The Australian saltwater crocodile (Crocodylus porosus) provides evidence that the capacitation of spermatozoa may extend beyond the mammalian lineage

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Although mammalian spermatozoa only acquire functional maturity as they are conveyed through the male (epididymal maturation) and female (capacitation) reproductive tracts, the degree of post-testicular development necessary to achieve fertilization in other vertebrate species remains far less clear. Indeed, despite reports that the epididymis of birds and reptiles is capable of secreting proteins that bind and modify the sperm surface characteristics, it remains unclear whether capacitation is a prerequisite for fertilization in these species. Using the ancient reptilian Australian saltwater crocodile as a model, this study was undertaken to explore whether reptile sperm do undergo capacitation-like changes following ejaculation. Our studies revealed that crocodile spermatozoa experienced a rapid and sustained, cyclic-AMP mediated increase in progressive motility following incubation under conditions optimized for the induction of capacitation in mammalian species such as the mouse and human. This response was coupled with elevated levels of phosphorylation associated with both protein kinase A and tyrosine kinase substrates, the latter of which were predominantly localized within the sperm flagellum. In findings that also accord with mammalian spermatozoa, we confirmed a homologue of outer dense fibre 2 as one of the principal substrates for tyrosine phosphorylation. Overall, our findings support the concept that crocodile spermatozoa do undergo a process that is homologous to capacitation in preparation for fertilization of an ovum.

1. Introduction

The spermatozoa of mammalian species acquire the competence to engage in fertilization during their extra-testicular development within both the male and female reproductive tracts. The latter of these events is termed capacitation and encompasses an intricate suite of biochemical and biophysical changes in the spermatozoon [1–3]. These changes are initiated shortly after ejaculation following alterations in the membrane architecture brought about by exposure to increased concentrations of bicarbonate, and concomitant cholesterol efflux and loss of decapacitation factors [4,5]. They subsequently proceed through the activation of complex signalling cascades that eventually converge on the cAMP-dependent protein kinase A (PKA), a key intermediary kinase in the capacitation cascade reported in eutherian vertebrates [6,7]. PKA, in turn, modulates the activity of a myriad of downstream targets including the reciprocal activation of tyrosine kinase(s) and deactivation of protein phosphatases. Such changes culminate in a global increase in tyrosine phosphorylation across an impressive array of sperm proteins that have been variously linked to oocyte recognition and adhesion, the ability to undergo acrosomal exocytosis, and an altered pattern
of movement referred to as hyperactivation [1]. The ability to mimic capacitation by incubating sperm in an appropriate *in vitro* culture medium has heralded unique insights into this important phase of sperm maturation, and enabled us to begin to explore the evolutionary origin and biological relevance of this process in sub-therian vertebrate species.

In this context, there have been several studies on sperm capacitation in birds, but a process homologous to capacitation in mammals has not been recorded [8,9]. Indeed, early *in vitro* studies indicated that fowl and turkey sperm do not require a period of capacitation in the female tract in order to fertilize an ovum [8,9], and that the acrosome reaction may be rapidly induced after incubation of fowl sperm *in vitro* in the presence of the inner perivitelline membrane of the oocyte and extracellular calcium [10]. These findings accord with our recent work on quail spermatozoa, which found no evidence of a need for capacitation and revealed that up to 90% of testicular sperm could bind to a perivitelline membrane and acrosome react [11]. However, conflicting evidence suggests that PKA may potentially be involved in stimulating the acrosome reaction in fowl spermatozoa [12].

In the case of reptiles, it has been argued that capacitation is also not necessary to permit the transition to functional maturity [13]. Nevertheless, early studies have provided evidence that the non-motile spermatozoa leaving the testes of the lizard *Lacerta vivipara* can be induced to swim progressively upon incubation in the presence of the phosphodiesterase inhibitor, caffeine [14]. These data suggest that sperm motility in this representative species of lizard, as in mammals, can respond favourably to elevation of intracellular cyclic-AMP concentrations. However, whether this response extends to other elements of sperm function and is conserved across reptilian phyla remains to be fully explored. In concert with the development of a sophisticated suite of techniques and functional assays with which to assess capacitation, it is now timely to revisit the premise that capacitation is a phenomenon that is confined to mammals. Thus, this study was designed to investigate whether reptile sperm do undergo capacitation-like changes following ejaculation using the ancient reptilian species of the Australian saltwater crocodile (*Crocodylus porosus*) as a model.

### 2. Material and methods

#### (a) Chemicals and reagents

Unless otherwise specified, chemical reagents were obtained from Sigma (St Louis, MO, USA). Anti-phosphoserine (P5747), phosphothreonine (P6623), phosphotyrosine (P3300) and tubulin (T5168) antibodies were purchased from Sigma. Anti-PKA (4782S) and anti-phospho (Ser/Thr) PKA substrate antibodies (9621) were from Cell Signaling (Danvers, MA, USA). Anti-ODF2 (ab120123) and anti-ADCY10 (ab203204) antibodies were from Abcam (Cambridge, UK). Anti-rabbit IgG-horseradish peroxidase (HRP) was purchased from Merck Millipore (Billerica, MA, USA), and anti-mouse IgG and anti-goat IgG HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All fluorescently labelled (Alexa Fluor) secondary antibodies were from Thermo Fisher Scientific (Cheshire, VIC, Australia). Fluorescein isothiocyanate conjugated *Pismum sativum* agglutinin (PSA) (FL-1051) was from Vector Laboratories (Burlingame, CA, USA). H89 (B1427) and herbimycin A (375 670) were from Sigma and Merck Millipore, respectively.

#### (b) Animals and semen collection

Semen used throughout this study was collected by digital massage [15] from mature (more than 3.0 m) saltwater crocodiles during the breeding season (November 2014, 2015).

#### (c) Semen evaluation

The mass activity of undiluted semen samples was assessed in accordance with the following criteria [16]: 0 = no swim—no oscillation of individual sperm; 1 = very slow swim—generalized oscillation of individual sperm only; 2 = very slow distinct swim; 3 = slow distinct swim; 4 = moderately fast distinct swim; 5 = fast distinct swim. Sperm concentration (>10^11 ml^-1) was estimated using a Makler sperm counting chamber (Makler Inc., Haifa, Israel), the percentage of motile spermatozoa was determined using a phase-contrast microscope equipped with a warm stage (30°C), and the rate of sperm movement was determined using criteria defined by Barth [17]: 0 = no sperm movement; 1 = slight tail undulation without forward motion; 2 = slow tail undulation with slow or stop and start forward motion; 3 = forward progression at a moderate speed; 4 = rapid forward progression; 5 = very rapid progression in which cells are difficult to follow visually. Nigrosin-eosin staining was used to assess sperm vitality [18]. A total of 200 spermatozoa were evaluated in each assay.

#### (d) Capacitation

To assess the response of crocodile spermatozoa to incubation under capacitating conditions, raw semen samples were diluted 1:4 into one of three modified formulations of Biggers, Whitten and Whittingham (BWW) medium [19], namely: (i) non-capacitating BWW media control (NC) (comprising 120 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl_2·2H_2O, 12.2 mM KH_2PO_4, 1.2 mM MgSO_4·7H_2O, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U ml^-1 penicillin, 5 mg ml^-1 streptomycin and 20 mM HEPES buffer and 3 mg ml^-1 BSA (pH 7.4, osmolality of 300 mOsm kg^-1)); (ii) complete BWW (BWW; as above but with the inclusion of 25 mM NaHCO_3); or (iii) capacitating BWW (CAP; an equivalent formulation to that of complete BWW, with additional supplementation of a phosphodiesterase inhibitor (pentoxifylline, 1 mM) and a membrane permeable cAMP analogue (dibutyryl cyclic-AMP, dbcAMP, 1 mM)) designed to elevate intracellular CAMP levels, thereby stimulating PKA and downstream hallmarks of capacitation including tyrosine phosphorylation [20,21]. Following dilution, an aliquot of sperm was immediately assessed for viability, motility characteristics, acrosomal integrity and phosphorylation status. The remainder of the sample was incubated at 30°C for up to 240 min. At hourly intervals throughout this incubation, sperm suspensions were sampled and assessed using the suite of functional assays described below. To substantiate capacitation-like changes in crocodile spermatozoa, a sub-population of these cells were co-incubated with either 10 μM H89 (a PKA inhibitor) or 10 μM herbimycin A (a broad spectrum inhibitor of tyrosine kinase activity).

#### (e) SDS-PAGE, immunoblotting and protein identification

After incubation, crocodile spermatozoa were pelleted (400g, 1 min), washed in NC media and re-centrifuged (400g, 1 min). The sperm pellet was resuspended in SDS extraction buffer (0.375 M Tris pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, protease inhibitor cocktail), incubated at 100°C for 5 min and equivalent amounts of protein (10 μg) were separated by SDS-PAGE [22]. Gels were either stained with silver reagent or transferred onto nitrocellulose membranes (Hybond C-extra;
GE Healthcare) [23]. Membranes were blocked for 1 h in Tris-buffered saline (TBS) containing 5% v/v skimmed milk powder. After rinsing with TBS containing 0.1% v/v Tween-20 (TBST), membranes were sequentially incubated with appropriate primary antibody at 4°C overnight and its corresponding HRP-conjugated secondary antibody for 1 h. Following three washes in TBST, labelled proteins were detected using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

Gel bands of interest were excised, subjected to tryptic digestion, separated by reversed-phase nano-LC (Dionex Ultimate 3000 RSLCnano, Idstein, Germany) and sequenced by tandem mass spectrometry on an electrospray ionization 3D Ion Trap Mass Spectrometer (AmaZon ETD, Bruker Daltonik, Bremen, Germany) [24]. Raw MS/MS files were converted into MASCOT generic format and imported into Bruker’s Proteinscape platform for database searching. Searches were performed using in-house licensed MASCOT server (v. 2.3.02, Matrix Science, London, UK) and sequenced peptides were aligned with those of the Archosauromorpha crown group comprising birds and crocodilians within the UniprotKB database (Archosauromorpha, January 2015, 612,062 sequences) using previously defined search criteria [24].

(f) Immunocytochemistry
Spermatozoa were fixed in 4% paraformaldehyde, washed three times with 0.05 M glycine in PBS and then applied to poly-L-lysine-coated glass coverslips. The cells were permeabilized with 0.2% Triton X-100 and blocked in 3% BSA/PBS for 1 h. Coverslips were then washed in PBS and incubated in a humidified chamber with appropriate primary and secondary antibodies (1 h at 37°C). Following this, coverslips were washed (3 x 5 min) in filtered PBS, counterstained with DAPI, before mounting in antifade reagent comprising 10% Mowiol powder. After rinsing with TBS containing 0.1% v/v Tween-20 (TBST), membranes were sequentially incubated with appropriate primary antibody at 4°C overnight and its corresponding HRP-conjugated secondary antibody for 1 h. Following three washes in TBST, labelled proteins were detected using enhanced chemiluminescence reagents (GE Healthcare, Buckinghamshire, UK).

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(g) Duolink proximity ligation assay

In situ proximity ligation assays (PLA) were conducted according to manufacturer’s instructions (OLINK Biosciences, Uppsala, Sweden; as described by Dun et al. [25]) using anti-ODF2 and anti-phosphotyrosine antibodies. Coverslips were mounted as previously described for immunocytochemistry and visualized by fluorescence microscopy (Carl Zeiss, Sydney, Australia). If target proteins reside within a maximum distance of 40 nm, this reaction results in the production of discrete fluorescent foci that appear as red spots [26]. PLA fluorescence was quantified for 200 cells per slide.

(h) Statistics
All experiments were replicated with material collected from at least five different animals and the graphical data presented as means ± s.e.m. Statistical significance was determined by analysis of variance (ANOVA).

3. Results
(a) Seminal characteristics
The saltwater crocodile semen used in this study displayed similar overall seminal characteristics of volume (1.15 ± 0.3 ml), sperm concentration (2.6 ± 0.3 x 10⁹ ml⁻¹) and mass activity (1.4 ± 0.2) (electronic supplementary material, table S1) to those previously recorded [15]. These spermatozoa were filiform with a slightly curved head (electronic supplementary material, figure S1) and a prominent acrosomal vesicle that stained with Pasadena (PSA), a lectin with specificity towards α-linked mannose-containing oligosaccharides. Extending distally, an elongated cylindrical nucleus terminated at a relatively short mid-piece with microtubules comprising tubulin extending throughout the principal piece. An analysis of the soluble sperm proteome revealed a unique profile that appeared to share few similarities with that of either mouse or human spermatozoa (electronic supplementary material, figure S1).

(b) Assessment of sperm capacitation
(i) Motility
Immediately after collection, all samples displayed excellent motility characteristics, with overall motility of 75–90% and rates of motility scores of between 4 and 4.5 (figure 1). Dilution into each medium initially supported strong motility; however, within the first hour of incubation in BWW a significant decrease was noted in the rate of sperm movement (figure 1). This was not associated with a commensurate decrease in sperm viability, which remained above 80% following their dilution into capacitating BWW. The motility characteristics of these ‘re-activated’ populations were comparable with those of sperm held in capacitating BWW media for the duration of their incubation (data not shown).
To determine whether enhanced motility parameters were associated with activation of capacitation-like signalling cascades, we initially assessed the rate of motility of spermatozoa incubated in capacitation media supplemented with either H89 or herbimycin A. These pharmacological inhibitors of PKA and tyrosine kinase(s) both led to a significant reduction in the rate of motility without any corresponding loss of sperm viability (figure 1). In the view of these data, the levels of sperm serine, threonine and tyrosine phosphorylation achieved under capacitation conditions were assessed (figure 2). These analyses revealed relatively high levels of serine phosphorylation in association with numerous proteins (approx. 15–200 kDa), with the predominant substrates resolving as a doublet of approximately 17–18 kDa and a higher molecular weight band of approximately 60 kDa. However, the majority of these targets were constitutively phosphorylated and their labelling intensity did not change under the incubation conditions employed in this study. Despite a far more restricted profile of threonine-phosphorylated proteins, these also displayed no clear increases during incubation under capacitation conditions. Rather, the predominant phosphothreonine substrate of approximately 90 kDa appeared to undergo a moderate decrease in labelling intensity by 120 min of incubation. In the case of tyrosine, only three predominant protein bands were labelled in freshly isolated cells and these underwent modest increases following incubation under capacitation conditions. However, unlike serine and threonine phosphorylation, we did detect an additional three high molecular weight proteins of approximately 200, 230 and 250 kDa that were only phosphorylated in capacitating media (figure 2). These additional bands were evident at 60 min and remained at similar levels at 120 min (figure 2a).

(ii) Capacitation-associated cell signalling
To determine whether enhanced motility parameters were associated with activation of capacitation-like signalling cascades, we initially assessed the rate of motility of spermatozoa incubated in capacitation media supplemented with either H89 or herbimycin A. These pharmacological inhibitors of PKA and tyrosine kinase(s) both led to a significant reduction in the rate of motility without any corresponding loss of sperm viability (figure 1). In the view of these data, the levels of sperm serine, threonine and tyrosine phosphorylation achieved under capacitation conditions were assessed (figure 2). These analyses revealed relatively high levels of serine phosphorylation in association with numerous proteins (approx. 15–200 kDa), with the predominant substrates resolving as a doublet of approximately 17–18 kDa and a higher molecular weight band of approximately 60 kDa. However, the majority of these targets were constitutively phosphorylated and their labelling intensity did not change under the incubation conditions employed in this study. Despite a far more restricted profile of threonine-phosphorylated proteins, these also displayed no clear increases during incubation under capacitation conditions. Rather, the predominant phosphothreonine substrate of approximately 90 kDa appeared to undergo a moderate decrease in labelling intensity by 120 min of incubation. In the case of tyrosine, only three predominant protein bands were labelled in freshly isolated cells and these underwent modest increases following incubation under capacitation conditions. However, unlike serine and threonine phosphorylation, we did detect an additional three high molecular weight proteins of approximately 200, 230 and 250 kDa that were only phosphorylated in capacitating media (figure 2). These additional bands were evident at 60 min and remained at similar levels at 120 min (figure 2a).
Incubation of spermatozoa with either H89 or herbimycin A led to a marked reduction, but did not eliminate, the labelling of these three phosphotyrosine substrates (figure 2).

Immunofluorescent labelling of phosphoserine and phosphothreonine revealed that their respective substrates were primarily localized within the sperm head in freshly isolated crocodile spermatozoa (figure 2, arrow). In both instances, this staining was accompanied by additional labelling, albeit generally weak, of the flagellum (figure 2c). Consistent with immunoblotting data, we failed to detect notable increases in staining intensity of either serine or threonine phosphorylation. Instead, the latter was clearly reduced within sperm head by 120 min of incubation under capacitation conditions (figure 2c, arrow). The most pronounced changes in phospho-labelling were associated with tyrosine residues, which were initially detected as discrete foci within the periacrosomal region of the sperm head (figure 2c, arrow). Upon incubation in capacitation media, however, intense phosphotyrosine labelling was also detected in the principal piece of the sperm flagellum (figure 2c).

(iii) Identification of phosphotyrosine substrates in crocodile spermatozoa

The detection of tyrosine phosphorylation in crocodile spermatozoa suggests that these cells are capable of responding to elevated levels of intracellular cAMP in a similar manner to that of mammalian spermatozoa. This notion is supported by our demonstration that crocodile spermatozoa harbour a homologue of PKA (figure 3a) that is localized throughout the principal piece of the flagellum (figure 3b). Although the significance of the finding awaits further investigation, we also noted that the labelling intensity of PKA was higher in crocodile spermatozoa than that of human spermatozoa. Additionally, capacitation conditions promoted a selective increase in the phosphorylation of numerous PKA substrates (ranging from approx. 17 to 250 kDa; with

Figure 3. Examination of PKA localization and activity in crocodile spermatozoa. The presence and localization of a PKA homologue was assessed in crocodile spermatozoa by (a) immunoblotting and (b) immunolocalization of fixed and permeabilized cells with anti-PKA antibodies. (c) Sperm proteins targeted for phosphorylation by PKA were assessed immediately after isolation and at regular intervals (60 and 120 min) throughout incubation under conditions optimized to suppress (NC), support (BWW) or actively drive (CAP) capacitation. An additional treatment in which spermatozoa were co-incubated with H89 was also included. (d) Phospho-PKA substrates were also localized in populations of non-capacitated (NC) and capacitated (CAP) crocodile spermatozoa. All experiments were replicated on independent samples from five different crocodiles, and representative immunoblots and immunolabelling patterns are shown. Scale bar, 5 μm.
prominent bands of approx. 35, 40, 42, 65, 90, 110, 200 and 250 kDa; figure 3c). The phosphorylation of these PKA substrates also proved sensitive to H89 inhibition. These data stand in marked contrast to the overall levels of serine and threonine phosphorylation (figure 2a), thus identifying this assay as a more sensitive indication of PKA function. The phospho-PKA substrates were initially weakly detected at the base of the nucleus and extending into the mid-piece. However, strong labelling was noted throughout the head and principal piece of capacitated spermatozoa (figure 3d).

To identify those proteins targeted for tyrosine phosphorylation, protein bands aligning with those that were prominently labelled with anti-phosphotyrosine were excised from duplicate gels and subjected to mass spectrometry analysis (electronic supplementary material, table S2). This strategy identified several putative phosphotyrosine substrates, which collectively fell into four broad categories, comprising those associated with cytoskeleton function (dynein, filamin A and outer dense fibre of sperm tails 2), molecular chaperoning activity (heat shock protein (HSP) 90AA1 and HSPA5), mitochondrial function (aconitase 2) and cellular signalling (phosphatidylethanolamine binding protein 1 and RAB2A, member RAS oncogene family).

To begin to validate these findings, antibodies were used to demonstrate that crocodile spermatozoa do indeed harbour a homologue of the mammalian ODF2 protein. This protein resolved at a slightly higher molecular weight and also appeared to label somewhat less intensely than the human ODF2 homologue (figure 4a). Nevertheless, the ODF2 protein was strongly localized to the mid-piece of the crocodile sperm flagellum (figure 4b). Additional bright fluorescence extended distally into the principal piece before gradually being reduced to punctuate labelling within the lower half of the tail (figure 4b). A proximity ligation assay confirmed that ODF2 was targeted for capacitation-associated phosphorylation. In this regard, only modest PLA labelling was detected in the mid-piece of non-capacitated (NC) spermatozoa (figure 4c). However, in cells incubated under capacitating conditions, this was replaced by intense PLA labelling within the mid-piece and extending through the upper half of the principal piece, coincident with the distribution of the ODF2 protein itself (figure 4c).

4. Discussion

A central tenet of male reproduction is that the spermatozoa of all mammalian species acquire the ability to fertilize an ovum during their post-testicular maturation within the male and female reproductive tracts [27]. The precise molecular mechanisms governing these critical phases of functional maturation remain elusive, as do the reasons for why such a complex developmental strategy may have evolved [28]. This is particularly enigmatic as the balance of evidence suggests that post-testicular maturation is not essential for fertilization in sub-therian vertebrate species such as the birds [8,9]. Similarly, there are only a limited number of studies that have reported functional changes in reptilian spermatozoa after they leave the testes [13,14]. Herein, we have used the Australian saltwater crocodile as a model to analyse functional attributes associated with post-testicular maturation in reptilian species. Our study revealed two key observations. First, incubation of crocodile spermatozoa under conditions that have been optimized to facilitate mammalian sperm capacitation, promotes pronounced changes in sperm velocity and the ability to sustain active motility. Second, we show that these properties were associated with the activation of a cAMP-mediated signalling cascade, incorporating PKA as a key intermediary kinase and culminating in the downstream tyrosine phosphorylation of a small subset of sperm proteins. This work therefore provides the first evidence for capacitation-like changes in reptilian spermatozoa, and challenges the notion that such changes are restricted to mammalian species [13].

Consistent with work on mammalian spermatozoa [6,7], we were able to show that bicarbonate is important, but not in itself sufficient, to accelerate capacitation-like changes in crocodile spermatozoa. At present, we do not know whether bicarbonate influences the activity of adenyl cyclase(s), as has been reported in the spermatozoa of mice and other mammalian species, and/or is based on intracellular alkalinization [29]. However, we have shown that crocodile spermatozoa do harbour a homologue of ADCY10, a unique soluble adenyl cyclase that primarily localizes to the mid-piece (mitochondria) of these cells (electronic supplementary material, figure S2) [30]. Such a finding is of interest as ADCY10 has been implicated as a sensor for bicarbonate, pH and Ca$^{2+}$, and is involved in coupling energy homeostasis...
with cAMP/PKA-dependent signalling events [31], ultimately leading to the phosphorylation of mitochondrial proteins and stimulation of motility [32]. Consistent with such observations, the candidate proteins we identified as substrates for tyrosine phosphorylation broadly group into four main functional categories, namely those implicated in mitochondrial function (ACO2), cellular signalling (PEBP1, RAB2A), molecular chaperones (HSP90AA1, HSPA5), and stabilization, regulation and remodelling of the cytoskeleton (FLNA, Dnah17, ODF2). While the confirmation and significance of such targets remain to be validated, many of these substrates may be homologous to those that are activated during the capacitation of mammalian spermatozoa [33]. For instance, molecular chaperones are targeted for tyrosine phosphorylation during the latter stages of capacitation and subsequently play a key role in surface remodelling events that prime spermatozoa for their interaction with the outer vestments of the ovum [20,25,34]. Conversely, both HSPA5 and HSP90AA1 are among a cohort of molecular chaperones that reside on the human sperm surface [35] and form part of the sensing machinery that modulates intracellular calcium concentrations [36], and hence facilitates sperm capacitation. HSPA5 has also been identified on the extracellular surface of mouse spermatozoa where it associates with proteins of the ADAM (a disintegrin and metalloprotease) family [37], which have been implicated in regulating the ability of spermatozoa to ascend the female reproductive tract [38].

Such findings are of interest as crocodilians can support prolonged periods of sperm storage within the female reproductive tract. Illustrative of this phenomenon, female caiman have been recorded to lay eggs containing a viable embryo greater than 12 months after isolation from a male [39], and multiple paternity has been shown to be a feature of annual egg clutches of both caiman [40] and American alligators [41,42]. These findings accord with the ovulatory strategy of the alligator whereby each ovary ovulates approximately 20 megalecithal eggs into the oviduct in quick succession [43]. Owing to the distention of the oviduct that ensues as the eggs progress through the reproductive tract [44], it is considered likely that any spermatozoa residing within the lumen would be displaced [45]. By contrast, the sequestering of spermatozoa within the caudal terminations of the large glandular areas of the oviduct may help to ensure a reservoir remains to fertilize all eggs within a clutch [45]. As an added advantage, this adaptation could help to ensure paternal diversity in the egg clutch oviposited each year. Indeed, recent evidence has raised the prospect that sperm–oviduct interactions may not only facilitate sperm storage but also play a role in post-copulatory sperm selection [46,47]. For instance, there is mounting evidence that the oviduct can discriminate spermatozoa based on DNA integrity [48].

However, as an important caveat to this notion, studies on alligator spermatozoa suggest that despite their entry into the lumina of the oviductal glands, upon arrival they fail to form intimate contact with the underlying cells [45]. This strategy contrasts with other species in which direct contact between the spermatozoa and oviductal epithelium suppresses sperm function, and hence facilitates their long-term storage [49]. It has thus been suggested that sperm longevity in the female alligator may be a property of the male gamete [45]. This agrees with our data demonstrating that crocodile spermatozoa rapidly lose motility, but not vitality, in the absence of specific stimulatory factors.

Another curiosity of the reproductive strategy adopted by the alligator, and presumably the crocodile, is the extended distance that separates the putative sites of sperm storage from that of the fertilization [45]. In this context, alligator sperm are primarily stored within the junctions of the posterior uterus/vagina and of the tube/isthmus, regions separated by as much as approximately 1–2 m from the infundibulum. This differs from avian species, where sperm reside in storage tubules located in the utero-vaginal junction, a short passage from the site of fertilization [50]. Thus, sperm movement and/or transport in crocodilian species would need to be rapid to facilitate their timely delivery to the predicted site of fertilization within the infundibulum. Such a model aligns well with our ability to stimulate high velocity and sustained sperm motility through elevation of intracellular cAMP, and also with our identification of candidate proteins targeted for tyrosine phosphorylation as a consequence of cAMP signalling. These include Dnah17, part of an evolutionarily conserved family of axonemal motor proteins that act as critical regulators of sperm motility [51]. Such regulation reflects their ability to propagate flagellar bending following activation via a kinase cascade involving both cAMP and downstream tyrosine kinase(s) [52,53]. Similarly, outer dense fibre of sperm tails 2 (ODF2) also serves as an important element of the cytoskeletal structures that surround the axoneme within the mid- and principal piece of the sperm tail [54]. This protein is instrumental in regulating the elasticity and recoil of the sperm tail, with defects leading to abnormal sperm morphology and compromised fertility [55]. As an important proof-of-concept, ODF2 is also recognized as an important target for cAMP-driven phosphorylation [56]. Coupled with activation of proteins involved with capacitation-associated transformation of the sperm surface and the putative enhancement of cellular respiration, such changes may not only enable sperm to navigate the female tract but also ensure they have the capacity to interact with the oocyte upon arrival at the site of fertilization.

At present, the nature of the in vivo cues responsible for stimulating the activity of stored alligator/crocodile spermatozoa and thus promoting their dispersal from an oviductal storage reservoir remains to be determined. On the basis of our data, it is conceivable that the process of ovulation leads to localized increases in bicarbonate that may be responsible for stimulating similar functional changes to those we have documented in vitro. While an important qualification to this hypothesis is that bicarbonate is a naturally abundant component of oviducal fluid, work in the pig has established that the ability of sperm to respond to bicarbonate may be modulated, at least in part, by complex interplay with protein secretions derived from oviduct epithelial cells [57]. Conversely, it has also been argued that progesterone serves as an important stimulus to trigger the controlled release of spermatozoa from the sperm-storage tubules in birds [58]. However, the inability to elicit capacitation-like changes in avian spermatozoa [11] suggests that this response probably acts via cAMP-independent signalling pathways.

5. Conclusion

Overall our findings provide the first evidence that the functional profile of crocodile spermatozoa is able to be influenced by exposure to capacitation stimuli. Furthermore, we have correlated the induction of enhanced motility to the post-translational modification of a relatively small subset of
sperm proteins, many of which are conserved in eutherian mammalian species. We contend that such changes may be due to sperm storage occurring within the female reproductive tract, and thus the need to alternatively silence and activate these genes in order to promote their longevity and fertilization competence, respectively. In addition to providing important mechanistic insights into the evolutionary significance of post-testicular sperm maturation, such findings have practical implications for the development of innovative assisted reproductive strategies for genetic management of commercial farmed crocodiles and for the conservation and propagation of endangered crocodilians, almost half of which are currently listed as vulnerable or endangered by the IUCN Red List [59].

Ethics. The study was undertaken at Koorana Crocodile Farm, Queensland, Australia with the approval of the University of Queensland Animal Ethics Committee (SAS/361/10) and Queensland Government Scientific Purposes Permit (WISP0374911).

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. B.N. conceived the study, participated in the laboratory work and data analysis, and drafted the manuscript; A.L.A. participated in the laboratory work and data analysis; N.D.S. conducted the proteomic analyses; R.M. coordinated access to crocodiles and collection of semen samples; S.D.J. participated in the study design, collection of semen samples, data analysis and drafting of the manuscript. All authors gave final approval for publication.

Competing interests. The authors declare that we have no competing interests.

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References


