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**Effect of Hoechst 33342 on stallion spermatozoa incubated in KMT or Tyrodes modified
INRA96**

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ABSTRACT

The only known means of effectively separating populations of X and Y bearing sperms is the
Beltsville sexing technology. The technology implies that each individual sperm is
interrogated for DNA content, measuring the intensity of the fluorescence after staining the
spermatozoa with Hoechst 33342. Because there are no data regarding the effect of the
staining on stallion sperm, ejaculates were incubated up to 90 minutes in presence of 0, 4.5, 9,
22.5, 31.5, 45, 54, 67.5, 76.5 and 90 μ M of Hoechst 33342, in two media, KMT or INRA-

Tyrodes. After 40 and 90 minutes of incubation, motility (CASA) and membrane integrity (flow cytometry after YoPro-1/Eth staining) were evaluated. In KMT extender sperm motility significantly decreased after 45 minutes of incubation when sperm were incubated in the presence of concentrations of Hoechst of 45 μ M or greater ($P<0.05$). When incubated in modified INRA96, stallion spermatozoa tolerated greater concentrations of Hoechst, because sperm motility only decreased when incubated in presence of 90 μ M ($P<0.05$) and membrane integrity was not affected. After 90 minutes of incubation the same effect was observed, but in this case at concentrations over 45 μ M the percentage of total motile sperm was also reduced although only in samples incubated in KMT. To produce this effect in samples incubated in Tyrodes modified INRA 96, Hoechst had to be present at concentrations over 67.5 μ M. Apparently, the detrimental effect of Hoechst to stallion spermatozoa varies depending on the media, and INRA modified extender may be an alternative to KMT.

Keywords: Stallion, Sperm, CASA, Hoechst 33342, Sex sorting

1. Introduction

The selection of the sex of the foal will offer obvious advantages for the horse industry because colts are preferred for dressage and endurance while fillies are desired as polo ponies. To date the only effective methodology validated in numerous laboratories is based on measuring the relative DNA content of X and Y chromosome bearing spermatozoa (Keeler et al., 1983; Morrell et al., 1988; Johnson, 1995; 1997; Johnson et al., 1999). This technology involves staining spermatozoa with the probe Hoechst 33342 (Morrell et al., 1988; Garner, 2009), a non-intercalating permanent nucleic acid stain that binds to the minor groove of the

49 DNA helix (Teng et al., 1988). Stallion sperm are stained, in most studies, with
50 concentrations of Hoechst 33342 ranging from 15 to 90 μM (Buss et al., 2005; Mari et al.,
51 2010; Gibb et al., 2011) although occasionally larger dosages have been used (Buchanan et
52 al., 2000) for up to 90 minutes at 34 or 35 $^{\circ}\text{C}$. After staining, a flow cytometer cell sorter is
53 used to detect the difference in the relative amount of DNA and subsequently to separate the
54 spermatozoa. Sex sorting is commercially available in the cattle industry (Frijters et al.,
55 2009), however developments in sex sorting technology in the horse industry are much less
56 developed (Gibb et al., 2011), due amongst many other reasons to a lesser fertility of the
57 sorted sperm and a reduced efficiency of the sex sorting procedure in horses, owing at least
58 partially to the opaque skim milk based media used during Hoechst 33342 staining. To
59 improve the efficacy of this technology in horses, all the stresses that the spermatozoa suffer
60 during the procedure should be critically evaluated (Morris, 2005; Rath et al., 2009). These
61 stresses include the amount of dilution, high pressure in the flow cytometer, laser exposition
62 and the possible detrimental effect of the staining with Hoechst 33342. The latter has not been
63 critically evaluated with horses. There are, however, some controversial reports in swine and
64 in cattle studies. In the first case a potential protective effect to boar sperm during sorting has
65 been proposed (Guthrie et al., 2002), while in bulls Hoechst 33342 at a concentration of 90
66 μM reduced oxygen consumption of thawed sperm (Downing et al., 1991), however, 900 μM
67 Hoechst 33342 completely abolished human sperm motility, while little effect was noted at 90
68 μM (Watkins et al., 1996), however, in boars, motility significantly decreased at 60 μM , and
69 was completely abolished at 90 μM (Vazquez et al., 2002). The improvement of this
70 technology in horses requires an in-depth knowledge of all the potential factors affecting the
71 process, especially the identification of the causes of any damage that the stallion
72 spermatozoa may suffer during the process to enable the latter to be minimized. Because

there are few data on the possible detrimental effect of Hoechst 33342 for stallion sperm, the present study was conducted to investigate the effect of a wide range of concentrations of Hoechst 33342 on stallion spermatozoa incubated in two different extenders. One of the extenders was modified to reduce opacity of the media without reduction the capacity to preserve spermatozoa during incubation.

2. Materials and methods

2.1. Semen collection

Semen (four ejaculates per stallion) was obtained from two Pure Spanish Stallions (PRE), one cross-breed, one Spanish Arabian horse and one Spanish Sports Horse individually housed at the Veterinary Teaching Hospital of the University of Extremadura, Cáceres, Spain. The stallions were maintained according to institutional and European regulations, and ejaculates were collected on a regular basis (two collections/week) during the 2010 breeding season, using a Missouri model artificial vagina with an inline filter to separate the gel fraction, lubricated and pre-warmed at 45 to 50 °C. The collected ejaculate was immediately transported to the laboratory for evaluation and processing.

2.2. Semen processing

The filtered ejaculate was then divided in two, each part being extended 1:1 (v/v) in one of two extenders: INRA96 (IMV, L'Aigle, France)-Tyrodes (65% of INRA96 and 35% of modified Tyrodes solution, yielding 71.86 mM NaCl, 1.0 mM NaPyruvate, 24.99 mM NaHCO₃, 3.1 mL Lactic Acid (60%), 1.99 mM CaCl₂, 25.08 mM KCl, 0.84 mM MgCl₂*6H₂O, 0.406 mM NaH₂PO₄ and 9.98 mM HEPES) and Kenney Modified Tyrodes (65% of Kenney extender, consisting of 49g of Skim Milk Powder in one liter of Nanopure

water and 133 mM of glucose, and 35% of modified Tyrodes solution, with 163 μ M of Penicilin G and 68.4 μ M of Streptomycin Sulfate added). Samples were centrifuged at 400 *g* for 10 minutes at room temperature (22 °C) and the resulting sperm pellet was re-extended each in the same medium to a final concentration of 111×10^6 spermatozoa/mL, measured on a Bürker chamber. All products were bought from Sigma-Aldrich Corporation, St. Louis, MO, USA, and pH adjusted to 7.2.

The two samples obtained were each split into 10 aliquots, placing 1.8 mL in each tube. To achieve a concentration of 100×10^6 spermatozoa/mL, 200 μ L of the corresponding extender with 0, 1, 2, 5, 7, 10, 12, 15, 17 and 20 μ L of a stock solution of 8,89 mM Hoechst 33342 (Sigma-Aldrich Corporation, St. Louis, MO, USA), prepared in Nanopure water, were added to the aliquots. This permitted obtaining final concentration values ranging from 0 to 90 μ M.

Ejaculates were then incubated up to 90 minutes in presence of 0, 4.5, 9, 22.5, 31.5, 45, 54, 67.5, 76.5 and 90 μ M Hoescht 33342, in two media, KMT or INRA-Tyrodes. After 40 and 90 minutes of incubation, motility (CASA) and membrane integrity (flow cytometry after YoPro-1/Eth staining) were evaluated.

2.3. Sperm motility analysis

Motility was measured using a computer-assisted sperm analysis system (CASA System, ISAS® Proiser, Valencia, Spain), based on the examination of 25 consecutive, digitalized images obtained from a single field using a 10x negative phase contrast objective in a light microscope (Olympus CX41, Tokyo, Japan), as previously described for stallion sperm (Macias Garcia et al., 2009; Ortega-Ferrusola et al., 2009). Images were taken with a time lapse of 1s, being image capture speed of one every 40ms. The number of objects

incorrectly identified as spermatozoa was minimized on the monitor by using the playback function. Regarding the setting parameters of the program, a spermatozoon was considered immotile when presenting a VCL $<10 \mu\text{m/s}$, and motile if it was $>15 \mu\text{m/s}$. Cells which deviated $<45\%$ from a straight line were designed as progressive motile. Cells with a VCL $>45 \mu\text{m/s}$ were considered as rapid sperm. Sperm motion absolute and re-calculated kinematic parameters measured by CASA included the following:

Curvilinear Velocity (VCL)	$\mu\text{m/s}$	Measures the sequential progression along the true trajectory
Linear Velocity (VSL)	$\mu\text{m/s}$	Measures the straight trajectory of the spermatozoa per unit time
Mean Velocity (VAP)	$\mu\text{m/s}$	Measures the mean trajectory of the spermatozoa per unit time
Linearity Coefficient (LIN)	%	$\text{VSL/VCL} \times 100$
Straightness Coefficient (STR)	%	$\text{VSL/VAP} \times 100$
Wobble Coefficient (WOB)	%	$\text{VAP/VCL} \times 100$
Average lateral head displacement (ALH)	μm	Measures the mean head displacement along the curvilinear trajectory
BCF	Hz	<i>Number of times the sperm head crosses the mean path/second</i>

2.4. Assessment of subtle sperm membrane changes and viability

Early sperm membrane changes and viability were determined as described in Peña et al. (Pena et al., 2005) with modifications for adaptation to the equine species (Ortega-Ferrusola et al., 2008; Ortega Ferrusola et al., 2009b; Ortega Ferrusola et al., 2009c). In brief, one mL of sperm suspension (5×10^6 spermatozoa/mL) was loaded with 3 μ L of Yo-Pro-1 (25 μ M) and one μ L of Ethidium Homodimer-1 (1.167 mM) (Molecular Probes Europe), which -after thorough mixing- was incubated at 37 °C in the dark for 16 min. This staining distinguishes four sperm subpopulations. The first is the subpopulation of unstained spermatozoa. These spermatozoa are considered alive and without any membrane alteration. Another sperm subpopulation consists of Yo-Pro-1 positive cells emitting green fluorescence. In the early stages of apoptosis there is a modification of membrane permeability that selectively allows entry of some non-permeable DNA-binding molecules. This subpopulation groups spermatozoa which may show a shift to another physiological state or early damage, since membranes become slightly permeable during the first steps of injury, enabling Yo-Pro-1 but not ethidium homodimer to penetrate the plasma membrane (Idziorek et al., 1995). None of these probes enters intact cells. Finally, two subpopulations of necrotic spermatozoa were easily detected: early necrotic, spermatozoa stained both with Yo-Pro-1 and ethidium homodimer (emitting both green and red fluorescence), and late necrotic spermatozoa, cells stained only with ethidium homodimer (emitting red fluorescence).

2.5. Flow cytometry analysis

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami, FL, USA) flow cytometer equipped with standard optics, an argon-ion laser (Cyonics, Coherent, Santa Clara, CA, USA) performing 15 mW at 488 nm and

EXPO 2000 software. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. Non-sperm events (debris) were identified and eliminated from the analysis as described in (Petrunkina et al., 2010). Forward and sideways light scatter were recorded for a total of 10,000 events per sample. Samples were measured at flow rate of 200 to 300 cells/sec. Green fluorescence was detected in FL1 (525 nm band pass filter) red fluorescence was detected in FL3 (620 nm band pass filter), and orange fluorescence in FL2 (570 nm band pass filter).

2.6. Statistical analysis

Data were first examined using the Kolmogorov-Smirnov test to determine their distribution, a multivariate analysis of variance was performed (ANOVA) and when significant differences were found, the non-parametric Mann-Whitney U-test was used to compare pairs of values directly if data did not adjust to a normal distribution. All analyses were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL). The Spearman non-parametric test was used to study the correlations among apoptosis and autophagy and the results of the sperm analysis. Significance was set at $P < 0.05$

3. Results

3.1. Effect on sperm motility and kinematics at the beginning of incubation at 35 °C

At concentrations equal or above 31.5 μM Hoechst 33342 affected some parameters of sperm kinematics, however, there was no effect on the percentages of total motile sperm, progressive motile or the percentage of rapid sperm, these changes related to linearity, straightness and wobble. VCL was reduced by Hoechst 33342 at 45 μM and 67.5 μM ($P < 0.01$) but only in those spermatozoa incubated in KMT extender. However at 76.5 and 90

179 μM , Hoechst 33342 VCL was reduced in both extenders ($P<0.05$). VAP was reduced when
180 spermatozoa were incubated in presence of 67.6 ($P<0.05$) and 90 μM ($P<0.01$) Hoechst
181 33342 but only in those samples extended in KMT (Figures 1-3).

183 3.2. Effect on sperm motility and kinematics after 40 minutes of incubation at 35 °C

184 The first changes were observed in those spermatozoa incubated at a concentration of
185 31.5 μM . The changes observed were a decrease in ALH and an increase in the linearity in
186 spermatozoa incubated in Tyrodes modified INRA 96. Incubation in the presence of 45 and
187 55 μM resulted in a decrease in the percentage of progressive, rapid sperm and VAP, but only
188 in samples extended in KMT ($P<0.05$), VCL was reduced in both extenders ($P<0.05$) while
189 linearity was increased in samples extended in Tyrodes-Modified INRA ($P<0.05$). At
190 concentrations of 67.5 and 76.5 μM , there was also a decrease in the percentage of total motile
191 sperm ($P<0.05$) but only in samples extended in KMT. At the concentration of 90 μM the
192 decrease was observed in both extenders ($P<0.05$; Figures 1-3).

194 3.3. Effect on sperm motility and kinematics after 90 minutes of incubation at 35 °C

195 Hoechst 33342 staining produced a dose-dependent effect on sperm motility and
196 kinematics. Concentrations above 31.5 μM resulted in a significant effect, reducing the
197 percentage of progressive motile sperm, the percentage of rapid progressive sperm and VCL
198 in both extenders. At a concentration of 45 μM and 54 μM , the staining also reduced the
199 percentage of total motile sperm, but only when incubated in KMT media and not in Tyrodes
200 Modified INRA 96. Similarly, the concentration of 45 μM reduced VAP, but only in
201 spermatozoa incubated in KMT media. Concentrations equal or above 67.5 μM also resulted
202 in a reduction in the percentage of total motile sperm in both extenders. Overall motility

values were superior in samples incubated in Tyrodes modified INRA 96 both at 40 and 90 minutes of incubation ($P<0.01$; Figures 1-3).

3.4. Effect on early membrane changes and viability after incubation up to 90 minutes

There was no effect of the Hoechst 33342 in the sperm membranes of spermatozoa at any time considered in spermatozoa incubated in both media (Figure 4).

4. Discussion

In the present study, the effect of Hoechst 33342 on stallion spermatozoa incubated up to 90 minutes at 35° C was evaluated. This is the first report in stallions evaluating the effect of the dye on sperm motility and kinematics using CASA analysis and sperm membranes evaluated using flow cytometry. Hoechst 33442 exerted a detrimental effect on sperm motility and kinematics that was time- and dose-dependent, but had no effect on the percentage of intact sperm or those showing early or late membrane damage. Interestingly the detrimental effects were reduced in Tyrodes modified INRA 96 extender. Even at the beginning of the incubation period, the dye was able to modify sperm kinematics at concentrations above 31.5 μM , however, the most striking effects were at concentrations above 67.5 μM when circular velocity was affected; after 40 minutes of incubation also an effect on the percentage of rapid sperm was observed at concentrations above 45 μM , but only in KMT extender. This parameter was only significantly affected in samples extended in Tyrodes modified INRA 96 if Hoechst was present at 90 μM .

After 90 minutes of incubation, differences in the sperm movement characteristics disappeared, but there was a clear effect of the probe on the percentages of motile, rapid sperm and velocities at concentrations above 31.5 μM . It is difficult to establish comparisons

because the amount of information available is limited. However, Clulow et al. (2009) reported a decrease in total motility and an increase in the number of dead spermatozoa after incubation for 90 minutes in the presence of Hoechst 33342 at concentrations ranging from 22.5 to 67.5 μ M, but the exact concentration of the dye for every individual stallion was not given. Kenney's modified Tyrodes media (KMT; Padilla and Foote, 1991) has been the main media used for incubation and staining stallion sperm to date (Buchanan et al., 2000; Buss et al., 2005; Mari et al., 2010), although recently it has been demonstrated that clear diluents increases the sex sorting efficiency of stallion sperm (Gibb et al., 2011). However in the latter study the effect of the staining on stallion sperm was not included in the experimental protocol. In the present study, the detrimental effect of Hoechst 33342 varied with the extender and thus can be minimized using defined extenders. Minimizing sperm damage along all the steps of the sorting procedure will improve the efficiency of this technology and will facilitate a greater use of sexed semen (Rath et al., 2009). In this regard, Tyrodes modified INRA 96 appeared superior to KMT, being able to keep the percentage of total motile, rapid sperm and velocities more effectively than KMT. These sperm parameters have been recently related to stallion fertility (Love, 2011). The fact that effects on sperm motility and kinematics were seen using Hoechst 33342 concentrations on the range used to stain stallion spermatozoa is noteworthy. Studies in pigs demonstrated that Hoechst 33342 was not detrimental in the range used to stain pig sperm (Vazquez et al., 2002). In bull sperm, apparently the staining does not affect the characteristics of sperm movement as determined using CASA analysis (Penfold et al., 1998). Differences among species are difficult to interpret although factors such as oxygen consumption may be involved (Downing et al., 1991). In relation to this, the greater impact on sperm motility in stallions compared with pigs may be related to the different management of energy resources in boar and stallion sperm.

While boar sperm obtains energy for motility mainly from anaerobic pathways (Marin et al., 2003), stallion sperm appear to be more dependent on oxidative phosphorylation (Pena et al., 2009; Ortega Ferrusola et al., 2010). Furthermore, differences in lipid composition of the sperm membranes may be involved in differing susceptibility to Hoechst 33342, because the major fatty acid present varies amongst boars (Cerolini et al., 2000), bulls (Schiller et al., 2003) and stallions (Macias Garcia et al., 2011).

The most striking finding in the present study, is the extent of the deleterious impact of Hoechst 33342 varies depending on the media used to extend the sperm. As a first practical application, one of the damages of the sex sorting procedure can be diminished simply by changing the media of incubation with the probe. In this regard, Tyrodes modified INRA-96 was able to maintain greater sperm velocities and percentages of total and progressive motile sperm at concentrations and times when KMT was not able to sustain motility. Greater sperm motilities are related to greater fertility in stallions (Love, 2011), in addition to human studies indicating that the maintenance of greater sperm velocities are essential for fertilization to occur (Olds-Clarke, 1996; De Geyter et al., 1998). Furthermore, a recent study in red deer clearly relates sperm velocity and fertility (Malo et al., 2005). Thus a steady increasing body of scientific evidence stresses the importance of sperm velocity as an attribute of fertile sperm.

In the present study the effect of Hoechst 33342 of sperm membrane intactness and early changes were also evaluated. A combination of probes was used that allows the identification of early damage on the sperm membrane (Ortega Ferrusola et al., 2009a; Ortega Ferrusola et al., 2009c) in an attempt to disclose subtle changes. Hoechst had no effect in sperm membrane intactness or in early damage.

In summary, Hoechst 33342 exerts a negative effect on sperm motility and kinematics, that is dose and time dependent without affecting the intactness of sperm membranes. Interestingly these detrimental effects can be minimized by incubating the spermatozoa in a modified INRA 96 media, in comparison to the traditionally used KMT extender, thus opening an approach to minimize the damage that stallion spermatozoa experience during the sorting process.

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References

- Buchanan, B.R., Seidel, G.E., Jr., McCue, P.M., Schenk, J.L., Herickhoff, L.A., Squires, E.L., 2000. Insemination of mares with low numbers of either unsexed or sexed spermatozoa. *Theriogenology* 53, 1333-1344.
- Buss, H., Clulow, J., Sieme, H., Maxwell, W.M., Morris, L.H., Sieg, B., Struckmann, C., Rath, D., 2005. Improvement of the freezability of sex-sorted stallion spermatozoa. *Anim. Reprod. Sci.* 89, 315-318.
- Cerolini, S., Maldjian, A., Surai, P., Noble, R., 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim. Reprod. Sci.* 58, 99-111.
- Clulow, J.R., Evans, G., Morris, L.H., Maxwell, W.M., 2009. Factors influencing the "sortability" of stallion spermatozoa into X- and Y-chromosome bearing populations. *Anim. Reprod. Sci.* 113, 220-228.
- De Geyter, C., De Geyter, M., Koppers, B., Nieschlag, E., 1998. Diagnostic accuracy of computer-assisted sperm motion analysis. *Hum. Reprod.* 13, 2512-2520.
- Downing, T.W., Garner, D.L., Ericsson, S.A., Redelman, D., 1991. Metabolic toxicity of fluorescent stains on thawed cryopreserved bovine sperm cells. *J. Histochem. Cytochem.* 39, 485-489.

- Frijters, A.C., Mullaart, E., Roelofs, R.M., van Hoorne, R.P., Moreno, J.F., Moreno, O., Merton, J.S., 2009. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? *Theriogenology* 71, 64-67.
- Garner, D.L., 2009. Hoechst 33342: the dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. *Theriogenology* 71, 11-21.
- Gibb, Z., Morris, L.H., Maxwell, W.M., Grupen, C.G., 2011. Use of a defined diluent increases the sex-sorting efficiency of stallion sperm. *Theriogenology* 75, 610-619...
- Guthrie, H.D., Johnson, L.A., Garrett, W.M., Welch, G.R., Dobrinsky, J.R., 2002. Flow cytometric sperm sorting: effects of varying laser power on embryo development in swine. *Mol. Reprod. Dev.* 61, 87-92.
- Idziorek, T., Estaquier, J., De Bels, F., Ameisen, J.C., 1995. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. *J. Immun. Meth.* 185, 249-258.
- Johnson, L.A., 1995. Sex preselection by flow cytometric separation of X and Y chromosome-bearing sperm based on DNA difference: a review. *Reprod. Fertil. Dev.* 7, 893-903.
- Johnson, L.A., 1997. Advances in gender preselection in swine. *J. Reprod. Fertil. Suppl.* 52, 255-266.
- Johnson, L.A., Welch, G.R., Rens, W., 1999. The Beltsville sperm sexing technology: high-speed sperm sorting gives improved sperm output for in vitro fertilization and AI. *J. Anim. Sci.* 77 Suppl 2, 213-220.
- Keeler, K.D., Mackenzie, N.M., Dresser, D.W., 1983. Flow microfluorometric analysis of living spermatozoa stained with Hoechst 33342. *J. Reprod. Fertil.* 68, 205-212.
- Love, C.C., 2011. Relationship between sperm motility, morphology and the fertility of stallions. *Theriogenology*. [76: 547-557](#)
- Macias Garcia, B., Gonzalez Fernandez, L., Morrell, J.M., Ortega Ferrusola, C., Tapia, J.A., Rodriguez Martinez, H., Pena, F.J., 2009. Single-layer centrifugation through colloid positively modifies the sperm subpopulation structure of frozen-thawed stallion spermatozoa. *Reprod. Domest. Anim.* 44, 523-526.
- Macias Garcia, B., Gonzalez Fernandez, L., Ortega Ferrusola, C., Morillo Rodriguez, A., Gallardo Bolanos, J.M., Rodriguez Martinez, H., Tapia, J.A., Morcuende, D., Pena, F.J., 2011. Fatty acids and plasmalogens of the phospholipids of the sperm membranes and their relation with the post-thaw quality of stallion spermatozoa. *Theriogenology* 75, 811-818.
- Malo, A.F., Garde, J.J., Soler, A.J., Garcia, A.J., Gomendio, M., Roldan, E.R., 2005. Male fertility in natural populations of red deer is determined by sperm velocity and the proportion of normal spermatozoa. *Biol. Reprod.* 72, 822-829.
- Mari, G., Rizzato, G., Merlo, B., Iacono, E., Bucci, D., Seren, E., Tamanini, C., Galeati, G., Spinaci, M., 2010. Quality and fertilizing ability in vivo of sex-sorted stallion spermatozoa. *Reprod. Domest. Anim.* 45, 331-335.
- Marin, S., Chiang, K., Bassilian, S., Lee, W.N., Boros, L.G., Fernandez-Novell, J.M., Centelles, J.J., Medrano, A., Rodriguez-Gil, J.E., Cascante, M., 2003. Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. *FEBS Lett.* 554, 342-346.
- Morrell, J.M., Keeler, K.D., Noakes, D.E., Mackenzie, N.M., Dresser, D.W., 1988. Sexing of sperm by flow cytometry. *Vet. Rec.* 122, 322-324.
- Morris, L.H., 2005. Challenges facing sex preselection of stallion spermatozoa. *Anim. Reprod. Sci.* 89, 147-157.

354 Olds-Clarke, P., 1996. How does poor motility alter sperm fertilizing ability? *J. Androl.* 17,
355 183-186.

356 Ortega Ferrusola, C., Gonzalez Fernandez, L., Macias Garcia, B., Salazar-Sandoval, C.,
357 Morillo Rodriguez, A., Rodriguez Martinez, H., Tapia, J.A., Pena, F.J., 2009a. Effect
358 of cryopreservation on nitric oxide production by stallion spermatozoa. *Biol. Reprod.*
359 81, 1106-1111.

360 Ortega Ferrusola, C., Gonzalez Fernandez, L., Macias Garcia, B., Salazar-Sandoval, C.,
361 Morillo Rodriguez, A., Rodriguez Martinez, H., Tapia, J.A., Pena, F.J., 2009b. Effect
362 of cryopreservation on nitric oxide production by stallion spermatozoa. *Biol. Reprod.*
363 81, 1106-1111.

364 Ortega Ferrusola, C., Gonzalez Fernandez, L., Morrell, J.M., Salazar Sandoval, C., Macias
365 Garcia, B., Rodriguez-Martinez, H., Tapia, J.A., Pena, F.J., 2009c. Lipid peroxidation,
366 assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa,
367 is stallion-dependent and is related to apoptotic-like changes. *Reproduction* 138, 55-
368 63.

369 Ortega Ferrusola, C., Gonzalez Fernandez, L., Salazar Sandoval, C., Macias Garcia, B.,
370 Rodriguez Martinez, H., Tapia, J.A., Pena, F.J., 2010. Inhibition of the mitochondrial
371 permeability transition pore reduces "apoptosis like" changes during cryopreservation
372 of stallion spermatozoa. *Theriogenology* 74, 458-465.

373 Ortega-Ferrusola, C., Macias Garcia, B., Suarez Rama, V., Gallardo-Bolanos, J.M., Gonzalez-
374 Fernandez, L., Tapia, J.A., Rodriguez-Martinez, H., Pena, F.J., 2009. Identification of
375 sperm subpopulations in stallion ejaculates: changes after cryopreservation and
376 comparison with traditional statistics. *Reprod. Domest. Anim.* 44, 419-423.

377 Ortega-Ferrusola, C., Sotillo-Galan, Y., Varela-Fernandez, E., Gallardo-Bolanos, J.M.,
378 Muriel, A., Gonzalez-Fernandez, L., Tapia, J.A., Pena, F.J., 2008. Detection of
379 "apoptosis-like" changes during the cryopreservation process in equine sperm. *J.*
380 *Androl.* 29, 213-221.

381 Padilla, A.W., Foote, R.H., 1991. Extender and centrifugation effects on the motility patterns
382 of slow-cooled stallion spermatozoa. *J. Anim. Sci.* 69, 3308-3313.

383 Pena, F.J., Rodriguez Martinez, H., Tapia, J.A., Ortega Ferrusola, C., Gonzalez Fernandez, L.,
384 Macias Garcia, B., 2009. Mitochondria in mammalian sperm physiology and
385 pathology: a review. *Reprod. Domest. Anim.* 44, 345-349.

386 Pena, F.J., Saravia, F., Johannisson, A., Walgren, M., Rodriguez-Martinez, H., 2005. A new
387 and simple method to evaluate early membrane changes in frozen-thawed boar
388 spermatozoa. *Int. J. Androl.* 28, 107-114.

389 Penfold, L.M., Holt, C., Holt, W.V., Welch, G.R., Cran, D.G., Johnson, L.A., 1998.
390 Comparative motility of X and Y chromosome-bearing bovine sperm separated on the
391 basis of DNA content by flow sorting. *Mol. Reprod. Dev.* 50, 323-327.

392 Petrunikina, A.M., Waberski, D., Bollwein, H., Sieme, H., 2010. Identifying non-sperm
393 particles during flow cytometric physiological assessment: a simple approach.
394 *Theriogenology* 73, 995-1000.

395 Rath, D., Moench-Tegeder, G., Taylor, U., Johnson, L.A., 2009. Improved quality of sex-
396 sorted sperm: a prerequisite for wider commercial application. *Theriogenology* 71, 22-
397 29.

398 Schiller, J., Muller, K., Suss, R., Arnhold, J., Gey, C., Herrmann, A., Lessig, J., Arnold, K.,
399 Muller, P., 2003. Analysis of the lipid composition of bull spermatozoa by MALDI-
400 TOF mass spectrometry--a cautionary note. *Chem. Phys. Lipids* 126, 85-94.

- Teng, M.K., Usman, N., Frederick, C.A., Wang, A.H., 1988. The molecular structure of the complex of Hoechst 33258 and the DNA dodecamer d(CGCGAATTCGCG). *Nucleic Acids Res.* 16, 2671-2690.
- Vazquez, J.M., Martinez, E.A., Parrilla, I., Gil, M.A., Lucas, X., Roca, J., 2002. Motility characteristics and fertilizing capacity of boar spermatozoa stained with Hoechst 33342. *Reprod. Domest. Anim.* 37, 369-374.
- Watkins, A.M., Chan, P.J., Kalugdan, T.H., Patton, W.C., Jacobson, J.D., King, A., 1996. Analysis of the flow cytometer stain Hoechst 33342 on human spermatozoa. *Mol. Hum. Reprod.* 2, 709-712.

FIGURE LEGENDS

Fig. 1. Progressive motility (means $\pm$ SE) of stallion sperm incubated in presence of Hoechst 33342 up to 90 minutes in two different media KMT (Kenney's modified Tyrodes) and INRA-T (INRA modified Tyrode's). Comparisons are made against the controls at the beginning of the incubation period, after 40 minutes of incubation, and after 90 minutes of incubation. * $P < 0.05$; ** $P < 0.01$

Fig. 2. Percentage of rapid sperm ($VCL > 45 \mu\text{m/s}$) of stallion sperm incubated in presence of Hoechst 33342 up to 90 minutes in two different media KMT (Kenney's modified Tyrodes) and INRA-T (INRA modified Tyrode's). Comparisons are made against the controls at the beginning of the incubation period, after 40 minutes of incubation, and after 90 minutes of incubation. * $P < 0.05$; ** $P < 0.01$

Fig. 3. Sperm velocities: $VCL \mu\text{m/s}$ (circular velocity), $VSL \mu\text{m/s}$ (straight line velocity) and $VAP \mu\text{m/s}$ (average velocity) of stallion sperm incubated in presence of Hoechst 33342 up to

90 minutes in two different media KMT (Kenney's modified Tyrodes) and INRA-T (INRA modified Tyrode's). Comparisons are made against the controls at the beginning of the incubation period, after 40 minutes of incubation, and after 90 minutes of incubation.

P*<0.05; *P* <0.01

Fig. 4. Percentage of spermatozoa with intact membranes after YoPro-1/Eth staining as described in material and methods of stallion sperm incubated in presence of Hoechst 33342 up to 90 minutes in two different media KMT (Kenney's modified Tyrodes) and INRA-T (INRA modified Tyrode's). Comparisons are made against the controls at the beginning of the incubation period, after 40 minutes of incubation, and after 90 minutes of incubation.

Figure 1-4

*

■ KMT ■ INRA-T

■ KMT ■ INRA-T

■ KMT ■ INRA-T

FIG 4