

## Successful Fertility Experiments with Cryopreserved Spermatozoa of Barramundi, *Lates calcarifer* (Bloch), using Dimethylsulfoxide and Glycerol as Cryoprotectants

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### Abstract

The fertility of cryopreserved *Lates calcarifer* sperm was studied to increase the availability of semen for routine fertilization of stripped eggs and to provide a tool for selective breeding. Semen diluted (1:4 v/v) and frozen (-196°C) with 5% dimethylsulfoxide (DMSO) or 10% glycerol (final concentration) as cryoprotectants was used to inseminate freshly stripped ova. Frozen-thawed sperm were motile for about 4 min after being mixed with seawater. In the DMSO medium, post-thaw sperm activation was immediate after dilution with seawater, but in the glycerol medium maximum motility intensity was delayed for up to 1 min. When eggs and sperm were mixed before the addition of seawater, semen frozen with DMSO as cryoprotectant gave a mean hatch rate (84.1%) no different ( $P > 0.05$ ) from that of unfrozen semen diluted with Ringer's solution (80.7%) or with DMSO (83.7%), but higher ( $P < 0.05$ ) than that of semen frozen with glycerol (60.9%). Adding sperm to seawater 30 s before mixing with eggs did not improve the fertility of sperm cryopreserved with glycerol. Eggs inseminated with glycerol-cryoprotected sperm showed higher mortality during incubation than those inseminated with DMSO-cryoprotected sperm.

Sperm held in liquid nitrogen for 90 days with DMSO as cryoprotectant yielded acceptable fertilization and hatching rates with semen-to-ova ratios of up to 1:100 (v/v), and produced fish with no apparent abnormalities over a 29-day period after hatch. These results show that cryopreservation of *L. calcarifer* sperm is feasible and well suited to a variety of hatchery purposes.

### Introduction

The barramundi or sea bass, *Lates calcarifer* (Bloch) (Centropomidae), is an important food fish in subtropical and tropical regions of the Indo-West Pacific (Grey 1987). Hatchery production of this species in Australia has expanded in the last decade owing to interest in restocking and aquaculture (Rutledge and Rimmer 1991). However, the unreliable supply of semen for stripping from captive fish is a significant problem (Garrett and Rasmussen 1987; Hogan *et al.* 1987). Stress from handling both broodstock and fish caught in the wild appears to reduce the quantity of semen that can later be manually expressed by abdominal pressure. Hormone-induced spermiation has met with only limited success (Rimmer 1989; Schipp *et al.* 1989), and copious quantities of semen are available for only a short time before spawning (Hogan *et al.* 1987). In addition, because barramundi are protandrous hermaphrodites they undergo natural sex inversion from male to female (Moore 1979); this creates uncertainties from year to year in the number of functional males in broodstock populations and makes a back-up supply of sperm highly desirable. Hogan *et al.* (1987) developed methods for chilled storage of barramundi sperm for periods of up to 1 week, but a method for longer-term storage of sperm from *L. calcarifer* is required to improve the reliability of hatchery production.

Successful methods for the cryopreservation of fish sperm have been documented for many cultivated species (see reviews by Stoss and Donaldson 1982; Stoss 1983; Leung and Jamieson 1991). These long-term storage techniques offer operational and manipulative advantages when oocytes are artificially inseminated with previously collected sperm. Cryopreservation of *L. calcarifer* spermatozoa was first reported by Leung (1987). He assessed the effect of the concentration of three cryoprotectants (dimethylsulfoxide (DMSO), glycerol and methanol) on the motility of frozen-thawed spermatozoa using a 1:4 (v/v) semen:diluent ratio. The medium consisted of freshwater-teleost Ringer's solution (see Kurokurra *et al.* 1984) containing either skim milk powder or chicken egg yolk. Leung (1987) did not conduct fertility trials but, with all diluents tested, motile spermatozoa were obtained after thawing, the best results being obtained with 4.8% DMSO followed by 9.1–16.7% glycerol (v/v final concentration). In subsequent work, R. Pidgeon, A. W. Blackshaw, M. F. Capra and J. Glaister (unpublished results) achieved best post-thaw motility of *L. calcarifer* spermatozoa using 5% DMSO and 10% glycerol (v/v final concentration), without the addition of either skim milk powder or chicken egg yolk to a similar Ringer's-based diluent. In that study, ova were inseminated but rates of fertilization and subsequent development were low, producing inconsistent results. It was suggested that the cryoprotective media interfered with fertilization and that a more reliable incubation and testing procedure was required. Satisfactory post-thaw motility was demonstrated in these studies but the fertility of these sperm remained unknown.

Consequently, in the present study, DMSO and glycerol were used as cryoprotectants for barramundi sperm at the optimal concentrations mentioned above; fertilization and hatching rates were used as indicators of fertility. Special consideration was given to activation of cryopreserved sperm and insemination procedures. Experiments were conducted to determine whether the cryoprotectants interfered with fertilization processes and to determine appropriate cryopreserved semen-to-ova ratios. The feasibility of using cryopreservation to increase the availability of semen for routine fertilization of stripped eggs and as a tool for selective breeding was investigated.

## Materials and Methods

### *Sperm Collection and Freezing*

Ripe *L. calcarifer* were captured with gill-nets from the Mission River near Weipa (12°35'S, 141°56'E) in far north Queensland, Australia, during a November full-moon spawning peak (Garrett 1987). Semen was manually expressed from male fish and stored on ice in 5-mL syringes before being mixed with diluents in 2-mL-capacity cryovials at a 1:4 ratio (v/v). Contamination of the semen with urine or seawater was carefully avoided to help prevent premature activation. Two cryoprotectants (DMSO and glycerol) were mixed with marine teleost Ringer's solution (7.25 g NaCl, 0.38 g KCl, 0.24 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.27 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.01 g NaHCO<sub>3</sub>, 0.41 g NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, 1.01 g D<sup>+</sup> glucose, and distilled water to 1 L) so that, after the addition of semen, there was a final concentration (v/v) of either 5% DMSO (0.69 M) or 10% glycerol (1.36 M). Semen (0.2 mL) was added to the chilled cryopreservation medium (0.8 mL), and vials were tightly capped and inverted about 10 times to achieve complete mixing.

Cryovials were then placed inside a cooling chamber in liquid nitrogen vapour which gave an initial cooling rate of 10°C min<sup>-1</sup>, with cooling to -76°C after 30 min. Frozen cryovials were then plunged into liquid nitrogen for storage; they were thawed as required by immersion in a 30°C water bath, with rapid swirling for 2–3 min.

### *Post-thaw Activation*

To investigate post-thaw activation of sperm in seawater, using both cryoprotectants, two vials of frozen semen from each experiment were thawed and kept at an ambient temperature between 31.5 and 33°C. Sixteen aliquots were taken over the first post-thaw hour to determine the intensity of sperm motility after activation in seawater with a salinity of 36 g kg<sup>-1</sup>. Semen adhering to the tip of a probe was mixed with two drops of seawater in the cavity of a microscope slide. A coverslip was placed over

the cavity and its contents, and motility was observed under 400× magnification 10 s after mixing with the seawater. Motility intensity was assessed according to the relative 5–0 scale modified from Hogan and Nicholson (1987) as follows:

- 5 Most active sample observed for this species; sperm creating swirling currents obscuring the movement of individual sperm across the field of view.
- 4 Very active sample; all sperm visibly progressing rapidly across the field of view.
- 3 Less energetic head and tail movement; most with forward motion.
- 2 Slow head and tail movement; some individuals progressing slowly.
- 1 Head movement only; no progressive motion.
- 0 No activity.

#### *Fertility Experiments*

Inseminations were performed by mixing freshly stripped ova (in clean dry plastic jars) with one vial of sperm and an equal volume of seawater. After periodic swirling for 5 min, the volume was doubled by adding more seawater over a further 5 min. Eggs were then placed into aerated 3-L hemispherical bowls of seawater for incubation. Water changes (50%) were undertaken immediately and at 3–4-h intervals thereafter until hatching commenced. Viability was estimated at two visually distinct developmental stages prior to hatching, as well as 4–5 h after hatching had started. Treatment effects were tested for significance by analysis of variance using a probability level of 0.05%, and data are presented as means ± 1 s.e.

#### *Dry insemination and delayed wet insemination*

The first fertility experiments compared unfrozen sperm diluted with Ringer's solution only (control), unfrozen sperm diluted with Ringer's solution and either DMSO or glycerol, and sperm diluted and frozen with Ringer's solution and either DMSO or glycerol. Semen in each of the three unfrozen treatments was stored undiluted on ice for 22–23 h prior to dilution, and for a further 2 h before insemination. Semen in the two frozen treatments was stored on ice for 3–4 h, diluted 15 min before freezing, and held in liquid nitrogen for about 21 h before thawing and immediate use. Initially, all treatments used the dry insemination method, in which semen (0.2 mL in a total diluted volume of 1 mL) and ova (about 1500 eggs in 0.5 mL) were thoroughly mixed before adding seawater. Because of differences in activation patterns between sperm cryopreserved with DMSO or glycerol (see Results), insemination protocols were adjusted in a similar experiment so that ova in all treatments were immediately exposed to fully activated sperm. This gave rise to a dry insemination protocol (as above) for semen with Ringer's solution and Ringer's + DMSO, and a delayed wet insemination protocol for semen with Ringer's + glycerol, in which semen was mixed with seawater 30 s before being added to ova. Gametes used in each experiment in Weipa were obtained from one running-ripe female fish (about 4 kg), and one male fish (2–3 kg; 5 mL of semen).

#### *Semen-to-ova ratio*

In a subsequent fertility experiment, semen-to-ova ratios were tested at the Southern Fisheries Centre (SFC) with semen previously collected from the Mission River and stored in liquid nitrogen for 90 days. The cryoprotectant was DMSO (with dilution and freezing as described above). Eggs were stripped from one broodstock female (17 kg) 38 h after injection with 30 µg kg<sup>-1</sup> luteinizing hormone-releasing hormone analogue (LH-RHa), and were divided in triplicate into volumes of 0.5, 2, 8, 20 and 100 mL. Each aliquot was then inseminated with one vial of cryopreserved semen according to the dry insemination protocol. After 10 min, a 1-mL subsample was taken by pipette from each aliquot of inseminated ova and incubated. Incubation and viability assessments were undertaken as described above. Ova in replicates 1 and 2 of this experiment were inseminated with semen collected from one fish (2–3 kg; total of 5 mL collected), and replicate 3 with semen from a different male of similar size (total of 2.2 mL collected). Sperm concentration in the semen used in this experiment was estimated with a haemocytometer (Improved Neubauer, depth 0.1 mm) and a 1:100 dilution with Ringer's solution. Eggs that remained in the insemination jars after subsampling were pooled, and larvae hatching from these eggs were grown to 20 mm in total length according to standard methods practised in Australian hatcheries (MacKinnon 1987; Russell *et al.* 1987). This was done to identify any major problems that might occur in progeny produced from sperm cryopreserved in this manner.

The salinities and temperatures of filtered (5 µm) seawater used for inseminations and incubation in the study were 36 g kg<sup>-1</sup> and 29–31°C at Weipa, and 31 g kg<sup>-1</sup> and 27.5–28.5°C at the SFC.

## Results

### *Post-thaw Activation*

Semen used in the study showed little or no sperm motility prior to activation with seawater. A low level of motility was induced by diluting semen with the glycerol diluent at ambient temperature, but this was minimized for fertility experiments by holding diluted semen on ice. No motility was induced by the DMSO diluent at ambient temperature. The post-thaw motility of *L. calcarifer* sperm, activated in seawater is shown in Fig. 1. A mean maximum motility intensity of 3.0 was observed immediately in DMSO aliquots, suggesting that activation was complete within 10 s of the addition of seawater. Motility intensity then steadily declined over 4 min. In contrast, maximum motility intensity (mean level of 3.8) was not attained in glycerol aliquots until 1 min after the addition of seawater. Little variation in sperm motility occurred in aliquots taken from vials during the first hour after thaw as can be seen by the low standard error associated with the means (Fig. 1).

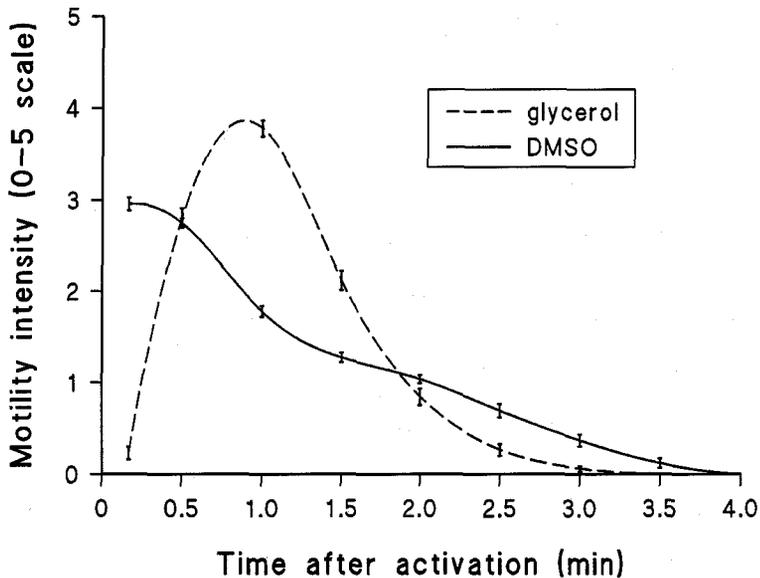


Fig. 1. Post-thaw motility in seawater of barramundi sperm cryopreserved using DMSO or glycerol. Data are means  $\pm$  1 s.e. ( $n=16$ ).

### *Dry Insemination and Delayed Wet Insemination*

At all developmental stages when dry insemination was employed, glycerol treatments gave the lowest mean viability estimates, with these becoming significantly lower ( $P < 0.05$ ) as incubation progressed to the late embryo stage and after hatching (Table 1). No significant differences in fertilization or hatch rates were found between the frozen or unfrozen semen + DMSO and the unfrozen semen + Ringer's treatments. Fewer ( $P < 0.05$ ) embryos hatched with the unfrozen semen + glycerol treatment than with the frozen semen + glycerol treatment.

The delayed wet insemination protocol did not improve the fertility of sperm cryopreserved with glycerol. In this experiment, differences between mean viability estimates in all treatments were again not significant ( $P > 0.05$ ) at the gastrula stage of development (85.5–89.7%). However, at the late embryo stage and after hatching, viability declined to

67.2 ± 3.74% and 47.4 ± 6.29% respectively for eggs inseminated with sperm frozen in glycerol ( $n=3$ ), compared with 81.7 ± 1.95% and 66.5 ± 1.20% respectively for eggs inseminated with sperm frozen in DMSO ( $n=2$ ).

**Table 1. Mean viability estimates (%) at three developmental stages of *Lates calcarifer* ova fertilized with spermatozoa diluted or cryopreserved using DMSO or glycerol**

Values are means ± s.e. ( $n=3$ ). Within columns, values with different superscripts are significantly different ( $P<0.05$ )

Insemination treatment <sup>A</sup>	Developmental viability estimates		
	Blastula-gastrula	Late embryo	Hatched larvae
Ringer's solution only; unfrozen semen	87.8 <sup>a</sup> ± 2.67	92.6 <sup>a</sup> ± 3.73	80.7 <sup>a</sup> ± 1.93
Ringer's + DMSO; unfrozen semen	87.3 <sup>a</sup> ± 3.66	83.4 <sup>a</sup> ± 4.96	83.7 <sup>a</sup> ± 0.81
Ringer's + DMSO; frozen semen	86.1 <sup>a</sup> ± 2.41	89.6 <sup>a</sup> ± 1.96	84.1 <sup>a</sup> ± 1.20
Ringer's + glycerol; unfrozen semen	71.1 <sup>a</sup> ± 6.70	59.6 <sup>b</sup> ± 7.30	42.0 <sup>c</sup> ± 8.74
Ringer's + glycerol; frozen semen	75.4 <sup>a</sup> ± 5.59	67.4 <sup>b</sup> ± 2.33	60.9 <sup>b</sup> ± 1.05

<sup>A</sup> Dry insemination protocol was used for all treatments.

#### Semen-to-ova Ratio

Viability estimates at all stages of embryo development were lower ( $P<0.05$ ) using the lowest semen-to-ova ratio of 1:500 (Table 2). The highest ratio (1:2.5) consistently gave the highest mean viability estimates, but the percentage of embryos that hatched was not significantly different from that obtained with the 1:100 ratio. Egg viability typically showed a slight decline during incubation in all treatments. Sperm counts of diluted semen in additional vials from the experiment for replicates 1 and 2 were  $7.04 \pm 0.06 \times 10^9$  per vial, and for replicate 3 were  $6.70 \pm 0.07 \times 10^9$  per vial.

**Table 2. Mean viability estimates (%) at three developmental stages of *Lates calcarifer* ova fertilized using different ratios of cryopreserved (DMSO) semen-to-ova**

Values are means ± s.e. ( $n=3$ ). Within columns, values with different superscripts are significantly different ( $P<0.05$ )

Semen:ova ratio	Developmental viability estimates		
	Blastula	Late embryo	Hatched larvae
1:2.5 (0.2:0.5 mL)	85.5 <sup>a</sup> ± 5.50	75.1 <sup>a</sup> ± 2.60	68.6 <sup>a</sup> ± 1.64
1:10 (0.2:2 mL)	83.4 <sup>ab</sup> ± 6.30	58.7 <sup>b</sup> ± 7.81	50.7 <sup>b</sup> ± 11.54
1:40 (0.2:8 mL)	72.5 <sup>ab</sup> ± 2.92	51.1 <sup>b</sup> ± 7.51	47.6 <sup>b</sup> ± 5.17
1:100 (0.2:20 mL)	67.4 <sup>b</sup> ± 12.64	48.8 <sup>b</sup> ± 11.03	53.2 <sup>ab</sup> ± 13.45
1:500 (0.2:100 mL)	33.2 <sup>c</sup> ± 9.15	20.4 <sup>c</sup> ± 7.60	16.7 <sup>c</sup> ± 2.94

#### Fish Produced with Frozen Sperm

The 8000 fish produced from frozen (DMSO) sperm in the study showed no apparent abnormalities during a 29-day culture period, growing to a total length of  $19.9 \pm 0.59$  mm ( $n=100$ ). Subsequent transfer to fresh water and air transport in plastic bags filled with water and oxygen (over approximately 5 h) appeared to have no unusual or detrimental effect on their health or condition.

## Discussion

### *Post-thaw Activation and Fertility*

No major interference with fertilization processes by either DMSO or glycerol was evident in this study and, in most cases, frozen and unfrozen semen had similar fertility. The cryoprotectants did not require the inclusion of adjuvants such as skim milk or egg yolk, as used by Leung (1987). The longer time needed for activation in seawater of glycerol-frozen sperm may stem from the slower permeation of glycerol in comparison with DMSO (Stoss 1983). This slower activation has special significance given the short period (<2 min) that most *L. calcarifer* eggs remain fertilizable after coming into contact with seawater (36 g kg<sup>-1</sup>) (Palmer, unpublished). Glycerol as cryoprotectant was tested under two different insemination protocols, taking into account this slower post-thaw activation. The results suggest that the slower post-thaw activation of sperm frozen in glycerol does not hinder fertilization with either insemination protocol, but that subsequent development of embryos is less successful when glycerol rather than DMSO is used in the medium. Premature partial activation upon dilution with the cryoprotectant is a possible cause of the lower success rate of the unfrozen glycerol treatment, and the overall results suggest that this medium was less suitable. Thus, DMSO was selected over glycerol for further fertility testing.

### *Semen-to-ova Ratio*

The results from the semen-to-ova ratio experiment indicate that a 1:100 (v/v) ratio can provide high fertilization and hatching rates in *L. calcarifer*. This finding does not allow for variability in semen quality between fish, but it does indicate scope for economic use of frozen and unfrozen semen stocks. Assuming that 1 mL of freshly stripped ova from *L. calcarifer* contains about 3000 eggs (volumetric determinations by A. Hogan, Walkamin Research Station, personal communication),  $1.2 \times 10^5$  sperm per egg were used in the 1:100 (v/v) ratio treatment. Rana and McAndrew (1989) reported satisfactory fertilization in tilapia (*Oreochromis* spp.) using similar numbers ( $1.4 \times 10^5$ ) of cryopreserved sperm per egg. In north Queensland, undiluted unfrozen semen-to-ova ratios of 1:10 (v/v) have commonly provided high (>90%) fertilization rates in *L. calcarifer* eggs stripped in the field. The demonstration, in the present study, of successful minimal sperm usage in *L. calcarifer* has application in the mass culture of this highly fecund species, since the numbers of running-ripe male fish at the start and finish of the hatchery season can be much reduced. Sperm extension through dilution (in this case with the cryopreservative medium) improves the mixing of a limited amount of semen with larger volumes of eggs, with possible associated improvements in fertilization rates using dry insemination techniques.

### *Fish Produced with Frozen Sperm*

Studies with other fish species have shown that there are few qualitative differences between fish produced from fresh and cryopreserved sperm (Chao *et al.* 1986; Rana and McAndrew 1989). The fish produced in this study also appeared normal. They were progeny from sperm collected in Weipa and ova from broodstock originating from the Burrum River in south-eastern Queensland, so they were crossed-strain fish from two genetically different natural populations (Shaklee and Salini 1985). Crossing of strains may improve vigour or provide desirable traits for aquaculture, and sperm cryopreservation could aid such research and production.

### *Aquaculture Implications*

There are currently three methods used by hatcheries in Australia to obtain fertilized *L. calcarifer* eggs. The first (and currently the most reliable) is hormone-induced tank

spawning of domesticated broodstock, for which sperm cryopreservation is of little direct benefit. One short-coming of spawning fish in this way is that hatchery managers, aside from observations of pre-spawning courtship displays, are often unsure how many, and to what degree, males present in the tank have participated in the spawning and therefore contributed to the progeny. In restocking programmes, this has particular relevance if the genetic diversity of stocked fish is to be controlled. The ability to fertilize a batch of eggs with sperm from a larger number of males is greatly enhanced by sperm cryopreservation, although stripping and artificial insemination of eggs would be necessary.

The second method commonly used for acquisition of fertilized eggs in *L. calcarifer* is to strip and fertilize eggs from wild ripe females captured during the spawning season. This method can provide high-quality ova, as demonstrated by the first fertility experiments in this study. These activities are strictly regulated by government authorities in Australia for protection of natural stocks, but semen collected under permit and then cryopreserved could provide new or additional genetic material for future breeding programmes and also be a safe-guard for sperm supply if few ripe males are captured with the female fish.

The third method available for egg acquisition is hormone-induced maturation of captive broodstock with ova stripped and fertilized soon after ovulation. This technique can be less reliable than tank spawning because of complications from different follicle sizes at injection, hormone dosage and latent time, water temperature, fish condition, and stress from handling. However, this method provides the best situation for selective breeding programmes, and studies of hormone induction and latent periods of *L. calcarifer* (Garrett and Rasmussen 1987; Garcia 1989, 1992) are useful in predicting appropriate stripping times. Sperm cryopreservation could form an integral part of such selective breeding exercises and, with barramundi, an individual fish could be crossed with itself after sex reversal. This could reduce the time taken for isolation of desirable traits for aquaculture and provide more precise estimates of heritability and the likelihood of inbreeding depression (C. P. Keenan, personal communication).

## Conclusion

The acquisition of high-quality gametes from *L. calcarifer* can be difficult and time consuming, making storage of excess gametes highly desirable. Cryopreservation of *L. calcarifer* sperm is feasible and well suited to specialized breeding. Poor egg quality coupled with incubation problems are the most likely causes of the low fertility of cryopreserved *L. calcarifer* sperm reported in earlier work, since both cryoprotectants used by R. Pidgeon, A. W. Blackshaw, M. F. Capra and J. Glaister (unpublished) routinely provided fertile spermatozoa in the present study. DMSO as a cryoprotectant of *L. calcarifer* sperm presented fewer complications, such as premature or delayed activation, and provided higher hatching rates than did glycerol. DMSO is potentially harmful to humans (Willhite and Katz 1984) and skin contact and inhalation of its vapours should be avoided, but it is currently one of the best cryoprotectants available for sperm of *L. calcarifer* and other species. Its use in cryopreservation of *L. calcarifer* sperm provides opportunities for natural resource conservation in Australia through preservation of the genetic diversity of existing barramundi wild stocks, and makes such genetic material more accessible for barramundi hatcheries on a global scale.

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