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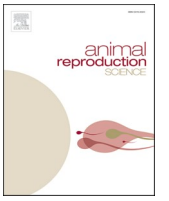

Refrigerated storage and cryopreservation of hormonally induced sperm in the threatened frog, *Litoria aurea*

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ABSTRACT

As sperm cryopreservation and other assisted reproductive technologies (ARTs) advance in common amphibian species, focus on applying non-lethal sperm collection methods to the conservation and genetic management of threatened species is imperative. The goal of this study was to examine the application of logistically practical ART protocols in a threatened frog (*Litoria aurea*). First, we tested the efficacy of various concentrations of human chorionic gonadotropin (hCG) (20, 40 IU/g bodyweight) and Gonadotropin releasing hormone antagonist (0.25 μg/g and 0.5 μg/g body weight GnRH-a) on the induction of spermatozoa. Using the samples obtained from the previous trials, we tested the effect of cold storage and cryopreservation protocols on long-term refrigerated storage and post-thaw sperm recovery. Our major findings include: (1) high quality sperm were induced with 20 and 40 IU/g bodyweight of (hCG); (2) proportions of live, motile sperm post-thaw, were recovered at higher levels than previously reported for *L. aurea* (>50%) when preserved with 15% v/v DMSO and 1% w/v sucrose; and (3) spermic urine stored at 5 °C retained motility for up to 14 days. Our findings demonstrate that the protocols developed in this study allowed for successful induction and recovery of high-quality spermatozoa from a threatened Australian anuran, *L. aurea*, providing a prime example of how ARTs can contribute to the conservation of rare and threatened species.

1. Introduction

Globally, amphibians are subject to threatening processes that are reducing population sizes below the critical thresholds required for the retention of genetic diversity beyond that observed for any other taxonomic group (Stuart et al., 2004; Gillespie et al., 2020; Campbell Grant et al., 2023). The resulting catastrophic rates of biodiversity loss highlight that species and populations will continue to decline through population fragmentation, inbreeding, and loss of heterozygosity and allelic richness (Ralls et al., 2020; Hu et al., 2021; Stock et al., 2023). As species decline, there is a pressing need for strategies that protect the genetic diversity of small and

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isolated populations (Ralls et al., 2020; Stock et al., 2023).

Assisted reproductive technologies (ARTs), including hormonal induction of gamete release, sperm cryopreservation and extended cold storage of sperm without cryopreservation, have the potential to bolster efforts to manage the most serious aspects of genetic loss in declining populations (Browne et al., 2019; Della Togna et al., 2020; Howell et al., 2020; Howell et al., 2021; Clulow et al., 2022). Sperm cryopreservation and biobanking can be utilised to manage genetics effectively through storage of founder genomes and the reduction of costs of captive breeding programs (Della Togna et al., 2020; Howell et al., 2020; Howell et al., 2021). Nevertheless, especially for threatened species, there is room for improving sperm collection methods so that they are minimally invasive.

Historically, many studies reporting sperm cryopreservation in amphibians have utilised testicular macerates, involving euthanasia, as a source of good quality, concentrated sperm that can be diluted out to large working volumes (Browne et al., 1998; Mugnano et al., 1998; Browne et al., 2002b; Upton et al., 2018). While testicular macerates have a place in laboratory studies and conservation programs in the case of opportunistic collection from recently deceased animals, development of non-lethal methods such as hormonal induction of spermic urine (release of mature sperm cells in urine) is critical when working with threatened species, because they do not harm the adult male and can be repeated several times. Most reports of hormonal induction of sperm release to date indicate a preference for the use of human chorionic gonadotropin (hCG) for producing concentrated high quality sperm samples in urine (i.e. from Pelodryadidae, Bufonidae and Limnodynastidae families; (Kouba et al., 2012; Silla and Roberts, 2012; Clulow et al., 2018; Arregui et al., 2019; Silla et al., 2019; Pham and Brannelly, 2022), with a smaller number of studies indicating a preference for gonadotropin-releasing hormone agonist (GnRH-a) (Trudeau et al., 2010; Silla and Roberts, 2012; Della Togna et al., 2017; Otero et al., 2023). Many studies report some success using exogenous hormones when inducing sperm release in a range of species, however subsequent cryopreservation of spermic urine has been explored as an alternative to testicular macerates in fewer species (Shishova et al., 2013; Uteshev et al., 2013; Hinkson et al., 2019; Arregui et al., 2020; Guy et al., 2020; Burger et al., 2022; Anastas et al., 2023).

Sperm cryopreservation can be challenging in some species and circumstances; thus, cold storage is an alternative method for short-term storage prior to use. Amphibian sperm, particularly from aquatic breeding temperate species, has a high tolerance to cooling and thermal shock with reports of sperm held at temperatures between 0–5 °C for periods of up to 21 days with varying degrees of retained motility and membrane integrity (Browne et al., 2002b; a; Browne et al., 2002c; Germano et al., 2013; Shishova et al., 2013; Silla et al., 2015; Keogh et al., 2017; Arregui et al., 2020; Figiel, 2020). Storing sperm at cool temperatures (0–5 °C) can be used as a standalone procedure, or as an intermediary step towards cryopreservation as an endpoint (Browne et al., 2002c; Campbell et al., 2021).

In this study, we chose the Green and Golden Bell frog, *Litoria aurea*, as a suitable candidate for the continued development of ARTs due to its status as a threatened and nationally vulnerable species. In Australia, *L. aurea* has undergone range contractions of more than 90% since the 1980's spurring extensive efforts to conserve the species through *ex situ* and *in situ* strategies (Mahony et al., 2013). These conservation efforts have resulted in developed *ex situ* breeding and management protocols as well as extensive characterisation of its reproductive biology making it a prime candidate for ARTs and gamete banking protocol development. Recently the application of ARTs in this species reported the production of reproductively viable young through IVF using opportunistically collected and cryopreserved testicular sperm (Upton et al., 2021). The development of protocols that would allow induction of sperm release using exogenous hormones would be a preferred non-lethal strategy. Thus, by building on existing knowledge, this study broadly aimed to: 1) Compare the efficacy of inducing spermic urine release using exogenous administration of 20 and 40 IU/g bodyweight of hCG and, 0.25 and 0.5 μg/g bodyweight GnRH-a; 2) Re-test previously developed cryopreservation protocols for sperm obtained from testes macerates and ensure an optimised post-thaw recovery when using spermic urine, and; 3) test short- and long-term rate of decay in motility in spermic urine when held in cold storage (5 °C), without cryopreservation, over a 14-day period.

2. Methods

2.1. Study species and Husbandry

Male *L. aurea* from the University of Newcastle breeding program were housed in plastic terraria (30 ×18 x 20 cm), with 25% terrestrial environment (autoclaved pebbles) and 75% aquatic environment (aged tap water), for the duration of the study (May and June 2020). Plastic aquaria plants were provided as refuges. Live crickets supplemented with calcium and vitamin powder (Multical Dust; Vetafarm, Wagga Wagga, NSW, Australia) were provided as food twice weekly for the duration of the experiment.

All animals underwent a precautionary protocol of heat treatment to ensure no chytridiomycosis infection was present. Animals were placed in UV- and temperature-controlled cabinets (TRISL – 1175, Thermoline Scientific Equipment, Wetherill Park, NSW) on a 12:12 UV light cycle started at 25 °C. Temperature was increased at 2 °C per day until 37 °C was reached, where the temperature was held for 6 h. This temperature has been shown to kill *Batrachochytrium dendrobatidis* within 4 h (Johnson et al., 2003). Subsequently, temperature was reduced at 2 °C per day until the original 25 °C was reached. Animals were kept at 25 °C until the conclusion of the experiment in June 2020. While experiments were outside the reported breeding season of *L. aurea*, all males included in experiments were in season, confirmed by the presence of darkened nuptial pads.

All experiments were carried out in accordance with International, National and Institutional standards for the care and welfare of animals and in accordance with the University of Newcastle's ethics approval, A-2013–328. Animals were collected and held under NSW Scientific Licence SL101269.

2.2. Experiment 1: Effect of hCG and GnRH-a concentrations on spermiation response of L. aurea

In order to determine the effect of hCG (Chorulon®, Intervet) and GnRH-a (des-Gly^{10}, D-Ala^{6}, Pro-LHRH, Bachem) concentration

on the spermiation response of *L. aurea*, 20 males ($n = 5$ per treatment) were assigned to one of 4 experimental treatments previously shown to be useful in a related pelodryadid species (see (Silla et al., 2019)): (1) 20 IU/g body weight hCG; (2) 40 IU/g of body weight hCG; (3) 0.25 μg/g body weight GnRH-a, and; (4) 0.5 μg/g body weight GnRH-a. Treatments were tested across 10 days between 6th May – 18th June 2020, with two males treated on each of the 10 days. As animals were originally sourced from wild populations, age was unknown (however males reach sexual maturity at around 1 year of age and all displayed secondary sexual characteristics indicative of sexual maturity (darkened nuptial pads and coloured throat). The weight range of males used in the study was 14.3 ± 2.3 g (mean and standard error, $n = 20$).

Prior to injection, a urine sample was collected from each male and found to be aspermic in all males used in the experiment. Males were weighed to the nearest 0.1 g for the determination of hormone concentration to be administered by body weight. Hormones were diluted in simplified amphibian ringer (SAR; 113 mM NaCl, 1 mM $CaCl_2$, 2 mM KCl, 3.6 mM $NaHCO_3$; ~220 mOsm/kg; recipe (Browne et al., 1998)) to a final volume of 200 μl. In addition, each day animals were treated, a male (total, $n = 10$) was administered with a control treatment of 200 μl of SAR to test for spermiation in negative controls. Hormones were administered subcutaneously via the dorsal lymph sac using a 31-gauge insulin syringe (BD, New Jersey, United States).

Once treated, frogs were placed in individual plastic containers (15 × 7 x 10 cm) with ~1 cm of water in the bottom to ensure hydration. At allotted collection intervals (1, 2, 3, 4, 5, 6, 24, 48 h post-injection), a thin plastic gel-loading tip (Corning, New York, United States), was used as a catheter to collect a spermic urine sample. The pipette tip was gently placed ~3–5 mm into the cloaca and gently moved in and out to facilitate urine collection by capillary action. The sample was transferred to a 0.5 mL Eppendorf tube and was kept on ice until volume, motility and sperm concentration could be determined (within the hour). Motility analyses were performed immediately after collection to reduce possible effects of time post-collection.

Concentration (cells/mL) of sperm in urine samples were assessed with an Improved Neubauer haemocytometer counting chamber at each collection interval. Ten microlitres was pipetted into each of two chambers, and the number of sperm in at least 5 secondary quadrats (per chamber) was counted and used to calculate total sperm per millilitre. For concentrated samples, sperm were diluted, as necessary, with SAR and the dilution factor was taken into account in the final calculation. For particularly dilute samples, all primary quadrats of the counting chamber were counted. The number of either primary or secondary quadrats was taken into account for the final calculation. The total number of sperm per sample was calculated using the volume of sample collected.

Due to small volumes of spermic urine available for assessment, motility in samples was analysed after a 1:5 dilution in SAR. While this was found to be less favourable than analysis of undiluted samples, it allowed sufficient volume for assessment of the effect of hormone type and dosage rate on motility where only small volumes were available. Sperm were categorised as either non-motile, or motile, and at least 100 sperm were counted at 400x magnification across at least four fields of view using a Kyowa Unilux-12 microscope and phase contrast optics. Due to small sample volumes, only one replicate count could be performed per sample.

2.3. Experiment 2: Cryopreservation of spermic urine of L. aurea

A further four males were used to test the efficacy of cryoprotectant solutions previously optimised using sperm derived from testicular macerates (see (Upton et al., 2021; Upton et al., 2023)) on the recovery of post-thaw spermic urine quality of *L. aurea*. Previous studies showed 15% v/v DMSO with 10% w/v sucrose provided sufficient recovery of sperm for fertilisation of ova (Upton et al., 2021), however a more recent study on related pelodryadid species shows 15% v/v DMSO in combination with 1% w/v sucrose could be a more appropriate cryoprotectant for Australian tree frogs (Upton et al., 2023). Thus, both of these cryoprotectants were chosen to test the recovery of spermic urine.

Males were injected with 20 IU/g body weight hCG and samples collected over 6 h until an accumulative volume of at least 50 μl was achieved. Forty microlitres of spermic urine were diluted 1:5 (to a final volume of 240 μl) with the appropriate ice-cold cryoprotectant and loaded into 0.25 mL Cassou straws (Minitube, Smythesdale, Victoria, Australia) at a volume of 120 μl per straw. Two straw replicates were produced per treatment, per animal. Straws were frozen in a programmable freezer (Freeze Control® CL-3300; CryoLogic Pty Ltd, Blackburn, Victoria, Australia), as per Upton et al., 2018.

Straws were thawed on a bench at room temperature (~21 °C) and assessed as per Upton et al., 2023. Briefly, motility was assessed by diluting samples 1:9 so that the final concentration of DMSO and sucrose in each sample was equal to 15% v/v DMSO and 1% w/v sucrose. This allowed assessment of motility at equal osmolalities across treatments whilst accounting for any dilution effects on motility. Following dilution, sperm were assessed as per Experiment 1. Sperm membrane integrity was assessed with an Eosin-Y dye exclusion assay (Upton et al., 2021; Upton et al., 2023). Sample was mixed 1:1 with 0.5% Eosin-Y in 0.9% saline to a final volume of 10 μl and viewed under a coverslip at 400x magnification. Sperm with clear cytoplasm (excluding the dye) were scored as live and sperm stained pink were scored as dead (membrane permeable to the dye). For both motility and membrane integrity, assessment of each straw counted at least 100 sperm across four fields of view, in duplicate. All sperm collections for this experiment occurred in June 2020, at which time a pre-freeze assessment was made, and post-thaw assessment occurred in September 2020, following three months of cryostorage.

2.4. Experiment 3: Cold storage of spermic urine without cryopreservation

To determine the longevity of motility in spermic urine samples at 5 °C, excess non-cryopreserved samples from Experiments 1 and 2 were kept in refrigerated storage for a period of up to 14 days. Aliquots (15–25 μl) of spermic urine from seven males were kept in 0.5 mL Eppendorf tubes. Where volume permitted ($n = 3$), three aliquots of spermic urine from the same animal were used and motility was analysed on days 1, 3, 5, 6, 8, 9, 10, 11, 12, 13 and 14 or until the sample ran out. Otherwise, a single aliquot from each animal (*n*

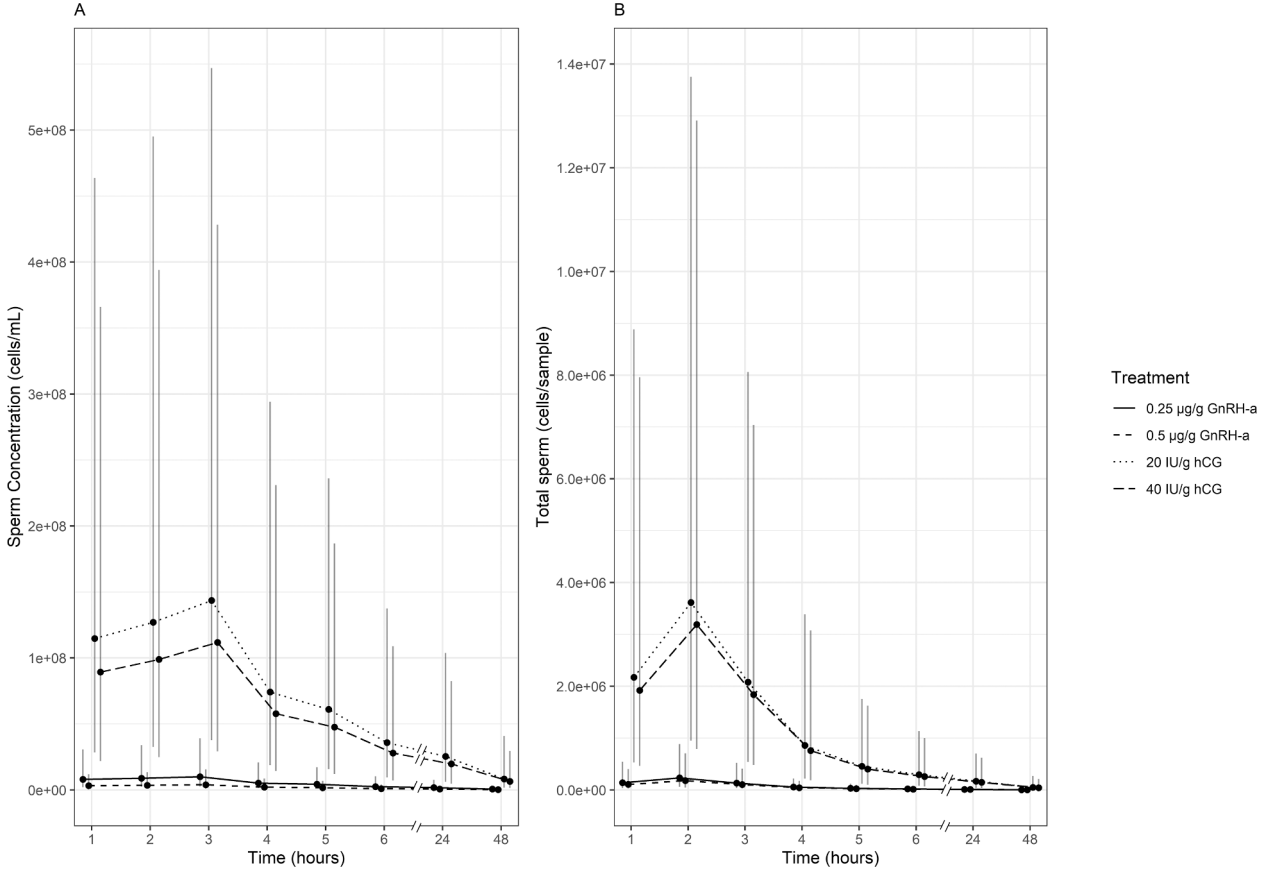


Fig. 1. Effect of treatment on induction of spermiation in *Litoria aurea* ($n = 5$ per treatment) for (a) sperm concentration (cells/mL) and (b) total sperm numbers (cells/sample) over a 48-hour collection period. Zero-inflated negative binomial GLMM; black dots represent estimated marginal means and error bars equal 95% confidence intervals.

= 4) was used and analysed on days 1, 2, 3 and 5. Initial motility was also determined on day 0. Motility of spermic urine was assessed undiluted, with no added media, by adding a 2 μl drop under a 12 mm diameter coverslip (Menzel-Gläser, Saarbrückener, Germany) at 200x magnification after allowing 1 min to reach room temperature. While osmolality couldn't be measured for each sample due to small volumes, a subset of urine samples gave an average osmolality of 50 mOsm/Kg ($n = 5$). Categorisation of motility was as per Experiments 1 and 2. Due to small volumes available, only a single count was completed, per sample, each day. Sperm were aerated by gently bubbling air into the sample with a pipette for about 5–10 s every day of storage, regardless of whether motility was being assessed, to account for any potential effects of aeration.

2.5. Statistics

Generalised Linear Mixed Models (GLMM) were used to fit Poisson regressions for sperm concentration (cells/mL) and total sperm numbers (cells/sample). The raw counts were used with an offset of the log reciprocal of an adjustment factor (based on dilution factor, volume and the number of quadrats counted as appropriate) to convert the raw counts to cells/mL or cells/sample respectively. Treatment and hour were utilised as main effects and animal ID as a random effect. Using AIC as a model fit criterion, it was found the model needed to be expanded to account for zero inflation as a function of time (hours) to account for excess zeros beyond those expected from the Poisson distribution and use the negative binomial distribution to account for overdispersion. Significance was determined by pairwise comparisons to determine ratios of expected counts between conditions and 95% confidence intervals.

To determine the effect of the four hormone treatments on motility, we used Generalised Linear Mixed Models (GLMM) to fit binary logistic regressions (interpreted as the proportion of successful cases (i.e. motile sperm) with total number of sperm equalling the weights for the model. Treatment and hour were used as main effects and overdispersion was addressed using an observation level random effect. The interaction between treatment and hour was not significant and was dropped from the model.

To determine the effect of the two cryoprotectants and time post-thaw on motility and membrane integrity, we used GLMMs to fit binary logistic regressions (interpreted as the proportion of successful cases (i.e. motile or live sperm) with total number of sperm equalling the weights for the model. We used a 2 × 2 factorial model design with cryoprotectant (15% v/v DMSO with either 1 or 10% w/v sucrose) and time post-thaw (0 and 30 min) as main effects and overdispersion was addressed using an observation level random effect. The interaction between cryoprotectant and time post-thaw was not significant and was dropped from the model.

To determine the effect of the days in cold storage (without cryopreservation) on motility, we used a GLMM with random slopes to fit a binomial logistic regression (interpreted as the proportion of successful cases (i.e. motile) with total number of sperm equalling the weights for the model. The number of days in storage was used as the model main effect (continuous) and day and animal ID were used as random effects to account for the random slopes analysis.

All analyses were completed using R (Version 3.6.2) with R packages glmmTMB used for zero-inflated negative binomials and lme4 used for all other GLMM modelling (Bates et al., 2015; Brooks et al., 2017). The package emmeans was used to model estimated marginal means (EMMs) and 95% confidence limits were calculated and back transformed to sperm concentrations (in cells/mL and cells/sample) and motility and membrane intact proportions. Ratios (for sperm concentration data) and odds ratios (for proportion data) comparing treatments were also generated using the package emmeans (Lenth et al., 2018). All graphing was completed using ggplot2 and gridExtra (Wickham, 2016; Auguie and Antonov, 2017).

3. Results

3.1. Experiment 1: Effect of hCG and GnRH-a concentrations on spermiation response of L. aurea

There was a significant effect of treatment and collection time on sperm concentration (likelihood ratio test (LRT) $\chi^2(3) = 15.0$, $P < 0.01$ and LRT $\chi^2(7) = 43.1$, $P < 0.001$ for treatment and collection time respectively) and total sperm numbers per sample (LRT $\chi^2(3) = 13.2$, $P < 0.01$ and LRT $\chi^2(7) = 77.7$, $P < 0.001$ for treatment and collection time respectively). The superior hormone was hCG across all time points with a peak in sperm concentration at 3 h and total sperm numbers at 2 h, for the time points measured. Ratios and 95% confidence intervals were generated to further compare the differences in sperm concentration in response to hormone treatments, with larger ratios indicating bigger differences in sperm concentration.

For sperm concentration, both hCG treatments were significantly better than the two GnRH-a treatments with the ratio of expected sperm concentrations ranging from the lowest for the comparison 40 IU/g hCG and 0.25 μg/g GnRH at 11.2, 95% CIs: [1.8, 70.3] to the highest for 20 IU/g hCG and 0.5 μg/g GnRH at 36.7, 95% CIs: [5.8, 232.3] (see Supplementary Table 1 for ratios and 95% CIs). Likewise, for total sperm numbers, hCG outperformed GnRH-a, with the ratio of expected sperm concentrations ranging from the lowest for the comparison 40 IU/g hCG and 0.25 μg/g GnRH at 13.8, 95% CIs: [2.2. 85.5] to the highest for 20 IU/g hCG and 0.5 μg/g GnRH at 20.3, 95% CI [3.3, 126.0] (Supplementary Table 2).

Predicted sperm concentration did not exceed 1.0×10^7 cells/mL in 0.25 μg/g GnRH-a or 3.91×10^6 cells/mL in 0.5 μg/g GnRH-a but reached as high as 1.44×10^8 and 1.12×10^8 cells/mL in 20 IU/g and 40 IU/g hCG respectively. Each of these maximum sperm concentrations occurred 3 h post-injection (Fig. 1a) and was significantly higher than sperm collected from 5 h onwards (see Table S3 for ratios and 95% CIs). Despite high concentrations, total volumes collected were low, with no statistically significant difference between treatments ($F(3,17) = 1.74$, $P = 0.20$). Total volumes collected ranged from 136.2 ± 28.8 to 400.5 ± 134.9 μl, with the highest volumes of urine in the treatments with the lowest concentrations (Table 1). For the times measured, model predicted total sperm numbers peaked at 2 h post-injection for all treatments, with 2.31×10^5 and 1.78×10^5 cells/sample for 0.25 μg/g GnRH-a and 0.5 μg/g GnRH-a respectively and 3.61×10^6 and 3.19×10^6 cells/sample in 20 IU/g and 40 IU/g hCG respectively (Fig. 1b). The total sperm

numbers derived at 2 h was significantly higher than sperm numbers collected from 4 h onwards (see Supplementary Table 3 for ratios and 95% CIs).

There was a significant effect of treatment (LRT $\chi^2(3) = 20.3$, $p < 0.001$), but not time (LRT $\chi^2(5) = 6.7$, $P = 0.24$) on the motility of sperm, with 20 IU/g hCG yielding sperm of significantly higher motility averaged across all time-points than all other treatments (EMM= 60.7%, 95% CIs: [43.6, 75.6]; see Table S4 for ratios and 95% CIs). The treatment 40 IU/g hCG had a much lower model predicted motility (EMM= 23.7%, 95% CIs: [13.3, 38.7]), which was not significantly different to either GnRH-a treatments (see Supplementary Table 4). Of the GnRH-a treatments, 0.25 μg/g had higher motility (EMM= 30.8%, 95% CIs: [17.2, 48.8]) than 0.5 μg/g (EMM=11.7%, 95% CIs: [5.7, 22.6]) which was significantly higher (Fig. 2, Supplementary Table 4).

3.2. Experiment 2: Cryopreservation of spermic urine of L. aurea

There was a significant effect of sucrose concentration on motility (likelihood ratio test (LRT) $\chi^2(1) = 31.9$, $P < 0.001$) and membrane integrity (LRT $\chi^2(1) = 23.1$, $P < 0.001$), but not time post-thaw (LRT $\chi^2(1) = 1.8$, $P = 0.2$ & LRT $\chi^2(1) = 0.9$, $P = 0.3$ for motility and membrnae intergity respectively). Fresh sperm before addition of cryoprotectants was higher in motility (EMM: 82.0%, 95% CIs: [60.1, 93.2]) and intact membranes (EMM: 75.3%, 95% CIs: [59.6, 86.3]) than all post-thaw parameters. Odds Ratios (OR) and (95% confidence intervals were used to further compare post-thaw parameters between treatments.

At both 0- and 30-minutes post-thaw, 15% v/v DMSO with 1% w/v sucrose achieved greater recovery of motility and intact membranes than when 10% w/v sucrose was used instead. For the best cryoprotectant, 15% v/v DMSO with 1% w/v sucrose, motility was 52.7% and 42.4% at 0- and 30-minutes post-thaw, and in membrane integrity from 53.1% and 48.8% at 0- and 30-minutes post-thaw, though these differences were not significant (Fig. 3). Likewise, when using 15% v/v DMSO with 10% sucrose, motility was 14.0% and 9.8% and membrane integrity was 30.1% and 26.6% at 0- and 30-minutes post-thaw (Fig. 3). Spermic urine cryopreserved with 15% v/v DMSO with 1% w/v sucrose were almost 7 times more likely to recover motility (OR: 6.9, 95% Confidence intervals (95% CI) [3.8, 12.4]) and 2.6 times more likely to recover with intact membranes (OR: 2.6, 95% CI [1.8, 3.8]) across both time points.

3.3. Experiment 3: Cold storage study

The predicted decline in motility was steady over the 14 day period ($b = -0.303$, $z(1) = -5.5$, $p < 0.001$). Predicted motility began at approximately 80% and declined to approximately 5% over a 14-day period. The random slopes analysis showed that samples from each of the frogs declined at similar rates (Fig. 4b). The fitted GLMM model with random slopes predicted motility declines would reach 50% by 4–5 days (Fig. 4b).

4. Discussion

This study adds to a growing number of successful protocols used to induce non-lethal release of sperm from the testes of amphibians by injection of exogenous hormones. There are a number of reports of hormonal induction protocols being extended to the next phase in ART protocol development, namely short-term cold storage of spermic urine by refrigeration (Germano et al., 2013; Keogh et al., 2017; Arregui et al., 2019; Arregui et al., 2020; Langhorne et al., 2021) and/or, cryopreservation (Shishova et al., 2011; Uteshev et al., 2013; Hobbs et al., 2023). This study has demonstrated that all three procedures, hormonal induction, refrigeration and cryopreservation, provide successful conservation approaches for *L. aurea.* High rates of motility were observed in freshly collected sperm samples when stored at 5 °C for up to 14 days and post-thaw after cryopreservation. The quality of the post-thawed samples was further evidenced by a large proportion of sperm being recovered with intact membranes indicating the sperm was alive. Therefore, the results of this study overall, largely completes the suite of technologies required for collecting, manipulating and storing male gametes in this threatened species.

We found large differences in the efficacy with which sperm could be induced after injection with the two hormonal regimes tested. Our study found GnRH-a to be relatively ineffective in inducing samples with a concentrated number of sperm when compared to hCG. The differences in spermiation response observed in samples obtained after induction with 20 IU/g BW of hCG compared to 40 IU/g BW may be indicative of receptor saturation since the sperm in samples obtained from the latter treatment were present in lesser, though not statistically significantly, lower concentrations. The aforementioned result warrants further exploration given the small

Table 1
Average volume of spermic urine (± standard error) collected per hormone treatment in Litoria aurea (n = 5 per treatment).

Treatment	Volume (μl)
0.25 μg/g GnRH-a[a]	219.4 ± 69.1
0.5 μg/g GnRH-a	400.5 ± 134.9
20 IU/g hCG[b]	193.3 ± 78.0
40 IU/g hCG	136.2 ± 28.8

[a] Gonadotropin-releasing hormone
[b] Human chorionic gonadotropin

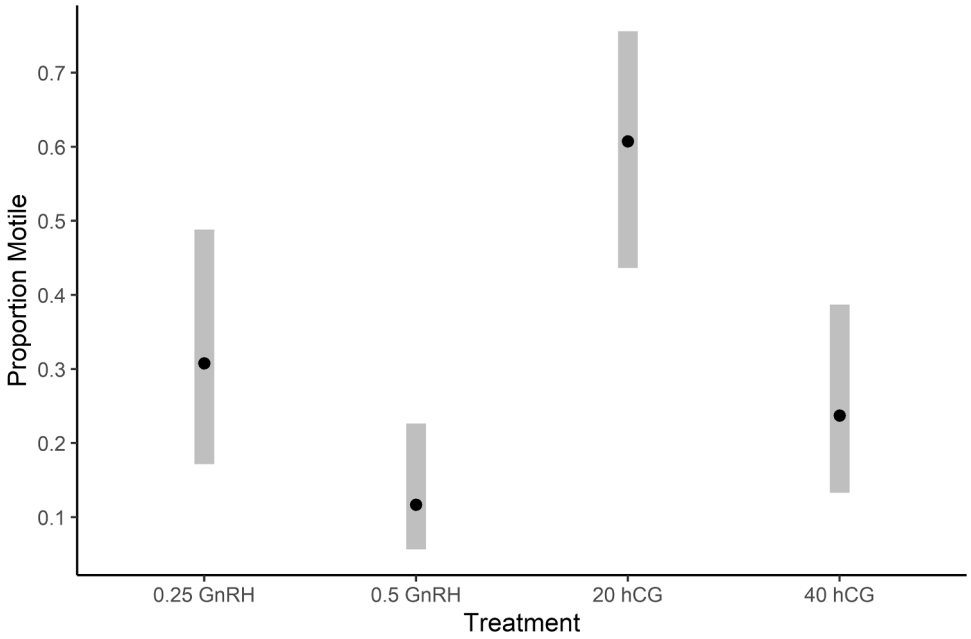


Fig. 2. Proportion motile for sperm induced by each treatment (0.25 μg/g body weight GnRH-a, 0.5 μg/g body weight GnRH-a, 20 IU/g bodyweight hCG, 40 IU/g body weight hCG) in *Litoria aurea* ($n = 5$ per treatment). Estimated marginal means determined by generalised linear mixed modelling. Error bars = 95% confidence intervals.

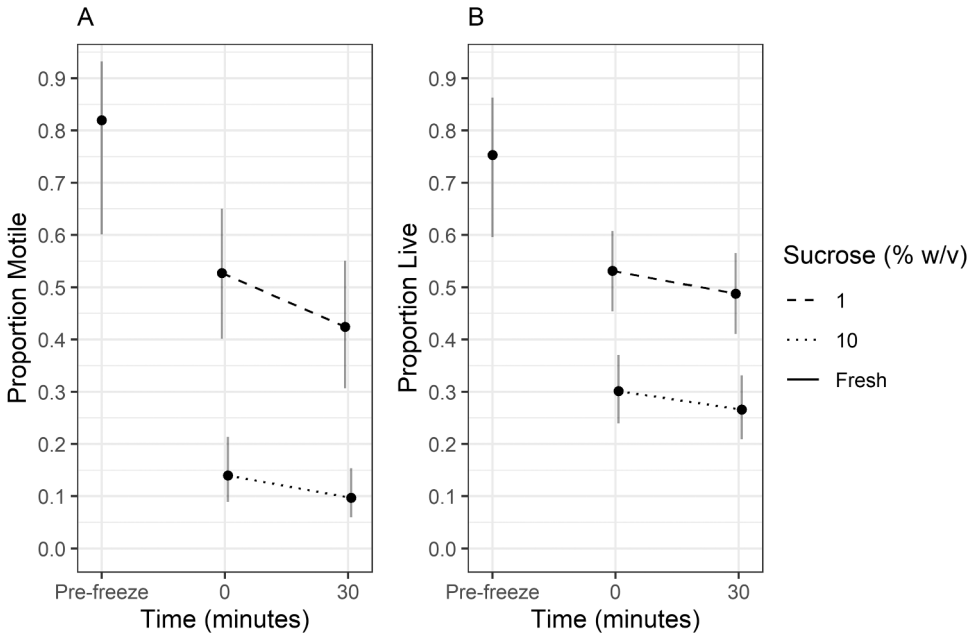
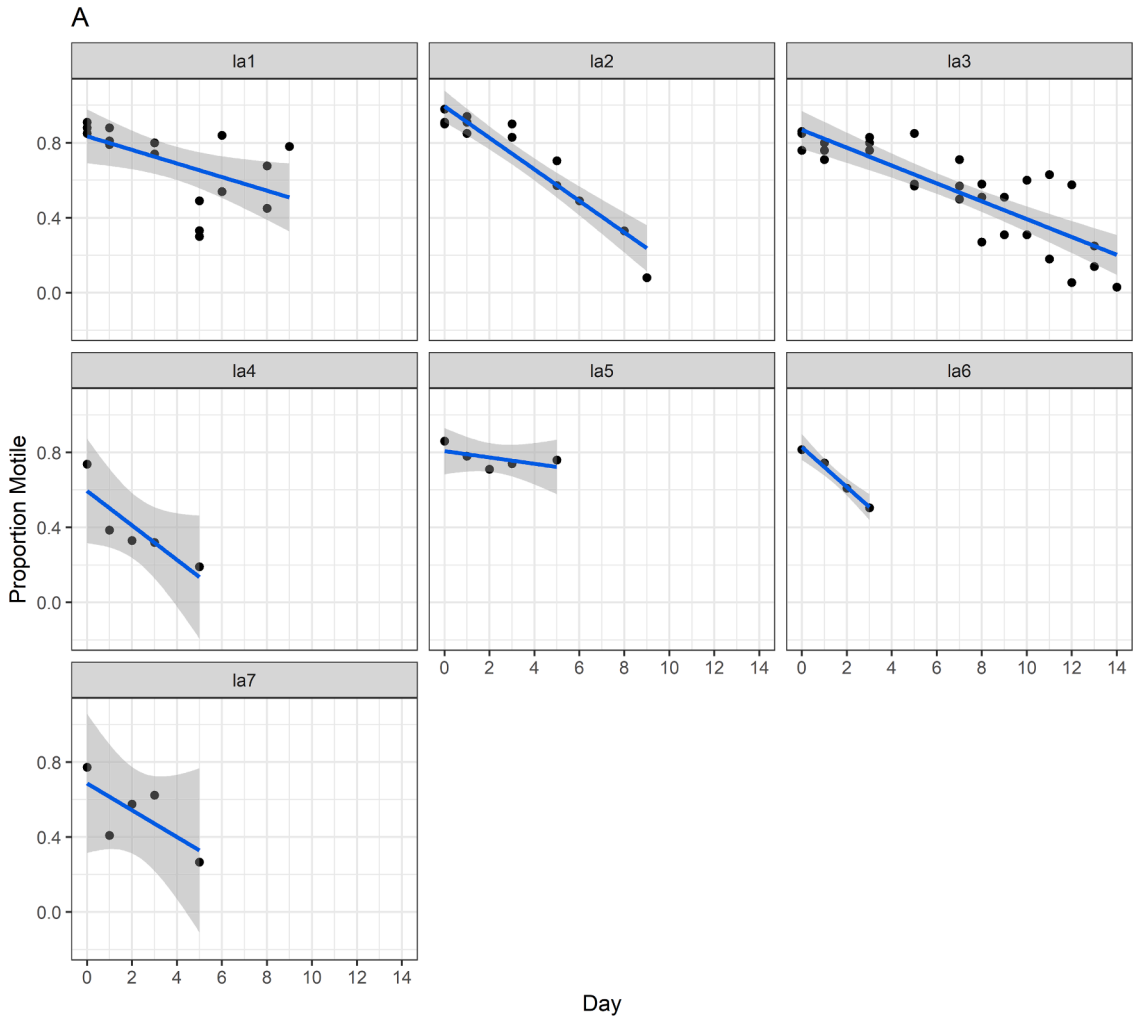


Fig. 3. Proportion motile and intact membranes for pre-freeze (cryoprotectant-free) spermic urine from *L. aurea* ($n = 4$), and each cryoprotectant (15% v/v DMSO with either 1 or 10% w/v sucrose) both 0- and 30-minutes post thaw. Estimated marginal means determined by generalised linear mixed modelling. Error bars = 95% confidence intervals. (a) Proportion motile (b) Proportion live.

sample size per treatment ($n = 5$). These data, however, support reports of hCG-induced spermiation in other pelodryadid species when administration occurred at a range of concentrations (~60–300 IU per whole animal injection) (Clulow et al., 2018; Silla et al., 2019; Pham and Brannelly, 2022; Silla et al., 2023), indicating the successful, though variable concentration-dependent response of many amphibian species to this synthetic mammalian gonadotropin. A recent study by Silla et al. (2019) on a pelodryadid species, *Litoria booroolongensis*, found a similar response to hCG at 20 and 40 IU/g body weight with sperm release also peaking after 2–4 h



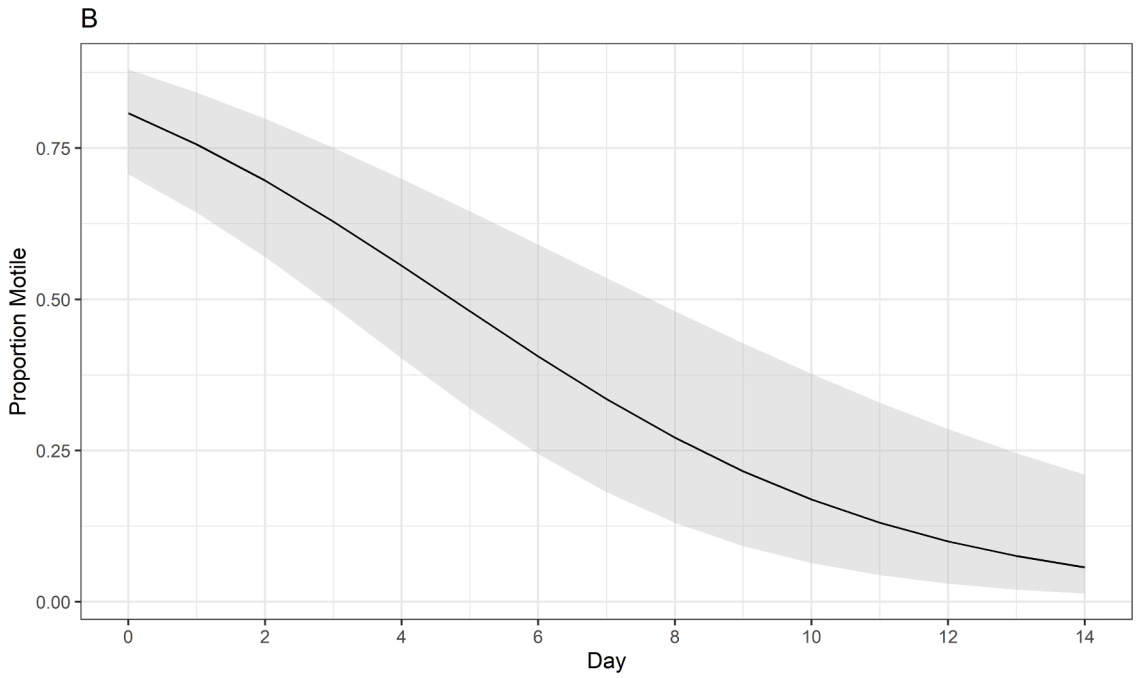


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Fig. 4. Reduction of motility of spermic urine samples of *L. aurea* ($n = 7$) stored at 5 °C. A. Individual slopes from each frog (la1-la7). Solid line indicates predicted decline by linear regression and grey shading indicates 95% confidence bands; B. Combined slopes analysis Solid line indicates predicted decline in motility determined by generalised linear mixed model (taking into account random slopes) and grey shading indicates 95% confidence band.

(Silla et al., 2019). Also noteworthy, the current study was performed at the end of the natural breeding season, indicating the induction of spermiation in *L. aurea* does not need to occur at the peak of the breeding season. This lengthy period of responsiveness is an interesting feature of this species and which may extend to a range of other Australian temperate frogs that are known to call over extended periods of the year (Lemckert and Mahony, 2008; Clulow et al., 2018).

The spermiation responses to GnRH-a presented in this study coincide with those reported by Silla et al. (2019) and Pham and Brannelly (2022). As with hCG, lower concentrations of GnRH-a induced higher numbers of sperm release in *L. aurea* (this study) and in *L. booroolongensis* (Silla et al., 2019), although the differences between GnRH-a concentrations weren't significant in the current study, possibly due to small sample size. The endocrinological mechanisms underlying the inverse relationship between declining sperm release in response to higher concentrations of GnRH-a and hCG is not resolved in either study; however, it is indicative of the importance of testing a number of concentrations to determine optimal dose-response curves when optimising such protocols. More research is required to examine whether seasonality affects spermiation in response to GnRH-a and hCG at earlier times in the breeding season. Possible explanations to the differential physiological responses to GnRH-a and hCG concentrations in this species as in others, could relate to complex events such as receptor affinity and saturation (physiological thresholds), and the induction of feed-back loops, site (testes and/or brain) and mechanism of action (inducing LH, FSH or both) (Clulow et al., 2018). Taken together, these data reinforce the view that hCG is likely to be the optimal choice for induction of spermiation across a range of pelodryadid species, in comparison to GnRH-a. Nevertheless, even though GnRH-a is less potent in inducing spermiation in *L. aurea*, both spermiation and breeding behaviours have also been induced in the Southern bell frog (*L. raniformis*) using GnRH-a (Mann et al., 2010), a closely related species to *L. aurea*.

Of the studies that examine the effects of cold storage on anuran sperm motility, fewer have utilised spermic urine than compared to testicular macerates (Germano et al., 2013; Keogh et al., 2017; Arregui et al., 2019; Arregui et al., 2020; Langhorne et al., 2021). Testicular macerates are in a totally different ionic and osmotic environment to spermic urine. This is important because sperm stored in the testes are usually macerated in isotonic media, such as SAR (~220 mOsm/kg) in which sperm demonstrate lower or complete immotility until activation by hypoosmotic shock (Inoda and Morisawa, 1987; Edwards et al., 2004). Cold storage of osmotically inactivated sperm is thought to reduce metabolic rates and energy utilisation by the sperm cell, allowing storage for longer periods of time (Browne et al., 2019). This has been the paradigm in studies of various amphibian species that utilise either whole testes or testicular macerates (Rostand, 1946, 1952; Browne et al., 2001; Browne et al., 2002b; Browne et al., 2002c; Shishova et al., 2013; Silla, 2013; Silla et al., 2015; Keogh et al., 2017).

In contrast to sperm derived from testicular macerates, spermic urine undergoes a hypoosmotic shock during its release in urine and is thus motile from the commencement of storage. In a range of Bufonid species (*Anaxyrus boreas boreas*, *A. fowleri* and *Peltophryne lemur*), spermic urine has been observed to range from 20–80 mOsm/Kg and in *Lithobates sevosa*, 45 mOsm/Kg (Langhorne, 2016). While *a priori* that this may be expected to pose a problem for longevity of motility during storage, the present study found that spermic urine of *L. aurea* can retain motility over 14 days at 5 °C with the predicted drop to 50% motile sperm at approximately 5 days. Similar studies utilising spermic urine have found retention of ~12–50% of motility in three species, after 2–6 days at 4–5 °C (Germano et al., 2013; Keogh et al., 2017; Arregui et al., 2020). While our study did not directly test the effects of aeration during cold storage, the aeration of samples daily (manually with a pipette tip) may still have had a beneficial effect, as found in some similar studies (Germano et al., 2013; Silla et al., 2015). While these results suggest some variation in preservation of motility for different species, taken together, the storage of both testicular and spermic urine at low temperatures for later use in IVF or for cryopreservation is probably feasible in a wide array of species.

There are increasing reports of cryopreservation of spermic urine in the literature (Shishova et al., 2013; Uteshev et al., 2013; Hinkson et al., 2019; Arregui et al., 2020; Hobbs et al., 2023; Lampert et al., 2023; Otero et al., 2023). Many of those species were from the families Ranidae and Bufonidae (not closely related to pelodryadid species) and produced large enough volumes of urinic sperm per animal to readily test and replicate a range of cryopreservation protocols, typically utilising either DMSO or N, N-dimethylformamide (DMFA) (Uteshev et al., 2013; Langhorne, 2016). In a previous study using testicular macerates, we noted post-thaw sperm membrane integrity was higher than post-thaw motility, indicative of non-lethal cryopreservation related injury to sperm (Upton et al., 2021). In the present study, this relationship remained true for the cryoprotectant previously used in this species (15% v/v DMSO with 10% w/v sucrose), but not true for the newly trialled cryodiluent, 15% v/v DMSO with only 1% w/v sucrose. This result indicates that the lower concentration of non-penetrating cryoprotectant (sucrose) in the cryodiluent minimises non-lethal damage and also improves on the previous method by a significant margin in terms of recovery of viable and motile sperm after cryopreservation.

This study further considered the practicality of using cryopreserved sperm for IVF by assessing post-thaw motility at both 0- and 30-minutes post-thaw. While there was a slight decrease in both motility and membrane integrity 30 min post-thaw in both treatments, this decrease was not significant, though further replication of this in future studies, as well as further time-points may be beneficial. The results suggest a reasonable window of time following thawing of sperm of at least half an hour that would provide enough time for post-thaw assessments, adjustment of sperm concentration, wash steps and addition of sperm to ova for fertilisation purposes. In addition to the ease with which spermiation responses can be induced in *L. aurea,* it is possible that extended sperm survival when

stored at 0–5 °C could also enhance the duration of post-thaw sperm viability when preparing for IVF experiments. Together with cold storage and cryopreservation, this study provides successful protocols for non-lethal collection and storage of sperm that could be successfully combined with fertilisation of ova when these are available.

5. Conclusions

Our study shows that sperm release can be induced in high concentrations with 20 IU/g bodyweight of human chorionic gonadotrophin (hCG). For the purpose of storing sperm we demonstrated that high levels of live, motile sperm could be recovered post-cryopreservation by treating the sperm with 15% v/v DMSO and 1% w/v sucrose pre-freeze and that spermic urine could be stored at 5 °C retaining motility over a 14-day period. Our findings demonstrate that it is possible to obtain and store large quantities of quality sperm from a threatened amphibian via non-lethal means, representing an important step forward for the use of ARTs in conservation programs for rare and threatened species.

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CRediT authorship contribution statement

Upton Rose: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Calatayud Natalie:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Clulow Simon:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Clulow John:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Data curation, Conceptualization. **Brett Darcie:** Writing – review & editing, Methodology, Data curation. **Burton Alana:** Writing – review & editing, Methodology, Data curation. **Colyvas Kim:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis. **Mahony Michael:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2024.107416.

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