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Research paper

The effect of gentamicin on sperm motility and bacterial abundance during chilled sperm storage in the Booroolong frog

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ABSTRACT

Antibiotics can inhibit bacterial contamination and extend sperm longevity during storage; a primary goal of captive facilities conducting biobanking and artificial fertilisation (AF). This study evaluated the effects of gentamicin on the short-term storage of Booroolong frog sperm. Sperm suspensions were obtained via either testis maceration, or as spermic urine, following hormonal induction of sperm-release. The effect of 0, 1, 2, 3 or 4 mg mL^{-1} gentamicin on bacterial abundance (CFU mL^{-1}) was determined and sperm motility assessed. In both testis macerate samples and spermic urine samples, gentamicin administered at intermediate-to-high doses (2, 3 & 4 mg mL^{-1}) eliminated, or significantly reduced, bacterial abundance. Sperm samples obtained via testis maceration exhibited significantly lower sperm motility at the highest doses (3 & 4 mg mL^{-1}). All remaining treatments (0, 1 & 2 mg mL^{-1}) were statistically similar and maintained sperm motility >55%. Sperm samples obtained as spermic urine exhibited no difference in sperm motility or velocity when treated with gentamicin at any dose. While antibiotic treatment did not improve sperm longevity as predicted, this is the first study to demonstrate that antibiotic treatment can reduce bacterial abundance without compromising sperm motility in an anuran amphibian. Antibiotic supplementation may be an important tool for reducing pathogen transmission where sperm samples are transferred between captive institutions for biobanking and AF.

1. Introduction

Artificial fertilisation (AF) techniques can maximise reproductive output and allow greater control of breeding designs for endangered amphibian species in captive facilities. Successful AF requires viable gametes (both sperm and eggs) to be available simultaneously (Silla, 2013). However, asynchrony of gamete-release is common in anurans (frogs and toads) and is usually attributed to sex-specific variation in gamete maturation rates, with females typically taking a longer and a more variable time to respond to hormone administration (Silla, 2011).

One technique used to improve the likelihood of successful fertilisation by ensuring the simultaneous availability of viable gametes from both sexes is sperm storage. Developing sperm storage protocols that maximise sperm longevity *in vitro* can ensure sperm is available and viable when eggs are released from females. Past studies investigating the influence of abiotic factors on sperm performance during short term storage have shown that the temperature and osmolality of the storage solution can greatly affect sperm longevity, with low temperature (0–5 °C) and isotonic osmolality (>220 mOsm kg^{-1}) improving sperm viability (Browne et al., 2002; Kouba et al., 2009). Under these conditions, the sperm of a number of Myobatrachid (Dziminski et al., 2010; Edwards et al., 2004; Silla, 2013), Hylid (Browne et al., 2002; Silla et al., 2015), Bufonid (Browne et al., 2001; Kouba et al., 2003) and Ranid (Mansour et al., 2010) species have been successfully stored for 6 to 15 days. For most anuran species, however, sperm viability falls below 50% in the first 6 days of storage. While the cause of this rapid decline remains unclear, there is emerging evidence that sperm viability during short-term storage may be significantly impacted by bacterial contamination.

In a variety of taxa ranging from invertebrates to mammals, bacterial flora has been associated with decreased sperm viability, reduced sperm motility and reduced fertilisation capacity (Aurich and Spergser, 2007; Viveiros et al., 2010; Yaniz et al., 2010). For example, in a study on Channel Catfish, sperm motility during chilled storage dropped from 93% to 1% within 72 h in contaminated samples, compared to sperm suspended in sterile buffers, which maintained motility for up to 10 days (Jenkins, 1997). Bacteria can impair sperm viability in two ways; first, bacteria can release enzymes (e.g. proteolytic enzymes), which invade the sperm cells causing cell rupture and death (Jenkins, 1997), and

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second bacteria can compete for resources within the storage medium (Saad et al., 1988).

The addition of antibiotics to stored sperm samples can potentially counter the detrimental effects of bacterial contamination and improve sperm longevity (Aurich and Spergser, 2007; Christensen and Tiersch, 1996; Saad et al., 1988; Segovia et al., 2000; Viveiros et al., 2010; Yaniz et al., 2010). For example, recent work on the storage of sperm of the Piracanjuba, *Brycon orbignyanus,* found that the addition of 0.1 mg mL^{-1} of gentamicin effectively inhibited bacterial growth, yielded higher sperm motility, and resulted in higher fertilisation capacity compared to untreated controls (Viveiros et al., 2010). In amphibians, the effect of antibiotic treatment on sperm during short-term storage is yet to be widely investigated. To date, only two published studies have quantified the impact of antibiotic treatment on sperm longevity. Germano et al. (2013) reported that the addition of the antibiotic penicillin-streptomycin to sperm samples from Fowler's toad, *Bufo fowleri,* negatively impacted sperm longevity after two to four days of storage. Similar results were reported by Silla et al. (2015) who found that 4 mg mL^{-1} of gentamicin was detrimental to sperm motility during short-term storage in the Booroolong frog, *Litoria booroolongensis*. At high concentration, antibiotics may have a toxic effect on sperm by reducing the sperms' mitochondrial function (Segovia et al., 2000), which, in turn impairs sperm motility and fertilisation capacity (Auger et al., 1989; Evenson et al., 1982; Segovia et al., 2000). Such negative effects of antibiotic supplementation on sperm are dose-dependent, and optimal doses for inhibiting bacterial contamination, without compromising sperm viability, may be species specific. As such, there is a need for additional studies that explore the effect of antibiotic dose on sperm performance and longevity during storage in a range of amphibian species.

The optimal antibiotic dose to administer to sperm samples during storage may also be correlated to the source of bacterial contamination, as well as the bacterial load within the sperm suspension. In anurans, two standard protocols are used to obtain mature sperm for use in AF. Traditionally, sperm have been obtained via the euthanasia of a male and the subsequent removal and maceration of the testes. Here, bacterial contamination can occur even when sterile surgical equipment and storage solutions are used, and is likely to be a result of contact with bodily fluids released during rupture of the bladder or intestinal gut (A.J. Silla, E. Love, P.G. Byrne, unpubl. Data). More recently, due to the decline of many amphibian species, focus has shifted toward the use of non-invasive techniques for acquiring sperm, with a specific focus on hormonally inducing spermiation (Clulow et al., 1999; Silla, 2011; Silla and Roberts, 2012). Following hormone injection, sperm are released into the cloaca and are collected in a fluid medium consisting of urine and cloacal secretions (spermic urine; Kouba et al., 2013). Bacterial contamination is a common problem in spermic urine samples because sperm are flushed through the cloaca, which is colonised by urinary and faecal bacteria. Therefore, sperm samples obtained via hormonal induction of spermiation harbour a greater diversity and abundance of bacteria compared with sperm samples obtained via testis maceration (A.J. Silla, E. Love, P.G. Byrne, unpubl. data), and may therefore benefit from supplementation with higher antibiotic doses.

The objective of this study was to investigate the dose-dependent effect of gentamicin on bacterial abundance and sperm performance in sperm samples obtained via testis maceration and hormonal induction in the critically endangered Booroolong frog, *Litoria booroolongensis*. Specifically, this study aimed to: 1) evaluate the efficacy of gentamicin at reducing bacterial abundance and improving sperm performance (sperm motility and velocity) during the chilled storage of sperm collected from testis macerates and; 2) evaluate the efficacy of gentamicin at reducing bacterial abundance and improving on sperm performance (sperm motility and velocity) during the chilled storage of sperm collected as spermic urine.

2. Material and methods

The procedures outlined below were performed following evaluation and approval by the University of Wollongong's Animal Ethics Committee (approval numbers AE12/17 and AE14/16).

2.1. Animal collection and husbandry

Male Booroolong frogs, *L. booroolongensis,* were generated from a captive colony held at Taronga Zoo (Sydney, NSW, Australia). Once reproductively mature, males were transported to the Ecological Research Centre at the University of Wollongong (Wollongong, NSW, Australia) where they were housed according to methods described elsewhere (see Silla et al., 2015). Briefly, animals were held in a constant temperature room maintained at 22 °C with a 13 h/11 h light/dark cycle including a half hour dim lighting phase at dawn and dusk. Males were housed in pairs in ventilated plastic terrariums (27 × 17 × 16.5 cm, L × W × H) containing a layer of aquarium gravel and sterilised PVC half-pipes for shelter. Animals had constant access to 1L of reverse osmosis (R.O.) water and were fed 10-day old crickets twice a week. At the commencement of experiments, males used in 'Experiment one' were approximately 3-years of age post-metamorphosis (weight ranged from 3.65 g to 7.80 g; mean mass ± SEM = 5.53 ± 0.23 g) and males used in 'Experiment two' were approximately 1-year of age post-metamorphosis (weight ranged from 3.14 g to 4.65 g; mean mass ± SEM = 3.95 ± 0.09 g). All frogs were deemed to be in breeding condition prior to the start of experiments, evident by the darkening of nuptial pads and initiation of calling behaviour.

2.2. Experiment one: Effect of gentamicin on sperm storage of testis macerate samples

2.2.1. Experimental design

To test the effect of various doses of the antibiotic gentamicin on bacterial abundance, a split-sample experimental design was used, whereby sperm suspensions from eight males were evenly divided among four experimental treatments (0, 1, 2, or 4 mg mL^{-1} gentamicin). Male frogs ($n = 8$) were euthanized via pithing and both testes were extracted and macerated post-mortem to generate sperm suspensions. Of note, frogs were not euthanized specifically for the present study, and available testis tissue was used post-mortem. The testis from each individual was macerated in 210-μL of chilled 1:1 simplified amphibian ringer (SAR: 113 mM NaCL, 1 mM $CaCL_2$, 2 mM KCl, 3.6 mM $NaHCO_3$; 220 mOsmo kg^{-1}) in 1.5 mL Eppendorf tubes. The 210-μL sperm suspension from each male was then homogenised and dived into four 50-μL subsamples. Each 50-μL subsample was then diluted in an additional 30-μL of 1:1 SAR containing varying concentrations of gentamicin, such that the final dose in each suspension was 0, 1, 2, or 4 mg mL^{-1}, respectively. The osmolality of each suspension medium remained at 220 ± 2 mOsmo kg^{-1}.

For each suspension, sperm concentration was quantified using an Improved Neubauer Haemocytometer (Bright Line, Optik Labor, Germany) according to procedures described previously (see Silla and Roberts, 2012). Briefly, a homogenised 2-uL subsample of sperm suspension was diluted in 18-uL of 1:1 SAR (1:10 dilution), homogenised and pipetted into a haemocytometer chamber. The number of spermatozoa present in five quadrats was recorded and used to calculate total sperm concentration. This was repeated