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Novel methods to detect capacitation-related changes in spermatozoa

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ABSTRACT

Prior to interaction with the oocyte, spermatozoa must undergo capacitation, which involves a series of physio-chemical transformations that occur in the female tract. As capacitation is a pre-requisite for successful fertilisation, it is a topic of great interest for sperm biologists, but the complexity of the numerous biochemical and biophysical processes involved make it difficult to measure. Capacitation is an extremely complex event that encompasses numerous integrated processes that can occur concurrently during this window of time. The identification of techniques to accurately assess and quantify capacitation is therefore crucial to gain a meaningful insight into this fascinating sperm maturation event. Whilst there are extensive reviews in the literature that focus on the functional changes to spermatozoa during capacitation, few have examined the methods required to measure these changes. The aim of this review is to highlight frequently used methods to quantify different stages of capacitation and identify promising novel techniques. Factors that are able to modulate various capacitation processes will also be discussed. The overall outcome is to provide researchers with a toolbox of methods that can be used to gain a deeper understanding of the intricacies of capacitation in spermatozoa.

1. Introduction to capacitation

Capacitation begins after ejaculation when spermatozoa are exposed to signalling factors within the female reproductive tract. It can also be mimicked in vitro by specifically formulated media that support the process. The composition of fluids in the female tract is biochemically distinct from that of the site of sperm storage (cauda epididymis) in the male reproductive tract, with observed changes in the concentration of certain ions, osmolarity and pH [1]. Bicarbonate is one such ion that substantially increases in concentration from the male to female tract [2,3], particularly in the oviduct environment around the time of ovulation [3]. The presence of elevated bicarbonate has been shown to activate an enzyme known as soluble adenyl cyclase (sAC), which is responsible for the production of cyclic adenosine monophosphate (cAMP) [4]. Cyclic AMP is an important second messenger in many cellular processes and it has a direct effect on protein kinase A activity (PKA; Fig. 1). This enzyme plays a crucial role in the phosphorylation of proteins with serine and threonine residues and this post-translational modification regulates protein function and allows spermatozoa to respond to their surrounding environment during capacitation [5] (Fig. 1).

During capacitation, the induction of the cAMP-PKA pathway by bicarbonate initiates several key plasma membrane events that are crucial to facilitate fertilisation. Firstly, the plasma membrane becomes destabilised as a result of phospholipid scrambling, which is mediated by the PKA-activated phospholipid translocase, scramblase [6–8] (Fig. 1). The enhancement in membrane fluidity and permeability is a vital pre-requisite for the redistribution of lipids, namely cholesterol, to the apical region of the sperm head [9]. Cholesterol is then actively transported from the plasma membrane, a process essential for fertilisation [9–12]. Because cholesterol is extremely hydrophobic, reverse cholesterol transport (RCT) from the cell membrane to the extracellular environment can only occur if it contains cholesterol acceptor molecules such as serum albumin or high-density lipoproteins (Fig. 1). The loss of cholesterol from the apical plasma membrane has been shown to assist in the formation of lipid rafts that harbour proteins required for zona pellucida binding and the acrosome reaction [13,14]. As a marker of the later stages of capacitation, proteins with tyrosine residues become increasingly phosphorylated by specific kinases that are also regulated by PKA [1]. In likeness to phosphorylation by PKA,

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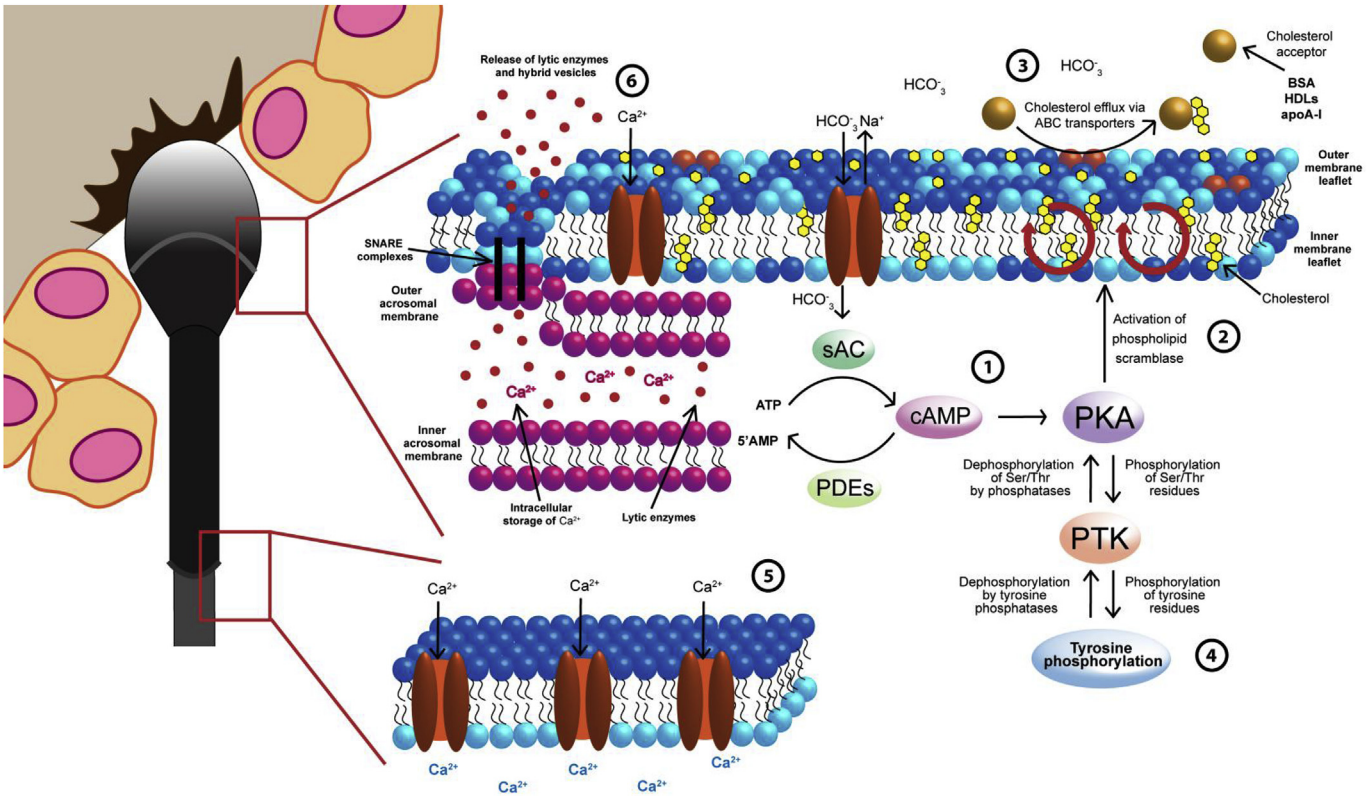

Fig. 1. The progression of events during capacitation. Bicarbonate concentrations increase as spermatozoa approach the oviduct, sAC becomes activated and that subsequently activates the cAMP-PKA pathway (**1**). The plasma membrane becomes increasingly destabilised owing to phospholipid scrambling mediated by PKA activity (**2**). This enhancement in membrane fluidity enables the redistribution of cholesterol to the apical sperm head (**2**) and the regulated loss of this sterol by cholesterol acceptors during a process known as cholesterol efflux (**3**). A downstream event of PKA action in the later stages of capacitation is an increase in the phosphorylation of proteins with tyrosine residues, leading to a modification of proteins associated with fertilisation (**4**). Hyperactivation is a calcium-dependent event that stimulates propulsive motility required for events leading to binding to the zona pellucida (**5**). Upon contact with the zona, spermatozoa undergo the acrosome reaction (**6**), another calcium-dependent process that enables the release of lytic enzymes that degrade the glycoprotein layer of the zona pellucida. This final stage leads to contact between the successful spermatozoon and the oolemma, and thus fertilisation is achieved.

tyrosine phosphorylation acts as a way to modulate sperm function, as many proteins that are identified as tyrosine phosphorylated are associated with events leading to fertilisation [15,16] (Fig. 1).

Exposure to effector molecules close to the site of fertilisation, such as progesterone released from the cumulus cells, also results in motility changes with spermatozoa acquiring a unique whip-like flagellar motion known as hyperactivation. Hyperactivation is dependent on calcium entry through specific channels known as voltage-sensitive calcium-selective channels or CatSper that are primarily functional in the principal piece of the flagellum [17]. The biological purpose of this propulsive motion is vital to assist with the release of spermatozoa from oviduct cells in the isthmic reservoir and the penetration of the zona pellucida (for an extensive review see Ref. [18]). Finally, as spermatozoa approach and interact with the zona pellucida of the oocyte, the calcium-dependent acrosome reaction is stimulated, causing a fusion between the outer acrosomal and overlying plasma membrane [19]. This fusion is regulated by the formation of soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) complexes and subsequently, the membranes begin to vesiculate and lytic enzymes that assist in the degradation of the zona are released [20,21] (Fig. 1). For spermatozoa that have successfully undergone capacitation-related processes, the initiation of hyperactivation and the acrosome reaction, the only event remaining is interaction with the oolemma and subsequent fertilisation.

The fact that capacitation is paramount for fertilisation makes it an extremely important area of sperm biology. Research conducted in vitro is our best model to understand the crucial physio-chemical changes that occur during capacitation. Based on the information collected from these investigations, we can then interpret the progression of events that are likely to occur in vivo during this incredible transformation in spermatozoa. In order to do this effectively, it is vital that researchers have access to a toolbox of reliable and informative assays that can detect the plethora of changes that occur during capacitation. Since it is such a complex process and the ability to define a spermatozoon as capacitated requires multiple criteria, it is crucial that there are resources available that will assist researchers in selecting the most appropriate assay to measure different stages of capacitation as well as gaining a deeper understanding of the results. The purpose of this review is to detail the various methods that can be used to identify the stages of capacitation outlined in the general introduction and examine the efficacy of each method for its designed purpose. This is by no means an all-encompassing list of methods to measure all capacitation-related processes. However, the review will aim to compile the most frequently used methods across capacitation research conducted in a number of species, including the boar. Furthermore, factors that may be supplemented to manipulate certain capacitation-related processes, such as the sAC-cAMP-PKA pathway or tyrosine phosphorylation, will be discussed to highlight their usefulness in distinguishing the likely progression of events during capacitation.

2. Methods to detect capacitation-related changes

2.1. The sAC-cAMP-PKA pathway

2.1.1. Quantifying sAC, cAMP and PKA

In early research on the study of this pathway, the measurement of sAC and PKA activity, or the production of cAMP, was commonly performed using radioactive enzymatic or immunoassays, respectively. For enzymatic assays, this generally involved the use of a radioactively labelled substrate that becomes modified by sAC or PKA activity and the resulting product was measured using a scintillation counter, an instrument that detects and measures ionising radiation [22,23]. In mouse spermatozoa, sAC activity under different capacitating conditions has been successfully measured with these radioactive assays whereby endogenous sAC was allowed to convert the substrate [α-^{32}P]ATP to [^{32}P]cAMP, which was then purified with chromatography and measured by scintillation counting [22,24]. A similar assay that harnesses the phosphorylation capability of PKA to transfer a radioactive phosphate to the substrate, kemptide, has also been implemented in mouse sperm capacitation protocols in order to measure the activity of this enzyme [23,25]. In the case of immunoassays like that used for cAMP, the assay works by first allowing radioactively labelled cAMP and endogenous sperm cAMP to competitively bind to plated anti-cAMP antibodies. The resulting measurement of labelled cAMP with scintillation counting is inversely proportional to the amount of endogenous cAMP, which can be determined using internal standards. For the most part, this was the assay of choice to quantify cAMP production and has been applied to a number of species including boar, bovine and human spermatozoa [26–28].

Whilst the use of radioactive techniques has proven to be effective and produce highly sensitive results, there are drawbacks that can outweigh these advantages, specifically the risk of handling radioactive material and the costs associated with safely disposing of waste. As a consequence, non-radioactive methods to measure this pathway are now more readily available as safe and useful alternatives for researchers specialising in sperm biology. Commercially available enzyme linked immunoassays (ELISA) have been used successfully to quantify cAMP production in spermatozoa in the mouse, boar and ram [25,29,30] As opposed to the use of radioactive enzymatic assays to measure PKA activity, researchers have developed a novel means to detect the function of this enzyme. Through the use of antibodies raised against the phosphorylated form of the target consensus sequence for PKA action [31], these target sequences or peptides can be detected by western blot analysis and have even been used to label spermatozoa to identify regions of PKA phosphorylation [31,32]. In both boar and human spermatozoa, the exposure to capacitating conditions produced a rapid increase in PKA phosphorylation as observed with western blotting. Furthermore, in boar spermatozoa this phosphorylation corresponded closely with cAMP production, reflecting the efficacy of this technique to detect PKA activity and the initial stages of capacitation [31,32].

2.1.2. Manipulating the sAC-cAMP-PKA pathway

In addition to the various methods in which the sAC-cAMP-PKA pathway can be measured, it may also be useful for researchers to be able to manipulate different aspects of this pathway and examine the consequences of these changes during capacitation. This is generally accomplished by simply supplementing capacitation media with antagonists or agonists of sAC, cAMP or PKA. For sAC, with the aim of identifying specific inhibitors, Hess et al. screened over 15,000 small lipophilic molecules that could provide temporal control of this enzyme as opposed to solely relying on knock-out mice models [33]. The compound KH7, which was highly selective for sAC was most effective at inhibiting cAMP production in mouse spermatozoa exposed to capacitating conditions [33]. Unlike the limited options to modulate sAC activity, researchers have a range of compounds that can be utilised to alter cAMP production and availability as well as PKA activity. For cAMP production, this can be achieved in two ways, either a cAMP analogue can be supplemented to increase its availability or alternatively, the enzymes responsible for the hydrolysis of cAMP, phosphodiesterases (PDEs), can be inhibited. The cAMP analogues, dibutyryl cAMP, 8-bromo-cAMP and cBIMPS are just a few of the most frequently used compounds that have successfully supported or enhanced capacitation in a number of species including the boar, bovine, mouse, ram and human [24,26,27,32,34]. The majority of PDE inhibitors are non-specific for particular subtypes of phosphodiesterases, yet these are still effective in a wide range of species, those being 1-isobutyl-1methylxanthine (IBMX), caffeine, papaverine and occasionally theophylline [29,34,35]. Finally, to manipulate PKA activity, H89 and Rp-cAMP are potent inhibitors of this enzyme, which have been shown to have a dramatic effect on capacitation-related processes that rely on the activity of this enzyme [27,32]. Together with the various ways in which the sAC-cAMP-PKA pathway can be measured, these tools provide the basis for studying the initial stages of capacitation and how this pathway influences the processes that depend on it, such as the modifications that occur within the plasma membrane.

2.2. Modifications to the plasma membrane

2.2.1. Changes in membrane fluidity

The change in membrane fluidity as a result of cAMP-PKA dependent phospholipid scrambling has been extensively studied, with many of these early investigations using the boar as a model species [7,29]. In these studies, researchers utilised a fluorescent probe known as merocyanine 540. Merocyanine 540 has been shown to have a high affinity for binding in the fluid phase of the membrane and thus became a sensitive tool to detect the degree of lipid packing [36]. Upon exposure to the capacitating agent, bicarbonate, it was found that boar spermatozoa became increasingly stained with merocyanine 540 as measured with flow cytometry owing to the rapid alterations in the plasma membrane architecture [7]. Since then, merocyanine 540 has been used to detect these early and rapid changes in the plasma membrane during capacitation in a range of species including the ram, stallion and canine [37–39]. Traditionally, merocyanine is combined with an indicator for cell viability, such as Yo-Pro, in order to isolate viable cells with high membrane fluidity, since the rearrangement of phospholipids in the plasma membrane can also be associated with apoptosis [40]. It should be noted that whilst this fluorescent probe is an extremely useful tool for detecting changes in membrane fluidity, the presence of lipid-containing components in capacitating media, like albumin, can compete for merocyanine binding when supplemented at increasing concentrations [41]. This characteristic of merocyanine 540 should not deter researchers from utilising this effective and reliable probe to measure changes in membrane fluidity, but it is important to be aware of how components in capacitation media may influence the results obtained.

The one limitation of merocyanine 540 is the fact that it detects non-specific membrane architectural changes. A phenomenon correlated with increased membrane fluidity in spermatozoa is the apparent translocation of specific phospholipids across the leaflets of the plasma membrane. The methods used to detect these alterations in the sperm membrane following exposure to capacitating conditions are further discussed in Supplemental Section 1.

2.2.2. Redistribution of cholesterol

Following the activation of cAMP-PKA dependent phospholipid translocation, and subsequent increase in membrane fluidity, cholesterol residing in the plasma membrane shifts from a uniform distribution across the sperm head to a concentration in the apical region of the head [8,9]. It is proposed that the redistribution of cholesterol to this area of the sperm head is likely where the proceeding event of regulated cholesterol removal takes place during the process of cholesterol efflux [12,42]. A technique that has enabled researchers to observe the movement of cholesterol to the apical sperm head is filipin labelling. Filipin is a multimeric compound produced by *Streptomyces filipinesis*, with the most commonly used isoform being Filipin III. This filipin isoform is able to bind to endogenous cholesterol within cell membranes to form complexes and it is also intrinsically UV fluorescent [9,43]. It is for this reason that filipin became a frequently used method to visualise the localisation of cholesterol in labelled spermatozoa with fluorescent microscopy (Fig. 2). This technique permitted the identification of filipin-cholesterol complex patterns that were frequently observed in non-capacitated and capacitated boar spermatozoa [8,9,44], and these were then later extrapolated for use in stallion spermatozoa to detect activated cells [45].

It is noteworthy that the method to prepare spermatozoa for filipin labelling prior to microscopic analysis can vary between researchers, with some choosing to fix cells in paraformaldehyde or glutaraldehyde prior to labelling [8,9,44] and others labelling unfixed, live spermatozoa [45,46]. Despite concerns that the fixation process may alter cholesterol localisation compared to that in live cells, the distinctive differences between non-capacitating and capacitating cells is indicative that the observed redistribution of cholesterol is likely a physiological response rather than an artefact. Since filipin has become a frequent tool to analyse cholesterol in the sperm plasma membrane, researchers began to quantify filipin fluorescence as a measure of cholesterol loss during capacitation. The application of filipin labelling for the purpose of measuring cholesterol efflux is further discussed in Supplemental Section 2.

2.3. Cholesterol efflux

Over the years that this process has been studied, researchers have implemented a range of methods to quantify cholesterol lost from the plasma membrane during capacitation. These can be classified under two categories, the first being methods that directly measure endogenous cholesterol efflux derived from spermatozoa, whilst the other methods utilise labelled cholesterol analogues as a means to measure the capacity for cholesterol loss. As for methods that measure endogenous cholesterol, the simplest uses the Amplex Red® enzymatic assay. This was originally developed for serum cholesterol [47], but has since been applied to capacitation studies to quantify cholesterol efflux from spermatozoa [38,48]. Unesterified cholesterol is first oxidised by the enzyme cholesterol oxidase to yield hydrogen peroxide; cholesterol is thence quantified based on the measurement of hydrogen peroxide. The presence of horseradish peroxidase is responsible for the oxidation of the hydrogen peroxide sensitive reagent, Amplex Red, which then produces a fluorescent end product known as resorufin that can be detected with fluorimetry [47].

Another means to quantify endogenous cholesterol efflux is lipid analysis, either with thin layer chromatography (TLC) or mass spectrometry. TLC analyses the separation of extracted lipids on a silica gel plate following development with suitable solvents [10,11]. Separated lipid bands can be quantified either by colorimetric measurement or by scraping selected lipid bands from the silica, re-extracting the separated lipids and measuring cholesterol with, for instance, the Amplex Red assay. Whilst TLC is a relatively simple technique to examine the changes in cholesterol in spermatozoa or the surrounding medium (Fig. 3), it is a crude form of lipid analysis in contrast to mass spectrometry.

Mass spectrometry allows the examination of all the lipid species in the same sample (lipidomics) that may be of interest and whether the composition of these lipids alters in the plasma membrane during capacitation [9]. Whilst mass spectrometry is extremely sensitive for detecting lipid changes in spermatozoa (Fig. 4), its major drawback is the requirement for specialised equipment and expertise, which is unavailable in many andrology laboratories. Moreover, the quantification of cholesterol efflux by lipid analysis or even enzymatic assays is generally performed on an entire sperm population including both viable and non-viable cells. This form of analysis is not ideal as the inclusion of non-viable, deteriorating cells can cause an increased detection of cholesterol loss that is more likely to be attributed to loss of the plasma membrane than the regulated efflux that occurs during capacitation [44].

Aside from methods that can directly measure cholesterol efflux from spermatozoa or the surrounding medium, labelled cholesterol analogues are another common way to quantify this process. This was often accomplished using radioactively labelled cholesterol or [^{3}H]cholesterol, although, much like enzymatic assays and lipid analysis, this technique only permits the measurement of cholesterol efflux from whole sperm populations. In addition, the high risk associated with radioactive material has limited the use of this method for cholesterol efflux analysis. Consequently, it was necessary to identify an alternative assay to measure cholesterol efflux in spermatozoa effectively, reliably and safely. Owing to the extensive research dedicated to cholesterol in somatic cells, a variety of cholesterol analogues could be tailored for use in spermatozoa. One that has shown potential for this purpose is BODIPY-cholesterol, which is composed of a boron dipyrromethene difluoride (BODIPY) fluorophore conjugated to a cholesterol molecule [49]. Until recently, its application to study the structure and dynamics of cholesterol in membranes was only documented in somatic cells. Upon exposure to large unilamellar vesicles, Milles et al. discovered, using Nuclear Magnetic Resonance (NMR) and fluorescence quenching experiments that the orientation of BODIPY-cholesterol was analogous to native cholesterol, with the

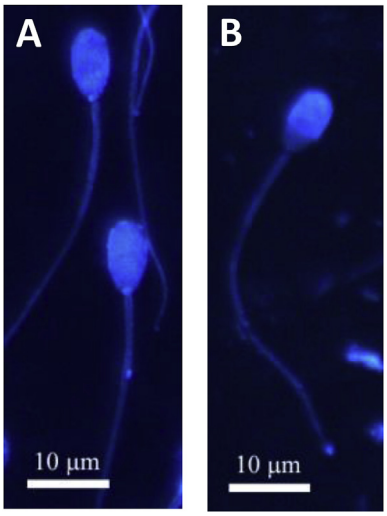

Fig. 2. Filipin labelled boar spermatozoa following fixation in paraformaldehyde [44]. In spermatozoa that are not exposed to capacitating conditions, filipin is frequently observed evenly across the entire sperm head (**A**). Once spermatozoa are incubated in capacitating conditions, cholesterol redistributes to the apical sperm head which is observed by the corresponding shift in filipin fluorescence (**B**).

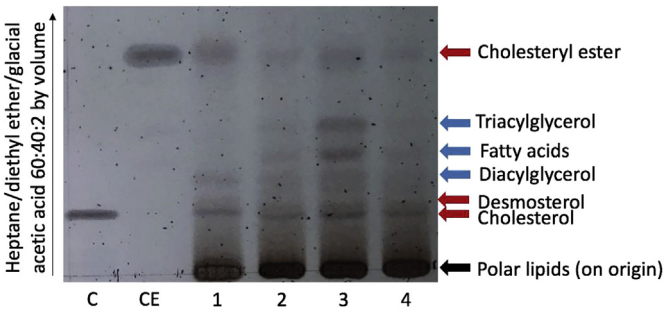

Fig. 3. An example of high-performance thin layer chromatography (HPTLC) that may be used as a tool to analyse cholesterol efflux. The first lanes are standards set by the researcher. In this case they are cholesterol (C) and cholesterol ester (CE), respectively. The remaining lanes are samples (cells or media) that have undergone lipid extraction and are loaded on the silica plate for development. In this example, the silica plate was developed with a mixture of heptane, diethyl ether and glacial acetic acid (60:40:2; v/v) until the solvent mixture reached 1 cm from the top of the plate. The bands corresponding to different lipids were visualised by spraying the plate with a mixture of sulfuric acid and acetic acid (1:1; v/v), then placing in an oven at 170 °C until the bands were visible. Lipids remaining on the origin have too high a polarity to migrate over the silica during development. The other various lipids that were able to migrate and separate on the silica are given in this example.

BODIPY-group inserted deeply into the hydrophobic membrane core [50]. This fluorescent cholesterol analogue was also able to preferentially partition into liquid ordered regions of model membranes, which were enriched with native cholesterol [51], only causing minor perturbations in model membranes [49]. BODIPY-cholesterol also successfully labelled live cells, even a developing zebrafish, further reflecting the simplicity of this analogue to label spermatozoa prior to in vitro capacitation and without the risk of negatively affecting viability [49].

With all the benefits of BODIPY-cholesterol, our laboratory developed an assay to study cholesterol efflux from boar spermatozoa during capacitation [44]. Spermatozoa are first labelled with BODIPY-cholesterol and, following incubation under capacitating conditions, the loss of BODIPY-cholesterol from the sperm surface can be detected using flow cytometry (Fig. 5). The BODIPY-cholesterol assay was successfully applied to boar spermatozoa to quantify cholesterol efflux akin to endogenous cholesterol measured with mass spectrometry and was a superior method compared to filipin labelling for this purpose [44]. Flow cytometric analysis enables researchers to counterstain for viability and segregate viable cells only for analysis. The results are biologically relevant, since only viable, capacitating spermatozoa are likely to reach the stage of fertilisation. The BODIPY-cholesterol assay has a number of potential applications in capacitation research, from elucidating the mechanisms and specific requirements for cholesterol efflux between species, to application in a clinical setting as a means to assess capacitation success.

2.4. Tyrosine phosphorylation

2.4.1. Detection of tyrosine phosphorylation

Mature spermatozoa are transcriptionally quiescent, meaning that in order to alter the function of proteins to support the processes of capacitation, these proteins undergo a cycle of phosphorylation and dephosphorylation. There are two commonly used methods to detect these capacitation-related changes in spermatozoa: immunofluorescence and Western blotting.

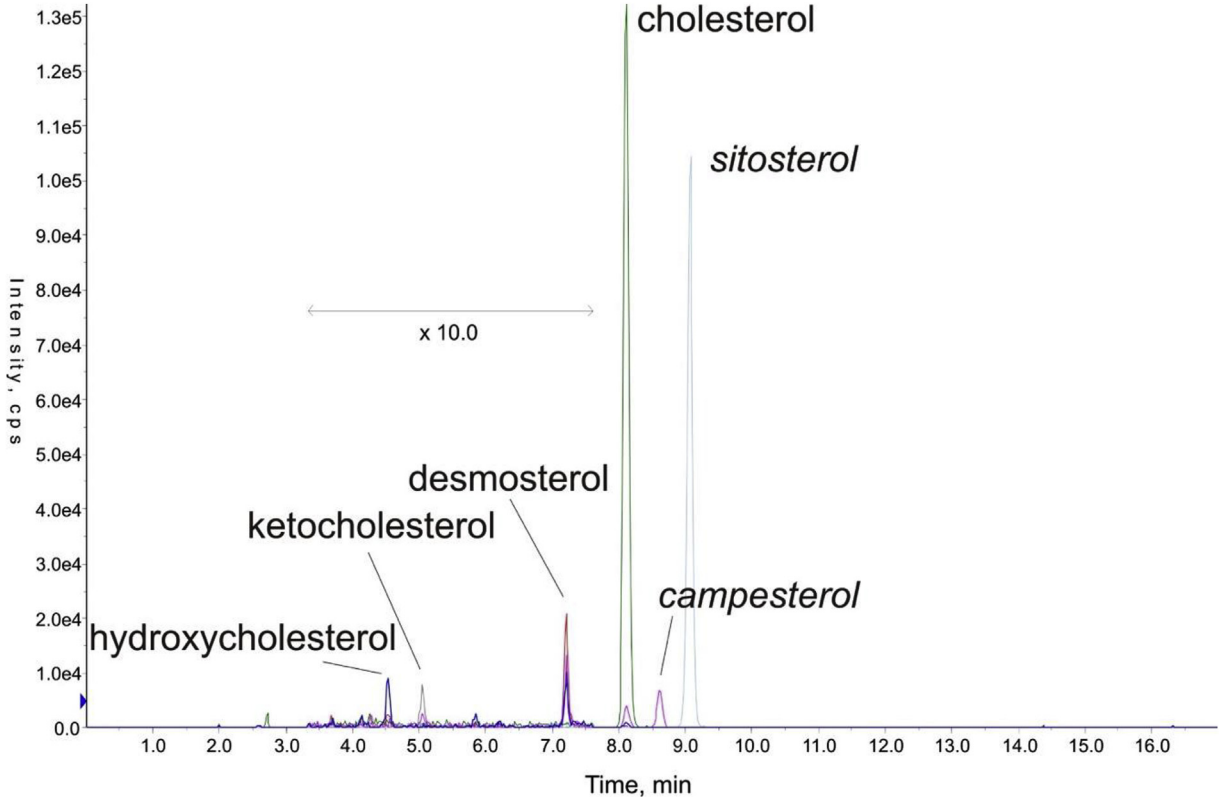

Fig. 4. HPLC-tandem mass spectrometry elution spectrogram of sterols that may be produced from washed sperm lipid extracts (1×10^6 spermatozoa total). The addition of reference sterols (in italics) allows for the absolute quantification of lipids and the presence of oxysterols in this example are enhanced 10X. The vertical axis represents the relative amount of *m*/*z* and MRM transition events per second. The horizonal axis is the elution time for the various lipids exiting the reverse phase HPTLC column before entering the MS/MS ionisation chamber in preparation for quantification.

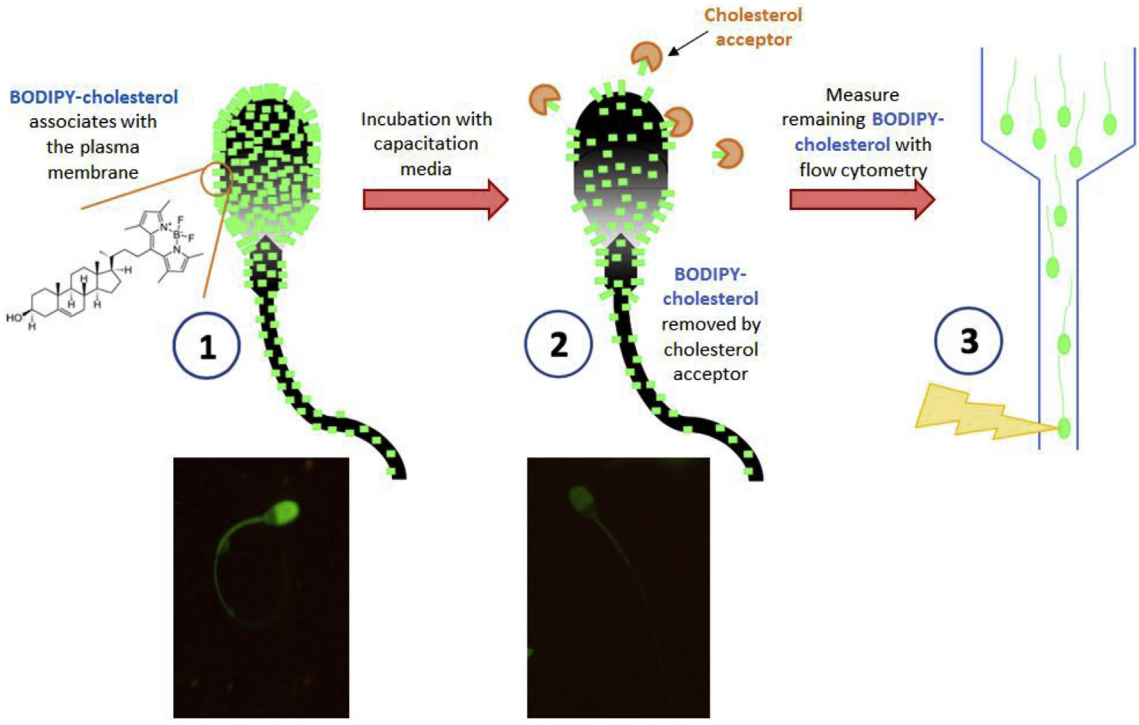

Fig. 5. Outline of the BODIPY-cholesterol assay methodology to quantify cholesterol efflux from spermatozoa [44].

Immunofluorescence allows the localisation of tyrosine phosphorylated proteins to be visualised by use of an antibody conjugated to a fluorophore like fluorescein isothiocyanate (FITC) [15,16,52,53]. This is particularly useful for observing the changes in location of phosphorylated proteins over the course of capacitation and how these may link to different functions. To demonstrate this, in mouse spermatozoa that were labelled with anti-phosphotyrosine, only 15% of cells presented with partial or full principle piece and mid-piece fluorescence prior to capacitation. When spermatozoa underwent capacitation and were exposed to zona pellucida, 90% of cells recovered from the zona showed a complete shift in fluorescence to the flagellum [15]. In another study, immunofluorescence was able to differentiate spermatozoa that were unable to bind to oviduct cells, and likely to be prematurely capacitated, based on their extensive phosphorylation across the equatorial and acrosomal regions, as well as occasionally in the flagellum [53]. While immunofluorescence can reveal the distribution of tyrosine phosphorylation and how this may change as a result of capacitation, the identification of particular proteins that have undergone phosphorylation can only be accomplished with Western blotting.

The application of Western blotting for protein tyrosine phosphorylation analysis in spermatozoa has been used across a range of species, including the boar, ram, mouse and human [11,35,38,54–56]. The degree of phosphorylation of particular bands under conditions of interest can be examined quantitatively with densitometry [38,52,55]. This method offers the possibility to identify particular proteins that have been protein tyrosine phosphorylated and explore their function relative to capacitation (Fig. 6). In mouse spermatozoa, the combined analysis of protein tyrosine phosphorylation with western blotting and immunofluorescence enabled Asquith et al. to isolate two molecular chaperones, heat shock protein 60 (hsp60) and endoplasmin (erp99), that are only phosphorylated under capacitating conditions and have been associated with facilitating sperm-zona recognition [16]. For a more comprehensive analysis, sperm proteins can instead be separated by 2D SDS-PAGE prior to western blotting and a duplicate gel can be used to isolate proteins for examination with tandem mass spectrometry [57]. In a novel approach, protein tyrosine phosphorylation has been investigated in specific regions of boar spermatozoa by isolating the plasma membranes of capacitated cells using nitrogen cavitation in a cell disruption bomb, and then subjecting them to gel electrophoresis and western blotting [54].

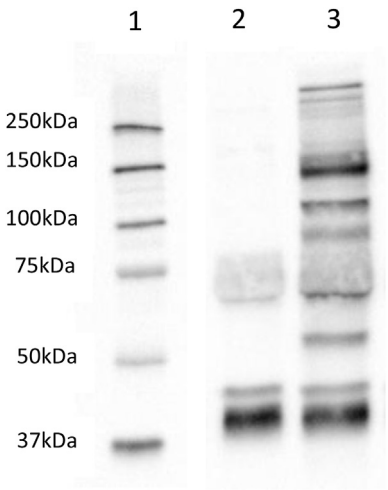

Fig. 6. An example of a Western blot for assessment of protein tyrosine phosphorylation. The first lane is a molecular weight marker which is used to determine the size of the sperm proteins that have undergone tyrosine phosphorylation. In this example, spermatozoa were exposed to non-capacitating (lane 2) and capacitating (lane 3) conditions and then prepared for Western blot analysis following 3 h of incubation. Note the extensive tyrosine phosphorylation present across sperm proteins of a range of sizes following capacitation (data unpublished).

While tyrosine phosphorylation is well known as a marker of

capacitation, some researchers have argued that it may not be necessary for successful fertilisation. Following the identification of the tyrosine kinase, FER, Andrew et al. developed a mouse model to gain insight into the biological function of this enzyme [58]. When spermatozoa were collected from male mice homozygous for the missense mutation of FER and were exposed to capacitating conditions, there was an absence of increased tyrosine phosphorylation that was otherwise observed in wild-type mice [59]. Interestingly, these spermatozoa also displayed a reduced ability to fertilise metaphase II oocytes in vitro (<10% fertilised oocytes compared with 30–40% in wild-type mice). However, in a similar study, homozygous males that mated naturally were still classified as fertile and produced similar litter sizes to wild-type mice [58]. It was proposed that upon exposure to the female environment, there may be unknown mechanisms that compensate for the lack of tyrosine phosphorylation and enables fertilisation to occur [59]. As it stands, tyrosine phosphorylation is still considered important for fertilisation in vitro and therefore may remain as an indicator for successful in vitro capacitation.

Like all methods used to detect or quantify different aspects of capacitation, there are some characteristics of tyrosine phosphorylation analysis that must be considered if this is to be used as an indicator of capacitation status. Firstly, the presence of protein tyrosine phosphorylation is not always associated with capacitation. Across many species, certain proteins of varying sizes have been tyrosine phosphorylated despite spermatozoa not having been exposed to capacitating conditions [16,52,60]. These proteins may be phosphorylated during the time spermatozoa reside in the epididymis, either by their direct incorporation with spermatozoa, or during their passage through this region of the tract [60]. Others suggest that it may be the result of processing spermatozoa for in vitro capacitation, such as washing to remove seminal plasma [55]. In addition to the capacitation-independent protein tyrosine phosphorylation, it is worth considering that phosphorylation is not a singular event but a continuous one. Tyrosine phosphorylation is regulated by kinases and phosphatases that are responsible for upregulating or downregulating the function of certain proteins and at different stages of capacitation. With this in mind, although protein tyrosine phosphorylation generally increases as capacitation ensues, some proteins may become dephosphorylated during this period which may be missed when samples are taken for analysis at specified time points. The dephosphorylation of several proteins is particularly evident following the acrosome reaction [52,54,60]. Therefore, upon detecting tyrosine phosphorylation by either immunofluorescence or western blotting, it should be remembered that the observations only reflect a snapshot in time and should be interpreted accordingly.

2.4.2. Manipulating tyrosine phosphorylation

Tyrosine phosphorylation is regulated by a series of enzymes beginning with protein kinase A (PKA), through to more specific enzymes that are responsible for the modification of proteins with tyrosine residues [5]. Much like the sAC-cAMP-PKA pathway, the pathway leading to tyrosine phosphorylation can also be manipulated by agonists or antagonists, which may be particularly useful to investigate tyrosine phosphorylation dependent processes. PKA is a known serine and threonine kinase and the phosphorylation of these residues may indirectly activate tyrosine kinases. Based on this proposal, most agonists or antagonists of PKA activity (see section 2.1.2) would be expected to affect tyrosine phosphorylation. The effect of cAMP analogues, PDE inhibitors or PKA inhibitors on the ability of these compounds to enhance tyrosine phosphorylation has been demonstrated in several studies, irrespective of whether spermatozoa were exposed to capacitating conditions or not [24,34,60,61]. The phosphorylation of serine and threonine residues that leads to tyrosine kinase activity, can also be manipulated by protein phosphatase inhibitors, which prevent the dephosphorylation of these proteins and ultimately increase tyrosine phosphorylation [29,35]. Calyculin A and okadiac acid are two frequently used phosphatase inhibitors that target protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) or PP2A only [62]. To modulate the activity of enzymes directly regulating the phosphorylation of tyrosine residues, it is possible to introduce an inhibitor for a specific tyrosine kinase known as Src family kinase (SFK). In human spermatozoa, SK1606 was able to inhibit tyrosine phosphorylation even in the presence of agonists that elevated PKA activity, clearly illustrating the targeted effect of this inhibitor on SFK [61]. Together, these agonists and antagonists may be useful for research on the functional relationship between the different components required to support tyrosine phosphorylation and how proceeding events may be influenced by changes to this pathway.

2.5. Hyperactivation

Hyperactivation is one of the hallmarks of the acquisition of fertilising ability in spermatozoa. For this reason, the mechanisms of sperm hyperactivation and its reliable measurement has been of interest to a number of researchers. Sperm hyperactivation was originally observed under phase-contrast microscopy through video recordings taken for defined periods. These recordings were either examined for the recognisable characteristics of hyperactivated motility and the percentage of cells expressing this motility subsequently determined [63,64], or the trajectories of spermatozoa were reconstructed manually in order to define their specific kinematic parameters [65,66]. Kinematic parameters explain different aspects of a spermatozoon's trajectory. A high amplitude of lateral head movement (ALH) and curvilinear velocity (VCL) with low linearity (LIN) are kinematic parameters representative of hyperactivated motility [67]. As technology advanced, the manual reconstruction of sperm motion was replaced with the automated computer assisted semen analysis (CASA) that is now widely used by andrologists. Such automated methods are more objective than manual measurement, but there are several aspects of CASA that must be considered in order to generate consistent hyperactivation data.

Firstly, the criteria to define hyperactivation are not “one size fits all”. Hyperactivation can differ considerably amongst species, likely due to structural differences in the flagellum [18]. Consequently, the threshold motility and kinematic parameters that classify a spermatozoon as hyperactive or not must be redefined for each species. As an example, hyperactive boar spermatozoa show an ALH >3.5 μm, VCL >97 μm/s and LIN <32%, whilst in the ram, ALH > 9 μm, VCL >250 μm/s and LIN ≤30% classifies a spermatozoon as hyperactive [65,66,68]. These studies clearly highlight the species-specific thresholds that define hyperactivation. They also demonstrate that this complex motility pattern cannot be characterised by a single kinematic parameter. Instead, the combination of several parameters is required to reliably detect hyperactivation [66].

The second factor that can influence CASA results is the image sampling frequency of video recordings, also known as the frame rate. Mortimer et al. manually tracked human sperm using 5–200 Hz frame rate recordings and also used CASA for an objective measure [69]. They concluded that certain kinematic parameters, such as VCL and ALH, were highly frame rate dependent. This indicates that researchers should use a consistent frame rate and select one that provides as much information on that trajectory as possible. Moreover, measurements across different CASA systems might not be comparable [69]. In addition to these factors, the chamber depth, media composition or the sperm concentration

used for analysis could also influence the detection and measurement of hyperactivation [18].

Despite the extensive research dedicated to understanding the processes of capacitation, it is still uncertain whether hyperactivation is indicative of the final stages of capacitation or if it is a separate event. It has been demonstrated in several species that hyperactivation is regulated by a different pathway to the well-defined cAMP-PKA pathway, particularly since factors other than those in capacitating media were necessary to support the process. In bull spermatozoa, for example, exposure to heparin and/or dibutyryl cAMP and IBMX was able to support capacitation, but these conditions were unable to initiate hyperactivation [64]. Instead, spermatozoa were induced to hyperactivate following treatment with procaine or caffeine without any evidence of capacitation, as indicated by the lack of tyrosine phosphorylation. The local anaesthetic procaine, which has been used to induce hyperactivation in other mammalian spermatozoa, is proposed to function by increasing the permeability of the plasma membrane to calcium [70]. In stallion spermatozoa, only the treatment with procaine following capacitation was able to support a drastic increase in the percentage of cells presenting hyperactivated motility (>50%) compared to spermatozoa that were not exposed to this inducer (<30%) [71]. In addition to procaine or caffeine, hyperactivation has been enhanced by progesterone [63,72], changes in temperature [72] and even induced by the calcium ionophore, A21387, owing to its ability to stimulate an increase in intracellular calcium [73] although with variable success [37,65,68]. The fact that hyperactivation can depend on factors other than those present in a capacitating medium, and that it can be induced independently of capacitation, indicates that it cannot be used as the sole measure of successful capacitation.

2.6. The acrosome reaction

2.6.1. Acrosome reaction inducers

The inducibility of the acrosome reaction is an absolute requirement for fertilisation to occur. The acrosome reaction does not occur spontaneously following in vitro capacitation unless stimulated, and when this response takes place it most likely the result of cell deterioration. For this reason, the ability to artificially induce the acrosome reaction and then measure it reliably, provides the opportunity to quantify the success of capacitation rather than the process itself. With respect to how the acrosome reaction can be effectively stimulated, there are a range of inducers, either non-physiological or physiological, that have been used by researchers investigating capacitation success in vitro. Lysophosphatidylcholine (LPC) and the calcium ionophore A23187 are non-physiological acrosome reaction inducers frequently used in a number of species. LPC is a fusogenic lipid, isolated from egg yolk, that renders bull, ram and human spermatozoa sensitive to the acrosome reaction, and is able to discriminate those cells that have capacitated [35,55,74,75]. Although LPC appears to have discriminatory action in different sperm populations, its use in human spermatozoa caused rapid acrosome reactivity in both freshly ejaculated and capacitated spermatozoa, whether in the presence or absence of extracellular calcium [75]. In contrast, the calcium ionophore A23187 was only able to induce the acrosome reaction in capacitated cells, and the response was more gradual than to LPC, suggesting it may be a better candidate to induce the acrosome reaction. Like LPC, A23187 has been used extensively across species [37,54,76,77]. Nevertheless, Liu and Baker [77] noted no observable correlation between the acrosome reaction in human spermatozoa induced by A23187 and the zona pellucida, indicating that the induction mechanism of A23187 is not comparable to that of physiological factors. Furthermore, A23187 and LPC should be used with caution under capacitating conditions, since too high a concentration of LPC can cause membrane damage (up to 400 μg/mL [74]) and A23197 can severely impair sperm motility (20 μM [78], 10 μM [79]). These factors should not discourage the use of LPC or A23187 if care is taken when applying them to capacitation protocols. For those who wish to induce the acrosome reaction using factors present in vivo, the zona pellucida and its associated proteins or progesterone may be the preferred options.

The acrosome reaction can be readily induced by whole zona pellucida or by solubilised and purified zona proteins, particularly ZP3 [77,80,81]. For domestic species like cattle or even mice, zona pellucidae are more readily available for inducing the acrosome reaction than from humans, where zonas are usually obtained post-mortem or from discarded oocytes destined for IVF [80]. To overcome this limitation, some researchers utilised cell expression systems to purify recombinant zona proteins that were able to support a population of acrosome reacted human spermatozoa [80,82]. Since progesterone is secreted at high concentrations by the cumulus oophorous surrounding the oocyte, it is not surprising that it can have a regulatory function with respect to the acrosome reaction in some species [83]. In boar and human spermatozoa, progesterone can stimulate the acrosome reaction and, in humans, the presence of this hormone was responsible for intracellular calcium oscillations in the sperm head that led to this acrosomal event [83–85]. In contrast, rhesus macaque spermatozoa cannot be directly acrosome reacted with progesterone. Nevertheless, the presence of this hormone is required to enhance the responsiveness of spermatozoa to the zona pellucida and in effect, primes the cell for the acrosome reaction [72]. While effectively stimulating the acrosome reaction in vitro is a vital aspect of capacitation research, this process is redundant without a reliable method to quantify it.

2.6.2. Quantifying the acrosome reaction

Much like every aspect of capacitation, there is more than one way to measure the acrosome reaction in spermatozoa. Some capacitation studies have opted for the use of phase contrast microscopy to examine any modifications or the loss of the acrosome from capacitated spermatozoa [10,76]. Despite efforts to create defined criteria to identify acrosome reacted cells [86,87], there is still the potential for subjectivity in this assessment. Fluorophores, which permit observation cellular functions combined with analysis by flow cytometry, have been used to measure capacitation-related events like the acrosome reaction. The chlorotetracycline (CTC) assay is one example of a fluorescence-based assessment of acrosome reactivity that has also been used to define different stages of sperm activation in a number of species, including the horse, ram and dog [37,76,88]. CTC is believed to bind to membrane calcium, whose distribution changes during capacitation [76], but exactly how this occurs at a molecular level is yet to be defined [37]. The CTC assay is able to quantify acrosome reactivity, in both ram and stallion spermatozoa, akin other methods such as the use of fluorescent conjugated lectins [37,55]. However, the fact that it is laborious and time-consuming does not make it a favourable option for this purpose.

One of the most common methods for quantifying changes in the acrosome following successful capacitation uses lectins or antibodies that bind to the acrosomal membrane after exocytosis. The exposure of the outer acrosomal membrane reveals glycosylated proteins that readily associate with specific lectins and/or antibodies conjugated to fluorophores, allowing the expression and distribution of the newly exposed glycoproteins to be detected with fluorescent microscopy or flow cytometry [89]. Moreover, these lectin and antibody conjugates are commonly combined with viability markers, such as propidium iodide, in order to detect

Table 1
Summary of methods to measure capacitation and factors that can be included to modulate the various processes in spermatozoa.

Capacitation-related process	Method of measurement	Modulators
sAC-cAMP-PKA pathway	Radioactive enzymatic or immunoassays [22, 24 (sAC); 23, 25 (PKA); 26–28 (cAMP)] cAMP enzyme linked immunoassays [25,29,30] Antibodies against target sequence for PKA action [31,32]	KH7 (*sAC inhibitor*) [33] Dibutyryl cAMP, 8-bromo-cAMP and cBIMPs (*cAMP analogues*) [24,26,27,32,34] IBMX, caffeine, papaverine and theophylline (*PDE inhibitors*) [29,34,35] H89 and Rp-cAMP (*PKA inhibitors*) [27,32]
Modifications to the plasma membrane	Detect membrane fluidity changes with merocyanine 540 (M540) using flow cytometry [7,29,37–39] Detect cholesterol redistribution with Filipin III using fluorescent microscopy [8,9,44–46]	N/A
Cholesterol efflux	Amplex® Red assay (*endogenous cholesterol*) [38,48] Lipid analysis by TLC or mass spectrometry (*endogenous cholesterol*) [10, 11 (TLC); 9, 44 (mass spectrometry)] BODIPY-cholesterol assay using flow cytometry (*cholesterol analogue*) [44]	N/A
Tyrosine phosphorylation	Observe regions of tyrosine phosphorylation in spermatozoa with immunofluorescence [15,16,52,53] Detect proteins of interest that have undergone tyrosine phosphorylation with western blotting [11,35,38,54–56]	Agonists or antagonists of PKA activity (**see sAC-cAMP-PKA pathway**) Calyculin A and okadaic acid (*protein phosphatase inhibitors*) [29,35] SK1606 (*Src family kinase inhibitor*) [61]
Hyperactivation	Computer assisted semen assessment (CASA) system	Procaine [64,71] Caffeine [64] Progesterone [63,72] Changes in temperature [72] A23187 [37,65,68]
Acrosome reaction	Chlorotetracycline (CTC) [37,55,76,88] Fluorophore conjugated peanut agglutinin (PNA) or other lectins [89–91] Antibodies against acrosomal membrane and vesicles, CD46 and CRB9 respectively [90,91]	Lysophosphatidylcholine (LPC) and A23187 (*non-physiological*) [35, 55, 74, 75 (LPC); 37, 54, 76–79 (A23187)] Whole zona pellucida or zona recombinant proteins and progesterone (*physiological*) [77, 80–82 (zona); 72, 83–85 (progesterone)]

viable spermatozoa that have undergone the acrosome reaction. There are numerous lectins that may be used to detect acrosome reacted spermatozoa, each with differential efficacies. Peanut agglutinin (PNA) is a commonly used lectin for identifying changes in the acrosome reaction. When compared to an antibody raised against the cell surface complement regulatory protein (CD46) that is localised on the inner acrosomal membrane, PNA was found to bind comparably without compromising sperm viability or membrane integrity [90]. Furthermore, Aitken and Brindle reported that, in human spermatozoa, PNA was better able to capture the elevated acrosome reaction rates, in contrast to the antibody CRB9 and lectin Pisum sativum agglutinin (PSA) that target acrosomal vesicles, likely owing to the earlier exposure of the inner acrosomal membrane prior to the dispersal of these vesicles [91]. The few inducers and methods to measure the acrosome reaction that have been outlined offer the opportunity to continue the investigation of capacitation that is beyond its intrinsic processes, and instead examines those that only occur as a result of successful capacitation and that lead to fertilisation.

3. Conclusions

The only true measure of progression through capacitation is the capacity for a spermatozoon to bind to the zona pellucida and fertilise the oocyte. Regardless of this, the various methods and techniques considered in this review (summarised in Table 1) will assist in piecing together the storyline of a spermatozoon's journey, starting with ejaculation, leading to extensive modifications during capacitation, and finishing with the end goal of interaction with the oocyte prior to fertilisation. With the advancement in technology and the development of novel assessment techniques, the possibilities for measuring capacitation-related processes and detection of the changes it induces will increase. This review serves to form an initial toolbox for researchers in the field of capacitation and one that can be continually updated with new tools as our knowledge of this process deepens.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2019.05.038.

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