

Capacitation, acrosome function and chromatin structure in stallion sperm[☆]

D.N. Neild^a, B.M. Gadella^{b,c}, A. Agüero^a,
T.A.E. Stout^d, B. Colenbrander^{c,d,*}

^a *Department of Theriogenology, School of Veterinary Sciences,
University of Buenos Aires, Argentina*

^b *Departments of Biochemistry and Cell Biology, Faculty of Veterinary Medicine,
Utrecht University, The Netherlands*

^c *Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University,
The Netherlands*

^d *Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University,
Yalelaan 12, 3584 CM Utrecht, The Netherlands*

Abstract

In general, fertility in breeding stallions is lower and more variable than in the other farm animal species, primarily because selection is based on pedigree, looks and/or athletic performance, with little consideration of fertility or fertility potential. Moreover, because the average stallion breeds only a limited number of mares per year and in-field fertility is influenced significantly by non-stallion factors such as management and mare fertility, meaningful fertility data are hard to come-by. Unfortunately, generating usable figures would involve impractically high costs, time and numbers of mares. Instead, a breeding soundness examination (BSE), based on assessments of sperm number, motility and morphological normality and of mating ability, is often carried out with the ostensible aim of identifying animals with the “potential for good fertility”. In fact, the BSE generally succeeds only in ruling out those stallions with a very clear reason for sub-fertility, and still fails to identify some seriously sub-fertile animals. Thus, the routine BSE has very limited use as a predictor of subsequent fertility. This paper reviews assays developed for identifying capacitated, acrosome-reacted and DNA-

[☆] This paper is part of the special issue entitled “Proceedings of the 4th International Symposium on Stallion Reproduction”, Guest Edited by Dr. Edward Squires.

* Corresponding author. Tel.: +31 30 253 1350; fax: +31 30 253 1054.

E-mail address: b.colenbrander@vet.uu.nl (B. Colenbrander).

damaged sperm, and assesses their utility for improving our ability to predict a stallion's fertility prior to the onset of his breeding career.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Stallion fertility; Acrosome reaction; Capacitation; DNA integrity

1. Introduction

Stallion reproductive performance depends on a number of inter-related factors, these include management, fertility of inseminated mares, freedom from venereal pathogens, libido, mating ability and semen quality (Amann, 2005). A breeding soundness examination (BSE) aims to examine the last three of these factors to give an indication of an individual's suitability to become a breeding stallion, i.e. to give an indication of fertility potential. The primary objective of the BSE is thus to determine whether a stallion has the desire and ability, under the conditions that he will subsequently have to operate, to successfully mount a mare or phantom, insert his penis into the vagina or artificial vagina, and ejaculate semen of sufficient quality (in terms of number, progressive motility and morphological normality) to achieve an acceptable pregnancy rate when he is used to breed a reasonable number of mares per season (Blanchard and Varner, 1996). In this latter respect, one of the parameters estimated during a BSE is sperm production capacity, and although this measure does not correlate closely with fertility per se (Parlevliet and Colenbrander, 1999), it does give valuable information on the number of mares a stallion can cover, or insemination doses he can produce in a day, week or breeding season; exceeding these limits will negatively affect apparent fertility. However, while a BSE is useful for identifying stallions that clearly do not meet the criteria for adequate fertility, it is of limited value for predicting fertility and, in some cases, fails to identify seriously sub-fertile animals (Colenbrander et al., 2003). Because of the limitations of the standard BSE for predicting fertility, a range of alternative tests have been developed and investigated in the hope of finding one that is simple, inexpensive and that correlates closely with in-field fertility. Recently, attention has been paid to assays that evaluate capacitation, the acrosome reaction (AR) and chromatin structure, since these are capacities or structures essential for successful fertilization and the establishment of a viable pregnancy.

2. Capacitation

After ejaculation, mammalian spermatozoa must still undergo a series of membrane-architectural and metabolic changes before they acquire the ability to bind to the zona pellucida (ZP) and fertilise an oocyte; these changes are collectively known as capacitation and normally take place within the female genital tract. The most important aspects of capacitation are the changes that enable a sperm to recognize the ZP and undergo the AR in response to ZP-binding; these changes include the facilitation of calcium entry into the cell (Baldi et al., 1991; Okamura et al., 1993; Parrish et al., 1999), increased intracellular calcium

and cAMP levels (Visconti and Kopf, 1998; Florman et al., 1989; Visconti et al., 1990), increased membrane fluidity secondary to alterations in lipid composition and architecture (Bearer and Friend, 1982; Langlais and Roberts, 1985; Harrison et al., 1996; Flesch and Gadella, 2000; Harrison and Gadella, 2005) and changes in metabolic activity leading to the acquisition of hypermotility (Yanagimachi, 1994; Nassar et al., 1999). Once capacitated, a sperm cell can bind to the ZP and respond to this binding by undergoing the AR, an exocytotic event that is instrumental to ZP-penetration and subsequent fusion of the sperm plasma membrane with the oolemma. The ability to undergo capacitation is thus a critical component of a sperm's fertilizing capacity. On the other hand, once capacitated, a sperm cell has reduced subsequent longevity (Watson, 1995).

Because *in vivo* capacitation takes place within the uterus and oviduct (Thomas et al., 1995), it is difficult to study. On the other hand, many aspects of sperm activation can be replicated by specific *in vitro* conditions/media, many of which (e.g. elevated bicarbonate concentrations) essentially replicate the physiological changes a sperm cell will encounter during its journey from the epididymis to the oviduct. One of the first events to be established as a signal that capacitation is in progress was sperm hyperactivation (Yanagimachi, 1994). Recently, it was shown that hyperactivation occurs within seconds of exposing a sperm to IVF medium, and that it is triggered by the phosphorylation of serine and threonine residues of specific sets of sperm proteins. This phosphorylation is a result of bicarbonate/soluble adenylate cyclase activation of protein kinase A (PKA; Wennemuth et al., 2003; Harrison, 2004). This suggests that, under IVF conditions at least, hyperactivation is a very early event in the capacitation process. However, while the vigorous non-progressive movement of hyperactive sperm is characteristic, it is interspersed with periods of immotility (Rathi et al., 2001) that may make estimations of number of hyperactive sperm rather inaccurate. Alternative ways of monitoring capacitation include changes detected by fluorescent labels such as chlortetracycline (CTC; Saling and Storey, 1979).

CTC is a fluorescent antibiotic whose distribution on the sperm changes during the transition from the non-capacitated, to the capacitated and then to the acrosome-reacted state, thereby allowing differentiation of various steps in the sperm activation process (Varner et al., 1993). The exact process visualized by CTC staining remains unclear, but it has been proposed to relate to the capacity of CTC to chelate calcium or to bind to a calcium-binding substance or an anionic peptide in the sperm plasma membrane (Yanagimachi, 1994). However, CTC staining alone gives no indication of sperm viability and, therefore, the protocol was recently combined with Hoechst 33258-labelling so that sperm identified as capacitated or acrosome reacted could simultaneously be classified as viable or degenerate (Neild et al., 2003); this approach is simple to replicate in-field conditions. On the down side, CTC staining is laborious because it is not amenable to flow cytometric evaluation, and it may be prone to artefacts because it involves fixing the sperm (Rathi et al., 2001). For these reasons, CTC staining has largely been superseded by more specific probes for capacitation whose presence can be evaluated flow cytometrically. One such stain is merocyanine 540, a reporter probe for phospholipid disorder which is often used in conjunction with a membrane impermeable nucleic acid stain, such as Yo-Pro-1, to allow simultaneous detection of capacitation and live-dead status (Rathi et al., 2001). In the resting sperm, phospholipids are distributed asymmetrically between the inner and outer leaflets of the sperm plasma membrane bilayer, this asym-

metry is maintained enzymatically and is largely responsible for the non-reactivity of the plasma membrane of non-capacitated sperm. Bicarbonate-induced capacitation involves a breakdown in phospholipid asymmetry ('scrambling') that leads to the plasma membrane becoming more 'fusogenic'; the relative phospholipid disorder also allows merocyanine intercalation, and it is this increased uptake of merocyanine that signals the occurrence of capacitation (Gadella et al., 1995). Rathi et al. (2001) found that the phospholipid scrambling detected by merocyanine 540 occurs earlier in capacitation than the changes detected by CTC staining and, because merocyanine 540 uptake can be measured flow cytometrically, the authors concluded that this was a quicker and more accurate way of detecting capacitation.

Another marker for a critical event in capacitation is the fluorescent antibiotic, filipin, that complexes unesterified cholesterol and can be used to monitor changes in sperm cholesterol distribution (Colenbrander et al., 2002). In non-capacitated sperm, cholesterol is distributed evenly over the entire sperm head. However, in the presence of bicarbonate, a proven inducer of capacitation, cholesterol leaves the equatorial and post-equatorial regions of the sperm head and becomes concentrated in the apical region. Subsequent to this bicarbonate-induced redistribution, cholesterol can be removed from the sperm plasma membrane by a cholesterol 'scavenger' such as albumin. This cholesterol depletion appears to be a rate limiting step in capacitation and critical to the activation of tyrosine kinases (Visconti et al., 1999). In turn, tyrosine phosphorylation of sperm proteins is critical to capacitation (Visconti and Kopf, 1998) because it leads to changes in protein conformation that contribute to increased ZP affinity (Pukazhenthil et al., 1998), hypermotility (Nassar et al., 1999) and the induction of the acrosome reaction (Benoff, 1998).

The significance of detecting capacitated sperm is two-fold. Sperm that are already capacitated prior to insemination are likely to be of reduced longevity and of questionable use for fertilisation *in vivo* unless insemination occurs very close to ovulation. On the other hand, because the ability to capacitate is absolutely critical to fertilising capacity, failure of a large proportion of sperm to undergo capacitation in response to a physiological inducer may be a useful indicator of likely sub-fertility (Samper, 2000). The importance of sperm remaining non-capacitated prior to insemination is supported by studies that separated capacitated and non-capacitated sperm by Sephadex filtration, where the ability of capacitated stallion sperm to bind to Sephadex appears to be due to stallion clusterin and four other, as yet unidentified, membrane proteins (Samper et al., 1995). Glass-wool–Sephadex filters combine the ability of Sephadex to trap capacitated cells with that of glass-wool to trap acrosome-reacted and membrane-damaged (dead) cells (Samper and Crabo, 1993; Samper et al., 1995). These authors reported a high correlation between the fertility of frozen stallion semen and the percentage of sperm passing through glass-wool/Sephadex ($r=0.93$) or just Sephadex filters ($r=0.84$), presumably because the trapped sperm were either dead, acrosome-reacted (and therefore no longer capable of fertilisation) or capacitated (and therefore of limited longevity). The correlation of glass-wool/Sephadex passage for fresh semen ($r=0.83$ and 0.64 , respectively) was lower, but still significant. Presumably the higher correlation of glass-wool–Sephadex passage to fertility for frozen-thawed semen is because the most significant source of 'infertile' sperm in this instance is membrane damage and capacitation-like changes induced by the freeze-thaw process.

3. Acrosome reaction and acrosomal function

The AR is an irreversible event that, under physiological conditions, is induced specifically by zona-binding and can only occur after sperm capacitation is complete. During the AR, the plasma membrane fuses with the outer acrosomal membrane at multiple sites over the anterior part of the sperm head. This results in the release of acrosomal contents (mainly enzymes) that promote sperm interaction with, and subsequent digestion and penetration of, the ZP. An important consequence of the AR is the acquisition of fusogenicity by the equatorial segment of the plasma membrane; this is required for the sperm to bind to and fuse with the oolemma (Yanagimachi, 1994). The AR can be induced artificially using calcium ionophores such as ionomycin and A23187, or lysophosphatidylcholine. However, the modes of action of these AR-inducers are considered non-physiological because they bypass normal intracellular regulatory mechanisms and instead, respectively, allow a massive influx of calcium or artificially enhance membrane fusogenicity (Gadella et al., 1995). Physiological inducers of the AR are still under study but it is believed that, as well as ZP, several components of ovarian follicular fluid come into this category (Bruker and Lipford, 1995). In the horse, follicular fluid has been shown to induce the AR in pre-incubated stallion spermatozoa, and progesterone was found to be involved in this process (Cheng et al., 1998a; Rathi et al., 2003). Indeed, it appears that progesterone enhances both sperm-ZP binding and ZP-mediated induction of the AR via specific receptors on the plasma membrane (Cheng et al., 1998b). Moreover, Rathi et al. (2000) found a high correlation ($r=0.73-0.84$) between stallion fertility and the percentage of sperm that exposed progesterone receptors after incubation in capacitation media. Similarly, Meyers et al. (1995) reported a correlation between the inability of sperm to expose progesterone receptors when stimulated by progesterone, and sub-fertility in some stallions. Thus, the ability to AR in response to a physiological inducing agent appears to be an important indicator of fertilizing capacity.

Column filtration techniques such as glass wool and glass bead filtration have been suggested as useful ways of evaluating acrosome integrity, because they trap membrane-damaged or acrosome-reacted cells but allow motile, membrane-intact sperm to pass through. As with Sephadex filtration, however, this is probably of more practical significance for frozen semen in which the proportion of acrosome-reacted or damaged sperm is generally much higher than in fresh semen.

Microscopic techniques described for evaluating acrosome integrity include transmission electron microscopy, and light microscopy combined with stains such as Spermac and Wells and Awa (Wells and Awa, 1970). The dual stain technique (DS: Trypan blue and Giemsa) is a simple procedure that generates four categories of sperm: either viable or non-viable combined with acrosome intact or reacted (Didion et al., 1989). A variation of this technique is the triple stain (TS: Trypan blue, Bismark brown and Rose bengal) that is used for evaluating evidence of the AR using bright-field microscopy. Varner et al. (1987) reported the detection of the AR in equine sperm using TS to be strongly correlated with that by TEM, but found that trypan blue uptake by sperm heads (to characterize non-viable sperm) was inconsistent. Indirect immuno-fluorescence techniques using monoclonal antibodies (Blach et al., 1988; Zhang et al., 1990) can also be used to investigate the acrosomal status of stallion sperm, but the antibodies are not easy to acquire.

The technique currently most widely used to analyse the acrosome reaction is the binding of fluorescently-labelled lectins such as fluorescein isothiocyanate conjugated concanavilin A (FITC-ConA: Blanc et al., 1991) or *Pisum sativum* agglutinin fluorescein conjugate (FITC-PSA: Farlin et al., 1992). Electron microscopy has been used to confirm that FITC-PSA labels the acrosome (Casey et al., 1993; Meyers et al., 1995) but, because it also binds to sugar residues in the ZP (Skutelsky et al., 1994), PSA cannot be used for assessing the AR during sperm-oocyte interaction. By contrast, fluorescein isothiocyanate conjugated *Arachis hypogea* agglutinin (FITC-PNA) binding is limited to the acrosomal cap of the sperm, or more specifically to the outer acrosomal membrane (OAM) with no binding to the plasma membrane (verified by immuno-electron microscopy; Cheng et al., 1996). Because PNA does not bind to the ZP, it can be used to assess the AR during gamete interaction (Cheng et al., 1996).

Another proposed in vitro assay of sperm function is acrosin amidase activity (Ball et al., 1997), where acrosin is a serine proteinase that is associated with the acrosome of mammalian sperm and is thought to be important to the AR, sperm-ZP binding and ZP penetration. However, while Ball et al. (1997) found significant inter-stallion and inter-ejaculate differences in acrosin amidase activity, it is not known how these differences relate to fertility. Another assay that provides information on whether sperm are capacitated and acrosome reacted is the zona-free hamster oocyte penetration test (HOPT), which tests the capacity of spermatozoa to penetrate an egg (Brackett et al., 1982; Okolski et al., 1987). However, this test is not very practical and is essentially used only for research purposes.

As with capacitation, the proportion of sperm that are acrosome reacted (and therefore no longer capable of fertilisation) is really only a meaningful indicator of likely fertility when frequent sperm damage is anticipated, e.g. after freezing and thawing. In fresh sperm, the ability to undergo the AR in response to a relatively physiological induction process (e.g. bicarbonate stimulation followed by exposure to ZP or progesterone) may be a more useful indicator of likely fertilising capacity; failure of a large proportion of sperm to respond may be indicative of an otherwise undetectable physiological defect.

4. DNA

Integrity and structural stability of sperm DNA is thought to be associated with male fertility. The sperm chromatin structure assay (SCSA) evaluates structural stability by measuring the susceptibility of sperm DNA to acid denaturation. The denatured sperm DNA is labelled with acridine orange, which stains single-stranded DNA (and in somatic cells, RNA) red (by excimer excitation) and double stranded DNA green (probe excited in monomere state). An increase in the amount of single-stranded DNA in fresh-frozen sperm has been associated with reduced fertility in species including man (Evenson et al., 1980), pig (Evenson et al., 1994), cow (Ballachey et al., 1988) and horse (Kenney et al., 1995; Love and Kenney, 1998). In the case of the horse, it has also been reported that rates of sperm chromatin denaturation are higher in semen from sub-fertile than fertile stallions (32% versus 16%), and that a stallion's denaturation score correlates negatively with his seasonal pregnancy rate (Kenney et al., 1995). In addition, in some stallions that exhibit a high level of compromised chromatin in fresh semen, the rate of chromatin quality decline

during chilled storage is markedly accelerated (Love, 2002). A newer technique for detecting DNA damage involves the use of single-cell gel electrophoresis (SCGE), and is known as the comet assay (Linfor and Meyers, 2002). At present, however, it is not clear whether the COMET assay detects the condensation status of DNA (normally extremely condensed due to protamines) or the incidence of DNA breaks (the latter could be a result of insufficient condensation). However, the condensation status of sperm DNA can also be detected using TEM of ultrathin plastic sections of sperm (Bartoov et al., 1999). Normal condensed DNA is seen as a homogenous black nucleus, while the presence of white holes (nuclear cavities or vacuoles) or of nuclear fragmentation is easy to distinguish and probably indicates inadequate DNA condensation. The drawbacks of this technique are its cost and elaborate, time-consuming nature. As with other features of sperm quality, however, while it is clear that stallions with a high percentage of DNA abnormalities are likely to be sub-fertile, the exact relationship between the level of DNA damage and fertility is not clear.

5. Field fertility trials

It is possible to carry out true fertility trials, but this is expensive, time-consuming and prone to the influence of many other factors (e.g. management, mare fertility, venereal disease: Varner et al., 1991b). Parameters that have been used for estimating fertility rates in the field include: non-return rate (%), foaling rate (%), pregnancy rate (%), per cycle pregnancy rate (%), first cycle pregnancy rate (%), and the number of services per pregnancy (Colenbrander et al., 2003). During a typical breeding season, however, the number of mares served by a single stallion averages around 40–50 mares for naturally-serving animals and 120–140 for those used via AI (Varner et al., 1991a). As a consequence, fertility data obtained per stallion are often based on a population of mares that is too small for meaningful statistical evaluation. Nevertheless, an ongoing rough indication of fertility during the breeding season can be obtained using first cycle or per cycle pregnancy rates, which therefore serve as an ‘early warning system’ for fertility problems; early pregnancy data does, however, exclude subsequent pregnancy losses which, in an admittedly modest number of cases, may be due to sperm chromosome or chromatin abnormalities (Kenney et al., 1991).

6. Conclusions

Predicting stallion fertility in the laboratory is far from an exact science but, because the acquisition of usable field data is impractically time-consuming and expensive, there is still a desire/need for laboratory tests to predict fertility with a reasonable degree of certainty before a stallion begins his breeding career (Colenbrander et al., 2003). Although the ideal would be to establish a single test that correlates strongly with in-field fertility, the wide array of attributes that a sperm needs to successfully reach and fertilise an oocyte means that this is likely to remain wishful thinking. Instead, the aim of current research is to identify a combination of tests that together analyse the most important sperm function parameters, and to use this combination to identify an ever-greater proportion of stallions with a clear

reason for sub-fertility. Given the simultaneous need to limit the complexity, labour and costs of fertility prediction, flow cytometry has emerged as a favoured tool. Stains are now available for assessing sperm viability, capacitation, acrosome status, chromatin stability and mitochondrial function, and a range of properties can be assessed simultaneously in a simple, rapid and objective manner. It is expected that refining the combination of stains to represent the most significant aspects of sperm fertilising capacity will allow increasingly accurate prediction of stallion fertility prior to breeding.

References

- Amann, R.P., 2005. Weaknesses in reports of “fertility” for horses and other species. *Theriogenology* 63, 698–715.
- Baldi, E., Casano, R., Falsetti, C., Krausz, C., Maggi, M., Forti, G., 1991. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J. Androl.* 12, 323–330.
- Ball, B.A., Fagnan, M.S., Dobrinski, I., 1997. Determination of acrosin amidase activity in equine spermatozoa. *Theriogenology* 48, 1191–1198.
- Ballachey, B.E., Saake, R.G., Evenson, D.P., 1988. The sperm chromatin structure assay: relationship with alternate tests of sperm quality and heterospermic performance of bulls. *J. Androl.* 9, 109.
- Bartoov, B., Eltes, F., Reichart, M., Langzam, J., Lederman, H., Zbuldovsky, N., 1999. Quantitative ultramorphological analysis of human sperm: fifteen years of experience in the diagnosis and management of male infertility factor. *Arch. Androl.* 43, 13–25.
- Bearer, E.L., Friend, D.S., 1982. Modifications of anionic lipid domains preceding membrane fusion in guinea pig sperm. *J. Cell Biol.* 92, 604–615.
- Benoff, S., 1998. Modelling human egg-sperm interactions in vitro: signal transduction pathways regulating the acrosome reaction. *Mol. Hum. Reprod.* 4, 453–471.
- Blach, E.L., Amann, R.P., Bowen, R.A., Sawyer, H.R., Hermeten, J.M., 1988. Use of a monoclonal antibody to evaluate integrity of the plasma membrane of stallion sperm. *Gamete Res.* 21, 233–241.
- Blanc, G., Magistrini, M., Palmer, E., 1991. Use of Concanavalin A for coating the membranes of stallion spermatozoa. *J. Reprod. Fertil., Suppl.* 44, 191–198.
- Blanchard, T.L., Varner, D.D., 1996. Evaluating breeding soundness in stallions-1: the basic examination. *Vet. Med.*, 54–63.
- Brackett, B.G., Cofone, M.A., Boice, W.J., Bousquet, D., 1982. Use of zona-free hamster ova to assess sperm fertilizing ability of the bull and stallion. *Gamete Res.* 5, 217–227.
- Bruker, C., Lipford, G.B., 1995. The human sperm acrosome reaction: physiology and regulatory mechanisms. An update. *Hum. Reprod. Update* 1, 51–62.
- Casey, P.J., Hillman, R.B., Robertson, K.R., Yudin, A.I., Liu, I.K.M., Drobnis, E.Z., 1993. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. *J. Androl.* 14, 289–297.
- Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Marks, A., Bevers, M.M., Colenbrander, B., 1996. Use of PNA (Peanut agglutinin) to assess the acrosomal status and the zona pellucida induced acrosome reaction in stallion spermatozoa. *J. Androl.* 17, 674–682.
- Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Tremoleda, J.L., Bevers, M.M., Colenbrander, B., 1998a. Progesterone in mare follicular fluid induces the acrosome reaction in stallion spermatozoa and enhances in vitro binding to the zona pellucida. *Int. J. Androl.* 21, 57–66.
- Cheng, F.P., Gadella, B.M., Voorhout, W.F., Fazeli, A.R., Bevers, M.M., Colenbrander, B., 1998b. Progesterone-induced acrosome reaction in stallion spermatozoa is mediated by a plasma membrane progesterone receptor. *Biol. Reprod.* 59, 733–742.
- Colenbrander, B., Brouwers, J.F.H.M., Neild, D.M., Stout, T.A.E., da Silva, P., Gadella, B.M., 2002. Capacitation dependent lipid rearrangements in the plasma membrane of equine sperm. *Theriogenology* 58, 341–345.
- Colenbrander, B., Gadella, B.M., Stout, T., 2003. The predictive value of semen analysis in the evaluation of stallion fertility. *Reprod. Domest. Anim.* 38, 305–311.
- Didion, B.A., Dobrinsky, J.R., Giles, J.R., Graves, C.N., 1989. Staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species. *Gamete Res.* 22, 51–57.

- Evenson, D.P., Darznkiewicz, Z., Melamed, M.R., 1980. Relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 210, 1131.
- Evenson, D.P., Thompson, L., Jost, L., 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* 41, 637–651.
- Farlin, M.E., Jasko, D.J., Graham, J.K., Squires, E.L., 1992. Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. *Mol. Reprod. Dev.* 32, 23–27.
- Flesch, F.M., Gadella, B.M., 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochim. Biophys. Acta* 1469, 197–235.
- Florman, H.M., Tombes, R.M., First, N.L., Babcock, D.F., 1989. An adhesion-associated agonist from the zona pellucida activates G-protein promoted elevations of internal Ca^{2+} and pH that mediate mammalian spermatozoa acrosomal exocytosis. *Dev. Biol.* 135, 133–146.
- Gadella, B.M., Lopes-Cardozo, M., van Golde, L.M.G., Colenbrander, B., Gadella Jr., T.W.J., 1995. Glycolipid migration from the apical to the equatorial subdomains of the sperm head plasma membrane precedes the acrosome reaction: Evidence for a primary capacitation event in boar spermatozoa. *J. Cell Sci.* 108, 935–945.
- Harrison, R.A.P., 2004. Rapid PKA-catalyzed phosphorylation of boar sperm proteins induced by the capacitating agent bicarbonate. *Mol. Reprod. Dev.* 67, 337–352.
- Harrison, R.A.P., Ashworth, P.J.C., Miller, N.G.A., 1996. Bicarbonate/ CO_2 , an effector of capacitation, induces a rapid, reversible change in the lipid architecture of boar sperm plasma membranes. *Mol. Reprod. Dev.* 45, 378–391.
- Harrison, R.A.P., Gadella, B.M., 2005. Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology* 63, 342–351.
- Kenney, R.M., Kent, M.G., Garcia, M.C., Hurtgen, J.P., 1991. The use of DNA index and karyotype analyses as adjuncts to the estimation of fertility in stallions. *J. Reprod. Fert., Suppl.* 44, 69–75.
- Kenney, R.M., Evenson, D.P., Garcia, M.C., Love, C.C., 1995. Relationship between sperm chromatin structure, motility and morphology of ejaculated sperm and seasonal pregnancy rate. *Biol. Reprod. Mono.* 1, 647–653.
- Langlais, J., Roberts, K.D., 1985. A molecular membrane model of sperm capacitation and the acrosome reaction of mammalian spermatozoa. *Gamete Res.* 12, 183–224.
- Linfor, J.J., Meyers, S.A., 2002. Detection of DNA damage in response to cooling injury in equine spermatozoa using single-cell gel electrophoresis. *Andrology* 23, 107–113.
- Love, C.C., Kenney, R.M., 1998. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology* 50, 955–972.
- Love, C.C., 2002. Stallion semen evaluation and interpretation. In: *Conference Proceedings Society for Theriogenology*. American College of Theriogenologists.
- Meyers, S.A., Overstreet, J.W., Liu, I.K.M., Drobnis, E.Z., 1995. Capacitation in vitro of stallion: comparison of progesterone induced acrosome reactions in fertile and sub-fertile males. *J. Androl.* 16, 47–54.
- Nassar, A., Mahony, M., Morshedi, M., Lin, M.H., Srisombut, C., Oehninger, S., 1999. Modulation of sperm tail protein phosphorylation by pentoxifylline and its correlation with hyperactivated motility. *Fertil. Steril.* 71, 919–923.
- Neild, D.M., Gadella, B.M., Chaves, M.G., Miragaya, M.H., Colenbrander, B., Agüero, A., 2003. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. *Theriogenology* 59, 1693–1705.
- Okamura, N., Tanba, M., Fukuda, A., Sugita, Y., Nagai, T., 1993. Forskolin stimulates porcine sperm capacitation by increasing calcium uptake. *FEBS Lett.* 316, 283–286.
- Okolski, A., Babusik, P., Tischner, M., Leitz, W., 1987. Evaluation of mare oocyte collection methods and stallion sperm penetration of zona-free hamster ova. *J. Reprod. Fert., Suppl.* 35, 191–196.
- Parlevliet, J.M., Colenbrander, B., 1999. Prediction of first season stallion fertility of 3-year-old dutch Warmbloods with prebreeding assessment of percentage of morphologically normal live sperm. *Equine Vet. J.* 31, 248–251.
- Parrish, J.J., Susko-Parrish, J.L., Graham, J.K., 1999. In vitro capacitation of bovine spermatozoa: role of intracellular calcium. *Theriogenology* 51, 461–472.
- Pukazhenthil, B.S., Wildt, D.E., Ottinger, M.A., Howard, J., 1998. Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucida penetration by tyrosine kinase inhibitors. *Mol. Reprod. Dev.* 49, 48–57.
- Rathi, R., Nielen, M., Cheng, F.P., van Buiten, A., Colenbrander, B., 2000. Exposure of progesterone receptors on the plasma membranes of stallion spermatozoa as a parameter for prediction of fertility. *J. Reprod. Fert., Suppl.* 56, 87–91.

- Rathi, R., Colenbrander, B., Bevers, M.M., Gadella, B.M., 2001. Evaluation of in vitro capacitation of stallion spermatozoa. *Biol. Reprod.* 65, 462–470.
- Rathi, R., Colenbrander, B., Stout, T.A.E., Bevers, M.M., Gadella, B.M., 2003. Progesterone induces acrosome reaction in stallion spermatozoa via a protein tyrosine kinase dependent pathway. *Mol. Reprod. Dev.* 64, 120–128.
- Saling, P.M., Storey, B.T., 1979. Mouse gamete interaction during fertilization in vitro: chlortetracycline as a fluorescent probe for the mouse sperm acrosome reactions. *J. Cell Biol.* 83, 544–555.
- Samper, J.C., Crabo, B.G., 1993. Assay of capacitated, freeze-damaged and extended stallion spermatozoa by filtration. *Theriogenology* 39, 1209–1220.
- Samper, J.C., Hamilton, D.W., Pryor, J.L., Loseth, K.J., Troedsson, M.H.T., Crabo, B.G., 1995. Mechanism of Sephadex trapping of capacitated stallion spermatozoa. *Biol. Reprod. Mono.* 1, 729–737.
- Samper, J.C., 2000. Artificial insemination. In: Samper, J.C. (Ed.), *Equine Breeding and Management and Artificial Insemination*. W. B. Saunders Company, Philadelphia, Pennsylvania, pp. 112–113.
- Skutelsky, E., Ranen, E., Shalgi, R., 1994. Variations in the distribution of sugar residues in the zona pellucida as possible species-specific determinants of mammalian oocytes. *J. Reprod. Fertil.* 100, 35–41.
- Thomas, P.G.A., Ball, B.A., Brinsko, S.P., 1995. Changes associated with induced capacitation influence the interaction between equine spermatozoa and oviduct epithelial cell monolayers. *Biol. Reprod. Mono.* 1, 697–705.
- Varner, D.D., Ward, C., Storey, B.T., Kenney, R.M., 1987. Induction and characterization of acrosome reaction in equine spermatozoa. *Am. J. Vet. Res.* 48, 1383–1389.
- Varner, D.D., Schumacher, J., Blanchard, T.L., Johnson, L., 1991a. Management of the breeding stallion. In: Prat, P.W., Thornwood, D.R. (Eds.), *Diseases and Management of Breeding Stallions*. American Veterinary Publications, Goleta, CA, Chapter 3, pp. 97–115.
- Varner, D.D., Schumacher, J., Blanchard, T.L., Johnson, L., 1991b. Breeding soundness examination. In: Prat, P.W., Thornwood, D.R. (Eds.), *Diseases and Management of Breeding Stallions*. American Veterinary Publications, Goleta, CA, Chapter 2, pp. 61–96.
- Varner, D.D., Bowen, J.A., Johnson, L., 1993. Effect of heparin on capacitation/acrosome reaction of equine sperm. *Arch. Androl.* 31, 199–207.
- Visconti, P.E., Muschietti, J.P., Flawia, M.M., Tezon, J.G., 1990. Bicarbonate dependence of cAMP accumulation induced by phorbol esters in hamster spermatozoa. *Biochim. Biophys. Acta* 1054, 231–236.
- Visconti, P.E., Kopf, G.S., 1998. Regulation of protein phosphorylation during sperm capacitation. *Biol. Reprod.* 59, 1–6.
- Visconti, P.E., Galantino-Homer, H., Ning, X., Moore, G.D., Valenzuela, J.P., Jorgez, C.J., Alvarez, J.G., Kopf, G.S., 1999. Cholesterol efflux-mediated signal transduction in mammalian sperm. Beta-cyclodextrins initiate transmembrane signaling leading to an increase in protein tyrosine phosphorylation and capacitation. *J. Biol. Chem.* 274, 3235–3242.
- Watson, P.F., 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod. Fertil. Dev.* 7, 871–891.
- Wells, M.E., Awa, O.A., 1970. New technique for assessing acrosomal characteristics of spermatozoa. *J. Dairy Sci.* 53, 227–232.
- Wennemuth, G., Carlson, A.E., Harper, A.J., Babcock, D.F., 2003. Bicarbonate actions on flagellar and Ca²⁺-channel responses: initial events in sperm activation. *Development* 130, 1317–1326.
- Yanagimachi, R., 1994. Mammalian fertilization. In: Knobil, E., Neill, J.D. (Eds.), *The Physiology of Reproduction*, second ed. Raven Press, New York, pp. 189–317.
- Zhang, J., Boyle, M.S., Smith, C.A., Moore, H.D.M., 1990. Acrosome reaction of stallion spermatozoa evaluated with monoclonal antibody and zona-free hamster eggs. *Mol. Reprod. Dev.* 27, 152–158.