa colonized the oviductal sperm reservoir, positively influencing fertility (pregnancy and farrowing rates), but not increasing the probability of these spermatozoa fertilizing ovulated oocytes. The implication of these results with respect to litter size requires further investigations.

In conclusion, the present results emphasize the importance of a precise timing of inseminations with respect to ovulation time on pig farms, in order to improve fertility when frozen-thawed spermatozoa are used. Moreover, differences between farms in farrowing rates can be explained, at least in part, by dissimilarities in ovarian status at the time of insemination, which could be associated with incorrect oestrus detection. Although these results are encouraging, further trials on a larger number of farms, and with a more precise detection of the insemination–ovulation interval, are needed to confirm them.

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Artículo 3

Use of frozen-thawed semen aggravates the summer-autumn infertility of artificially inseminated weaned sows in the Mediterranean region

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Use of frozen-thawed semen aggravates the summer-autumn infertility of artificially inseminated weaned sows in the Mediterranean region[1]

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ABSTRACT: Improvement of farrowing rate (FR) and litter size (LS) of sows that are AI with frozen-thawed (FT) semen can hardly be reached without identification of the factors behind the high variability seen among trials. Three experiments using weaned (4-d wean-to-estrus interval) multiparous (parity 2 to 7) sows were conducted to evaluate the effect of period of the year on FR and LS of FT-inseminated sows in southern Spain. Sows were grouped into 2 periods of the year: winter-spring (November to April; WS) and summer-autumn (May to October; SA). Ovarian status was monitored by transrectal ultrasonography to record how long before or after ovulation AI was performed (pre-, peri-, or postovulatory AI) and to determine the onset of estrus-to-ovulation interval (EOI). Inseminations were performed using deep intrauterine AI with 1.5×10^9 FT sperm per dose. The first experiment was designed to determine the influence of the period of the year on FR and LS of FT semen. Sows (116 in WS and 100 in SA) were AI at 33 and 39 h after the onset of estrus. The period of the year influenced the FR and LS ($P < 0.01$). Farrowing rate and LS were least in SA ($P < 0.05$). This pattern of annual variation was similar to that shown by sows on the same farm currently undergoing AI with liquid semen (cervical AI at 12 and 36 h after the onset of estrus with 3×10^9 sperm per dose). However, the FR reduction in SA respect to WS was more substantial in sows artificially inseminated with FT (77.6 vs. 50%, $P < 0.001$) than those artificially inseminated with liquid semen (83.9 vs. 71.8%, $P < 0.05$). More pre- and less periovulatory AI were performed in SA sows than in WS sows ($P \leq 0.05$). Experiment 2 was designed to evaluate whether the period of the year influenced EOI. Ovarian status was transrectal ultrasonography scanned every 6 h after the onset of estrus until the end of ovulation (WS: 30; SA: 31 sows). There were more sows with long EOI (>48 h) in SA than in WS ($P \leq 0.05$). Experiment 3 aimed to improve the reduced FR and LS recorded in SA sows when using FT semen (Exp. 1) by inducing ovulation with eCG + hCG. A single AI with FT semen was performed 5 h before the expected ovulation (55 sows). As a control, spontaneously ovulating sows (n = 53) were FT-inseminated as in Exp. 1. Hormonal induction of ovulation did not improve FR and LS ($P > 0.05$). In the Spanish Mediterranean area, a longer EOI during SA negatively influenced the FR and LS of weaned sows after AI. This effect was particularly evident when FT semen was used. These findings were not ameliorated by hormonal induction of ovulation.

Key words: fertility, frozen-thawed semen, ovulation, season, swine

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INTRODUCTION

Important advances in the production of frozen-thawed (**FT**) boar semen and its use for AI have been made in recent years, increasing the efficiency of FT semen in commercial swine enterprises (Roca et al., 2006a,b; Grossfeld et al., 2008; Rath et al., 2009). However, FT semen is not routinely used due to its variable reproductive performance [in terms of fertility, farrowing rate (**FR**), and prolificacy] under field conditions. Its fertility has ranged from 40 to 85% (Eriksson et al., 2002; Roca et al., 2003; Bolarín et al., 2006; Wong-

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tawan et al., 2006). Several factors might account for this variation, including differences in postthaw sperm quality, the number of spermatozoa used per AI dose, and the interval between AI and ovulation (Roca et al., 2006a). However, other factors known to affect swine reproductive performance, such as environment and season (Love et al., 1993; Peltoniemi et al., 2000; Peltoniemi and Virolainen, 2006), may also have contributed to these differences.

Seasonal impairment of pig reproduction is evident worldwide. A clear reduction in fertility occurs during summer and early autumn, when cervical AI with liquid semen is used (Peña et al., 1998; Peltoniemi et al., 1999; Gaustad-Aas et al., 2004; Suriyasomboon et al., 2006). Complementary studies using FT semen are not yet available. This lack of data may in part be due to the variable reproductive performance found in many studies. An improved understanding of the impact of period of the year on FT semen fertility and prolificacy could help to explain differences among trials in the reproductive performance of FT-inseminated sows. Such an understanding could also facilitate the development of refined AI strategies that aim to achieve a high and consistent fertility and prolificacy using FT semen throughout the year. Therefore, the present study examined the influence of the period of the year on the reproductive performance of FT-inseminated sows. Ovarian status was monitored by transrectal ultrasonography (**TRU**). This monitoring allowed us to record how long before or after ovulation the AI was performed. The ability of hormone-induced ovulation to improve fertility and prolificacy in FT-inseminated sows was also evaluated.

MATERIALS AND METHODS

The experimental protocols were reviewed and approved by the Ethical Committee for Experimentation with Animals of the University of Murcia, Spain.

Farm Location and Climatic Data

The study was carried out over a period of 3 yr (2005 to 2007) on a commercial pig breeding farm (Agropor SL Group) located in Murcia, Spain (37° 59′ NL, 1° 08′ WL). Pens and farm rooms were exposed to natural day length, which varied from 14 h, 54 min of light during the summer solstice to 9 h, 30 min of light during the winter solstice. Maximum outside air temperature varied from 16.5°C in January (mid-winter) to 34.3°C in August (mid-summer). The minimum air temperature ranged from 3.8°C in January to 19.5°C in August. Relative humidity was always above 50%. The humidity ranged from 51% in July to 69% in December. Sunshine ranged from 172 h in January to 338 h in July. Day length and meteorological data were obtained from the Spanish State Agency of Meteorology (http://www.aemet.es).

Animals and Farm Management

Multiparous (2 to 7 parities) crossbred (Landrace × Large White) sows with a lactation period of 18 to 25 d and showing a mean BCS of 3.3 ± 0.1 (mean ± SEM), with a range of 2.5 to 4 (on a scale of 1 to 5), were randomly selected to carry out the different field experiments at first estrus postweaning. The weaned sows were placed into individual crates in large rooms with windows exposed to the natural day length (from 14 h and 54 min of light at the summer solstice to 9 h and 30 min of light at the winter solstice). The sows had ad libitum access to water and were fed a commercial diet (16% CP and 12.2 MJ of ME/kg of DM) twice daily, receiving from 3.0 kg/d in mid-winter to 2.5 kg/d in mid-summer.

Ten healthy and mature (2 to 4 yr of age) Pietrain boars of proven fertility were housed at a commercial AI station. The station was owned by the above-mentioned swine company. These boars were used as the source of ejaculates for the study. The boars were housed in individual pens in a climate-controlled (15 to 25°C) building. The facility was exposed to natural daylight and supplementary electric light to provide a 16-h daily light regime. The boars were given ad libitum access to water and were fed a commercial diet according to the nutritional requirements of adult boars.

Semen Processing, Sperm Cryopreservation, and Postthaw Sperm Quality Assessments

The sperm-rich fractions of ejaculates were collected 1/wk over 5 mo (November to March, 2004) by the gloved-hand method. Only ejaculates with more than 75% motile spermatozoa and more than 80% intact acrosomal ridges were used. For cryopreservation, sperm-rich fractions from each ejaculate were extended (1:1, vol/vol, in Beltsville Thaw Solution; **BTS**). They were then refrigerated at 17°C for 3 h and centrifuged at 2,400 × *g* for 3 min at 17°C. The supernatant was removed, and the sperm pellet was resuspended in a lactose-egg yolk extender [80 mL (80%, vol/vol, 310 m*M* β-lactose) and 20 mL of egg yolk] to a concentration of 1.5×10^9 sperm/mL. The sperm suspension was slowly cooled to 5°C for 2 h. The sperm suspension was then re-extended (2:1, vol/vol) with lactose-egg yolk extender containing 9% glycerol, and 1.5% Orvus Es Paste. This extension yielded a final concentration of 3% glycerol, 0.5% Orvus Es Paste, and 1×10^9 sperm/mL (Hernandez et al., 2007). The spermatozoa were then packed into 0.5-mL straws. The straws were frozen in a computerized freezing machine (IceCube 1810, Minitüb, Tiefenbach, Germany) at a rate of −6°C/min from 5 to −5°C. The temperature was then reduced at −40°C/min from −5 to −80°C. Finally, the temperature was reduced at −70°C/min from −80 to −150°C. The frozen straws were placed in liquid nitrogen until used for AI.

During each AI trial, straws from the 10 boars were thawed by direct plunging into a 37°C water bath. They were then shaken vigorously for 20 s. Straws were emptied and contents mixed into prewarmed (37°C) tubes containing BTS to obtain the required AI doses (7.5 mL of total volume per dose). The thawed sperm suspensions were evaluated for motility and plasma membrane integrity. The suspensions were then stored at 21 to 23°C until inseminations were performed (within 1 h after thawing). Sperm motility was evaluated using a computer-assisted semen motility analysis system (sperm class analyzer, Microptic, Barcelona, Spain) following the procedure described by Hernandez et al. (2007). Plasma membrane integrity was cytometrically evaluated using the fluorescent probe SYBR-14 and propidium iodide according to the manufacturer's instructions (L-7011, Live/Dead Sperm Viability Kit, Molecular Probes Europe, Leiden, the Netherlands). The motility and plasma membrane integrity of the AI doses ranged from 45 to 60% and 51 to 65%, respectively.

Detection of Estrus and Ovulation

Estrus detection was performed by experienced personnel twice daily (0700 and 1900 h) beginning 3 d after weaning. Sows were checked for estrus in their crates 4 at a time using the back pressure test for approximately 5 to 7 min during nose-to-nose contact with a mature boar (randomly chosen from among the 4 teaser boars) located in the alleyway in front of the crates. Sows exhibiting a standing heat reflex in the presence of a boar were considered to be in estrus. The time of onset of estrus was defined as the first time a sow revealed a standing manual response minus 6 h. To standardize the AI strategy, only sows that started estrus at d 4 after weaning were selected. Most of the weaned sows on the farm came into estrus on this day (between 52 to 57%, without differences throughout the year).

Ovaries of sows exhibiting a standing reflex were examined by serial TRU to check for the presence of follicles and corpora lutea and to determine the moment of ovulation (Bolarín et al., 2009). The TRU examinations were performed by a single operator using a real-time, B mode scanner with a transrectal 7.5-MHz multi-angle transducer (Esaote Pie Medical, Maastricht, the Netherlands). Sows were removed from the study if they had a narrow anus or rectum or both (too narrow to insert the transrectal ultrasound device), if they were showing quiescent ovaries or abnormal ovarian structures, or if they had ovulated within 24 h from onset of estrus. In selected sows, the number of large follicles (≥6 mm in diameter) or corpora lutea was recorded at each scanning. Ovulation was defined as the sudden disappearance of the follicular structure in at least one-half of the previously identified large follicles.

Insemination Procedure

Frozen-thawed semen was deposited using a deep intrauterine insemination (**DUI**) procedure (Martinez et al., 2002). This insemination procedure involved placing semen in the proximal one-third of one uterine horn (Martinez et al., 2005). This procedure can achieve high fertility rates using as few as 1 to 2×10^9 total FT spermatozoa (Roca et al., 2006b). For each sow, DUI took place in gestation crates and was performed using a Deep Blue catheter (Minitüb, Tiefenbach Germany). The procedure involved the introduction of a long (180 cm), flexible, and thin device through a conventional insemination catheter into one uterine horn. The catheter was inserted into the cervical folds as a guide. A FT semen dose of 7.5 mL containing 1.5×10^9 spermatozoa was slowly infused through the flexible catheter using a temperate syringe. Before removing the device, an extra 2 mL of sperm-free BTS was flushed through the catheter to force any remaining spermatozoa into the uterus. All DUI were carried out by 2 experienced technicians.

Fertility Assessments

The AI sows were exposed once daily to teaser boars from d 18 through 35 after the onset of estrus. This exposure was used to identify nonpregnant sows. The sows that returned to estrus (**RE**) were divided into 2 groups. The first group was composed of those that showed regular (18 to 24 d) estrus intervals. The second group consisted of those that showed delayed estrus intervals (longer than 24 d). Pregnancy was confirmed by transabdominal ultrasonography at d 28 after AI (Martinez et al., 1992). Pregnant sows were monitored until term for abortions, farrowing, and number of piglets born; all of these variables were recorded. The FR was assessed as the proportion of sows that farrowed after AI. Litter size (**LS**) was defined as the total number of piglets born (dead and alive) per litter.

Experimental Design

Sows were arbitrarily grouped according to 2 different periods of the year (**SA**, summer-autumn; and **WS**, winter-spring). This division was done following the guidelines reported by Peltoniemi et al. (2000), with focus on seasonal sow infertility, being adapted to locally defined hot and cold months in the Mediterranean area (Murcia, Spain). The SA period ran from May to October (based on the prevalence of days with minimum and maximum air temperature up to 15 and 25°C, respectively) and WS from November to April (prevalence of days with minimum and maximum air temperature down to 10 and 20°C, respectively). This division in SA and WS was confirmed by the monthly variation on fertility and prolificacy recorded in the farm during 2 yr before the present experiments start-

ed. Sows showing health problems were removed from the experiments.

Exp. 1: Effect of the Period of the Year on the Reproductive Performance of Weaned Sows Inseminated with FT Semen. Given the variable reproductive performance of FT semen among trials, this experiment evaluated the effect of the period of the year on this variability. At the onset of estrus, multiparous sows with a weaning-to-estrus interval (**WEI**) of 4 d were randomly assigned (4 to 6 sows every week) to the WS (n = 116) or SA group (n = 100). The mean ± SEM of parity and that of the number of piglets born in the latest farrowing were similar ($P > 0.05$) in the 2 groups (for WS sows, 3.7 ± 0.1 parity and 10.9 ± 0.1 piglets; for SA sows: 3.8 ± 0.1 parity and 10.8 ± 0.1 piglets).

Sows were double FT inseminated at 33 and 39 h after the onset of estrus. The ovaries of each selected sow were TRU scanned at 24 h after the onset of estrus and just before each AI. Using this scan, the sows were grouped into 3 categories based on how long before or after ovulation the DUI inseminations were to be completed. Each AI was classified as 1) preovulatory, when large follicles were visible before the 2 AI; 2) periovulatory, when ovulation had occurred during or between the 2 AI; or 3) postovulatory, when corpora hemorrhagica were visible before the first AI. Thus, pre-, peri-, and postovulatory ovarian status indicated that the sows had ovulated >39, 33 to 39, and <33 h after onset of estrus, respectively.

The reproductive performance of sows undergoing AI using liquid semen on the same farm during the experiment period was recorded and used as a reference for the reproductive performance of FT semen. A similar number of weaned sows with the same WEI (WS, n = 112; SA, n = 110) were randomly selected each week and subjected to the usual AI program used on the farm. These sows were cervically inseminated at 12 and 36 h after the onset of estrus with liquid semen. Each AI dose consisted of 3.0×10^9 sperm extended in 80 mL of BTS. The liquid semen was a mixture of semen from the same boars that provided the FT semen doses (pooled from 3 to 4 boars). The reproductive performance of sows inseminated with FT and liquid semen was evaluated as explained above.

Exp. 2: Influence of the Period of the Year on the Estrus-to-Ovulation Interval. In an effort to explain the large decreased in FR and LS in FT-inseminated sows during SA, the occurrence of ovulation was monitored in weaned multiparous sows. Sows that had a WEI of 4 d were randomly selected (9 to 13 sows/wk) at the onset of estrus and assigned to the WS (n = 30) or SA groups (n = 31). The mean ± SEM parity and that of the number of piglets born in the latest farrowing, respectively, were as follows: for WS sows, 3.6 ± 0.3 and 11.2 ± 0.3; for SA sows, 3.5 ± 0.3 and 11 ± 0.3 ($P > 0.05$). The ovaries of each sow were scanned by TRU at intervals of 6 h from the onset of estrus until the completion of ovulation (when the number of large follicles was zero). According to the elapsed time from the onset of estrus to ovulation, the sows were grouped into 3 different intervals: <24 h, 24 to 48 h, and >48 h.

Exp. 3: Effect of Hormonal Induction of Ovulation on the Fertility and Prolificacy of Weaned DUI-Inseminated Sows Using FT Semen for SA. Based on Exp. 2, induction of ovulation seemed to be a reasonable way to improve the FR and LS of FT-inseminated sows during SA. A total of 108 weaned sows were randomly selected during SA (4 to 6 sows every week) and assigned to hormonal treatment (ovulation induction) or control (spontaneous ovulation). In the hormonal treatment group, the sows (n = 55) received intramuscularly 1,250 IU of eCG (Folligon, Intervet International B.V., Boxmeer, the Netherlands) 24 h after weaning and 750 IU of hCG (Veterin Corion, Divasa Farmavic S.A., Gurb-Vic, Barcelona, Spain) 72 h later to stimulate follicular development and induce ovulation. The sows underwent AI with FT semen at 37 h after hCG treatment. Control sows with a WEI of 4 d (n = 53) underwent AI with FT semen twice at the onset of estrus following the same schedule as was described for Exp. 1. The mean ± SEM parity and number of piglets born per farrowing were 3.8 ± 0.2 and 11 ± 0.2, respectively, for the sows that received hormonal treatment ($P > 0.05$). For the controls, the mean ± SEM parity and number of piglets born per farrowing were 3.6 ± 0.2 and 10.9 ± 0.2, respectively ($P > 0.05$). As in Exp. 1, the ovaries of each selected sow were TRU scanned 24 h after the onset of estrus and just before each AI. Sows were grouped into 3 categories based on how long before or after ovulation the DUI inseminations were to be completed (pre-, peri-, and postovulatory).

Statistical Analyses

The data were analyzed (SPSS Inc., Chicago, IL). The percentage of RE, abortion, and farrowing were modeled according to a binomial model of variables (Fisz, 1980). Fertility variables were analyzed by ANOVA using mixed models. In Exp. 1, the statistical model included the fixed effect of period of the year (WS vs. SA). In Exp. 3, the model included the fixed effect of the AI schedule (hormonally induced ovulation vs. spontaneous ovulation). Parity and lactation length did not affect any of the fertility variables evaluated in these experiments. Therefore, these effects were not included in the statistical models. The time frame of AI within the period of the year was random in the statistical models of both experiments. When ANOVA revealed a significant difference, values were compared using the Bonferroni test. The distribution of FT inseminations into pre-, peri-, and postovulatory groups (Exp. 1 and 3) and the differences in estrus-to-ovulation interval (**EOI**) between WS and SA (Exp. 2) were analyzed using a chi-square test. Differences were considered to be significant when $P \leq 0.05$.

Table 1. Reproductive variables of weaned sows receiving deep intrauterine insemination (twice at 33 and 39 h after onset of estrus) with frozen-thawed semen ($1{,}500 \times 10^6$ sperm per AI dose) during winter-spring (November to April) or summer-autumn (May to October) in southern Spain (Exp. 1)

Reproductive variable	Winter-spring	Summer-autumn
Sows inseminated, n	116	100
Returned to estrus, n (%)	25 (21.6)[a]	47 (47)[b]
Regular (≤24 d), n (%)	18 (72)	31 (66)
Delayed (≥25 d), n (%)	7 (28)	16 (34)
Aborted, n (%)	1 (1.1)	3 (5.7)
Farrowed, n (%)	90 (77.6)[a]	50 (50)[b]
Litter size,[1] mean ± SEM	9.9 ± 0.2[a]	9.0 ± 0.3[b]

[a,b]Values with different superscripts within a row differ by $P < 0.01$.

[1]Litter size indicates the total number of piglets born.

RESULTS

Exp. 1: Effect of the Period of the Year on the Reproductive Performance of Weaned Sows Inseminated with FT Semen

The period of the year affected the reproductive performance of weaned sows inseminated with FT semen ($P < 0.01$). The greatest RE, the least FR, and the smallest LS were obtained when AI was done during SA (Table 1). This pattern was similar to that shown by sows undergoing AI with liquid semen on the same farm. However, the magnitude of variation between periods of the year was different for FT and liquid semen (Figure 1). The increased RE and reduced FR with respect to WS during SA were more noticeable in sows undergoing AI with FT semen.

The FT-inseminated sows were separated according to how long before ovulation the DUI inseminations were to be performed (pre-, peri-, or postovulatory AI). Using these categories, the proportions of sows in each group varied depending on the period of the year ($P \leq 0.05$). More periovulatory and fewer preovulatory FT inseminations were seen in WS than in SA (Figure 2). Farrowing rates and LS achieved with pre-, peri-, or postovulatory FT AI in both periods of the year are compiled in Table 2. Pre- and postovulatory FT AI led to a reduced FR in SA than in WS ($P \leq 0.05$). Periovulatory FT AI consistently yielded greater FR, regardless of the period of the year ($P > 0.05$). Litter size did not vary according to ovarian status at the time of AI within the same period of the year, nor did it vary across periods of the year when the ovarian status was the same ($P > 0.05$).

Exp. 2: Influence of the Period of the Year on EOI

Figure 3 shows how the occurrence of ovulation after the onset of estrus varied between the 2 periods of the year in weaned sows with the same WEI. Overall, the EOI exhibited greater variability during SA than during WS. The longest ($P \leq 0.05$) EOI was observed during SA.

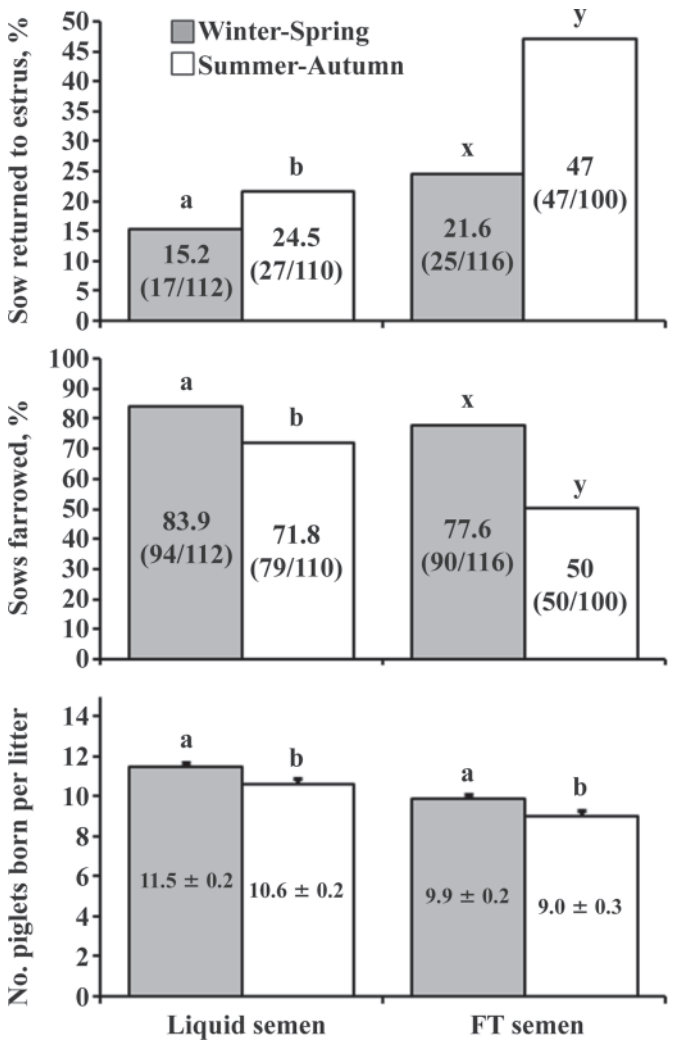

Figure 1. Reproductive variables (return to estrus, farrowing rate, and litter size) of weaned sows undergoing AI with liquid (cervically at 12 and 36 h after the onset of estrus with 3×10^9 sperm per dose) or frozen-thawed (FT, deep intrauterine insemination at 33 and 39 h after the onset of estrus with 1.5×10^9 sperm per dose) semen during winter-spring (November to April) or summer-autumn (May to October) in a farm located in southern Spain (Exp. 1). The numbers inside the bars indicate the percentage and total number (in parentheses) of sows. [a,b;x,y]Indicates differences at $P < 0.05$ and $P < 0.001$, respectively, between winter-spring and summer-autumn within the same semen source.

Exp. 3: Effect of Hormonal Induction of Ovulation on the Fertility and Prolificacy of Weaned DUI-Inseminated Sows Using FT Semen for SA

The distribution of FT-inseminated sows according to their ovarian status at the time of DUI did not differ ($P > 0.05$) between hormonally induced or spontaneously ovulated sows. There was no difference ($P > 0.05$) in FR or LS when spontaneous and hormonally induced ovulation were compared among FT artificially inseminated sows (Table 3). However, it is important

Table 2. Farrowing rates and litter sizes of weaned sows receiving deep intrauterine insemination (twice at 33 and 39 h after onset of estrus) with frozen-thawed semen ($1,500 \times 10^6$ sperm per AI dose) during winter-spring (WS; November to April) or summer-autumn (SA; May to October) in southern Spain[1]

	Farrowing rate, n		Litter size,[2] mean ± SEM	
Ovarian status at AI time	WS[3]	SA[4]	WS	SA
Preovulatory	77.4 (41/53)^{ab,x}	48.3 (29/60)^{b,y}	10 ± 0.4	8.6 ± 0.4
Periovulatory	87.8 (36/41)^{a}	79.2 (19/24)^{a}	10.2 ± 0.3	9.5 ± 0.5
Postovulatory	59.1 (13/22)^{b,x}	12.5 (2/16)^{c,y}	8.8 ± 0.6	9.0 ± 1
Overall	77.6 (90/116)	50 (50/100)	9.9 ± 0.2	9.0 ± 0.3

$^{a-c}$Values with different superscripts within a column differ by $P < 0.05$.

^{x,y}Values with different superscripts within a row differ by $P < 0.05$.

[1]The data are distributed according to ovarian status at the time of AI (Exp. 1). Preovulatory: large follicles visible before the 2 AI; periovulatory: ovulation occurred during or between the 2 AI; and postovulatory: corpus hemorrhagica visible before the first AI.

[2]Litter size indicates the total number of piglets born.

[3]WS = inseminations carried out from November to April.

[4]SA = inseminations carried out from May to October.

to highlight that those sows in which ovulation was induced showed the greatest fertility rates.

DISCUSSION

This study demonstrates that in southern Spain, under Mediterranean conditions, the outcome (in terms of fertility and prolificacy) of AI practiced on breeding sows under commercial conditions is clearly influenced by the period of the year when the AI was performed. This influence is more noticeable when the sows undergo AI with FT semen compared with the commonly used liquid semen. There was a considerable increase in RE and a subsequent reduction in FR and LS in sows that had been inseminated during SA compared with those inseminated during WS. This annual pattern of RE and FR has been well documented in naturally mated or liquid AI sows throughout the world (Peltoniemi and Virolainen, 2006). Some of these studies included similar environmental conditions to those in the Spanish Mediterranean area (Dominguez et al., 1996; Peña et al., 1998). Long photoperiod and high ambient temperatures are the main environmental cues determining seasonality in pigs, which is evidenced by a prolonged WEI and a reduction in fertility, particularly FR, during summer and early autumn (Martinat-Botte et al., 1984; Love et al., 1993; Peltoniemi et al., 1999, 2000) The decrease in the FR seems to be caused by an early disruption of pregnancy, with some sows showing delayed returns to estrus (Tast et al., 2002). Overall, these seasonality manifestations have been evidenced in the sows inseminated in the present study, regardless of whether they were inseminated with liquid or FT semen.

Unlike the above-mentioned studies, our experiments showed that the influence of period of the year was dependent on semen source. Whereas the outcome of pregnancy and prolificacy of sows undergoing AI with liquid or FT semen showed a similar pattern of annual variation, the drop in FR during SA was substantially greater for FT-AI sows than for LS-AI sows. To the best of our knowledge, no studies have reported similar data concerning seasonal effects on reproductive performance of FT-inseminated sows. The physiological basis for the increased sensitivity of FT semen to seasonality remains unknown. In the absence of possible variations in quality of FT spermatozoa (all AI doses used in the different experiments came from the same semen pool) and excluding the influence of some reproductive variables of sows, such as parity, lactation length, or WEI (they were the same for FT- and LS-inseminated sows), it is likely that the interaction between FT semen and the genital tract of the sow would be the main reason.

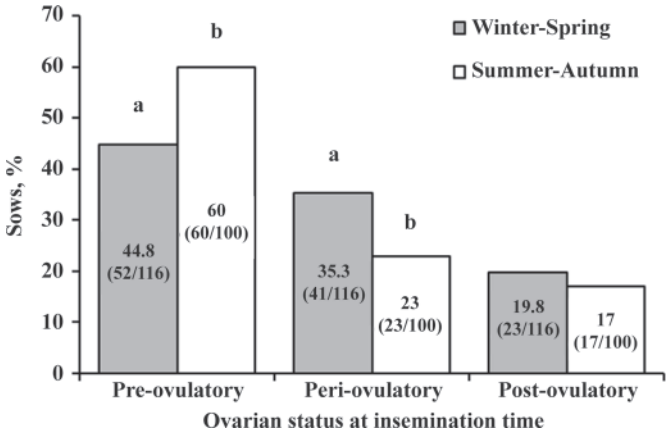

Figure 2. Distribution of weaned sows in each period of the year according to how long before or after ovulation the deep intrauterine inseminations were to be performed. Ovarian status was visualized at the moment of AI with frozen-thawed semen (at 33 and 39 h after onset of estrus). Pre-, peri-, and postovulatory ovarian status indicates that sows ovulated >39, 33 to 39, and <33 h after onset of estrus, respectively (Exp. 1). ^{a,b}Indicates statistical differences ($P < 0.05$) between winter-spring and summer-autumn within the same ovarian status. The numbers inside the bars indicate the percentage and total number (in parentheses) of sows.

Table 3. Farrowing rate and litter size of weaned sows with spontaneous (control) or hormonally induced (eCG+hCG) ovulation[1]

Ovarian status at AI time[2]	No. of sows, %		Farrowing rate, n		Litter size,[3] mean ± SEM	
	Control[4]	eCG+hCG[5]	Control	eCG+hCG	Control	eCG+hCG
Preovulatory	31 (58.5)	36 (65.5)	54.8 (17/31)	66.7 (24/36)	9.3 ± 0.6	10.4 ± 0.5
Periovulatory	14 (26.4)	11 (20)	71.4 (10/14)	90.1 (10/11)	9.5 ± 0.6	10.3 ± 0.4
Postovulatory	8 (15.1)	8 (14.5)	12.5 (1/8)	12.5 (1/8)	8.0 ± 0.0	7.0 ± 0.0
Overall	53	55	52.8 (28/53)	63.6 (35/55)	9.4 ± 0.4	10.3 ± 0.4

[1]Sows were once (hormonally induced ovulation) or twice (spontaneous ovulation) deep intrauterine inseminated (DUI) with frozen-thawed semen during summer-autumn (May to October) in southern Spain and distributed according to their ovarian status at the time of AI (Exp. 3).

[2]Preovulatory: large follicles visible before the 2 AI; periovulatory: ovulation occurred during or between the 2 AI; and postovulatory: corpus hemorrhagica visible before the first AI.

[3]Litter size indicates the total number of piglets born per litter.

[4]Weaned sows with spontaneous ovulation were twice DUI at 33 and 39 h after the onset of estrus.

[5]Weaned sows with induced ovulation [1,250 IU of eCG (Intervet International B.V. Boxmeer, the Netherlands) 24 h after weaning and 750 IU of hCG (Divasa Farmavic S.A., Gurb-Vic, Barcelona, Spain) 72 h later] were DUI at 37 h after hCG.

Liquid and FT spermatozoa differ in their functional lifespan within the female genital tract. This difference may explain the drastic decrease in fertility for FT semen during SA. In fertilization trials, it has been established that liquid preserved spermatozoa remain functional in the genital tract of sows for 18 h post-AI. In contrast, FT spermatozoa are functional for about 6 to 8 h (Waberski et al., 1994; Wongtawan et al., 2006). This difference explains why the interval between AI and ovulation is more important for FT-AI than for liquid-AI (Bolarín et al., 2006). By affecting the interval between AI and ovulation, seasonality might directly compromise the fertilizing ability of FT semen. In the current study, seasonal variation in the onset of EOI was deduced by evaluating the distribution of FT-inseminated sows in each of the 3 categories of ovarian status at AI time. The distribution pattern during SA showed a greater and lesser proportion of pre- and periovulatory AI, respectively, when compared with WS, regardless of the fact that AI was performed at the same time (counted from the onset of estrus) in both periods of the year. The overall fertility of FT semen depends on how close AI occurs relative to the moment of ovulation (Bolarín et al., 2006). This explains why

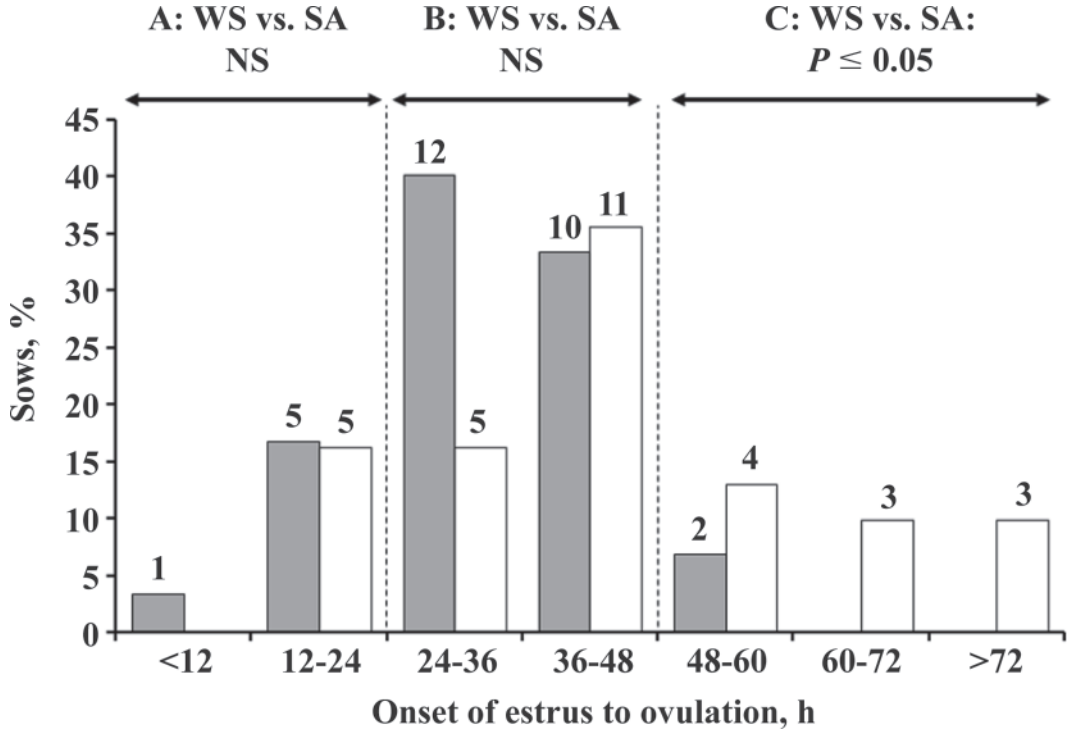

Figure 3. Distribution of multiparous sows (weaning-to-estrus interval of 4 d) during winter-spring (WS, bars in gray) or summer-autumn (SA, bars in white) in southern Spain according to estrus-to-ovulation interval (Exp. 2). The numbers above the bars indicate the number of sows in each group. For chi-square analysis, sows were grouped into 3 subjective categories: before (A), during (B), and after (C) the expected spontaneous ovulation time (24 to 48 h). NS = not significant.

reduced fertility was observed among FT-AI sows during SA than during WS. The preovulatory fertility difference between periods of the year was surprising and seems to indicate that the percentage of sows undergoing AI long before ovulation was greater during SA than during WS. To expand on this finding, a second experiment was carried out. The ovaries of weaned sows were evaluated by TRU in both periods of the year to identify differences in EOI. The period of the year influenced the EOI. It was longer during SA than WS. The tendency of sows to ovulate later in SA explains the reduced fertility of preovulatory AI during SA. This finding confirms that a large number of sows underwent AI too soon before ovulation during SA. The effect of season or period of the year on EOI remains controversial. Belstra et al. (2004) reported seasonal changes in EOI when weaned sows were studied using TRU every 6 h. Sows ovulated later in summer than they did during spring. However, other studies have not detected seasonal differences in EOI (Weitze et al., 1994; Knox and Zas, 2001). The factors responsible for seasonal differences in EOI are not well understood. However, differences in the duration of estrus could be a contributing factor. There are some reports indicating that the duration of estrus is, on average, longer in the SA months (Soede and Kemp, 1997), and it seems that the farm itself, particularly depending on its geographical location, and the genetics of the population, could contribute to this effect (Belstra et al., 2004). Elevations of body temperature at critical periods have been shown to influence ovarian function (Lucy et al., 2001). Such influence could lay behind the longer EOI recorded during SA than during WS in the present study. Furthermore, it has been reported that heat stress has a delayed effect on ovarian function, particularly on follicular development (Guzeloglu et al., 2001; Roth et al., 2001), which could explain the increased EOI variability exhibited during SA.

The results of the 2 first experiments demonstrated a drastic drop in the fertility of FT-inseminated sows during SA. This decrease could be related to increased variability of EOI during SA. This variability could cause FT-AI to fall outside of the preovulatory interval of 4 to 8 h. Therefore, it seems reasonable to suggest that specific AI strategies should be designed for FT semen during the SA months. These strategies might be particularly important in warm regions and could allow consistent fertility levels throughout the year. Performance of a third AI would be logical since it would increase the probability that at least one AI fell inside the safe interval of 4 to 8 h before ovulation (Wongtawan et al., 2006). Alternatively, induction of ovulation using hormones could provide an accurate AI ovulation interval. Such induction might be a better option because an additional AI would require considerable expense. Administration of eCG combined with hCG induced estrus and ovulation in weaned sows (Christenson and Teague, 1975; Hühn et al., 1996). These hormones allow single AI at a predetermined time without estrus detection, thus reducing expenses (particularly labor expenses). Furthermore, eCG + hCG treatment has been demonstrated to minimize the effects of season on fertility in weaned sows (Bates et al., 1991; Almond and Bilkei, 2006). A common protocol for inducing synchronized estrus and ovulation time in swine consists of administration of eCG at 24 h after weaning followed by hCG 60 to 72 h later (Hühn et al., 1996; Duanyai and Srikandakumar, 1998). The treatment schedule used in this study was 1,250 IU of eCG at 24 h after weaning followed by 750 IU of hCG 72 h later. This schedule was effective in weaned sows because it promoted simultaneous follicular development (Bolarín et al., 2009). This method kept most ovulations within a window of 40 to 42 h after hCG administration in weaned sows treated during winter (A. Bolarín and J. Roca, unpublished data) and resulted in an FR as great as 78% after a single DUI with 1×10^9 FT spermatozoa (Roca et al., 2003). However, the FR obtained during SA was just above 60%. Similar seasonal fertility responses to gonadotropin treatments in weaned sows have been detected (Bates et al., 2000). These seasonal differences in FR suggest that the effect of eCG + hCG treatment on ovulation synchronization is seasonally dependent. A less well synchronized response to eCG + hCG treatment during SA was confirmed in Exp. 3, which showed variable distribution of hormonally treated sows in pre-, peri-, and postovulatory conditions at the time of AI.

Hormone (eCG + hCG) treatment did not ameliorate the negative influence of climatic conditions during SA on the fertility of FT semen. However, the value of this treatment should not be underestimated because it led to a 20% (11 points) improvement in FR and 0.9 more piglets per litter. Both of these variables are of economic and productive relevance. As always, improved fertility should be balanced against the cost of hormone doses, labor fees, animal welfare issues, and consumer requirements. Ethical concerns related to the use of exogenous hormones in agriculture must be also considered.

In conclusion, the data obtained in this study demonstrate that, in southern Spain, under Mediterranean conditions, the outcome (in terms of fertility and prolificacy) of AI practiced on breeding sows under commercial conditions is clearly influenced by the period of the year when the AI was performed. The mean FR and LS size were less during SA than during WS. This seasonal pattern was similar to that in sows undergoing AI with liquid semen. However, the magnitude of variation was more noticeable for FT-inseminated sows. This finding could be related to the variation in the time of ovulation after onset of estrus observed in SA. Such variation makes it difficult to ensure that AI occurs near ovulation. The application of eCG and hCG together with a single AI at a fixed time did not substantially improve the fertility of FT-inseminated weaned sows during SA. Future studies on this matter are warranted.

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CONCLUSIONES

Conclusiones

1. La ecografía transrectal es una herramienta útil, inocua, precisa y eficaz para la detección y el contaje de folículos pre-ovulatorios y para la determinación del momento de ovulación en los ovarios de cerdas, aunque puede subestimar el número de folículos cuando éste es elevado.

2. La inseminación intrauterina profunda (DUI) es un procedimiento eficiente para el empleo comercial de los espermatozoides criopreservados en los programas de inseminación artificial de porcino. La DUI permite alcanzar excelentes resultados de fertilidad y prolificidad inseminando con un reducido número de espermatozoides criopreservados cuando son depositados momentos previos al momento de ovulación.

3. Las diferencias en el intervalo entre la inseminación y la ovulación explicarían gran parte de la variabilidad observada entre granjas en la fertilidad y prolificidad de los espermatozoides criopreservados.

4. El periodo del año influye de manera relevante en la fertilidad y prolificidad de las cerdas inseminadas con espermatozoides criopreservados, Ambos parámetros reproductivos son más bajos durante el verano y el otoño, lo cual estaría relacionado, en nuestra latitud geográfica, con la amplia variabilidad en el intervalo entre el inicio del estro y la ovulación que muestran las cerdas durante dichas estaciones.

ABREVIACIONES

Abreviaciones

IA/AI: Inseminación artificial.

DUI: Inseminación intrauterina profunda (Deep Intrauterine Insemination).

TRU: Ecografía transrectal (Transrectal Ultrasonography).

POFs: Folículos peri-ovulatorios (Peri ovulatory Follicles).

LAP: Laparoscopia.

LS: Tamaño de camada (Litter Size).

FT: Congelado-descongelado (Frozen-Thawed).

FR: Tasa de partos (Farrowing rate).

WS: Invierno-primavera (Winter-Spring).

SA: Verano-otoño (Summer-Autumn).

EOI: Intervalo estro-ovulación (Estrus-ovulation interval).

BTS: Beltsville Thawing Solution.

RE: Retorno al estro.

WEI: Intervalo destete-estro (Weaning-to-estrus Interval).

eCG: Gonadotropina coriónica equine.

hCG: Gonadotropina coriónica humana.

LEY: Diluyente con yema de huevo y lactosa (Lactose-egg yolk extender).

LEYGO: Diluyente LEY-Glicerol-Orvus-ES-Paste.

CL: Corpora lutea.

CH: Corpora hemorrágica.

ANEXO GRÁFICO

Fig. 1. a: Ecógrafo Pie Medical SC100 utilizado para llevar a cabo las ecografías transrectales en las cerdas. **b:** Sonda sectorial de 5 MHz. **c:** Sonda lineal de 7.5 MHz.

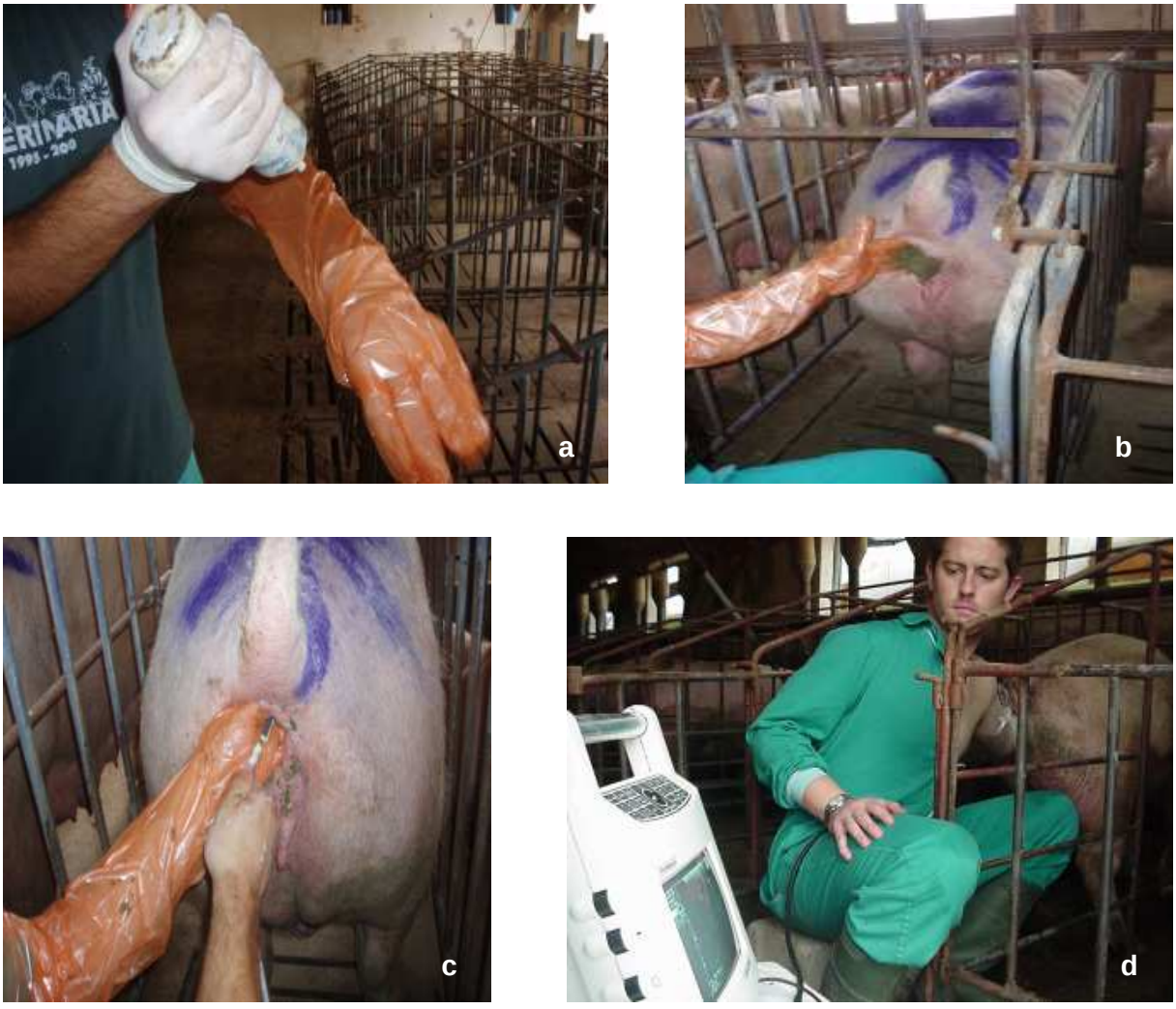


Fig. 2. **a:** Lubrificación del guante con vaselina, para facilitar la práctica de la ecografía transrectal, **b:** Retirada de heces para limpiar el recto y optimizar el contacto entre la sonda y la mucosa uterina, **c:** Inserción de la sonda vía transrectal, previa dilatación el músculo esfínter del ano, **d:** Ecografía transrectal.

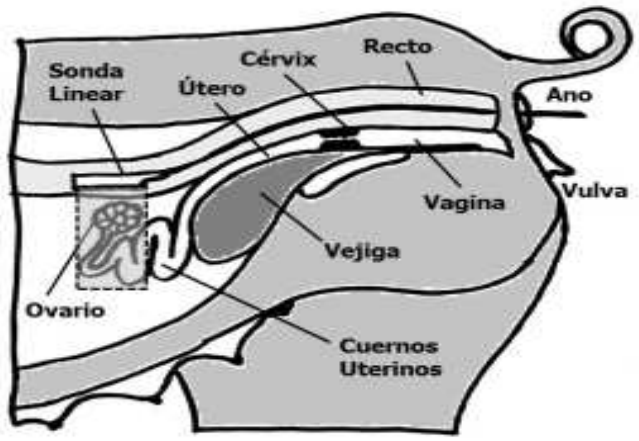


Fig. 3. Esquema de la técnica de ecografía transrectal para identificación de estructuras ováricas, y posiciones relativas de la vejiga de la orina como referencia, y del ovario respecto del recto.

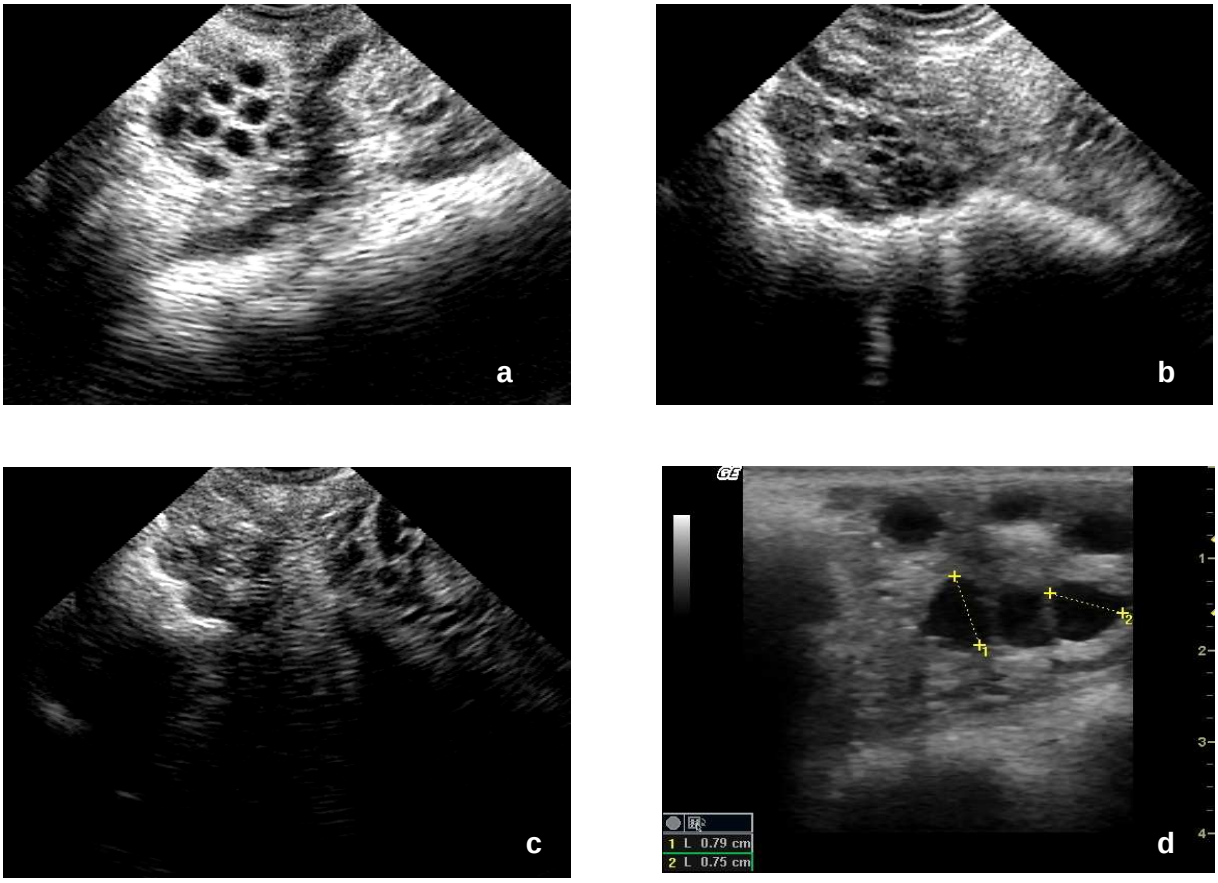


Fig. 4. a: Ovario pre-ovulatorio, con folículos mayores de 7 mm bien definidos, **b:** Ovario peri-ovulatorio, con folículos de tamaño irregular, mal definidos, y algunos cuerpos rojos evidentes, **c:** ovario post-ovulatorio, con evidencia de cuerpos lúteos, **d:** Medición de folículos. Sólo mediante ecografías seriadas logramos identificar la tendencia folicular al crecimiento, y el momento en que los folículos desaparecen.

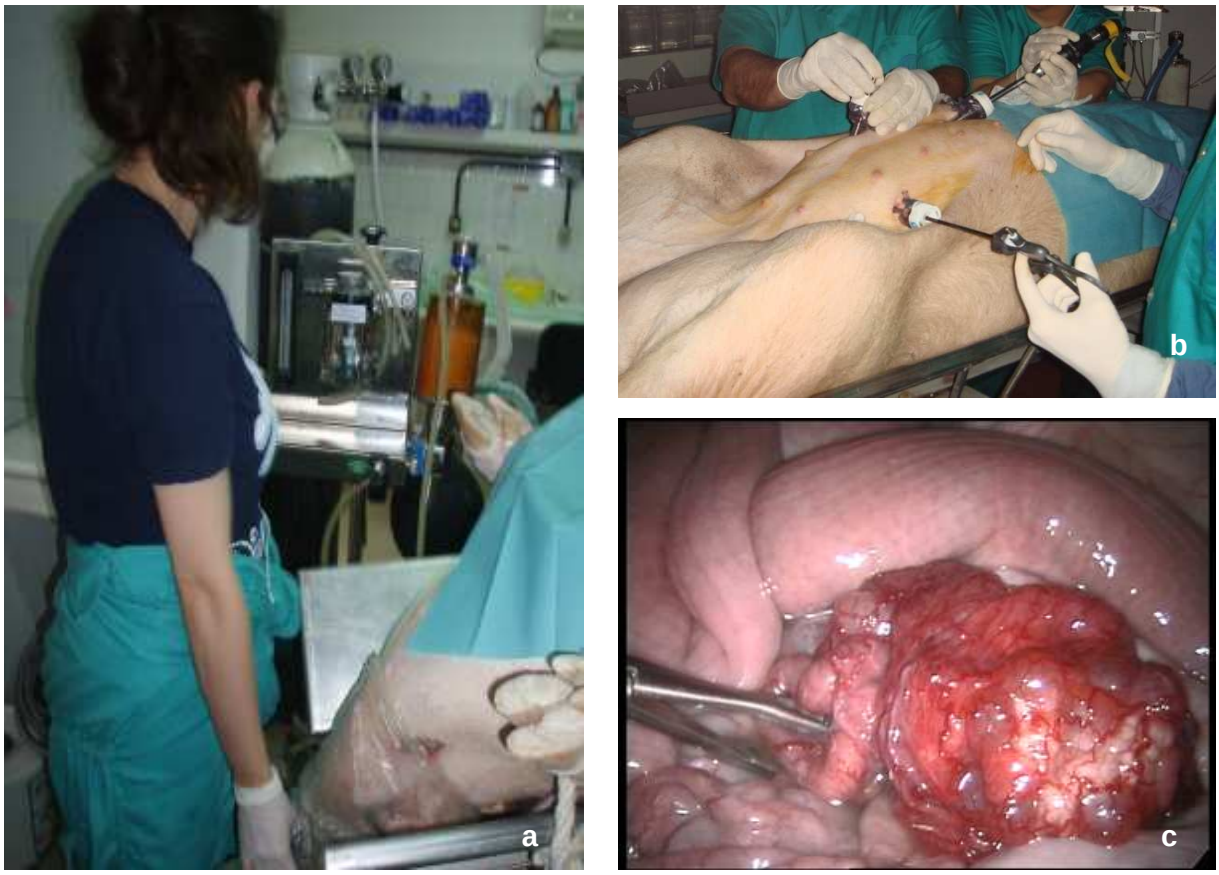


Fig. 5. a: Anestesia inhalatoria a una cerda en quirófano, **b:** Laparotomía, **c:** Contaje folicular mediante visualización directa del ovario por laparotomía.

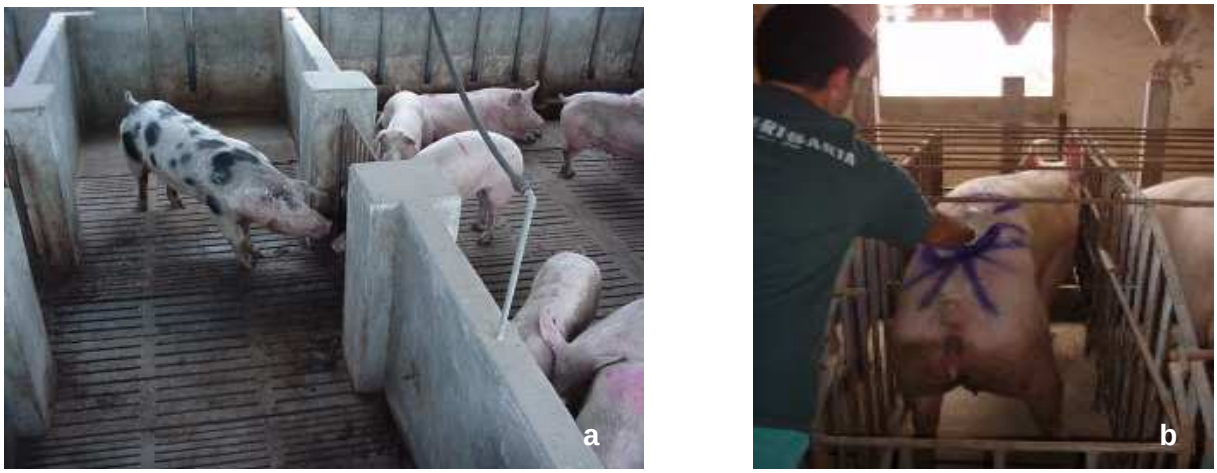


Fig. 6. **a:** Detección de estro en parques en presencia de un verraco recela, **b:** Confirmación de estro al hombre en jaula mediante reflejo de inmovilidad.

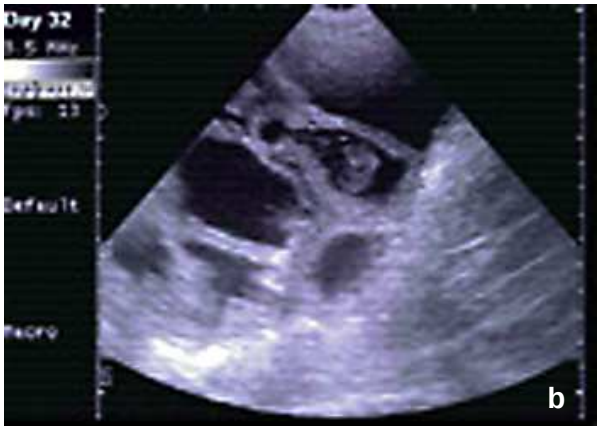
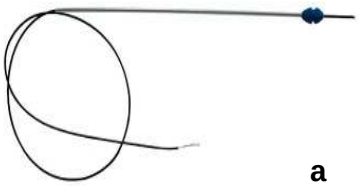
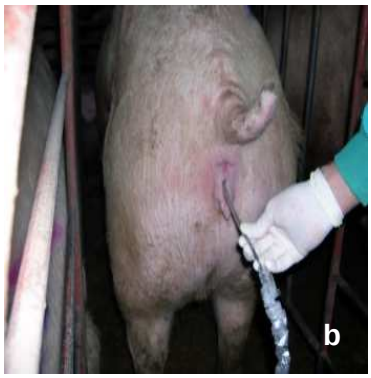


Fig. 7. a y b: Confirmación de gestación por visualización de la vesícula embrionaria, mediante ecografía trans-abdominal, **c:** control y seguimiento de partos y camadas.





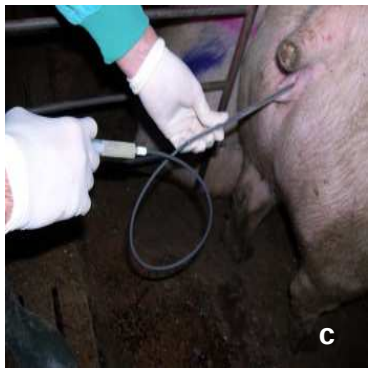


Fig. 8. a: Catéter de inseminación intrauterina profunda Deep Blue, Minitüb, **b:** Inserción higiénica de la sonda a través de un catéter tradicional de inseminación artificial, para inseminación intrauterina profunda (DUI), **c:** Técnica de inseminación intrauterina profunda con dosis de inseminación de 5 ml.

Fig. 9. a: Biocongelador (Icecube 1810, Minitüb), **b:** Racks de pajuelas para almacenaje de espermatozoides criopreservados, **c:** **E**tiquetadora de pajuelas de semen criopreservado..

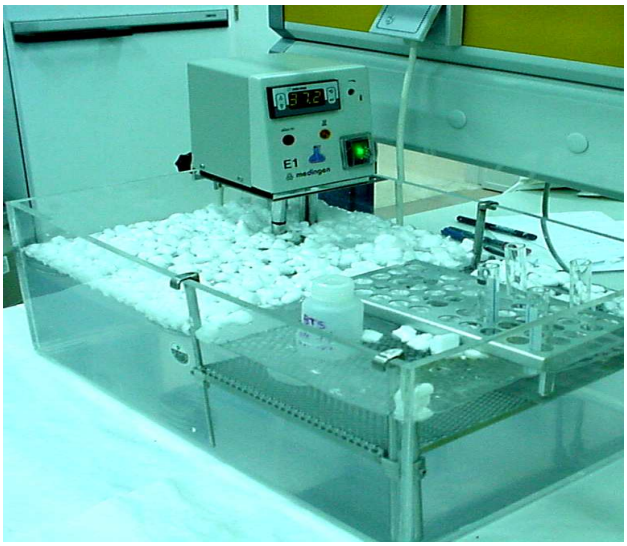


Fig. 10. Descongelación del semen criopreservado, en baño termostatizado a 38 ºC.

APÉNDICE

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Year	Abbreviated Journal Title (*linked to journal information*)	ISSN	JCR Data					
			Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life
2009	ANIM REPROD SCI	0378-4320	4274	1.563	2.107	0.322	264	6.3

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Year	Abbreviated Journal Title (*linked to journal information*)	ISSN	JCR Data				
			Total Cites	Impact Factor	Immediacy Index	Articles	Cited Half-life
2006	THERIOGENOLOGY	0093-691X	8036	1.898	0.290	427	6.8

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Year	Abbreviated Journal Title (*linked to journal information*)	ISSN	JCR Data					
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2009	J ANIM SCI	0021-8812	22105	2.466	2.760	0.425	463	>10.0