Generation of a sexually mature individual of the Eastern dwarf tree frog, *Litoria fallax*, from cryopreserved testicular macerates: proof of capacity of cryopreserved sperm derived offspring to complete development

Rose Upton¹*, Simon Clulow¹,², Michael J. Mahony¹ and John Clulow¹

¹ School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia
² Department of Biological Sciences, Macquarie University, Sydney, NSW 2109, Australia

*Corresponding author: School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia.
Email: rose.upton@uon.edu.au

Amphibians are the most threatened vertebrate class globally based on recent rates of decline and extinction. Sperm cryopreservation and other assisted reproductive technologies have the potential to help manage small and threatened populations and prevent extinctions. There are a growing number of reports of recovery of amphibian sperm after cryopreservation, but relatively few published reports of amphibian embryos generated from frozen sperm developing beyond metamorphosis to the adult stage and achieving sexual maturation. In this study on the Eastern dwarf tree frog (*Litoria fallax*), a temperate amphibian species from eastern Australia, a small number of viable metamorphs and one sexually mature male frog (itself producing sperm) were produced from cryopreserved sperm, demonstrating the capacity of embryos generated from cryopreserved sperm to complete the life cycle to sexual maturity. Low progression rates between developmental stages were not deemed to be due to effects of cryopreservation, since control embryos from unfrozen sperm had a similarly low progression rate through development.

Key words: ART, assisted reproductive technologies, conservation, cryopreservation, IVF, sperm

Introduction

Assisted reproductive technologies (ARTs) for amphibians are of great interest because of their potential to assist the conservation of threatened amphibians that now face decline and extinction at a rate that is unparalleled amongst the vertebrates (Clulow et al., 2014; Clulow and Clulow, 2016; Bower et al., 2017). There are a large number of species requiring direct intervention to prevent extinction through captive breeding, but this can be costly and leads to problems
of inbreeding and loss of genetic diversity (Snyder et al., 1996). The use of sperm cryopreservation to reduce captive colony costs by reducing the number of live animals required to maintain genetic diversity increases the efficiency and cost-effectiveness of such programmes (Clulow et al., 2014).

Considerable progress in cryopreservation of amphibian sperm has been reported since the first reports of the generation of embryos from cryopreserved sperm in 1998 (Browne et al., 1998; Mansour et al., 2009; Shishova et al., 2011; Langhorne et al., 2013; Uteshev et al., 2013; Clulow et al., 2014; Clulow and Clulow, 2016; Pearl et al., 2017). Nevertheless, most reports of amphibian sperm cryopreservation deal with the recovery of sperm assessed by parameters such as motility and vitality but do not report developmental success to metamorphosis or beyond.

There are only a small number of studies that report development from cryopreserved sperm to blastulae (Browne et al., 1998), tailbud stage (Praoath and Pérez, 2017) swimming larvae (Mansour et al., 2009; Shishova et al., 2011; Langhorne et al., 2013; Uteshev et al., 2013), one review reporting unpublished results of development past metamorphosis (Browne et al., 2011), and only one study that demonstrates the complete life cycle of amphibians generated from frozen sperm (Pearl et al., 2017).

The production of sexually mature adults from cryopreserved sperm is an important proof of concept in assisted reproduction generally, but especially for amphibians, since amphibian larvae produced by procedures such as nuclear transfer do not necessarily reach metamorphosis or develop to maturity (Di Berardino et al., 2003). Hence there is a need to demonstrate the completion of development to metamorphosis and beyond to the adult stage and sexual maturity in amphibians generated with cryopreserved sperm across a range of species to establish general applicability of the approach.

Here we report the completion of development to metamorphosis and sexual maturity of offspring produced from cryopreserved sperm of the Eastern dwarf tree frog.

**Materials and methods**

*Litoria fallax* is a pond-breeding species that spawns in a range of habitats across several months of the austral spring and summer (September to April), although males may call throughout the year. Thirty-five calling *L. fallax* males and seven gravid, ovulated females (found in amplexus following rain and storm events) were collected from ponds located in the Hunter Estuary (−32.84 S, 151.69 E), under NSW Scientific Licence SL101269, and euthanized by immersion in 0.4% w/v ethyl 3-aminobenzoate methanesulphonate (MS-222; Sigma Aldrich; E10521) buffered with 0.4% w/v sodium bicarbonate, followed by excision of the heart.

Males were collected ahead of time over December 2015 and housed in plastic terraria (30 cm × 18 cm × 20 cm) containing autoclaved gravel and aged tap water (to create an environment ~25% terrestrial and 75% aquatic) with no more than 15 individuals to a tank. Terraria were exposed to natural day light cycles and were supplied with crickets supplemented with Multical calcium/vitamin powder (Vetafarm, Wagga Wagga, NSW) three times weekly. Individuals were kept in this manner for at least a week prior to experiments taking place. Females found in amplexus on the 30 December 2015 were separated from the amplexing male and housed at 10°C in controlled temperature cabinets (TRSL-1175-2-SD; Thermoline Scientific, Wetherill Park, NSW) to delay oviposition (Sherman et al., 2010), allowing a delay in the commencement of IVF trials until 10–15 h following collection of animals from the field. All experimental work involving animal use was completed in accordance with the necessary guidelines under University of Newcastle ethics approval A-2013-328.

Four testicular macerates were prepared in 100 μl Simplified Amphibian Ringer (SAR; 113 mM NaCl, 1 mM CaCl₂, 2 mM KCl, 3.6 mM NaHCO₃; recipe as used by Browne et al., 1998) by teasing apart the testes within the specified volume using fine forceps. One of these was used as an unfrozen control macerate (UF1), used purely to demonstrate viability and fertility of oocytes, and pooled both testes from five male *L. fallax*. The remaining three macerates (CR1, CR2 and CR3) each pooled both testes of 10 male *L. fallax* per macerate (to adjust for loss of sperm and a decrease in sperm concentration following cryopreservation and washing procedure), and were cryopreserved in the following way: each macerate was extended at a dilution ratio of 1:6 with 15% v/v dimethyl sulfoxide (Me₂SO) and 1% w/v sucrose solution immediately after maceration, before being loaded into 250 μl cassou straws. The cryoprotectant solution was modified from that of Browne et al. (1998), following results of experimental observations indicating that 1% w/v sucrose was more appropriate for this species than 10% w/v sucrose in the extender (our unpublished work). Straws were loaded into the chamber of a controlled programmable freezing device (Cryologic, model CL3300) and frozen using a cooling ramp adapted from Browne et al. (1998); briefly, this included a 10 min hold step at 2°C followed by a −1°C min⁻¹ ramp to −8°C, a −3°C min⁻¹ ramp to −16°C and a −3.4°C min⁻¹ ramp to −80°C, followed by quenching in liquid nitrogen (Browne et al., 1998).

Straws were subsequently thawed at room temperature (after a minimum quench time of 30 min within liquid nitrogen) and taken through a wash protocol to remove cryoprotectants. Thawed macerates were centrifuged twice for 2 min at 1000 x g, first in 500 μl, and a second time in 100 μl of SAR. Following centrifugation, each macerate was activated by a 1:6 dilution in distilled water and added to ova (removed from the oviducts of euthanized, ovulated females) in a dry 3.5 mm diameter petri dish. Twenty minutes was allowed for fertilization (indicated by rotation of the animal pole to the upper surface of the ovum) before flooding the dish with 2 ml of 10% v/v SAR.
The ova from seven female *L. fallax* (found in amplexus) were used in the experiment, with 30–100 oocytes per petri dish depending on the number of ova recovered from each female. Ova from each female were fertilized with unfrozen macerate UF1 and with two of CR1, CR2 or CR3 frozen-thawed macerates. Sperm concentrations were not measured directly in individual dishes but were calculated from the macerates after cryopreservation and were in the order of $2.75 \times 10^6$ for the fresh macerate and $4.2 \times 10^3$ to $2.5 \times 10^6$ to cells per ml for two of the cryopreserved macerates, but were not measured in the third.

After the completion of IVF, the hatching, development and metamorphosis of the fresh control and experimental cryopreserved sperm treatments were monitored for 20 weeks. The sexual maturity of two adult (both male) frogs derived from the IVF procedures (observed –1 year after fertilization), one resulting from the use of thawed, cryopreserved sperm and one resulting from the control group using unfrozen sperm, were tested by determining the presence of sperm in urine after administration of human chorionic gonadotropin (hCG) (Chorulon; MSD Animal Health, Bendigo, Victoria) at a dosage rate of 3 IU/g body weight (a dosage rate within reported range causing sperm release in anurans (Kouba et al., 2012; Clulow et al., 2018)).

Frequency data were analysed by chi-square contingency analysis, subjected to Yates correction where cell numbers were below 10. Statistical calculations were computed via the Vassarstats portal (http://www.vassarstats.net/).

### Results

The number of embryos produced from each treatment (summed across the replicates) and the progression to later developmental stages are shown (Table 1). Progression to hatched tadpoles was significantly higher in cryopreserved replicates ($X^2, P < 0.001$), and progression to mature tadpoles (Fig 1B–C) from hatched tadpoles (Fig. 1A) was nominally higher (but not statistically higher with Yates corrected $X^2, P = 0.086$) in unfrozen macerate IVF’s.

Taken together, the data show a similar progression between unfrozen and cryopreserved macerate offspring, indicating that the relatively low progression to metamorphosis and sexual maturity (Fig. 1) was a function of loss between developmental stages and not due to deleterious effects of macerate cryopreservation.

### Discussion

The purpose of this study was to demonstrate that cryopreserved sperm of *L. fallax* are capable of producing viable larvae that reach metamorphosis and sexual maturity. Although, no adult females were produced in this study, and the overall survival rates were low, the production of sperm from the male generated from cryopreserved sperm indicates that sexual maturity and the production of gametes in offspring generated from frozen sperm in this species is feasible. Low survival rates observed during the study were most likely due to sub-optimal IVF conditions or ova quality that impacted later development stages, resulting in few individuals reaching metamorphosis and beyond. Nevertheless, as embryos experienced similar rates of abnormalities within the unfrozen treatment compared to the cryopreserved sperm treatment, the cause of the problems are not related to cryopreservation. It is likely that further optimization of the IVF protocol used here may result in a higher hatching rate from both unfrozen and cryopreserved sperm IVF in this species. Further investigation should result in improved developmental success after IVF in this species. IVF conditions that need to be investigated to increase embryo survival include holding times for ova and sperm prior to mixing, ionic composition and osmolality of media (Edwards et al., 2004) and possibly inclusion of anti-oxidants, since reactive oxygen species damage to gametes and embryos is a possible cause of embryonic loss (Guerin et al., 2001).

| Stage | Unfrozen macerates—no. surviving (7 IVF replicates) | Cryopreserved macerates—no. surviving (14 IVF replicates) |
|-------|-----------------------------------------------------|----------------------------------------------------------|
| Embryo (Gosner Stages 1–19) | 351/413 (84.9%) | 216/603 (35.8%) |
| Hatched Tadpole (Gosner Stage 20) | 105 (29.9%) | 133 (61.5%) |
| Mature Tadpoles (pre-metamorphosis; Gosner stage 41) | 10 (9.5%) | 4 (3.0%) |
| Metamorphosis (undergoing metamorphosis; Gosner stages 42–45) | 1 | 4 |
| Juvenile Frog (tail absorbed; Gosner Stage 46) | 1 | 3 |
| Sexually Mature Frog | 1 | 1 |

Percentage values indicate percent at previous stage proceeding to the following stage in each treatment.
Even though there are reports of the cryopreservation of sperm from a growing number of amphibian species, and the generation of embryos and tadpoles for some of those species (Browne et al., 1998; Mansour et al., 2009, 2010; Shishova et al., 2011; Langhorne et al., 2013; Uteshev et al., 2013; Clulow et al., 2014; Clulow and Clulow, 2016; Pearl et al., 2017; Proaño and Pérez, 2017), this study provides one of only two reports for amphibians in which the production of sexually mature adult frogs derived from cryopreserved sperm has been demonstrated, either through the production of a mature individual producing gametes (as in this study), or through the generation of fertile adults that subsequently reproduced (Pearl et al., 2017). Consequently, this study provides additional proof of concept support for the capacity to use ARTs in declining amphibian species in captive breeding and conservation programmes. Nevertheless, the authors know of only one captive breeding programme involving an endangered species that has incorporated the use of cryopreserved sperm to add back lost wild-type genes to the captive bred population (Howard et al., 2015).

Given the serious decline of amphibian species globally, there is pressure to apply efficient and optimized captive breeding to amphibian conservation using best practice approaches that avoid adverse outcomes such as selection for domestic traits and loss of genetic fitness associated with captive breeding (Snyder et al., 1996; Kraaijeveld-Smit et al., 2006; Griffiths and Pavaujeau, 2008; Bowkett, 2009). There is a strong argument that the proof of concept of capacity to generate sexually mature animals from cryopreserved sperm demonstrated here and elsewhere should be considered by amphibian conservation programme managers for incorporation into their breeding protocols to maximize retention of wild-type genetic diversity.

Acknowledgements

We thank Stephen Mahony for providing photographs. We also thank Lesley Wong and Rebecca Seeto who provided laboratory assistance. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

Bower DS, Lips KR, Schwarzkopf L, Georges A, Clulow S (2017) Amphibians on the brink. Science 357: 454.

Bowkett AE (2009) Recent captive-breeding proposals and the return of the ark concept to global species conservation. Conserv Biol 23: 773–776.

Browne RK, Clulow J, Mahony MJ, Clark AK (1998) Successful recovery of motility and fertility of cryopreserved cane toad (Bufo marinus) sperm. Cryobiology 37: 339–345.

Browne R, Li H, Robertson H, Uteshev V, Shishova N, McGinnity D, Nofs S, Figiel C, Mansour N, Lloyd R (2011) Reptile and amphibian
conservation through gene banking and other reproduction technologies. Russ J Herpetol 13: 165–174.

Clulow J, Clulow S (2016) Cryopreservation and other assisted reproductive technologies for the conservation of threatened amphibians and reptiles: bringing the arts up to speed. Reprod Fertil Dev 28: 1116–1132.

Clulow J, Pomering M, Herbert D, Upton R, Calatayud N, Clulow S, Mahony MJ, Trudeau VL (2018) Differential success in obtaining gametes between male and female Australian temperate frogs by hormonal induction: a review. Gen Comp Endocrinol. doi:10.1016/j.ygcen.2018.05.032.

Clulow J, Trudeau VL, Kouba AJ (2014) Amphibian declines in the twenty-first century: why we need assisted reproductive technologies. Adv Exp Med Biol 753: 275–316.

Di Berardino MA, McKinnell RG, Wolf DP (2003) The golden anniversary of cloning: a celebratory essay. Differentiation 71: 398–401.

Edwards DL, Mahony MJ, Clulow J (2004) Effect of sperm concentration, medium osmolality, and oocyte storage on artificial fertilisation success in a myobatrachid frog (Limnodynastes tasmaniensis). Reprod Fertil Dev 16: 347–354.

Griffiths RA, Pavajeau L (2008) Captive breeding, reintroduction, and the conservation of amphibians. Conserv Biol 22: 852–861.

Guerin P, El Mouatassim S, Menezo Y (2001) Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7: 175–189.

Howard J, Lynch C, Santymire RM, Marinari PE, Wildt DE (2015) Recovery of gene diversity using long-term cryopreserved spermatozoa and artificial insemination in the endangered black-footed ferret. Anim Conserv 19: 102–111.

Kouba AJ, delBarco-Trillo J, Vance CK, Milam C, Carr M (2012) A comparison of human chorionic gonadotropin and luteinizing hormone releasing hormone on the induction of spermiation and ampheplex in the American toad (Anaxyrus americanus). Reprod Biol Endocrinol 10: 59.

Kraaijeveld-Smit FJL, Griffiths RA, Moore RD, Beebee TJC (2006) Captive breeding and the fitness of reintroduced species: a test of the responses to predators in a threatened amphibian. J Appl Ecol 43: 360–365.

Langhorne CJ, Calatayud NE, Kouba AJ, Feugang JM, Vance CK, Willard ST (2013) 026 cryoconservation: successful sperm cryopreservation and develop-mental outcomes using endangered north American amphibians. Cryobiology 67: 405.

Mansour N, Lahnsteiner F, Patzner RA (2009) Optimization of the cryopreservation of African clawed frog (Xenopus laevis) sperm. Theriogenology 72: 1221–1228.

Mansour N, Lahnsteiner F, Patzner RA (2010) Motility and cryopreservation of spermatozoa of European common frog, Rana temporaria. Theriogenology 74: 724–732.

Pearl E, Morrow S, Noble A, Lerebours A, Horb M, Guille M (2017) An optimized method for cryogenic storage of xenopus sperm to maximise the effectiveness of research using genetically altered frogs. Theriogenology 92: 149–155.

Proaño B, Pérez OD (2017) In vitro fertilizations with cryopreserved sperm of Rhinella marina (anura: Bufonidae) in ecuador. Amphib Reptile Conserv 11: 1–6.

Sherman CD, Wapstra E, Olsson M (2010) Sperm competition and offspring viability at hybridization in Australian tree frogs, Litoria peronii and L. tyleri. Heredity (Edinb) 104: 141–147.

Shishova NR, Uteshev VK, Kaurova S, Browne RK, Gakhova EN (2011) Cryopreservation of hormonally induced sperm for the conservation of threatened amphibians with Rana temporaria as a model research species. Theriogenology 75: 220–232.

Snyder NF, Derrickson SR, Beissinger SR, Wiley JW, Smith TB, Toone WD, Miller B (1996) Limitations of captive breeding in endangered species recovery. Conserv Biol 10: 338–348.

Utessheh VK, Shishova N, Kaurova S, Manokhin A, Gakhova E (2013) Collection and cryopreservation of hormonally induced sperm of pool frog (Pelophylax lessonae). Russ J Herpetol 20: 105–109.