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Abstract

Effective activation of sperm motility is fundamental to successful artificial fertilisation; however, studies investigating optimal procedures in amphibians are lacking. This study found the optimal osmolality of activation media for sperm motility activation and evaluated the effect of phosphodiesterase (PDE) inhibitors on sperm activation and longevity in the critically endangered booroolong frog, *Litoria booroolongensis*. To assess the effect of medium osmolality (10, 25, 50, 75, 100 and 200 mOsmol kg⁻¹) and PDE inhibitors (control, 2.5 mM caffeine, 5 mM caffeine, 2.5 mM pentoxifylline, 5 mM pentoxifylline, 2.5 mM theophylline and 5 mM theophylline) on initial activation, percentage sperm motility and sperm velocity were quantified using computer-assisted sperm analysis. To assess the effect of PDE inhibitors (control, 2.5 mM caffeine and 2.5 mM theophylline) on sperm longevity, percentage motility and velocity were assessed hourly until 10 h after activation. High (>60%) percentage motility was achieved in a broad range of activation-medium osmolalities (10-75 mOsmol kg⁻¹). PDE inhibitors did not have an effect on initial sperm motility or velocity, but caffeine and theophylline improved sperm longevity, significantly increasing motility and velocity at 8, 9 and 10 h after activation. Data also show that sperm longevity in *L. booroolongensis* is extreme, with spermatozoa remaining motile more than twice as long as those of any other anuran amphibian.

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**Sperm motility activation in the critically endangered booroolong
frog: the effect of medium osmolality and phosphodiesterase-
inhibitors**

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Running title: sperm motility activation in the Booroolong frog

Abstract

Effective activation of sperm motility is fundamental to successful artificial fertilisation, however studies investigating optimal procedures in amphibians are lacking. This study found the optimal osmolality of activation media for sperm motility activation, and evaluated the effect of phosphodiesterase (PDE)-inhibitors on sperm activation and longevity in the critically endangered booroolong frog, *Litoria booroolongensis*. To assess the effect of medium osmolality (10, 25, 50, 75, 100 & 200 mOsmol kg⁻¹) and PDE -inhibitors (control, 2.5mM caffeine, 5mM caffeine, 2.5mM pentoxifylline, 5mM pentoxifylline, 2.5mM theophylline & 5mM theophylline) on initial activation, sperm motility (%) and sperm velocity (VCL) were quantified using computer assisted sperm analysis (CASA). To assess the effect of PDE-inhibitors (control, 2.5mM caffeine and 2.5mM theophylline) on sperm longevity, percentage motility and velocity were assessed hourly until 10 hours post-activation. High (>60%) percent motility was achieved in a broad range of activation-medium osmolalities (10 - 75 mOsmol kg⁻¹). PDE-inhibitors did not have an effect on initial sperm motility or velocity, but caffeine and theophylline improved sperm longevity, significantly increasing motility and velocity at 8, 9, and 10 hours post-activation. Data also show that sperm longevity in *L. booroolongensis* is extreme, with sperm remaining motile more than twice the duration of any other anuran amphibian.

Keywords: amphibian, antioxidant, artificial fertilisation, assisted reproduction, IVF, sperm activation, reproduction technologies, spermatozoa

Introduction

Reproduction Technologies (RTs) have the potential to bypass the behavioural and physical impediments to natural mating and fertilisation that animals in captivity often encounter (Durrant 2009). Of particular value, are chilled sperm storage and cryopreservation technologies, which facilitate the transfer of genetic material between breeding institutions for use in artificial fertilisation (AF) trials (Kouba *et al.* 2013; Keogh *et al.* 2017). The success of artificial fertilisation trials largely depends on two characteristics of male quality: 1) the maintenance of high sperm viability during storage, and 2) the effective activation of sperm motility. Development of protocols for anuran sperm storage has recently received increased research attention (Kouba *et al.* 2013; Silla *et al.* 2015; Keogh *et al.* 2017), while research identifying optimal procedures for the activation of sperm motility post-storage is notably lacking.

Anuran sperm motility, as in many external fertilisers, is activated by a change in osmolality between the seminal fluid and that of the external fertilisation medium (Browne *et al.* 2015). A sudden decrease in osmotic strength, or hypotonic shock, triggers the activation of flagellar movement in anurans by promoting an increase in intracellular cyclic adenosine monophosphate (cAMP) which in turn promotes the phosphorylation of protein kinase A (PKA) substrates (O'Brien *et al.* 2011). To ensure sperm motility activation, anuran artificial fertilisation trials are typically conducted by diluting sperm suspensions in fertilisation media with osmolalities that do not exceed 50mOsmol kg⁻¹ (Wolf and Hedrick 1971; Edwards *et al.* 2004; Byrne and Silla 2010; Silla 2011). While it is accepted that a low osmolality medium is required to activate sperm motility during artificial fertilisation, few studies have attempted to identify osmolality optima, and those that have demonstrate that optimal osmolalities may be species-specific (Costanzo *et al.* 1998; Kouba *et al.* 2003; Byrne *et al.* 2015).

Previous research dating back to the 1980's, on external fertilising fish, has shown that activation of sperm motility can be further enhanced by manipulating osmolality-induced cAMP-dependent signalling cascades (Cosson 2004). Phosphodiesterases (PDEs) are a family of enzymes that hydrolyse cyclic nucleotides and are consequently involved in the regulation of intracellular levels of cAMP (Boswell-Smith *et al.* 2006). The addition of PDE inhibitors, such as caffeine, theophylline, and pentoxifylline, to sperm activation media has been shown to inhibit PDE activity, increasing levels of cAMP, and resulting in improved sperm motility in a variety of taxa ranging from invertebrates to mammals (Benau and Turner 1980; Kopf *et*

al. 1983; Roth *et al.* 1994; Yunes *et al.* 2005; Fitzsimmons *et al.* 2007). Despite relatively widespread use of PDE inhibitors to improve male fertility in mammals (Roth *et al.* 1994; Yunes *et al.* 2005; Mostafa 2008; Pankaj *et al.* 2009; Stephens *et al.* 2013), only a single study has tested the effect of PDE inhibition on the activation of anuran sperm motility (Fitzsimmons *et al.* 2007). Here, the addition of theophylline to the activation medium was shown to have a positive effect on percentage sperm motility in the Cane Toad, *Rhinella marina* (Fitzsimmons *et al.* 2007).

In addition to their inhibitory effect on PDE, methylxanthines, including caffeine, theophylline, and pentoxifylline, have been recognised for their ability to act as antioxidants, suppressing reactive oxygen species (ROS) formation (Yovich 1993; Devasagayam *et al.* 1996). Sperm spontaneously produce ROS, which is necessary for normal physiological sperm function, however oxidative stress ensues when ROS production exceeds the limited endogenous antioxidant defences that sperm possess (Guthrie and Welch 2012). Oxidative stress results in peroxidative damage to sperm lipids, membranes and DNA, reducing sperm viability and fertilisation capacity (Guthrie and Welch 2012; Dias *et al.* 2014). Addition of pentoxifylline to the activation medium has been previously shown to suppress ROS formation and improve sperm motility in humans (Yovich 1993; Safarinejad 2011). The combined action of phosphodiesterase inhibition and ROS scavenging may similarly improve percentage sperm motility activation and longevity in a variety of amphibian species and warrants investigation.

The aim of this study was to identify optimal conditions for sperm motility activation in the critically endangered booroolong frog, *Litoria booroolongensis*. Our specific objectives were to evaluate: 1) the effect of medium osmolality on the activation of sperm motility, 2) the effect of PDE-inhibitors (caffeine, theophylline, and pentoxifylline) on the activation of sperm motility, and 3) the effect of PDE-inhibitors (caffeine and theophylline) on sperm longevity.

Materials and methods

All procedures described herein were approved by the University of Wollongong's Animal Ethics Committee (protocols AE11/23 and AE12/17). Please note that frogs were not specifically euthanized for the purpose of the present study, but freshly available testis tissue was used post-mortem.

Animals

Frogs were bred and reared in captivity at Taronga Zoo (Sydney, NSW, Australia). A total of 32 males were transported to the Ecological Research Centre at the University of Wollongong (Wollongong, NSW, Australia), where they were held until the commencement of this experiment. Frogs were housed in ventilated plastic terrariums (27 x 17 x 16.5 cm, L x W x H; Mayvic, Lansvale, NSW, Australia), two frogs per terrarium. Enclosures contained a layer of aquarium gravel and sterilised Eucalypt leaves for shelter. Animals had constant access to approximately 200mL reverse osmosis water and were fed 10-day-old crickets once a week. Frogs were maintained in an artificially illuminated constant temperature room set to 22°C with a 13 h/11 h light/dark cycle that included a 30 min twilight (dim lighting) phase at both dawn and dusk. These conditions were chosen to reflect natural field conditions during late austral spring. Prior to the initiation of experiments, frogs were determined to be in breeding condition by the darkening of their nuptial pads and initiation of calling behaviour. All frogs were 1 - 2 years of age at the time of the study and ranged in mass from 1.44 – 7.11 grams (mean $\pm$ sem = 3.62 ± 0.29 g).

Preparation of sperm suspensions

Sperm suspensions were generated via testes extraction and maceration. Male frogs (n=32) were killed via pithing (brain destruction), after which both testes were removed and placed in a 1.5mL eppendorf tube. The testes of each individual were then thoroughly macerated in 200- 300 μ L chilled 1:1 simplified amphibian ringer (SAR; composition: NaCl 113mM; CaCl₂ 1mM; KCl 2mM; NaHCO₃ 3.6mM; ~200mOsmol kg⁻¹). Males with a body mass of < 3g had both testes macerated in 200 μ L SAR, while testes removed from males weighing > 3g were macerated in 300 μ L SAR. For each suspension, sperm concentration was quantified using an Improved Neubauer Haemocytometer (Bright Line, Optik Labor, Germany). A homogenised 2 μ L subsample of sperm suspension was diluted in 18 μ L SAR (1:10 dilution), homogenised and pipetted into the haemocytometer chamber. The number of spermatozoa present in five quadrats was recorded and used to calculate total sperm concentration within the suspension. The dilution and counting protocols were repeated twice per suspension, and sperm counts averaged. Sperm suspensions (n=32) ranged in concentration from 1.50 – 15.18 x 10⁷ sperm L⁻¹.

Standard protocols for conducting amphibian artificial fertilisation (AF), typically involve preparing and storing sperm suspensions prior to obtaining fresh oocytes (Silla 2011; 2013; Silla *et al.* 2015). Sperm suspensions are stored in a high osmolality solution ($\geq 200\text{mOsmol kg}^{-1}$) at low temperature ($0-5^{\circ}\text{C}$) until motility is activated in a fertilisation medium (Browne *et al.* 2001; Silla *et al.* 2015). The present study aimed to replicate these standard protocols and all sperm suspensions were therefore refrigerated at 5°C for 20-21 hours prior to the commencement activation of experiments. Experiments were conducted from November 9, 2012 to January 21, 2013.

Experiment 1: Effect of medium osmolality on the activation of sperm motility

To determine the effect of medium osmolality on sperm motility activation, a split-sample experimental design was adopted. This approach involved activating aliquots of sperm suspension from each individual male ($n=10$) across all experimental treatments. In total, six activation media were applied: 10, 25, 50, 75, 100, and $200 \pm 2\text{mOsmol kg}^{-1}$. Activation media were generated by diluting SAR in distilled water. The osmolality of each medium was confirmed using a freezing-point depression osmometer (Osmomat[®]030, Gonotec, Germany). To activate sperm motility, each sperm suspension was homogenised, and six discrete $2\mu\text{l}$ subsamples were removed and diluted in $18\mu\text{l}$ of each of the six chilled activation media (1:10 dilution). The order in which each activation treatment was applied was randomized for each sperm suspension using a random number generator. Percentage sperm motility and sperm velocity (curvilinear velocity; VCL) were assessed using a computer assisted sperm analysis (CASA) system (CEROS version 12; Hamilton Thorne, Beverley, MA). Percentage sperm motility and VCL were recorded five-minutes post-activation, including a two minute settling period, where the suspension was pipetted into a haemocytometer chamber (exact depth 0.1mm) and placed on the microscope stage to allow fluid to settle prior to analysis. A fluid settlement period was necessary to ensure accurate sperm motility readings that were unaltered by fluid dynamics. All sperm motility assessments were conducted in quadruplicate and averaged. Sperm suspensions were activated and motility parameters assessed, in a constant temperature room set to 22°C (mean $\pm$ sem= 21.21 ± 0.12 , range= $21.1 - 21.8^{\circ}\text{C}$).

Experiment 2: Effect of phosphodiesterase inhibition on the activation of sperm motility

The effect of PDE inhibition on sperm motility activation was determined by activating sperm suspensions in media containing one of three PDE inhibitors (caffeine [C0750; Sigma-Aldrich, Castle Hill, NSW, Australia], pentoxifylline [P1784; Sigma-Aldrich], and theophylline [T1633; Sigma-Aldrich]), at two concentrations (2.5mM and 5mM). Aliquots of sperm suspension from each replicate male (n=10) were activated across all experimental treatments (split-sample experimental design). PDE activation media were generated by diluting each PDE inhibitor in a 1:16 dilution of SAR ($25 \pm 2\text{mOsmol kg}^{-1}$) to the appropriate concentration. A control treatment consisted of sperm activated in pure 1:16 SAR. To activate sperm motility, each sperm suspension was homogenised and seven discrete 2 μ l subsamples were removed and diluted in 18 μ l of each of the seven PDE activation media (2.5mM caffeine, 5mM caffeine, 2.5mM pentoxifylline, 5mM pentoxifylline, 2.5mM theophylline, 5mM theophylline, or control). The order in which each PDE activation treatment was applied was randomized. Percentage sperm motility and VCL were recorded five-minutes post-activation according to the methods described in 'Experiment 1' above. All sperm suspensions were activated, and motility assessments conducted, in a constant temperature room set to 22°C (mean $\pm$ sem= 21.74 ± 0.12 , range= 21.0 -22.3 °C).

Experiment 3: Effect of phosphodiesterase inhibition on sperm longevity

To determine the effect of PDE inhibition on the longevity of sperm motility activation, three treatments were applied (control, caffeine, and theophylline) and sperm motility measured up to 10h post-activation. PDE activation media were generated by diluting each PDE inhibitor (caffeine and theophylline) in a 1:16 dilution of SAR ($25 \pm 2\text{mOsmol kg}^{-1}$) to a final concentration of 2.5mM. A control treatment consisted of sperm activated in pure 1:16 SAR. Aliquots of sperm suspension from each replicate male (n=12) were activated across all experimental treatments (split-sample experimental design). Sperm motility was activated by homogenising each sperm suspension, removing three discrete 2 μ l subsamples, and diluting each subsample in 18 μ l of each of the three PDE activation media. Activated subsamples were pipetted into independent semen analysis slide chambers (exact depth 0.02mm; 4-cell chambered, Leja, Nieuw- Venne, The Netherlands). Percentage sperm motility and VCL were recorded five-minutes post-activation (0h) and every hour thereafter until 10hrs post-activation. Chambered semen analysis slides were covered with laboratory film wax (Parafilm®, Chicago, IL, USA) between each motility analysis time interval in order to prevent any evaporation of the activation fluid medium. Sperm motility assessments were

conducted in quadruplicate and averaged. All sperm suspensions were activated, and motility assessments conducted, in a constant temperature room set to 22°C (mean $\pm$ sem= 20.4 $\pm$ 0.14, range= 19.4 -21.6 °C).

Computer assisted sperm analysis (CASA)

The CASA system used throughout experiments 1-3 inclusive, was set to detect a cell size of 7 pixels (minimum cell size 4 pixels), minimum cell detection contrast of 30, static cell size of 10 pixels, and a static cell intensity of 80. VAP cut-off was 5 m/s and slow moving spermatozoa (those with VAP < 5 m/s) were considered motile and contributed to percent motility measures.

Statistical analyses

In order to test the effect of osmolality and PDE inhibition on sperm motility activation, one-way ANOVA models were run. For each model, the dependent variable was either percent sperm motility or velocity (VCL) and the independent variable was treatment. Comparisons among treatment means were conducted using Tukey-Kramer Honestly Significant Difference (HSD) *post hoc* tests. Prior to analyses, all percent motility data was arcsine transformed using the transformation $\sin^{-1}(\sqrt{x})$. Brown-Forsythe tests were used to verify homogeneity of variances. To test the effect of PDE inhibition on the longevity of sperm motility activation two repeated measures MANOVAs were performed. Within each model, treatment and sampling time (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h) were fixed factors, and the response variable was either percent motility (arcsine transformed) or VCL. Prior to analysis, assumptions of sphericity for within subject effects were tested using the Mauchly criterion. A Greenhouse-Geisser correction for sphericity was applied. Following the MANOVA model, the effect of treatment at each sampling time (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 h) was examined using separate one-way ANOVAs and Tukey-Kramer HSD *post hoc* tests. All statistical analyses were performed using JMP® pro 11.0 software package (SAS Institute Inc. North Carolina, USA). Significance was accepted at $p < 0.05$ for all statistical analyses.

Results

Experiment 1: Effect of medium osmolality on the activation of sperm motility

The osmolality of the activation medium had a significant effect on both percent sperm motility (one-way ANOVA, $F_{5,54} = 12.689$, $p < 0.0001$; Fig. 1a) and sperm velocity (one-way

ANOVA, $F_{5,54} = 5.998$, $p = 0.0002$; Fig. 1b). The percentage of motile sperm was significantly lower (35.9%) at 200 mOsmol kg^{-1} , than all other osmolality treatments (Fig. 1a). Activation media with osmolalities ≤ 50 mOsmol kg^{-1} exhibited percent motilities of greater than 70% (Fig. 1a), with the highest percent motility (73.61%) observed at an osmolality of 25 mOsmol kg^{-1} . Sperm velocities were consistently greater than $22\mu\text{m s}^{-1}$ in all osmolality treatments, with the exception of 200 mOsmol kg^{-1} , which exhibited a significantly lower mean velocity ($16.58\mu\text{m s}^{-1}$; Fig. 1b).

Experiment 2: Effect of phosphodiesterase inhibition on the activation of sperm motility

Percent sperm motility did not differ significantly among PDE activation treatments (one-way ANOVA, $F_{6,63} = 0.813$, $p = 0.564$; Fig. 2a). Overall, mean sperm motilities ranged from 59.26 to 68.51%. Sperm velocity was lowest in the control treatment ($25.69\mu\text{m s}^{-1}$) and highest in activation media containing 2.5mM theophylline ($28.14\mu\text{m s}^{-1}$), though differences among PDE activation treatments were not statistically significant (one-way ANOVA, $F_{6,63} = 1.106$, $p = 0.369$; Fig. 2b).

Experiment 3: Effect of phosphodiesterase inhibition on sperm longevity

Percent sperm motility declined over the 10-hour activation period in all experimental treatments (MANOVA; Greenhouse-Geisser corrected $F = 208.85$, $p < 0.0001$; Fig. 3a), with initial mean motilities of greater than 76% falling to below 35% by the 10th hour. PDE treatment had a significant effect on average sperm motility at 8 hours (one-way ANOVA, $F_{2,33} = 9.42$, $p = 0.0006$), 9 hours (one-way ANOVA, $F_{2,33} = 22.13$, $p < 0.0001$), and 10 hours (one-way ANOVA, $F_{2,33} = 26.77$, $p < 0.0001$) post activation (Fig. 3a). At each of these three time periods (8, 9, and 10h), the control treatment showed significantly lower percent sperm motility than samples treated with either caffeine or theophylline (Tukey-Kramer HSD, $p > 0.05$; Fig. 3a). Sperm motility was statistically similar among PDE treatments from hours 0- 7 post-activation (one-way ANOVAs, $p > 0.05$; Fig. 3a).

Sperm velocity declined over the 10-hour activation period in all experimental treatments (MANOVA; Greenhouse-Geisser corrected $F = 83.77$, $p < 0.0001$; Fig. 3b). As with percent motility, sperm velocity was statistically similar among PDE treatments between 0-7 hours post-activation (one-way ANOVAs, $p > 0.05$; Fig. 3b). PDE treatment had a significant effect on average sperm velocity at 8 hours (one-way ANOVA, $F_{2,33} = 5.01$, $p =$

0.013), 9 hours (one-way ANOVA, $F_{2,33} = 4.86$, $p = 0.014$), and 10 hours (one-way ANOVA, $F_{2,33} = 7.12$, $p = 0.003$) post activation (Fig. 3b). At each of these three time periods (8, 9, and 10h), the control treatment showed significantly lower sperm velocity than activation media containing either caffeine or theophyllene (Tukey-Kramer HSD, $p > 0.05$; Fig. 3b).

Discussion

Identifying properties of the activation medium required for optimal recovery of anuran sperm motility post-storage is fundamental to achieving successful artificial fertilisations. Despite this, research on the topic has been limited. The present study identifies activation-medium osmolality optima and shows that PDE-inhibitors enhance sperm longevity in the booroolong frog.

While it is generally accepted that a low osmolality medium is required to activate anuran sperm motility (Browne *et al.* 2015), results from the present study show that sperm from the booroolong frog achieves high (>60%) percent motility in a broad range of activation-medium osmolalities (10 - 75 mOsmol kg⁻¹). Successful sperm activation in response to a broad range of activation-medium osmolalities has similarly been reported in the common eastern froglet, *Crinia signifera* (>30% sperm motility in media ranging from 10- 75 mOsmol kg⁻¹, Byrne *et al.* 2015) and the African clawed frog, *Xenopus laevis* (>30% motility in media ranging from 37- 75 mOsmol L⁻¹, Christensen 2002). These results are in contrast to those of Kouba *et al.* (2003), who reported a rapid decline in percent motility in activation media with osmolalities ≥ 20 mOsmol L⁻¹ (solutions of 10 - 50 mM NaCl) in the American toad, *Anaxyrus americanus*. Of note, all studies to date have tested the effect of medium-osmolality on anuran sperm activation by employing saline solutions at various dilutions. It is unclear from this approach whether differences in sperm activation are a consequence of overall osmolality of the activation medium, or differences in the concentration of specific cations in solution. Regardless of the mechanism, data suggest that there is species-specificity in the activation response of anuran spermatozoa. It has previously been suggested that osmolality optima and osmotic tolerance of anuran sperm may reflect the level of osmotic stochasticity of the fertilisation environment experienced by a species (Byrne *et al.* 2015). Given that the majority of anuran species have a long evolutionary history of breeding in osmotically dynamic waterbodies, selection may have favoured broad osmotic activation tolerance in a greater number of anuran species than is currently realised.

Results from the present study show that the addition of PDE-inhibitors to the activation medium did not have an effect on initial percent sperm motility or velocity, but that Caffeine and Theophylline improved sperm longevity, increasing percent motility and velocity at 8, 9, and 10 hours post activation. PDE-inhibition has been shown to improve flagellar motility by increasing intracellular cAMP content, however previous studies report an immediate effect on sperm motility that declines with increasing time post-activation (Morisawa and Ishida 1987; Calogero *et al.* 1998). One explanation for the positive effect of Caffeine and Theophylline being delayed, is that sperm longevity was enhanced, not as an outcome of PDE-inhibition, but as a result of the antioxidant properties of these additives. The rate of intracellular damage to sperm, and concomitant decrease in overall percent sperm motility of the sperm suspension, would be expected to increase over time as the production of ROS exceeds sperm's endogenous antioxidant capacity (Dias *et al.* 2014). As a consequence, the effects of exogenous antioxidants may only become apparent once ROS production surpasses a threshold and disrupts intracellular redox balance. Indeed, previous research on brown trout (*Salmo trutta*) revealed a 50% increase in ROS concentrations in both spermatozoa and seminal plasma following a 48 hour incubation period, which was significantly reduced in the presence of the antioxidant uric acid (Lahnsteiner *et al.* 2010). It is unclear whether the improvements to sperm motility characteristics observed in the present study were an outcome of the antioxidant properties of Caffeine and Theophylline. Further research would benefit from the use of luminal chemiluminescence assays which allow the direct measurement intracellular and extracellular ROS generation by spermatozoa (Kashou *et al.* 2013).

Activation of sperm motility occurs within seconds in externally fertilising fish and amphibians and is followed by a rapid to moderate decline in velocity and percent motility (Cosson 2004; Browne *et al.* 2015). To date, sperm longevity has been reported to be shortest in freshwater fish (mean = 2.5 mins, n = 54 species), followed by marine fish (mean = 9.2 mins, n = 13 species), and finally anurans (mean = 68.3 mins, n = 21 species)(reviewed by Browne *et al.* 2015). Of the 21 anuran species previously investigated, sperm longevity ranged from 2.8 mins in the chinese giant salamander (*Andrias davidianus*) to a maximum duration of 4.5 hours in the broad palmed frog (*Litoria latopalmata*)(Browne *et al.* 2015). Data from the present study show that 10 % of sperm from the booroolong frog remain motile at 10 hours post-activation, representing unprecedented sperm endurance that more than doubles the maximum sperm activation duration previously recorded in an anuran. It is unclear why extreme sperm longevity may have evolved in *L.booroolongensis*, but

evolutionary theory suggests that it may be linked to sperm competition risk (Snook 2005), ovum diameter or water turbulence (Stockley *et al.* 1996). Given that little is known about the adaptive benefit of increased sperm longevity in anurans, and external fertilisers in general, this is an area that requires research attention.

Conclusions

Overall, this study shows that sperm from the booroolong frog are activated in a broad-range of activation medium osmolalities and that PDE-inhibitors improved sperm longevity, increasing percent motility and velocity at 8, 9, and 10 hours post activation. We postulate that the stimulatory effect of PDE-inhibitors may be a result of their antioxidant properties, and this warrants further investigation. We also show that sperm longevity in *L. booroolongensis* is extreme compared to what has been previously reported in other anurans.

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Competing interests

The authors declare that they have no competing interests

Authors' contributions

AS conceived the study. AS and PB designed the experiments. AS and LK collected the data. AS, LK and PB performed the statistical analyses. AS wrote the manuscript. All authors read and approved the final manuscript.

References

- 405 Benau, D., and Turner, C. (1980) Initiation, prolongation, and reactivation of the motility of salmonid
406 spermatozoa. *Gamete Research* **3**(3), 247-257
- 407
- 408 Boswell-Smith, V., Spina, D., and Page, C.P. (2006) Phosphodiesterase inhibitors. *British Journal of*
409 *Pharmacology* **147**(S1), S252-S257
- 410
- 411 Browne, R., Clulow, J., and Mahony, M. (2001) Short-term storage of cane toad (*Bufo marinus*)
412 gametes. *Reproduction* **121**(1), 167-173
- 413
- 414 Browne, R., Kaurova, S., Uteshev, V., Shishova, N., McGinnity, D., Figiel, C., Mansour, N., Agnew, D.,
415 Wu, M., and Gakhova, E. (2015) Sperm motility of externally fertilizing fish and amphibians.
416 *Theriogenology* **83**(1), 1-13. e8
- 417
- 418 Byrne, P.G., Dunne, C., Munn, A.J., and Silla, A.J. (2015) Environmental osmolality influences sperm
419 motility activation in an anuran amphibian. *Journal of Evolutionary Biology* **28**(3), 521-534
- 420
- 421 Byrne, P.G., and Silla, A.J. (2010) Hormonal induction of gamete release, and *in-vitro* fertilisation, in
422 the critically endangered Southern Corroboree Frog, *Pseudophryne corroboree*. *Reproductive Biology*
423 *and Endocrinology* **8**:144
- 424
- 425 Calogero, A.E., Fishel, S., Hall, J., Ferrara, E., Vicari, E., Green, S., Hunter, A., Burrello, N., Thornton, S.,
426 and D'Agata, R. (1998) Correlation between intracellular cAMP content, kinematic parameters and
427 hyperactivation of human spermatozoa after incubation with pentoxifylline. *Human reproduction*
428 **13**(4), 911-915
- 429
- 430 Christensen, J.R. (2002) The Effects of Environmental Contaminants on Metamorphosis in *Rana*
431 *Catesbeiana* and Sperm Motility in *Xenopus Laevis*. *Retrospective Theses and Dissertations, 1919-*
432 *2007* doi:<http://dx.doi.org/10.14288/1.0075091>
- 433
- 434 Cosson, J. (2004) The ionic and osmotic factors controlling motility of fish spermatozoa. *Aquaculture*
435 *International* **12**(1), 69-85
- 436
- 437 Costanzo, J.P., Mugnano, J.A., Wehrheim, H.M., and Lee, R.E. (1998) Osmotic and freezing tolerance
438 in spermatozoa of freeze-tolerant and-intolerant frogs. *American Journal of Physiology-Regulatory,*
439 *Integrative and Comparative Physiology* **275**(3), R713-R719
- 440
- 441 Devasagayam, T.P.A., Kamat, J.P., Mohan, H., and Kesavan, P.C. (1996) Caffeine as an antioxidant:
442 inhibition of lipid peroxidation induced by reactive oxygen species. *Biochimica et Biophysica Acta*
443 *(BBA) - Biomembranes* **1282**(1), 63-70
- 444
- 445 Dias, T.n.R., Alves, M.G., Tomás, G.a.D., Socorro, S., Silva, B.M., and Oliveira, P.F. (2014) White tea as
446 a promising antioxidant medium additive for sperm storage at room temperature: a comparative
447 study with green tea. *Journal of agricultural and food chemistry* **62**(3), 608-617

- Durrant, B.S. (2009) The importance and potential of artificial insemination in CANDES (companion animals, non-domestic, endangered species). *Theriogenology* **71**(1), 113-122
- Edwards, D.L., Mahony, M.J., and Clulow, J. (2004) Effect of sperm concentration, medium osmolality and oocyte storage on artificial fertilisation success in a myobatrachid frog (*Limnodynastes tasmaniensis*). *Reproduction, fertility and development* **16**, 347-354
- Fitzsimmons, C., McLaughlin, E., Mahony, M., and Clulow, J. (2007) Optimisation of handling, activation and assessment procedures for *Bufo marinus* spermatozoa. *Reproduction, Fertility and Development* **19**(4), 594-601
- Guthrie, H., and Welch, G. (2012) Effects of reactive oxygen species on sperm function. *Theriogenology* **78**(8), 1700-1708
- Kashou, A.H., Sharma, R., and Agarwal, A. (2013) Assessment of Oxidative Stress in Sperm and Semen. In 'Spermatogenesis: Methods and Protocols.' (Eds. TD Carrell and IK Aston) pp. 351-361. (Humana Press: Totowa, NJ)
- Keogh, L.M., Byrne, P.G., and Silla, A.J. (2017) The effect of gentamicin on sperm motility and bacterial abundance during chilled sperm storage in the Booroolong frog. *General and Comparative Endocrinology* **243**, 51-59
- Kopf, G.S., Lewis, C.A., and Vacquier, V.D. (1983) Regulation of abalone sperm cyclic AMP concentrations and the acrosome reaction by calcium and methylxanthines. *Developmental biology* **98**(1), 28-36
- Kouba, A.J., Lloyd, R.E., Houck, M.L., Silla, A.J., Calatayud, N., Trudeau, V.L., Clulow, J., Molinia, F., Langhorne, C., and Vance, C. (2013) Emerging trends for biobanking amphibian genetic resources: The hope, reality and challenges for the next decade. *Biological Conservation* **164**, 10-21
- Kouba, A.J., Vance, C.K., Frommeyer, M.A., and Roth, T.L. (2003) Structural and functional aspects of *Bufo americanus* spermatozoa: effects of inactivation and reactivation. *Journal of Experimental Zoology-Part B: Molecular and Developmental Evolution* **295**(2), 172-182
- Lahnsteiner, F., Mansour, N., and Plaetzer, K. (2010) Antioxidant systems of brown trout (*Salmo trutta* f. *fario*) semen. *Animal reproduction science* **119**(3), 314-321
- Morisawa, M., and Ishida, K. (1987) Short-term changes in levels of cyclic AMP, adenylate cyclase, and phosphodiesterase during the initiation of sperm motility in rainbow trout. *Journal of Experimental Zoology* **242**(2), 199-204

- 490 Mostafa, T. (2008) Oral phosphodiesterase-5 inhibitors and sperm functions. *International Journal of*
491 *Impotence Research* **20**(6), 530-536
- 492
- 493 O'Brien, E.D., Krapf, D., Cabada, M.O., Visconti, P.E., and Arranz, S.E. (2011) Transmembrane
494 adenylyl cyclase regulates amphibian sperm motility through protein kinase A activation.
495 *Developmental biology* **350**(1), 80-88
- 496
- 497 Pankaj, P., Raina, V., Roy, B., Mohanty, T., and Mishra, A. (2009) Effect of antioxidant preservative on
498 cold protection ability of low grade riverine buffalo (*Bubalus bubalis*) bull spermatozoa. *Asian-*
499 *Australasian Journal of Animal Sciences* **22**(5), 626-635
- 500
- 501 Roth, T., Howard, J., Donoghue, A., Swanson, W., and Wildt, D. (1994) Function and culture
502 requirements of snow leopard (*Panthera uncia*) spermatozoa in vitro. *Journal of reproduction and*
503 *fertility* **101**(3), 563-569
- 504
- 505 Safarinejad, M.R. (2011) Effect of pentoxifylline on semen parameters, reproductive hormones, and
506 seminal plasma antioxidant capacity in men with idiopathic infertility: a randomized double-blind
507 placebo-controlled study. *International Urology and Nephrology* **43**(2), 315-328
- 508
- 509 Silla, A.J. (2011) Effect of priming injections of luteinizing hormone-releasing hormone on
510 spermiation and ovulation in Günther's Toadlet, *Pseudophryne guentheri*. *Reproductive Biology and*
511 *Endocrinology* **9**:68(1)
- 512
- 513 Silla, A.J. (2013) Artificial fertilisation in a terrestrial toadlet (*Pseudophryne guentheri*): effect of
514 medium osmolality, sperm concentration and gamete storage. *Reproduction, Fertility and*
515 *Development* **25**(8), 1134-1141
- 516
- 517 Silla, A.J., Keogh, L.M., and Byrne, P.G. (2015) Antibiotics and oxygen availability affect the short-
518 term storage of spermatozoa from the critically endangered booroolong frog, *Litoria*
519 *booroolongensis*. *Reproduction, Fertility and Development* **27**(8), 1147-1153
- 520
- 521 Snook, R.R. (2005) Sperm in competition: not playing by the numbers. *Trends in ecology & evolution*
522 **20**(1), 46-53
- 523
- 524 Stephens, T.D., Brooks, R.M., Carrington, J.L., Cheng, L., Carrington, A.C., Porr, C.A., and Splan, R.K.
525 (2013) Effects of Pentoxifylline, Caffeine, and Taurine on Post-Thaw Motility and Longevity of Equine
526 Frozen Semen. *Journal of Equine Veterinary Science* **33**(8), 615-621
- 527
- 528 Stockley, P., Gage, M.J.G., Parker, G.A., and Moller, A.P. (1996) Female Reproductive Biology and the
529 Coevolution of Ejaculate Characteristics in Fish. *Proceedings of the Royal Society of London. Series B:*
530 *Biological Sciences* **263**(1369), 451-458
- 531

532 Wolf, D.P., and Hedrick, J.L. (1971) A molecular approach to fertilization 11. Viability and artificial
533 fertilization of *Xenopus laevis* gametes. *Development and Biology* **25**, 360-376

534
535 Yovich, J.L. (1993) Pentoxifylline: actions and applications in assisted reproduction. *Human*
536 *reproduction* **8**(11), 1786-1791

537
538 Yunes, R., Fernández, P., Doncel, G.F., and Acosta, A.A. (2005) Cyclic nucleotide phosphodiesterase
539 inhibition increases tyrosine phosphorylation and hyper motility in normal and pathological human
540 spermatozoa. *Biocell* **29**(3), 287-293

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Figure legends

Fig. 1: Effect of activation medium osmolality on a) percentage sperm motility and b) sperm velocity (VCL). Data shown are untransformed mean $\pm$ s.e.m. (n=10). Letters displayed are the result of Tukey–Kramer HSD post hoc tests on $\sin^{-1}(\sqrt{x})$ transformed percent motility and untransformed velocity data. Treatments that share a letter are not significantly different ($P>0.05$).

Fig. 2: Effect of phosphodiesterase inhibitors on a) percentage sperm motility and b) sperm velocity (VCL). Data shown are untransformed mean $\pm$ s.e.m. (n=10). Letters displayed are the result of Tukey–Kramer HSD post hoc tests on $\sin^{-1}(\sqrt{x})$ transformed percent motility and untransformed velocity data. Treatments that share a letter are not significantly different ($P>0.05$).

Fig. 3: Effect of phosphodiesterase inhibitors on the duration of sperm motility activation. Shown are: a) percentage sperm motility and b) sperm velocity (VCL). * denotes time periods where treatments are significantly different ($P>0.05$).

Figure 1

Figure 2

Figure 3