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Synergism between albumin, bicarbonate and cAMP upregulation for cholesterol efflux from ram sperm

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Abstract

Compared to other mammalian species, ram spermatozoa are difficult to capacitate *in vitro*. Dibutyryl cAMP (db-cAMP) and the phosphodiesterase (PDE) inhibitors, caffeine and theophylline (cAMP up-regulators), must be added to traditional capacitation media (containing bicarbonate, calcium and BSA) to elicit a capacitation response. In this exploratory study, we assessed whether bicarbonate was still required for ram spermatozoa if cAMP is up-regulated by the addition of db-cAMP and PDE inhibitors and what role BSA plays in cholesterol efflux under these conditions. In this study, the validated BODIPY-cholesterol assay was used for the first time in ram spermatozoa to quantify cholesterol efflux by tracking the loss of BODIPY-cholesterol from the sperm plasma membrane using flow cytometry. The results show that under cAMP up-regulated conditions, an increase in membrane fluidity and tyrosine phosphorylation of sperm proteins remain as bicarbonate-dependent processes. In fact, the supplementation of bicarbonate under these conditions was necessary to further enhance cAMP production in ram spermatozoa, which correlated with the presence of these capacitation-related processes. When BSA was supplemented with cAMP up-regulators (as well as bicarbonate), there was a loss of approximately 20–23% of BODIPY-cholesterol (79.5 ± 30.5% to 76.9 ± 12.3% remaining from 10 min), indicating that BSA is essential for mediating cholesterol efflux in ram spermatozoa as measured by the BODIPY-cholesterol assay. The current study identifies the functional relationship between bicarbonate, BSA and cAMP up-regulators that is required to support capacitation-related processes in ram spermatozoa, specifically cholesterol efflux.

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Introduction

Despite the extensive maturation that spermatozoa have already undergone in the epididymis at the time of ejaculation (Dacheux & Dacheux 2014), these cells still lack the capacity to fertilise. It is only when spermatozoa are exposed to the cocktail of fluids in the oviduct that they are functionally and structurally transformed for fertilisation during a process known as capacitation (Austin 1951, Chang 1951). Capacitation involves a series of intensely regulated events that render spermatozoa competent for hyperactivation, interaction with the cumulus cell layer and the zona pellucida surrounding the oocyte as well as preparing cells to acrosome react for effective penetration of these layers to reach the oolemma (Yanagimachi 1994, 2011). The ability to successfully capacitate spermatozoa *in vitro* using a defined medium is not only a requirement for effective *in vitro* fertilisation (IVF), but it also helps to delineate the precursors required for fertilisation *in vivo*.

While the exact composition of capacitation media may vary across mammals to suit species-specific capacitation requirements, there are three key additives that are universally fundamental for supporting this process, bicarbonate, serum albumin and calcium (for reviews see Bailey 2010, Aitken & Nixon 2013, Leemans *et al*. 2019). Bicarbonate is imperative for the initiation of capacitation via the activation of soluble adenylyl cyclase (sAC) and subsequent stimulation of the cAMP-protein kinase A (PKA) pathway (Harrison *et al*. 1996, Harrison & Miller 2000). Many capacitation-related processes rely on this pathway, including the increase of membrane fluidity, which is a very early step during *in vitro* sperm capacitation (Harrison & Miller 2000). This modification in the plasma membrane becomes a preparatory step for enabling cholesterol efflux mediated by serum albumin. The most common serum albumin used for *in vitro* capacitation, fatty acid free BSA, acts as a cholesterol acceptor in the extracellular environment. The removal of cholesterol enables the migration of lipid rafts to the anterior sperm head, permits zona binding and the acrosome reaction (Shadan *et al*. 2004, van Gestel *et al*. 2005, Gadella *et al*. 2008, Leahy & Gadella 2015). Finally, calcium is also required for a number of capacitation-related processes, specifically

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hyperactivation and the acrosome reaction (Suarez *et al*. 1993, Suarez & Dai 1995, Parrish *et al*. 1999).

Traditional capacitation media (e.g. Tyrode's albumin lactate and pyruvate or TALP) with bicarbonate and BSA is frequently used to successfully capacitate a range of species *in vitro*, yet the ram appears to be an exception as additional stimulation is required to support this process. Previous research has indicated that increasing concentrations of bicarbonate (up to 25 mM) have a minimal or variable effect on the proportion of capacitated ram spermatozoa (based on chlorotetracyclin (CTC) staining and the assessment of protein tyrosine phosphorylation (Pérez-Pé *et al*. 2002, Grasa *et al*. 2006, Colas *et al*. 2008)). This is in striking contrast to the mouse, hamster, boar and human whereby the exposure to bicarbonate is necessary to trigger and/or support *in vitro* capacitation (Visconti *et al*. 1995*a*, 1999, Harrison *et al*. 1996, Harrison & Miller 2000, Flesch *et al*. 2001, de Vries *et al*. 2003, Battistone *et al*. 2013, Soriano-Úbeda *et al*. 2019). It is only upon the addition of the cAMP up-regulators, dibutyryl cAMP (db-cAMP, a membrane permeable and metabolic inert cAMP analog) caffeine and theophylline (phosphodiesterase (PDE) inhibitors), that there is evidence of successful capacitation in ram spermatozoa, including an increase in membrane fluidity (as assessed by merocyanine 540 staining) and tyrosine phosphorylation (Colas *et al*. 2008, Leahy *et al*. 2016, Pini *et al*. 2018). The capacitation response only under cAMP upregulation suggests that ram spermatozoa require an extreme elevation of cAMP production, perhaps at an even higher rate relative to other species, and one that may not be stimulated by bicarbonate alone. While the exposure of spermatozoa to bicarbonate may seem redundant when under the influence of cAMP up-regulators, the exact role of bicarbonate under high cAMP conditions has not been investigated in the ram.

Following the initial stimulatory response to bicarbonate in other mammalian spermatozoa, this typically ensues with cholesterol efflux to serum albumin, yet it is unclear whether such a response can be evoked in ram spermatozoa. Previous studies that have examined cholesterol efflux in ram spermatozoa suggest that this process is not supported when in the presence of BSA or methyl-β-cyclodextrin in traditional capacitation media (Grasa *et al*. 2006, Colas *et al*. 2008), but there is currently a lack of quantifiable evidence to confirm this. This is due, in part, to the absence of a simple and reliable means by which cholesterol efflux can be quantified. Currently used methods to quantify this process, such as lipid analysis with mass spectrometry (Flesch *et al*. 2001, Bernecic *et al*. 2019) or the assessment of filipin labelling (Gadella & Harrison 2002, Shadan *et al*. 2004, Takeo *et al*. 2008, Bromfield *et al*. 2014), can require the use of specialised reagents and equipment that may not be readily accessible in an andrology laboratory or is a suboptimal method that can produce subjective results.

For this reason, we developed a flow cytometric assay that utilises the fluorescent cholesterol analogue, BODIPY-cholesterol, to quantify cholesterol efflux from spermatozoa during *in vitro* capacitation (Bernecic *et al*. 2019). The BODIPY-cholesterol assay was validated in boar spermatozoa and was able to measure cholesterol efflux from spermatozoa akin to endogenous cholesterol loss quantified with mass spectrometry. Furthermore, it was a superior method of measuring cholesterol efflux when compared to filipin labelling. Like described in somatic cells, BODIPY-cholesterol causes minimal disruption to sperm membranes, it can be used to label live, unfixed cells and it mimics the characteristic properties of native cholesterol (Hölttä-Vuori *et al*. 2008, Sankaranarayanan *et al*. 2011, Bernecic *et al*. 2019).

In order to resolve the uncertainty regarding the functional role of commonly supplemented media components on ram sperm capacitation, the objective of this study was to examine the effects of bicarbonate and BSA with cAMP up-regulators to elucidate their importance for capacitation-related processes in ram spermatozoa, specifically whether these components are necessary to support cholesterol efflux.

Materials and methods

Chemicals

Unless otherwise stated, products were sourced from Sigma-Aldrich and were of the highest reagent grade available. Fatty acid free BSA fraction V (catalogue no. 10775835001) and complete ultra mini EDTA-free protease inhibitor tablets (catalogue no. 05892791001) were sourced from Roche. BODIPY-cholesterol (TopFluor Cholesterol) was purchased from Avanti Polar Lipids, Inc. (catalogue no. 810255; Ablaster, AL, USA). Propidium iodide (PI; catalogue no. P3566), fluorescein isothiocyanate-peanut agglutinin (FITC-PNA; catalogue no. L21409) and MagicMark XP Western Blot Standards (catalogue no. LC5602) were sourced from Invitrogen and merocyanine 540 (M540; catalogue no. M24571) and Yo-Pro-1 (catalogue no. Y3603) from Molecular Probes. For density gradients, BoviPure and BoviDilute (catalogue no. BP-100 and BD-100) were purchased from NidaCon (Gothenberg, Sweden) and Parameter cAMP Assay Kit (catalogue no. KGE002B) from In Vitro Technologies (Victoria, Australia).

Incubation media

The basal medium used for Experiment 1 and 2 was modified Tyrode's medium supplemented with BSA, lactate and pyruvate (TALP) (Parrish *et al*. 1988). TALP consisted of 2 mM $CaCl_2$, 3 mM KCl, 0.4 mM $MgCl_2$, 90 mM NaCl, 0.3 mM NaH_2PO_4, 10 mM HEPES, 21.6 mM Sodium lactate, 5 mM D-glucose, 2 mM Sodium pyruvate, 25 mM $NaHCO_3$ and 3 mg/mL fatty acid free BSA. Where necessary, the pH of media was adjusted to 7.3 with NaOH and the osmolarity was measured as

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300 ± 10 mOsm by freezing point depression using an osmometer (Fiske 210 Micro Osmometer; Advanced Instruments).

For Experiment 1, the TALP medium was modified by omitting 25 mM bicarbonate and adjusting NaCl concentration from 90 mM to 115 mM to ensure the osmolarity of the media was maintained. In Experiment 2, TALP medium was also modified by omitting BSA and replacing it with 10 mg/mL polyvinyl alcohol (PVA). To all TALP media, 1.5 mM D-penicillamine (PEN) was supplemented to eliminate agglutination of ram spermatozoa during *in vitro* capacitation (Leahy *et al*. 2016).

Sperm preparation

For all *in vitro* capacitation measurements in Experiment 1 and 2, ram semen was collected from a total of four Merino and two Poll Dorset rams using an artificial vagina (minimum of two ejaculates) with project approval from the University of Sydney's animal ethics committee (Project No: AEC No: 2016/1106). Only ejaculates that had a wave motion score of 4 (out of 5) or higher were used in the study (Evans 1987) and none of the ejaculates presented indicators of a possible subclinical infection (i.e. elevated levels of white blood cells). Rams were housed at the animal house at the Faculty of Science, The University of Sydney, Camperdown, NSW, Australia. For both experiments (excluding the BODIPY-cholesterol assay), seminal plasma-free spermatozoa were obtained by a modified swim-up procedure by layering 200 µL of raw semen below a 2 mL layer of medium followed by an incubation for 60 min at 37°C. For Experiment 1, a swim up with TALP and TALP devoid of bicarbonate was prepared in order to obtain a bicarbonate-free sperm population and the same was performed for Experiment 2, except BSA was omitted from TALP. Following incubation, the top 1 mL of the medium containing the washed spermatozoa was carefully removed and the concentration was determined using a Neubauer Improved haemocytometer (Marienfield Superior).

For the BODIPY-cholesterol assay, raw semen was diluted in TALP modified without BSA and bicarbonate to 250×10^6 sperm/mL prior to labelling. Subjective motility was evaluated by light microscopy following dilution and only those samples with 70% total motility and higher were used for the BODIPY-cholesterol assay.

Experimental design

Experiment 1: Bicarbonate dependency for initiating capacitation and cholesterol efflux from ram spermatozoa exposed to cAMP upregulated conditions

Experiment 1 was designed to assess the necessity of bicarbonate for *in vitro* capacitation of ram spermatozoa under upregulated cAMP conditions. Following the swim up, washed spermatozoa were diluted to a final concentration of 50×10^6 sperm/mL in either TALP with or without bicarbonate and 1 mM of db-cAMP, caffeine and theophylline (cAMP up-regulators). All conditions were still in presence of the cholesterol acceptor, BSA. Total motility was objectively monitored throughout the incubation period using computer-assisted sperm analysis (CASA) (Supplementary Table 1, see section on supplementary materials given at the end of this article). Experiment 1 was replicated ten times and the BODIPY-cholesterol assay for this experiment replicated eight times. Samples were assessed immediately after exposure to various capacitating and non-capacitating conditions (10 min) and after 180 min of incubation (3 h) at 37°C.

Experiment 2: BSA dependency for cholesterol efflux from ram spermatozoa exposed to cAMP upregulated conditions

Experiment 2 was designed to assess the effect of BSA as a cholesterol acceptor under upregulated cAMP conditions during *in vitro* capacitation. In likeness with Experiment 1, washed spermatozoa were diluted to 50×10^6 sperm/mL in either TALP with or without BSA and 1 mM of all three cAMP up-regulators. All conditions were still in the presence of bicarbonate. Total motility was objectively monitored throughout the incubation period using computer-assisted sperm analysis (CASA) (Supplementary Table 2). Experiment 2 was replicated seven times and the BODIPY-cholesterol assay for this experiment replicated eight times. Samples were assessed immediately after exposure to various capacitating and non-capacitating conditions (10 min) and after 180 min of incubation (3 h) at 37°C.

Flow cytometric evaluation of sperm function

For analysis of sperm function during *in vitro* capacitation, an Accuri flow cytometer with a standard argon ion laser was used (488 nm; Accuri C6; BD Accuri Cytometers, Ann Arbor, MI, USA). A sperm cell specific population was gated based on forward light scatter and sideward light scatter profiles. Changes in membrane fluidity and integrity were assessed through dual fluorescent staining with 0.83 µM merocyanine 540 (M540) and 25 nm Yo-Pro-1 for 10 min at 37°C. M540 fluorescence was detected on FL-2 (585/40 nm band-pass filter) and Yo-Pro-1 fluorescence detected on FL-1 (533/30 nm band-pass filter). When analysing this data, only spermatozoa with an intact plasma membrane (Yo-Pro-1-) and either low or high M540 fluorescence (corresponding to low and high membrane fluidity, respectively) were assessed. Acrosome and membrane integrity were examined by dual fluorescence staining with 0.4 µg/mL PNA-FITC and 6 µM PI for 10 min at 37°C. PNA-FITC detects acrosome reactivity by binding to the sugar moieties present on both the OAM and IAM (outer and inner acrosomal membrane, respectively) of ram spermatozoa. Though PI is traditionally used to assess membrane integrity, otherwise quoted as viability, capacitating spermatozoa have been shown to uptake PI (when compared to non-capacitating spermatozoa) but at a lower rate compared to those cells deemed as having a damaged plasma membrane (Kerns *et al*. 2018). This distinction was crucial for this study to ensure that spermatozoa with an intact plasma membrane (PI-), regardless of capacitation status, were identified and gated appropriately for analysis. For flow cytometric assessment, FITC-PNA fluorescence was detected on FL-1 (533/30 nm band-pass filter) and PI fluorescence was detected on FL-3 (670 nm

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long-pass filter). These two parameters were measured to monitor sperm quality over the course of the incubation period (see Supplementary Figs 1 and 2 for Experiment 1 and 2, respectively). For each sample, 10,000 events were recorded and BD ACCURI software was used for further analysis.

BODIPY-cholesterol assay

For the BODIPY-cholesterol assay, ram spermatozoa were labelled with BODIPY-cholesterol and prepared for *in vitro* capacitation as previously described (Bernecic *et al*. 2019). Briefly, following dilution to 250×10^6 sperm/mL with TALP devoid of BSA and bicarbonate, spermatozoa were labelled with 1 µM BODIPY-cholesterol (1 mM stock solution dissolved in DMSO) for 10 min at 37°C. Excess BODIPY-cholesterol was removed via centrifugation (300 ***g***, 20 min, 25°C) through a two-way step discontinuous gradient of 40% and 80% isotonic Bovipure diluted with Bovidilute. A gradient for each ram was performed in duplicate and the sperm pellets for a single ram were pooled then washed of density gradient medium with TALP devoid of BSA and bicarbonate via centrifugation (300 ***g***, 10 min, 25°C). For this assay, an initial swim up was not necessary as spermatozoa were processed with a density gradient during the removal of excess BODIPY-cholesterol. The concentration of the resulting sperm pellet was determined using a haemocytometer and was diluted to a final concentration of 20×10^6 sperm/mL with the respective media conditions outlined for up to 180 min at 37°C.

For the reliable assessment of cholesterol efflux with the BODIPY-cholesterol assay, only spermatozoa with an intact plasma membrane (PI-) were analysed in order to avoid the detection of potential intracellular labelling in membrane damaged cells (PI+; Bernecic *et al*. 2019). To do this, BODIPY-cholesterol labelled spermatozoa were counterstained with 6 µM PI for 10 min prior to analysis on an Accuri flow cytometer. A sperm cell specific population was gated based on forward light scatter and sideward light scatter profiles. BODIPY-cholesterol fluorescence was detected on FL-1 (533/30 nm band-pass filter) and PI fluorescence on FL-3 (670 nm long-pass filter). For each sample, 10,000 events were recorded for further analysis in the BD ACCURI software. To determine cholesterol efflux induced by the various media conditions, the percent change in BODIPY-cholesterol fluorescence from 10 min to 180 min (3 h) was calculated for the PI- population.

Extraction of ram sperm proteins

To extract proteins for analysis of tyrosine phosphorylation in the whole sperm population, aliquots of 10×10^6 sperm were washed (600 ***g***, 10 min) in PBS to remove media derived BSA. The sperm pellet was suspended 1:1 in lysis buffer (62.6 mM Tris, 1 mM sodium orthovanadate (added to specifically inhibit tyrosine phosphatases), 2% w/v SDS, complete ultra mini EDTA-free protease inhibitor tablet) and kept at room temperature for 1 h. Following this lysis period, samples were then centrifuged (7500 ***g***, 15 min) and the lysate was retained. The protein concentration of lysates was estimated using a bicinchoninic acid assay (BCA; catalogue no. 23225; Pierce) and standardised to 1 mg/mL with Milli-Q water before further dilution with loading buffer (final concentration of 62.5 mM Tris, pH 6.8; 5% (v/v) 2-mercaptoethanol; 2% (v/v) SDS; 10% glycerol (v/v); 0.2% (w/v) bromophenol blue). Samples were then incubated for 5 min at 95°C and then stored at −80°C until required.

SDS-PAGE and tyrosine phosphorylation western blot

All reagents and equipment used for SDS-PAGE and Western blot analysis were purchased from Bio-Rad unless otherwise specified. Precision Plus Kaleidoscope standard (catalogue no. 1610375), MagicMark XP Western Blot Standards (Invitrogen) and processed sperm protein lysates (30 µg) were loaded into 10% Mini-PROTEAN TGX stain-free gels (catalogue no. 4568033) and electrophoresis was carried out initially at 75 V for 10 min then increased to 200 V for a further 20–30 min. To validate gel loading, quantification of protein bands was achieved using a stain-free analysis protocol on a Chemi-Doc MP Imaging System. Proteins were then transferred overnight to an Immuno-Blot PVDF membrane (catalogue no. 1620177) at 30 V in Towbin's transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol (v/v)). Non-specific sites on the membrane were blocked for 1 h in TBS (20 mM Tris, 150 mM NaCl, pH 7.6) supplemented with 0.1% Tween 20 (TBS-T) containing 5% (w/v) BSA. Blots were incubated with MAB HRP-conjugated anti-phosphotyrosine (monoclonal AB; clone 4G10, Upstate, catalogue no. 16-105) diluted 1:2000 in TBS-T + 0.1% BSA (w/v) with agitation, for 1 h at room temperature. After washing three times in TBS-T for 15 min, tyrosine phosphorylated proteins were visualised on a Chemi-Doc system using enhanced chemiluminescence (Clarity; catalogue no. 1705061) as per manufacturer's instructions.

Measurement of cAMP production

To further elucidate the role of bicarbonate under cAMP up-regulated conditions, sperm cAMP production was quantified only following exposure to media with or without bicarbonate and supplemented with cAMP up-regulators (BSA included in all conditions). Spermatozoa exposed to TALP alone were also included as a control, as was a media sample containing a known concentration of db-cAMP to check whether the presence of this analogue alone could influence the assay results. The production of cAMP under these conditions was quantified using a Parameter cAMP Assay Kit. In brief, aliquots of 10×10^6 sperm were washed three times with cold PBS (900 ***g***; 10 min; 4°C) and resulting sperm pellets were lysed with the provided Cell Lysis Buffer 5 before storage at −80°C until required. Lysates were assayed for cAMP as per the manufacturer's instructions and the results were analysed using the online data analysis tool, MyAssays (MyAssays Ltd. 2018). The cAMP assay was replicated six times for all samples and intra and inter-assay variation were calculated from the results. In addition, parallelism and spike/recovery were performed as quality controls.

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Determining the individual and additive effect of db-cAMP, caffeine and theophylline on ram spermatozoa

In an attempt to further understand the individual effects of db-cAMP and PDEs with bicarbonate on the early stages of capacitation, semen was collected from three Merino rams twice ($n=6$) and either diluted in TALP alone or supplemented with either 1 mM db-cAMP or 1 mM caffeine and theophylline. To determine the additive effects of cAMP up-regulators, semen ($n=6$) was diluted with TALP alone or supplemented with the additive combination of 1 mM db-cAMP, caffeine and theophylline. Spermatozoa in all conditions were incubated at 37°C for up to 180 min (3 h). To observe the effects of these conditions on ram sperm capacitation, changes in membrane fluidity and integrity were quantified as described in 'Flow cytometric evaluation of sperm function' section.

Statistical analysis

All experiments and the respective functional tests and assays were analysed using linear mixed model regression (REML; R version 3.4.1). For all *in vitro* capacitation measures including cAMP production, the media condition and incubation time were set as fixed effects in the model, replicate and ram were included as nested random effects. The same was applied for the BODIPY-cholesterol assay results excluding the individual effect of time. Interactions between the fixed effects were assessed where appropriate. Pairwise comparisons between fixed effects were determined by lsmeans with a Tukey adjustment. Normality and homoscedasticity of the residuals was assessed for all models and manipulation of the model to reduce heteroscedasticity of the residuals was performed if necessary. In some cases, this required the application of a log transformation and, if so, the results were back-transformed and presented as the geometric mean ± 95% CIs. Otherwise, data are presented as the mean ± S.D. and results that are $P<0.05$ are considered significant.

Results

The presence of cAMP up-regulators does not omit the need for bicarbonate to support capacitation-related processes in ram spermatozoa

When both bicarbonate and cAMP up-regulators were present in media, there was a significant increase in the percentage of spermatozoa with high membrane fluidity compared to media without these additional stimulants (Fig. 1A; bicarbonate + up-regulators: 30.9 ± 27.9%; bicarbonate alone: 1.3 ± 0.8%; $P<0.01$). Contrastingly, the supplementation of cAMP up-regulators in absence of bicarbonate (including BSA) was insufficient to support an equivalent percentage of cells with high membrane fluidity as compared to when bicarbonate was also present (Fig. 1A; up-regulators alone: 12.0 ± 12.4%; $P<0.05$).

For the first application of the BODIPY-cholesterol assay in a species other than the boar (Bernecic *et al.* 2019), it was interesting to observe that cholesterol efflux corresponded strongly to changes in membrane fluidity (as measured with M540) but only when ram spermatozoa were exposed to specific conditions (Fig. 2). Following incubation in TALP with bicarbonate and cAMP up-regulators (including BSA), spermatozoa lost approximately 20% of BODIPY-cholesterol when compared to BSA containing media with bicarbonate alone (Fig. 2A; bicarbonate + up-regulators: 79.5 ± 30.5% remaining from 10 min; bicarbonate alone: 103.7 ± 13.0% remaining from 10 min; $P<0.05$). In contrast, exposure to BSA containing medium with only cAMP up-regulators was unable to cause an equivalent response in ram spermatozoa with respect to cholesterol efflux (up-regulators alone: 101.6 ± 52.0% remaining from 10 min).

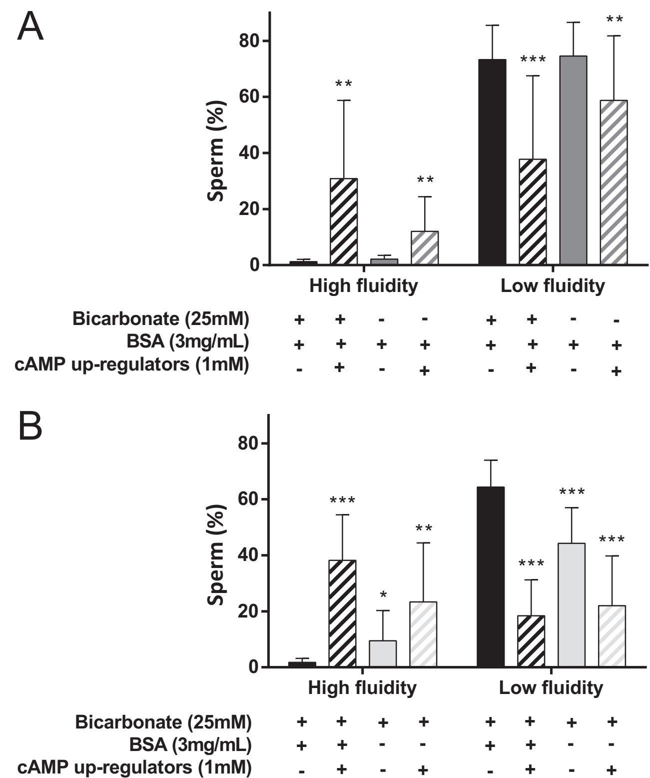

Figure 1 The percentage of Yo-Pro-1- ram spermatozoa with high or low membrane fluidity following exposure to media with or without bicarbonate and db-cAMP, caffeine and theophylline (cAMP up-regulators; A) or BSA and cAMP up-regulators (B) for up to 180 min. Changes in membrane fluidity over the incubation period were detected with merocyanine 540 using flow cytometry. The inclusion of bicarbonate, BSA and cAMP up-regulators was essential to stimulate high fluidity in the plasma membrane of a significant population of ram spermatozoa (A and B; black hatched bar). Data corresponds to mean response over time ± S.D. for ten or seven independent samples for (A) and (B), respectively. Results presented at each time point are included as Supplementary information (Supplementary Figs 3 and 4). $*P<0.05$, $**P<0.01$ and $***P<0.001$ indicate the difference from TALP (bicarbonate and BSA).

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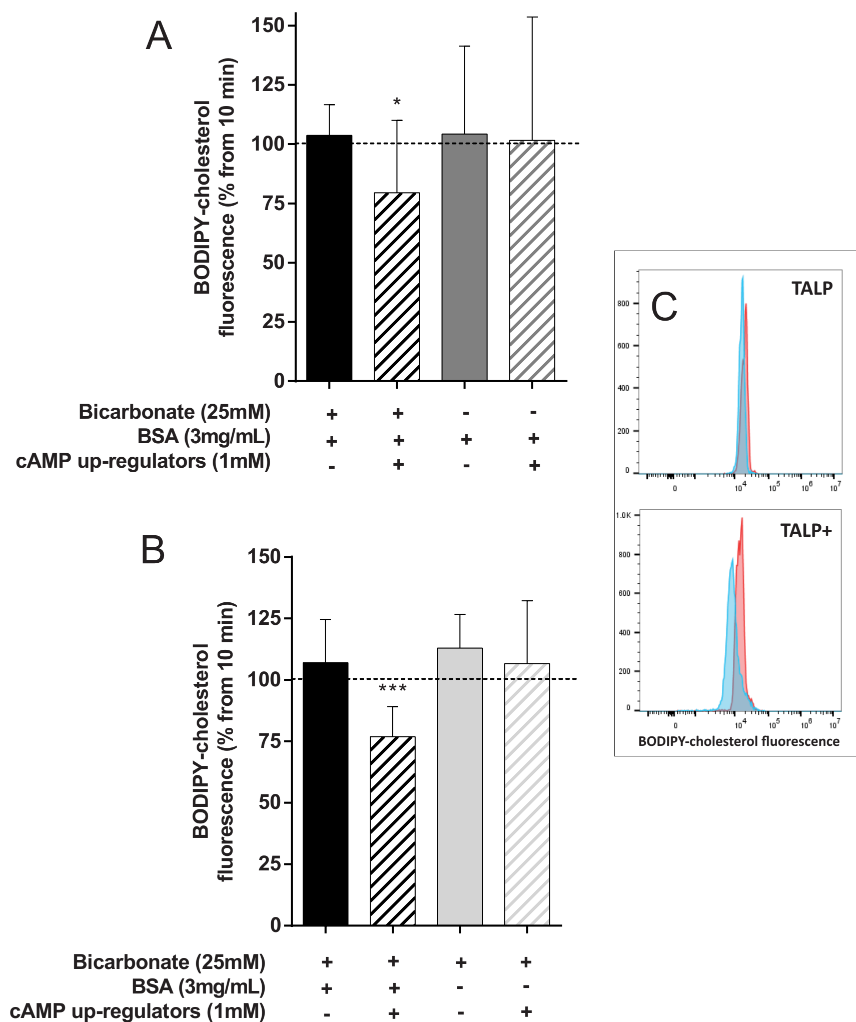

Figure 2 The percentage of BODIPY-cholesterol fluorescence remaining following exposure to media with or without bicarbonate and db-cAMP, caffeine and theophylline (cAMP up-regulators; A) or BSA and cAMP up-regulators (B). Ram spermatozoa were initially labelled with BODIPY-cholesterol prior to incubation and the loss of this fluorophore in the PI-population was tracked with flow cytometry immediately following exposure to treatment conditions (10 min) and 180 min. BODIPY-cholesterol efflux was only observed in the presence of cAMP up-regulators with the addition of bicarbonate and BSA (A and B; black hatched bar). Representative histograms illustrate this loss over time (10 min: red histogram, 180 min: blue histogram) from spermatozoa when exposed to bicarbonate, BSA and cAMP up-regulators (C; TALP+) when compared to bicarbonate and BSA alone (TALP). Data corresponds to mean $\pm$ s.d. for nine independent samples for (A) and (B). $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate the difference from TALP (bicarbonate and BSA).

In this study, tyrosine phosphorylation of proteins (from the whole sperm population) between the sizes of 38 and 44 kDa appeared to be constitutively phosphorylated as they were present across all media conditions and at both 10 min and 180 min (Fig. 3A and B for bicarbonate and BSA, respectively). This consistent finding indicates that these proteins were potentially already phosphorylated prior to *in vitro* capacitation (Pérez-Pé *et al*. 2002, Grasa *et al*. 2004), which could be due to previous exposure to bicarbonate in seminal plasma (Okamura *et al*. 1985). However, tyrosine phosphorylation of high molecular weight sperm proteins (50–250 kDa) only occurred following the exposure of spermatozoa to BSA containing media supplemented bicarbonate and cAMP up-regulators for 180 min (Fig. 3A). The omission of bicarbonate and replacement with cAMP up-regulators in BSA containing media did not induce high molecular weight protein tyrosine phosphorylation but instead resulted in a protein phosphorylation banding pattern analogous to non-capacitated sperm (Fig. 3A).

The functional relationship between bicarbonate and cAMP up-regulators in presence of BSA

Since bicarbonate was identified as an important addition along with the cAMP up-regulators (db-cAMP, caffeine and theophylline) in order to support *in vitro* capacitation, it was of interest to further explore the action of bicarbonate in ram spermatozoa. To do this, the production of cAMP was quantified in ram spermatozoa exposed to TALP with or without bicarbonate and cAMP up-regulators (including BSA). In order to significantly maximise cAMP production, it appeared that the presence of cAMP up-regulators was the critical factor, as without these stimulants, cAMP remained low throughout the incubation period (Fig. 4). It is therefore not unexpected why TALP alone is unable to initiate ram sperm capacitation *in vitro* as this medium fails to sufficiently stimulate an increased production of cAMP. Though the presence of bicarbonate with cAMP up-regulators was responsible for increasing the cAMP concentration by up to 22% relative to up-regulators

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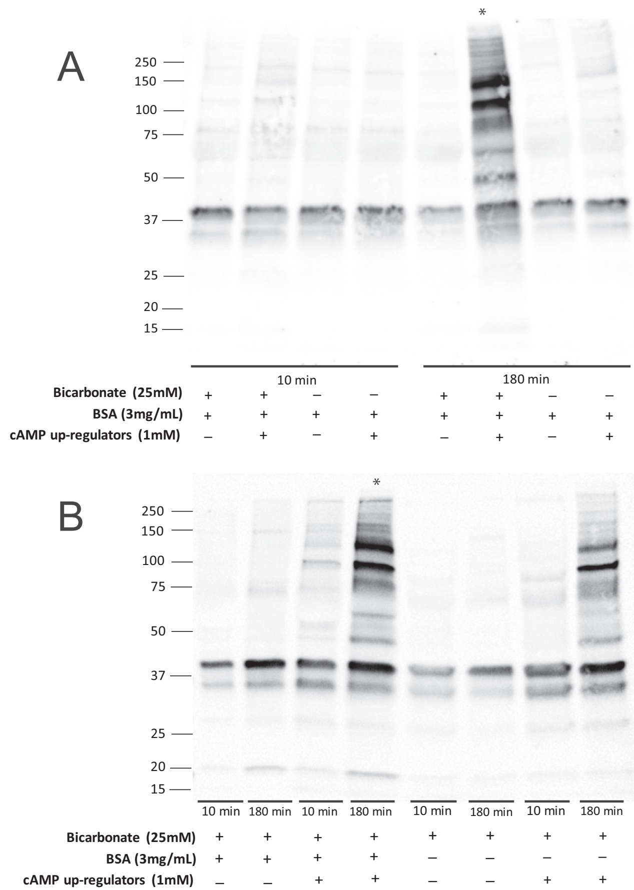

Figure 3 Capacitation-associated tyrosine phosphorylation of sperm proteins following exposure to media with or without bicarbonate and db-cAMP, caffeine and theophylline (cAMP up-regulators; A) or BSA and cAMP up-regulators (B). Samples of the whole sperm population were taken for assessment immediately following exposure to media (10 min) and 180 min of incubation. Maximal phosphorylation of high molecular weight proteins (50–250 kDa) was evident when ram spermatozoa were exposed to cAMP up-regulators with bicarbonate and BSA for 180 min (A and B, respectively; indicated with a star (*)). Western blot analysis of each experiment was repeated three times and a representative blot is shown.

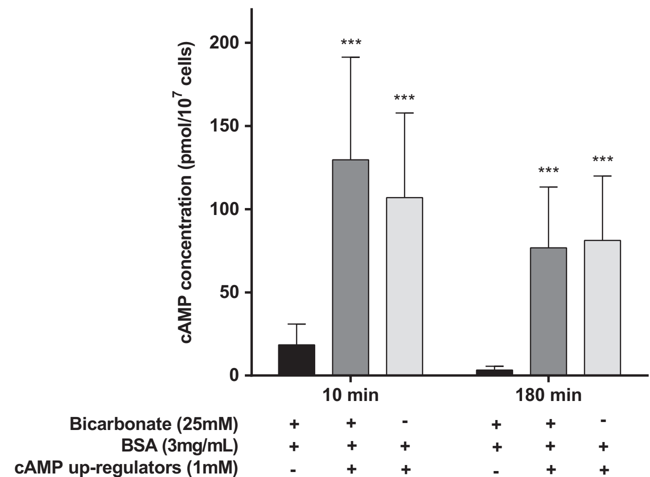

Figure 4 Cyclic AMP (cAMP) production in spermatozoa exposed to TALP with or without bicarbonate and db-cAMP, caffeine and theophylline (cAMP up-regulators). Samples were taken for assessment of cAMP production immediately following exposure to treated conditions (10 min) and at 180 min of incubation. Endogenous cAMP was significantly elevated when compared to TALP for the duration of the *in vitro* capacitation period in both TALP with cAMP up-regulators and TALP devoid of bicarbonate with cAMP up-regulators. Data corresponds to geometric mean ± 95% CIs for six independent samples. ***$P < 0.001$ indicates the difference from TALP.

alone at 10 min, this difference was insignificant (Fig. 4; $P > 0.05$). For the measurement of cAMP, the intra-assay CV was 9.7 and 11.2% for plate 1 and 2, respectively, inter-assay CV was 1.7% and the average recovery of the low, mid and high spiked samples was 142.1%, 89.2% and 78.6%, respectively. For the db-cAMP control, the average recovery was 26.1%, meaning that, while the assay does show some capability of detecting this analogue, any influence this may have on the results will be the same for all the conditions tested (with the exception of TALP alone).

With evidence that bicarbonate was able to enhance cAMP production in ram spermatozoa, the next step was to examine whether activation of the cAMP-PKA pathway (as indirectly measured by changes in membrane fluidity) was due to the combination of bicarbonate with db-cAMP, PDE inhibitors (caffeine and theophylline) or both. Interestingly, the presence of db-cAMP or PDE inhibitors with bicarbonate was only able to induce a marginal but significant increase in membrane fluidity over time when compared to spermatozoa exposed to TALP alone (Fig. 5A and B). Under these conditions the percentage of cells with high membrane fluidity remained below 12% and was considerably variable. Once these components were added sequentially to media, the percentage of cells with high membrane fluidity doubled following exposure to caffeine in combination with db-cAMP and bicarbonate when compared to TALP alone at both 10 and 180 min (Fig. 5C; $P < 0.001$). The supplementation of theophylline further supported an increase in cells with high membrane fluidity to triple the percentage of that present in TALP alone ($P < 0.001$).

In a cAMP upregulated environment, BSA is required to mediate cholesterol efflux and support capacitation

Much like bicarbonate, the presence of cAMP up-regulators with BSA was essential to stimulate an increase in the percentage of cells with high membrane fluidity (Fig. 1B; BSA + up-regulators: 38.2 ± 16.3%), induce a subsequent 23% loss of BODIPY-cholesterol over the incubation period (Fig. 2B; BSA + up-regulators: 76.9 ± 12.3% remaining from 10 min) and enhance tyrosine phosphorylation in high molecular weight

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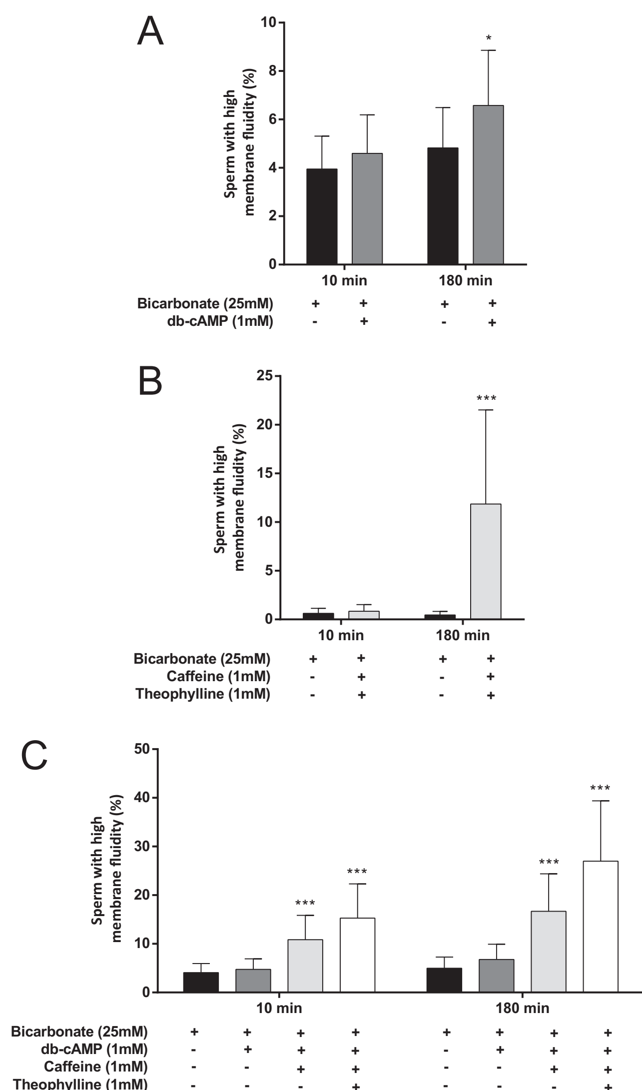

Figure 5 The percentage of Yo-Pro-1- ram spermatozoa with high membrane fluidity following incubation in TALP supplemented either with or without db-cAMP (A), phosphodiesterase (PDE) inhibitors, caffeine and theophylline (B) or the additive combination of all cAMP up-regulators (C). Samples were assessed for changes in membrane fluidity as an early indicator for capacitation immediately following exposure to treated conditions (10 min) and at 180 min of incubation. Data corresponds to geometric mean ± 95% CIs for six or five independent samples for (A and C) and (B), respectively. **P* < 0.05 and ****P* < 0.0001 indicate the difference from TALP.

proteins (Fig. 3B). Only upon the incubation of spermatozoa in TALP devoid of BSA supplemented with cAMP up-regulators (including bicarbonate) is the importance of BSA as a cholesterol acceptor truly observed, since cholesterol efflux was not supported from spermatozoa exposed to this condition (Fig. 2B; up-regulators alone: 106.6 ± 25.6% remaining from 10 min). The removal of BSA under cAMP-upregulated conditions did not have the same drastic effect on membrane fluidity (Fig. 1B; up-regulators alone: 23.4 ± 21.0%) or tyrosine phosphorylation (Fig. 3B; visible attenuation of band intensity in cAMP up-regulators alone) as it did on cholesterol efflux, so it is more likely that BSA is enhancing these processes as opposed to regulating them.

Discussion

The exposure to increased levels of bicarbonate present in either the female reproductive tract or formulated media is an absolute requirement for capacitation to occur in a multitude of species. This ion is known to promote alkalinisation of the cytoplasm and membrane hyperpolarisation (Demarco *et al.* 2003), both of which are linked to the regulation of Ca^{2+} channel activity during capacitation (Arnoult *et al.* 1999). In addition, bicarbonate is responsible for the activation of soluble adenylyl cyclase (sAC) (Chen *et al.* 2000), an enzyme whose main function is to produce cAMP that subsequently stimulates protein kinase A (PKA) activity (Harrison & Gadella 2005). Initiation of the cAMP-PKA pathway is the crux of capacitation as many processes rely on it to proceed. In the current study, we found in likeness to previous research in the ram that bicarbonate alone was unable to trigger a capacitation response. Only when spermatozoa were exposed to an environment where cAMP was maintained at high levels, which was achieved through the exogenous supplementation of a cAMP analogue (db-cAMP) and multiple PDE inhibitors (caffeine and theophylline), did this initiate and support the capacitation-related processes, high membrane fluidity and tyrosine phosphorylation of high molecular weight proteins (Colas *et al.* 2008, Leahy *et al.* 2016, Pini *et al.* 2018). In fact, the response following exposure to an up-regulated cAMP environment could be observed almost immediately. This was demonstrated by the elevation in cAMP production and increase in membrane fluidity, which is known to be induced rapidly by bicarbonate and/or cAMP up-regulators (Harrison *et al.* 1996, Harrison & Miller 2000). It is important to note here that there are other ways in which endogenous cAMP production can be regulated, such as that via multi-drug resistance associated protein 4 (MRP4) transporter activity (Osycka-Salut *et al.* 2014). This transporter has been localised in bovine spermatozoa and is responsible for extruding cAMP into the extracellular environment, which can then go onto activating various signalling pathways including the cAMP-PKA pathway (Osycka-Salut *et al.* 2014, Alonso *et al.* 2017). Although, further research is necessary to better understand the role of this transporter in ram spermatozoa during capacitation, specifically under up-regulated cAMP conditions.

It has been previously reported that exposure of spermatozoa to cAMP up-regulators are able to support capacitation in various species and that these stimulants can in fact replace the action of bicarbonate. In boar,

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human and mouse spermatozoa, the omission of bicarbonate prevents changes in membrane fluidity and the presence of tyrosine phosphorylation, but these processes can be readily recovered following the addition of various cAMP analogues and PDE inhibitors (Visconti *et al*. 1995*b*, Harrison *et al*. 1996, Gadella & Harrison 2000, de Vries *et al*. 2003). Interestingly, this was not observed in ram spermatozoa, since the exposure to cAMP up-regulators alone was unable to stimulate high membrane fluidity and there was no evidence of tyrosine phosphorylation, indicating that these are bicarbonate-dependent processes. Based on these findings alone, it would seem that the activation of sAC by bicarbonate is still a critical part of the cAMP-PKA pathway for ram spermatozoa, even if this ion alone is insufficient to support capacitation. The enzyme sAC is known to be a key element for sperm function, with studies in knock-out mice for sAC revealing that spermatozoa are unable to undergo capacitation in suitable *in vitro* conditions and that overall intracellular cAMP is decreased (Hess *et al*. 2005, Xie *et al*. 2006). Following a deeper investigation into the role of bicarbonate, it was found in this study that it contributed up to a 22% increase (at 10 min) in overall cAMP produced by ram spermatozoa exposed to cAMP up-regulators alone. While this increase in cAMP did not reach statistical significance, it did correlate with an enhanced capacitation response in this species, specifically the presence of maximal tyrosine phosphorylation by 180 min of incubation. Though there is extensive evidence in literature that one of the downstream events of increased cAMP production is the activation of signalling pathways leading to tyrosine phosphorylation, the current study would benefit from measuring PKA activity to solidify this connection (Visconti *et al*. 1995*b*, Leclerc *et al*. 1996, Galantino-Homer *et al*. 1997, Buffone *et al*. 2014). Nevertheless, this finding continues to highlight the importance of bicarbonate during *in vitro* capacitation of ram spermatozoa and how cAMP up-regulators are unable to completely bypass its function.

The requirement of bicarbonate and multiple stimulants with the capability of upregulating cAMP production is atypical for the majority of species, particularly since these components are known to act on the same pathway. What then makes ram spermatozoa so different that they need such extreme cAMP stimulation to provoke a capacitation response? One possibility is that ram spermatozoa may have unusually high phosphodiesterase activity in relation to other species (Colas *et al*. 2008). Phosphodiesterases (PDEs) are a family of enzymes with 11 known types that play a dominant role in the degradation of cAMP and/or cyclic GMP (cGMP) (Dimitriadis *et al*. 2008). Several of these PDEs have been identified in mature spermatozoa of humans, mice and bovine and their function confirmed by the use of specific PDE inhibitors (Fisch *et al*. 1998, Richter *et al*. 1999, Lefievre *et al*. 2000, Baxendale & Fraser 2005, Bajpai *et al*. 2006, Bergeron *et al*. 2016). During capacitation of mouse and bovine spermatozoa, PDE activity is either lowered or maintained during capacitation (Monks & Fraser 1987, Galantino-Homer *et al*. 2004), thus enabling increases in cAMP production upon stimulation of sAC. For ram spermatozoa, even before exposure to capacitating conditions, the hydrolysis of cAMP by PDEs is ~100 times greater than its synthesis by sAC (Tash 1976). Although what appears to contradict this conclusion in the current study was that bicarbonate with PDE inhibitors alone could only elicit a minor increase in cells with high membrane fluidity, which was considerably lower than what was observed for spermatozoa exposed to full cAMP stimulation. When bicarbonate and cAMP up-regulators were added sequentially to media, an extreme response in membrane fluidity was noted following the addition of caffeine and theophylline when cells were already exposed to bicarbonate and db-cAMP. So, while these components cannot induce strong responses alone, it is likely that bicarbonate, db-cAMP and PDE inhibitors are required to concurrently activate sAC, boost cAMP availability and inhibit excessive phosphodiesterase activity in order to overcome the difficulty in driving the cAMP-PKA pathway in ram spermatozoa.

The primary role of serum albumin in traditional capacitation media is to act as a cholesterol acceptor in the extracellular environment and mediate cholesterol efflux from the sperm plasma membrane (Osheroff *et al*. 1999, Visconti *et al*. 1999, Flesch *et al*. 2001). In ram spermatozoa, attempting to stimulate cholesterol efflux with BSA has been unsuccessful without cAMP upregulation, even the use of the cholesterol-sequestering agent, methyl-β-cyclodextrin, was unable to induce maximal cholesterol efflux as it does in other species (Grasa *et al*. 2006, Colas *et al*. 2008). Based on the results of this study, we were able to show that the BODIPY-cholesterol assay quantified up to 23% loss of BODIPY-cholesterol from ram spermatozoa exposed to BSA under cAMP upregulated conditions. The ability for BSA to act as a cholesterol acceptor is further confirmed by the absence of cholesterol efflux from spermatozoa exposed to media only with cAMP up-regulators. This strongly demonstrates that BSA mediates cholesterol efflux from the plasma membrane of ram spermatozoa under these specific upregulated conditions.

Previous research in the ram suggested that the reason for the lack of cholesterol efflux from spermatozoa could be due to the lower ratio of cholesterol to phospholipids in the plasma membrane when compared with other species (sheep: 0.38 vs humans: 0.99; (Darin-Bennett & White 1977)), and as a result, a further reduction in cholesterol may be detrimental to the cell (Grasa *et al*. 2006, Colas *et al*. 2008). While this is a valid point, it is noteworthy that the presence of cAMP up-regulators (and bicarbonate) with BSA was all that was required to stimulate cholesterol efflux in this species. In addition to

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evidence of cholesterol efflux in these media conditions, approximately 38% of spermatozoa also exhibited high membrane fluidity, which was significantly greater than spermatozoa exposed to BSA (and bicarbonate) alone. Therefore, these findings suggest that the inability for ram spermatozoa to efflux cholesterol following incubation in TALP alone was simply due to the fact that the cells were inadequately prepared for this process, but it can be readily supported by the presence of bicarbonate and cAMP up-regulators along with a cholesterol acceptor like BSA.

In conclusion, the findings of this study have confirmed the importance of bicarbonate and BSA for *in vitro* capacitation of ram spermatozoa within a cAMP up-regulated environment. Bicarbonate was responsible for further elevating cAMP production in ram spermatozoa exposed to cAMP up-regulators (db-cAMP, caffeine and theophylline) to subsequently drive the cAMP-PKA pathway. In addition, the exposure to bicarbonate was crucial to enhance membrane fluidity and permit tyrosine phosphorylation. The cholesterol acceptor, BSA, was able to successfully mediate cholesterol efflux from ram spermatozoa, as measured by the BODIPY-cholesterol assay, but only when the plasma membrane was adequately prepared under cAMP upregulated conditions. The irreplaceable nature of bicarbonate and BSA, even with cAMP upregulation, illustrates the significance of the synergism between these components to facilitate many capacitation-related processes that are essential for the preparation of ram spermatozoa for fertilisation.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/REP-19-0430.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

N B performed and analysed all experiments, as well as prepared the manuscript and figures. The manuscript was critically reviewed and approved for submission by B M G, S P de G and T L.

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References

Aitken RJ & Nixon B 2013 Sperm capacitation: a distant landscape glimpsed but unexplored. *Molecular Human Reproduction* **19** 785–793. (https://doi.org/10.1093/molehr/gat067)

Alonso CAI, Osycka-Salut CE, Castellano L, Cesari A, Di Siervi N, Mutto A, Johannisson A, Morrell JM, Davio C & Perez-Martinez S 2017 Extracellular cAMP activates molecular signalling pathways associated with sperm capacitation in bovines. *Molecular Human Reproduction* **23** 521–534. (https://doi.org/10.1093/molehr/gax030)

Arnoult C, Kazam IG, Visconti PE, Kopf GS, Villaz M & Florman HM 1999 Control of the low voltage-activated calcium channel of mouse sperm by egg ZP3 and by membrane hyperpolarization during capacitation. *PNAS* **96** 6757–6762. (https://doi.org/10.1073/pnas.96.12.6757)

Austin CR 1951 Observations on the penetration of the sperm in the mammalian egg. *Australian Journal of Scientific Research B* **4** 581–596. (https://doi.org/10.1071/bi9510581)

Bailey JL 2010 Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine* **56** 334–348. (https://doi.org/10.3109/19396368.2010.512377)

Bajpai M, Fiedler SE, Huang Z, Vijayaraghavan S, Olson GE, Livera G, Conti M & Carr DW 2006 AKAP3 selectively binds PDE4A isoforms in bovine spermatozoa. *Biology of Reproduction* **74** 109–118. (https://doi.org/10.1095/biolreprod.105.043588)

Battistone MA, Da Ros VG, Salicioni AM, Navarrete FA, Krapf D, Visconti PE & Cuasnicu PS 2013 Functional human sperm capacitation requires both bicarbonate-dependent PKA activation and down-regulation of Ser/Thr phosphatases by Src family kinases. *Molecular Human Reproduction* **19** 570–580. (https://doi.org/10.1093/molehr/gat033)

Baxendale RW & Fraser LR 2005 Mammalian sperm phosphodiesterases and their involvement in receptor-mediated cell signaling important for capacitation. *Molecular Reproduction and Development* **71** 495–508. (https://doi.org/10.1002/mrd.20265)

Bergeron A, Aragon JP, Guillemette C, Hébert A, Sullivan R, Blondin P & Richard FJ 2016 Characterization of cAMP-phosphodiesterase activity in bovine seminal plasma. *Andrology* **4** 1123–1130. (https://doi.org/10.1111/andr.12267)

Bernecic NC, Zhang M, Gadella BM, Brouwers JFHM, Jansen JWA, Arkesteijn GJA, de Graaf SP & Leahy T 2019 BODIPY-cholesterol can be reliably used to monitor cholesterol efflux from capacitating mammalian spermatozoa. *Scientific Reports* **9** 9804. (https://doi.org/10.1038/s41598-019-45831-7)

Bromfield EG, Aitken RJ, Gibb Z, Lambourne SR & Nixon B 2014 Capacitation in the presence of methyl-beta-cyclodextrin results in enhanced zona pellucida-binding ability of stallion spermatozoa. *Reproduction* **147** 153–166. (https://doi.org/10.1530/REP-13-0393)

Buffone MG, Wertheimer EV, Visconti PE & Krapf D 2014 Central role of soluble adenylyl cyclase and cAMP in sperm physiology. *Biochimica et Biophysica Acta* **1842** 2610–2620. (https://doi.org/10.1016/j.bbadis.2014.07.013)

Chang MC 1951 Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature* **168** 697–698. (https://doi.org/10.1038/168697b0)

Chen Y, Cann MJ, Litvin TN, Iourgenko V, Sinclair ML, Levin LR & Buck J 2000 Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. *Science* **289** 625–628. (https://doi.org/10.1126/science.289.5479.625)

Colas C, James P, Howes L, Jones R, Cebrian-Perez JA & Muiño-Blanco T 2008 Cyclic-AMP initiates protein tyrosine phosphorylation independent of cholesterol efflux during ram sperm capacitation. *Reproduction, Fertility, and Development* **20** 649–658. (https://doi.org/10.1071/RD08023)

Dacheux JL & Dacheux F 2014 New insights into epididymal function in relation to sperm maturation. *Reproduction* **147** R27–R42. (https://doi.org/10.1530/REP-13-0420)

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Darin-Bennett A & White IG 1977 Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold-shock. *Cryobiology* **14** 466–470. (https://doi.org/10.1016/0011-2240(77)90008-6)

de Vries KJ, Wiedmer T, Sims PJ & Gadella BM 2003 Caspase-independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human sperm cells. *Biology of Reproduction* **68** 2122–2134. (https://doi.org/10.1095/biolreprod.102.012500)

Demarco IA, Espinosa F, Edwards J, Sosnik J, De la Vega-Beltrán JL, Hockensmith JW, Kopf GS, Darszon A & Visconti PE 2003 Involvement of a Na+/HCO3 – cotransporter in mouse sperm capacitation. *Journal of Biological Chemistry* **278** 7001–7009. (https://doi.org/10.1074/jbc.M206284200)

Dimitriadis F, Giannakis D, Pardalidis N, Zikopoulos K, Paraskevaidis E, Giotitsas N, Kalaboki V, Tsounapi P, Baltogiannis D, Georgiou I *et al.* 2008 Effects of phosphodiesterase 5 inhibitors on sperm parameters and fertilizing capacity. *Asian Journal of Andrology* **10** 115–133. (https://doi.org/10.1111/j.1745-7262.2008.00373.x)

Evans G 1987 *Salamon's Artificial Insemination of Sheep and Goats*. Sydney: Butterworths.

Fisch JD, Behr B & Conti M 1998 Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. *Human Reproduction* **13** 1248–1254. (https://doi.org/10.1093/humrep/13.5.1248)

Flesch FM, Brouwers JFHM, Nievelstein PFEM, Verkleij AJ, van Golde LMG, Colenbrander B & Gadella BM 2001 Bicarbonate stimulated phospholipid scrambling induces cholesterol redistribution and enables cholesterol depletion in the sperm plasma membrane. *Journal of Cell Science* **114** 3543–3555.

Gadella BM & Harrison RAP 2000 The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development* **127** 2407–2420.

Gadella BM & Harrison RAP 2002 Capacitation induces cyclic adenosine 3′,5′-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. *Biology of Reproduction* **67** 340–350. (https://doi.org/10.1095/biolreprod67.1.340)

Gadella BM, Tsai PS, Boerke A & Brewis IA 2008 Sperm head membrane reorganisation during capacitation. *International Journal of Developmental Biology* **52** 473–480. (https://doi.org/10.1387/ijdb.082583bg)

Galantino-Homer HL, Visconti PE & Kopf GS 1997 Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3',5'-monophosphate-dependent pathway. *Biology of Reproduction* **56** 707–719. (https://doi.org/10.1095/biolreprod56.3.707)

Galantino-Homer HL, Florman HM, Storey BT, Dobrinski I & Kopf GS 2004 Bovine sperm capacitation: assessment of phosphodiesterase activity and intracellular alkalinization on capacitation-associated protein tyrosine phosphorylation. *Molecular Reproduction and Development* **67** 487–500. (https://doi.org/10.1002/mrd.20034)

Grasa P, Cebrián-Pérez JA & Muiño-Blanco T 2006 Signal transduction mechanisms involved in ram sperm capacitation. *Reproduction* **132** 721–732. (https://doi.org/10.1530/rep.1.00770)

Grasa P, Perez-Pe R, Baguena O, Forcada F, Abecia A, Cebrian-Perez JA & Muino-Blanco T 2004 Ram sperm selection by a dextran/swim-up procedure increases fertilization rates following intrauterine insemination in superovulated ewes. *Journal of Andrology* **25** 982–990. (https://doi.org/10.1002/j.1939-4640.2004.tb03171.x)

Harrison RAP & Gadella BM 2005 Bicarbonate-induced membrane processing in sperm capacitation. *Theriogenology* **63** 342–351. (https://doi.org/10.1016/j.theriogenology.2004.09.016)

Harrison RAP & Miller NGA 2000 cAMP-dependent protein kinase control of plasma membrane lipid architecture in boar sperm. *Molecular Reproduction and Development* **55** 220–228. (https://doi.org/10.1002/(SICI)1098-2795(200002)55:2<220::AID-MRD12>3.0.CO;2-I)

Harrison RAP, Ashworth PJC & Miller NGA 1996 Bicarbonate/CO2, an effector of capacitation, induces a rapid and reversible change in the lipid architecture of boar sperm plasma membranes. *Molecular Reproduction and Development* **45** 378–391. (https://doi.org/10.1002/(SICI)1098-2795(199611)45:3<378::AID-MRD16>3.0.CO;2-V)

Hess KC, Jones BH, Marquez B, Chen Y, Ord TS, Kamenetsky M, Miyamoto C, Zippin JH, Kopf GS, Suarez SS *et al.* 2005 The 'soluble' adenylyl cyclase in sperm mediates multiple signaling events required for fertilization. *Developmental Cell* **9** 249–259. (https://doi.org/10.1016/j.devcel.2005.06.007)

Hölttä-Vuori M, Uronen RL, Repakova J, Salonen E, Vattulainen I, Panula P, Li Z, Bittman R & Ikonen E 2008 BODIPY-cholesterol: a new tool to visualize sterol trafficking in living cells and organisms. *Traffic* **9** 1839–1849. (https://doi.org/10.1111/j.1600-0854.2008.00801.x)

Kerns K, Zigo M, Drobnis EZ, Sutovsky M & Sutovsky P 2018 Zinc ion flux during mammalian sperm capacitation. *Nature Communications* **9** 2061. (https://doi.org/10.1038/s41467-018-04523-y)

Leahy T & Gadella BM 2015 New insights into the regulation of cholesterol efflux from the sperm membrane. *Asian Journal of Andrology* **17** 561–567. (https://doi.org/10.4103/1008-682X.153309)

Leahy T, Rickard JP, Aitken RJ & de Graaf SP 2016 Penicillamine prevents ram sperm agglutination in media that support capacitation. *Reproduction* **151** 167–177. (https://doi.org/10.1530/REP-15-0413)

Leclerc P, de Lamirande E & Gagnon C 1996 Cyclic adenosine 3',5'monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biology of Reproduction* **55** 684–692. (https://doi.org/10.1095/biolreprod55.3.684)

Leemans B, Stout TAE, De Schauwer C, Heras S, Nelis H, Hoogewijs M, Van Soom A & Gadella BM 2019 Update on mammalian sperm capacitation: how much does the horse differ from other species? *Reproduction* **157** R181–R197. (https://doi.org/10.1530/REP-18-0541)

Lefievre L, de Lamirande E & Gagnon C 2000 The cyclic GMP-specific phosphodiesterase inhibitor, sildenafil, stimulates human sperm motility and capacitation but not acrosome reaction. *Journal of Andrology* **21** 929–937. (https://doi.org/10.1002/j.1939-4640.2000.tb03424.x)

Monks NJ, Fraser LR 1987 Phosphodiesterase activity of mouse sperm incubated under conditions that modulate fertilizing potential in vitro. *Gamete Research* **18** 85–96. (https://doi.org/10.1002/mrd.1120180110)

MyAssays Ltd. 2018 'Four parameter logistic curve' online analysis tool. (available at: http://www.myassays.com/four-parameter-logistic-curve.assay)

Okamura N, Tajima Y, Soejima A, Masuda H & Sugita Y 1985 Sodium bicarbonate in seminal plasma stimulates the motility of mammalian spermatozoa through direct activation of adenylate cyclase. *Journal of Biological Chemistry* **260** 9699–9705.

Osheroff JE, Visconti PE, Valenzuela JP, Travis AJ, Alvarez J & Kopf GS 1999 Regulation of human sperm capacitation by a cholesterol efflux- stimulated signal transduction pathway leading to protein kinase A-mediated up-regulation of protein tyrosine phosphorylation. *Molecular Human Reproduction* **5** 1017–1026. (https://doi.org/10.1093/molehr/5.11.1017)

Osycka-Salut CE, Diez F, Burdet J, Gervasi MG, Franchi A, Bianciotti LG, Davio C & Perez-Martinez S 2014 Cyclic AMP efflux, via MRPs and A I adenosine receptors, is critical for bovine sperm capacitation. *Molecular Human Reproduction* **20** 89–99. (https://doi.org/10.1093/molehr/gat053)

Parrish JJ, Susko-Parrish JL, Winer MA & First NL 1988 Capacitation of bovine sperm by heparin. *Biology of Reproduction* **38** 1171–1180. (https://doi.org/10.1095/biolreprod38.5.1171)

Parrish JJ, Susko-Parrish JL & Graham JK 1999 In vitro capacitation of bovine spermatozoa: role of intracellular calcium. *Theriogenology* **51** 461–472. (https://doi.org/10.1016/S0093-691X(98)00240-4)

Pérez-Pé R, Grasa P, Fernández-Juan M, Peleato ML, Cebrián-Pérez JA & Muiño-Blanco T 2002 Seminal plasma proteins reduce protein tyrosine phosphorylation in the plasma membrane of cold-shocked ram spermatozoa. *Molecular Reproduction and Development* **61** 226–233. (https://doi.org/10.1002/mrd.1152)

Pini T, de Graaf SP, Druart X, Tsikis G, Labas V, Teixeira-Gomes AP, Gadella BM & Leahy T 2018 Binder of sperm proteins 1 and 5 have contrasting effects on the capacitation of ram spermatozoa. *Biology of Reproduction* **98** 765–775. (https://doi.org/10.1093/biolre/ioy032)

Richter W, Dettmer D & Glander HJ 1999 Detection of mRNA transcripts of cyclic nucleotide phosphodiesterase subtypes in ejaculated human spermatozoa. *Molecular Human Reproduction* **5** 732–736. (https://doi.org/10.1093/molehr/5.8.732)

Sankaranarayanan S, Kellner-Weibel G, De La Llera-Moya M, Phillips MC, Asztalos BF, Bittman R & Rothblat GH 2011 A sensitive assay for ABCA1-mediated cholesterol efflux using BODIPY-cholesterol. *Journal of Lipid Research* **52** 2332–2340. (https://doi.org/10.1194/jlr.D018051)

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Shadan S, James PS, Howes EA & Jones R 2004 Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biology of Reproduction* **71** 253–265. (https://doi.org/10.1095/biolreprod.103.026435)

Soriano-Úbeda C, Romero-Aguirregomezcorta J, Matás C, Visconti PE & García-Vázquez FA 2019 Manipulation of bicarbonate concentration in sperm capacitation media improves in vitro fertilisation output in porcine species. *Journal of Animal Science and Biotechnology* **10** 19. (https://doi.org/10.1186/s40104-019-0324-y)

Suarez SS & Dai X 1995 Intracellular calcium reaches different levels of elevation in hyperactivated and acrosome-reacted hamster sperm. *Molecular Reproduction and Development* **42** 325–333. (https://doi.org/10.1002/mrd.1080420310)

Suarez SS, Varosi SM & Dai X 1993 Intracellular calcium increases with hyperactivation in intact, moving hamster sperm and oscillates with the flagellar beat cycle. *PNAS* **90** 4660–4664. (https://doi.org/10.1073/pnas.90.10.4660)

Takeo T, Hoshii T, Kondo Y, Toyodome H, Arima H, Yamamura K-i, Irie T & Nakagata N 2008 Methyl-beta-cyclodextrin improves fertilizing ability of C57BL/6 mouse sperm after freezing and thawing by facilitating cholesterol efflux from the cells. *Biology of Reproduction* **78** 546–551. (https://doi.org/10.1095/biolreprod.107.065359)

Tash JS 1976 Investigations on adenosine 3',5'-monophosphate phosphodiesterase in ram semen and initial characterization of a sperm specific isoenzyme. *Journal of Reproduction and Fertility* **47** 63–72. (https://doi.org/10.1530/jrf.0.0470063)

van Gestel RA, Brewis IA, Ashton PR, Helms JB, Brouwers JFHM & Gadella BM 2005 Capacitation-dependent concentration of lipid rafts in the apical ridge head area of porcine sperm cells. *Molecular Human Reproduction* **11** 583–590. (https://doi.org/10.1093/molehr/gah200)

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P & Kopf GS 1995*a* Capacitation of mouse spermatozoa. 1. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* **121** 1129–1137.

Visconti PE, Moore GD, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P & Kopf GS 1995*b* Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. *Development* **121** 1139–1150.

Visconti PE, Ning X, Fornés MW, Alvarez JG, Stein P, Connors SA & Kopf GS 1999 Cholesterol efflux-mediated signal transduction in mammalian sperm: cholesterol release signals an increase in protein tyrosine phosphorylation during mouse sperm capacitation. *Developmental Biology* **214** 429–443. (https://doi.org/10.1006/dbio.1999.9428)

Xie F, Garcia MA, Carlson AE, Schuh SM, Babcock DF, Jaiswal BS, Gossen JA, Esposito G, van Duin M & Conti M 2006 Soluble adenylyl cyclase (sAC) is indispensable for sperm function and fertilization. *Developmental Biology* **296** 353–362. (https://doi.org/10.1016/j.ydbio.2006.05.038)

Yanagimachi R 1994 Mammalian fertilization. In *The Physiology of Reproduction*, pp. 189–317. Eds E Knobil & JD Neill. New York: Raven Press.

Yanagimachi R 2011 Mammalian sperm acrosome reaction: where does it begin before fertilization? *Biology of Reproduction* **85** 4–5. (https://doi.org/10.1095/biolreprod.111.092601)

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