



APPLICATION OF INNOVATIVE BIOTECHNOLOGIES REGARDING AQUACULTURE AND FISHERIES SECTOR IN MALAYSIA : CRYOPRESERVATION PROGRAMME

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General ideas of cryopreservation

- **Semen cryopreservation** : Storage of semen samples in the ultra-low temperature in liquid nitrogen (-196° C).
- A technology for storage of semen samples indefinitely without deterioration
- It is new among the aquaculture industries and also in conservation of our fisheries genetic resources in Malaysia.

General ideas of cryopreservation

- Semen cryopreservation is important either for aquaculture or in conserving our fisheries genetic resources for future and repeated use
- To date, there isn't any standard protocol available for fish semen cryopreservation.
- Standardization in the semen cryopreservation protocols in fish species is not an easy task (Tiersch 2000).
- Different fish species gives different response to the so called excellent cryopreservation protocol /extender formulation/cryoprotectants.

Background

- Cryopreservation of fish semen was started at Freshwater Fisheries Research Centre (FFRC) in 2006 as one of the research projects in its 9th Malaysia Plan (2006-2010) research programme “**Conservation of Inland Fisheries Resources**”
- Project duration: Five years (2006 -2010)
- Source of funding: Developmental fund of the 9th Malaysia Plan

Background

- Programme “**Conservation of Inland Fisheries Resources**” consisting of 4 main projects:-
 1. [Ecology, Biology and Genetics of Selected Freshwater Fishes](#)
 2. [Conservation and Management of Freshwater Fishes](#)
 3. [Repository Centre for Freshwater Fishes](#)
 4. [Sanctuary of Freshwater Fishes](#)

Conservation of Inland Fisheries Resources Programme

No	Research Activities	Estimated Cost (RM million)					
		2006	2007	2008	2009	2010	Total
1.	<p>Ecology, Biology and Genetic of Selected Freshwater Fishes (<i>Probarbus julieni</i>, <i>Pangasius nasutus</i>, <i>Tor</i> sp.)</p> <p>1.1. Monitoring/ Field work : Jan 2006 – Jun 2008</p> <p>1.2. Taxonomy / morphology and genetics studies: Jan 2006 – Dec 2009</p> <p>1.3. Cryopreservation of gametes: Jan 2006 – Dec 2010</p> <p>1.4. Reproductive Biology : Jan 2006- Dec 2009</p>	0.186	0.406	0.348	0.148	0.098	1.186

Cryopreservation of fish gametes

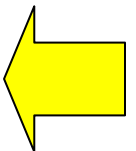
Project activities.....

- Collection of mature male broodstocks
- Evaluation of sperm concentration and quality prefreezing
- Optimization of extender suitability, equilibration, freezing and thawing (rates, duration and temperature)
- Post-thawing evaluation

Expected output.....

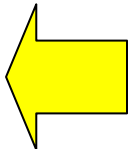
- Establishment of gene / sperm bank for threatened / endangered species (conservation) and superior breed (aquaculture)

Funding (5 years): RM 500,000 (143,000 USD)



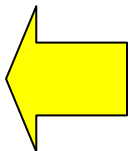
Conservation of Inland Fisheries Resources Programme

No	Research Activities	Estimated Cost (RM million)					
		2006	2007	2008	2009	2010	Total
2.	Conservation and Management of Freshwater Fishes 2.1. Broodstock development : Jan 2006 – Dec 2009 2.2. Spawning : Jan 2007- Dec 2009 2.3. Public release of fish fry: Jan 2008 – Dec 2009 2.4. Tagging and monitoring : Jan 2008 – Dec 2010	0.026	0.039	0.102	0.077	0.067	0.311



Conservation of Inland Fisheries Resources Programme

No	Research Activities	Estimated Cost (RM million)					
		2006	2007	2008	2009	2010	Total
3.	Repository Centre for Freshwater Fishes 3.1. Technical visit, courses and consultation services: Jan 2006-Jun 2006 3.2. Modification: Jun 2006-Jan 2007 3.3. Purchasing of equipment and collection of specimens: Jan 2007- Dec 2010 3.4. Cataloging – computer and software: Jun 2006 – Dec 2010	0.038	0.160	0.020	0.015	0.015	0.248



Conservation of Inland Fisheries Resources Programme

No	Research Activities	Estimated Cost (RM million)					
		2006	2007	2008	2009	2010	Total
4.	Sanctuary of Freshwater Fishes 4.1. Ecosystem Studies: Jan 2007 – Dec 2008 4.2. Public awareness programme and Management: Jan 2007 – Dec 2010	0.000	0.070	0.080	0.110	0.130	0.390
	Grand total	0.250	0.675	0.550	0.350	0.310	2.135

MAIN OBJECTIVES OF THE GAMETE CRYOPRESERVATION PROJECT

- To develop the species-specific protocols for spermatozoa cryopreservation of selected indigenous freshwater fish species
- To establish a cryogenic sperm bank for the threatened and endangered species (conservation purposes) and also the potential indigenous species for aquaculture

SPECIES OF OUR FOCUS

- Conservation : -

- [Probarbus jullieni](#)

- *Tor tambroides*, *T. douronensis*



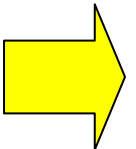
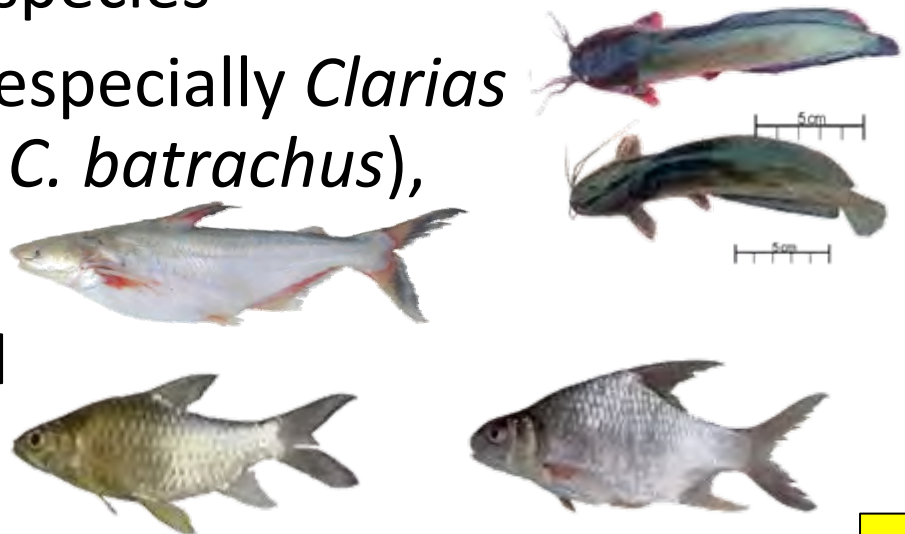
- Potential aquaculture species

- Indigenous catfish (especially *Clarias macrocephalus* and *C. batrachus*),

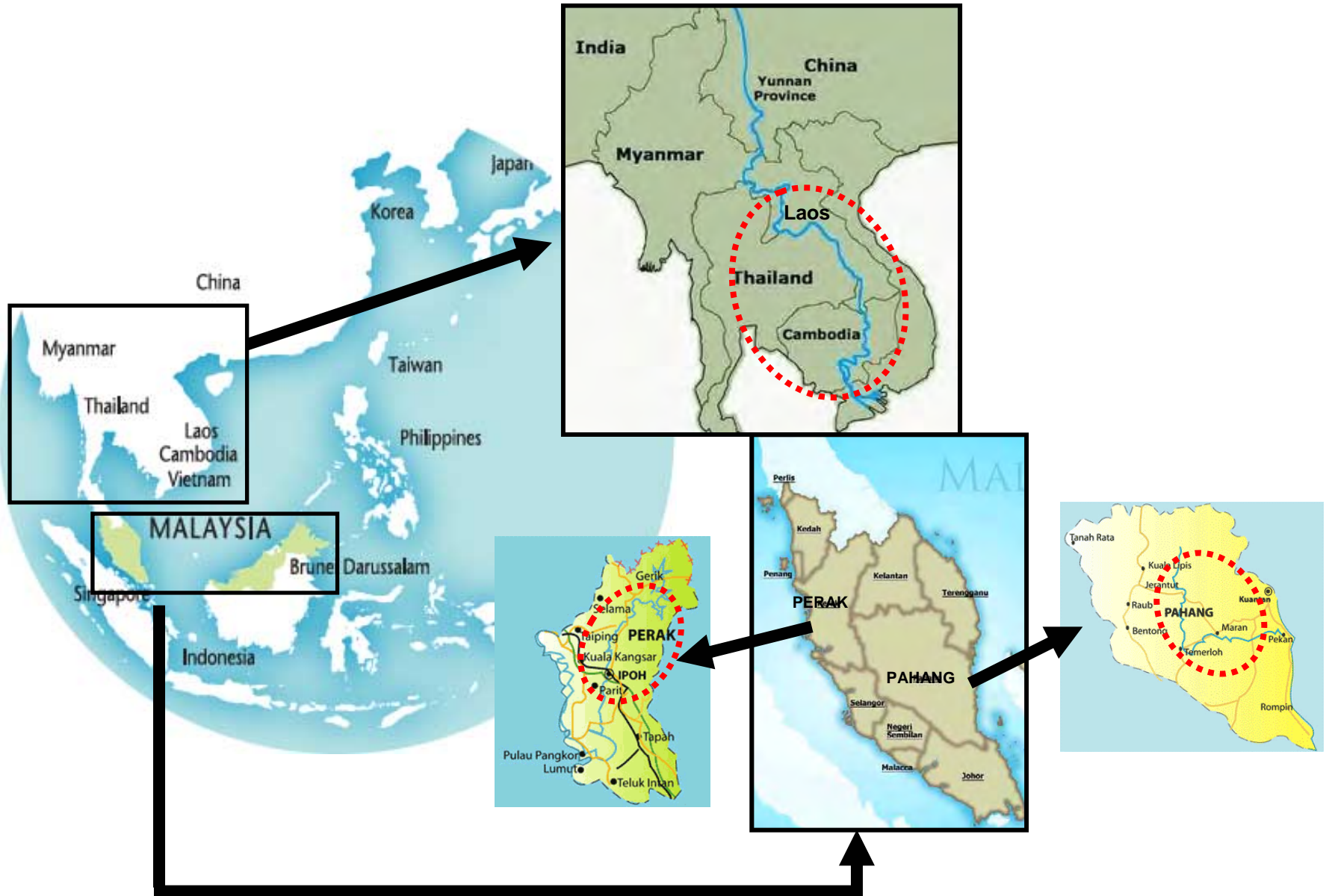
- *Pangasius nasutus*

- *Hypsibarbus* sp. and

- *Puntiopletis bulu*



Populations distribution of *P. jullieni*

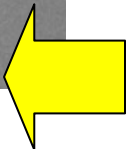
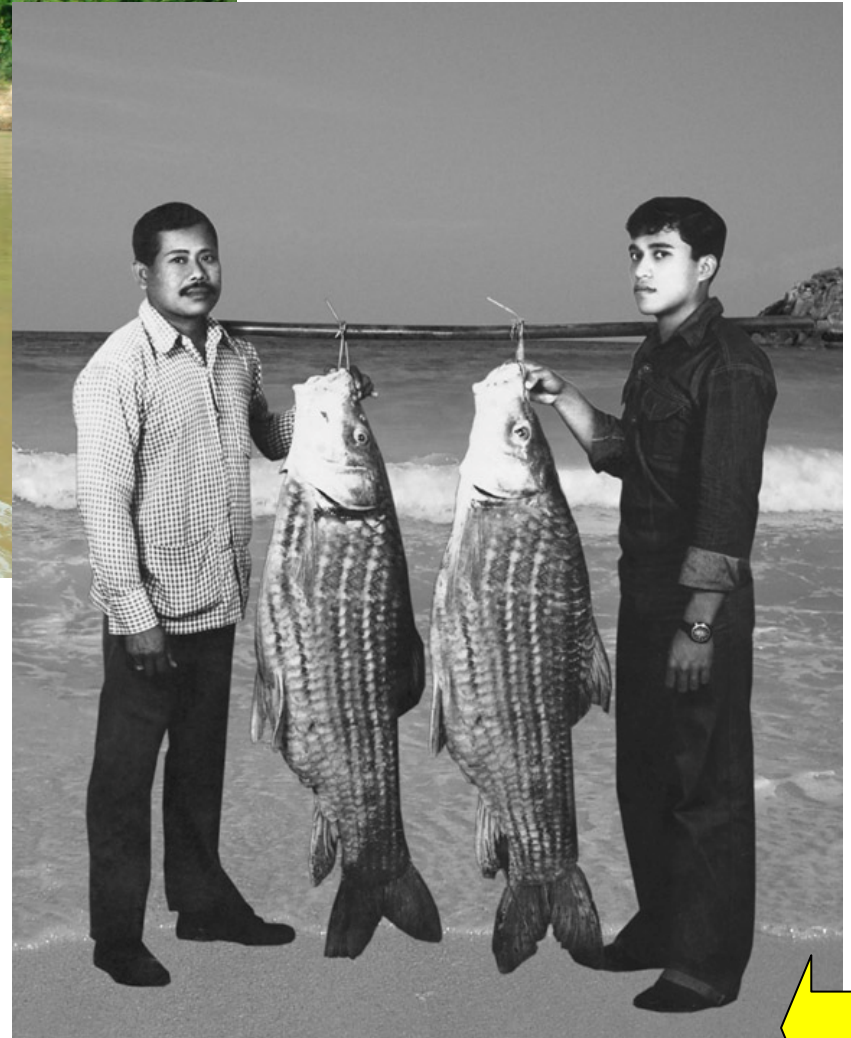




Annual total landing:

2002 – 16 MT

2007 - 2.1 MT



Study material

- Cultured brood fish



Methods

Collection of semen

Evaluation of
fresh semen
quality

Optimization of the
sperm cryopreservation
protocol:-

- a) Extender solution
- b) Cryoprotectant
- c) Optimum sperm
diluent ratio
- d) Freezing procedures
- e) Thawing procedure

Evaluation of egg
fertilization and
hatching ability

- a) CS VS FS

RESULTS

Table 1. Quality of fresh semen of *P. jullieni*

Parameters	Results
Density	$9.6 \times 10^8 - 6.2 \times 10^{10}$ sperm cells / mL (mean 9.4×10^9 sperm cells / mL)
Semen volume	2 mL – 16 mL during peak season in October
Motility	70% to 99% Duration: 20 s, slowed down after 10s of progressive movement
pH	7.14 - 7.85
Osmolality of the seminal plasma	270 ± 20 mmol/kg

Optimization of Protocols in Sperm Cryopreservation

- ✓ Type of extender formulation
- ✓ Type of cryoprotectant and its optimum concentration
- ✓ Optimum sperm: diluent ratio
- ✓ Optimum freezing procedure
- ✓ Optimum thawing procedure
- ✓ Post-thawed sperm activation media
- ✓ Fertilization trials/ Hatching (*P. jullieni* & *Tor tambroides*)

Table 2. Sperm motility (%) between pre-freezed and post-thawed samples in the fish species studied

Species	Motility %	
	Before freezing	Post-thawed
<i>Probarbus jullieni</i> (Isok barb)	>85%	30-83% (Mean: 49%)
<i>Tor tambroides</i> & <i>T. douronensis</i> (Malaysian Mahseer)	>85%	35-89% (Mean: 55%)
<i>Pangasius nasutus</i> (River catfish, Patin Buah)	>90%	35-70%
<i>Hypsibarbus wetmorei</i> (Kerai Kunyit)	>90%	35-80%

Table 3. Frequency of breeding trials using cryopreserved semen in *P. jullieni* and *T. tambroides* (Dec 2007– Dec 2009)

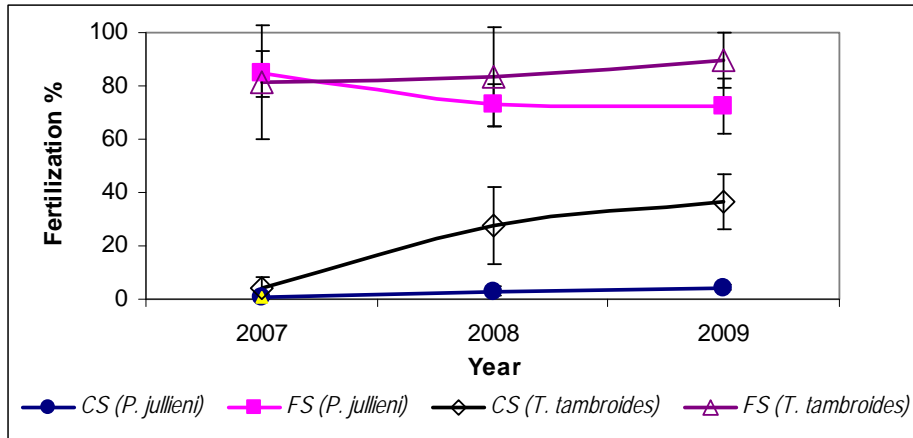
Year	<i>Probarbus jullieni</i>*	<i>T. tambroides</i>
2007	2	1
2008	3	2
2009	2	1

* The breeding season for *P. jullieni* normally falls between end of November to January each year.

Table 4. The highest fertilization and hatching percentages obtained by using cryopreserved semen

Species	Fertilization	Hatching
<i>P. jullieni</i>	9.9%	5.2%
<i>T. tambroides</i>	56.0%	55.2%

Figure 2. Fertilization (a) and hatching (b) performances: Cryopreserved semen (CS) VS Fresh semen (FS)



(a) Fertilization percentages by using cryopreserved semen (CS) and fresh semen (FS) both in *P. jullieni* and *Tor tambroides*.

(b) Hatching percentages by using cryopreserved semen (CS) and fresh semen (FS) both in *P. jullieni* and *Tor tambroides*.

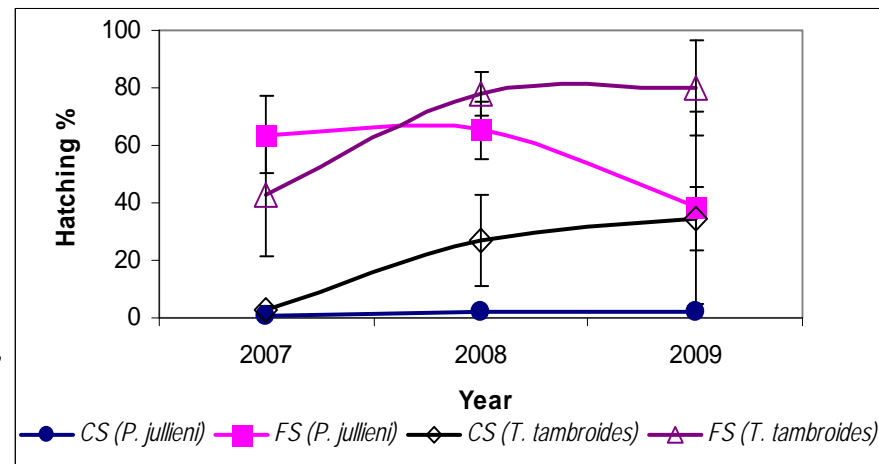


Figure 3. Fertilization, hatching and survival percentages between cryopreserved (CS) and fresh semen (FS) in *T. tambroides*

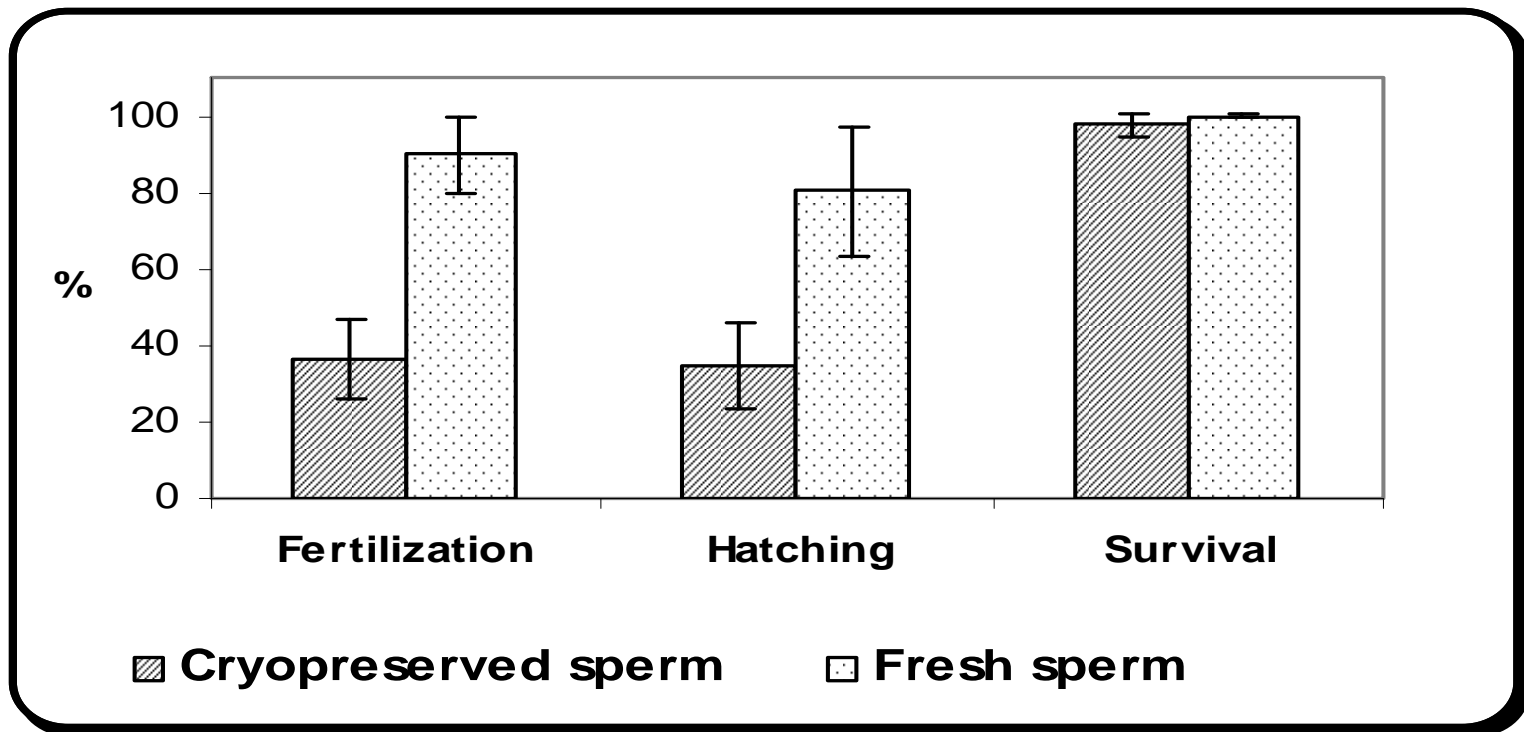


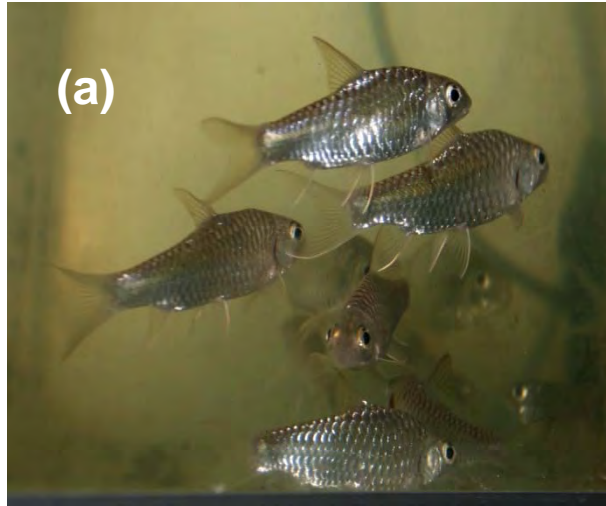
Table 5. Performance of egg fertilization and hatching by frozen semen after 5-month, 9-month and 12-month of cryostorage (*T. tamboides*)

Duration of cryostorage	Fertilization % (Mean \pm SD)	Hatching % (Mean \pm SD)
5-month	13.4 \pm 13.3 ^a	2.1 \pm 1.0 ^b
9-month	36.8 \pm 13.1 ^a	34.0 \pm 15.5 ^a
12-month	29.8 \pm 8.9 ^a	27.9 \pm 10.6 ^{ab}

Mean value with the same alphabet in the same column indicates no significant difference ($P > 0.05$) via LSD test. n = 120.

- **Patent No: PI 20093726, dated 8 Sept 2009 - Method for semen cryopreservation and *in vitro* fertilization of *Tor* spp.**

Figure 4. Fingerlings fertilized by cryopreserved semen



(a) & (b) *T. tambroides*

(c) *P. jullieni*

Table 6. Status of semen cryobanking of *P. jullieni* and *Tor* spp. (started in 2008)

Species	Number of fish	Volume
<i>Tor</i> spp.	88	150 mL
<i>P. jullieni</i>	43	350 mL

CONCLUSIONS

- Workable protocol for the semen cryopreservation of selected freshwater fish species is developed.
- The overall protocols are promising for successful cryopreservation of the species studied



CONCLUSIONS

- The present study has provided valuable information and significant baseline on *ex situ* conservation via sperm cryopreservation
- The information gathered from the study gave better understanding of the sperm cryobanking and will contribute to the on going research on semen cryobanking of other fish species.

Successes in gamete cryopreservation: Key Factors

- Breeding / reproductive biology of the particular species
- Comprehensive and long term programme with proper and clear targets
- Funding
- Human resource development: skilled personnel / technical worker
- Political will / government policy

ACKNOWLEDGEMENT

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Thank you

