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Effect of sperm concentration, medium osmolality and oocyte storage on artificial fertilisation success in a myobatrachid frog (*Limnodynastes tasmaniensis*)

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Abstract. The present study optimised artificial fertilisation and oocyte storage conditions in *Limnodynastes tasmaniensis* (Myobatrachidae). Data on general reproductive biology, the effect of sperm motility and concentration, medium osmolality and oocyte storage on artificial fertilisation success are presented. Egg number was most strongly correlated with bodyweight ($r = 0.819$). Sperm yield was correlated with testes weight ($r = 0.827$), which was strongly correlated with snout–vent length ($r = 0.772$). Optimal artificial fertilisation occurred in 0–7 mOsm kg⁻¹ amphibian Ringer, similar to ranid, bufonid and hylid species. High fertilisation rates were achieved using spermatozoa with little forwards progressive motility at comparatively low concentrations (3×10^4 sperm cells mL⁻¹) and with no relationship between percentage sperm motility and fertilisation success (correlation of fertilisation rate with sperm motility after activation: $r = -0.145$). Oocytes stored in 5 mOsm kg⁻¹ solutions showed no significant decline in fertilisability after 2 h, showing that swelling of the jelly surrounding the eggs does not prevent sperm from fusing with the oocyte in this species. Fertilisability of oocytes was extended to >4 h in medium to high osmolality solutions (124–271 mOsm kg⁻¹). These data allow for the future use of *L. tasmaniensis* in developing assisted reproductive technology protocols for foam-nesting myobatrachid species, many of which are now threatened with extinction in the wild.

Introduction

Myobatrachids (Australasian ground frogs) are the most diverse Australian anuran family, comprising up to 50% of the Australian amphibian fauna (Cogger 1992). They can be distinguished from other anuran families most specifically by aspects of their reproductive biology, which are unique among the Amphibia. These include a unique sperm ultra-structure (Lee and Jamieson 1992) and a lack of forwards progressive sperm motility (Clark 1994; Browne *et al.* 2002). The reduced motility of myobatrachid sperm may in part be explained by the displacement of the mitochondria from the axoneme and rigidity engendered by the axial fibre, which is deeply embedded in the nuclear fossa (Lee and Jamieson 1992). The Myobatrachidae also contains a startling array of reproductive strategies, including direct development, terrestrial egg deposition followed by an obligate aquatic tadpole, as well as aquatic egg deposition and development (with and without the construction of a foam-nest) (Cogger 1992).

Limnodynastes tasmaniensis is a foam-nesting species that breeds throughout the year, except for a short period during the middle of winter (June) (Horton 1982). Breeding occurs in open water pools in grassland or swampland, although

populations that do not construct foam-nests (south-eastern region of South Australia only) spawn among vegetation to support oocyte masses (Seymour and Roberts 1991). The foam-nest is formed when the female ‘whips’ a jelly-like substance with modified flanges on each hand during spawning (Horton 1982). Upon aeration, this jelly-like substance creates a foam-nest that floats on the surface of the water. The foam-nest aerates oocytes, prevents desiccation and predation, provides insulation, and suspends embryos on the surface above the anoxic bottom of pools (Seymour and Roberts 1991).

Limnodynastes tasmaniensis is an extremely common species, but there are many other myobatrachids suffering range contractions and population declines (Alford and Richards 1999). Assisted reproductive technologies (ART) have been considered in a last effort to maintain genetic diversity in such declining wildlife populations (Benirschke 1984; Ralls and Ballou 1986; Holt *et al.* 1996; Bawa *et al.* 1997). For amphibian ART to be successful, artificial fertilisation and short-term oocyte holding protocols must be used in conjunction with sperm banks for the maintenance of genetic diversity, as amphibian oocytes cannot currently

be cryopreserved (Clulow *et al.* 1999; Mahony *et al.* 1999). Sperm cryopreservation protocols have been developed for gene banking purposes in several myobatrachids and other anuran groups (Browne *et al.* 1998; Browne *et al.* 2002).

The differences in artificial fertilisation protocols developed for *Xenopus* (high salt requirements, Wolf and Hendrick 1971; Moriya 1976) and *Rana* species (Rugh 1962) have proven that despite a uniform reproductive strategy in these species, protocols may have to vary for different anuran groups. This may particularly hold true for myobatrachid frogs, which display a diverse array of reproductive modes. Optimising artificial fertilisation and short-term oocyte storage in representative myobatrachid species, covering all developmental modes, would allow these protocols to be applied in conjunction with protocols for cryopreservation and storage of sperm in future assisted reproductive programmes for vulnerable and endangered species.

The present study aimed to optimise artificial fertilisation and oocyte storage in a foam-nesting species, *L. tasmaniensis*. The effect of sperm concentration and medium osmolality on fertilisation success was assessed, thus providing an optimal artificial fertilisation protocol. In addition, a series of oocyte holding experiments were carried out with solutions of various osmolalities to find the optimum conditions for storing oocytes for short periods. Data were also collected on the relationship between body length and mass and gamete productivity, and between sperm quality and fertilisation success.

Materials and methods

Animals

Animals were collected after the required permits were obtained from the New South Wales National Parks and Wildlife Service, and all experiments were conducted with approval of The University of Newcastle Animal Care and Ethics Committee (approval no. 706 0601). Adult male and female *L. tasmaniensis* were collected from Kooragang Island in the Hunter River estuary (32.50–32.55°S, 151.42–151.47°E). Frogs were housed in 1.30 × 60.6-m polyethylene containers and fed mealworms *ad libitum*. Randomly selected males and gravid females (identified by an observable accumulation of mature oocytes through the ventral body wall) were then transferred to 17 × 116-cm polyethylene containers (Combined Packaging, Newcastle, Australia) immediately before use in experiments.

Ovulation was induced with two injections of homogenised male cane toad (*Bufo marinus*) pituitaries into the dorsal lymph sac (three pituitaries/200 µL amphibian Ringer) 20 h apart. Amphibian Ringer (113 mM NaCl, 2 mM KCl, 1.35 mM CaCl₂, 1.2 mM NaHCO₃) is isotonic to amphibian somatic tissue with an osmolality of 217 mOsm kg⁻¹ (Rugh 1962). Females were responsive to pituitary injections throughout the study (March–October 2001), except for a few weeks in June (mid winter). Both male and female animals were killed by injection of 1 mL of 5 mg L⁻¹ MS-222 (3-aminobenzoic acid ethyl ester; Sigma, St Louis, MO, USA). Bodyweight (0.005 g with Ohaus Precision Standard Scales; Ohaus, Pine Brook, NJ, USA) and length (0.05 mm with callipers) measurements were recorded for each individual. Bodyweight was measured before the frogs were killed as the volume of anaesthetic used would alter the measurement for such small frogs. Snout–vent length was recorded after death.

Gamete collection and quality assessment

After ovulation, oocytes pass through the oviduct where the jelly layers required for fertilisation are acquired (Caputo *et al.* 2001). In *L. tasmaniensis*, the oocytes accumulate in the ovisac (the posterior section of the oviduct) to await oviposition. Oocytes were collected directly from the ovisac 20 h after the second pituitary injection and placed into 35 × 35-mm polystyrene weigh boats (Bacto Laboratories, Liverpool, NSW, Australia). Oocyte yields are stated as the number of oocytes extracted from the ovisac of individual females (851 ± 50; mean ± s.d.).

Sperm suspensions were prepared by macerating testes in 100 µL of chilled amphibian Ringer and suspensions were kept on ice until use (Browne *et al.* 1998). Sperm quality was assessed by scoring the percentage of motile sperm in a 1-µL subsample of original suspension, which was diluted 20:1 with distilled water to activate spermatozoa. Flagellar movement of spermatozoa was scored using the Emmens ranking system (Emmens 1947) and provides a measure of forwards progressive motility.

Artificial fertilisation system

The methods employed were similar to those previously used for artificial fertilisation of *B. marinus* by Browne *et al.* (1998). Oocyte masses were divided approximately equally between treatments and placed in the centre of 60 × 15-mm Petri fertilisation dishes (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA). Concentrated sperm suspension was added to the edge of each fertilisation dish (separate to oocyte masses) and 3–5 mL of fertilisation medium was then added to each dish. The volume of concentrated sperm suspension was controlled (determined by haemocytometry) so that the desired sperm concentration would be present in each dish after the addition of media. Dishes were agitated frequently for 2–3 min following the addition of media to provide an even mix of gametes, to disperse oocyte masses, and to simulate maternal mixing of egg masses following egg deposition. Adding sperm to the edge of the dish, followed by the addition of media and agitation, ensured that all oocytes were evenly exposed to the intended sperm concentration (generally 3.16 × 10⁴ sperm mL⁻¹ unless otherwise stated). Fertilisation rates were recorded as the proportion of oocytes that developed to the blastula stage. All artificial fertilisation experiments were conducted at room temperature (21°C).

Experiment 1: optimal sperm concentration

Sperm concentrations were increased on a log scale from 10³ to 3.16 × 10⁵ sperm mL⁻¹ to determine optimum sperm concentration during artificial fertilisation. A zero sperm control was included to correct for parthenogenetic cleavage, which may be initiated by defined artificial fertilisation protocols (e.g. by pricking, which activates the oocyte (Rugh 1962) and can lead to parthenogenetic development in some urodeles (Lofts 1974)). For each treatment, there were 94 ± 8 oocytes per dish, with four or five replicates per treatment (mean ± s.e.m.).

Experiment 2: effect of media osmolality on fertilisation rate

The osmolality of the fertilisation medium was varied from 0 to 217 mOsm kg⁻¹ amphibian Ringer, by dilution of 1 × Ringer with distilled water, to determine the effects of osmolality on fertilisation. The following proportional dilutions of full strength amphibian Ringer were used: 0.025× for 5 mOsm kg⁻¹; 0.05× for 7 mOsm kg⁻¹; 0.15× for 25 mOsm kg⁻¹; 0.3× for 56 mOsm kg⁻¹; 0.6× for 124 mOsm kg⁻¹; 1× for 217 mOsm kg⁻¹. These dilutions were chosen to represent a range of osmolalities from low to high ionic strengths. The osmolality of each dilution of amphibian Ringer was measured using a Vapour Pressure Osmometer 5520 (Wescor Inc., Logan, UT, USA). Amphibian Ringer was selected in this experiment because it is commonly used in artificial fertilisation experiments in anurans (Rugh 1962; Cross and Elinson 1980; Browne *et al.* 1998) and is similar to De Boer's solution

(Wolf and Hendrick 1971), another solution commonly used for artificial fertilisation in anurans (Katagiri 1962; del Pino 1973; Hollinger and Corton 1980). For each treatment, there were 79 ± 4 oocytes per dish, with between three and five replicates per treatment (mean \pm s.e.m.).

Experiment 3: effect of short-term oocyte storage on fertilisation rate

The aim of these experiments was to determine the effects of short-term oocyte storage on the oocyte fertilisability and the optimum osmolality for holding oocytes before fertilisation. Oocytes were held in 15 mL of 5 mOsm kg⁻¹ (0.025 \times) Ringer for 0.5, 1 and 2 h; in 124 mOsm kg⁻¹ (0.6 \times) Ringer for 0.5, 1, 2, 3 and 4 h; in 217 mOsm kg⁻¹ (1 \times) Ringer for 4, 8, 12 and 16 h; or in 271 mOsm kg⁻¹ (1.25 \times) Ringer for 4, 8 and 12 h before insemination. (1.25 \times Ringer was made by increasing the ionic strength of the standard Ringer by 25%.) There were 65 ± 3 oocytes per dish in the 5 and 124 mOsm kg⁻¹ treatments, and 112 ± 4 oocytes per dish in the 217 and 271 mOsm kg⁻¹ treatments (four replicates per treatment, mean \pm s.e.m.).

Statistical analysis

Pearson's correlation coefficient (r) was used to determine correlations between bivariate data, which were arcsine-transformed before analysis if data were percentages or not normally distributed. Univariate (one-way) analysis of variance was used to test for significant differences between means, with the Student–Newman–Keuls *post hoc* test used to indicate where specific differences occurred. The data from oocyte holding experiments were arcsine-transformed and analysed using a repeated-measures (multivariate) analysis of variance. Where significant differences were detected, a Student–Newman–Keuls *post hoc* test was used to determine which treatment means were significantly different. All analyses were performed using the STATISTICA 5.5 (StatSoft, Inc, Tulsa, OK, USA) software package.

Results

Indicators of reproductive output

Data for indicators of reproductive output are plotted in Fig. 1 (egg yield) and Fig. 2 (sperm yield). Egg yield was strongly correlated with bodyweight (gravid weight) ($r = 0.819$; $P < 0.01$) and length ($r = 0.663$; $P < 0.01$), with gravid weight being the stronger indicator of egg yield (female: bodyweight = 6.57 ± 1.41 g, snout–vent length = 37.66 ± 3.14 mm; mean \pm s.d.). Both external (bodyweight and length) and internal (testes weight) parameters were significant indicators of sperm yield. Sperm yield was most strongly correlated with testes weight ($r = 0.827$; $P < 0.01$), but moderate correlations were also found with bodyweight ($r = 0.505$; $P < 0.01$) and snout–vent length ($r = 0.602$; $P < 0.01$). Snout–vent length was also highly correlated with testes weight ($r = 0.772$; data not shown). (Male: bodyweight = 3.81 ± 1.04 g, snout–vent length = 33.12 ± 2.54 mm; mean \pm s.d.).

Gamete viability

Fertilisation rate was not correlated with the percentage of motile spermatozoa after activation ($r = -0.145$; data not shown). Sperm flagellar movement very rarely exceeded 1.5 on the Emmens scale (Emmens 1947), which equated to very slow motility with medium flagellar movement in the majority of spermatozoa (results not shown).

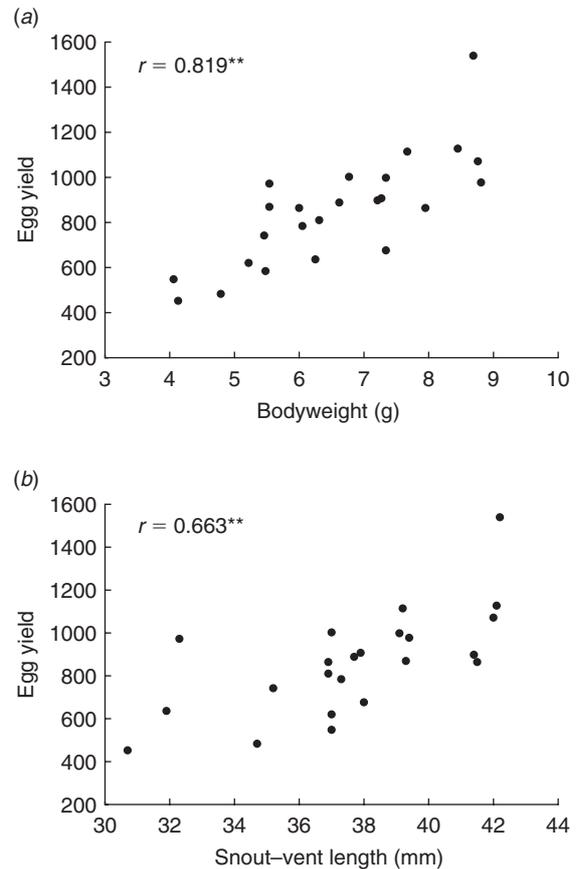


Fig. 1. Indicators of female reproductive output. (a) Egg yield per female against bodyweight ($r = 0.819^{**}$). (b) Egg yield per female against snout–vent length ($r = 0.663^{**}$). $^{**}P < 0.01$; $^{n.s.}P > 0.05$; $n = 24$.

Experiment 1: optimal sperm concentration

A plot of fertilisation rate against sperm concentration (Fig. 3) shows an increase in fertilisation rate with increasing sperm concentration to an asymptote of 3.16×10^4 sperm mL⁻¹ ($F = 39.423$; d.f. = 6; $P < 0.01$). Fertilisation rates were significantly higher when sperm concentration was greater than 10^4 sperm mL⁻¹. No cleavage (parthenogenetic activation) occurred without the addition of sperm.

Experiment 2: effect of medium osmolality on fertilisation rate

Fertilisation rate decreased with increasing osmolality of the medium and fell to 0% in iso-osmotic amphibian Ringer (217 mOsm kg⁻¹) (Fig. 4). Significantly higher rates of fertilisation were achieved using osmolalities below 7 mOsm kg⁻¹ compared with osmolalities above 124 mOsm kg⁻¹ ($F = 7.960$; d.f. = 6; $P < 0.01$). Increases in the variability of fertilisation rates also occurred at higher osmolalities. Polyspermy, measured by the appearance of multiple and irregular cleavage furrows (Elinson 1986), was not observed at any osmolality. Embryos were observed for 6 days (Stage 25; Gosner

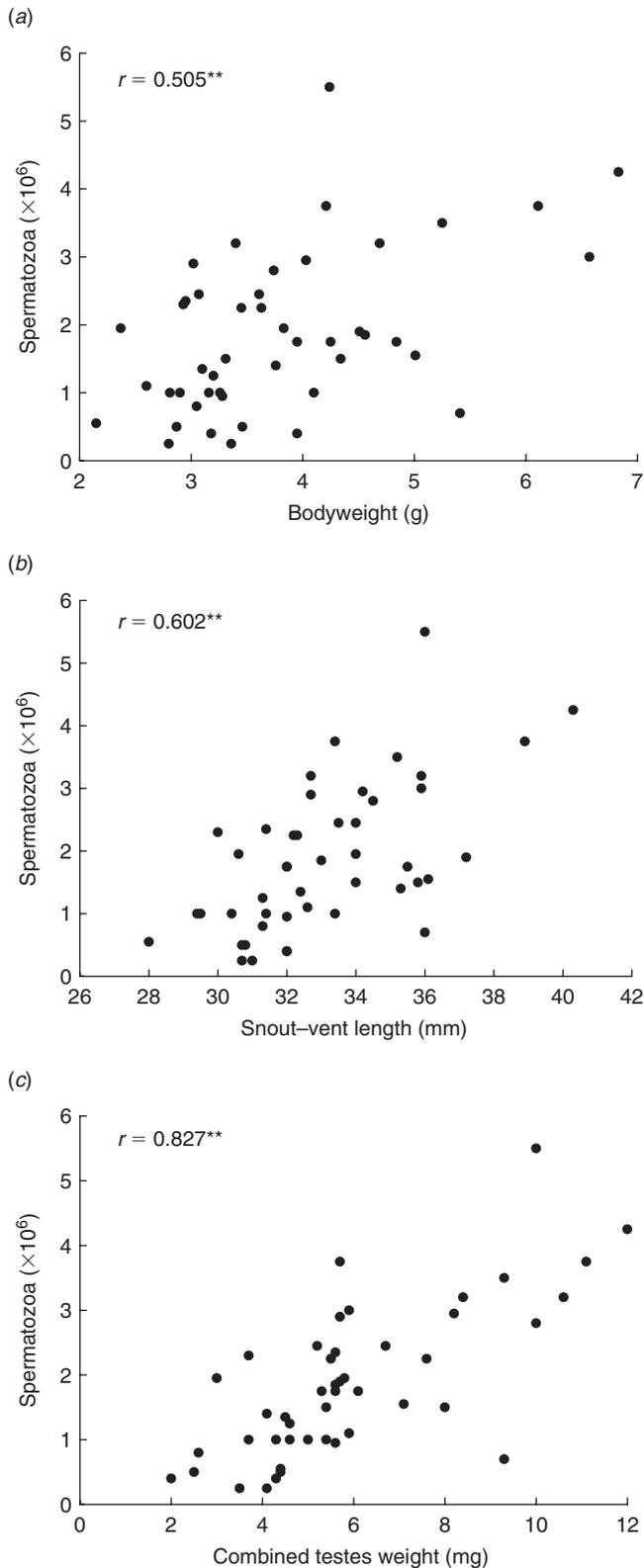


Fig. 2. Indicators of male reproductive output. Sperm yield per male against (a) bodyweight ($r = 0.505^{**}$; $n = 47$); (b) snout-vent length ($r = 0.602^{**}$; $n = 46$); (c) combined testes weight ($r = 0.827^{**}$; $n = 48$). $**P < 0.01$.

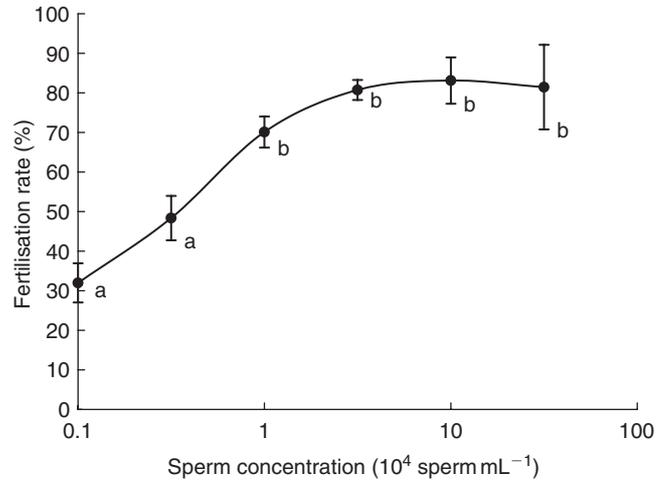


Fig. 3. Fertilisation rates at various sperm concentrations. Sperm concentration is on a log scale from 0.10^4 to 100×10^4 sperm mL^{-1} . Values are means \pm s.e.m.; n (number of females contributing oocytes) = 4–5 at each sperm concentration. Fertilisation rates with different letters are significantly different ($P < 0.05$).

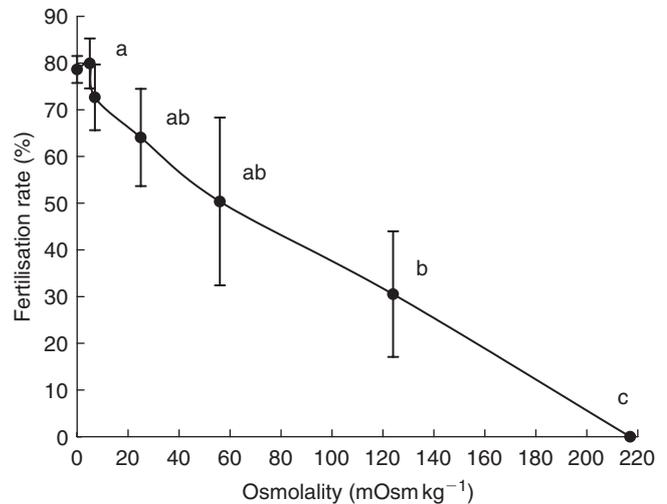


Fig. 4. Declining fertilisation rates in solutions of increasing osmolality (adjusted Ringer). Values are means \pm s.e.m.; n (number of females contributing oocytes) = 3–5 at each osmolality. Fertilisation rates with different letters are significantly different ($P < 0.05$).

1960) with no significant differences in survival in any of the osmolality treatments (data not shown).

Experiment 3: effect of short-term oocyte storage on fertilisation rate

The response of oocytes to storage at low-to-medium osmolality (5 and 124 mOsm kg^{-1} Ringer) and high osmolality is shown in Fig. 5. The latter was tested at two osmolalities (217 and 271 mOsm kg^{-1} Ringer) and in a separate experiment to the low-to-medium osmolality experiment owing

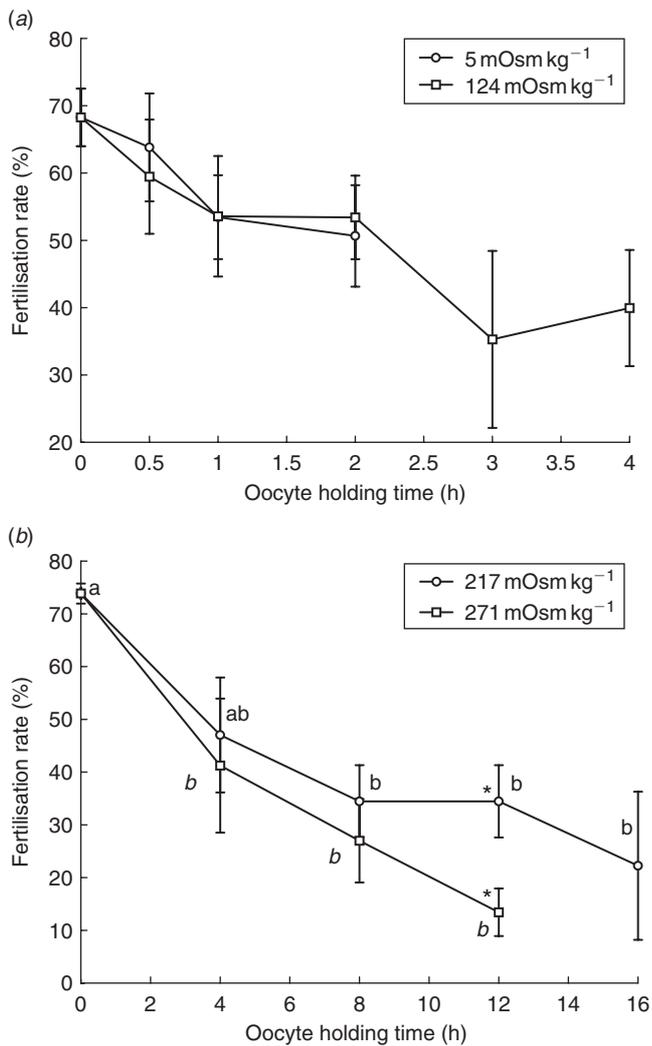


Fig. 5. Declining fertilisation rates with increased oocyte holding time in (a) low-to-medium ($n = 5-6$) and (b) high ($n = 4$) osmolality Ringer before insemination. Values are means \pm s.e.m. In treatments in (a), there was no significant decline in fertilisation rate with time. In treatments in (b), fertilisation rate declined significantly with time ($P < 0.05$ to 0.01). In (b), within treatments, means with different letters are significantly different across time, and, between treatments, means with an asterisk are significantly different at the same time point ($P < 0.05$).

to the differing time scales involved and because the number of oocytes available per female was limited. Analysis of the low-to-medium osmolality data shows there was no significant decline in fertilisation rate after holding oocytes in 5 mOsm kg^{-1} for 2 h ($F = 1.564$; d.f. = 3; $P > 0.05$) or in 124 mOsm kg^{-1} for 4 h ($F = 2.063$; d.f. = 5; $P > 0.05$) when compared with oocytes fertilised at the beginning of the experiment. There were no significant differences between 5 and 124 mOsm kg^{-1} treatments at any time point.

Analysis of the high osmolality data shows significant declines in fertilisation rates with increasing holding time in both the 217 mOsm kg^{-1} ($F = 4.699$; d.f. = 4; $P < 0.05$)

and 271 mOsm kg^{-1} ($F = 10.951$; d.f. = 3; $P < 0.01$) treatments. However, there were no statistical differences between the 217 and 271 mOsm kg^{-1} treatments, except at the 12-h time point ($F = 6.570$; d.f. = 1; $P < 0.05$). *Post hoc* analysis revealed that fertilisation rates in 217 mOsm kg^{-1} Ringer had declined significantly at the 8-h time point and continued to decline (8 h, $P < 0.05$; 12 h, $P < 0.01$; 16 h, $P < 0.01$). Similarly, in 271 mOsm kg^{-1} Ringer, fertilisation rates declined significantly after 4 h storage (4 h, $P < 0.05$; 8 h, $P < 0.01$; 12 h, $P < 0.01$), when compared with oocytes that were fertilized at time zero.

Moderate rates of fertilisation were retained for up to 2 h in 5 mOsm kg^{-1} Ringer ($59 \pm 13.49\%$; mean \pm s.e.m.) and up to 4 h in 124, 217 and 271 mOsm kg^{-1} Ringer ($42.79 \pm 15.08\%$, $55.29 \pm 18.98\%$ and $46.92 \pm 21.03\%$ respectively). Low levels of oocyte fertilisability were maintained for at least 16 h in 217 mOsm kg^{-1} ($22.47 \pm 22.13\%$), with fertilisation capacity all but lost after 12 h of oocyte storage in 271 mOsm kg^{-1} Ringer ($6.96 \pm 4.96\%$). There was no evidence of polyspermy observed in any treatment. Embryos were observed for 6 days (Stage 25, Gosner 1960) with no significant differences in survival, or abnormality, between any of the osmolality holding treatments (data not shown).

Discussion

Indicators of reproductive output

Positive correlations have been reported between egg yield and gravid weight and snout-vent length in several temperate anuran species (Pettus and Angleton 1967; Kuramoto 1978). Such measures allow for the prediction of the potential gamete yield of animals selected for breeding programmes. In *L. tasmaniensis*, snout-vent length was a less effective indicator of egg yield than gravid weight, which is not unexpected owing to the extensive contribution mature oocytes make to the weight of gravid females (13.5–19.5% in *L. tasmaniensis*; data not shown). There are relatively few reports of correlates of sperm yield in amphibians and the results of those reports have been at variance. Some studies have shown correlations between bodyweight and testes weight (various Australian species, Byrne *et al.* 2002; *Xenopus*, Hollinger and Corton 1980), whereas others have shown no association between these variables (*Rana*, Rugh 1937). Body length and weight were only moderately correlated with sperm yield in *L. tasmaniensis*. In contrast to egg yield in females, snout-vent length was a better indicator of gamete yield in males than bodyweight (snout-vent length was more strongly correlated than bodyweight with testes weight).

Gamete viability

Sperm samples of *L. tasmaniensis* with percentage motility as low as 10% and little to no forwards progressive motility, were able to achieve high rates of fertilisation. These results are in contrast to results of studies using *Xenopus laevis* in which

positive correlations between percentage motility and fertilisation rate were reported (Wolf and Hendrick 1971). This anomaly may be specific to myobatrachid species, because studies carried out on *Limnodynastes peronii* (a close relative of *L. tasmaniensis*; Schäuble *et al.* 2000) also showed a lack of correlation between percentage motility and fertilisation rate (Clark 1994). This is a promising finding for the application of sperm cryopreservation protocols developed for myobatrachids. These protocols have so far not achieved high recovery of sperm motility after thawing (Browne *et al.* 2002), nevertheless our results show that suspensions with low levels of motility can still achieve high levels of fertilisation.

Experiment 1: optimal sperm concentration

Byrne *et al.* (2002) have suggested that fertilisation in foam-nesting myobatrachids may be more efficient than in non-foam-nesting myobatrachids, based on the presence of comparatively smaller testes in foam-nesters. It was argued the ejaculate volume required for fertilisation might be reduced in species ovipositing in foam because of an increased fertilisation efficiency (Byrne *et al.* 2002), possibly owing to the mixing action employed by females to construct the foam-nest. Byrne *et al.* (2002) also argued that a reduced requirement for gamete production in foam-nesting myobatrachids led to the evolution of comparatively smaller testes. To add to these findings, the present study reports that optimum sperm concentrations during fertilisation in *L. tasmaniensis* (3.16×10^4 sperm mL⁻¹) were several orders of magnitude lower than concentrations reported for other amphibians using similar methods (*X. laevis*, 5×10^5 to 3×10^7 sperm mL⁻¹, Hollinger and Corton 1980; *B. marinus*, 10^6 sperm mL⁻¹, Browne *et al.* 1998). The fact that low sperm motility and a lack of forwards progressive motility have no effect on the efficiency of fertilisation in two foam-nesting species (*L. tasmaniensis*, present study; *L. peronii*, Clark 1994) adds further support to the increased fertilisation efficiency hypothesis.

Experiment 2: effect of medium osmolality on fertilisation rate

Fertilisation rates in *L. tasmaniensis* were highest in low ionic strength media (0–7 mOsm kg⁻¹) in the present study, similar to optimum fertilisation osmolalities reported for most anuran species, but differing from *X. laevis*, where optimum fertilisation rates are achieved in solutions with osmolalities ≥ 100 mOsm kg⁻¹ (Wolf and Hendrick 1971). The deleterious effects of high osmolality at and above 124 mOsm kg⁻¹ on fertilisation rates probably result from a lack of sperm activation above 100 mOsm kg⁻¹. This suppressive effect of high osmolality on sperm motility has been reported for several amphibian species (Morisawa and Morisawa 1990; Browne *et al.* 1998), including some myobatrachids (Browne *et al.* 2002). The decreases in mean fertilisation rates above 7 mOsm kg⁻¹ in *L. tasmaniensis* in the present

study may be explained by a reduction in sperm activation below 100 mOsm kg⁻¹. Alternatively, the decline in fertilisation rates above 7 mOsm kg⁻¹ may also be associated with increasing Ca²⁺ concentrations in the high osmolality media. An inhibitory effect of Ca²⁺ on artificial fertilisation has been reported in *X. laevis* using media containing >0.25 mM Ca²⁺ (Hollinger and Corton 1980), which is close to the level of Ca²⁺ in the 25 and 56 mOsm kg⁻¹ media (0.203 and 0.41 mM respectively) used with *L. tasmaniensis* in the present study.

Experiment 3: effect of short-term oocyte storage on fertilisation rate

Previous studies on anurans have reported that fertilisation rates of oocytes held in solutions of low (5 mOsm kg⁻¹) osmolality before insemination decline to zero within 0.5–1 h of storage (Elinson 1986). Other studies on anurans have also shown that the fertility of oocytes may be preserved for ≤ 2 h up to several hours in solutions of higher osmotic strength (Katagiri 1961; Wolf and Hendrick 1971; Hollinger and Corton 1980) when held at room temperature. The results of the *L. tasmaniensis* oocyte-holding experiments conducted at low osmolality (5 mOsm kg⁻¹) in the present study differ substantially from the findings of studies carried out under similar conditions on several other anuran species (Katagiri 1961; Hollinger and Corton 1980; Elinson 1986), in which it was found that anuran oocytes lose the ability to be fertilised within an hour of exposure to low ionic strength solutions. The prevailing hypothesis to explain the loss of fertilisability in hypotonic solutions is that the oocyte jelly swells, resulting in structural changes within the jelly that prevent passage of spermatozoa through the jelly to the oocyte (Olson and Chandler 1999). The present study has shown that the model of rapid loss of fertilisability of oocytes held in low osmolality medium does not apply to *L. tasmaniensis*, and it is possible that there may be no rapid loss of fertilising capacity at low osmolality in other foam-nesting myobatrachid species.

The egg jelly matrix of foam-nesters, such as *L. tasmaniensis*, consists of the egg jelly capsule that surrounds individual oocytes and a unique matrix that envelops the entire clutch. It is the presence of this unique mucous matrix, used in the construction of the foam-nest, which makes fertilisation in foam-nesters functionally different to other anuran species (Salthe and Mecham 1974). Nevertheless, subsequent studies indicate that low ionic strength solutions do induce swelling in *L. tasmaniensis* egg jelly (Herbert, D., Mahony, M., and Clulow, J., unpublished observations), as in other anurans (Wolf and Hendrick 1971; del Pino 1973). It is therefore possible that the evolution of the 'foam-nest' system in *L. tasmaniensis* may have resulted in structural differences in the egg jelly that alter its ability to block sperm entry upon swelling. It is uncertain from the results of the present study how long the fertilisability of *L. tasmaniensis* oocytes may last past 2 h at low osmolality. The observation of this anomaly in *L. tasmaniensis* in which spermatozoa

are able to penetrate oviducal jelly, even after the jelly has swollen in hypotonic solutions, has not been reported in other amphibians and is worthy of further investigation.

The results from the experiment on the storage of *L. tasmaniensis* oocytes in medium osmolality solutions ($\sim 124 \text{ mOsm kg}^{-1}$) is consistent with data for the storage of *Rana temporaria* oocytes in such solutions (Katagiri 1961), but not with data from *X. laevis*, where fertilisation capacity rapidly declined at a similar medium osmolality (Wolf and Hendrick 1971; Hollinger and Corton 1980). In amphibian species other than *X. laevis* (including *L. tasmaniensis*, the present study), isotonic solutions (around 220 mOsm kg^{-1}) have generally proven more effective than hypertonic solutions (e.g. 271 mOsm kg^{-1}) in extending oocyte life (Barberi and Raisman 1969; Hollinger and Corton 1980). Taken together, the data from various species indicates that oocyte storage is improved with increasing osmolality to an optimum, beyond which further rises in osmolality are associated with a loss of stored oocyte viability. It has been argued that loss of viability when storing oocytes in medium to high osmolality solutions may be owing to leaching of diffusible protein jelly components (Barberi and Raisman 1969; Olson and Chandler 1999) responsible for sperm activation (Omata 1993). It has also been suggested that extended *in vitro* storage of the anuran oocyte may result in a reduction in the level of cortical contraction (Elinson 1977), thereby reducing the ability of pronuclei to fuse (Elinson 1975), or, alternatively, that extended storage in these solutions may lead to changes in protein synthesis affecting oocyte metabolism and hence reducing the ability of the oocyte to cleave (Buhler and Zelarayan 1997).

Conclusions

The present study has described basic reproductive function and some novel reproductive traits in *L. tasmaniensis*. Artificial (*in vitro*) fertilisation protocols have been refined and oocyte storage conditions have been optimised. Further experiments are needed to investigate the mechanisms behind the unusual ability of *L. tasmaniensis* oocytes to maintain the capacity to be fertilised after extended storage in hypotonic solutions. The increased fertilisation efficiency hypothesis for foam-nesting species should also be investigated further. The information presented here allows for this species to be used as model for the development of ART protocols to be used in conservation programmes for foam-nesting and other myobatrachid amphibians. Protocols for non-invasive gamete collection, nuclear transfer and androgenesis will also need to be refined across several species and genera with different reproductive modes to make ART a viable option in widespread amphibian conservation programmes. Once procedures are refined in this manner, they may then be more confidently applied to vulnerable and highly endangered species to ensure against extinction and loss of genetic diversity.

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