Short-term storage of cane toad (Bufo marinus) gametes

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The responses of cane toad (Bufo marinus) gametes, used as a model for the development of assisted reproduction techniques for rare and endangered amphibians, to short-term storage at temperatures > 0°C were studied. Whole excised testes were stored at 0° or 4°C for 15 days, and sperm motility was measured at excision and after storage for 2, 5, 7, 10, 12 and 15 days. Spermatozoa showed > 50% motility for 7 days at 0°C and for 5 days at 4°C. At 15 days, only spermatozoa stored at 0°C still showed some motility (3%). Sperm suspensions were prepared at 5 day intervals over 30 days in simplified amphibian ringer (SAR) at dilutions of 1:1, 1:5 and 1:10 (w/v) testes:SAR. Aliquots from each dilution were stored at 0°C in Eppendorf tubes opened at 5 day intervals of storage (aerated) or kept sealed (unaerated) (treatments: aerated or unaerated; 5, 10, 15, 20, 25 and 30 days storage).

After 30 days, sperm motility and fertilizing capacity were determined. The optimal protocol for sperm storage up to 10 days, as assessed by the retention of fertilizing capacity, was as a 1:5 testis:SAR (w/v) suspension, whereas the longest absolute retention of both motility and fertilizing capacity was observed in concentrated (1:1 dilution), anaerobic suspensions (up to 25–30 days). Oviductal oocytes placed in SAR at 5, 10, 15, 20 and 25°C immediately after ovulation lost viability when cooled rapidly to 5°C and stored for 2 h. However, oocytes retained viability for up to 8 h at the optimum storage temperature of 15°C. Thus, it is concluded that during short-term storage spermatozoa retain viability for longer than oocytes, and that spermatozoa in suspensions retain viability for longer than spermatozoa stored in situ in excised testes.

Introduction

In the last two decades ten species of Australian amphibians are presumed to have become extinct, and a large number of extant species have decreased markedly in numbers and distribution (Mahony, 1996). Decreases in numbers of amphibians have also been recorded in Europe, and North and South America, with epidemic pathogens implicated in many cases (Blaustein et al., 1994; Berger et al., 1998). For some species, populations survive as only a few individuals in wild or captive populations (Denton et al., 1997). There is considerable scope for the application of assisted reproductive technologies to manage and conserve highly endangered amphibian species using models developed for fish. For example, in fish, stored spermatozoa may be used to maintain genetic diversity in small populations (Maitland, 1995; Holt et al., 1996) such as that of the Formosan landlocked salmon (Oncorynchus masou formosanus) (Gwo et al., 1999). The use of short-term storage of spermatozoa is practised widely in the conservation of Atlantic sturgeon (Acipenser huso) (DiLauro et al., 1994).

The handling and storage of gametes is central to many assisted reproductive procedures and broadly may be considered as either short-term storage at temperatures above 0°C, or long-term storage at temperatures below 0°C. Short-term storage of amphibian gametes may be necessary when the availability of male and female gametes for in vitro fertilization is asynchronous (Lahnsteiner et al., 1997) or the sources of gametes are separated spatially. Short-term storage may also be necessary during transport to a facility with the capacity to cryopreserve gametes for long-term storage when spermatozoa and oocytes are removed from moribund or recently dead animals in the field. There are few reports describing the short-term storage of amphibian spermatozoa and oocytes. Rostand (1946) described the storage of spermatozoa from edible frog (Rana esculenta) at –4°C in a high concentration of glycerol for 10 days. Hollinger and Corton (1980) examined the effect of storage of spermatozoa from African clawed toads (Xenopus laevis) for 1 and 6 days at 4°C at different concentrations on fertility using dose–response relationships, and found a decrease in relative fertility of spermatozoa stored in suspensions to 60% of that of fresh spermatozoa after 1 day and to 25% after 6 days.

The capacity of amphibian oocytes for extended short-term storage appears to be lower than that of spermatozoa. Storage of X. laevis oocytes in low ionic strength solution (0.05 × DB) (DB contains 0.11 mol NaCl l⁻¹, 0.0013 mol KCl l⁻¹, 0.00044 mol CaCl₂ l⁻¹, and NaHCO₃ to pH 7.2 (Wolf and Hedrick, 1971)) at 24°C resulted in low fertility...
after 30 min, but with higher osmolality (3 × DB) eggs maintained fertility for 2 h and, in some cases, for 12–14 h (Wolf and Hedrick, 1971). Hollinger and Corton (1980) observed an increase in embryo abnormality and a decrease in viability with time of oocyte storage before fertilization.

The use of short-term storage of spermatozoa in the conservation and management of wild fish stocks has received increased attention (DiLauro et al., 1994; Satterfield, 1995; Tiersch et al., 1998). Fish spermatozoa may be stored as seminal fluid or stripped from the testis by maceration in various diluents (Wayman et al., 1997). The ratio of dilution of seminal fluid or testis mass to diluent has been shown to affect viability over time, and diluents of different ionicity, and lipid and protein content have either reduced or increased sperm storage time in fish (Erdahl and Grahan, 1987). Studies of freshwater fish spermatozoa that have similar motility activation mechanisms to those of amphibian spermatozoa (activation by lowered osmolality) may provide a useful model for the short-term storage of amphibian spermatozoa (Hollinger and Corton, 1980; Stoss et al., 1983). However, studies have reported osmotic damage to fish (Billard et al., 1990) and amphibian spermatozoa (Wolf and Hedrick, 1971; Costanzo et al., 1998) only minutes after activation in fresh water, for example, only 50% of activated wood frog (Rana sylvatica) spermatozoa survive after 5–10 min in 5 mosmol kg⁻¹ (Costanzo et al., 1998). Cane toad (Bufo marinus) spermatozoa typically maintains a high degree of motility and membrane integrity for 4–6 h when activated in 40 mosmol kg⁻¹ (R. Browne, unpublished).

This study investigated the short-term preservation of gametes from the cane toad (Bufo marinus). The effects of temperature, aeration and dilution on the motility and fertilizing capacity of stored spermatozoa and the short-term storage of oocytes at different temperatures were investigated.

Materials and Methods

Animals

Wild caught, adult B. marinus were obtained from Mareeba (17.00°S, 145.43°E), North Queensland. Testes were collected from males and oocytes from ovulated females between September and May. At Mareeba, B. marinus is an exotic species that spawns during the tropical wet season from October to May. Toads were killed by injection with 5 ml MS222 (Tricaine methanesulphonate, Ruth Consolidated Industries P/L., Annandale, NSW) at a concentration of 2 g l⁻¹ into the dorsal lymph sac and the heart was removed to ensure death before the surgical removal of testes or oocytes.

After removal, testes were placed in 10 ml of simplified amphibian ringer (SAR) (Rugh, 1962) (113.0 mmol NaCl l⁻¹, 1 mmol CaCl₂ l⁻¹, 2.0 mmol KCl l⁻¹, 3.6 mmol NaHCO₃ l⁻¹; 220 mosmol kg⁻¹, prepared in milli Q water, pH 7.2) in Petri dishes before allocation to experimental treatments (see below). The isotonic osmolality of SAR prevented activation of sperm motility. After experimental treatments, samples were activated by dilution of one part sperm suspension to two parts distilled H₂O (70 mosmol kg⁻¹). The percentage of motile spermatozoa and the vigour of motility on a scale of 0–4 (Emmens, 1947) in activated samples before and after experimental treatments were determined under a phase-contrast microscope (× 400).

In preparation for the induction of ovulation, female toads were conditioned in a temperature-controlled room under a 12 h light: 12 h dark cycle at 28–30°C, and fed high protein dog food pellets (15% (w/w) protein (Luv® Tender Chunks, Friskies Pet Care, Sydney, NSW) twice a week for at least 8 weeks. The high temperature and high protein diet of this protocol markedly improved the percentage of female toads ovulating after induction. Female toads held at 21°C were induced to ovulate by the injection of six freshly collected male B. marinus pituitaries per female (homogenized in 1 ml of SAR) into their dorsal lymph sacs using a 1 ml syringe with a 23 gauge needle (Browne et al., 1998). Females were killed 8–12 h after induction of ovulation when oocytes were expressed from the cloaca, and the oocyte masses were removed surgically from the oviducts.

Experiment 1: storage of whole testes in vitro at 0°C and 4°C

Testes removed from six male toads were placed in 1.5 ml Eppendorf tubes and immediately stored on ice (0°C) or at 4°C in a refrigerator (n = 6 per treatment) with one testis from each toad in each treatment. An initial 0.01 g sample was removed as a 1.5 mm slice from the middle of the testes. Subsequently, a 0.75 mm slice from the central end of one half and the opposite end of the other half (about 10% of the testis mass) was removed at each of the sampling intervals. These samples were then macerated in 0.01 ml SAR, activated and the sperm motility was determined as described above.

Experiment 2: storage of aerated and unaerated sperm suspensions at 0°C at three dilutions

At 5 day intervals over 30 days, 24 testes (from 12 toads) were individually macerated in 0.1 ml SAR and the resulting suspensions were pooled and immediately stored on ice. The sperm concentration in the pooled suspension at each time interval was determined with a Neubauer haemocytometer. From the pooled suspension, 24 × 0.1 ml aliquots were placed in 1.5 ml Eppendorf tubes. Eight of these aliquots were not diluted any further; eight were diluted with 0.4 ml SAR (1:5 dilutions) and eight were diluted with 0.9 ml SAR (1:10 dilutions), giving a total of eight replicates of each dilution at each time interval. All samples were stored at 0°C (on ice) between processing. Four suspensions of each dilution were exposed to air in a refrigerated room (4°C) at 5 day intervals (aerobic) and the
other four were kept sealed (anaerobic) from day 0. At 30 days after preparation of the first sample, the percentage of motile, activated spermatozoa in each sample was determined under a light microscope at ×400 magnification and the fertilizing capacity of each sample was determined.

The fertilization rate of sperm suspensions was determined by flushing the samples with 10 ml distilled water into Petri dishes containing freshly collected oocytes (63 ± 3.2, n = 88; mean ± SEM per dish). Only samples showing some motility were tested for fertilizing capacity. Sub-samples of oocytes from each female incubated without spermatozoa were negative controls for parthenogenetic activation (n = 4) and controls were fertilized with optimal sperm concentrations as for Expt 1 (n = 4). The mean sperm concentration per dish was 1.06 ± 0.04 × 10^7 ml^-1 (mean ± SEM) for all treatments.

**Experiment 3: effect of temperature on storage of oocytes**

Oviductal oocytes from different females were used in each of three replicated fertilization trials. The fertilization rate of the oviductal oocytes in each trial (time 0 controls) was determined immediately after removal of the oocytes from the oviduct, and before division of the oviductal egg mass into sub-samples that were stored at 5, 10, 15, 20 and 25°C. Samples of approximately 1500 oviductal oocytes (mass 7 g) from each female were stored in five containers containing 100 ml SAR. The temperature of each container was maintained by immersion in a water bath set at the appropriate temperature. Temperature in the containers was monitored with a mercury thermometer and maintained within ± 1.0°C. Fertilization rate was determined at time 0 for controls and at 1 and 2 h, and at 2 h intervals subsequently for treatments until 12 h had elapsed. Strips of oviductal oocytes (oviductal eggs of *Bufo marinus* are held in gelatinized strips) from each control (time 0) or treatment container were cut into appropriate lengths (121 ± 22, n = 96; 126 ± 16, n = 96; 137 ± 17.2, n = 63; mean ± SD oocytes for trials 1, 2 and 3, respectively, n = number of Petri dishes) and placed in three separate Petri dishes to determine the fertilization rate. Both testes from two male *Bufo marinus* were macerated in 0.4 ml SAR, producing an initial sperm concentration of 0.5–1.0 × 10^9 ml^-1. Fertilization in each Petri dish was achieved by the addition of 0.025 ml sperm suspension in 10 ml distilled water at 21°C to give a final sperm concentration of 1.25–2.5 × 10^9 ml^-1. The concentration of spermatozoa needed to achieve optimal fertilization rates with *Bufo marinus* is 1 × 10^6 ml^-1 (Browne et al., 1998). The percentage of fertilized oocytes in each Petri dish was determined 3 h after fertilization from the proportion of oocytes showing first cleavage. Samples of oocytes from each female were also left without the addition of spermatozoa at each sampling period as negative controls to test for parthenogenetic activation. The relative fertilization rate (RF) for each oocyte trial was calculated as 

\[ RF = \frac{TF}{CF} \times 100 \]  

(TF: treatment fertilization rate; CF: control fertilization rate).

**Statistical analyses**

Percentage fertilization data from Expt 1 was subjected to chi-squared analysis after arcsine transformation because of non-parametric distribution. Motility and fertilization data for Expts 2 and 3 were arcsine transformed before testing for normality and homogeneity of variance, and then analysed by one-way ANOVA. Individual means were ranked with the Tukey–Kramer HSD (honestly significant difference) test. All analyses were performed with JMP 3.2 software package (SAS Institute Inc.).

**Results**

**Experiment 1**

Spermatozoa stored in whole testes retained the capacity for motility for up to 12 days at 4°C and 15 days at 0°C. Motility of > 40%, vigour 3, was evident for up to 7 and 10 days at 4°C and 0°C, respectively. Motility values for spermatozoa from testes maintained at 0°C were significantly higher than for spermatozoa from testes maintained at 4°C testes, except in treatments after 7 days. Testicular spermatozoa at 0°C showed a linear decrease in motility for 7 days with a 10% decline between 2, 5 and 7 days, then a 20% decline between 7, 10 and 12 days. In contrast, the motility of testicular spermatozoa at 4°C dropped 40% by day 2, maintained a value of approximately 50% until day 7, and then declined precipitously to 1.5% at day 12 (Fig. 1).

![Fig. 1. Mean percentage and degree of motility of activated cane toad (*Bufo marinus*) testicular spermatozoa. Testes were stored at 0°C or 4°C before release of spermatozoa by maceration. (●) 0°C, percentage motile; (■) 0°C, degree of motility; (○) 4°C, percentage motile; (□) 4°C, degree of motility. Letters indicate significant differences between 0°C and 4°C means for the same parameter (percentage motile or degree of motility) at the same storage period: a: P < 0.001; b: P < 0.01; and c: P < 0.05.](attachment:image.png)
**Experiment 2**

There was no cleavage in parthenogenic control oocytes. At day 10, concentrated sperm suspensions showed a significantly ($P < 0.05$) higher capacity for motility than did diluted suspensions. Sperm clumping was observed in activated 1:1 suspensions, and the degree of clumping increased with storage time and time after activation (Fig. 2a). The fertilization rate for controls ($t = 0$) was 96.8 ± 0.4% (mean ± SEM). At day 5, there were significantly higher ($P < 0.05$) relative fertilization rates in 1:5 dilutions (about 85%) than in the other four treatments (about 55%). At day 10, the mean values had formed into four significantly different ($P < 0.05$) fertilization rate clusters. In contrast to day 10 percentage motilities, day 10 fertilization rates of undiluted suspensions were lower than they were for 1:5 diluted suspensions. Spermatozoa from anaerobic undiluted suspensions stored for up to 30 days fertilized oocytes (Fig. 2b). There was a significant ($r^2 > 0.50$) correlation between mean motility and fertilization rate at days 0, 5 and 25 of storage (Fig. 3).

**Experiment 3**

Control (time zero) fertilization rates of fresh oocytes with untreated spermatozoa for trials 1, 2 and 3 were 88.8 ± 9.0, 84.0 ± 6.6 and 80.9 ± 5.5, respectively (mean ± SD, $n = 3$). The greatest retention of oocyte viability over time was observed at 15°C, with > 90% of the initial relative fertilization rate being maintained at 6 h after ovulation, a rate significantly higher than in the other treatments ($P < 0.05$). Storage at 5°C resulted in a precipitous decline to zero relative fertilization rate at 2 h (Fig. 4).

**Fig. 2.** Mean percentage motility of cane toad (*Bufo marinus*) activated spermatozoa (a) and relative percentage fertilization of oocytes (b) from stored suspensions at dilutions of 1:1, 1:5, or 1:10 (w/v, testes: simplified amphibian ringer), at 0°C in aerated and unaerated conditions: (□) aerated, 1:1; (○) aerated, 1:5; (△) aerated, 1:10; (■) unaerated, 1:1; (●) unaerated, 1:5; (▲) unaerated 1:10. Individual means or clusters with different letters are significantly different ($P < 0.05$). Mean percentage motility pooled $\bar{se} = 2.48$, $n = 23$. Mean relative percentage fertilization pooled $\bar{se} = 5.63$, $n = 21$.

**Fig. 3.** Correlation between mean relative percentage sperm motility and mean relative percentage fertilization of cane toad (*Bufo marinus*) oocytes fertilized by spermatozoa stored in sperm suspensions for 5 days (●) $r^2 = 0.52$ (-----); 10 days (○) $r^2 = 0.02$; 15 days (□) $r^2 = 0.23$; and 20 days (▲) $r^2 = 0.84$ (----).

**Fig. 4.** Relative percentage fertilization of cane toad (*Bufo marinus*) oocytes held at 5 (■), 10 (○), 15 (▲), 20 (○) and 25°C (●) in simplified amphibian ringer against time after collection ($n = 3$ for 10, 15 and 20°C; $n = 2$ for 5 and 25°C). Pooled $\bar{se} = 7.6$, $n = 74$. Means that share the same letter are not significantly different ($P < 0.05$).
The present study showed that it is possible to maintain high sperm motility and fertilizing ability after storage of testis and sperm suspensions from *B. marinus* for up to 10 days *in vitro* at 0°C (on ice), although storage in diluent suspensions was more effective than storage in whole testes. After storage for 10 days of storage, higher sperm motility and fertilizing capacity were maintained in SAR suspensions than in whole testes. Some sperm motility and fertilizing capacity was still evident in some samples after 30 days of storage. The effect of aerobic versus anaerobic conditions had only a minor effect on sperm viability in stored suspensions. There was evidence that maintenance of spermatozoa in suspensions at higher concentrations provided the most favourable conditions for short-term storage. The finding that amphibian spermatozoa, *in situ* in excised testes or *in vitro* in suspensions, may be maintained unfrozen for a number of weeks at low temperature indicates that it is feasible to recover viable spermatozoa from recently dead or moribund amphibians and store them for some time. The spermatozoa may then be cryopreserved or used for *in vitro* fertilization. These findings should be of use in the management of small, captive or wild populations that contain valuable or rare animals.

The longevity of unaerated *B. marinus* sperm suspensions indicates that either metabolic rate is lowered sufficiently for there to be an adequate supply of oxygen, or that anaerobic metabolism maintains metabolism and cellular viability at low temperatures in this species. In fish, the effect of gaseous exchange on sperm storage varies widely. Some species require aerobic respiration while spermatozoa from other species may persist with energy derived only from glycolysis or stored ATP (Büyükătipoglu and Holtz, 1978). With turbot (*Scophthalmus maximus*) spermatozoa, an atmosphere of air rather than pure oxygen increases viability with storage (Chereguini et al., 1997); however, an atmosphere of pure oxygen increases the storage time of rainbow trout (*Oncorhynchus mykiss*) spermatozoa (Billard, 1981). Oxygen dependence increases the potential for damaging oxidative effects on membrane integrity and cell metabolism (Ciereszko and Dabrowski, 1994). For some species, a deeper layer of sperm suspensions in aerated containers, producing hypoxic conditions, increases storage time (Clemens and Hill, 1969). It is not clear from the present study whether the improved longevity of *B. marinus* spermatozoa in concentrated suspensions was due to an increased availability of glycolytic substrate. Dilution of fish sperm suspensions has also resulted in a reduction of storage life (Stoss and Holtz, 1983) but further studies will be required to determine whether the capacity to maintain anaerobic metabolism is a general characteristic of amphibian sperm metabolism, and whether addition of glycolytic substrates improves sperm storage.

There was no evidence of cold shock in *B. marinus* spermatozoa with the rate of cooling of samples on ice used in the present study, even though *B. marinus* is a tropical–warm temperate species. There are no reports to our knowledge of amphibian or fish (Stoss et al., 1983) spermatozoa displaying cold shock, a phenomenon that is common in eutherian mammal (White, 1993) but not marsupial spermatozoa (Taggart et al., 1996). Costanzo et al. (1998) demonstrated that the spermatozoa of *R. sylvatica* can survive sub-zero temperatures of –4°C with naturally conferred cryoprotection. Consequently, there may be optimism for short-term, low temperature storage with other temperate climate anurans and tropical–warm temperate species.

There was a positive correlation ($r^2 > 0.5$) between the motility and fertilizing capacity of stored *B. marinus* spermatozoa at the beginning (day 5) and end (day 20) of the storage period. The greatest deviation from this correlation occurred on day 10 when the fertilization rate of spermatozoa in undiluted suspensions was lower than it was in 1:5 dilutions, while the opposite was the case for sperm motility. The high fertilizing capacity of the 1:5 dilutions, even though they displayed low motility, is explained by the high sperm concentration ($10^7$ ml$^{-1}$) which is one order of magnitude above the saturation concentration of spermatozoa needed to achieve 100% fertilization ($10^6$ ml$^{-1}$) and two orders of magnitude higher than that required for 80% fertilization ($10^5$ ml$^{-1}$) rates in fresh samples (Browne et al., 1998). The absence of the sperm clumping that occurred predominantly in undiluted suspensions may also have increased the fertilization rate in this treatment.

The present study showed that the motility of spermatozoa retained in whole testes was much higher over time at 0°C than at 4°C, with substantial motility (28–44%) retained for 10–12 days at 0°C. This period of storage *in situ* in excised *B. marinus* testes was longer than that reported for some fish species. Testicular spermatozoa only retained viability for 2 days at 0°C in herring (*Clupea pallasi*) (Dushkina, 1975) and for 18 h at 4°C in brown trout (*Salmo trutta*), which was far less than for sperm suspensions of the same species (Billard et al., 1981). In amphibians, the testes are the site of sperm storage in males. Testicular spermatozoa are embedded in Sertoli cells until they are released by testicular hydration induced by gonadotrophins (Burgos and Vitale-Calpe, 1967). Spermatozoa may be stored for many months in the testes until the occurrence of suitable spawning conditions (Lofts, 1974). The reason for the higher survival of spermatozoa in whole testes at 0°C than at 4°C, and the rapid decrease in sperm motility after 2 days storage at 4°C was not determined in the present study. However, with testicular excision, Sertoli and other somatic cells in the testes that are dependent on aerobic metabolism may die more rapidly at a relatively higher metabolic rate and oxygen demand. Higher temperature may also reduce enzyme activity and promote greater release of toxic metabolites at 4°C. With maceration, a proportion of spermatozoa released are immature with cytoplasmic droplets still present. Further studies may determine the susceptibility of
these cells to stress from testicular storage or whether greater proportions of immature, immotile cells are released during maceration at 4°C than at 0°C after 2 days.

The present study showed that the storage of B. marinus oocytes (in SAR) is limited to hours at the optimal temperature of 15°C, with temperatures at 10°C or lower and 20°C or above resulting in a rapid loss of viability. This temperature range is lower than the optimum of 28°C or above resulting in a rapid loss of viability. This temperature range is lower than the optimum of 28°C or above.

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