



Project Number 4915

Application of Cryopreservation Techniques for the
Genetic Management of Commercially Important
Tropical Fish Species

Final Report

April 1992 to March 1995

K.J. Rana
B. J. McAndrew

Institute of Aquaculture
University of Stirling
Stirling
Scotland
FK9 4LA

CONTENTS

	Page
1. EXECUTIVE SUMMARY	1
2. OBJECTIVES AS SET OUT IN THE PROJECT MEMORANDUM	3
3. BACKGROUND OF THE PROJECT	3
4. WORK CARRIED OUT DURING THIS PERIOD	4
4.1 Procurement and incubation of tilapia gametes	4
4.2 Development and improvement of cryopreservation protocols	4
4.2.1 Sperm fitness and integrity	4
4.2.2 Reduction of cryoprotectant toxicity and use of sperm motility enhancers	5
4.3 Cryopreservation of tropical fish species	6
4.4 Transportation of unfrozen milt	6
4.5 Development of a portable field cooler for cryopreservation of tilapia spermatozoa	7
4.5.1 Laboratory trials	7
4.5.2 Field cryopreservation trials	8
4.5.3 Patent for the FRPC and design modifications	9
4.6 Uptake and dissemination of information	9
5. RESULTS OF FINDINGS OBTAINED BY THE PROJECT	9
5.1 Procurement and incubation of tilapia gametes	9
5.2 Development and improvement of cryopreservation protocols	10
5.2.1 Sperm fitness and integrity	10
5.2.2 Reduction of cryoprotectant toxicity and use of sperm motility enhancers	11
5.3 Cryopreservation of tropical fish species	14
5.4 Transportation of unfrozen milt	15
5.5 Current cooling methods and development of a portable field cooler for cryopreserving tilapia spermatozoa	16

5.5.1	Laboratory trials	16
5.5.2	Field cryopreservation trials	21
5.5.3	Patent for the FRPC and design modifications	23
5.6	Uptake and dissemination of information	23
5.6.1	Uptake of cryopreservation technology	23
5.6.2	Training	24
5.6.3	Publications and meetings	24
6.	IMPLICATIONS OF RESULTS	24
6.1	Development and evaluation of cryopreservation protocols	25
6.2	Transportation of unfrozen tilapia milt	25
6.3	Portable field cooler	26
7.	PRIORITY TASKS FOR FOLLOW UP	26
8.	APPENDICES	28
Appendix 1	Patent application paper for the FRPC	29
Appendix 2	Site specific cryomanual for cryounit, ICLARM	36
Appendix 3	Publications and meetings attended	47

5.5.1	Laboratory trials	16
5.5.2	Field cryopreservation trials	21
5.5.3	Patent for the FRPC and design modifications	23
5.6	Uptake and dissemination of information	23
5.6.1	Uptake of cryopreservation technology	23
5.6.2	Training	24
5.6.3	Publications and meetings	24
6.	IMPLICATIONS OF RESULTS	24
6.1	Development and evaluation of cryopreservation protocols	25
6.2	Transportation of unfrozen tilapia milt	25
6.3	Portable field cooler	26
7.	PRIORITY TASKS FOR FOLLOW UP	26
8.	APPENDICES	28
Appendix 1	Patent application paper for the FRPC	29
Appendix 2	Site specific cryomanual for cryounit, ICLARM	36
Appendix 3	Publications and meetings attended	47

1. EXECUTIVE SUMMARY

- i) The prime objective of this study was to develop the technology necessary for establishing cryopreserved tilapia sperm banks. This was achieved through applied laboratory and field trials and a manual on the cryopreservation of tilapia spermatozoa.
- ii) To simplify cryopreservation technology and promote its uptake this project investigated, developed and improved tilapia protocols, widened the use of cryopreservation protocols to other tropical species, developed appropriate techniques for the collection and transportation of milt and developed a reliable and robust portable cooler for the field cryopreservation of tilapia milt. Despite Dr Rana's 6 month illness most of these objectives and tasks were achieved.

Methods for the objective assessment of spermatozoa fitness and cryodamage were developed and verified for tilapia; computer assisted semen analysis (CASA) and fluorescent probes for use with fluorometry and microscopy.

The Hobson Sperm Tracker (HST) for CASA was verified for use with tilapia spermatozoa. The repeatability of sperm fitness characteristics of the same section of a video tape was high. Of the six fitness parameters investigated, the HST showed a high degree of consistency. The CV's(%) of most parameters were less than 5%.

The CASA studies clearly showed that cryopreservation reduced the fitness of *O. niloticus* spermatozoa. The speed of post-thaw cells was significantly ($P < 0.05$) reduced. The significant ($P < 0.05$) reduction in linearity and head amplitude and increase in angular movement of cryopreserved spermatozoa indicated that the post-thaw cells were more unidirectional and demonstrated more lateral head movement.

- iv) A more detailed perspective of cryodamage was obtained by using a novel fluorometric technique. These studies showed that each progressive step of the cryopreservation protocol for *O. niloticus* spermatozoa significantly ($P < 0.05$) increased the proportion of cells with damaged membranes. Fresh untreated milt contained only 3% damaged cells. Diluting cells with the tilapia cryodiluent increased the proportion of damaged cells to 35% and controlled cooling increased this damage to over 60%. Uncontrolled cooling resulted in over 90% of cell death.
- v) Trials were conducted to ameliorate the unexpectedly high precooling cryoprotectant toxicity and maximise the opportunity for post-thaw activation of the partially

damaged cells. Step wise addition of cryoprotectant increased the proportion of motile cells. The post-thaw scores were on average higher by 2-3 units.

To maximise post-thaw activation of tilapia spermatozoa enhancers which are usually used in insemination fluids were successfully incorporated into cryodiluents for the first time. Theophylline and caffeine either in the cryodiluent or in the activator increased the proportion of post-thaw motile cells. Overall, theophylline was superior to caffeine and 10mM theophylline in cryodiluent increased post-thaw motility scores from 3 to over 5. Such improvements increased the number of activated post-thaw cells by up to 60%.

- vi) Milt from *Puntius conchoni* (minor carp species) was successfully collected in 25 μ l capillary tubes and cryopreserved. Post-thaw milt yielded motility scores of 3-5. In replicated fertilization trials mean hatch rates of 91 and 94 % of control values were achieved.
- vii) Tilapia milt can be successfully transported in an unfrozen state both nationally and internationally. The success, however, depended on transport conditions. Fresh undiluted milt was successfully couriered from Stirling to Sri Lanka (4-5days) and cryopreserved on site in the Fixed Rate Portable Cooler (FRPC). Such milt yielded a minimum of 60% eyed eggs.
- viii) To develop reliable field technology for tilapia milt the various existing field cryopreservation methods were evaluated. All the cooling methods tested were unpredictable and unreliable resulting in low repeatability and therefore a different approach was considered for this project.
- ix) Two sizes (KB18-10 and KB 70-35) of a novel FRPC was designed and extensively tested under laboratory and field conditions. The FRPC generated ideal linear cooling rates with low variance (<10%) between straws and between runs. Minor changes in the use of the FRPC has now reduced this variation to 5-7%.
- x) The KB18-10 FRPC was successfully tested during field trips to Sri Lanka, Mexico and Philippines. All three trips highlighted several problems associated with transport of cryopreserved fish semen. Milt cryopreserved in Sri Lanka and Mexico and tested at IOA were highly fertile (60-90%).
- xi) Following advice from the University of Stirling and ODA, a UK patent application was lodged under the company and inventors name as ODA and Dr Rana, respectively.

- xii) During this project high priority was placed on transferring and disseminating cryopreservation technology. The tilapia cryopreservation technology developed at Stirling was successfully transferred to ICLARM in the Philippines and is now an integral part of the tilapia GIFT programme. One post doctorate from Hungary, 2 PhD and 4 MSc students have been involved or trained at IOA in cryopreservation technology during this project.

Currently, a paper is being considered for publication in Reproduction, Fertility and Development. In addition, two monographs have been written, one published and the second is still in press. Dr Rana presented invited papers and lectures in 7 countries.

- xiii) A manual on cryopreservation of tilapia semen was drafted to promote the uptake of the technology.
- xiv) It is strongly recommended that the FRPC be developed further into a self contained kit for field or laboratory cryopresevation to promote the standardisation of the technology.

2. OBJECTIVES AS SET OUT IN THE PROJECT MEMORANDUM

- i) Procurement of broodstock and gametes from selected model species of aquacultural or commercial interest.
- ii) Evaluate and modify techniques for the transportation of fresh milt.
- iii) Evaluate, modify and widen cryopreservation protocols for other tropical species such as Chinese and Indian carp and apply various techniques to assess sperm quality.
- iv) Develop equipment and techniques for the freezing of milt under field conditions.
- v) Produce a user friendly manual for the field application of milt storage and cryopreservation.

3. BACKGROUND OF THE PROJECT

The culture of many tropical finfish species now relies on the seed produced from closed farmed hatchery stocks. There is growing evidence that many of these cultured fish stocks are genetically degraded due to poor broodstock management practices. In these circumstances it is possible to improve these populations by the introduction of genetic

variation from unrelated conspecific wild or cultured stocks.

The maintenance of wild or cultured fish stocks in a genetically stable form may therefore be crucial for sustained long term development of fish production. The cryopreservation and storage of milt will enable germplasm to be stored in a genetically stable form. This technology for the tilapias has been developed under earlier ODA funded programmes (R4151 and R4523) at the Institute of Aquaculture (IOA), University of Stirling. The accessibility of cryopreservation technology to researchers, farmers and conservationists alike, however, is likely to be dependant upon the availability of suitably equipped laboratories and the logistics of obtaining material for cryopreservation. Accessibility to cryopreservation technology therefore demands an alternative approach. This has provided the rationale for this project.

4. WORK CARRIED OUT DURING THIS PERIOD

To promote the uptake of cryopreservation technology for the tilapias and other commercially important and endangered tropical finfish species this project investigated and developed appropriate techniques and protocols aimed at improving and extending the cryopreservation protocols to other tropical species, developed appropriate techniques for the collection and transportation of milt and developed a simple yet reliable portable cooler for the cryopreservation of milt under field conditions and laboratory conditions..

4.1 Procurement and incubation of tilapia gametes

The application of cryopreservation technology depends on artificial procurement of gametes and the subsequent incubation of eggs and hatchlings. For the tilapias these technologies have been developed at the IOA.

4.2 Development and improvement of cryopreservation protocols

4.2.1 Sperm fitness and integrity

Robust evaluation of cryopreservation protocols require an objective assessment of sperm viability. Two novel methods were therefore developed and verified for quantifying fish spermatozoa fitness; the Hobson Sperm Tracker (HST) for CASA and fluorescent probes for use with fluorometry and microscopy.

The HST which was developed for the objective assessment of mammalian spermatozoa fitness was modified and verified to track tilapia spermatozoa. Mammalian spermatozoa are about 4-5 times larger than fish spermatozoa. Therefore the initial task was to adapt the computer software programme to optimise confident tracking of individual tilapia sperm cells. Since tilapia spermatozoa are motile for only a few minutes at best, swift activation, focusing and videotaping methods were developed to capture motility sequences on video tape. The sperm concentration giving optimal numbers of spermatozoa for computer analysis was established. Following the ratification of sperm dilution for optimal digitisation, the swimming speed and proportion of motile cells and fitness characteristics such as linearity, path velocity, curvilinear velocity, average angular movement, and head amplitude of fresh and cryopreserved spermatozoa were objectively calculated and statistically compared using the Student's t test.

Cell membranes are a primary site for cryoinjury. To obtain an objective assessment of membrane integrity during the key stages of cryopreservation a novel fluorometric technique using propidium iodide was developed and optimised for tilapia sperm cells. Since such probes bind specifically to intracellular DNA of only damaged cells the proportion of damaged cells can be quantified. Variables such as excitation and emission spectra for the probe, linearity of DNA bound fluorescent intensity, optimal concentration of propidium iodide and digitonin, effect of seminal plasma and formalin on fluorescent intensity were examined and optimal conditions used to quantify membrane damage in comparable samples of untreated fresh, cryodiluent equilibrated and post-thawed tilapia spermatozoa. The cell viability was compared statistically using Student's t test and one way ANOVAR.

4.2.2 Reduction of cryoprotectant toxicity and use of sperm motility enhancers

The unexpectedly high level of damaged cells observed in the above trials prior to cooling prompted the consideration of methods for ameliorating the precooling toxicity of the cryodiluent and improving post-thaw activation of cells by using enhancers such as theophylline and caffeine.

Evaluation of prefreezing motility scores over several trials using the normal tilapia cryopreservation protocol revealed that milt equilibrated in the cryodiluent are typically 2-5 points lower than undiluted milt. This decline was attributed to the universal use of a single step procedure for cryoprotectant addition and the intrinsic toxicity of the cryoprotectant, methanol. Therefore, the effect of stepwise addition and equilibration of cryoprotectant on post-thawed viability was studied. *Oreochromis niloticus* milt was equilibrated over either 1, 2, 3 or 4 steps at equal time intervals and methanol volumes to give a final concentration of 10% methanol and then cryopreserved as normal. The viability of replicated post-thaw samples were evaluated as motility scores and fertility rates.

To maximise the yield and duration of motility of post-thaw cells the use of motility enhancers was reconsidered. Unlike all previously reported studies where enhancers are used in insemination media, the enhancers in this study were included in the cryodiluents. Either 5-20mM caffeine or theophylline was added to the cryodiluent and post-thaw activation compared with the normal *O. niloticus* protocol and with normal protocol in which post-thaw samples were activated with the 5-20mM caffeine and theophylline.

4.3 Cryopreservation of tropical fish species

As milt from Indian and Chinese carps was unavailable another cyprinid model was used. Cryopreservation of milt from a related threatened *Puntius* species, *P. conchoni*, was attempted. Milt collection techniques from these very small (5-10g) fish were successfully developed. Milt was diluted 10 fold with four extenders (see Table 4) containing a final concentration 10% methanol (MeOH) and cooled at a rate of 5°C/min. Post-thaw motility scores were evaluated and the best protocols tested using, *in vitro* fertilization trials. Post-thaw viability of milt was tested as motility scores and hatch rates (90-100 eggs/treatment).

4.4 Transportation of unfrozen milt

To widen the possible use of cryopreservation technology and distribute material from centres which do not have the facilities or infrastructure for cryopreservation the feasibility of transporting unfrozen milt was considered. Three related aspects of milt transportation were studied. Laboratory storage, transportation and subsequent cryopreservation and viability testing of transported milt were investigated.

Milt collected from a minimum of five *Oreochromis niloticus* males was pooled and replicated samples of either neat or diluted samples were transferred to capped 1.5ml Eppendorfs and stored under a range of laboratory conditions aimed at optimising their longevity. Optimal conditions were used for transportation trials. To test the feasibility of milt transportation, national and international transport trials were conducted.

For national transport undiluted and diluted (1:10) milt pooled from 5 males was transported (UK Summer) in air and oxygen filled plastic bags and in Eppendorfs within the UK using the normal first and second class postal service. Samples were placed with cooler packs in a polystyrene box and were self addressed to the IOA and posted first class (2 days). To simulate longer transportation periods milt was also sent second class (4-5 days).

To compare treatments samples of milt destined for transport were cryopreserved and also

held at 4°C under similar packaged conditions. On arrival milt quality of all treatments was evaluated. On arrival samples with motile spermatozoa were noted and cryopreserved and each 0.5ml straw was used to fertilize between 150-180 eggs. The optimum conditions were used to establish the feasibility of international transport of tilapia semen.

For international transport of milt three species were studied. For all species undiluted milt was dispensed in 1.5ml Eppendorfs taped to precooled (<4°C) gel packs. *Oreochromis niloticus* milt was shipped to Sri Lanka from IOA by DHL (5 days), checked for quality and useable samples frozen on site in the Fixed Rate Portable Cooler (FRPC) and returned to IOA for fertility evaluations. In addition, *O. niloticus*, *Puntius gonionotus* and *Dicentrarchus labrax* milt was dispatched to IOA from Mexico, Thailand and Spain, respectively. On arrival the viability of samples were assessed as motility scores.

4.5 Development of a portable field cooler for cryopreservation of tilapia spermatozoa

The main objective of this component of the programme was to develop a simple and effective protocol for cryopreserving tilapia milt under field conditions. A multichannel temperature logger was developed to monitor cooling profiles and rates within straws. Considerable effort was devoted to developing reproducible linear cooling rates of around 5°C/min optimum for tilapias and as generated by the programmable controlled rate cooler.

4.5.1 Laboratory trials

Current methods utilizing liquid nitrogen (LN) vapour to cool mammalian and fish semen in polystyrene boxes and within goblets suspended in refrigerator necks were extensively evaluated for their reliability in generating predictable cooling rates and the logistics of using these and other methods under tropical fields conditions. The effect of variables such as surface area of boxes and height of straws above LN on cooling rates within straws were thoroughly investigated. In addition, the cooling rates and their variation among straws and among trials were examined to establish the degree of reproducibility. These methods were deemed unsuitable for generating reliable and predictable cooling rates and characteristics. In order to develop a robust system for field cryopreservation alternative approaches were considered and systems were designed and tested after taking into consideration field related problems such as air temperature and pressure in the tropics, available space and facilities in the field, limited supply of coolant, duration of field cryopreservation work, safety, reliability and cost of apparatus.

In view of the above considerations the concept of cooling straws within the neck of a portable LN refrigerator was developed. As no relevant technical data were available on LN refrigerators, the likely variables influencing cooling rates were investigated. Given the very low boiling point of LN (-196°C) the level of LN in any refrigerator will decline with time. Under tropical conditions this may occur at a faster rate and may influence cooling rates within straws and dictate the maximum period possible for field cryopreservation. It was therefore important to establish the minimal acceptable LN level in the refrigerator required to generate the required cooling rates. The temperature variation within the neck was therefore established under a range LN levels. To minimise the dramatic differences in cooling rates between the pre and post freezing phases typically experienced in other methods, a heat sink design was conceptualised.

Following several prototypes two sizes (KB18-10 and KB 70-35) of a novel Fixed Rate Portable Cooler (FRPC) was designed for cooling 0.5ml straws and was extensively tested under laboratory and field conditions. The final design consisted of a series of specially designed individual straw holders (see Appendix 1). Since straws in the FRPC are cooled unconventionally in a vertical plane the cooling rates were measured at different heights within the straws to resolve the likelihood of any vertical cooling rate gradient along the length of the straw.

The FRPC was rigorously tested under laboratory conditions to confirm consistency of cooling rates within straws at different positions in the FRPC and between several cooling runs. Cryoviability of *O. niloticus* milt cooled in the FRPC and programmable cooler (KRYO 10, Planer Biomed.) was compared.

4.5.2 Field cryopreservation trials

Following several successful laboratory trials, the portable KB18-10 FRPC was tested during field trips to Sri Lanka, Mexico and Philippines.

Samples of milt were cryopreserved under tropical indoor, outdoor and farm conditions. In addition, *O. niloticus* semen was extracted from surgically removed testes and cryopreserved in the FRPC. The cryosuccess of half the samples was evaluated as post-thaw motility scores in the field and remainder air freighted to IOA for fertility trials.

Part of the field exercise was also to identify, evaluate and highlight the type and nature of problems that are likely to be encountered with customs, airlines and government departments in such field cryopreservation exercises.

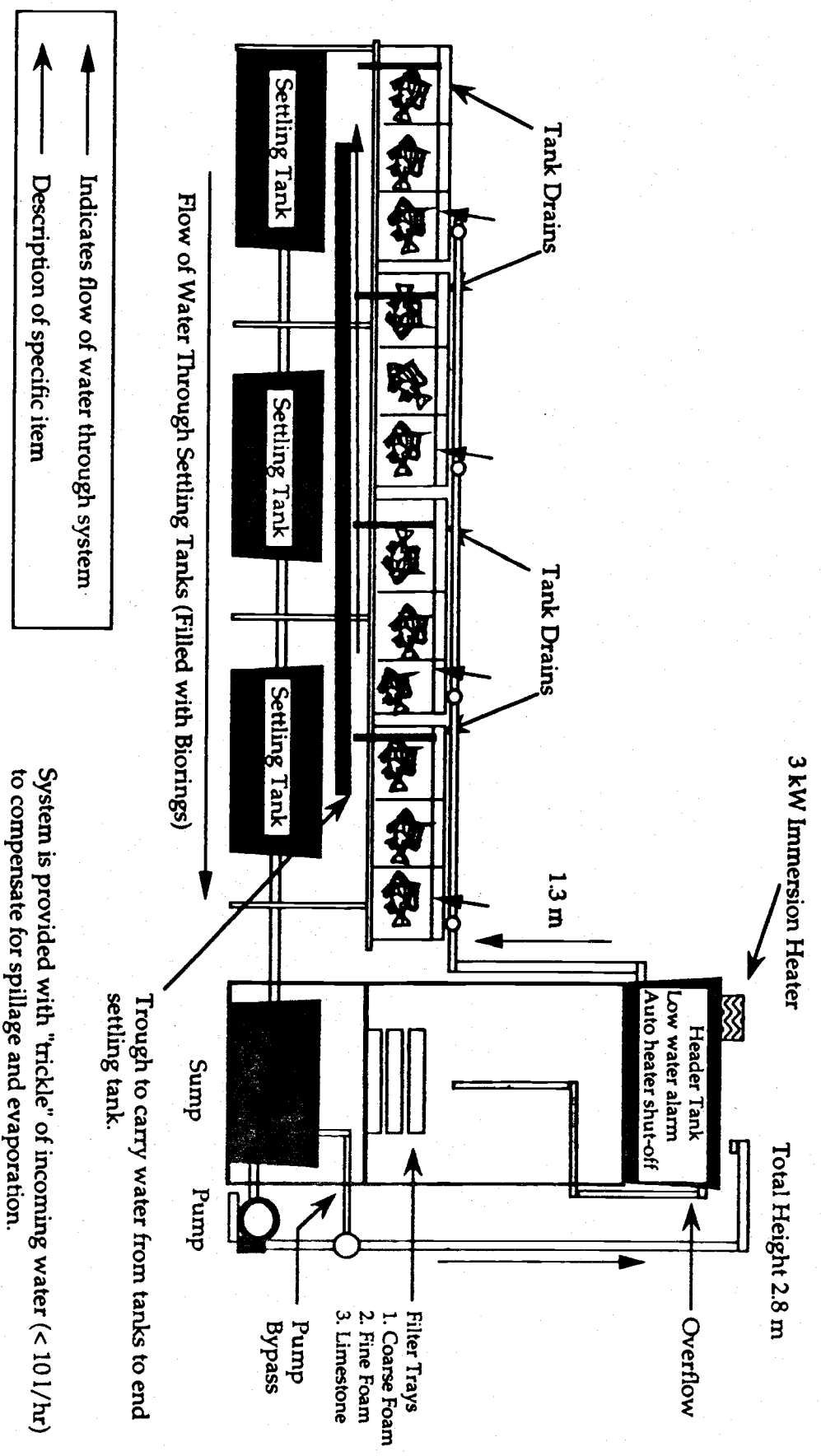


Fig. 1 Schematic diagram of recirculating *Oreochromis* broodstock system

4.5.3 Patent for the FRPC and design modifications

The concepts and the design of the FRPC were considered sufficiently novel for a patent and therefore an application was lodged at the UK Patent office to facilitate appropriate exploitation of the FRPC in Europe and lesser industrialised countries. Discussions are underway to lodge an application for a world wide patent. The design was also modified to accommodate 0.25ml straws.

4.6 Uptake and dissemination of information

Dissemination of information to promote the uptake of the cryopreservation technology was given high priority. In addition to freezing, the application of cryopreservation technology is dependant on the total control of the reproductive cycle and on *in vitro* culture of embryos. These aspects which are often neglected were also investigated and disseminated.

Efforts were focused on transferring cryopreservation technology developed at the IOA to ICLARM, training and dissemination of cryopreservation technology at conferences and through publications. For wider dissemination a manual on cryopreservation and related technologies was also drafted.

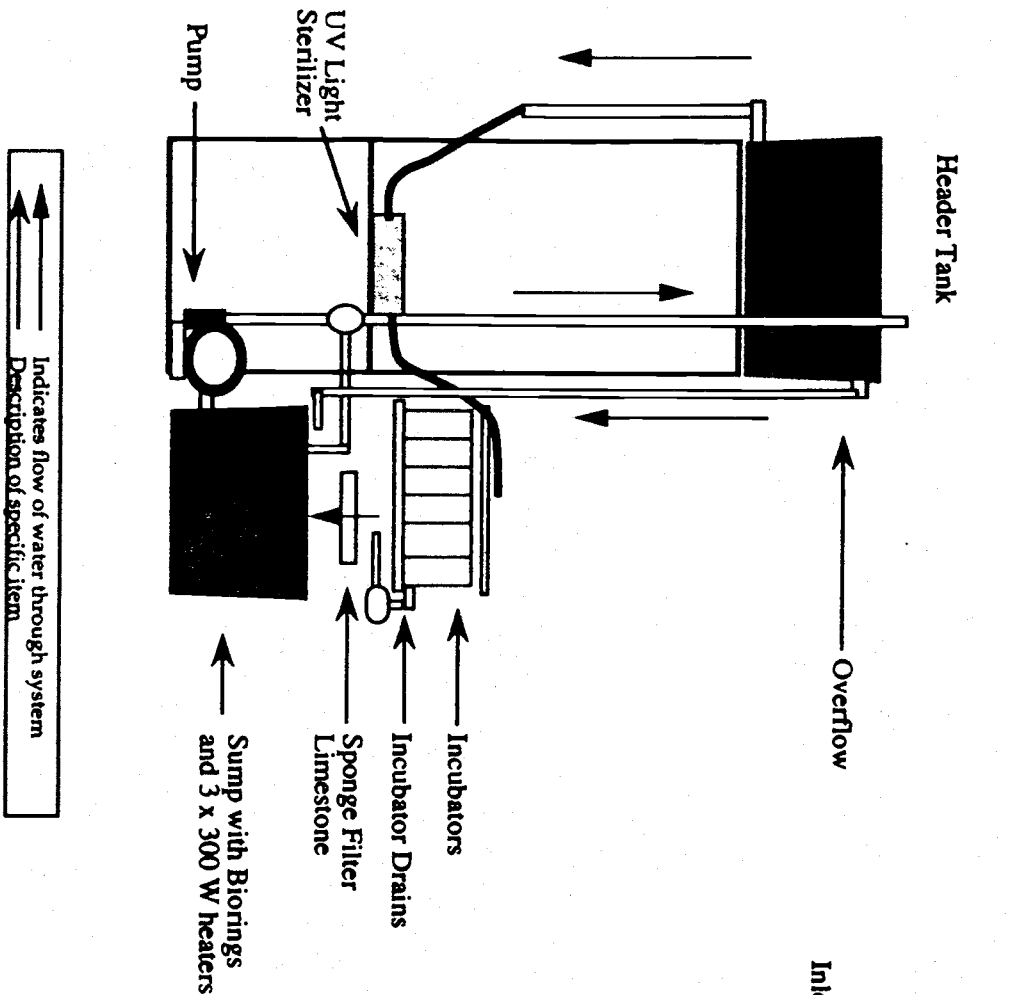
5. RESULTS OF FINDINGS OBTAINED BY THE PROJECT

5.1 Procurement and incubation of tilapia gametes

The schematic drawing of the recirculatory system developed at IOA is shown in Fig 1. Maintaining fish in isolation was the most efficient method for procuring milt. The holding facility composed of a battery of 4ft glass tanks. Each tank was partitioned into 3 sections to hold three fish of either sex of up to 500g. Males maintained in such isolated sections could be manually stripped of their milt weekly. Females in spawning condition were easy to recognise and ovulated females were maintained in nets suspended in the tanks to prevent natural spawning.

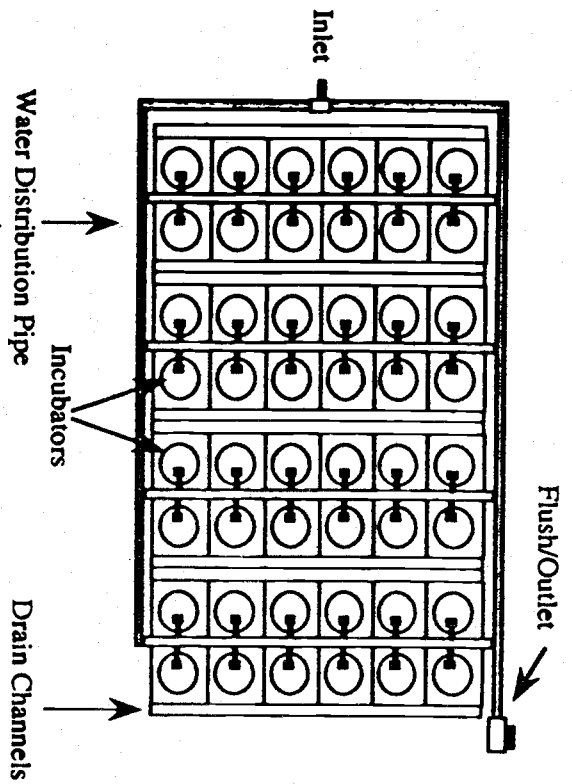
The incubation unit developed at the IOA is shown in Fig. 2. The system contains a 15W UV steriliser to provide a minimum dose of $30000\mu\text{W}/\text{cm}^2/\text{sec}$. Up to a 1000 eggs could be incubated in 0.75l incubators. Overall success rate to swim-up were in excess of 80%. For fertility estimates eggs were incubated for 24h and fixed in Bouin's fluid.

Water Recirculating System for Incubator



Indicates flow of water through system
Description of specific item

Top View of 48 Incubator Battery



Tilapia Egg Incubator

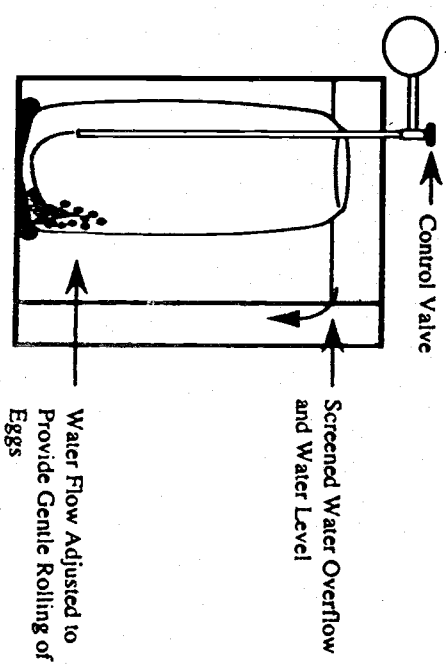


Fig. 2. Schematic drawing of the *Oreochromis* incubating system.

5.2 Development and improvement of cryopreservation protocols

5.2.1 Sperm fitness and integrity

Computer analysis of sperm fitness

The Hobson Sperm Tracker (HST) was used to quantify sperm fitness. The accuracy of the computer generated data depended on the configuration of the microscope and computer set up parameters.

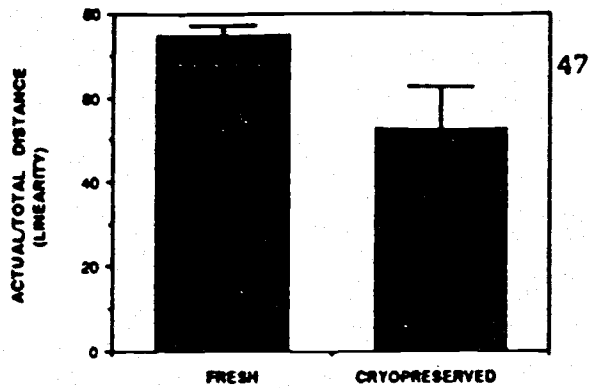
Of all the stage and photoeyepiece magnification combinations tested to provide optimal clarity and size of sperm head the x20 or x40 objective in combination with 3.3 photoeyepiece proved most suitable and these settings were used throughout the project. The dilution factor of milt during activation varied with milt samples. The optimum number of cells on the monitor screen for computer tracking was between 80-150. At these densities the computer programme could comfortably distinguish individual cell tracks to provide meaningful results.

The HST velocity measurements of an oscilloscope signal of known speed ($49.9 \mu\text{msec}^{-1}$) confirmed the accuracy ($P < 0.05$) of computer generated velocities. Using the x20 objective and optimal computer settings the proportion of motile cells on the same section of a video tape was compared with manual determination and were found to be significantly ($P < 0.05$) different. This difference, however was not significant ($P > 0.05$) at x 40 objective setting.

Using the optimal settings the repeatability of sperm fitness characteristics of the same section of the video tape was evaluated. These data shown in Table 1 indicate that the six parameters investigated showed a high degree of consistency with most CV's(%) being less than 10%.

Table 1 Repeatability of results of sperm fitness characteristics generated by the Hobson Sperm Tracker

Run no	No. sperm	Linearity	Mean speed	Absolute angle	Beat Cross Frequency	Head amplitude
1	65	77	51	41	5	10
2	59	73	50	41	4	9
3	57	77	51	39	5	9
4	56	72	50	42	4	7
5	52	75	51	37	4	9
mean	57.8	74.8	50.8	40	4.4	8.8
SEM	2.12	1.01	0.24	0.89	0.24	0.49
CV (%)	8.2	3.0	1.0	5	12.2	11



47

FIGURE 3: Linearity of fresh and cryopreserved sperm

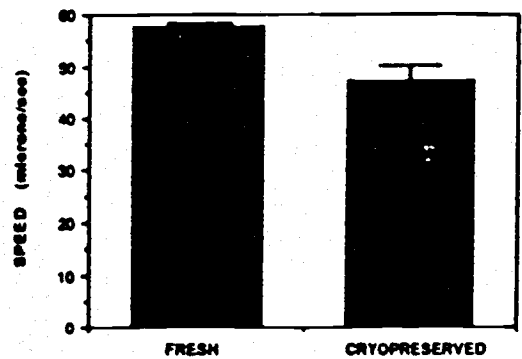


FIGURE 4: Speed of fresh and cryopreserved sperm

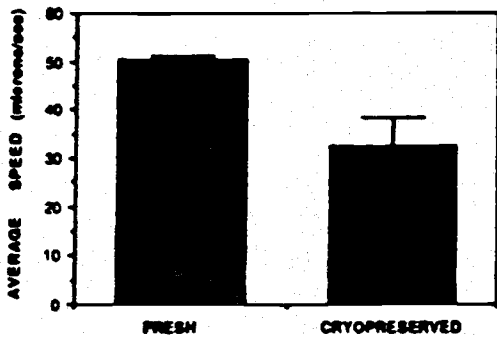


FIGURE 5: Average speed of fresh and cryopreserved sperm

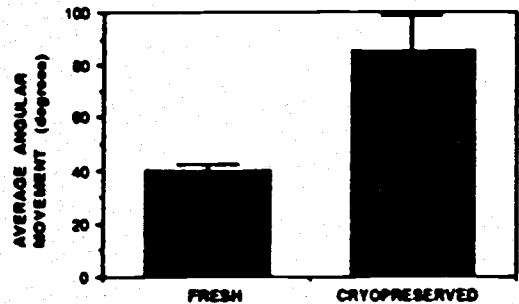


FIGURE 6: Average angular movement of fresh and cryopreserved sperm

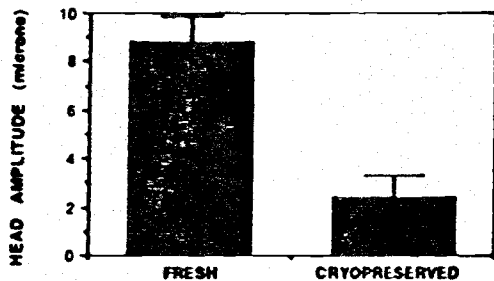


FIGURE 7: Head amplitude of fresh and cryopreserved sperm

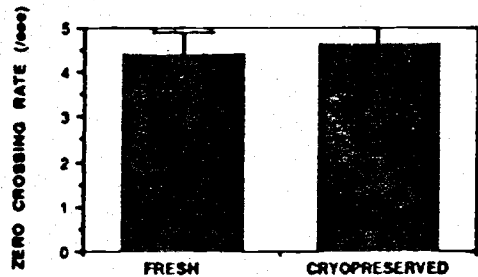


FIGURE 8: Zero crossing rate (BCF) of fresh and cryopreserved sperm

Therefore the HST was considered to be a reliable alternative to the subjective evaluation of sperm fitness. Of the three treatments, fresh, controlled rate cooled and plunge cooled in LN, the latter showed no post-thaw motility and therefore all statistical comparisons were conducted using Student's t test. The means and SEM of both fresh and cryopreserved *O. niloticus* milt for six computer generated fitness parameters are given in Figs. 3-8. Overall, cryopreservation reduced the fitness of *O. niloticus* spermatozoa. The speed of cells were reduced. The significant ($P < 0.05$) reduction in linearity and head amplitude and increase in angular movement of cryopreserved spermatozoa suggest that the post-thaw spermatozoa were more unidirectional and demonstrated more lateral head movement.

The above analysis provides an overall effect of cryopreservation and therefore does not help to elucidate when the viability of cells may be compromised. Therefore to obtain a more detailed perspective of cryodamage a new fluorometric technique was developed using propidium iodide.

Optimum fluorometric settings (excitation and emission of 309 and 597nm and slit width of 10nm) for the probes were established and used. The optimal concentration of the propidium iodide and digitonin, were established and used to establish if seminal plasma influenced the intensity reading. These studies showed that *O. niloticus* seminal plasma significantly ($P < 0.05$) increased the intensity of the fluorescence and therefore all subsequent samples were centrifuged to remove the seminal plasma prior to fluorometric assays. After establishing the linearity between fluorescence intensity and fish DNA concentration the assay was used to estimate and compare membrane damage of *O. niloticus* spermatozoa at key stages of the cryopreservation protocol with that of fresh untreated cells.

Each progressive step in the protocol for cryopreserving *O. niloticus* spermatozoa significantly ($P < 0.05$) increased the level of damaged cells. The proportion of damaged cells in fresh untreated, precooled cells equilibrated in the tilapia cryodiluent for 30min and post-thaw cells cooled either in the programmable controlled rate cooler at $5^{\circ}\text{C}/\text{min}$ or by plunging samples directly into LN are given in Fig. 9. Fresh milt contained only 3% damaged cells. Diluting cells with the cryodiluent increased the damaged cells to 35% and controlled cooling increased this damage to over 60%. Given the highly coupled nature of cooling and warming it was not possible to establish the proportion of damage attributable to cooling and to thawing. Uncontrolled cooling resulted in over 90% of cell death.

5.2.2 Reduction of cryoprotectant toxicity and use of sperm motility enhancers

Given the high level of damage both before and during cooling and warming described above efforts were focused to ameliorate prefreezing cryoprotectant toxicity and maximise the

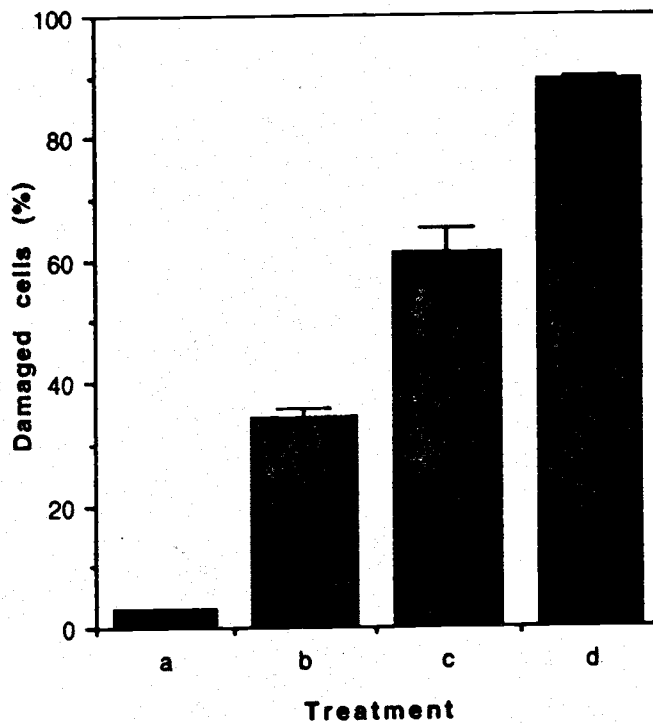


Fig .9 Fluorometric assessment of membrane integrity of *Oreochromis niloticus* spermatozoa during key phases of cryopreservation. (a) fresh undiluted samples (b) spermatozoa equilibrated for 25 - 30 min in cryodiluent (c) cells cooled at 5 °C / min in a controlled rate cooler and (d) samples cooled by purging directly into liquid nitrogen. Means given with SeM based on 4 replicates.

opportunity for post-thaw activation of the partially damaged cells.

The addition of cryoprotectant in multiple steps of equal volumes reduced the trauma on cells. Unfortunately, the video recorder which contained the video tape of activation sequences for these treatments was stolen and therefore quantitative motility estimates were not possible. The outcome of multiple step addition of cryodiluents on pre and post-thaw motility scores and fertility rates are given in Table 2.

Table 2 Viability of *O. niloticus* milt following multiple step equilibration of cryoprotectant

No of steps	n	No. of eggs	Mean (SEM) motility score		Fertilisation rate (%control) x(SEM)	Sperm:egg ratio x10 ⁶ x(SEM)
			Precooling	Post-thaw		
1	2	213	6	3.8 (0.3)	78.7 (14.6)	0.16(0.01)
1	2	418	6	2.8 (0.8)	98.8 (8.3)	0.11(0.00)
2	3	201	7	4.7 (0.3)	93.0 (3.9)	0.17(0.01)
2	2	415	7	4.0 (0.5)	105 (3.1)	0.11(0.000)
3	3	173	7.5	6.3 (0.2)	96.4 (2.1)	0.19(0.02)
3	2	381	7.5	3.8 (0.8)	98.4 (1.1)	0.11(0.00)
4	3	171	7	6.5 (0.0)	96.6 (2.5)	0.19(0.03)
4	1	321	7	5.0 (-)	96.3 (-)	0.14(-)

Increasing the number of steps improved the pre cooling motility score marginally. The post-thaw scores were on average higher by 2-3 units. The improvement in the yield of motile cells, however, was not reflected in the fertility rates. This was most probably due to the high sperm density and low egg numbers used. In view of this the number of eggs/ treatment were doubled (Table 2) but even these were not high enough for the lower motility yield from the single step addition to be expressed as lower fertility rates. Nevertheless, protocols now advocated for tilapias should include multiple step addition of cryoprotectants.

In tilapias and other fish species cooling and thawing reduces sperm fitness (Fig 3-8) and the duration of motility. To maximise the post-thaw activation of tilapia spermatozoa enhancers which are usually used in insemination fluids were incorporated in cryodiluents. As seen in Table 3 theophylline and caffeine in the cryodiluent enhanced

Table 3. Influence of theophylline (TH) and caffeine (CA) in cryodiluent and in activating solutions on mean¹ post-thaw *O. niloticus* spermatozoa motility

Extender ³	Activator	Motility Score ²	Time (min) to:	
			50% motility	0% motility
MFR	Water	3.7 (0.3)	2.8(0.31)	5.8(.7)
	5mM TH	4.0 (-)	2.3 (0.3)	4.8 (0.1)
	10mM TH	5.7 (0.3)	3.0(-)	4.8 (0.2)
MFR + 5mM TH	Water	3(-)	1.7 (0.2)	3.3(0.2)
	5mM TH	5(-)	2(-)	3.5 (-)
	10mM TH	5.3(0.2)	3(0.5)	5.6(0.3)
MFR + 10mM TH	Water	5.3(0.7)	2.3(0.3)	4.2 (0.2)
	5mM TH	3.3(0.3)	2.7(0.3)	3.3(0.2)
	10mM TH	5.0	2.0(-)	4.7(0.2)
MFR + 5mM CA	Water	4.7(0.3)	2.5(-)	4.1(0.2)
	5mM TH	4.7(0.3)	2.0(-)	4(-)
	10mM TH	4.0(-)	2.5(-)	4(-)
MFR + 10mM CA	Water	4.3(0.3)	3.3(0.3)	4.5(-)
	5mM TH	3.3(0.3)	1.8(0.2)	3.8(0.2)
	10mM TH	5.0(-)	2.0(-)	3.8(0.2)

¹ Means given with SeM. n=3.

² Motility score based on 0-10 scale, 0 being no and 10 total motility.

³ MFR= Modified fish Ringers

the proportion of post-thaw motile cells. Overall, theophylline was superior to caffeine and the use of 10mM theophylline in the cryodiluent increased motility scores from 3 to over 5. Although the velocity of the spermatozoa could not be estimated all microscopic observations indicated that the speed of spermatozoa with enhancers was greater and this is reflected in shorter times to 100% inactivation in relevant treatments (Table 3). Overall enhancers in either post-thaw activators or in cryodiluent were beneficial but for simplicity is recommended that it be added to the cryodiluent. Such improvements in efficacy will be invaluable particularly in instances where only small volumes, dilute and poor quality milt are available for gene banking eg. smaller tilapia species, substrate and old spawners and rare individuals.

5.3 Cryopreservation of tropical fish species

Milt from *P. conchoni* (5-10g) was successfully collected in 25 μ l capillary tubes. Males were successfully anaesthetized in benzocaine and up to 20 μ l milt/male was collected. Milt was successfully maintained in an immotile state in physiological saline for up to 4 days at 4°C. Extenders tested for this species are given in Table 4.

Table 4 Chemical composition of extenders evaluated for the cryopreservation of rosy barb (*Puntius conchoni*) and common carp (*Cyprinus carpio*) milt

CHEMICALS	EXTENDER NO (g/100ml)			
	1	2	3	4
Glucose	3	3	-	-
Sodium citrate	1	2	-	-
Potassium chloride	0.03	0.06	0.02	0.62
Sodium chloride	0.4	-	0.75	0.44
Sodium bicarbonate	0.015	-	0.02	0.02
Lecithin	0.3	-	-	-
Calcium chloride	-	-	0.02	0.22
Magnesium chloride	-	-	-	0.008
Distilled water (mls)	100	100	100	100

Post-thaw milt yielded motility scores of 3-5. In replicated fertilization trials using post-thaw milt and up to 100 eggs/treatment, mean hatch rates of 91 and 94 % were obtained for extenders 2 and 3, respectively (Table 5).

Table 5 Mean (SEM) viability of cryopreserved¹ rosy barb (*Puntius conchoni*) milt

	EXTENDER NUMBER ²		CONTROL
	2	3	
Post-thaw score	5-6	5-6	7
Mean no. of eggs used	97	90	107
Sperm: egg ratio x 10 ⁶	0.95	1.1	0.9
Mean fertility (% of control)	91 (2.4)	94(5.3)	-

¹ Milt cooled at a rate of 5°C/min in programmable chamber.

² Modified fish Ringer also tried but post-thaw motility scores were low (2) and therefore not used in fertility trials. Composition of extenders given in Table 4.

5.4 Transportation of unfrozen milt

Transportation of tilapia milt and subsequent cryopreservation was feasible but success depended on transportation conditions and on there being no delay.

Initial trails were conducted at IOA to define optimum storage conditions. Milt was best stored undiluted at 4°C. In view of the small volumes of tilapia milt, storage of up to 200µl of undiluted milt in 1.5ml Eppendorfs was most effective. This storage method was subsequently used in both national and international milt transportation trials.

Milt was successfully transported for up to 4 days using conventional mail (Table 6). Transportation of diluted milt (1:10 in MFR) in plastic bags in either air or oxygen and in Eppendorfs was unsuccessful. Undiluted milt, however, gave satisfactory post-transport motility scores of 3-5 following 3-4 days of transport. In replicated trails using pooled milt, the viability of cryopreserved milt following national transportation and laboratory storage was similar ($P > 0.05$). Eighty four percent of the eggs inseminated with transported and cryopreserved milt were fertile (Table 6).

Table 6 Viability of post-thawed *Oreochromis niloticus* milt transported in the national mail for 4 days and then cryopreserved.

Treatment	Motility score		Mean (SEM) sperm:egg ratio x 10 ⁶	Mean ^{1,3} (SEM) fert. rate (% of controls)
	Post transport/storage	Precooling		
Transported	3-4	3	0.63(0.09)	84.0(0.6)
Storage at 4°C	5-6	4-5	0.61(0.04)	82.0(9.0)
Cryo control ²	-	4-5	0.80(0.11)	82.0(2.1)

¹ Fresh controls = 88% eyed eggs. Between 150-180 eggs/treatment used.

² Samples frozen prior to transport

³ Means based on triplicate treatments

Four international transportation trials were conducted using three species. *Oreochromis niloticus* milt dispatched from IOA to Sri Lanka took 5 days to reach the field station. The prechilled gel packs helped to keep the maximum temperature to below 15°C during transport. Following transportation 50% of the cells in 50% of the samples could be activated. These samples were pooled and cryopreserved on site in the FRPC and post-thaw motility in all samples tested were 3-4. Samples which were returned to IOA for fertility trials yielded 60-90% eyed eggs (see Table 14).

Puntius gonionotus milt samples were successfully hand carried from Thailand to IOA. On arrival, motile cells were present in all samples but only about 50% of the samples contained

milt of suitable quality for cryopreservation. The quality of milt from the same male varied between the various samples. In the majority of samples, between 30-60% of cells were motile (Table 7).

Table 7 Post-transport activation of *Puntius gonionotus* milt hand carried (36h) from Thailand to IOA.

Fish Number	Motility score range ¹
1	4-6
2	2-4
3	2-3
4	1-2
5	2-6
6	3-5

¹ Three samples/fish transported. Motility score based on 0-10; 0 being no motility and 10 total activation.

Sea bass milt samples couriered from Spain with DHL arrived at IOA within 30h of dispatch. All samples were evaluated for quality. As with tilapia, only 50% of the samples contained 60-70% motile cells upon activation.

Not all attempts to transport unfrozen tilapia semen were successful. Milt shipped from Mexico (5 days) could not be revived. In this particular case unexpected delays at the Mexican customs and high tropical temperatures in which they were held were the most likely contributory factors for the failure. Overall, milt transportation trails demonstrated that provided semen of fish can be maintained cool and transported within 4-5 days they can be used for gene banking purposes.

5.5 Current cooling methods and development of a portable field cooler for cryopreserving tilapia spermatozoa

5.5.1 Laboratory trials

Evaluation of current methods

In total, 28 laboratory based trials were conducted to evaluate the various existing field cryopreservation methods while developing a new cooling system for tilapia spermatozoa.

Cooling profiles within straws can be typically divided into two phases the pre and post-freezing phases (see Fig 10). Cooling straws in the programmable controlled rate cooler results in reproducible cooling rates during the pre and post freeze phases both within and among cooling runs (see Fig 13a). This uniformity and cooling rate was considered ideal and was used as the bench mark for evaluating currently used methods.

One of the most widely used methods for field cryopreservation is LN vapour cooling in polystyrene boxes. The temperature dynamics and the cooling profiles within straws cooled in polystyrene boxes containing known volumes of LN revealed several unacceptable variables. The ambient temperature above LN and the cooling rates between the pre and post freezing rates were highly variable. In addition, it was demonstrated that the cooling rates vary with the height of straws above LN as well the surface area of the LN or size of the box used. (Table 8).

Table 8 Effect of height and surface area of coolant on the ambient temperature and mean cooling rates ($^{\circ}\text{C}/\text{min}$)¹ within 0.5 ml straws in insulated boxes

Height above LN (cm)	Surface area (cm ²)					
	490			784		
	Ambient T $^{\circ}\text{C}$	Pre-freeze cooling rate	post-freeze cooling rate	Ambient T $^{\circ}\text{C}$	Pre-freeze cooling rate	Post-freeze cooling rate
8	-46.5(4.8)	37.7(7.0)	7.1(1.2)	-65.8(2.9)	40.1(5.1)	7.8(0.6)
12	-27.0(1.7)	19.4(3.3)	3.7(0.3)	-38.3(2.8)	27.7(5.0)	4.9(0.7)
16	-20.8(1.5)	10.8(2.6)	2.5(0.2)	-27.5(2.9)	8.8(1.7)	2.5(0.2)

¹ Means given with SEM based on 7 replicates

The ambient temperature at different heights and cooling rates were significantly affected by the height and area of LN. For any given height a larger surface area generated higher ambient temperature and cooling rates (Table 8). A 60% increase in surface area raised the ambient temperature by between 35-45%. Similarly, the differences in cooling rates between the pre-cooling and post-freezing phases varied by as much as 500%. In addition to the variables of height and surface area a co-related variable is apparent from Table 8; the higher variability (indicated by the larger standard error of the means) of cooling rates between straws placed closer to LN surface. Clearly, such a cooling method is unpredictable and unreliable.

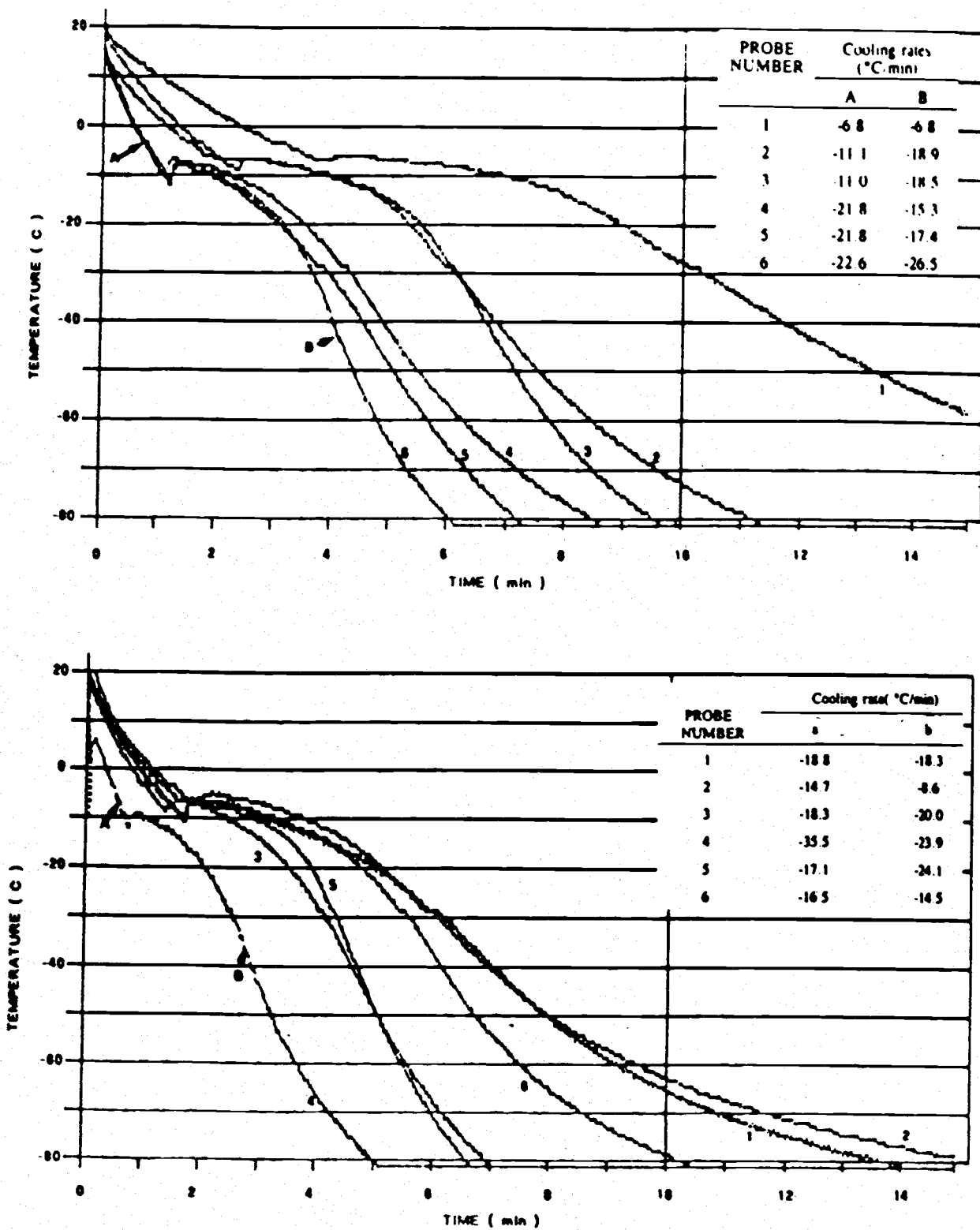


Fig. 10

Cooling profiles within straws cooled in goblets demonstrating unpredictability and variability of cooling rates. Cooling rates at the pre (A) and post (B) freezing phases within each straw are given for two typical trials.

Cooling straws in a goblet within the neck of a LN refrigerator was equally unsatisfactory. In common with the above methods the cooling rates between the pre and post-freeze phase was markedly different. Moreover, the cooling rates between straws in any given cooling run was highly variable. Such trials of which two typical runs are illustrated in Fig 10 show that no two cooling runs even under similar conditions are the same and that the cooling rates are highly unpredictable and follow a random pattern. The cooling rates between the pre and post-freezing phases varied by as much as 70%.

Development of the Fixed Rate Portable Cooler (FRPC)

Following several options two sizes (KB18-10 and KB 70-35) of a novel FRPC were designed for use in a 10 and 35l LN refrigerator, respectively, and extensively tested under laboratory (KB18-10 and KB 70-35) and field (KB18-10) conditions. The ambient temperature within the neck at any give height of both refrigerators were similar irrespective of the LN level in the vessel (Table 9; Fig 11).

Table 9 Vapour temperature within a 10l refrigerator containing varying levels of liquid nitrogen

Level of LN (cm)	Mean (SEM) LN vapour temperature (°C) within the neck of the refrigerator ¹ (cm from top of refrigerator neck)		
	6	8	10
21	-59 (1.7)	-99 (2.9)	> -150
23	-78 (2.9)	-101 (4.2)	> -150
27	-50 (-)	-105 (9.6)	> -150
31	-70 (-)	-110 (8.5)	> -150

¹ Positions correspond to the midway distance of the FRPC when the upper surface of cooler is positioned at either 0, 2 or 4 cm from the top of the refrigerator neck, respectively.

For field application the 10l refrigerator will be used for cooling and storing samples. Therefore the rational for choosing the level of 21cm was based on minimum level of LN required to keep straws at the bottom of the canister submerged at all times. The stability of the neck temperature with LN levels as low as 21cm will enable the 10l refrigerator to be

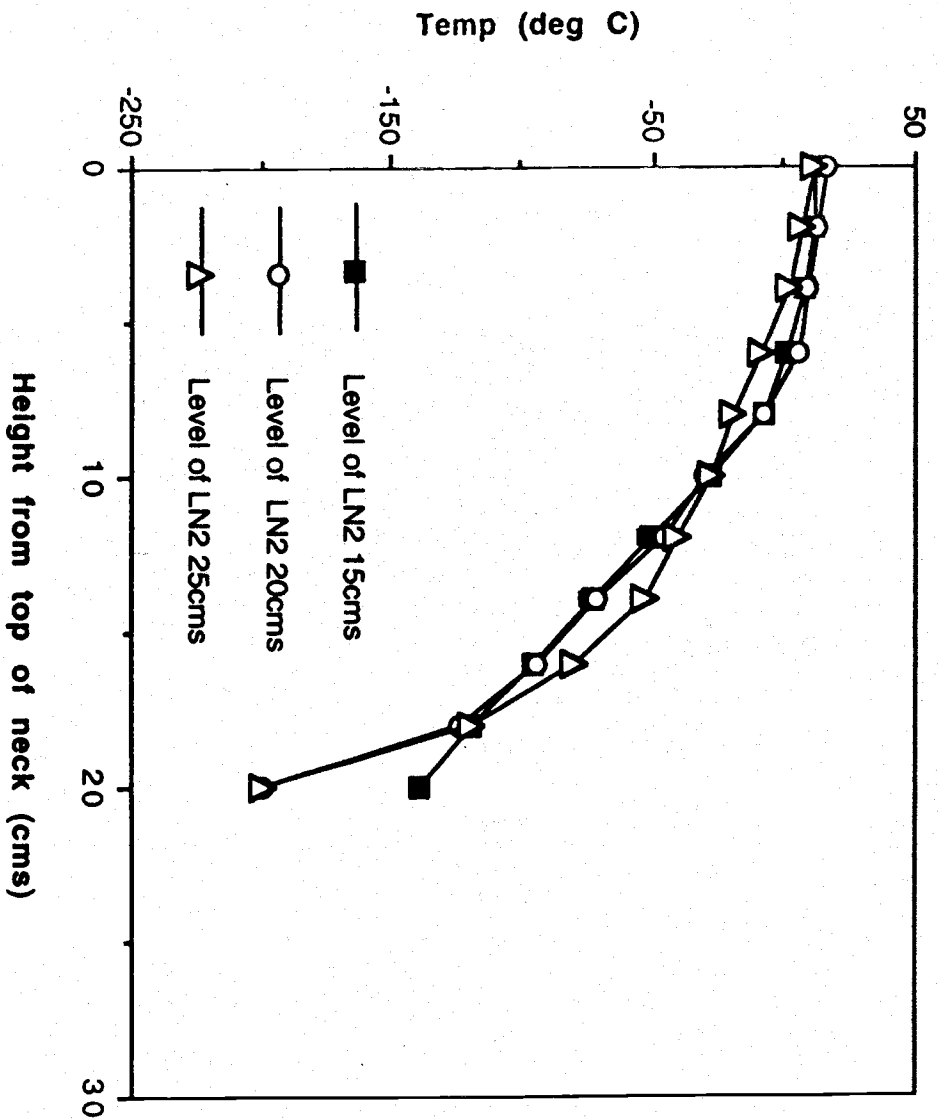


Fig.11 LN vapour temperature at different positions within neck of a 35 l. LN refrigerator

used for a minimum of two weeks in the field. Similarly, the temperature profiles within the larger 35l refrigerator were unaffected by decline in LN levels down to 15cm (Fig 11).

Having established that ambient temperature within the refrigerator neck is unlikely to be a variable during field or laboratory conditions factors such as the position of the FRPC within the neck and the cooling profiles within straws under a range of user conditions were elucidated. Higher cooling rates could be generated by positioning the KB18-10 at 4 cm below the refrigerator neck (Table 10). For tilapia, the ideal position for the FRPC was 2 cm below the neck and this position was used throughout this study.

Table 10 Summary of cooling rates within straws cooled in the FRPC at varying LN refrigerator conditions

Height of LN (cm)	Height of FRPC below neck (cm)	Mean (SEM) ¹ cooling rates within straws (°C/min)	
		Pre-freezing	Post-freezing
29	0	8.3(1.1)	5.9(0.9)
	2	8.6(0.3)	6.1(0.3)
	4	12.7(0.4)	9.9(0.7)
27	0	4.7(0.5)	3.2(0.2)
	2	9.2(0.3)	6.4(0.3)
	4	13.2(0.4)	9.9(0.2)
25	0	4.4(0.1)	2.9(0.2)
	2	8.2(0.8)	5.7(0.4)
	4	11.2(0.7)	8.4(0.4)
23	0	4.2(0.1)	2.7(0.2)
	2	7.7(0.3)	5.6(0.2)
	4	10.5(1.0)	8.0(0.6)
21	0	4.1(0.2)	2.8(0.1)
	2	7.4(0.3)	5.4(0.3)
	4	9.8(2.0)	7.3(1.8)

¹ Data based on 4 separate trials at each height. Each trial tested with 8 temperature probes. Similar evaluation of the KB70-35 shown in Table 11 indicated that for tilapias the FRPC

used for a minimum of two weeks in the field. Similarly, the temperature profiles within the larger 35l refrigerator were unaffected by decline in LN levels down to 15cm (Fig 11).

Having established that ambient temperature within the refrigerator neck is unlikely to be a variable during field or laboratory conditions factors such as the position of the FRPC within the neck and the cooling profiles within straws under a range of user conditions were elucidated. Higher cooling rates could be generated by positioning the KB18-10 at 4 cm below the refrigerator neck (Table 10). For tilapia, the ideal position for the FRPC was 2 cm below the neck and this position was used throughout this study.

Table 10 Summary of cooling rates within straws cooled in the FRPC at varying LN refrigerator conditions

Height of LN (cm)	Height of FRPC below neck (cm)	Mean (SEM) ¹ cooling rates within straws (°C/min)	
		Pre-freezing	Post-freezing
29	0	8.3(1.1)	5.9(0.9)
	2	8.6(0.3)	6.1(0.3)
	4	12.7(0.4)	9.9(0.7)
27	0	4.7(0.5)	3.2(0.2)
	2	9.2(0.3)	6.4(0.3)
	4	13.2(0.4)	9.9(0.2)
25	0	4.4(0.1)	2.9(0.2)
	2	8.2(0.8)	5.7(0.4)
	4	11.2(0.7)	8.4(0.4)
23	0	4.2(0.1)	2.7(0.2)
	2	7.7(0.3)	5.6(0.2)
	4	10.5(1.0)	8.0(0.6)
21	0	4.1(0.2)	2.8(0.1)
	2	7.4(0.3)	5.4(0.3)
	4	9.8(2.0)	7.3(1.8)

¹ Data based on 4 separate trials at each height. Each trial tested with 8 temperature probes. Similar evaluation of the KB70-35 shown in Table 11 indicated that for tilapias the FRPC

used for a minimum of two weeks in the field. Similarly, the temperature profiles within the larger 35l refrigerator were unaffected by decline in LN levels down to 15cm (Fig 11).

Having established that ambient temperature within the refrigerator neck is unlikely to be a variable during field or laboratory conditions factors such as the position of the FRPC within the neck and the cooling profiles within straws under a range of user conditions were elucidated. Higher cooling rates could be generated by positioning the KB18-10 at 4 cm below the refrigerator neck (Table 10). For tilapia, the ideal position for the FRPC was 2 cm below the neck and this position was used throughout this study.

Table 10 Summary of cooling rates within straws cooled in the FRPC at varying LN refrigerator conditions

Height of LN (cm)	Height of FRPC below neck (cm)	Mean (SEM) ¹ cooling rates within straws (°C/min)	
		Pre-freezing	Post-freezing
29	0	8.3(1.1)	5.9(0.9)
	2	8.6(0.3)	6.1(0.3)
	4	12.7(0.4)	9.9(0.7)
27	0	4.7(0.5)	3.2(0.2)
	2	9.2(0.3)	6.4(0.3)
	4	13.2(0.4)	9.9(0.2)
25	0	4.4(0.1)	2.9(0.2)
	2	8.2(0.8)	5.7(0.4)
	4	11.2(0.7)	8.4(0.4)
23	0	4.2(0.1)	2.7(0.2)
	2	7.7(0.3)	5.6(0.2)
	4	10.5(1.0)	8.0(0.6)
21	0	4.1(0.2)	2.8(0.1)
	2	7.4(0.3)	5.4(0.3)
	4	9.8(2.0)	7.3(1.8)

¹ Data based on 4 separate trials at each height. Each trial tested with 8 temperature probes
 Similar evaluation of the KB70-35 shown in Table 11 indicated that for tilapias the FRPC

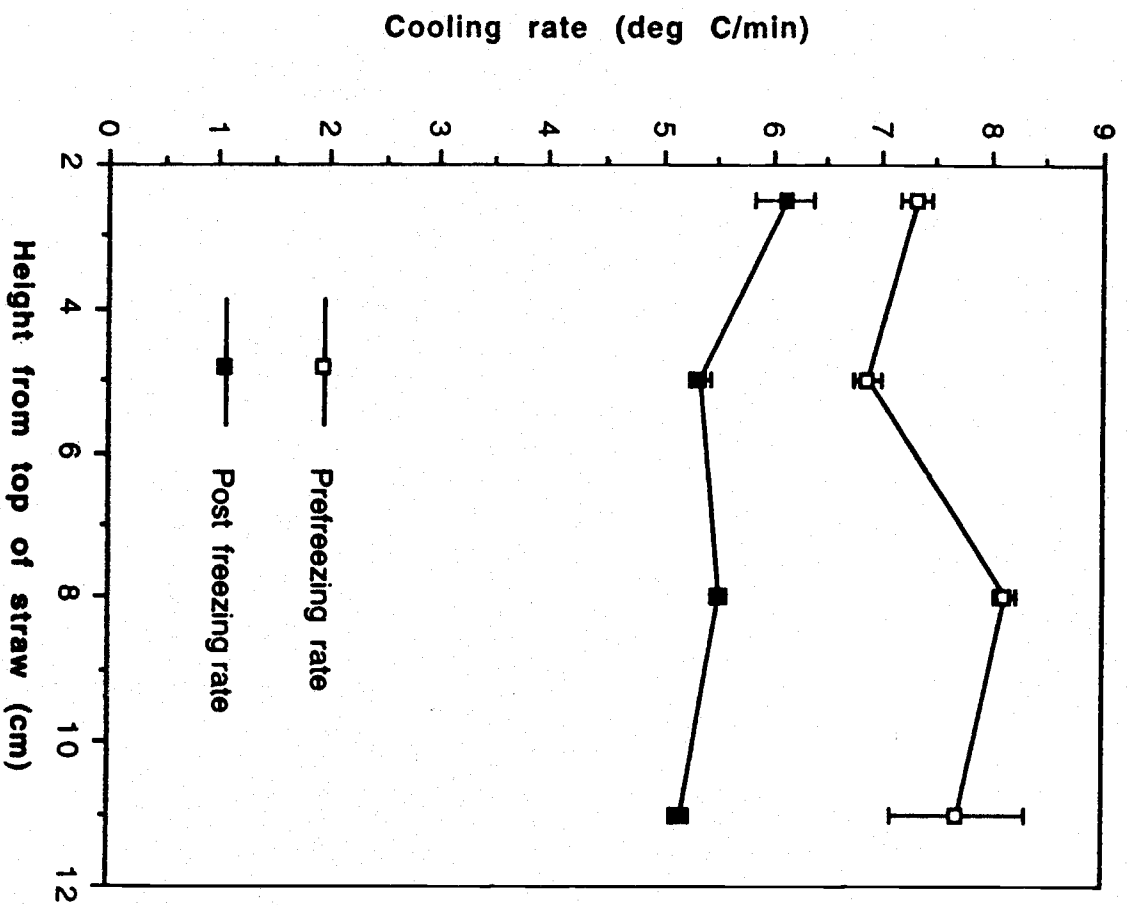


Fig12 Variation of cooling rates at different heights within 0.5 ml straw cooled in the FRPC

Table 11 Cooling rates within 0.5ml straws generated at two potentially useful heights in the neck of a 35L LN refrigerator using the KB 70-35 FRPC.

Height of FRPC from neck (cm)	cooling phase	cooling rate (°C/min) ¹
7	prefreezing	3.1 (0.12)
	postfreezing	3.3 (0.06)
9	prefreezing	5.3 (0.12)
	postfreezing	4.1 (0.06)

¹ means given with SEM based on three replicates.

need to be positioned at 9cm below the neck. The lower position was due to the greater influence of air temperature on the wider neck of the 35l refrigerator (Fig. 11). For both sizes of LN refrigerators an ambient temperature of around -100°C was required to generate suitable uniform linear cooling rates for tilapias.

The cooling rates at different heights within straws cooled in the vertical plane were not significantly ($P < 0.05$) different. The air jacket between the cooling tubes and the straws eliminated any vertical cooling rate gradients and cooling rates at the top and bottom of straws of the pre and post freeze phase were 7.2 (0.27) and 6.1 (0.54) and 7.7 (0.87) and 5.2 (0.11) °C/min, respectively (Fig. 12).

In summary, laboratory trials demonstrated the highly variable cooling profiles of currently used methods and the uniformity of cooling rates within the FRPC (Table 12, Fig. 13a-d).

Table 12 Comparison of mean pre and post freezing cooling rates within straws cooled using varying methods.

	Programmable ¹ cooler (KRYO 10)		Polystyrene box ²		Goblet ³		FRPC (KB18-10) ³	
	Pre-freezing rate	Post-freezing rate	Pre-freezing rate	Post-freezing rate	Pre-freezing rate	Post-freezing rate	Pre-freezing rate	Post-freezing rate
Mean (°C/min)	5.0	6.5	34.3	8.1	16.1	17.8	8.4	6.5
Std. deviation	0.22	0.5	4.9	2.8	7.1	6.0	0.51	0.63
Range (°C)	4.7-5.3	5-7.5	25.3-41.3	5.4-12.9	6-36	6-27	7.7-8.9	4.9-6.7
No. of straws	12	12	12	12	28	28	25	25
CV (%)	4.4	7.6	14.2	34.5	44	34	6.1	9.7

¹ Straws cooled at 5°C/min

² Straws positioned 8cm above LN surface

³ Goblet and FRPC positioned 2cm below the neck of a 10l LN refrigerator.

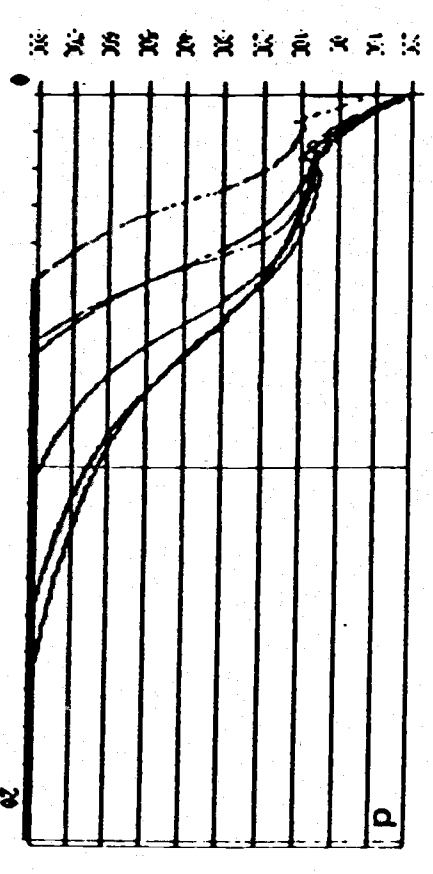
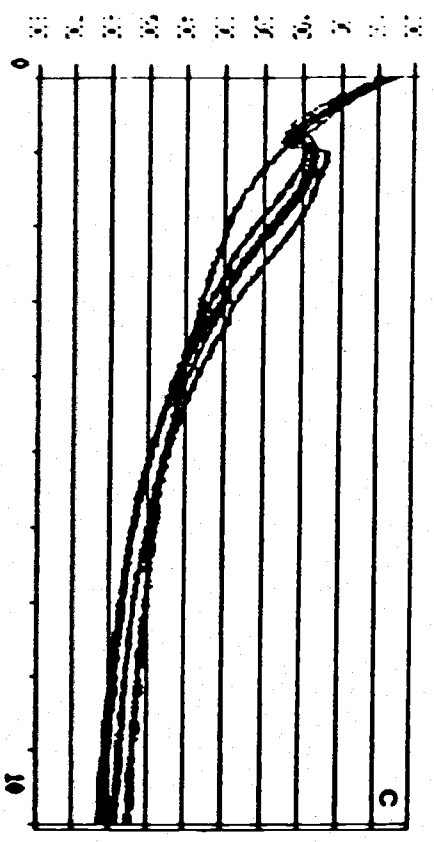
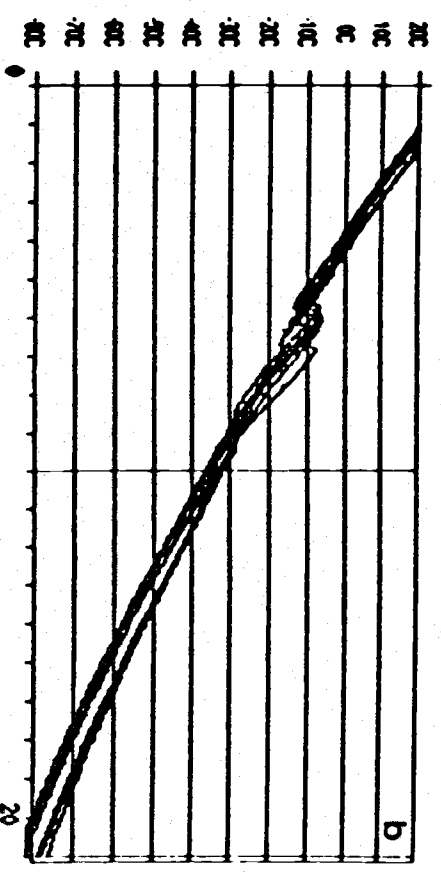
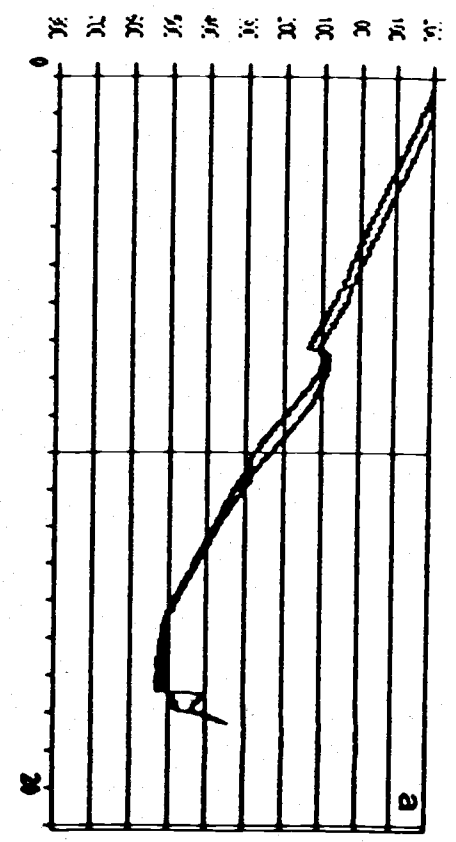


Fig 13. Typical cooling profiles within straws cooled in (a) Programmable controlled rate cooler (b) FRPC (c) Polystyrene box and (d) Goblet held in neck of LN refrigerator

The pre and post freezing cooling characteristics within straws cooled in the FRPC were ideal in that the cooling rates of both cooling phases were linear and the variation among straws and runs was less than 10%. Since the above trials minor changes in the use of the FRPC has reduced this variation to 5-7% (Fig 13b).

Laboratory trials comparing the post-thaw motility of *O. niloticus* cryopreserved using three methods indicated that the FRPC produced similar motility scores to those of the KRYO 10. While milt cooled in the goblet was also viable but motility was lower and more variable (Table 13).

Table 13 Cooling rates and post-thaw motility scores of *O. niloticus* milt cryopreserved using three cooling methods.

	COOLING METHOD ¹		
	Programmable cooler	FRPC	Goblet
Mean cooling rates(°C/min) ¹	5	6.9 and 5.1	36 and 6
Post-thaw motility	5-6	4-5	1-4

¹ Values for goblet and FRPC refers to mean cooling rates in the pre and post freezing cooling phases, respectively.

5.5.2 Field cryopreservation trials

The KB18-10 FRPC was successfully tested during field trips to Sri Lanka, Mexico and Philippines but all three trips highlighted several problems associated with the transportation of cryopreserved fish semen.

On all trips milt was successfully cryopreserved in laboratories, outdoors, on the banks of rivers and on commercial tilapia farms. In total milt from 109 *O. niloticus* males were cryopreserved; of these only 4 fish could not be successfully cryopreserved. In Sri Lanka post-thaw motility scores of all samples were >4. Unfortunately as milt was limited most of the samples were evaluated on site. Milt samples which were shipped from IOA, however, were cryopreserved on site, returned to IOA and yielded between 60-90% eyed eggs (Table 14).

Table 14 Viability of post-thawed *Oreochromis niloticus* milt cryopreserved in the tropics using the KB18-10 fixed rate portable cooler

Run No	No of eggs x (SEM)	No of replicates	Fertilisation rate (% of control) x (SEM)	Sperm:egg ratio No x10 ⁶ /egg	Post-thaw motility score x(SEM)
1 ^a	120	-	60.0	0.5	3
2 ^a	110	-	69.7	0.55	3
3 ^a	115	-	60.1	0.52	3
4 ^a	189	-	90.0	0.32	2
5 ^a	131	-	77.1	0.46	3
6 ^b	239.0(17.0)	6	88.2(5.0)	0.29	5.3(0.4)
7 ^b	241.0(21.0)	6	94.2(3.0)	0.29	6.0(0.2)
8 ^b	230.0(19.0)	9	40.0(9.0)	0.06	3.0(0.4)

^a Fresh milt couriered from Stirling to Sri Lanka and cryopreserved in FRPC and cryopreserved samples transported to Stirling for evaluation. Transport time, 4-5 days.

^b Samples collected and cryopreserved in Mexico in FRPC. Samples evaluated at Stirling.

Milt freshly collected and cryopreserved on site yielded higher post-thaw viability. Milt cryopreserved in Mexico and tested at IOA were highly fertile. Fertility rates of between 40-95% were achieved (Table 14). The fertility rate of 40% was due to the very low sperm concentration.

The variation in cooling rates within straws cooled in the KB18-10 under a range of tropical conditions was extremely low. Typical cooling profiles obtained under field condition were almost linear and were similar in all trials. In the Mexican trials mean pre and post freezing cooling rates averaged 5.3(0.4) and 4.3(0.25) °C/min, respectively (Fig 14a). Similarly in the Philippines cooling rates within straws in all 16 runs were highly consistent (Fig 14b). The mean (SEM) of the pre and post freezing rates of 5.4 (0.095) and 4.2 (0.15) °C/min were almost identical to those of the Sri Lankan and Mexican trip. The overall cooling rate averaged 4.8 (0.5)°C/min and compared favourably with ideal 5°C/min attainable in the programable cooler.

The final and largest field trial was conducted at the ICLARM cryounit in the Philippines installed by IOA. Milt from 80 males was cryopreserved with a 95 % success rate. Post-thaw motility scores of the same samples cooled in the KYRO 10 and FRPC at ICLARM gave comparable results (Fig 15). Of the 106 samples cryopreserved and tested in the FRPC over 90 gave post-thaw scores of over 3 (Fig 16).

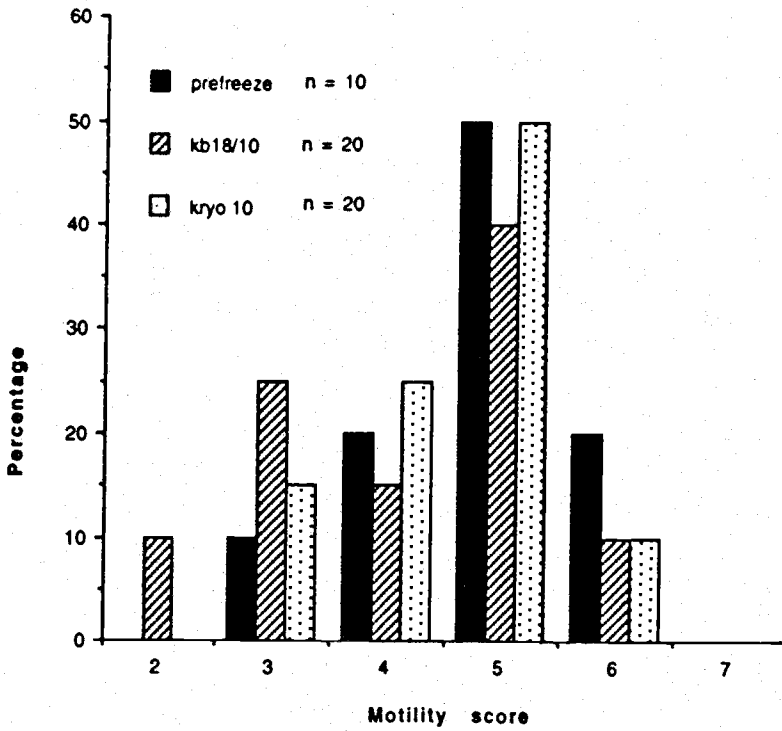


Fig 15. Comparison of post - thaw motility of *O. niloticus* spermatozoa cooled in the FRPC and programmable controlled rate cooler

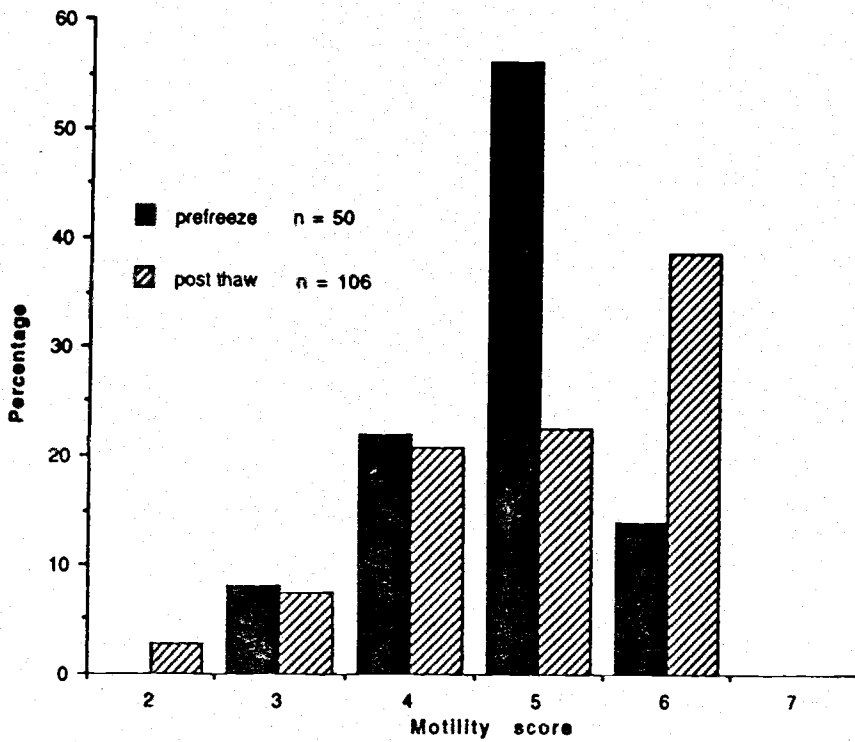


Fig 16. Post - thaw motility scores of *O. niloticus* spermatozoa cooled in the FRPC and programmable controlled rate cooler.

5.5.3 Patent for the FRPC and design modifications

The design of the FRPC was considered novel and following several highly successful laboratory and field trials a patent application was lodged at the UK office. Following advice from the University and ODA, the patent application was lodged under the company and inventors name as ODA and Dr Rana, respectively. The original patent application was filed for the UK only and may be widened to a world wide patent. The paper accompanying the patent application stating the features, design considerations and technical drawings submitted to the UK Patent office is given in Appendix 1.

Since the original patent application the FRPC has been modified. An interchangeable secondary plate has now been successfully designed to enable the FRPC to be used for cooling 0.5 as well as 0.25ml straws. This should widen the application to include those groups of animals whose milt is conventionally stored in 0.25 ml straws.

5.6 Uptake and dissemination of information

High priority was placed on transferring and disseminating cryopreservation technology.

5.6.1 Uptake of cryopreservation technology

The technology developed at Stirling was successfully transferred to ICLARM in the Philippines. The cryo unit was successfully designed, installed and tested, staff trained and frozen sperm bank of the founder broodstock and base populations of the UNDP funded GIFT tilapias initiated.

The broodstock facility which will be used to procure eggs for post-thaw evaluation of cryopreserved milt is capable of holding up to 24 females. The dedicated artificial incubation system which can house up to 20 incubators was successfully installed and used.

Four staff at ICLARM were trained in cryopreservation technology. Staff were trained in collection techniques of milt by manual stripping and gonadal extraction, evaluation of milt quality, processing, handling and packaging of neat and extended milt, operation of the programmable cooler and FRPC, thawing procedures for cryopreserved milt and *in vitro* culture of embryos, handling of LN and LN refrigerators and record keeping. Post-thaw *O. niloticus* milt cryopreserved during the mission was evaluated. Motilities of post-thawed milt for all attempts (n=16) ranged between 4-6. As the facilities at Munoz, Philippines were new

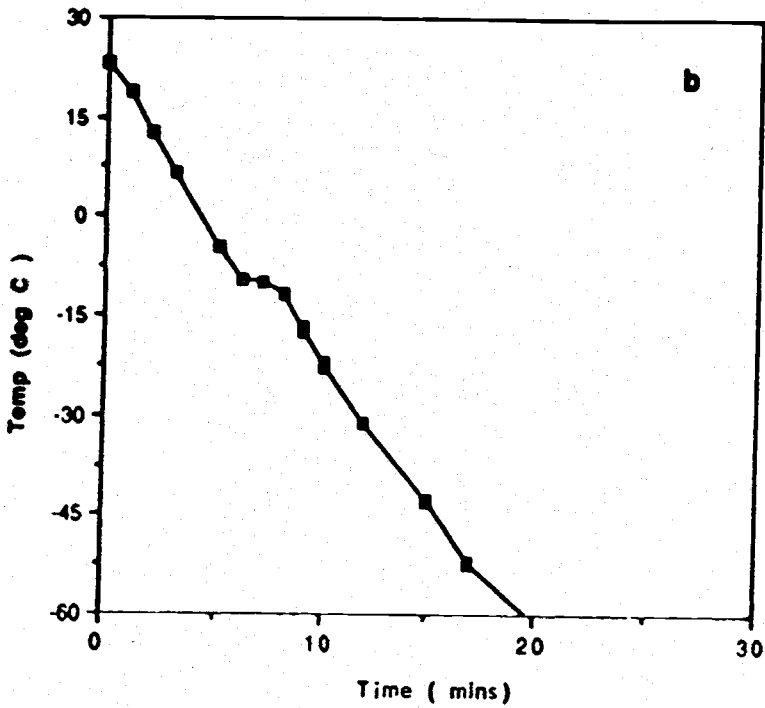
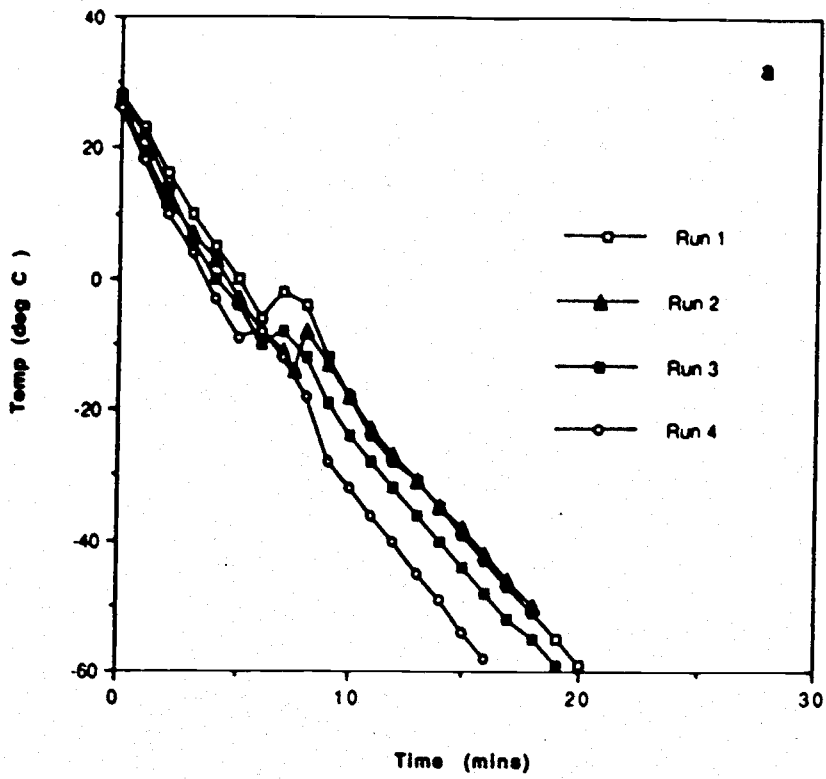


Fig.14 Mean cooling rates within straws cooled under field conditions in
 a) Mexico b) Philippines. means given with SeM based on (a) n = 4 (b) n = 16

the availability of eggs were limited. Only one batch of eggs was obtained and tested with post-thaw milt. In this trial cryopreserved *O. niloticus* milt tested on 200 eggs produced 88% eyed eggs.

A practical manual was also produced specifically for the cryounit at ICLARM, Philippines (Appendix 2).

5.6.2 Training

During 1993-1994 post-doctoral training on the cryopreservation of gametes was extended to a visiting scientist from the Fish Culture Institute, Szarvas, Hungary. Four staff at ICLARM were trained in cryopreservation technology. In addition, 2 PhD and 4 MSc students received training in cryobiology.

5.6.3 Publications and meetings

Papers on the use of fluorometry to monitor tilapia sperm damage and on the use of the Hobson Sperm Tracker in evaluating *O. niloticus* sperm fitness were drafted. The former paper is currently being considered for publication in *Reproduction, Fertility and Development*. In addition, a new monograph on "Preservation of Gametes" has been published in "Broodstock Management and Larval Quality". A second monograph on "Cryopreservation of Fish Spermatozoa" is currently in press to appear in the series "Methods in Molecular Biology".

Information associated with the application of cryopreservation technology developed at Stirling was disseminated at national and international meetings. Dr Rana gave invited papers and lectures in Saudi Arabia, Singapore, Philippines Senegal, Belgium, Scotland and England.

The titles of the monographs and meetings attended are given in Appendix 3.

6. IMPLICATIONS OF RESULTS

The overall emphasis of this project was to objectively evaluate the various stages of cryopreservation and to broaden the uptake of cryopreservation technology by developing

new protocols for different species and by developing a robust and reliable field cryopreservation technology for tilapia spermatozoa. Most of these objective were achieved. Unfortunately, the field trips were delayed due to Dr Rana's illness and the fertility trials of milt from the final trip could not be completed.

6.1 Development and evaluation of cryopreservation protocols

Objective assessment of sperm fitness based on computer assisted semen analysis technique and fluorometry showed conclusively that the fitness of cryopreserved tilapia is reduced and consequently the proportion and duration of post-thaw motile cells is reduced. In most previous studies, however, it was assumed that cryoinjury was inflicted during the cooling phase. Therefore the observation that about 50% of cells were damaged prior to cooling was of particular significance. Without exception all published protocols for all fish spermatozoa cryopreservation use a single step addition of cryoprotectant. In this study multiple or step-wise equilibration of cells with cryoprotectant reduced precooling toxicity and improved the post-thaw yield of motile cells.

Given that the cryopreservation processes reduces the viability and fitness of post-thaw cells the use of motility enhancers may be beneficial in improving the efficacy of protocols. Enhancers such as theophylline and caffeine have been used for salmonids but its use was limited to insemination fluids. In this project motility enhancers were successfully incorporated into the tilapia cryodiluent.

These overall improvements in post-thaw motility will enable efficient use of milt of marginal quality. Since acceptable fertility rates can be achieved by using lower sperm:egg ratios dilution of milt can be increased thereby making more economical use of small volumes of milt. This will be crucial in cases where small volumes of milt are available for genebanking.

6.2 Transportation of unfrozen tilapia milt

The transport (3-5 days) and subsequent successful cryopreservation of tilapia milt has an obvious utility in gene banking. Tilapia milt from a much wider geographical region can be cryopreserved. Since tilapia milt does not have to be cryopreserved immediately after collection multiple samples of the milt can be transported, cryopreserved and held in different repositories for safe keeping at minimal transport and maintenance costs. This method of transport, if successfully used, can substantially reduce transport costs. Delay and high

storage temperatures, however, are likely to affect the reliability of this approach. In this study delay during customs clearance and high tropical temperatures were key causes of failure.

6.3 Portable field cooler

This study demonstrated that currently used field cryopreservation techniques are highly variable and cooling characteristics are unsuitable for tilapia. Whilst these techniques can at times be successfully used to cool tilapia cells their cooling rates are unreliable and unpredictable and therefore not advocated for the tilapias.

During this project a novel cooler, the FRPC, was developed at IOA and successfully tested under field conditions. The FRPC offers a relatively cheap but reliable low technology alternative for the cryopreservation of tilapia spermatozoa thus creating the opportunity for widening the uptake of these technologies. Institutions and laboratories not primarily engaged in basic cryobiology should be able to generate reproducible cooling rates under both laboratory (KB70-35) and field conditions (KB18-10). The widespread use of a reliable cooling method and protocol will make a significant contribution towards international standardisation and towards improving cryopreservation and conservation technology between institutions.

7. PRIORITY TASKS FOR FOLLOW UP

To promote our understanding and uptake of cryopreservation technology for the tilapias data generated during this project on the following key aspects should be disseminated;

objective assessment of sperm fitness and viability at key stages during cryopreservation,

development of techniques for ameliorating cryoprotectant toxicity,

feasibility of transporting unfrozen tilapia semen and

field cryopreservation of tilapia semen using the FRPC.

A paper on objective assessment of membrane damage of tilapia spermatozoa during key phases of cryopreservation is currently being considered for publication and a second paper on sperm fitness is drafted.

The development of reliable field cryopreservation technology is to be published in aquaculture magazines, news letters etc. To this end an article has just appeared in NAGA, the ICLARM quarterly journal and a second article is due to be published in the January issue of the same publication. To extend the information further, two articles on cryopreservation have also been written for the ODA section of Aquaculture News. The manual on tilapia cryopreservation technology should be upgraded and published in book format and made readily available to prospective users of the technology at moderate cost.

Although the development of a cooling apparatus was not a specific objective of this project it was deemed necessary following the evaluation of currently used approaches. The FRPC was considered novel and patented in the UK to enable the cooling system to be properly exploited. At present only the FRPC has been developed. Standardised tilapia cryopreservation technology is best promoted as a unified package. The FRPC should be incorporated into a compact portable kit which comprises the FRPC portable LN refrigerator, essential equipment, chemicals etc. Given the history of its development to date the IOA would be the logical place to develop such a kit.

It should be borne in mind that the programmable control rate cooler alone costs in excess of £10000.00. It is estimated that the complete mobile kit will be only about half the price of the programmable cooler. At this stage it is envisaged that the ODA may bear the cost of further development and the kit can be used in relevant ODA and other aquaculture development projects. To widen the scope of the kit its suitability for cryopreserving spermatozoa of other fish species should be explored.

8. APPENDICES

Appendix 1. Patent application paper for the FRPC

FIXED RATE PORTABLE COOLER FOR CRYOPRESERVING GAMETES AND EMBRYOS

K. J. Rana, Institute of Aquaculture, University of Stirling, Stirling, Scotland

1 INTRODUCTION

Cryopreservation is the process whereby biological material suspended in an appropriate buffered saline medium containing a cryoprotectant is cooled at a rate within the optimum range for the biological system. It is widely acknowledged and published since the 1950s that the cooling rate the biological system is subjected too, is crucial for successful cryopreservation and that reproducibility of the desired cooling rates has a significant bearing on overall cryopreservation success. A linear cooling rate over the entire cooling phase is most desirable.

Spermatozoa, eggs and embryos are most commonly cryopreserved for germplasm conservation. Under current practices sperm or egg samples are packaged in plastic straws (0.25 - 5ml) and cooled using varying methods. Following cooling to the desired subzero temperature the straws are removed and plunged into LN and then transferred to canisters for storage under LN until required.

Several approaches are used to achieve the desired cooling rates during cryopreservation. The accuracy and reproducibility of the cooling rate depend on the system used. Prior to the use of controlled rate coolers (CRCs) biological samples were cooled either in alcohol baths or more simply on a rack mounted at a predetermined distance above liquid nitrogen or as sperm pellets on dry ice blocks.

The cooling method has a profound effect on the cooling rates. In most methods cooling rates are non-linear and when samples are cooled in LN nitrogen vapour the pre and post-freeze cooling rates can vary considerably. Typical examples of such rates determined at the Institute of Aquaculture, Stirling University are given in Fig 4c. In such methods of cooling, the cooling rates are driven by the difference between ambient and sample temperatures. Since LN vapour temperature is low (-100°C) samples cool rapidly. During this process the temperature difference between coolant and sample reduces and the post-freeze temperature therefore is typically lower than the preefreeze cooling rate (Fig 4c)

The above methods all have some limitations under both laboratory and particularly field conditions. For example, dry ice blocks have a very short life span (1-2 days) and this can limit the range of field cryopreservation. The use of LN vapour cooling in an insulated box requires larger volumes of LN, increases the number of procedural steps and risk of LN spillage and burns. Moreover, the cooling rates during the cryopreservation are not linear.

The advent of CRCs has increased the accuracy of the experimentation in determining optimal cooling rates for specific cells and tissues such as spermatozoa, eggs and embryos. This system, however, is largely laboratory based, is frightfully costly and may require a dedicated laboratory for the application of cryopreservation technology.

Given these constraints an alternative approach is being pursued. The following sections provides information on the development of a portable fixed rate field cooler for laboratory or field use. The design features and its application are described using fish spermatozoa to demonstrate its capability.

2 THE FIXED RATE PORTABLE COOLER (FRPC) FOR 0.5ML STRAWS

2.1 Claims of this invention

This invention for which patent is filed addresses several of the above limitations and seeks to protect the cryopreservation kit and method. Such a kit will not necessary require a costly laboratory nor depend on electricity and can be used under field conditions for a few weeks.

This invention does not claim discovery of the ability of cells to be cryopreserved nor does it claim discovery to some of the equipment used in the kit. It does, however, claim a unique cooling apparatus and an accompanying series of steps and procedures that can be used to successfully cryopreserve mammalian and aquatic gametes and embryos. For this submission the kit is tested for fish spermatozoa but will be widened to other cell types.

2.2 Considerations for developing a field cryopreservation system.

Several factors for field use or less privileged laboratories have been considered in developing the steps of this invention.

- i) Limited air volume and extremely high vertical temperature gradient in the LN refrigerator neck.

- ii) Limited availability of liquid nitrogen under field conditions and the need to spend several days in the field.
- iii) Speed of operation and maximising number of straws to be cooled in a run.
- iv) High ambient temperatures in tropics resulting in rapid premature warming during post-freeze transfers.
- v) Limited space and equipment.
- vi) Need for ice to keep gametes chilled during processing.
- vii) Rapid transfer for storage following cryopreservation.

2.3 Design and features of the fixed rate portable cooler (FRPC) for 0.5ml straws

The full technical drawing describing the cooling cylinder is shown in Fig. 1-3. This FRPC, designated as KB18-10, is designed to hold 18 - 0.5ml straws and to fit a standard 10l LN refrigerator. The cooling rates generated within straws in the KB18-10 were linear and averaged 5-6°C/min both under laboratory and field conditions.

The configuration and number of tubes comprising the FRPC can be varied to suit individual need and size of LN refrigerator.

This modular system claims the following unique features:

- i) The straws are cryopreserved in the vertical plane within the neck of the LN refrigerator.
- ii) To reduce costs and space requirements the cooler can be used with a standard LN refrigerator used for storing the cryopreserved material. It is easily removed or installed.
- iii) The position of the cooler in the LN refrigerator neck is easily controlled by an adjustable collar (Fig 1c). The storage canisters need not be removed during cryopreservation. Therefore the storage vessel can serve a dual function; storage and cryopreservation.
- iv) Heat sinks are often engineered as a block from a metal of high thermal conductivity eg. copper, aluminum, brass. Given the height of the straws (13cm) such heat sinks

1	2	3	4	5	6	7	8	9	10	
DESCRIPTION		APPRO. DATE	TITLE				DRAWING No.			
							DRAWN	TRACED	CHECKED	APPROVED
										DATE

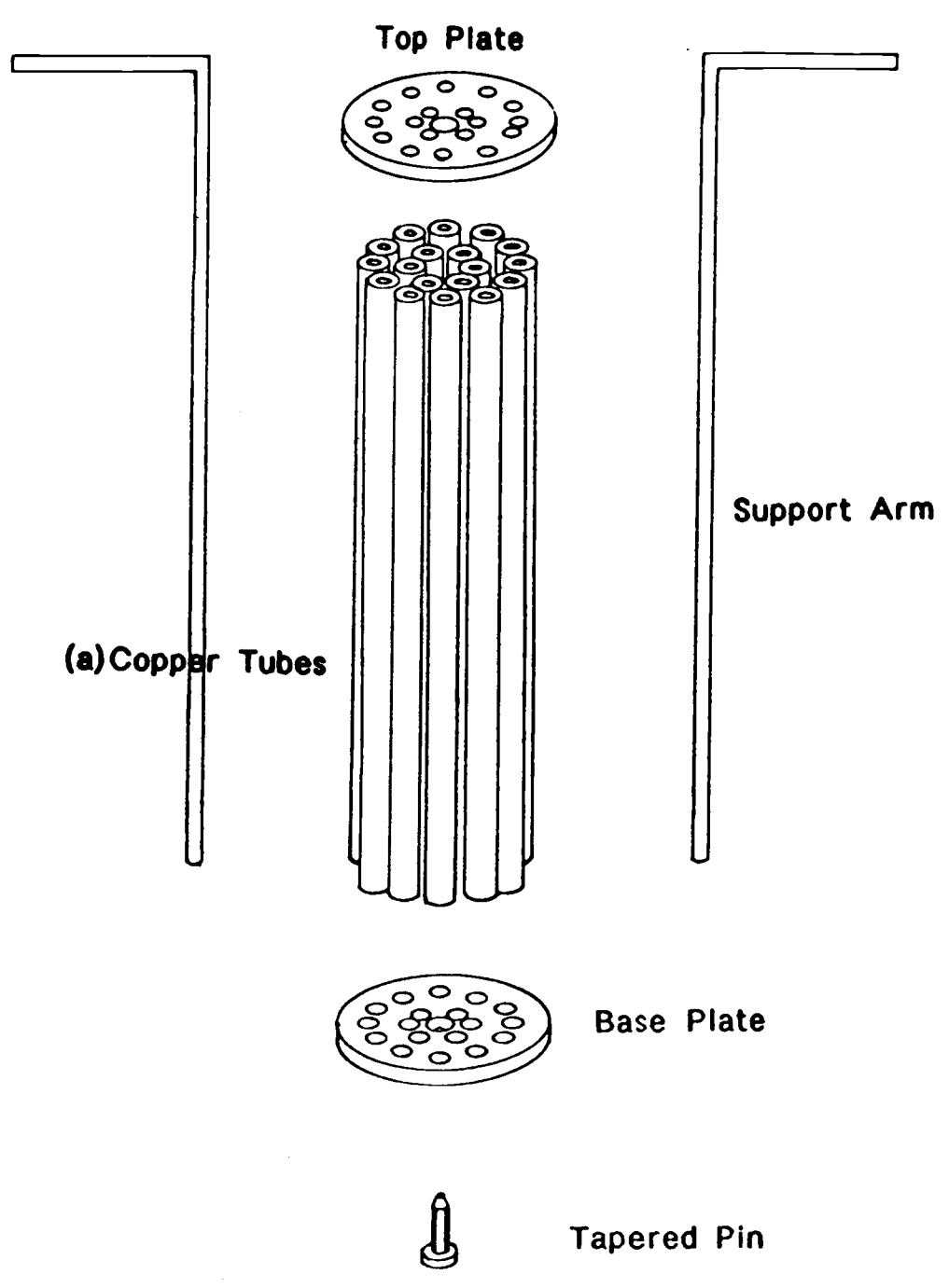


Fig 2 EXPLODED VIEW OF THE FIXED RATE COOLER

DRG.No.

when placed in the neck of the refrigerator will result in a high vertical gradient.

Instead, to obtain a more uniform cooling rate within the straw this invention uses a series of engineered copper tubes each housing a single straw. This configuration allows for a more uniform coolant (LN vapour) distribution around each straw (Fig 2a).

- v) A unique feature of this invention is that the straws containing the biological material are not in direct contact with the heat sink, the copper tubes. Instead, a precision tapered pin (Fig 3d) mounted on the base plate and projecting internally into the copper tube and a corresponding aperture (Fig 3a) positioned on the top engaging plate hold the straw at a fixed distance from the walls of the copper tubes (Fig 3b). The air break between the straw and the heat sink cools more quickly and uniformly along its vertical axis thereby minimising any variation in cooling rate within the straw.
- vi) To save space, time and LN in the field, the overall diameter of the FRPC is reduced to allow for the easy removal and positioning of the required storage canister directly under the cooler.
- vii) To conserve LN under field conditions the cooler has a central aperture (Fig 1b) through which straws can be passed directly into the canister (Fig 1d) below via a removable shoot. Thus the need to plunge straws in LN prior to storage is eliminated.

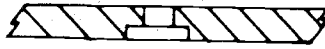
2.4 The procedure for cryopreserving aquatic gametes or embryos using the FRPC

The above invention is central to the portable kit developed to cryopreserve fish spermatozoa, in this case *Oreochromis niloticus*.

Under field conditions chilled temperature was achieved by using hydrated polyacrylamide gel. A predetermined quantity of hydrated gel pellets were frozen by plunging them in LN and used with unfrozen gel as ice to maintain a temperature of between 4-10°C.

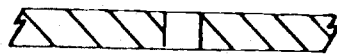
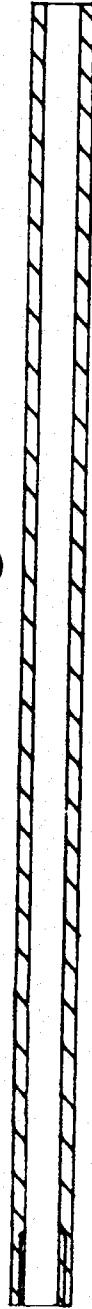
Milt was collected from males in 1.5ml Eppendorfs and maintained either separately or pooled under chilled conditions. The milt was then diluted with the appropriate cryodiluent

(a) Hole in Top Plate
3.5mm



Top Plate

(b) Bore of copper Tube
3.8mm



(c) Base Plate

(d) Tapered Pin

Fig 3 SECTION OF COOLING TUBE

DRG.No.

DO NOT SCALE

SHEET OF

11	1	DESCRIPTION	APPRO. DATE	TITLE	DRAWN	TRACED	CHECKED	APPROVED	DATE
	2								
	3								
	4								
	5								
	6								
	7								
	8								
	9								
	10								

DRAWING No.

7/11/2011

and drawn into 0.5ml plastic straws and sealed with colour coded powder or beads.

The filled straws were kept chilled on the gel. Prior to cooling the straws were wiped and loaded into the FRPC. The loaded cylinder was then lowered to the required height in the LN refrigerator neck for 15 minutes. Following cryopreservation the canister for storing the straws was manoeuvred into position beneath the cooler and the frozen straws guided into the canister through a shoot inserted through the central aperture of the cooler. The canister was then be returned to its original storage position and the cooler removed, dried and the whole procedure was repeated if required.

The ability to identify individual samples is also not compromised. Using the full range of canister, straw and bead colour combinations up to 50 individual fish samples/canister (300/LN refrigerator) can be processed under field conditions.

2.5 Results of field and laboratory trials using the FRPC under tropical field conditions for *O. niloticus*

Typical cooling profiles within straws in different cooling systems are shown in Fig 4 and indicate that cooling rates in the FRPC are almost linear across the entire cooling process. The mean cooling rates between ambient and -50°C determined under laboratory and field conditions are illustrated in Fig 4b and Fig 5.

When samples are cooled over LN vapour using conventional methods the pre and post freezing rates are considerably higher and vary considerably (Fig 4c).

Post-thaw viability of tilapia spermatozoa cryopreserved in the fixed rate portable cooler is given in Table 1 and these are similar to those obtained in CRCs.

The FRPC will now be tested on a range of fish and other aquatic species.

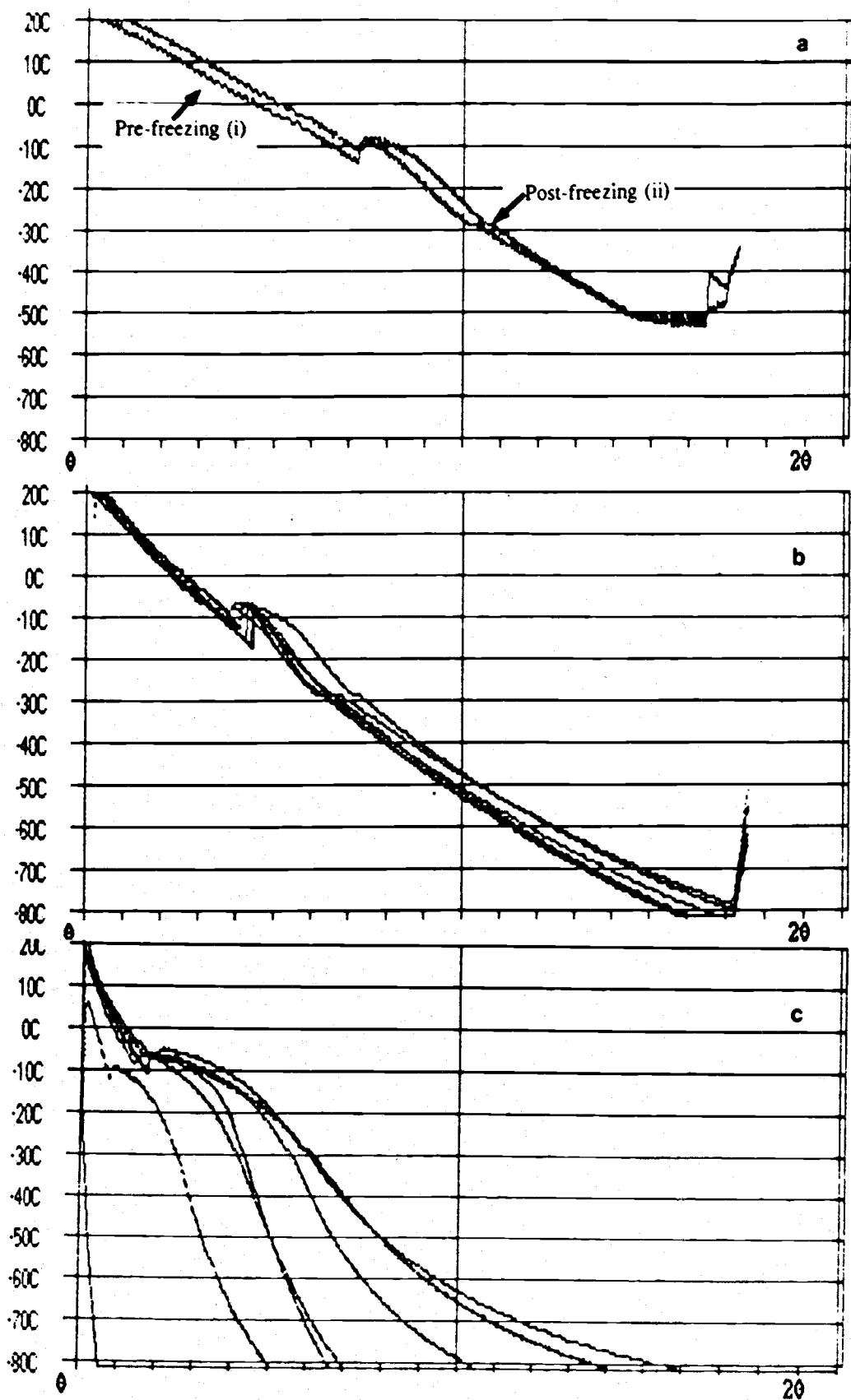


Fig 4 Typical cooling curves obtained within straws cooled in a) Programmable chamber b) Fixed rate portable cooler and c) Goblet held 2cm below neck of the LN refrigerator. Note high cooling rates and their variability between straws in (c).

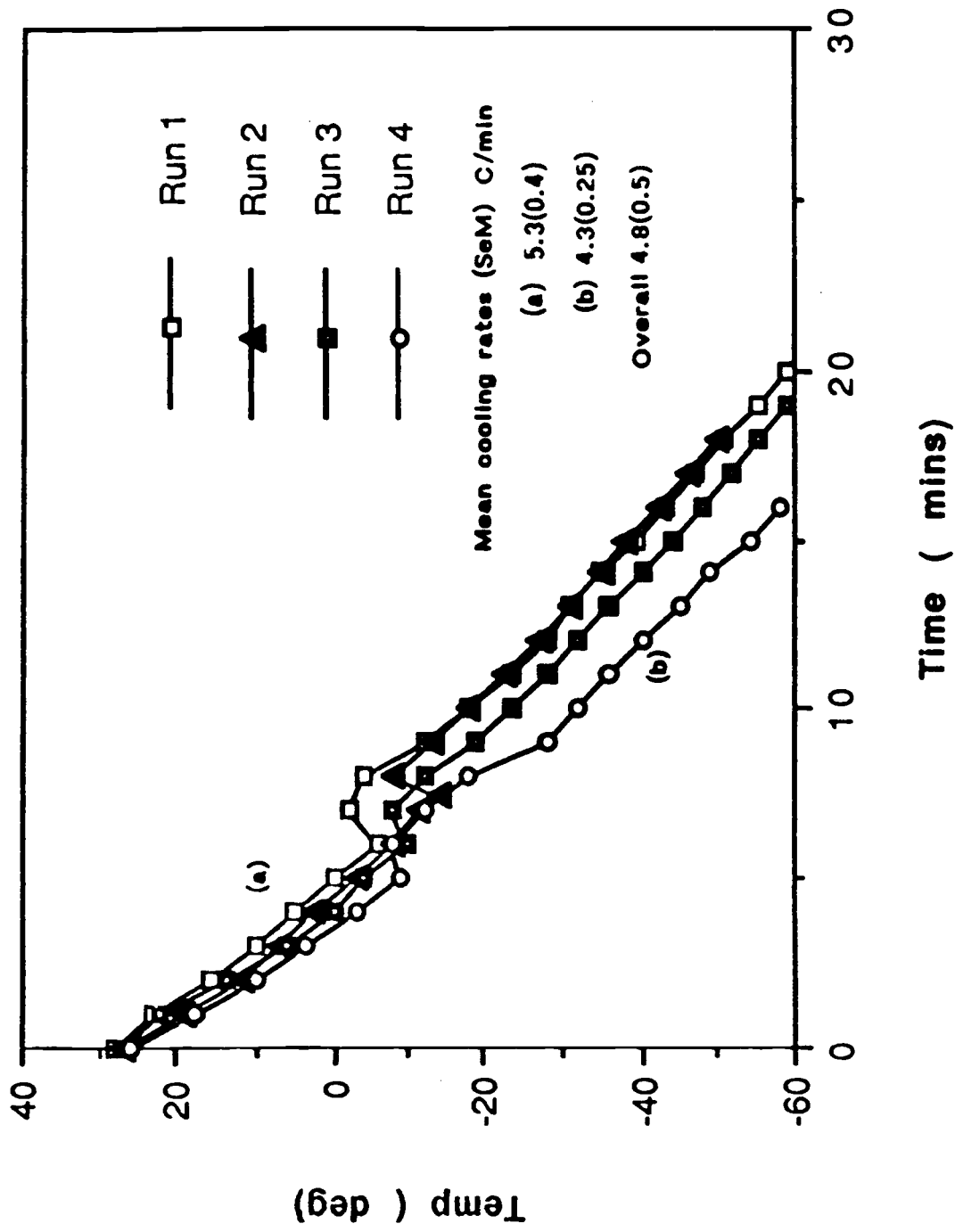
Table 1 Viability of post-thawed *Oreochromis niloticus* milt cryopreserved in the tropics using a fixed rate portable cooler (FRPC)

Run No	Mean (SEM) no of eggs	No of replicates	Mean (SEM) fertilisation rate (% of control)	Sperm: eggs ratio No.x10 ⁶ /eg g	Post-thaw motility score X (SeM)
1 ^a	120	-	60.0	0.5	3
2 ^a	110	-	69.7	0.55	3
3 ^a	115	-	60.1	0.52	3
4 ^a	189	-	90.0	0.32	2
5 ^a	131	-	77.1	0.46	3
6 ^b	239.0(17.0)	6	88.2(5.0)	0.29	5.3(0.4)
7 ^b	241.0(21.0)	6	94.2(3.0)	0.29	6.0(0.2)
8 ^b	230.0(19.0)	9	40.0(9.0)	0.06	3.0(0.4)

^a Fresh milt couriered from Stirling to Sri Lanka and cryopreserved in FRPC and cryopreserved samples transported to Stirling for evaluation. Transport time, 4-5days.

^b Samples collected and cryopreserved in Mexico in FRPC. Samples evaluated at Stirling.

Fig 5 Freeze runs using 10 L dewar in Mexico



Appendix 2. Site specific cryomanual for cryounit, ICLARM

MANUAL FOR ICLARM CRYOUNTIT

A) PREPARATION AND PROTOCOLS FOR CRYOPRESERVATION OF TILAPIA MILT

1a Equipment required for preparation of solutions

250 ml Measuring cylinder
500 ml Beaker
Spatula
Weighing boats/tinfoil
Distilled Water
Bottle for solution
Chemicals

1b Preparation of Modified Fish Ringer

CHEMICAL	g/l
NaCl	6.5
KCl	3.0
NaHCO ₃	0.2
CaCl ₂ ·6H ₂ O	0.3
pH	7.9

Prepare 500mls in distilled water and store for 1 month in the refrigerator

1c Precautions during preparation

- i) Use clean dry glassware.
- ii) Weigh as accurately as possible.
- iii) Always use distilled water.
- iv) If chemical remains on boat/foil wash carefully into container and make up with required volume of distilled water.

- v) Label storage bottle clearly with name date and contents.
- vi) Store in refrigerator.
- vii) Before use shake well to aerate.

2a Equipment for milt collection and evaluation

Capillary tubes

Soft absorbent paper

Microscope slide

Marker pen

Small amount of tap water at 25-28°C for motility check

200-1000 μ l finpipette set at 200 μ l

2b Collection of milt

- i) Select fish and ensure careful handling.
- ii) Apply abdominal pressure to clear bladder of urine.
- iii) Gently remove all moisture and mucus with tissue.
- iv) Take care when applying pressure during the stripping of the milt to avoid urine contamination.
- v) Hold the capillary tube against the tip of the genital papillae and apply gentle pressure across the abdomen.
- vi) Check sample by placing small amount on a glass slide and view under the microscope.
- vii) Sperm should be immotile at this stage. Keep samples cool preferably below 20°C.
- viii) Only immotile samples should be used (>90%).
- ix) Activate by the addition of about 100-200 μ l of tap water and observe motility. Only milt with > 90% motile sperms should be used.
- x) Now store in the fridge(+4°C) and use as soon as possible.

3a Equipment required for estimation of sperm concentration

Tally counter

Neubauer slide counter

Deactivator solution prepared as follows:

NaCl 0.7g

KCl 0.6g

Distilled water 100ml

Microscope

5-40 μ l and 200-1000 μ l finpipettes

3b Estimation of sperm count

- i) Make a dilution with deactivator which is practical for counting. A range between 1 in 500 and 1 in 1000 is suggested.
- ii) Carefully place one drop of this solution on the Neubauer slide counter and cover with the designated cover slip.
- iii) Leave the slide for approximately 10 min to allow the sperm to settle into one plane.
- iv) Count 5 large squares. This should be replicated and the average no. of cells calculated.
- v) To estimate conc /ml use average count x dilution x 50000.

Example: If average no = 200 and dilution = 500, then cell conc. (no./ml) = $200 \times 500 \times 50000 = 5.0 \times 10^9$

4a Filling of the dewar and connection to the Kryo 10

- i) Using the face mask and protective gloves, fill the dewar 1/2 full with liquid nitrogen.
- ii) Attach the pump to the dewar with the valve in an open position.
- iii) Switch on the heater, close the valve lever and wait until the pressure rises to 5 on the gauge.
- iv) Initiate the programme (See Kryo 10 manual).

4b Programming the Kryo 10

- i) Using the instructions provided with the machine set the following parameters.
- ii) Start temperature at 20 - 25°C.
- iii) Set cooling rate at - 5°C/min.
- iv) Target temperature - 60°C.
- v) Hold step here for 2 min.
- vi) Remove samples as quickly as possible to liquid nitrogen.
- vii) Dry chamber to avoid condensation rusting the compartment.

5a Equipment required for preparation of milt sample for cryopreservaton

Modified fish Ringer + 10% Methanol

Appropriate sizes of finpipettes

Small plastic or glass containers to make up milt dilutions

5b Preparation of milt with extender

- i) Decide on required dilution and prepare number of straws desired.
- ii) A maximum time of 30 minutes should be allowed from mixing the milt with the extender to placing in the straws in the Kryo 10. The number of straws which can be reasonably filled within this time span should be calculated.

6a Equipment required for straw filling

Appropriate size and colour of straw

Beads or sealing powder depending on size of straw required

Cryomarker pen

Tissue paper

200 -1000 µl finpipette set at 200µl

200 -1000 μ l finpipette tips

Small amount of water for sealing the powder

6b Procedure for straw filling and sealing

0.5 ml straws

- i) Choose straw and bead to be used ensuring the colours are compatible.
- ii) Label straws using cryomarker at sealed end for easier identification when removing straws from the LN.
- iii) Place unplugged end of straw into prepared milt sample, insert 200 - 1000 μ l finpipette into plugged end of straw.
- iv) Depress pipette plunger fully drawing sample into the straw.
- v) Quickly turn the straw to a horizontal position replacing the plunger into the straw tip.
- vi) Depress the plunger again drawing the sample through until the plug has gelled.
- vii) Seal at the opposite end with a bead applying enough pressure to ensure that the bead is firmly inserted into the straw.

0.25 ml straws

- i) Follow steps i-vi for 0.5 ml straws.
- vii) Dip the unplugged end of the straw into a small amount of water and then immediately into a small container of tightly packed powder.
- viii) Dry straws with absorbent paper to avoid ice forming during the freezing process and the straws adhering to one another.
- ix) The time that is spent on this procedure should be kept within the 30 minute equilibration time.
- x) Arrange the straws in the Kryo 10 prior to freezing.

7 Storage of straws in LN refrigerator

- i) The level of liquid nitrogen must always cover the samples.
- ii) Record the no of the dewar, no. of the canister and position in the canister top or bottom for future recovery of the sample.
- iii) Complete record cards for each sample and file.
- iv) Ensure low level liquid nitrogen alarm is plugged in and switched on. Test alarm.

8a Equipment required for thawing straws

Water bath at 40°C. This can simply be a bucket

Thermometer

Timer

Scissors

Slides and microscope

Tap water at approximately 27°C

Face visor, protective gloves and tweezers

8b Thawing procedure

- i) Use visor and gloves.
- ii) Tweezers can be useful here as gloves may be too clumsy to handle the straws.
- iii) Identify the straw and immediately plunge into the water bath and gently agitate for 8 seconds.
- iv) Wipe the straw and cut near to the unmarked end.
- v) Place drop on to a microscope slide and activate with the addition of 100µl tap water by carefully mixing.
- vi) Give motility score from 1 -10 when 10 is equivalent to total no. of sperm moving.

B) RECOMMENDED SAFE LABORATORY PROCEDURES TO BE OBSERVED WHILE WORKING IN THE CRYOUNIT

1a Personal safety

- i) Laboratory coat should always be worn.
- ii) When dealing with hazardous chemicals use disposable gloves and if required mask or breathing apparatus.
- iii) Mouth pipetting is not permitted.
- iv) Eye wash station to be located nearby.
- v) First aid kit to be easily accessible.

1b General laboratory procedures

- i) Before handling hazardous chemicals precautions for safe handling should be taken.
- ii) Glass should be disposed of into glass waste bin only.
- iii) Sharps and blades should be disposed off into sharp disposal bin only.
- iv) Spillage should be dealt with immediately as other people will subsequently use the bench and equipment.
- v) Solutions should be kept in bottles clearly labelled with the date, solution name and owners name.

1c Liquid nitrogen

Great care is required when handling liquid nitrogen, use cryogloves and mask. Avoid splashes and spillage.

1d Kryo 10 freezing chamber

- i) Before refilling the dewar, ensure that the release valve has been opened and when removing pump ensure pressure gauge is at 0.
- ii) When filling the dewar great care should be taken to avoid damage to the heating element.
- iii) The dewar should be 1/2 full of liquid nitrogen before operating the machine.
- iv) When chamber returns to re-start temperature, wipe the chamber and leave the lid open to avoid condensation rusting the chamber.

1e Finpipettes

- i) Instruction must be given to new users. This equipment needs very careful handling to ensure accurate operation.
- ii) Tips should be removed immediately and not left attached otherwise harmful chemicals may find their way into the mechanics of the pipette.
- iii) If possible pipette should be left in an upright position.

C) LIQUID NITROGEN INFORMATION

- i) Liquid nitrogen will vaporise rapidly to nearly 830 times its liquid volume.
- ii) Spilt liquid or cold gas can result in cold "burns".
- iii) It also reduces the oxygen content in the vicinity, therefore the room should be well ventilated when transferring liquid nitrogen.
- iv) Extreme care is essential when handling liquid nitrogen.

D) PERSONAL PROTECTION

Items that should be worn when handling liquid nitrogen:

Face mask

Cryogenic gloves

Protection on feet

E) THINGS TO REMEMBER

- i) Check that there is adequate liquid nitrogen in dewar.
- ii) Always take care when handling the liquid nitrogen and use protection provided.
- iii) Reset circuit breaker before starting Kryo chamber.
- iv) Pressure gauge on LN₂ pump must read about 5 lb/in² before chamber can be used.
- v) Store milt samples in fridge until required and keep on ice while making dilutions and filling straws.
- vi) Bring extender out and let it come to room temperature if used straight from refrigerator it may cause cold shock to the cells.
- vii) Make extender + methanol **first** and add the milt only when all the other procedures have been completed.
- viii) When calculating amount of solution required for filling straws make slightly more than required.
- ix) Have liquid nitrogen in polystyrene box for removal of straws from liquid nitrogen.
- x) Use visor when thawing straws. Should the plugged end blow out then plunge the bead end into the water bath to salvage the sample.
- xi) Dry the freezing chamber when finishing the freezing run.

- xii) Remember to release the valve on pump and ensure pressure gauge is reading zero before removing from the dewar.
- xiii) Note the colour code, fish number and location of straw in the dewar for future cataloguing.
- xiv) When changing the microscope bulb handle with tissue and not bare hands.
- xv) Cover microscope objective and lens when not in use.
- xvi) Finpipettes to be kept in an upright position when possible. If they require to be dismantled they must be re-calibrated before use - instructions in the leaflet in packaging box.

Appendix 3. Publications and meetings attended

PUBLICATIONS AND MEETINGS ATTENDED:

Monographs:

- Rana K J (1994) Preservation of fish gametes. Niall Bromage and Ronald Roberts, eds In: Broodstock Management and Larval Quality, Blackwell Science, Chapter 3. 53-75.
- Rana K J (1995) Cryopreservation of fish spermatozoa. Day G. D. and McLellan M. R. eds: Cryopreservation and freeze-drying protocols. Chapter 16 (in press).

Invited meetings:

- Myers J M, Penman D J, Rana K J & McAndrew B J (1992) Genetic manipulations and gene transfer in tilapia International workshop on genetics in aquaculture. August 31st to September 4th 1992. University of Stirling.
- Rana K J (1992) Cryopreservation of aquatic gametes and embryos. International workshop on genetics in aquaculture. August 31st to September 4th 1992. University of Stirling.
- Rana K J and Suliman E M (1993) Artificial incubation of tilapia eggs and fry under semi-intensive and intensive aquaculture systems. Aquaculture symposium - Technology and Investment Opportunities, Riyadh, Saudi Arabia. 11-14th April 1993.
- Rana K J (1993) Ex-situ conservation of tropical fish genetical resources current status and future prospects. The third international aquarium and fish accessories exhibition and conference. 24-27th June 1993, Singapore.
- Rana K J (1994) Applications of new technologies for the aquatic germplasm technologies. Taxonomic and Advisory Group Conference. 29th April 1994. Chester Zoo, England.
- Rana K J, McAndrew B J and Gilmour Ann (1994) Preliminary observations on a compact field freezer for cryopreservation of tilapia milt. International Society for Low Temperature Biology, Leuven, Belgium. 19-23 July 1994.
- Rana K J (1994) Recent Progress in the Cryopreservation of Fish Spermatozoa. British Andrology Society, London. September 1994.

Post graduate training

Danting J C (1992) Optimization of UV irradiation treatment and cryopreservation of *O. niloticus* spermatozoa for induced gynogenesis. University of Stirling. 74pp.

Lawrence C S (1993) Development of computer image analysis and fluorometry to assess the fitness of cryopreserved tilapia spermatozoa. MSc Thesis. Institute of Aquaculture, University of Stirling. 79pp.

FINAL FINANCIAL STATEMENT

Application of cryopreservation for the genetic management of commercially important species.
R4915

GRANT AWARDED

PERSONAL EMOLUMENTS	BUDGET	ACTUAL EXPENDITURE 1/4/92 - 31/3/93	ACTUAL EXPENDITURE 1/4/93 - 31/3/94	ACTUAL EXPENDITURE 1/4/94 - 31/3/95	TOTAL EXPENDITURE
Technician	£50,471.00	£13,339.55	£14,591.27	£15,409.41	£43,340.23
CAPITAL EQUIPMENT	£3,525.00	£2,357.41	£1,177.11	£0.00	£3,534.52
OTHER COSTS					
Broodstock, feed	£1,200.00	£1,182.00	£300.00	£0.00	£1,482.00
Liquid nitrogen	£2,200.00	£797.15	£938.14	£432.00	£2,167.29
Disposables	£4,000.00	£1,586.78	£2,115.86	£1,393.36	£5,096.00
Travel & Subsistence:					
U.K	£875.00	£354.30	£4,139.75	£73.85	£4,567.90
Overseas	£8,000.00	£0.00	£2,292.15	£1,806.00	£4,098.15
Overhead	£20,189.00	£5,335.82	£5,836.51	£6,163.76	£17,336.09
	£90,460.00	£24,953.01	£31,390.79	£25,278.38	£81,622.18