The degenerate primers MAR124F and MAR276R were initially used by various groups[4,6,7,11] to scan the *B. mori* genome for the MLEs. In all the cases it amplified only one type of MLE, despite the feature that these primers are capable of amplifying multiple types of mariner elements[2]. But in the current study, RGF–PCR amplified three different types of MLEs (Table 5), which was not possible in the earlier studies. The probable reason for this is that in conventional PCR, while there are numerous copies of a gene, some copies might be amplified in the earlier cycles and those copies will be amplified further and further. Moreover in a given PCR condition, some copies may amplify better than their counterparts of the same genome. But in RGF–PCR, different regions of the template DNA are accessible to primers in different fractions and so all the copies have a chance for amplification from one or the other individual fractions. Further, using different restriction enzymes and different combinations of restriction enzymes, many regions of the genomic DNA can be fractionated and more individual copies can be amplified.

Limitation in this technique may be due to the following: (i) partial or incomplete digestion of the genomic DNA; (ii) shearing of DNA during extraction process, and (iii) molecular trapping of part of the smaller fragments of DNA in large fragments in gel electrophoresis. These factors can result in the amplification of the same copy from different fractions. However, such kind of limitations can be eliminated by careful extraction of the genomic DNA, complete digestion of the DNA with restriction enzyme and by complete fractionation of the fragments. Despite these features, RGF–PCR will be a useful technique to isolate, amplify and analyse the intragenomic polymorphism of different copies of a multi-copy gene. Apart from analysing the multi-copy genes, this technique can be used to (i) analyse the repetitive DNA elements, and (ii) locate and analyse the integration site of the transgene in different regions of the genome of a transgenic animal.

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Live sperm from post-mortem preserved Indian catfish*

R. Koteeswaran and T. J. Pandian[†]

Centre for Advanced Studies in Functional Genomics, Madurai Kamaraj University, Madurai 625 021, India

In the 240-day post-mortem preserved (at –20°C) specimens of *Heteropneustes fossilis,* live, fertile sperm are present. Motility and initiation of development were adduced as evidence for survival and fertility of the cadaveric sperm. Of the normal eggs that were fertilized by the cadaveric sperm, 97% underwent cortical reactions, 95% first few cell divisions, 40% blastulation, 3% gastrulation and 2% alone successfully hatched.

THIS communication reports about the discovery of live, fertile sperm from dead but preserved (at –20°C) specimens of the teleostean fish *Heteropneustes fossilis* for 240 days. Investigations on mammalian organ transplantation, especially humans have shown that non-vascularized cornea of the eye can be *in vitro* preserved for the longest duration of 4.6 years[1]; however, transplantation of vascularized organs like kidney and heart is directly made from the donor; the recently developed perfusion techniques have extended the life of cadaver kidney (33 days)[2] or heart (2 days)[3] for a limited duration prior to its transplantation. Hence, the life of vascularized organs can be prolonged under special conditions for a known period of time, even after their removal from live or just-dead donors. However, the need for such transfer of other vascularized organs like testis seems not to have arisen; for a literature search indicated that except for a stray report of Dushkina[4],

*Dedicated to Prof. M. S. Swaminathan, scientist, scholar and humanist, on his 76th birthday.

[†]For correspondence. (e-mail: tjpandi@pronet.net.in)

which claimed the fertility of sperm drawn from dead herring, *Clupea pallasi* stored at 0.8°C for 2 days, no report is available on survival and fertility of cadaveric sperm of vertebrates. This report presents experimental evidences for survival and fertility of the cadaveric sperm drawn from specimens of *H. fossilis*, that were post-mortem preserved at –20°C for 240 days.

Short-term storage of fish sperm, ranging from hours to weeks, is a technique profitably used in hatcheries to overcome problems such as asynchrony in maturation, transportation of gametes and/or selective use of spawners[5]. The males of silurids, a group to which *H. fossilis* belongs, are generally not amenable for stripping[6]; many catfishes like *H. fossilis* are amenable for multiple spawning[7] and/or *in vivo* preservation of eggs[8]. Therefore, the need for short- and long-term sperm preservation of silurids is obvious. Cryopreservation of fish sperm has been on increasing demand with the development of genetic selection and manipulation (e.g. ploidy[9], transgenic[10]) and the unpredictable risk of losing valuable strains. Briefly, sperm cryopreservation is an important means to preserve biodiversity. However, the need for costly equipment, including liquid nitrogen facility and sophisticated techniques has been a bottleneck in the development of protocols for cryopreservation of fish sperm, especially in developing countries like India. Therefore a simple, easily practicable technique of post-mortem preservation of fish sperm may prove a boon.

The death of fish is recognized, when it helplessly sinks to the bottom of the aquarium and fails to make spontaneous opercular movement, allowing the outgoing water from the gill chamber. Being a facultative air-breathing fish, *H. fossilis* may be kept out of water for a maximum duration of 10 h at 28°C. Its death can be recognized when it fails to make spontaneous opercular movement or shows no response on being disturbed.

A collection consisting of freshly dead adult *H. fossilis* males (< 18 ± 1.5 cm standard length) was preserved at –20°C within 6 h following death on 30 January 2001. This post-mortem storage temperature was chosen, as there are indications that at storage temperature below –18°C, no appreciable biochemical changes occur over a period of 10 months[11]. Viability and fertility of vertebrate sperm are recognized by one or more of the following: (i) tail movement, (ii) acrosome reaction[12] and (iii) initiation of development. An acrosome is absent in teleostean fishes[5]. Therefore, physiological evidence for viability and fertility of fish sperm can be obtained only from microscopic observation of tail movement and initiation of development.

The teleostean sperm may be motile for a relatively longer duration, owing to external fertilization and obligate need for the sperm to enter the egg through the micropyle, as the acrosome is absent. Frequency of sperm displaying motility was observed and traced in a selected area of a haemocytometer (Neu-Bauer, Germany) on the screen of the system-integrated video console. Microscopic observations of sperm, drawn from specimens that were post-mortem preserved for

Table 1. Sperm motility and fertility in *Heteropneustes fossilis* post-mortem preserved for different durations. Each value represents the mean (± SD) of a minimum of 25 estimations made on sperm drawn from a minimum of 5 post-mortem preserved specimens

Preservation duration (days)	Motility (%)	Fertility (%)
10	95.7 ± 2.134	94.2 ± 2.482
20	95.4 ± 2.059	96.0 ± 1.673
30	97.4 ± 0.800	94.2 ± 2.713
60	95.0 ± 1.414	89.6 ± 2.577
90	97.0 ± 1.095	88.2 ± 1.939
120	97.4 ± 1.200	94.0 ± 2.366
150	93.4 ± 2.577	93.0 ± 1.897
180	96.4 ± 2.059	96.4 ± 1.200
210	94.8 ± 2.135	97.0 ± 1.095
240	96.4 ± 1.855	93.6 ± 1.855
Control	98.4 ± 1.367	98.3 ± 0.914

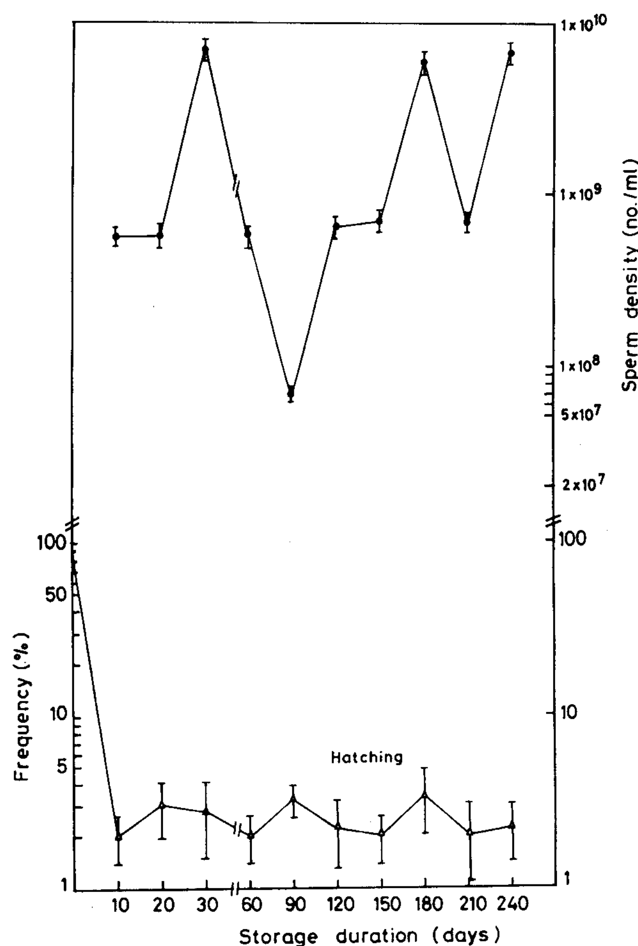


Figure 1. Sperm density (upper panel) and frequency of hatching (lower panel) of *H. fossilis* as function of the duration of post-mortem preservation. Each value represents the mean (± SD) of a minimum of 25 estimations made on sperm drawn from a minimum of 5 post-mortem preserved specimens.

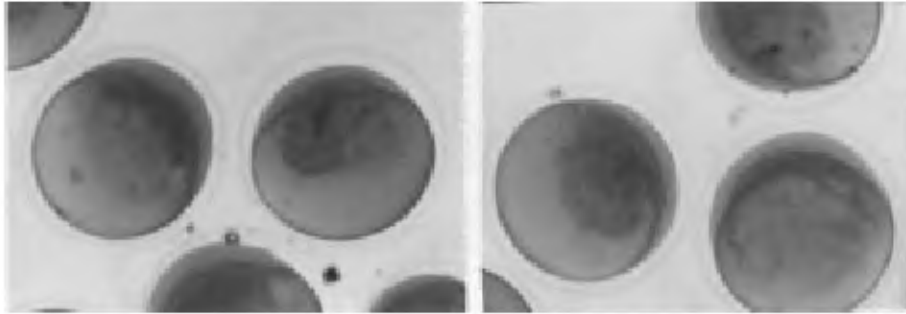


Figure 2. Eggs undergoing cortical reactions on being fertilized by sperm drawn from live (left panel) and 240-day post-mortem preserved (right panel) specimens of *H. fossilis.*

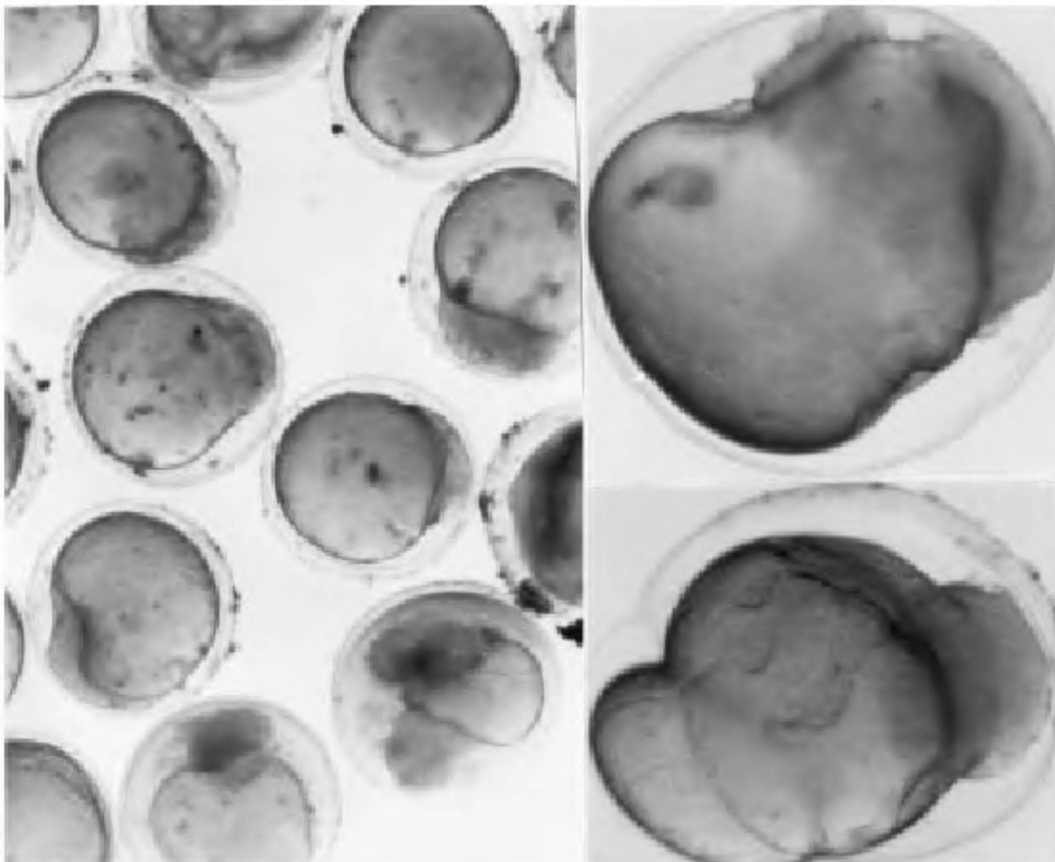


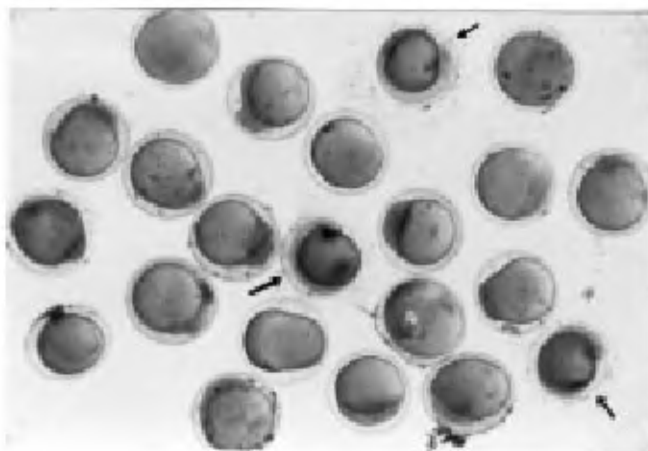
Figure 3. Asynchronized cleavage (right panel) and disintegrating blastomeres (left panel) of *H. fossilis*. These eggs were fertilized by the 240-day post-mortem preserved sperm.

10, 20, 30, 60, 90, 120, 150, 180, 210 and 240 days showed that they were motile, immediately following the addition of freshwater to the milt. Counting of the frequency of motile sperm, drawn from live and cadaveric specimens clearly indicated that almost all the sperm of both cases were motile. This observation confirmed that the sperm remained alive, even when the specimen was post-mortem (within 6 h following death) preserved for a duration of 240 days (Table 1).

Since *H. fossilis* is not amenable for stripping, 0.1 g (wet weight) tissue of testis was removed from live and post-mortem preserved specimens, and macerated in 1 ml of 0.9% NaCl. From this solution containing the macerated testis, 25 μl was transferred to haemocytometer for microscopic counting (Nikon, Japan) of sperm density, following the standard procedure described by Gunasekaran[13]. For artificial fertilization studies, cadaveric sperm density of 1×10^5/egg was used. This is higher than the minimum sperm density of 8×10^3, that is known to ensure successful activation/fertilization in eggs of *H. fossilis*[14]. Estimation of the sperm density of the live and post-mortem preserved specimens, despite wide fluctuations, showed no significant difference; life continues to persist in the cadaveric sperm even after 240 days (Figure 1). Briefly, almost equal number of sperm are alive and fertile in live and post-mortem preserved specimens of *H. fossilis*.

Batches of 50 *H. fossilis* eggs were dry fertilized, each with saline containing sperm from 0.1 g of macerated testis of live or post-mortem preserved cadaveric specimens. Within 35 s following the addition of freshwater to the eggs–milt complex, cortical reactions were apparent (Figure 2). As entry of the conspecific irradiated sperm, or the heterospecific intact or irradiated sperm can activate the egg and initiate cortical reaction[9], the formation of pre-vitelline space in *H. fossilis* egg owing to the entry of cadaveric sperm by itself cannot be taken as an evidence for the genetic viability of the sperm. However, the cortical reaction was followed by the cleavages indicating that the cadaveric sperm remained genetically as fertile as the sperm of live specimens. Thus in the eggs that were fertilized by the cadaveric sperm, the first cell division was completed within 28 min. Considering cell cleavage as an index of egg fertilization, it was noted that all the eggs (100%) were fertilized when the sperm from live specimens were used. Equally, high values (≈ 95%) of fertilization were recorded, when sperm from cadaveric specimens preserved for 10 to 240 days were used (Table 1). This clearly indicates that the cadaveric sperm, irrespective of short or long duration of preservation, remained equally fertile, as those from the live specimens.

Embryonic development of the eggs fertilized by normal or cadaveric sperm was followed. Figure 3 shows that the eggs fertilized by cadaveric sperm began to suffer from asynchronous cleavage and disintegrating blastomeres; therefore, < 40% of the eggs fertilized by cadaveric sperm alone successfully completed blastulation. The impairment of development in these embryos became more and more apparent as development proceeded. Consequently, ≈ 3% eggs underwent gastrulation (Figure 4) and 2% alone hatched as normal fry. In these fry, the yolk was reabsorbed 3 days after hatching, as it happened in the normal ones. The fry hatched from eggs fertilized by the cadaveric sperm began to feed on the fourth day. Hatchability values perceptibly decreased and oscillated between 2 and 4%, even when 10-day-old cadaveric sperms were used (Figure 1). The reason(s) for the massive failure of blastulation of the developing eggs fertilized by the 10-day-old cadaveric sperm are not known, but are being investigated.



Figur 4. Developing eggs fertilized by the 240-day old cadaveric sperm of *H. fossilis*. Note the somite formation in embryos that are marked by arrows; the other embryos suffer impairment during different developmental stages of *H. fossilis*.

This report has shown the possibility of using a simple and widely practicable method of post-mortem preservation of sperm of teleostean fish. A taxonomic survey clearly indicated the successful use of cadaveric sperm to initiate development in many freshwater and marine teleostean fishes. For instance, 51% sperm of *Ephinephleus merra* were live and motile, even after the post-mortem preservation of the grouper at –20°C for a period longer than 112 days (Koteeswaran, David and Pandian, unpublished observation).

The sting of death is perhaps first manifested in the heart and brain, but its manifestation in other vascular organs may occur sooner or later. Due to the total absence of motility, the vascularized organs like muscle and testis may meet the subdued energy requirement by partial or complete anaerobiosis. Therefore they remain alive for a longer duration, when the respective organ is post-mortem preserved. Yet the fact that more and more eggs of *H. fossilis*, which were fertilized by the cadaveric sperm, succumbed during blastulation and gastrulation may indicate the impairment/death of critical number of genes or strategically important genes, responsible for blastulation and/or gastrulation even within 10 days of post-mortem preservation.

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