REPRODUCTION
REVIEW

Focus on Determinants of Male Fertility

# Determinants of sperm quality and fertility in domestic species

A M Petrunkina[1], D Waberski[2], A R Günzel-Apel[3] and E Töpfer-Petersen[4]

*Unit for Reproductive Medicine of Clinics, [1]Clinic for Horses, [2]Clinic for Swine and Small Ruminants, [3]Small Animal Clinic and [4]Institute of Reproductive Biology, University of Veterinary Medicine Hannover Foundation, Bünteweg 15, 30559 Hannover, Germany*

*Correspondence should be addressed to A M Petrunkina; Email: anna.petrunkina@tiho-hannover.de*

## Abstract

**Fertilization success cannot be attributed solely to the absolute number of vital, motile, morphologically normal spermatozoa inseminated into the female but more especially to their functional competence. A range of *in vitro* tests has therefore been developed to monitor crucial aspects of sperm function: their ability to adapt to changing osmotic conditions, to bind to the oviductal epithelium, and to undergo capacitation in an appropriate and timely manner. The tests employ flow cytometry in conjunction with fluorescent techniques, electronic cell counting, and computer-assisted image area analysis. The highly quantitative analysis provided by electronic sizing and flow cytometry enables assessment of representative cell numbers in a very short time with high reproducibility. More importantly, it allows the detection of physiological heterogeneity within an ejaculate in terms of the development of cell subpopulations and enables the kinetic analysis of changes in living cell suspensions. The tests offer a promising strategy for evaluating fertility in domestic animals. The capability for volume regulation ensures that sperm recover from the tonic shocks experienced at ejaculation and during cryopreservation. Assessment of capacitation *in vitro* provides valuable information on both the sperm's ability to respond to fertilizing conditions and the sequence and rates of ongoing capacitation/destabilization processes. The monitoring of response to capacitating conditions in kinetic terms allows the sensitive and adequate detection of sperm populations expressing fertilization attributes and their ability to respond to external stimuli in a timely manner. However, subfertility is likely to be associated with a suboptimal response (i.e. too high or too low) rather than a minimal response.**

*Reproduction* (2007) **134** 3–17

## Are current parameters for semen evaluation adequate?

Laboratory assessment of sperm quality is an essential procedure in many aspects of assisted reproduction in domestic species. The choice of adequate parameters by reproducible, fast, and sensitive methods is of increasing concern, because it has become more and more apparent that standard seminal parameters like motility, morphology, and sperm concentration are insufficient, not only to predict fertility but even to identify subfertile individuals (Gadea *et al.* 2004). In the last decade, increasingly sophisticated methods of examining what might be termed basal or structural characteristics have been developed. Amongst these are sperm kinematics assessed by computer-assisted motility analysis, osmotic resistance tests, plasma membrane integrity evaluation with fluorescent membrane-impermeable dyes, evaluation of acrosomal status with fluorescein–isothiocyanate-conjugated lectins, investigation of DNA integrity using the sperm chromatin structure assay, or assessment of membrane architectural status (Gillan *et al.* 2005, Silva & Gadella 2006). Most methods require the application of fluorescence microscopy and/or flow cytometric techniques. Being highly quantitative, repeatable, and sensitive, these techniques are beginning to find their place in the modern spermatological laboratory; some are already routinely applied to the evaluation of commercially produced semen in boars and bulls (Christensen *et al.* 2004, 2005*a*). However, although these parameters help to explain some of the sources of individual variation in animal fertility (Holt *et al.* 1997, Christensen *et al.* 2004, 2005*b*, Quintero-Moreno *et al.* 2004, Gil *et al.* 2005, Hallap *et al.* 2006), they still appear to be too insensitive for the evaluation


ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-07-0046
Online version via www.reproduction-online.org

of fertility and portraying biological heterogeneity between individuals, ejaculates, and cells within an ejaculate. The failure to find strong correlations between *in vivo* or *in vitro* fertility and quantitative assessments using either standard spermatological parameters or the more sophisticated tests described above has been reported in several domestic species (e.g. for pig, horse, and goat: Quintero-Moreno *et al.* 2004, Gil *et al.* 2005, Petrunkina *et al.* 2005*a*, Kuisma *et al.* 2006, Sancho *et al.* 2006, Peterson *et al.* 2007). A new approach is clearly needed (see, for example, Rodriguez-Martinez 2006, 2007*a*).

## Functional prerequisites for fertilization are the key evaluation parameters

In recent years, increasing attention has been paid to the tests of the capacitation process, as an immediate precursor to fertilization (egg penetration). However, on the path to fertilization there are many preliminary steps prior to capacitation (Töpfer-Petersen *et al.* 2002). During its lifetime between testis and egg, the fertilizing sperm cell encounters several different environments to which it must respond (Fig. 1). It undergoes maturational processes as it passes through the epididymis, during which molecular and macromolecular components exchange in the extracellular milieu as well as on or within the cell. At ejaculation, it passes from a hyperosmotic environment to an iso-osmotic one, coming in contact with not only the secretions of accessory glands but also uterine fluid. Within the female tract, it is stored by binding to a special region of the oviduct, which process assists it to survive in what appears to be a hostile environment. Then in coordination with changing conditions brought about by hormonal changes around the time of ovulation, it acquires the active fertilizing competence (capacitation) needed for fertilization. Finally, it must respond to a new 'structural' environment (cumulus cells and zona pellucida) as it encounters the egg. The sperm's fertilizing competence therefore depends upon its ability to: adapt to a new environment, bind to the oviductal epithelium, survive in the female genital tract, capacitate/respond to fertilizing conditions, and interact with the female gamete.

It would seem, therefore, that sperm assessment should involve tests of sperm response to particular environmental conditions related to the overall fertilization process. In choosing the parameters to be tested, the following questions should be addressed: a) is the sperm parameter relevant for fertilization? b) does the female tract select according to the parameter? and c) does the parameter portray the physiological heterogeneity of sperm?

This latter aspect is extremely important. Recent studies using tests of sperm response to environmental changes have revealed considerable functional heterogeneity in all species examined, not only between individuals and ejaculates but also within the sperm population of a given ejaculate (Holt & Van Look 2004). This heterogeneity may stem from intercellular differences during spermatogenesis but may stem more particularly from intercellular difference emanating from maturational processes within the epididymis, when major changes in membrane architecture are brought about (Jones 1989). Such functional heterogeneity is very likely to impact strongly on the individual sperm's ability

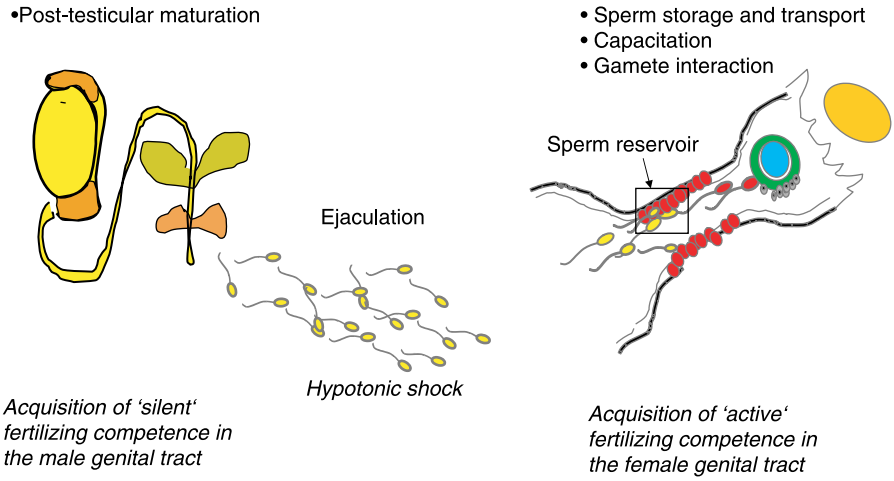


**Figure 1** The sperm's route to fertilization. After achieving 'silent' fertilizing competence during epididymal maturation, spermatozoa are exposed to accessory gland secretions at ejaculation and to female genital tract fluids after deposition in the female. Resistance to the hypotonic shock at ejaculation through volume regulatory ability is thus an important strategy of adaptation. As the sperm reach the oviduct, those with potential fertilizing competence bind to the oviductal epithelium to establish the 'sperm oviductal reservoir' so as to achieve later 'active' fertilizing competence after release and capacitation. Only cells able to bind to the oviductal epithelium, undergo capacitation, and interact with the oocyte possess full fertilizing ability. Thus, the testing of volume regulatory ability, oviductal binding, and capacitatory response appears to be a promising strategy for sperm quality evaluation.

a) to respond adequately to the changing environments it encounters during passage in the female reproductive tract and b) to eventually fertilize the egg. Thus, functional assessment tests should be sensitive enough to reveal not only differences between ejaculates and males but also, in particular, intercellular heterogeneity with special focus on the development of subpopulations showing different temporal or quantitative responses.

In this review, we present information on three independent tests of sperm function, which we have been studying: volume regulation (response to osmotic stress), binding to the oviductal epithelium, and response to capacitating conditions. Each of these tests reveals physiological heterogeneity, each is related to the fertilizing process, and each can be regarded as a measure of interaction with the female tract which may lead to selection. Although much biological knowledge has been accumulated about these processes during the last decade, the tests are still not widely accepted, and we believe that their importance for sperm functional assessment has not so far attracted sufficient attention.

## Sperm cell volume regulation

### *Physiological importance of cell volume regulation*

When cells encounter hypo- or hyper-tonic environments, they tend to swell or shrink due to the influx or efflux of water during reestablishment of osmotic equilibrium. However, a wide range of animal cell types, including spermatozoa, are able to maintain their volume after osmotic shock, thereby avoiding the consequences of excessive volume changes (Lang *et al.* 1998). During its life, the spermatozoon experiences considerable changes in its environment, most notably during maturation within the epididymis and at ejaculation. During epididymal transit, an uptake of osmolytes from epididymal secretions takes place, and spermatozoa acquire the ability to regulate cell volume (Yeung *et al.* 2004*a*). At ejaculation, they transfer from the hypertonic epididymal environment to the isotonic conditions of seminal plasma and the female genital tract fluids, at which time the spermatozoa experience a considerable osmotic gradient (Yeung *et al.* 2004*a*, 2004*b* and references therein). Moreover, under the artificial conditions of semen cryopreservation, the cells are exposed to major osmotic challenges: during freezing, they become dehydrated and shrink due to local hypertonicity; during thawing, when rehydration takes place, they are submitted to hypotonic shock. To be able to maintain cellular functionality in the face of such osmotic changes, spermatozoa of several mammalian species (boar, mouse, bull, human) have been found to exhibit volume regulatory abilities, particularly regulatory volume decrease (RVD) in response to hypotonic challenge (Petrunkina *et al.* 2004*a*, 2004*b* and references therein); after exposure to hypertonic conditions, the cells are able to recover their volume after initial shrinking, a process known as regulatory volume increase (RVI: Petrunkina *et al.* 2005*b*).

### *Mechanisms of cell volume regulation*

Spermatozoa, like many other mammalian cell types, control their volume in the face of hypotonic challenge through the opening of independent $K^+$ and $Cl^-$ channels. As water enters the cell to achieve equilibrium between internal and external tonicities, the channels open to allow loss of $K^+$ down the concentration gradient (intracellular $K^+$ concentration is normally much higher than the extracellular concentration). To maintain electrical neutrality, $Cl^-$ exits in parallel. The overall ion loss reduces intracellular tonicity and consequently water influx, so that swelling is reduced or even reversed (Fig. 2A). Other osmolytes may also be lost in a controlled way to assist this process. It has been reported for a variety of somatic cell types that the activation of the $K^+$ and $Cl^-$ channels is swelling sensitive, as is that of organic osmolyte channels (Lang *et al.* 1998). Similar swelling-dependent activation of these channels has been demonstrated for spermatozoa of a variety of species such as cattle, swine, dog, and human (Kulkarni *et al.* 1997, Petrunkina *et al.* 2001*a*, 2004*a*, 2004*b*, Barfield *et al.* 2005, Yeung *et al.* 2005*a*). The major candidates for these channels have been identified in boar and human sperm, in which the chloride channel (CLC)-3 of the CLC-family, the voltage-gated $K^+$ channel Kv1.5, and the β-subunit minK protein appear to be the most likely to be involved (Petrunkina *et al.* 2004*a*, Barfield *et al.* 2005, Yeung *et al.* 2005*a*).

It should be pointed out that sperm must also regulate their volume under isotonic conditions. It has been found that for boar sperm $Na^+$ transport (together with $Cl^-$ ions) is largely responsible in this situation (Petrunkina *et al.* 2005*b*). However, other transport mechanisms may also be involved in volume regulation in mammalian spermatozoa, such as sodium–hydrogen exchanger, potassium chloride co-transporter, $Na^+/K^+$-ATPase, as well as the transport of different osmolytes (e.g. taurine flux). There are distinct differences in ion transport mechanisms between sperm of mouse, human, and domesticated species (Petrunkina *et al.* 2001*a*, 2004*a*, 2004*b*, Yeung *et al.* 2004*a*, 2004*b*, 2005*a*, 2005*b*, Klein *et al.* 2006, and references therein).

In somatic cells, swelling activation of $K^+$ and $Cl^-$ currents is known to involve signaling cascades mediated by protein phosphorylation and dephosphorylation (Klein *et al.* 1993, Voets *et al.* 1998, Thoroed *et al.* 1999). Little is known about whether and how phosphorylation processes are linked to the activation of osmosensing regulatory mechanisms in spermatozoa. Work in our laboratory has provided an evidence that

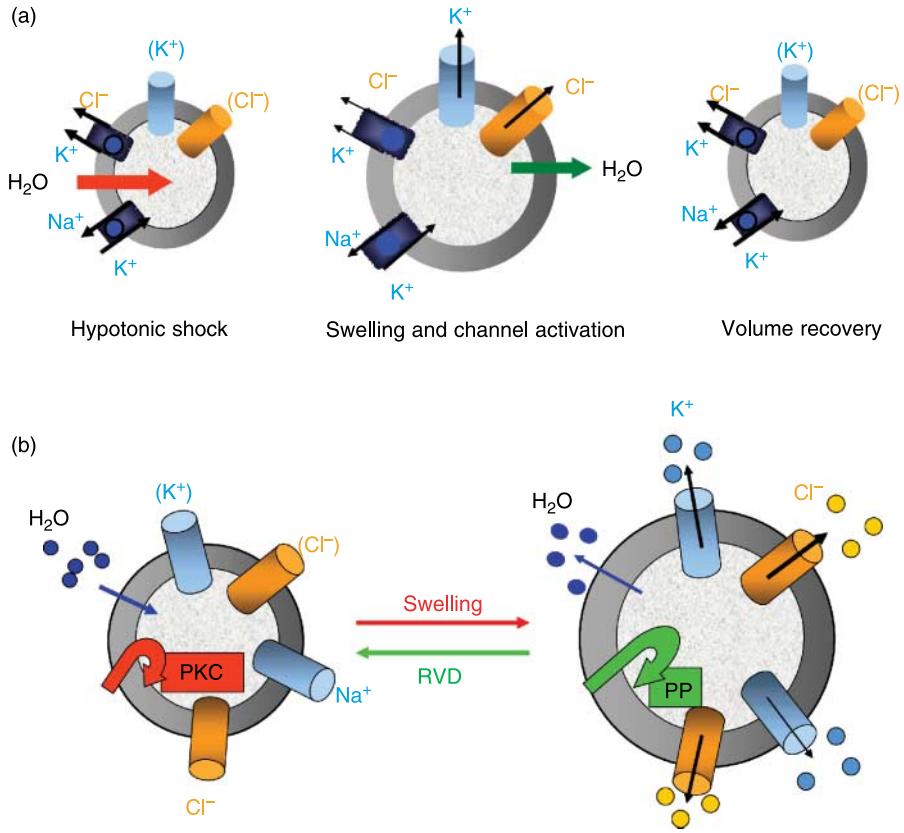


**Figure 2** Mechanisms and signaling pathways of volume regulation in mammalian spermatozoa. (A) Hypotonic shock causes water to enter the cell to dilute the intracellular environment and reestablish osmotic balance. The main ion channels are inactive under 'steady-state' conditions, but they are activated when swelling occurs, with the result that an efflux of major intracellular ions takes place. $K^+$ and $Cl^-$ must leave the cell in tandem in order to maintain electroneutrality. Subsequently, coupled water transport takes place and the cell volume decreases. This recovery of cell volume under hypotonic conditions is referred as to regulatory volume decrease (RVD; Note that under physiological, i.e. isotonic, conditions other mechanisms such as the $K^+$–$Cl^-$ co-transporter (mouse) or the $Na^+/K^+$ pump (dog) are additionally involved in volume regulation). (B) Activation of volume regulatory transport mechanisms is mediated by changes in phosphorylation–dephosphorylation balance. Under hypotonic conditions, the activation of transport mechanisms to regulate cell volume is mainly mediated through protein kinase C (PKC)- and protein phosphatase (PP)-dependent pathways. By maintaining serine and threonine residues in a phosphorylated state, PKC activity appears to keep the ion channels closed, while inhibition of PKC or increased activity of PP causes channels to open and initiate the RVD process. A cAMP-dependent pathway (not shown) is also involved because elevation of cAMP levels appears to activate transport mechanisms in a similar way to enhanced PP activity.

volume regulation under hypertonic conditions is mediated by protein tyrosine kinase-dependent pathways (Petrunkina *et al.* 2005*b*), while volume regulation following hypotonic stress is activated through protein kinase C- and cAMP-dependent pathways (Petrunkina *et al.* 2007; see Fig. 2B). Modification of cytoskeleton organization seems also be involved in the sperm volume regulation (Petrunkina *et al.* 2004*c*, 2004*d*).

### *Testing sperm cell volume regulation in domestic species*

Male gametes react to hypoosmolality by developing bent or curled sperm tails. In the classic hypoosmotic swelling test (HOST), this property is used to characterize membrane integrity (Jeyendran *et al.* 1984). Swollen sperm with curled tails are considered as intact. However, this evaluation is somewhat subjective, and it is not quantitatively rigorous. Since sperm integrity can only be detected via the possession of curled tails, it is not possible with this test to determine either the percentage of intact sperm under isotonic conditions or distinguish between the intact sperm that have not developed curled tails under hypoosmotic conditions and defect cells that did swell but then 'exploded' (it is of course possible to gather such information by using a membrane-impermeable fluorescent dye in conjunction with flow cytometry and fluorescent microscopy).

However, response to osmotic challenge can be measured quantitatively using an electronic cell counter. In this instrument, cell volume is determined by changes in electrical resistance as sperm pass through a capillary pore; the resulting data are expressed on the basis of cell frequency distribution. The methodology, described in detail in our previous publications (Petrunkina *et al.*

2001*a*, 2004*b*, 2004*c*, 2004*d*, 2005*b*), guarantees high accuracy and resolution; the electronic signals are neither dependent on the orientation of the sperm cells as they pass through the capillary pore nor on the geometrical shape of the cells.

The volumetric data are presented for analysis as histograms (Figs 3 and 4). Swelling can be monitored via the relative volume shift $V_r$

$$V_r = \frac{V_{hypotonic}}{V_{isotonic}}$$

and its temporal changes under different osmotic conditions, e.g. after 5 and 20 min: $V_{r,5}$ and $V_{r,20}$. The relative volume shift (response after 5 min) shows to which extent the volume distribution curve has been shifted to the right, i.e. how much swelling has taken place. The RVD is calculated as recovery of cell volume during 20 min:

$$RVD = \Delta V_r(t) = V_{r,5} - V_{r,20}$$

Either modal or mean values of the cell volume distribution can be used to monitor cell volume changes, but the modal value has been shown to be more sensitive to sample heterogeneity (Petrunkina & Töpfer-Petersen 2000).

Electronic cell counting technology produces exact measurement of sperm cell volume in physical units (unlike when flow cytometers are used to assess volume changes by measuring light scatter). Derivation of the RVD allows one to measure to what extent the cell volume has recovered after the initial hypotonically

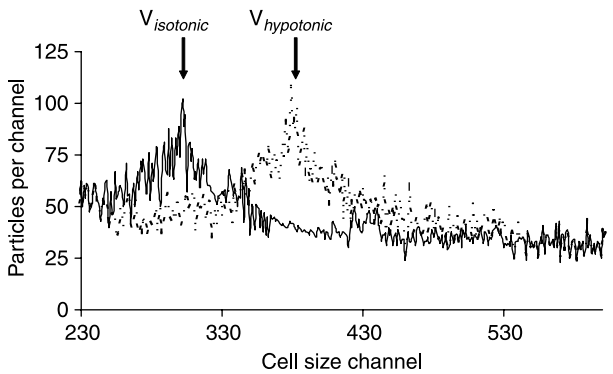


**Figure 3** Histograms of volumetric data from an electronic cell counter. The curves represent data obtained using an electronic cell counter CASY1 (Schärfe System GmbH). The solid curve represents an isotonic volume distribution with modal volume $V_{isotonic}$, the dashed curve represents the hypotonic volume distribution with modal volume $V_{hypotonic}$. The shift of the hypotonic volume distribution curve to the right reflects the progressive swelling of the cell population in response to hypotonic conditions. The degree of swelling is given by the ratio $V_r = V_{hypotonic}/V_{isotonic}$. The recording of volumetric curves at different times of exposure to anisotonic conditions and the consequent calculation of the kinetics of $V_r$ quantifies the environmental adaptability of the sperm sample.

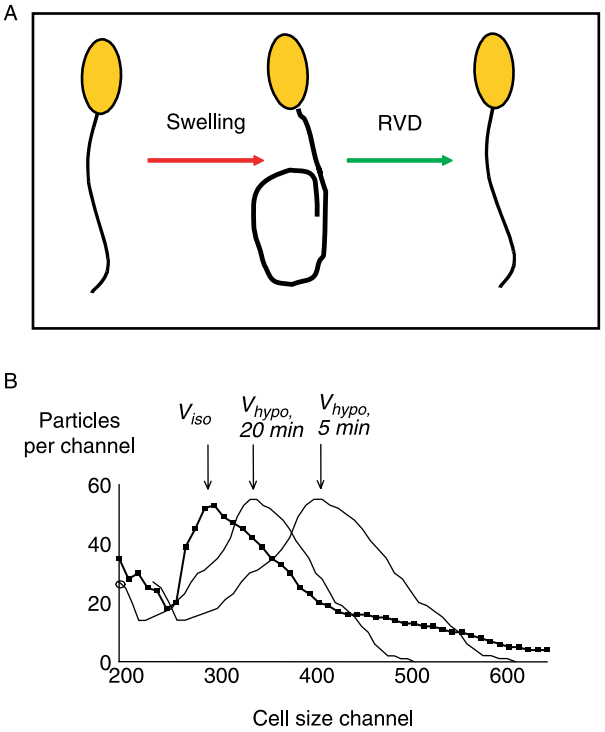


**Figure 4** The hypoosmotic swelling test (HOST) compared with the volume regulatory test. (A) Swelling of spermatozoa in response to hypotonic conditions. In the classic hypoosmotic swelling test, sperm respond to reduced osmolality with a characteristic curling of the tail which can be assessed morphologically. After longer exposure to hypotonic conditions, volume recovery will take place. This recovery may be too subtle to be detected and quantified by microscopical evaluation; moreover higher levels of cell death may result from the prolonged incubation used for classic HOST. (B) Using electronic cell counting, the response of the entire population can be assessed via the displacement of the volume distribution curve and its modal value. A heterogeneous primary response (relative volume shift) resulting in several subpopulations can thereby be sensitively assessed and quantified. Moreover, electronic volume measurement offers a straightforward means of recording kinetic changes in the cell volume. After 20 min of exposure to hypotonic conditions, the cell volume distribution is shifted to the left relative to that taken after 5 min of exposure, yielding a much lower modal value close to the isotonic initial value. The degree of volume regulation is given by the formulation $RVD = V_{r,5} - V_{r,20}$.

induced swelling (Fig. 4B). Such data cannot be provided at all by classic HOST (Fig. 4A).

It should be pointed out that although the major physiological osmotic stress to spermatozoa would appear to take place during ejaculation, i.e. as epididymal cells are mixed with accessory fluids and female tract secretions, it is not necessary to work only on epididymal spermatozoa by exposing them to an isotonic environment (e.g. Yeung *et al.* 2004*b*). The ejaculated cells continue to express osmotic regulating ability, and testing this ability against exposure to a hypotonic environment will reveal functional deficiencies, if present. Such deficiencies would suggest a suboptimal osmotic response to ejaculation and possibly

deficiencies in other functions. In particular, poor osmotic regulatory ability would imply inability to withstand cryopreservation (Petrunkina *et al.* 2004*b*, 2005*b*, 2005*c*).

### *Biological heterogeneity of cell volume response*

Within a given species, sperm volumetric response to changing osmotic conditions shows a high level of heterogeneity. Some ejaculates develop several peaks under isotonic, hypotonic, and hypertonic conditions. Whereas the differences under hypotonic or hypertonic conditions can be mostly attributed either to ongoing cell damage (plasma membrane degeneration) or deficiencies in RVD or RVI, the changes under isotonic conditions remain poorly understood. Spermatozoa corresponding to the different populations obviously differ in functional status, most likely in their cytosolic ion content and the functionality of their membrane channels. We have shown recently that the occurrence of additional subpopulations under isotonic conditions is associated with an uncontrolled ion uptake from the external medium and is related to a deficiency in the signaling pathways that control cell volume (Petrunkina *et al.* 2005*b*, 2007). Because the presence of such peaks is an early sign of progressive instability in the ejaculate, their development is referred to as necrotic volume increase (NVI). During NVI, the dephosphorylation–phosphorylation balance within the controlling signaling system is disturbed, the swelling-stimulated channels which are usually closed under isotonic conditions become activated, ion uptake and coupled water transport takes place, and the cells swell. In this situation, the volume distribution curve is shifted greatly to the right, and the peak is located beyond the reference area appropriate for physiological conditions (Petrunkina *et al.* 2005*b*, 2007). The changes are accompanied by membrane leakage to propidium iodide. In human, as reviewed by Yeung *et al.* (2006), failure to regulate sperm volume under physiological conditions may be one of the sources of infertility. Such findings indicate that it is important to evaluate the ejaculate's volumetric response under all three conditions: isotonic, hypertonic, and hypotonic; moreover, besides evaluating the primary $V_r$ response, the degree of RVD or RVI must be estimated (Petrunkina *et al.* 2004*b*, 2005*b*, 2005*c*). Ejaculates can thereby be classified according to their volume regulatory responses. Using this approach, it has been found both in human and boar that differences in the volume distribution curves observed under isotonic and hypotonic conditions can be associated with different spermatological characteristics, thereby reflecting differences in the physiological status between ejaculates (Petzoldt & Engel 1994, Petrounkina *et al.* 2000). Individual differences in volume regulatory ability have also been observed in dog and bull (Petrunkina *et al.* 2001*b*, 2004*b*, Khalil *et al.* 2006). These findings strongly suggest the potential usefulness of this parameter for advanced spermatology.

### *Volume regulation and fertility*

Yeung *et al.* (1999) hypothesized that the ability to regulate volume is important for achieving fertility. As mentioned above, sperm that have undergone swelling have curled or bent tails; this has a detrimental effect on motility expression, with the result that sperm transport and egg penetration may be compromised. They also suggested that tyrosine phosphorylation may be involved in RVD, because sperm from c-ros tyrosine kinase receptor knockout mice were infertile and showed impaired motility and other characteristics commensurate with volume regulatory disturbance (though as pointed out by the authors later (Yeung *et al.* 2004*a*), these changes may have been due to an effect of c-ros knockout on the epididymis itself and thence disturbance of the maturation process. In domestic species (bull, boar, and dog), it has been demonstrated that control of sperm cell volume is closely related to sperm fertilizing competence *in vivo* (Petrunkina *et al.* 2001*b*, 2005*b*, Khalil *et al.* 2006). For example (Table 1), in bulls and boars grouped according to fertility, the level of relative swelling after 20 min of exposure to hypotonic conditions was still relatively high in subfertile animals, while in fertile animals $V_{r,20\ min}$ was significantly lower. Nevertheless, the actual mechanistic link between RVD ability and fertilizing competence remains to be elucidated.

## Sperm interaction with the oviduct

In many mammalian species, the oviduct plays two crucial roles. It is, of course, the region in which

**Table 1** Volume recovery after osmotically induced swelling in fertile and subfertile animals.

| | Bull | | Boar | |
|---|---|---|---|---|
| **Parameter** | $V_r$ (20 min) | NRR (%) | $V_r$ (20 min) | FR (%) |
| Fertile individuals | 1.13±0.16* | 72.3±3.0* | 1.50±0.22* | +2.60±0.8* |
| Subfertile individuals | 1.42±0.24* | 58.9±6.6* | 2.33±0.57* | −0.32±3.19* |

$V_r$, 20 min: degree of swelling after 20 min of exposure to hypotonic conditions; the higher the figure, the poorer the recovery (see text for definition of $V_r$). NRR, non-return rate (expressed as percentage of cows not returned for repeated insemination); FR, farrowing rates expressed as percentage deviation from the average farrowing rates of the AI producer. *$P<0.05$ (presented values differ significantly between the rows; data sources: (Petrunkina *et al.* 2004*a*; boar); (Khalil *et al.* 2006; bull); fertility data as supplied by the commercial AI centers).

spermatozoa become capacitated. However, because sperm are generally deposited in the female genital tract in advance of ovulation, the oviduct has developed as a region in which spermatozoa are sequestered to await the arrival of the eggs. The biological significance and role of the oviduct has been highlighted in numerous reviews (e.g. Bedford 1999, Rodriguez-Martinez *et al.* 2001, Suarez 2002, Hunter 2003, Suarez & Pacey 2006, Rodriguez-Martinez 2007*b*). A recent review by Suarez (2007) gives current views regarding sperm–oviduct interactions and *in vivo* oviduct studies, especially with respect to assisted reproduction.

### *Sperm storage – binding to the oviductal epithelium*

Sperm storage in the oviduct involves adhesion to and controlled release from the epithelium. Of the several millions of sperm normally ejaculated, only a small proportion becomes trapped within the distal portion of the isthmus, in which they reside under protective conditions until ovulation. This sperm storage site in the mammalian oviduct, designated as a 'functional sperm reservoir' (Hunter 1981), selects the fertilization-competent sperm population, modulates sperm capacitation, and regulates sperm transport to minimize polyspermic fertilization. Of particular, importance is the concept that binding to the oviductal epithelium stabilizes the spermatozoa and greatly enhances their survival in a relatively hostile environment. It is therefore obvious that ability to bind to the oviduct is a very important sperm function with respect to fertilizing ability.

The binding process inevitably takes place through receptor–ligand interaction. These latter have yet to be identified and characterized in detail, though it has been demonstrated that the mechanism involves specific terminal carbohydrate residues (Suarez 2002, Töpfer-Petersen *et al.* 2002, Suarez & Pacey 2006 and references therein). Obviously, correct sperm membrane architecture is required for the binding process to take place efficiently. Development of the correct architecture may depend on adequacy of the maturation process, during which extensive remodeling of the sperm membrane takes place (see Jones 1989). Testing of sperm ability to bind to the oviductal epithelium, therefore, not only measures what is considered to be a key sperm function but may also reveal indirectly other functional shortcomings.

### *Sperm capacitation – modulation by the oviduct*

Capacitation is a collective series of changes that take place naturally in the female reproductive tract, particularly in the oviduct. These changes 'prime' the spermatozoa for fertilization. The essential importance of capacitation has induced a very great deal of research, but only rather recently have significant advances in our knowledge of the process become available. Since studies of sperm capacitation within the oviduct itself are obviously extremely difficult, *in vitro* approaches have had to be developed. From these (and those described below in the section relating to *in vitro* capacitation), it has become clear that capacitation involves many steps, though the sequence of these is not yet completely understood. Nevertheless, it has emerged that a bicarbonate/$CO_2$-rich environment is an essential trigger (see below). The molecular aspects of capacitation have been discussed in many reviews, particularly recently by Brewis *et al.* (2005) and Harrison & Gadella (2005), and physiological issues have been dealt with in other recent reviews which have focussed especially on the comparative assessment of sperm–oviduct interactions *in vivo* and *in vitro* (Hunter 2003, Hunter & Rodriguez-Martinez 2004). As pointed out earlier, only normal viable uncapacitated sperm are selected by the oviductal epithelium. Numerous studies in different homologous and heterologous *in vitro* models have shown that sperm survive longer when in contact with the oviductal epithelium, and that capacitation-related changes are modulated by the oviduct in the human, canine, porcine, equine, and bovine species (Ellington *et al.* 1998, 1999, Gualtieri & Talevi 2000, Kawakami *et al.* 2001, Petrunkina *et al.* 2001*d*, 2003). Furthermore, proteins derived from oviduct epithelial plasma membranes suppress response to bicarbonate (see below) and modify sperm movement in a subpopulation-specific and dose-dependent manner (Satake *et al.* 2006). Thus, it appears that during binding sperm are made to wait for the trigger that causes their release and that sets in train the completion of capacitation. *In vivo* capacitation is synchronized with ovulation (Hunter & Rodriguez-Martinez 2004). Most viable sperm detected *in vivo* in sperm reservoir before ovulation are uncapacitated. Under the influence of local and systemic ovarian control mechanisms, conditions within the oviduct are generated that reverse the inhibitory influence on sperm function, allowing the sperm to be released from the oviductal storage site and to progress toward the site of fertilization, so the capacitation rates increase after ovulation (Töpfer-Petersen *et al.* 2002, Hunter & Rodriguez-Martinez 2004, Tienthai *et al.* 2004, Rodriguez-Martinez 2007*b*). Little is yet known about the factors and mechanisms that regulate the inhibiting and activating effects of the oviduct on inseminated sperm. However, evidence has been presented to show that slow capacitational changes take place during binding, and it has been hypothesized that release of bound sperm results from changes in the sperm surface brought about by capacitation (Ellington *et al.* 1999, Gualtieri & Talevi 2000, Petrunkina *et al.* 2003, 2004*e*, Gualtieri *et al.* 2005). In pig, capacitation does not occur *in vivo* to any great degree during the pre- and peri-ovulatory period unless spermatozoa are exposed to bicarbonate which appears to be the key effector of the membrane

destabilizing changes *in vitro* and *in vivo* (Tienthai *et al.* 2004, Rodriguez-Martinez 2007*b*), although addition of isthmic oviductal fluid to the pre- or peri-ovulatory sperm maintains sperm viability. Post-ovulatory isthmic oviductal fluid, on the other hand, significantly increases sperm release (Tienthai *et al.* 2004). In cattle, heparin, and other sulfated glycoconjugates induce the synchronous release of sperm adhering to the tubal epithelium *in vitro* (Gualtieri *et al.* 2005, Bergqvist *et al.* 2006).

From the body of evidence outlined previously, there is an indication that the caudal isthmic environment *in vivo* may not support capacitation before ovulation in the way that artificial capacitation media do for sperm co-incubated with oviductal epithelial cells or explants *in vitro.* Knowledge of the releasing mechanisms derived from *in vitro* studies may therefore be limited because the culture media thus far commonly used may represent a post-ovulatory environment (Hunter & Rodriguez-Martinez 2004). Nevertheless, *in vitro* models are useful to monitor the sequence of modifications occurring in sperm bound to the oviduct or in released sperm and to understand how the oviduct protects the selected sperm population over the longer time period. To understand how the 'active' fertilizing competence of the sperm is synchronized with the arrival of the ovulated egg to ensure successful fertilization, however, one needs to consider the whole system over a broad time period, not at limited time points. Since the capacitation process is considered to be a controlled destabilization process which reduces the life span of sperm (Harrison 1996), maintenance of sperm viability within a certain window of time and control of capacitation are mutually associated events. Use of stepwise dynamic analysis of the diversity of kinetic changes in sperm parameters during interaction with the oviductal epithelium and in response to capacitating conditions would help to understand how specific coordination and completion of these processes ensure maximal rates of fertilization *in vivo.*

### *The sperm–oviduct binding assay as an estimation of the potentially fertilization-competent sperm subpopulation*

To characterize sperm ability to bind to the oviductal epithelium, a sperm–oviduct binding assay has been established (Suarez *et al.* 1991, Lefebvre *et al.* 1995). In our laboratory, this assay has been adapted to characterize the binding ability as an individual ejaculate property which could be relevant to the assessment of fertility in domesticated species (cattle, pig, dog; Petrunkina *et al.* 2001*c*, 2004*e*, Khalil *et al.* 2006, Waberski *et al.* 2006). Since only potentially competent sperm bind to the oviduct, inability to establish a sufficiently high number of sperm in the functional sperm reservoir would result in failure of fertilization (Hunter & Rodriguez-Martinez 2004). A comparison of the bound and unbound subpopulations reveals differential characteristics relevant to survival and capacitation. It has been shown for several domestic species that the oviduct selects for binding only sperm which are mature, viable, motile, morphologically intact, have low $Ca^{2+}$ content, no head protein phosphorylation, and have not undergone capacitation (as indicated by chlortetracycline staining; Fazeli *et al.* 1999, Töpfer-Petersen *et al.* 2002, Gualtieri & Talevi 2003). Sperm subpopulations that are not bound have increased percentages of morphologically altered cells, possess higher calcium levels, and have other markers of ongoing destabilization, for example, enhanced tyrosine phosphorylation, increased levels of capacitated cells, and reduced levels of membrane integrity. Moreover, sperm with attached cytoplasmic droplets have a reduced ability to bind, so that immature sperm are 'rejected'. It has been reported by different groups that epididymal sperm do not bind to the oviduct in cattle and pigs to the same extent as do ejaculated (Petrunkina *et al.* 2001*c*, Gwathmey *et al.* 2003), and it has recently been shown that cells with superior volume regulation have better ability to bind to the oviductal epithelium: RVD correlated significantly with binding index (Khalil *et al.* 2006). All the evidence taken together points to the hypothesis, that development of the ability to bind to the oviduct, like volume regulatory ability, may be one of the final stages of epididymal maturation.

Technically, in the sperm–oviduct assay, set numbers of small pieces of oviductal epithelium (explants) and sperm are co-incubated together for 15 min after which sperm binding to the explants is recorded by computer videomicrography (for technical details of approach see Petrunkina *et al.* 2001*c*). The sperm number bound to selected regions is counted, and the corresponding surface areas of oviductal epithelium are measured using computer-assisted software. The average number of sperm bound to 0.01 $mm^2$ of explant surface (the binding index, BI) is calculated as

$$\mathrm{BI} = \frac{1}{N}\sum_{i}^{N}\mathrm{BI}_i$$

from

$$\mathrm{BI}_i = \frac{\sum_{k=1}^{3} N_k}{\sum_{k=1}^{3} S_k}$$

where $\mathrm{BI}_i$ is the binding index calculated for each videotaped explant, $N$ is the number of explants, $N_k$ is the sperm number bound to each particular region of the explant, and $S_k$ is the area of this region; in this way, variability between different regions of an explant is taken into account. Because sperm binding to the oviductal epithelium is dose dependent, it is important to maintain standard conditions with respect to sperm

concentration when comparing sperm donors or carrying out inhibitor studies on sperm–oviduct interactions (e.g. Petrunkina *et al.* 2001*a*).

The parameter has been shown to detect differences between individual boars and bulls, and between fresh and stored semen (Petrunkina *et al.* 2001*c*, De Pauw *et al.* 2002, Khalil *et al.* 2006, Waberski *et al.* 2006), and pilot studies by our group have shown that the ability of sperm to bind to the oviductal epithelium is different in fertile and subfertile boars and bulls. In bulls (Khalil *et al.* 2006), sperm from subfertile animals ('non-return rate' NRR = 58.9 ± 6.6%) had a significantly lower binding index (BI = 23.5 ± 2.6 sperm/0.01 $mm^2$) than the sperm of fertile animals (BI = 19.3 ± 3.6, NRR = 72.3 ± 3.0%; $P < 0.05$). Similarly, in group of four subfertile boars (Waberski *et al.* 2006), the binding index was considerably (at least mean minus one S.D.) lower (BI < 17.4 sperm/0.01 $mm^2$) than in the average population (26.4 ± 8.9 sperm/0.01 $mm^2$), though in this case, the difference was not tested statistically. De Pauw *et al.* (2002) also found in bulls a positive association between the non-return rate of the respective sires and the log-transformed number of spermatozoa bound to 0.01 $mm^2$ oviductal epithelium after 24 h of co-incubation.

It is important to realize that the *in vitro* binding test does not resemble in detail conditions *in vivo*. Quite apart from likely differences in milieu and the problems of mimicking the physiological changes that occur within the oviduct around the time of ovulation, the numbers of spermatozoa which bind to oviductal cells *in vitro* are much higher than that are found bound *in vivo*. However, one should bear in mind that the *in vitro* binding assay tests the binding capacity of an isolated oviductal cell culture or explant with respect to a standardized number of spermatozoa from the test sample under standard conditions. *In vivo* due to the barrier-like nature of the uterotubal junction, the number of sperm available for binding is much less per unit area of oviductal epithelium. Nevertheless, despite its physiological shortcomings, the *in vitro* binding test is proving very useful for the assessment of sperm quality, given its high sensitivity for detecting individual differences.

## Sperm response to capacitating conditions *in vitro*

It is axiomatic that spermatozoa undergo capacitation during co-incubation with eggs to achieve *in vitro* fertilization. Thus, molecular studies of capacitation can be carried out using *in vitro* incubation in IVF media. Such studies have revealed that the essential component of such media with respect to supporting capacitation is bicarbonate/$CO_2$; indeed, transfer of sperm from an IVF medium devoid of bicarbonate to one including it triggers the capacitation sequence. The key sperm property crucial to fertilization which can be tested *in vitro* is thus the ability of sperm to respond to bicarbonate by undergoing capacitatory changes (Silva & Gadella 2006).

The response of sperm from different individuals to capacitating conditions varies highly in its degree. This variability has been reported in many domestic species with respect to most parameters associated with capacitation: increase in cytosolic $Ca^{2+}$ levels, changes in motility, changes in cAMP levels, rapid collapse of phospholipid transverse asymmetry, phosphorylation of tyrosine residues, and response to calcium ionophore A23187 (Harrison *et al.* 1993, Holt *et al.* 1997, Harrison 2003, Harrison & Gadella 2005, Piehler *et al.* 2006). Examination of the specific responses to capacitating conditions also reveals, in all species, considerable functional heterogeneity within the sperm population. Several populations of cells exist within a single ejaculate, which differ in their capacitational behavior. The development of the subpopulation can be followed and assessed (e.g. sperm motility and velocity parameters, sperm volume, merocyanine binding, surface exposure of endogenous aminophospholipids and lectin binding; Petrunkina & Töpfer-Petersen 2000, Gadella & Harrison 2002, Holt & Harrison 2002, Baker *et al.* 2004, Holt & Van Look 2004, Harrison & Gadella 2005). Activation of motility and changes in lipid architecture of membranes with bicarbonate appear to be especially suitable responses to assess, because they take place within a very short time after exposure to capacitating conditions, and a high level of sensitivity towards individual differences and ejaculate heterogeneity is achieved (Gadella & Harrison 2002, Harrison 2003, Satake *et al.* 2006). The ability to detect such differences between individuals argues strongly for the inclusion of capacitation tests in the spectrum of advanced methods for evaluating sperm quality.

However, although assessing capacitational changes during incubation under capacitating conditions would seem to be a particularly appropriate way of predicting sperm fertilizing capability, results so far have not been sufficiently successful. Many studies in different domesticated species have been performed in recent years, measuring parameters such as the response of sperm to calcium ionophore or to the zona pellucida (intact and solubilized proteins), increase in cytosolic calcium content, stimulation of motility by bicarbonate, merocyanine binding, lectin binding, or protein tyrosine phosphorylation, and redistribution of membrane proteins (reviewed by Aitken 2006, Rodriguez-Martinez 2006). Use of these has certainly led to substantial improvements in the assessment of semen quality in domestic species, but their direct relationship to fertility remains poorly defined (Colenbrander *et al.* 2003, Rijsselaere *et al.* 2005), and only a few of them have displayed significant correlations with fertility in bulls, boars, and stallions (Januskauskas *et al.* 2003, Gil *et al.* 2005, Kirk *et al.* 2005, Kuisma *et al.* 2006). A major

problem with such assessment methods is that the fertilization process is multifunctional, and it is clear that several sperm fertility attributes must be assessed in parallel and the results analyzed multiparametrically. Indeed, various groups have shown that multiple regression analysis improves the prediction level of fertility (Holt *et al.* 1997, Kirk *et al.* 2005, Petrunkina *et al.* 2005*a*, Rodriguez-Martinez 2006, Garcia-Macias *et al.* 2007). However, even when data from several parameters are combined, these data apply to the global sample and not to individual cells. For a particular cell to have adequate fertilizing ability, several or more processes must be able to function correctly within that given cell. As yet, however, it is not possible to test several capacitational parameters at the same time in a single cell.

Simultaneously, currently used assessment tests often suffer from methodological shortcomings. First, in many cases sperm response to non-capacitating conditions has not been tested adequately as a control comparison. Given our knowledge about the key importance of bicarbonate as a capacitation trigger, the control medium should be *identical* to the capacitating medium save that it should be devoid of bicarbonate; moreover, in order to ensure this latter, incubation in the non-bicarbonate-containing medium must be carried out in a $CO_2$-free atmosphere – otherwise the $CO_2$ will dissolve in the medium and form bicarbonate! Secondly, insufficient attention has been paid to pH control in the bicarbonate-containing medium, with the result that capacitation has been carried out under various pH conditions; maintenance of a given pH in a medium when bicarbonate/$CO_2$ is part of the buffer system requires careful attention to chemical principles (see comments on use of bicarbonate in Suzuki *et al.* 1994). Thirdly, given that bicarbonate triggers capacitation, it is clearly a mistake to extract, wash, or otherwise process sperm in bicarbonate-containing media prior to submitting them to the test incubations; in such situations, capacitation is likely to have been triggered prematurely in both control and test samples.

However, even assuming that tests of capacitational response are carried out with due attention to the foregoing points, consideration of the physiological situation implies that further developments in assessment procedures are needed if fertilizing ability is to be predicted with useful accuracy.

### *Capacitation as a 'continuum': the 'uncertainty principle' and fertility evaluation*

In his seminal papers on the Uncertainty Principle in quantum mechanics, Heisenberg (1927*a*,1927*b*) wrote: 'It seems to be a general law of nature that we cannot determine position and velocity simultaneously with arbitrary accuracy'. That is to say, it is not possible to determine simultaneously the position and momentum of an atomic particle. Moreover, the better the position is known, the less well is the momentum known and vice versa (Heisenberg 1927*a*).

Strange though it may seem, there are parallels between this major physical law and the determination of sperm response to capacitating conditions. The complexity of the biological process, involving both male and female, is such that an absolute quantitative value can in all likelihood never be provided, i.e. uncertainty will always exist (c.f. Amann 1989, Amann & Hammerstedt 1993). Nevertheless, at the present time, this uncertainty is unnecessarily high because evaluation is based only on the degree of response as measured by a given parameter after a given period of incubation ('single parameter – single time point' approach). The current view is that a 'low' response to stimuli (capacitating conditions) is associated with in- or subfertility, whereas a 'high' response is associated with good fertility (e.g. Amann & Hammerstedt 1993, Herrera *et al.* 2002, Brinsko *et al.* 2007). However, in the light of available evidence, it appears that the ability to respond to capacitating conditions in terms of absolute values or percentages of responding cells is not of itself the key factor, but the occurrence of a certain response level within a certain interval of time. The definition of capacitation as a process of positive controlled destabilization (Harrison 1996) implies that too high or too rapid a response can lead to a destabilization too great to allow the sperm to fertilize the egg. In other words, dealing merely with a high value of response at a particular time point ignores the rate of change, which will be too high if the destabilization of the plasma membrane is continuing to progress at the same high rate. Similarly, although a total lack of ability to respond to capacitating conditions would allow the elimination of non-responding samples, slowly responding samples may well prove to be adequately (or even optimally!) fertile. As outlined above, sperm capacitation must be synchronized with the processes in the female. However, very tight synchrony cannot be achieved and so a continuous supply of sperm capable of fertilizing must be provided so that enough are available to the oocyte at the actual time of ovulation. Thus, around the time of ovulation, successive cohorts of capacitating cells must release from the oviductal epithelium. This in turn implies that within the responding sperm population, individual cells must capacitate and/or release at different rates. Indeed, it has been found that the rates of sperm capacitational response in parameters such as motility, merocyanine binding, and levels of cAMP vary greatly, not only between individuals and ejaculates, but also between the cells within one ejaculate (Harrison 1996, 2003, Flesch *et al.* 2001, Holt & Harrison 2002). Moreover, the rates at which different functions are expressed differ greatly (e.g. changes in merocyanine binding occur rapidly whereas changes in tyrosine phosphorylation occur slowly, c.f. Piehler *et al.* 2006).

We believe, therefore, that capacitational changes must be assessed on the basis of several parameters measured over a suitable period of time, within which several subpopulations may develop. While a positive response to capacitating conditions is important, the rate of capacitation as a destabilization process is crucial, because under *in vivo* conditions capacitation must be considered as a conflict between destabilization and sperm survival (Harrison 1996). Due to this dichotomy and the high degree of biological variation, one needs to determine the response limits for expression of capacitational changes. Not only must the degree and rate of positive responsiveness of the sperm be characterized, but also this must be related to natural restrictions like the position of the change parameter in the capacitation sequence, the life span of the spermatozoa, and the intended interval between insemination and ovulation. Evaluation of sperm quality is then based on these limits: if the capacitational changes are either too slow or too rapid (the latter also associated with excessive cell death) that response may be associated with subfertility (Fig. 5). It must nevertheless be remembered that while such an approach will be a much more sensitive way of assessing fertility likelihood, precise fertility prediction will very likely remain an uncertainty.

### *Proposed future methodology for fertility assessment via in vitro capacitation tests*

A graphical representation and mathematical details of the procedure are shown in Fig. 5 and its legend. The first stage would be to measure the time courses of several selected capacitational change parameters ($P_1$,…, $P_N$). These parameters would be chosen on the basis of different overall kinetics (i.e. compare fast changes, medium rate changes, and slow changes) and should be carried out on a suitable number of males (e.g. 20, but the number of individuals should be established based on the between- and within-individual variations of the trials). From these kinetics studies, time limits $T_1$ and $T_2$ would be chosen, according to the particular parameter's kinetic characteristics. For fast changes, the limits would be relatively close together and begin soon after the start of incubation whereas for slow changes the limits would be much further apart and begin only once the change became detectable. Note that it would be important to ensure that sperm were not exposed to capacitating conditions until the start of the test incubations. Once the time limits have been chosen, a more extensive examination of the chosen parameters would be required based on data from a large number of males. This would enable reliable mean values and standard deviations to be calculated for each parameter within its chosen time period. Finally, threshold values for each parameter (lower and upper limit) would be set at the start ($T_1$) and end ($T_2$) of its time period. For each parameter, the time limits and thresholds would thus form a 'window', from which we propose that any semen sample giving a test result for the particular parameter that fell outside the window's borders would be considered to be of questionable fertility.

As an addendum to the idea that subfertility may be associated with opposite extremes of capacitatory response, we found in a recent preliminary study of eight subfertile boars (Petrunkina *et al.* 2005*a*) that the relationship between responsiveness and fertility was not linear but dichotomic: the changes in the sperm parameters induced by exposure to capacitating conditions were either high or low (Table 2) although all eight showed satisfactory levels of motility. Moreover, the response to capacitating conditions assessed on a group of fertile and subfertile boars ($n=18$) correlated with the farrowing rates and litter sizes within a multiple regression model. Both the response in terms of

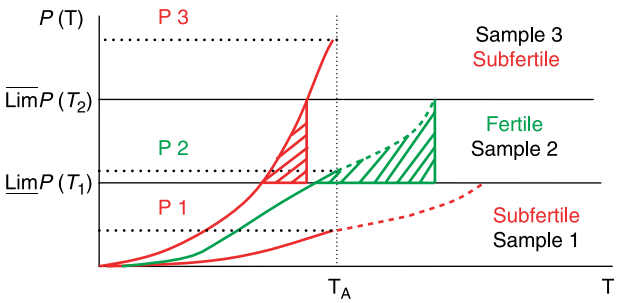


**Figure 5** The capacitation 'continuum' – comparison of three different hypothetical ejaculates. In this model, three different ejaculates have been tested for their capacitation response; the kinetics of their responses is plotted. The $P(T)$ axis indicates the absolute level of a given capacitation response parameter (e.g. intracellular $Ca^{2+}$ increase), while the $T$ axis indicates the time of exposure to capacitating conditions. The responsiveness threshold for $T_1$ would be the parameter's mean value at $T_1$ minus its S.D. at $T_1$ ($\underline{\lim} P(T_1) = \overline{P(T_1)} - SD_{T_1}$) and for $T_2$ its mean value at $T_2$ plus the S.D. at $T_2$ ($\overline{\lim} P(T_2) = \overline{P(T_2)} + SD_{T_2}$). Prior experimentation (on other samples – see text) has determined that samples showing a level of response greater than the upper limit $\overline{\lim} P(T_2)$ or less than the lower limit $\underline{\lim} P(T_1)$ are subfertile. In the former situation, the destabilization that is a part of the capacitation response has progressed too far whereas in the latter the response is simply insufficient. $T_1$ and $T_2$ indicate the period of incubation within which a response must be shown. In sample 1, the response to capacitating conditions after assessment time $T_A$ ($T_1 < T_A < T_2$) is very low (less than the lower designated limit of responsiveness $\underline{\lim} P(T_1)$, i.e. $P_1(T_A) < \underline{\lim} P(T_1)$). In samples 2 and 3, capacitation progresses at higher rates. However, the response of sample 3 after time $T_A$ is too high, above the upper designated limit of responsiveness $\overline{\lim} P(T_2)$: $P_3(T_A) > \overline{\lim} P(T_2)$. Moreover, the prolongation of capacitation as a 'positive' destabilization process must be taken into account (i.e. sufficient numbers of sperm must survive and remain functionally competent). Sample 3 is destabilizing at a high rate, so its 'capacitation window' is very short (red-hatched area). On the other hand, in sample 1 capacitation progresses so slowly that it does not attain the required lower limit of response before $T_2$. In sample 2, however, capacitation takes place at a suitable rate so that at time $T_A$ its response $P_2(T_A)$ falls between the upper and lower response limits (i.e. $\underline{\lim} P(T_1) < P_2(T_A) < \overline{\lim} P(T_2)$), and its capacitation window is consequently relatively long (green-hatched area).

**Table 2** Dichotomic response to capacitating conditions shown by spermatozoa from subfertile boars.

| | **Motility** (%) | **MAS** (%) | **AR** (%) | **Ca, live** (%) | **PI** (%) |
|---|---|---|---|---|---|
| High responders | 83.1±1.6 | 15.8±1.4* | 35.8±4* | 22.0±2.9* | 22.9±3.9* |
| Low responders | 79.5±1.5 | 36.4±10.8* | 18.5±4.9* | 12.0±2.6* | 12.0±0.9* |

Motility assessed by subjective microscopic analysis of extended semen. MAS, morphologically abnormal cells; AR, percentage of cells undergoing the ionophore-induced acrosome reaction after 2-h exposure to capacitating conditions; Ca, live: percentage of live cells with increased intracellular $Ca^{2+}$ content; PI, percentage of propidium iodide positive (i.e. dead) cells. *$P<0.05$ (presented values differ significantly between the rows; data source: (Petrunkina *et al*. 2005*a*); fertility data were supplied by the commercial AI centers). Responsiveness to capacitating conditions was assessed in eight subfertile animals that showed a negative deviation in farrowing rate relative to the AI average (−5.4±2.5%, $n=8$). Two distinct patterns of responsiveness were observed: 'high' and 'low'. In four of the animals, the levels of acrosome reactions, calcium influx, and cell death were significantly higher than in the other four. Whereas low responding boars had a significantly higher percentage of morphological abnormalities, other standard sperm parameters of the subfertile boars were within the normal range.

emerging sustainable population of live cells with high cytosolic calcium content and the maintenance of sufficiently large numbers of membrane-intact cells were compounds of this model, demonstrating that although the sperm population must respond, destabilization should not progress too rapidly.

## Concluding remarks

It is now clear that fertilization success does not simply depend on the absolute number of vital, motile, morphologically normal spermatozoa inseminated in the female but more importantly on their functional competence. To evaluate male fertility, therefore, sensitive tests must be made of specific sperm properties that are prerequisite for fertilization. Three such properties are: a) the ability to regulate cellular volume in the face of changing environments, b) the ability to bind to the oviductal epithelium, and c) the ability to undergo capacitation in a timely and appropriate manner. Tests of these properties have been found to offer promising new strategies for evaluating fertility in domestic animals, due to their ability to reveal physiological heterogeneity within the ejaculate and their relevance to the establishment of the oviductal sperm reservoir and subsequently to fertilization. A capability for volume regulation implies adequate maintenance of cell volume during tonic stress, not only at ejaculation and in the female genital tract but also during cryopreservation and thawing. Ability to bind to the oviductal epithelium implies that an adequate population of sperm can remain protected in the oviductal reservoir prior to ovulation. *In vitro* tests of capacitation provide valuable information on the sperm population's ability to respond to fertilizing conditions in a suitable physiological manner. Of particular relevance in these latter tests are the sequential manner in which capacitational changes occur and the rate of the accompanying ongoing destabilization process. The capacitational response must be considered as an overall process. Rather than measuring a single parameter after an arbitrary period of incubation, several parameters need to be measured in parallel over time periods appropriate to the rate at which they develop. It is much more likely that good fertilizing capability can be predicted from an 'optimal' response, within limits and within a certain time period, rather than from a maximal and rapid response.

Overall, it is clear that semen samples must be subjected in parallel to several different tests, selected on the basis of the criteria outlined in the introductory paragraphs. In order to provide the highest level of fertility prediction from such tests (and from any others carried out), their outcome should be subjected to multiparametric analysis.

## Acknowledgements

Authors wish to thank to Dr R A P Harrison for the critical reading of the manuscript, constructive discussions, and English revision. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 27 January 2007
First decision 2 April 2007
Revised manuscript received 13 May 2007
Accepted 16 May 2007