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Sperm cryopreservation in an Australian skink (*Eulamprus quoyii*)

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Abstract. Assisted reproductive technologies for population and genetic management for threatened herpetofauna have grown substantially in the past decade. Here we describe experiments to optimise sperm cryopreservation in a model squamate, the eastern water skink *Eulamprus quoyii*. Small, concentrated volumes of highly motile spermatozoa were reliably collected from adult male *E. quoyii* by non-lethal ventral massage. Samples were used to: (1) test whether protein-rich diluents, namely Beltsville poultry semen extender (BPSE) and TES and Tris (TEST) yolk buffer (TYB), improve post-thaw quality metrics compared with Dulbecco's phosphate-buffered saline (DPBS); and (2) compare the efficacy of these diluents in combination with either 1.35 M glycerol or 1.35 M dimethyl sulfoxide (DMSO) at two freezing rates, fast (approximately –20°C min^{-1}) versus slow (–6°C min^{-1}). Glycerol and DMSO performed equally well in preserving spermatozoa under slow freezing rates. Under these conditions, the use of the complex diluents BPSE and TYB significantly improved post-thaw total motility compared with DPBS. Complex interactions occurred between cryodiluent type, cryoprotectant and freezing rate when testing fast versus slow freezing rates among treatment groups. Under slow freezing rates, DMSO was better at preserving membrane integrity and motility, regardless of diluent type, but successful fast freezing required complex diluents to support motility and membrane integrity, which has implications for implementation in a field setting.

Keywords: conservation breeding programs, controlled-rate freezing, dry shipper, genome resource bank, reptile, spermatozoa, wildlife.

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Introduction

Genetic management within animal production systems is enhanced by using assisted reproductive technologies, in particular the collection and cryopreservation of spermatozoa combined with artificial insemination (AI). Although these management practices are commonplace for many commercially relevant mammalian and avian species, their application to genetic management within conservation breeding programs remains underused, and research on how to expand their use is comparatively rare. Although this theory and approach is generally accepted as integral to future herpetofauna breeding programs (Clulow and Clulow 2016; Strand *et al.* 2020), relatively few species have been studied with the aim of adequately understanding fundamental reproductive physiology, a necessary precursor for progressing these technologies.

Sperm extraction from living specimens has been successful in squamates using both electrostimulation and manual massage techniques (Zacariotti *et al.* 2007; Molinia *et al.* 2010; Zimmerman *et al.* 2013; López Juri *et al.* 2018; Martínez-Torres *et al.* 2019*a*). Most of these studies have resulted in the basic characterisation of male gametes and some have demonstrated the handling and use of fresh or cooled semen for AI (Mattson *et al.* 2007; Oliveri *et al.* 2018). Yet, far fewer have progressed to the study of cryopreservation (see Clulow and Clulow 2016;

Campbell *et al.* 2020, 2021; Young *et al.* 2021). Perhaps the most comprehensive characterisation of reptilian sperm physiology and cryobiology are studies on the saltwater crocodile *Crocodylus porosus* (Johnston *et al.* 2017); however, crocodilians are more closely related to birds than squamates and represent only one species of over 8000 reptilian species in existence today.

Skinks (Scincidae) are the largest and most diverse group of lizards and are a taxon of conservation concern, with approximately 270 species being listed as imperilled on the International Union for Conservation of Nature (IUCN) Red List (i.e. 'Near Threatened' or higher status) of the more than 1715 species worldwide (Uetz *et al.* 2020). Australia is a centre for Scincidae diversity (Chapple *et al.* 2021). Skinks exhibit diverse reproductive strategies and physiology, from viviparous to oviparous, as well as mixed strategies between the two (Blackburn 2015; Laird *et al.* 2019). In light of this diversity, a model skink species is needed for developing foundational sperm cryopreservation and AI techniques that can be refined for each species.

In this study we adapted and used a non-lethal sperm collection technique with an abundant skink species, the eastern water skink *Eulamprus quoyii*, with the purpose of developing sperm collection and cryopreservation techniques for potential use in the conservation and rescue of threatened and endangered Australian skink species. *E. quoyii* is a mid-sized (maximum snout–vent length (SVL) 133 mm; Salkeld *et al.* 2008), viviparous skink that is widely distributed across eastern Australia. The skinks are seasonal breeders, with testicular recrudescence beginning in late autumn, before winter brumation. Testis weights are maximal immediately before and during emergence from winter brumation, with copulation usually occurring in September–October (Veron 1969). Targeting this period of maximum sperm production and storage, our preliminary experiments identified dimethyl sulfoxide (DMSO) and glycerol as equally suitable cryoprotectants for slow rate (6°C min^{-1}) sperm cryopreservation (see Hobbs *et al.* 2019). Here, we attempted to further optimise *E. quoyii* sperm cryotolerance by investigating the dilution of spermatozoa in complex diluents and the use of a fast freezing technique that would simplify logistics for field collections from wild-living animals. Specifically, our aims were to determine: (1) whether the use of complex sperm diluents improves post-thaw motility parameters and membrane integrity; (2) whether a field-friendly, fast cryopreservation technique is as effective as controlled-rate slow freezing; and (3) the combination of diluent, cryoprotectant and freezing protocol for optimal sperm preservation.

Materials and methods

Animals

The adult male *E. quoyii* ($n = 54$; mean (±s.d.) SVL 114.5 ± 4.6 mm (range 103–126 mm)) used in this study were wild caught in the spring of 2010 from five separate sites throughout the Sydney region (Noble *et al.* 2013) and had been in captivity for 4 years before our first attempts at sperm collection. Individuals were identified using microchips (passive integrated transponder tags) that were previously inserted as part of an unrelated study (see Noble *et al.* 2013). For the duration of the present sperm cryopreservation study, we housed the skinks under seminatural conditions within large outdoor circular bins (diameter 3200 mm, height 500 mm) containing bark mulch substrate with logs and roofing tiles for refuges. No more than six males were held in any individual enclosure. Skinks had constant access to water in a container large enough for submersion and were fed crickets or dog food 3 days per week with the potential for additional free-living novel prey *ad libitum*.

On each experimental day, the skinks were hand caught and transferred to the laboratory in individual calico bags, no more than six males at a time. The skinks were kept at room temperature (19.0–21.0°C) before sperm collection and immediately transferred back to their enclosures after recovering from the procedure. For reference, the historical range for mean high temperature at Macquarie University for September and October is between 19.7°C and 24.1°C (BOM 2020).

All our animal handling and experimental procedures were approved by the Macquarie University Animal Ethics Committee (ARA2015-024/10). Furthermore, all our work was conducted under the principles and guidelines of The Code (National Health and Medical Research Council 2013) and relevant state legislation (*Animal Research Act 1985* (NSW), *Animal Research Regulation 2010* (NSW)).

Sperm collection and handling

Unless stated otherwise, chemicals were purchased from Sigma Aldrich.

Spermatozoa were collected using a non-lethal manual collection technique adapted from Zacariotti *et al.* (2007; Viperidae) and Molinia *et al.* (2010; Scincidae). Ejaculates were collected only once per male per breeding season. A local anaesthetic (Lignocaine 20; Troy Laboratories) was administered craniolateral to the cloacal opening across two injection sites (2 × 3 units; total dose ≤10 mg kg^{-1}), and animals were rested for 5 min. It was necessary to maintain hand restraint during this time because increased abdominal pressure, through voluntary movement, was enough to expel spermatozoa following cloacal relaxation, resulting in the loss or contamination of the sample.

After 5 min, we gently opened the cloaca and flushed it with warm, sterile Ca^{2+}/Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS; D8537) to remove contaminating faeces and urates. Approximately 100 µL DPBS was then placed in the cloaca to help in the collection of spermatozoa by ventral massage, which consisted of gentle pressure applied in a rocking motion between the ischium and pubis. Skink spermatozoa did not readily suspend in DPBS and could be picked up as a pellet using a sterile 20 µL pipette tip and transferred to an Eppendorf tube. Any remaining DPBS supernatant was removed and the pellet was immediately overlaid with an equal volume of fresh, warmed (24°C) DPBS to reduce evaporation. Total sperm volume per animal was cumulative from two to three massage attempts.

Previously, we determined that spermatozoa do not freely disperse in DPBS and remain as a negatively buoyant sperm pellet with mass activity; this motility is maintained at room temperature for up to 16 h (Hobbs *et al.* 2019). Consequently, DPBS was chosen as the base diluent for sperm handling, membrane assessment and as a control for subsequent

cryopreservation experiments in *E. quoyii*. Previously, DPBS has been shown to support saltwater crocodile *C. porosus* (Johnston *et al.* 2014*a*) and yellow-spotted monitor *Varanus panoptes* (Campbell *et al.* 2020) sperm dilution and cryopreservation. Tris–citrate–glucose (TCG) buffer (composition: 36.3 g L^{-1} Tris (hydroxymethyl) aminomethane, 19.9 g L^{-1} citric acid (monohydrate) and 10 g L^{-1} D-glucose; pH 7.4; 280 mOsmol; Johnston *et al.* 2000) was the only buffer tested in which *E. quoyii* spermatozoa became free swimming. Motility was maintained at room temperature for 60 min, but motility deteriorated significantly over the course of 3 h, reaching a nadir by 16 h (Hobbs *et al.* 2019). Thus, TCG was used across all experiments to assess progressive motility, total motility and cell counts.

Sperm quality metrics

All raw, prefreeze and post-thaw sperm assessments were performed on a microscope warm stage at 28–32°C. All counts were conducted in duplicate or triplicate for each sample on five or more random fields of view until ≥100 cells were counted.

Membrane integrity assessment

A 1-μL aliquot of the sperm sample (initial dilution or prefreeze cryoprotectant (CPA) treated) was diluted in 99 μL DPBS containing ×1 SYBR Green I (S9430) and 0.001 ng mL^{-1} propidium iodide (PI; P4864) and incubated in the dark for 5 min at 28–32°C. Post-thaw samples were diluted 1 : 9 (sperm : staining solution, v/v) due to low sperm concentrations. A wet mount slide was prepared and viewed under fluorescence at a magnification of ×400 to determine the proportion of green (intact)- versus yellow/red (disrupted)-stained sperm cells.

Sperm motility

A 1-μL aliquot of the sperm sample was diluted in 99 μL TCG to assess sperm motility. After 5 min incubation in TCG in the dark at 28–32°C, wet mounts were prepared and viewed at a magnification of ×400 with phase contrast. Sperm motility was objectively categorised as progressive (forward moving), non-progressive (stationary movement or non-linear) or non-motile for a minimum of 100 cells across at least five random fields of view. Total motility was calculated as the sum of progressive and non-progressive motile spermatozoa.

Sperm concentration

Sperm concentration was calculated for individual animals ($n = 33$) and pooled aliquots using the same method. A single sperm dilution was prepared for each assessment by diluting 1 μL sperm sample in 99–499 μL TCG; the volume varied in order to create a single cell suspension of appropriate concentration to ensure accurate and rapid counting. Diluted spermatozoa were loaded into two chambers of an improved Neubauer chamber and intact sperm cells were counted at a magnification of ×400 under phase contrast.

Sperm cryopreservation

Across all cryopreservation experiments, raw sperm aliquots were pooled from four to six males for each experimental replicate to provide enough spermatozoa to distribute equally across treatments. Each sperm collection and assessment procedure lasted approximately 10 min. Sperm samples were held in the dark at 24°C for a maximum of 60 min before pooling (Hobbs *et al.* 2019) and only sperm samples exhibiting >60% total motility were included in pooled aliquots for cryopreservation experiments. Individual males contributed to one pooled sperm aliquot for each experiment. All cryopreservation experiments were repeated a minimum of three times, with a minimum of two replicate straws per treatment group. Following cryopreservation, sperm samples were held in a dry shipper for up to 5 days before being transferred to Taronga's CryoDiversity Bank in Dubbo, NSW, Australia, and stored under liquid nitrogen. Samples were held for a minimum of 3 months before post-thaw assessment.

Experiment 1: effects of sperm diluent (constant CPA) on post-thaw sperm quality metrics

Two complex sperm diluents containing different protein sources (egg yolk (EY) or albumin) were selected and tested for their ability to improve cryopreservation outcomes compared with DPBS, used successfully in the yellow-spotted monitor (Campbell *et al.* 2020) and the saltwater crocodile (Johnston *et al.* 2014*a*), in the presence of 1.35 M DMSO or 1.35 M glycerol under controlled rate freezing. Sperm diluents, namely Beltsville poultry semen extender (BPSE; composition 51.27 mM monosodium glutamate, 27.75 mM D-fructose, 8.51 mM N-[Tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid (TES), 1.42 mM magnesium chloride, 1.97 mM potassium citrate, 72.91 mM potassium phosphate monobasic, 4.78 mM potassium phosphate dibasic, 52.42 mM sodium acetate, 4.1 μM penicillin G, 4.6 μM streptomycin and 0.3% (w/v) bovine serum albumin (BSA); pH 7.2; mean (±s.d.) 335 ± 2 mOsmol kg^{-1}) and TES and Tris (TEST) yolk buffer (TYB; composition 176.01 mM TES, 8.99 mM D-fructose, 79.99 mM Trizma base, 4.1 μM penicillin G, 4.6 μM streptomycin and 20% (v/v) chicken EY; pH 7.2; mean (±s.d.) 335 ± 2 mOsmol kg^{-1}), have previously been successfully used to support non-domestic avian (Gee 1995; O'Brien *et al.* 2016), testudine (Sirinarumitr *et al.* 2010; Zimmerman and Mitchell 2017) and squamate (Zacariotti *et al.* 2012; Zimmerman *et al.* 2013; Young *et al.* 2017) sperm dilution and cryopreservation.

Pooled sperm samples (mean (±s.d.) total motility 89.0 ± 2.8%, progressive motility 5.0 ± 1.4% and 94.5 ± 5.7% membrane intact) were split evenly between the six treatments and diluted 1 : 1 (sperm : diluent, v/v) with either DPBS, BPSE or TYB and then cooled to 6°C over 2 h in a refrigerated waterbath (approximately –0.1°C min^{-1}). Samples cooled in DPBS had a mean (±s.d.) total motility of 81.4 ± 8.7% and progressive motility of 8.8 ± 6.8%, with 71.6 ± 6.5% of cells having intact membranes. Samples cooled in DTYB had a mean (±s.d.) total motility of 89.6 ± 5.1% and progressive motility of 6.3 ± 3.5%, with 92.9 ± 5.4% of cells having intact membranes. Samples cooled in BPSE had a mean (±s.d.) total motility of 80.7 ± 10.4% and progressive motility of 5.5 ± 3.1%, with 70.3 ± 14.3% of cells having intact membranes. The cooled samples were mixed gently, and precooled concentrated CPA diluted in the corresponding diluent was added in a stepwise manner (¼ volume, ¼ volume, ½ volume), with 10-min

incubation after each addition of CPA, to reach the final desired CPA concentration (1.35 M). Samples were gently mixed with a pipette and 10 µL of the sample was loaded onto 0.2-mL French straws (two or more straws per treatment). Straws were transferred to a programmable freezer (Cryobath 2000; Cryologic) and held at 4°C for 5 min then cryopreserved using a linear cooling ramp of –6°C min^{-1} to –80°C and then plunged into liquid nitrogen (slow freezing rate).

The experiment was repeated five times across three separate days using pooled sperm samples ($n = 4$–6 males per replicate; $n = 25$ males total).

Experiment 2: effects of freezing rate on post-thaw sperm quality metrics

We compared the efficacy of the same diluent/CPA combinations examined in Experiment 1 (DPBS, TYB, BPSE) in supporting sperm quality and survival using the controlled-rate slow-freezing method versus a field-friendly, fast-freezing, dry shipper cryopreservation method.

Pooled sperm samples (mean (±s.d.) total motility 69.2 ± 6.5%, progressive motility 30.1 ± 19.2%, 88.3 ± 5.0% membrane intact) were overlaid with an equal volume of DPBS and then cooled to 6°C over 2 h in a refrigerated waterbath (approximately –0.1°C min^{-1}). Following cooling in DPBS, the samples had a mean (±s.d.) total motility of 55.8 ± 7.7% and progressive motility of 18.1 ± 10.4%, with 83.7 ± 6.7% of spermatozoa having intact membranes. To further standardise between experimental replicates, the sperm concentration in pooled samples was adjusted to 400×10^6 spermatozoa mL^{-1} using cooled DPBS before the addition of complex diluents. Cooled sperm aliquots were split equally between the 12 treatments and incubated with complex diluents for 2 min at 6°C before drop-wise addition of precooled concentrated CPA.

Following the final addition of CPA, the samples were gently mixed with a pipette and then 10 µL was loaded onto 0.2-mL French straws (four or more straws per diluent/CPA) and transferred to either: (1) a programmable freezer (Cryobath 2000) and cryopreserved using the same ramp protocol as in Experiment 1; or (2) a pre-cooled (4°C) cryocane and goblet, then lowered gently (2 s) into a precharged dry shipper (Roth *et al.* 1999). Cooling rates in the dry shipper were monitored using thermocouple probes inserted into duplicate straws containing 1.35 M DMSO in DPBS. The temperature of ice nucleation was typically between –10°C and –14°C; the mean (±s.d.) dry shipper freezing rate across experimental replicates was –19.1°C ± 3.8°C min^{-1}.

Experiment 2 was repeated four times across four separate days using pooled sperm samples ($n = 4$–6 males per replicate; $n = 22$ males total).

Sample thawing: all experiments

All sperm samples were stored under liquid nitrogen for a minimum of 3 months before thawing. All the post-thaw assessments were performed in a blinded manner by the same assessor (RH).

For Experiment 1, individual straws were thawed for 10 s in air and for 30 s in a waterbath at 30°C. For Experiment 2, the straws were immediately transferred to a waterbath at 39–40°C and warmed for 10 s to thaw.

A 1- to 2-µL aliquot of spermatozoa was diluted 1 : 5 (sperm : diluent, v/v) with TCG containing 0.3% w/v BSA and incubated for 5 min at 30°C for motility assessment. Similarly, a 1- to 2-µL aliquot of spermatozoa was diluted 1 : 9 (v/v) in SYBR/PI in DPBS with 0.3% w/v BSA for assessment of membrane integrity. In Experiment 2, a 10-µL aliquot of DPBS-diluted thawed spermatozoa was also placed under mineral oil in a 35-mm culture dish and maintained at 28°C for 60 min to reassess motility status and membrane integrity 60 min after thawing.

Statistical analysis

To determine the effect of the permeating cryoprotectant and diluent on motility and membrane integrity (Experiment 1), we used generalised linear mixed models (GLMM) using the package *lme4* ver. 1.1.23 (Bates *et al.* 2015) to fit binary logistic regressions, interpreted as the proportion of successful cases (i.e. motile or membrane-intact spermatozoa), with the total number of spermatozoa equalling the weights for the model. We used a 2 × 3 factorial model design with permeating cryoprotectant (DMSO or glycerol) and diluent (BPSE, DPBS and TYB) as main effects. The interaction between the main effects was not significant and was dropped from the model. To determine the effects of freeze rate (Experiment 2; fast or slow), permeating cryoprotectant (DMSO or glycerol) and diluent (BPSE, DPBS and TYB), we used a GLMM to fit beta-binomial logistic regressions using the package *glmmTMB* ver. 1.0.1.2 (Brooks *et al.* 2017). Freeze rate, permeating cryoprotectants and diluent were used as main effects.

Overdispersion was dealt with in all models using sperm pool ID, aliquot number and the number of straws as nested random effects. All analyses were completed using R ver. 3.6.2 (R Core Team 2020). Model estimated marginal means (EMMs) and 95% confidence intervals (CIs) were determined for each condition and back-transformed to proportions. Odds ratios (ORs) comparing treatments were also generated using the package *emmeans* ver. 1.4.8 (Lenth 2020). All graphing was completed using the package *ggplot2* ver. 3.3.2 (Wickham 2016) and *gridExtra* ver. 2.3 (Auguie 2017).

Results

Sperm characteristics

We were able to extract spermatozoa on 89 of 91 attempts across all years using the adapted ventral massage technique. Samples were obtained for cryopreservation experiments from 30 animals across two breeding seasons; 14 males were sampled in both consecutive breeding seasons. Sperm volume and concentration were not correlated with SVL ($r^2 = 0.0035$ and 0.0029 respectively), despite a 23-mm length difference between the smallest (SVL 103 mm) and largest (SVL 126 mm) male (mean (±s.d.) SVL 114.5 ± 4.6 mm; median 114.0 mm).

Using light microscopy, it was seen that the gross morphology of *E. quoyii* spermatozoa (Fig. 1; Table 1) conformed to that described previously for other *Sphenomorphus* and *Egernia*-group skinks (Jamieson *et al.* 1996). The filiform spermatozoa

have a curved head with elongated acrosome, a mid-piece with four ring structures presumed to separate the columnar mitochondrion and a long tail (~78% of total sperm length).

Sperm quality metrics were highly variable between aliquots (Table 1), although most samples were of suitable quality (>60% total motility, >80% intact) for inclusion in cryopreservation experiments. The sperm concentration of raw samples ranged between 45 and 2675 $\times 10^6$ spermatozoa mL^{-1}, with a median sperm concentration of 925 $\times 10^6$ spermatozoa mL^{-1}.

Experiment 1: effects of sperm diluent (constant CPA) on post-thaw sperm quality metrics

Diluent type had a significant effect on all post-thaw sperm quality metrics, including post-thaw total motility (likelihood ratio test (LRT), $\chi^2_2 = 17.8$; $P < 0.001$), forward progressive motility (LRT, $\chi^2_2 = 17.8$; $P < 0.001$) and membrane integrity (LRT, $\chi^2_2 = 6.5$; $P = 0.039$; Fig. 2), with TYB having the highest recovery of total motility (EMM = 15.4%) and BPSE having the highest recovery of intact spermatozoa (EMM = 29.1%). There was no significant effect of CPA type on post-thaw sperm total motility (LRT, $\chi^2_1 = 0.43$; $P = 0.51$), forward progressive motility (LRT, $\chi^2_1 = 0.34$; $P = 0.56$) and membrane integrity (LRT, $\chi^2_1 = 0.18$; $P = 0.67$).

ORs were used to further compare results between treatments (see Supplementary Table S1). Among diluent treatments, 1.35 M glycerol and 1.35 M DMSO were equally effective at preserving total sperm motility (OR 1.1; 95% CI 0.9–1.4), progressive motility (OR 1.1; 95% CI 0.8–1.7) and membrane integrity (OR 1.1; 95% CI 0.8–1.6).

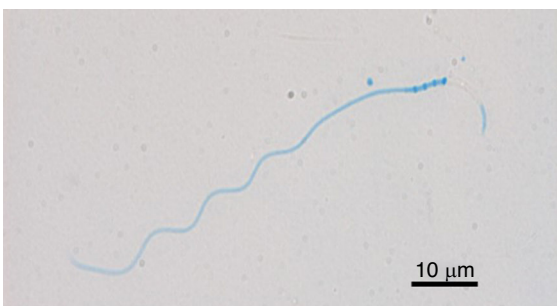

Fig. 1. Mature spermatozoon of *Eulamprus quoyii*. Spermatozoa were fixed in 4% paraformaldehyde in phosphate-buffered saline and visualised using Coomassie G250 (Larson and Miller 1999).

When results were averaged across cryoprotectant type, TYB (EMM = 15.4%) was twofold more likely and BPSE (EMM = 10.2%) 1.5-fold more likely than DPBS (EMM = 8.2%) to have a positive effect on total motility (OR 2.0 (95% CI 1.5–2.8) and 1.5 (95% CI 1.1–2.2) respectively; Fig. 1*a*; Table S1). TYB (EMM = 4.3%) was twofold more likely than BSPE (EMM 2.1%; OR 2.1; 95% CI 1.2–3.7) and fourfold more likely than DPBS (EMM = 1.1%; OR 4.0; 95% CI 2.1–7.6) to have a positive effect on progressive motility (Fig. 2*b*). In contrast, BPSE was 1.8-fold more likely to have a positive effect on membrane integrity than TYB (OR 1.8; 95% CI 1.1–3.1); the effects of DPBS were intermediate (Fig. 2*c*).

Experiment 2: effects of freezing rate on post-thaw sperm quality metrics

There were complex interactions between diluent type, CPA type and cryopreservation rate on post-thaw total motility (LRT, $\chi^2_2 = 5.5$; $P = 0.06$), progressive motility (LRT, $\chi^2_2 = 17.7$; $P < 0.001$) and membrane integrity (LRT, $\chi^2_2 = 8.2$; $P < 0.02$; Fig. 3), with little difference between DMSO and glycerol when a fast freezing rate was used, and DMSO in combination with DPBS outperforming glycerol when a slow rate was used. ORs were used to further compare results between treatments (Table S2), revealing additional significant differences between the performance of each diluent under fast freezing rates and CPA type during slow freezing on total sperm motility.

During slow-freezing cryopreservation, the use of 1.35 M DMSO in BPSE (EMM = 15.1%) or DPBS (EMM = 17.0%) was three- to fivefold more likely to have a positive effect on total sperm motility than the use of the same diluent with the alternative CPA, namely 1.35 M glycerol (EMM = 5.1% (OR 3.3; 95% CI 2.1–5.4) and EMM = 3.9% (OR 5.1; 95% CI 3.1–8.4) respectively). CPA did not affect the efficacy of TYB under slow freezing. Compared with DPBS, the use of complex diluents (TYB and BPSE) increased the likelihood of positive effects on total sperm motility using a fast freezing rate. TYB or BPSE diluents combined with either CPA (EMM 13.4–15.6%) were twofold more likely to have a positive effect on total motility than either DPBS/CPA combination (EMM 6.4–6.9%; OR 2.1–2.7; 95% CI range 1.3 to 1.7, 3.4 to 4.3).

The likelihood of retaining progressive motility under a slow freezing rate reflected the results for total motility. Using 1.35 M DMSO in either BPSE (EMM = 2.7%) or DPBS (EMM = 5.5%) was significantly more likely to have a positive effect on progressive motility than using the same diluent with the

Table 1. Characteristics of initial sperm aliquots used across all experiments

	No. aliquots assessed	Mean± s.d.	Range	Median
Volume (μL)	89	9.7± 4.2	1.5–19.0	10.0
Concentration ($\times 10^6$ cells mL^{-1})	33	945.4± 722.8	45.0–2675.0	925.0
Total motility (%)	46	63.9± 24.4	10.0–100.0	68.5
Membrane intact (%)	43	82.2± 17.6	42.0–100.0	88.5
Spermatozoa total length (μm)	4	78.3± 1.6	70.7–83.1	78.2
Head length (μm)		10.5± 0.4	9.4–12.5	10.5
Mid-piece length (μm)		6.5± 0.3	5.3–7.2	6.5

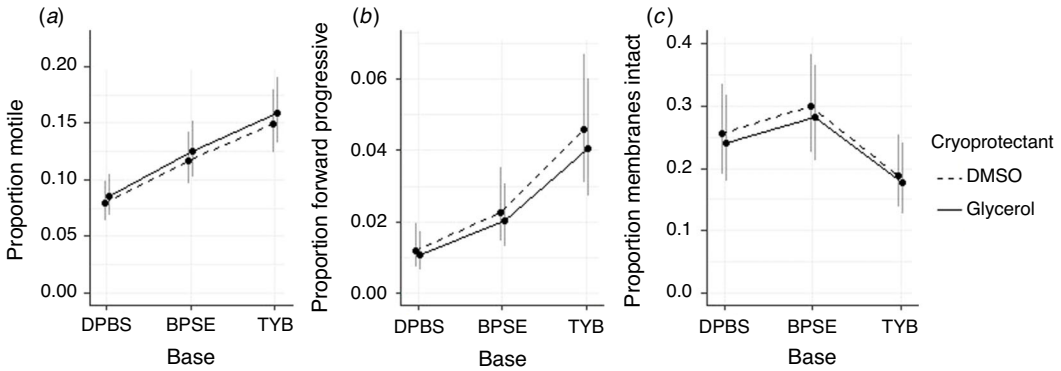

Fig. 2. Improved sperm motility following cryopreservation in complex diluents BPSE and TYB using either DMSO or glycerol as a cryoprotectant compared with a simple salt buffer (DPBS; Experiment 1). Data show predicted (*a*) total sperm motility, (*b*) progressive sperm motility and (*c*) sperm membrane integrity for each combination of cryoprotectant and diluent, based on estimated marginal means. Error bars are 95% confidence intervals.

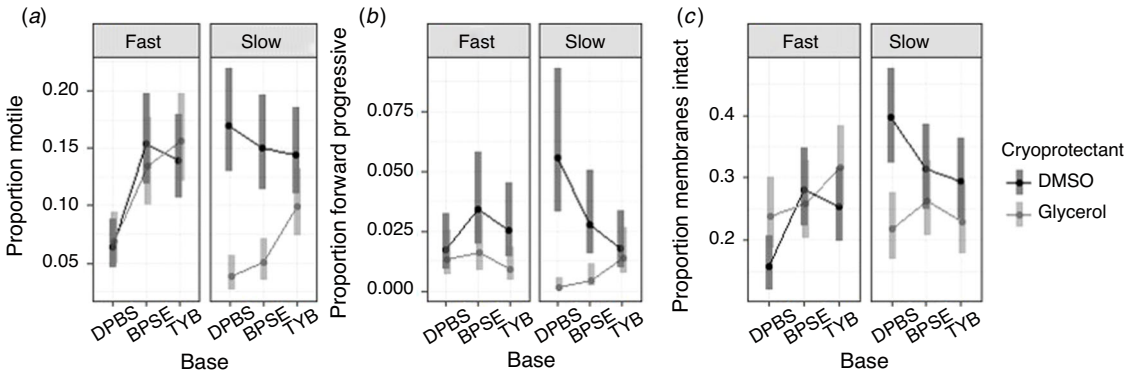

Fig. 3. Complex interactions between cryoprotectant and diluent type depending on cryopreservation rate (Experiment 2). Data show predicted (*a*) total sperm motility, (*b*) progressive sperm motility and (*c*) sperm membrane integrity for each combination of cryoprotectant, diluent and freezing rate, based on estimated marginal means. Error bars are 95% confidence intervals.

alternative CPA, namely 1.35 M glycerol (EMM = 0.4% (OR 6.4; 95% CI 2.2–18.4) and EMM = 0.1% (OR 48.3; 95% CI 10.1–231.9) respectively). Moreover, 1.35 M DMSO in DPBS was more likely to have a positive effect on progressive motility than all treatments at all freezing rates, except for 1.35 M DMSO in BPSE under a fast freezing rate (OR 1.7; 95% CI 0.8–3.5).

No single treatment was significantly better at preserving membrane integrity than all other treatments. However, 1.35 M DMSO in DPBS under a slow freezing rate (EMM = 39.7%) was more likely to result in positive membrane integrity than most other treatment combinations, ranging from the lowest for the comparison of 1.35 M DMSO in DPBS under a slow freezing rate with 1.35 M DMSO in TYB under a slow freezing rate (OR 1.5; 95% CI 1.1–2.3) to the highest for the comparison of 1.35 M DMSO in DPBS under a slow freezing rate with 1.35 M DMSO in DPBS under a fast freezing rate (OR 3.5; 95% CI 2.3–5.2). Further, 1.35 M DMSO in DPBS under a slow freezing rate was not different from 1.35 M DMSO in BPSE under a slow freezing rate and 1.35 M glycerol in TYB under a fast freezing rate. Conversely, the use of 1.35 M DMSO in DPBS at a fast freezing rate (EMM = 15.9%) was less likely to have a positive effect on membrane integrity than all treatments, except 1.35 M glycerol in DPBS under a slow freezing rate, ranging from the lowest for the comparison of 1.35 M DMSO in DPBS at a fast freezing rate with 1.35 M glycerol in TYB under a slow freezing rate (OR 1.6; 95% CI 1.1, 2.4) to the highest for a comparison of 1.35 M DMSO in DPBS at a fast freezing rate with 1.35 M DMSO in DPBS under a slow freezing rate (OR 3.5; 95% CI 2.3–5.2; Table S2).

Preservation of membrane integrity was similar between the initial and 60-min post-thaw assessment time points within groups. There was an effect of treatment, but not time (initial vs 60 min) after thawing (LRT, $\chi^2_{11} = 43.8$ ($P < 0.001$) and $\chi^2_1 = 0.43$ ($P = 0.51$) for treatment and time respectively; Fig. S1). However, only TYB-diluted spermatozoa and those cryopreserved at a fast freezing rate in 1.35 M glycerol in BPSE maintained any motility (non-progressive, <5% total spermatozoa) following 60-min post-thaw incubation in DPBS at 28°C.

Discussion

This study shows that: (1) *E. quoyii* semen can be collected in small concentrated aliquots from conscious animals at the onset

of the breeding season; (2) the functional characteristics of spermatozoa can be supported by dilution in several buffers; and (3) these spermatozoa can survive cryopreservation while maintaining minimal motility parameters using both fast and slow freezing rates.

Fresh semen samples collected in our study contained similar sperm concentrations to those obtained from South American rattlesnakes *Crotalus terrificus* using an almost identical method (see Zacariotti *et al.* 2007). These *E. quoyii* samples were also 3- to 10-fold more concentrated than samples collected by manual extraction from wild-caught *Sceloporus* (Martínez-Torres *et al.* 2019*b*) and *Anolis* species (Kahrl and Cox 2015), yet substantially less concentrated than manual extraction from saltwater crocodiles (Johnston *et al.* 2014*a*, 2014*b*). Of note, unlike some previous snake (Mengden *et al.* 1980) and crocodile (Johnston *et al.* 2014*b*) sperm extraction methods, eversion of the hemipenes or penis was not necessary for collection of ejaculate from *E. quoyii*.

We found the extracted semen had a minimal fluid component and spermatozoa in raw samples could not be separated from significant amounts of fluid by centrifugation. The small ejaculate volumes and minimal seminal plasma are consistent with the lack of accessory glands in squamates (Gist 2011). Instead, seminal fluid is derived from the sexual segment of the kidney (SSK), a mechanism that may not be stimulated by the simple manual extraction method used in this study. SSK-derived seminal fluid has been shown to support sperm function and, in some species, contributes to the formation of either aspermic or spermic copulatory plugs in squamates (Friesen *et al.* 2020). During initial, mostly unsuccessful, attempts to recover natural ejaculates from female *E. quoyii* after mating, we did observe one recently mated female eject a sperm pellet from her cloaca when handled. Under microscopic observation, this pellet appeared to consist of tightly packed spermatozoa held in a gel-like matrix; only those spermatozoa at the edges of the matrix were free to exhibit motility. We can only speculate whether this matrix formed as a result of SSK contribution or as a result of contact with, or contribution from, the female reproductive tract. However, it may go some way to explain why we found only one diluent in which ejaculated spermatozoa became free swimming. Motile spermatozoa formed tight pellets in all but one buffer tested (TCG; Hobbs *et al.* 2019). This pelleting behaviour occurred even during incubation in the presence of cryoprotectant and protein-rich diluents TYB and BPSE, and could be observed following straw loading. When *E. quoyii* semen was suspended in TCG, rapid and linear sperm movement was observed, and motility could be accurately assessed. However, and as in crocodilians (Johnston *et al.* 2014*a*), motility rapidly and significantly deteriorated over the course of a few hours (Hobbs *et al.* 2019), and so TCG was deemed unsuitable as a diluent for short-term storage or cryopreservation of *E. quoyii* spermatozoa.

Phosphate-buffered saline has proven a simple and suitable diluent for sperm extension (Tourmente *et al.* 2007, 2011; Mozafari *et al.* 2012) and cryopreservation (Johnston *et al.* 2017; Campbell *et al.* 2020) in several non-avian reptiles. Ca^{2+}/Mg^{2+}-free DPBS supports *E. quoyii* spermatozoa at room temperature (Hobbs *et al.* 2019) and we report here that spermatozoa survive and remain motile through cryopreservation at a controlled slow freezing rate and subsequent thawing in DPBS in the presence of both 1.35 M DMSO and 1.35 M glycerol with similar efficacy, although in low proportions. Although we did not statistically compare results for slow freezing between Experiments 1 and 2 due to slight methodological advances between years, most of the difference in the efficacy of treatments between the years can be attributed to improved membrane integrity and motility in DPBS-/DMSO-treated spermatozoa in Experiment 2. It is worth noting, spermatozoa cryopreserved at a slow freezing rate were thawed at a faster rate in Experiment 2, which may account, in part, for the improved membrane integrity in DMSO-treated groups compared with the matching treatment in Experiment 1.

Dilution of *E. quoyii* spermatozoa in complex diluents of similar osmolality and pH to DPBS but containing additional components (including animal protein/lipids, D-fructose and antibiotics) significantly improved sperm motility parameters under a slow freezing rate in samples where the sperm concentration was not adjusted to a standard value before freezing (Experiment 1). When sperm concentrations were adjusted before the addition of a cryodiluent (Experiment 2), the complex diluents only had a significant effect on sperm cryotolerance during fast freezing (–19°C min^{-1}), such that sperm motility was significantly reduced in their absence (DPBS).

The exact mechanisms underlying the positive effects of BSA, EY and EY-derived low-density lipoproteins on sperm tolerance to freezing is still debated, complex and multifaceted, and appear to be most effective in species or genetic lines whose spermatozoa are susceptible to cold shock (Holt 2000; Bergeron and Manjunath 2006). In the present study, when spermatozoa were diluted in complex diluents before cooling and the addition of CPA (Experiment 1), sperm motility was superior in both complex diluents compared with DPBS when spermatozoa were cryopreserved at a slow freezing rate, regardless of the CPA used. In contrast, spermatozoa diluted in complex diluents after cooling and before the addition of the CPA (Experiment 2), a time point at which cold shock-induced damage would likely already be present in all treatment groups, sperm progressive motility was highest in the DPBS–DMSO treatment, a diluent absent of protein or lipid supplements, compared with all other treatments. This suggests that, as in the saltwater crocodile (Johnston *et al.* 2014*a*), EY may not be a major contributor to sperm cryotolerance in skinks. In mammals, the addition of EY to ejaculates has been shown to affect sperm membrane cholesterol efflux (Bergeron and Manjunath 2006). Although this mechanism has not been directly demonstrated in birds, the addition of EY has been shown to improve cryosurvival and motility in domestic chicken spermatozoa (Santiago-Moreno *et al.* 2012) in the absence of cold shock sensitivity in this species (Parks and Lynch 1992). Moreover, sperm membrane fluidity is one predictor of sperm cryotolerance in birds and is directly related to the cholesterol : phospholipid ratio (Blesbois *et al.* 2005), such that lower cholesterol content, and thus higher membrane fluidity, increases spermatozoa survival after thawing. Artificial removal of cholesterol from domestic chicken spermatozoa before cryopreservation also induces significant increases in sperm post-thaw survival (Partyka *et al.* 2016).

Determination of skink sperm membrane sterol and phospholipid content in fresh and cryopreserved spermatozoa could inform the need for and identity of protein and lipid components in future cryodiluents.

The positive effects of complex diluents may also be attributed to the presence of TES and/or the cryoprotective properties of the non-penetrating sugar (i.e. fructose) present in both complex diluents tested. Mono-, di- and trisaccharides show varying degrees of cryoprotection that can be species specific in nature (Yildiz *et al.* 2000; Gómez-Fernández *et al.* 2012). Osmotically active non-penetrating agents, such as fructose, can alter membrane phase and permeability during cooling in a similar manner to permeable cryoprotectants (Sieme *et al.* 2016). Moreover, fructose is a metabolic substrate in spermatozoa (although lower in affinity than glucose) and may directly contribute to the measurement of increased motility parameters (Rodríguez-Gil 2013). The addition of the non-penetrating cryoprotectant sucrose in combination with glycerol improves crocodilian sperm cryotolerance (Johnston *et al.* 2017), with no significant differences in sperm motility between similar fast (~20°C min^{-1})- and slow (6°C min^{-1})-freezing methods. A combination of penetrating cryoprotectants is also more effective for sperm preservation than single CPAs in Burmese pythons *Python bivittatus* (Young *et al.* 2018). Investigating combinations of cryoprotectants is an obvious next step in lizard sperm cryopreservation.

Under conditions of slow freezing, DMSO was more effective at maintaining sperm quality parameters than glycerol. A similar finding has been reported for epididymal spermatozoa from the Argentine black and white tegu *Salvator merianae* (Young *et al.* 2017). However, *E. quoyii* spermatozoa treated with glycerol maintained motility in similar proportions to DMSO-treated spermatozoa under fast freezing conditions, but only when diluted in complex diluents. Although post-thaw sperm membrane integrity was more negatively impacted during fast freezing and thawing, our results show that both methods offer similar efficacy in sperm cryopreservation for this species if certain combinations of diluent and CPA are selected. This offers greater flexibility in the application of such methods for conservation purposes. Dry shippers are simple, portable 'freezing machines' that do not require electricity, computer connection or the transport of liquid nitrogen. This offers a field-friendly option for the study and preservation of spermatozoa, and thus genetic diversity, from wild-living animals. If a laboratory, or field station, is available to conduct slow-freezing protocols, then the optimal choice would be the use of DMSO in a simple diluent, namely DPBS.

There is clearly room for further optimisation, with the optimum diluent/CPA/freeze rate combination yielding a maximum of approximately 40% membrane intact spermatozoa and 17.5% total motility (from initial median values of approximately 90% and 70% respectively). Although DMSO and glycerol are both promising candidates for skink sperm cryopreservation, their use for conservation biobanking purposes will be guided by future investigations in squamates to understand whether either CPA exhibits contraceptive properties in the female reproductive tract, as has been reported in domestic chicken hens for glycerol-treated spermatozoa (e.g. Long and Kulkarni 2004).

Establishing a non-lethal semen collection and preservation technique for *E. quoyii* is a gateway to longitudinal studies of male reproductive health and fertility, sexual selection mechanisms and mating systems, as well as providing foundational information that can be applied for the conservation of other, more threatened, skink species. Optimising and adapting assisted reproductive technologies may assist gene flow across increasingly fragmented landscapes, such as those affected by agriculture, natural resource extraction and urbanisation (all major threats to reptile persistence; see Böhm *et al.* 2013), whether that be by establishing frozen reserves, cool storage or fresh collection for direct AI.

Conflicts of interest

The authors declare no conflicts of interest.

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Reproduction, Fertility and Development

Supplementary Material

Sperm cryopreservation in an Australian skink (*Eulamprus quoyii*)

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Table S1. Odds Ratios and Confidence Intervals of Total Motility (Motile), Forward Progressive Motility, Non-progressive Motility and Membrane Integrity (Membrane Intact) for sperm cryopreserved using different combinations of base diluent (DPBS, BPSE, TYB), and cryoprotectant (DMSO or glycerol) at a slow-freezing rate only. OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; DMSO = 1.35 M DMSO; Glycerol = 1.35 M glycerol.

Table S2. Odds Ratios and Confidence Intervals of Total Motility (Motile), Forward Progressive Motility, Non-progressive Motility and Membrane Integrity (Membrane Intact) for sperm cryopreserved using different combinations of base diluent (DPBS, BPSE, TYB), cryoprotectant (DMSO or glycerol) and either a fast- or slow-freezing rate. OR = odds ratio; LCL = lower confidence limit; UCL = upper confidence limit; DMSO = 1.35 M DMSO; Glycerol = 1.35 M glycerol.

Fig. S1. Predicted sperm membrane integrity at zero and 60 minutes post-thaw for each combination of cryoprotectant, diluent, and freezing rate; based on estimated marginal means. Error bars = 95% confidence intervals.