

Short-term Cold Storage of Sperm from Six Neotropical Characiformes Fishes

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ABSTRACT

Sperm of the following Neotropical Characiformes fish species were tested for cold storage: Brycon lundii, Piaractus mesopotamicus, Leporinus elongatus, Leporinus friderici, Prochilodus lineatus and Prochilodus marggravii. Each sperm sample was split into two aliquots. The first was placed into a plastic bag with air or oxygen and the second, in a plastic tube with air. The samples were maintained at temperatures between 1.7-4.9 °C. The rate of sperm motility was estimated using a 50 mM NaCl solution as the activating solution. The shortest sperm storage duration (7 h) was recorded for L. friderici, when the sperm motility rate reached ~ 30%, whereas the longest duration (20 h) was obtained with the sperm of P. lineatus. A fertilisation test using Prochilodus marggravii sperm refrigerated for 8 h yielded 88-90% of viable embryos. The refrigerated storage method could be of practical applications, especially in fish reproductive management at hatchery stations.

Key words: Cool storage of sperm, sperm motility, semen, Neotropical fishes

INTRODUCTION

The storage of fish sperm at temperatures above freezing and without the addition of cryoprotectants has long been used as a successful method for refrigerated preservation of sperm (McNiven et al., 1993). This technique is seldom used by Neotropical fish culturists; however, it could be relevant to reproductive management of their fishes, specially given the widespread applications of artificial propagation techniques. Despite its practical importance (Barret, 1951; Billard and Legendre, 1982; Kavamoto et al., 1987), this is only the second study to be published in which this storage method is used with Neotropical fishes. In the only publication using one of these species, Ferraz de Lima et al. (1989) reported good results after storing

Piaractus mesopotamicus (Holmberg) sperm for 30h in a domestic refrigerator, under oxygen atmosphere. Cold storage is a simple technique that allows sperm to be available at various time intervals, for the fertilisation of eggs produced by hormone-induced females, which ensures greater productivity in the reproductive process. By facilitating the use of sperm from a larger number of males per female, it can help improve the genetic quality of the resulting offspring, in addition to allowing the characteristics of the sperm to be studied (Ciereszko and Dabrowski, 1994). An additional advantage is the ease of transport in appropriate containers (Henderson and Dewar, 1959). Plastic bags (Billard, 1981; Billard and Legendre, 1982; Stoss and Refstie, 1983) and plastic tubes (Chereguini et al., 1997; Wayman et al., 1998) are widely used as storage containers

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to which gases may be added (i.e., oxygen: Billard, 1981; Cieresko and Dabrowski, 1994; Dilauro et al., 1994; Chereguini et al., 1997; Lanhsteiner and Patzner, 1998; Bencic et al., 2000; Jenkins-Keeran et al., 2001; air: Billard, 1981; Billard and Legendre, 1982; Bencic et al., 2000; Jenkins-Keeran et al., 2001; nitrogen: Bencic et al., 2000; Jenkins-Keeran et al., 2001). The quality of the preserved sperm can be evaluated by sperm motility rate, motility duration, and by its fertilising capacity (Rana, 1995).

The Characiformes species used in this study were medium- to large-size fishes of great relevance to commercial fishing and sports. For this reason, they are widely used in fry mass production destined to commercial farms and restocking programs. *Brycon lundii* Reinhardt have had its stocks greatly reduced and its natural populations are currently endangered (Lins et al., 1997). The objective of this study was to determine the effectiveness of cold storage of sperm from Neotropical species by assessing sperm motility and its fertilising capacity.

MATERIAL AND METHODS

All storage experiments were conducted using small (23 x 18 x 14 cm) styrofoam boxes containing a 3-cm layer of ice cubes or a frozen thermogel bag. To help maintain the temperature and facilitate transport, these boxes were placed inside a larger (80 x 50 x 58 cm) styrofoam box.

Storage temperature determination

In three preliminary assays, the storage temperatures inside the small styrofoam boxes containing ice cubes or a frozen thermogel bag were hourly recorded with the help of a thermopar thermometer. The assays with ice cubes lasted for 6 h; at this time, the ice was melting. The assays with thermogel bags extended for 12 h.

Fish and sperm collection

The following species were used (with the respective number of individuals used and their weight and total length, in parenthesis): *Brycon lundii* Reinhardt (4; 577.5±121.2 g; 37.4±2.4 cm), *Piaractus mesopotamicus* (Holmberg) (6; 2971.7±987.3 g; 51.0±4.6 cm), *Leporinus elongatus* Valenciennes (4; 378.3±110.0 g; 34.1±2.0 cm), *Leporinus friderici* (Bloch) (5;

472.0±150.0 g; 28.0±9.5 cm), *Prochilodus lineatus* (Valenciennes) (6; 1470.0±706.5 g; 47.9±0.6 cm) and *Prochilodus marggravii* (Walbaum) (6; 414.2±162.6 g; 32.8±3.6 cm). The donors, originated from hatchery station, were previously maintained in earthen ponds where they received commercial feed daily, containing a minimum of 22% crude protein. From November, 1999 to January, 2000, the fish were ready to yield sperm. At this time, the sperm oozed from the genital papilla under slight manual pressure. The coelomic wall of the fish was massaged gently and the extruded sperm collected in 12-mL plastic capped tubes. In collecting the sperm, great care was taken to prevent contamination with urine, faeces, blood and/or water. Test for contamination was performed by observing a small drop of each fresh sperm sample on a glass slide under the light microscope at 400x. Samples showing no sperm motility were used in the experiments.

Sperm storage

Sperm from each individual was divided into two aliquots of 0.5 to 2 mL each. One of the aliquots was placed in a 10 x 20-cm plastic bag inflated with oxygen (sperm from *P. mesopotamicus*, *L. elongatus*, *L. friderici*, *P. lineatus* and *P. marggravii*) or air (*B. lundii*) and the other one was kept in the original capped tube. The ratio sperm: air or oxygen (v:v, mL) was 1:110-440. The aliquots were stored in the small styrofoam boxes containing either ice cubes or a frozen thermogel bag. Oxygen or air and ice cubes were renewed at intervals of approximately 6 h, whereas the frozen thermogel bags were renewed at 12-h intervals.

Sperm motility activation

Before the storage tests, NaCl solutions of different osmolalities were tested for sperm motility activation. The sperm samples were pre-diluted at a ratio of 1:100 with NaCl 200 mM (previously established as non-activator of sperm motility). This mixture was again diluted (final dilution ratio = 1:1000) with NaCl solutions at various concentrations (150, 100, 75, 50, 25 mM), and immediately analysed. Motility rates were obtained under a light microscope, using 400 x magnification. The two-stage dilution of the sperm was carried out as recommended by Billard and Cosson (1992) for adequate motility evaluation.

The NaCl concentration at which the highest sperm motility rate occurred, i.e. 50 mM, was used for activation of the spermatozoa during the storage experiments. The sperm motility analyses were performed at intervals of ~5-9h and continued until the rate of sperm motility reached ~30%.

Fertilisation with stored sperm

A fertilisation test was made with stored sperm from *Prochilodus marggravii* of a hatchery station following its routine protocol (Sato et al. 1996). Sperm from two fishes was pooled (1.5 mL), kept cool for 8 h in a plastic tube on a frozen thermogel bag, as described before, and used for the fertilisation of 240 g of eggs obtained by artificially induced spawning. Approximately one h prior to fertilisation, sperm motility rate was estimated (%). Eggs and sperm were placed in a plastic bowl and gently mixed together, with water from the incubators being added. After being washed and hydrated, the eggs were divided into two portions and each portion was transferred to a funnel-type, 60-L capacity plastic incubator. Egg samples from both incubators were collected 5 h after fertilisation and immediately fixed in 4% formalin buffered solution. The percentage of viable eggs, as identified under a stereomicroscope, was then determined as a measure of the fertilising success. Fertilisation rates obtained with fresh sperm in the routine work of the hatchery station was used as control. Historical hatchery station of *P. marggravii* fertility rate was 68.8 ± 18.7 (Sato et al., 1996).

Statistical analyses

The sperm motility rates of sperm stored in both containers were compared using the Mann-Whitney U. test. Kruskal-Wallis test was applied to detect any differences in sperm motility rate at the different time intervals. Median differences were considered to be significant at $P < 0.05$ (Statistica for Windows, Tulsa, OK, 1996).

RESULTS

Storage temperatures

The assays made with ice cubes or frozen thermogel bag showed temperatures averaging from 1.7 to 4.9°C over the zero - 12h interval. During these assays, room temperature was 21.8 to 24°C.

Motility rate of stored sperm

The rates of sperm motility gradually declined over the course of the experiments, with variations occurring between species and individuals of the same species (Fig. 1). Such a decline in sperm motility rates was significant for all species ($P < 0.05$). However, there were no significant differences in sperm motility rates between samples from either storage environment - plastic bag and plastic tube ($P > 0.05$) - except for *P. marggravii* at 6 h. Considering a sperm motility rate of 30%, which could still have practical applications, this value was reached at 7 h in *L. friderici* and at 20 h in *P. lineatus* (Fig. 1); representing, respectively, the shortest and longest storage duration.

Fertilisation

The percentages of *Prochilodus marggravii* eggs fertilised with 8h stored sperm were 88 and 90%, respectively, for the two incubators.

DISCUSSION

Sperm immotility in all species of this study, when incubated in NaCl 200 mM, suggested that the osmolality of their seminal fluid probably did not differ from that of teleosts in general (Morisawa and Suzuki, 1980). Sperm from all species studied was induced to motility by a reduction in the osmotic pressure of the diluent as demonstrated in other species (Morisawa and Suzuki, 1980; Billard and Cosson, 1992; Cosson et al., 1999). Considering that the dilution rate was a determining factor in the characterisation of sperm motility, a relatively high rate of dilution was used to produce simultaneous activation of almost all spermatozoa, following the recommendation of Billard and Cosson (1992). The method described in this work allowed sperm storage in cooled plastic bags and capped tubes for at least 7h, at temperatures averaging from 1.7 - 4.9°C. With frozen thermogel bags, temperatures up to 4.9°C were maintained during 12 h of storage, which made them more advantageous than ice cubes. Sperm motility rates gradually declined over the period of storage, with marked individual variation. No attempt was made to determine the probable causes of such variations, yet the following could explain them: i) use of a subjective scale for motility evaluation, which might have led to measurement errors; ii)

difficulty in adequately evaluate contamination of the samples with urine or faeces (Saad and Billard, 1995; Legendre et al., 1996), undetected *a priori*; iii) differences in pH levels - not evaluated in our

study - between the seminal plasma and the diluting solution (Cosson et al., 1999); and iv) thermal shock (Billard et al., 1993).

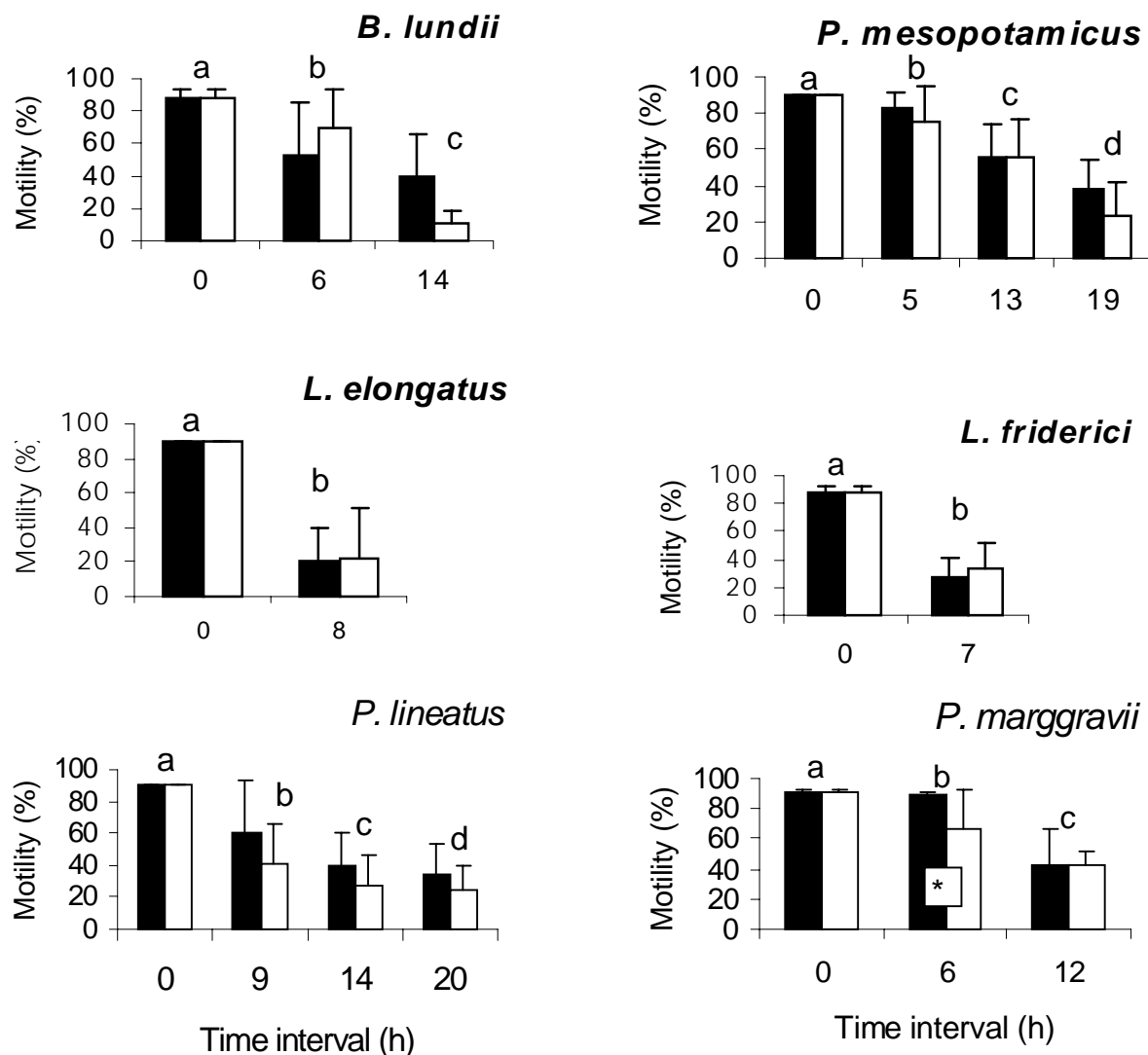


Figure 1 - Sperm motility (%) at different time intervals (h) using NaCl 50 mM as activating solution; dark column (■): sperm stored in plastic bag; blank column (□): sperm stored in plastic tube; different letters above columns indicate significant differences ($P < 0.05$) between time intervals in each storage recipient; * = significant difference between storage recipients ($P < 0.05$).

The environments in which sperm is stored are critical to successful preservation, as they must be capable of ensuring that spermatozoa remain viable outside the genital tract. Plastic bags gassed with air or oxygen and plastic tubes stoppered to prevent drying (Billard et al., 1993) both proved useful for the preservation of sperm from our

species. The presence of oxygen is considered to be important for the sperm preservation process (Billard, 1981; McNiven et al., 1993; Bencic et al., 2000; Jenkins-Keeran et al., 2001). Although the ratio of sperm:oxygen or air was as recommended by Stoss and Refstie (1983), the addition of oxygen or air did not result in significant

improvement of sperm viability of our fish, except in the case of *P. marggravii*. Its sperm, kept for 6 h in plastic bags gassed with oxygen, exhibited significantly higher sperm motility when compared with the sperm stored in the original capped tubes (Fig. 1).

Although sperm may be preserved in diluted form (Legendre et al., 1996), in this study we used it undiluted, fresh and integral. According to Cosson et al. (1999), sperm for preservation should show initial sperm motility rates of more than 90%. All sperm used in this study had an initial motility rate of at least 80% (see Fig. 1). Duration of sperm viability, considering the minimal practical rate of 30%, varied from 7 to 20 h in the different species used in this work. Sperm from *L. friderici* and *L. elongatus*, both of the Anostomidae family, had the shortest duration of motility, i.e. 7-8 h, whereas that from *P. mesopotamicus* and *P. lineatus* remained motile until 19-20 h, when a motility rate of ~40% was reached. The 30-h period of survival reported by Ferraz de Lima et al. (1989) for *P. mesopotamicus* sperm cannot be compared with the data obtained in this study, since sperm motility rate at the end of the survival period was not mentioned by those authors. Sperm from fishes of temperate regions can remain viable for much longer periods - days or weeks - (Billard, 1981; Billard and Legendre, 1982; Hara et al., 1982; Stoss and Refstie, 1983; Ciereszko and Dabrowski, 1994; Dilauro et al., 1994; Bencic et al., 2000; Jenkins-Keeran et al., 2001), as compared to the results reported here for Neotropical fishes.

The high percentages of *P. marggravii* eggs that were fertilised with 8 h stored sperm, comparable to those obtained with fresh sperm under routine hatchery conditions (Sato et al., 1996), confirmed the applicability of the cooling preservation technique described herein. Thus, sperm of the species studied here could be preserved for up to 20 h in some cases, maintaining its fertilising capacity, when stored in plastic bags, with or without oxygen addition, or in capped tubes, at temperatures between 1.7 to 4.9 °C.

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RESUMO

O sêmen das seguintes espécies de Characiformes neotropicals foi testado para armazenamento em ambiente resfriado: *Brycon lundii*, *Piaractus mesopotamicus*, *Leporinus elongatus*, *Leporinus friderici*, *Prochilodus lineatus* e *Prochilodus marggravii*. Amostras de sêmen, obtidas por massagem da parede celômica, foram armazenadas em saco plástico com ar ou oxigênio ou em tubo plástico com ar, e mantidas resfriadas entre 1,7-4,9°C. A taxa de motilidade espermática foi estimada usando-se NaCl 50 mM como solução ativadora. O sêmen com menor duração de viabilidade (7 h) foi o de *L. friderici*, quando a taxa de motilidade espermática alcançou ~30%, enquanto que o de maior duração (20 h) foi o de *P. lineatus*. A fertilização de ovócitos utilizando sêmen refrigerado por 8 h de *Prochilodus marggravii* produziu 88-90% de embriões viáveis. O método de armazenamento desenvolvido neste trabalho tem aplicações práticas, especialmente no manejo reprodutivo de peixes em estações de piscicultura.

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