THE CRYOPRESERVATION AND MANIPULATION OF OVA AND MILT IN COMMERCIALLY IMPORTANT TROPICAL SPECIES.

R 4523

FINAL REPORT

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1 OBJECTIVES

The major aims and objectives as set out in the original project memorandum are stated below:

a) Investigate the major factors inhibiting the cryopreservation of fish eggs and embryos. These will include factors such as egg volume, membrane permeability and bound water. The importance of these major constraints will be investigated on a range of invertebrate (10-100 μm) and fish eggs and embryos. Membrane permeability will be tested by dechorionation, microinjection of cryoprotectants into eggs and electroporation.

b) Following the construction of the facilities for maintaining and breeding carps at Stirling by BAFRU the second major objective was to develop and improve protocols and methodologies for a range of carp species and to develop techniques for the quality assessment of stored milt.

c) Increase the species and strains of tropical species held at Stirling using cryopreservation of milt and extend the cryopreservation technology in ODA and other related projects.

Following the comments and concerns of the ODA review panel in April 1991 that

oysters were not an acceptable model (despite invertebrates being in the project memorandum), the emphasis of the project was redirected earlier than was planned to fish eggs and embryos with the agreement of Prof. Roberts of the Institute of Aquaculture. This, however, had meant that investigations into the fundamental questions on the cryobiology of a successful model could not be pursued and our efforts had to be diverted to establishing new holding facilities and models much earlier than we had anticipated.

2 EXECUTIVE SUMMARY OF WORK CARRIED OUT IN THIS PERIOD

1. The project began, using tilapia and catfish as the models for the cryopreservation of finfish eggs. Experiments on cold tolerance, membrane permeability and estimates of unbound water showed that the volume and membrane structure of these eggs did not allow sufficient movement of water out or cryoprotectant into the egg to avoid intracellular freezing.

2. A change to invertebrate model species for egg cryopreservation was made because of the large numbers of relatively small ova they produce compared to finfish. The species chosen were the Pacific oyster and the brine shrimp because of their suitability, availability and economic importance in developing countries.

3. Attempts at the cryopreservation of these organisms resulted in the first reproducible and authenticated evidence for the frozen storage of embryos in these organisms.

4. This work showed the main block to the cryopreservation of eggs to be the perivitelline and to a lesser extent the chorionic egg membranes.

5. The *Artemia salina* work showed that the level of unbound water may not be as critical a factor as was first thought.

6. It was hoped that future studies on these successful models would enable us to assess techniques for overcoming membrane permeability. The enforced, earlier than expected, move to finfish species, without the development and assessment of the proposed membrane manipulations as well as the lower fecundity of the finfish models did delay subsequent progress.

7. Basic protocols for the management and husbandry of the new finfish models have been developed from scratch.

8. Having established the cold tolerance of the new species, the membrane permeability was assessed at differing developmental stages, after enzyme treatment, electroporation, and at differing cryoprotectant concentrations.

9. The rate of water and cryoprotectant uptake was assessed using the appropriate radiolabelled compound. One implication of this study was that all potentially successful treatments resulted in the egg being in contact with the unfrozen cryoprotectant for long periods (2-3h) which resulted in measurable toxicity effects.

10. No finfish eggs or embryos have yet been successfully frozen, although this work suggests several potentially successful future directions.

11. The milt from the carp, *Carassius auratus*, was successfully frozen.

12. Variability in freezing rates within the programmable tissue cooler and the importance of urine contamination in milt were identified as major factors in reducing the reproducibility of protocols.

13. Better techniques for assessing milt quality were developed using video analysis.

14. The development of improved activators should improve the fertility and therefore the yield from cryopreserved milt or from marginal samples.

15. The work of this project has been widely disseminated to other scientists through a series of publications, conference presentations, the taught MSc course, and training of overseas workers through the British Council.

2.1 Detailed Summary of Research Studies (In chronological order)

a) Studies were conducted into membrane permeability of unfertilized and water hardened tilapia eggs, storage of unfertilized tilapia eggs, subzero tolerance and cryopreservation of fish and shellfish embryos.

b) Cold tolerance of various embryonic stages of *Oreochromis niloticus* and *Clarias gariepinus* cooled at 0.3°C/min in dimethyl sulphoxide (DMSO) and methanol (MeOH) was investigated. At best pigmented embryos could tolerate being cooled to -10 to -20°C

c) Membrane permeability of unfertilized and fertilized tilapia eggs was determined using (radiolabelled) tritiated water ($^3H_20$). Unfertilized eggs had a higher rate of water uptake than water hardened eggs. In both cases, the dehydration was insufficient to prevent intracellular freezing, resulting in no post-thawed survival. Eggs remained viable when stored in tap water for up to four hours.

d) In total, 9 cryotrials were conducted using the shellfish, *Crassostrea gigas* as a model. Protocols used were reliable and results reproducible. The cryoprotectant DMSO at 1.0 and 1.5 M yielded the highest number of moving post-thawed oyster embryos. The use of embryos from larger eggs improved overall success by 15%. Post thawed embryos were kept for 5 days and survival in the controls and post-thawed groups was 1% (to be

published). Further studies to elucidate these findings were abandoned following the advice of the ODA review panel and efforts were made to divert resources to investigations on fish eggs and embryos.

e) Broodstock of new model species were obtained and established. Techniques for rearing, handling and artificial breeding for some of the new species were determined. Gametes and embryos were used in prefreezing cryoprotectant toxicity trials and permeability studies.

f) Six trials were initiated using *Artemia* cysts as models to evaluate the importance of intracellular water during freezing. *Artemia* could be successfully cryopreserved in the cysted and decapsulated forms and the high percentage of internal water present was not a limitation to successful cryopreservation. Up to 60 % of decapsulated and cysted embryos remained viable after cryopreservation even though they were fully hydrated prior to cryopreservation. The results from this model raises new fundamental questions on the freezing of eggs and embryos.

g) Three new tropical fish groups, *Barbus spp., Betta spp., Brachydanio spp.,* were selected as models and reared in the new ODA recirculation system. In vitro and natural spawning techniques were successfully developed for *Barbus* and *Brachydanio* model species. Up to 95% hatch rates were achieved in artificial stripping and incubation trials. The consistency of

spawning in some model species limited the rate with which results could be followed up.

h) Dechorionation of eggs and embryos depended on fish species, embryonic stage and concentration of enzyme. All the eggs could be successfully dechorionated. Trypsin concentration of 2.5 mg/l resulted in 100% dechorionation but was lethal to embryos at the blastula stage.

i) Data on the permeability of eggs are incomplete. Results to date suggest that the permeability or rate of uptake of water decreases with embryonic development and large differences exist between fish species. The uptake of cryoprotectant in the earlier embryonic stages was still insufficient to be successful. A window for the successful application of electroporation has now been identified.

j) The viability of embryos was affected by the cryoprotectant concentration, equilibration time and the mode of administration. The use of a step wise method of equilibration increased the viability of embryos subjected to high cryoprotectant concentration for more than an hour.

k) In view of the long contact time between cryoprotectant and gametes and embryos, a series of trials on prefreezing toxicity of cryoprotectants was initiated. Results to date suggest that the commonly used cryoprotectant DMSO, does have a denaturing effect on the activity of the enzyme Lactate

dehydrogenase (LDH) in intact embryos and that this effect is positively correlated with concentration. Therefore, in addition to freeze damage, the viability of the embryo may also be influenced by the toxicity of the cryoprotectant prior to freezing.

l) A multichannel temperature recorder was developed for monitoring cooling profiles within freezing containers such as straws and cryovials during cooling. Preliminary results to date show a high degree of variation. The implication of these results on the reproducibility of protocols needs testing. The data logger will be used to develop a reliable field based cryopreservation protocol.

m) Urine contamination can significantly affect the chemical components of the seminal plasma and reduce the post collection activation of the milt.

n) Carp milt was successfully cryopreserved. Higher post-thaw motility scores were obtained with MeOH as the cryoprotectant than with DMSO. With the best protocol, between 50-60% of the post-thawed spermatozoa were motile upon activation and motility was improved by using sodium citrate as the extender. These findings will form the basis for future work on other carp species.

o) The duration of motility of activated *O. niloticus* spermatozoa can be manipulated. The time for sperm motility in the sample (1:200 dilution) to drop to 10% of the original activation of unfrozen milt was increased 45 fold from 3 to 130 minutes by adding 2% sodium citrate to the extender. Similarly the duration of motility of post-thaw tilapia spermatozoa was increased about forty fold from 2 to over 75 minutes.

p) Studies on the activation of demembranated sperm cells were inconclusive. Spermatozoa could be successfully demembranated and could be stimulated to vibrate by means of an external energy source such as ATP and cAMP, but, in contrast to published studies on salmonids no progressive movement was observed.

3 RESULTS AND FINDINGS

3.1 Background

Previous studies funded by ODA on the cryopreservation of tilapia spermatozoa resulted in the successful development of protocols for 10 different species of tilapia (R4151). The current project (R4523) which is an extension of the original project aims to understand the various parameters for the successful cryopreservation of fish eggs and embryos by investigating a range of aquatic models and to develop, improve, expand and understand the observed variability of post-thawed milt and to develop cryopreservation protocols for carps.

Mammalian eggs and embryos are very small (< 100 um), lack any significant yolk reserves and in the last 5 years have proved relatively easy to freeze. In contrast all attempts to date to cryopreserve fish eggs have been unsuccessful. The major obstacles are thought to be associated with the relatively large volume, yolk reserves and impermeable chorionic and vitelline membranes found in many fish eggs.

The lack of any significant advance to date in the sub-zero storage of fish eggs and embryos may be a reflection of the species used. The majority of the work having been on the commercially important salmonid species. The eggs from these species have therefore tended to exacerbate the problem as they are all

characterized by having large egg and yolk volumes and well developed membrane structures. New fish and shellfish models with more tractable characteristics were needed to investigate the cryobiology and cryosurvival of eggs. These new model species required the development of suitable holding facilities and in vitro techniques of gamete procurement and rearing.

Attempts to freeze tilapia and carp eggs (2-3 mm) came up against similar problems of egg volume as those associated with salmonids. A new model species which produces large number of of small eggs and embryos was needed.The commercially important oyster, *Crassostrea gigas,* was chosen as it can produces many millions of 30-40 µm diameter eggs and 60-100 µm long larvae and was readily available from local sources. Successful protocols for freezing larvae were developed, but, the work had to be stopped as the ODA review committee considered the work to be irrelevant and the remainder of the project time should be devoted to fish eggs and embryos.

Studies on the cryopreservation of spermatozoa focused on attempts to develop and improve cryopreservation protocols for carp. It was intended that Indian and Chinese carp broodstock from the BAFRU unit would be available but unfortunately this was delayed and so another related carp, *Carassius auratus* , was used as a model to develop a protocol and identify some of the likely problems associated with carp milt activation and cryopreservation.

One objective of the project was to reduce the variability in success observed in

milt cryopreservation protocols developed for the various species and to re-examine the relevant components of the cryopreservation procedure. The importance of urine as a contaminant and the variability of cooling rates in straws within the programmable cooling chamber were investigated as two possible sources of variation. The possibility of extending the duration of sperm motility was also investigated.

Cryopreservation protocols were modified for the freezing of milt of special importance. The milt from sex-reversed females or UV irradiated milt used in genetic manipulation experiments is now of great importance in aquaculture and research. Techniques were developed for the extraction of milt from the testes dissected from unstrippable fish species.

3.2 Studies on Aquatic Eggs and Embryos

3.2:1 Invertebrates (30-400μm)

3.2.1:1 Cryopreservation of Oyster eggs and embryos : A model species with a small egg volume.

Initial studies focused on developing basic husbandry techniques and reviewing the embryonic stages of oysters. A total of 9 cryopreservation trials were conducted. Five development stages, three cooling rates, three cryoprotectants at two concentrations and two thawing temperatures were evaluated (Table 1).

Samples were extended in fish Ringers in 0.5 ml straws and held under liquid nitrogen.

Table 1 Variables investigated during oyster cryopreservation trials

a) Five developmental stages:	unfertilized eggs fertilized eggs , 4 - 8 cell embryos (2.5-3.0 h) early blastula (5h) trochophore (7h)
b) Three cryoprotectants at two concentrations	DMSO, MeOH and glycerol (1.0 and 1.5M)
c) Three cooling rates	0.5, 1.0 and 2.0°C / min
d) Two thawing temperatures	15°C & 40°C (water bath).

Samples incubated in 30mm Petri dishes containing 26% SW. Survival was monitored after 6 h.

The summary results of the initial cryopreservation trials are given in Table 2 .

Table 2 Summary [1] of cryosurvival of oyster embryos

Embryonic stage	Cryo	Conc (M)	Cooling rate(°C/min)	Thawing regime	Recovery[2] Score
Blastula	DMSO	1.0	0.5	15	2-3
	DMSO	1.0	0.5	40	3-4
Trochophore	DMSO	1.0	0.5	15	1-2
	DMSO	1.0	0.5	40	3
	DMSO	1.5	0.5	15	4-5
	DMSO	1.5	0.5	40	5
	MeOH	1.5	1.0	15	1-2
	MeOH	1.5	1.0	40	1
	Glycerol	1.5	1.0	15	1-2
	Glycerol	1.5	1.0	40	2-3
	DMSO	1.0	2.0	15	1-2
	DMSO	1.0	2.0	40	1
	DMSO	1.5	1.0	15	1
	Glycerol	1.0	2.0	15	5
	Glycerol	1.0	2.0	40	4
	DMSO	1.5	2.0	15	3
	DMSO	1.5	2.0	40	4
	Glycerol	1.5	2.0	15	5
	Glycerol	1.5	2.0	15	3

1) All other treatments were unsuccessful
2) Score based on 0 being no recovery and 10 total recovery

In the second trial swimming oyster larvae were recovered in 80% of the samples thawed (n= 32). In all trials DMSO at either 1.0 or 1.5 M gave consistently better post-thaw viability than glycerol and good recovery was obtained for the more advanced D-larvae (Tables 3 & 4).

Table 3. Effect of cooling rates, cryoprotectants and concentration on mean[1] post-thaw recovery (%) of trochophore oyster larvae

CRYOPROTECTANT		COOLING RATE (°C/min)		
Type	Conc(M)	0.5	1.0	2.0
METHANOL	1.0	0	15 (3)	0
	1.5	0	15 (9)	0
GLYCEROL	1.0	0	15 (6)	45 (7)
	1.5	0	25 (7)	40 (14)
DMSO	1.0	12.5 (3.5)	10	35 (7)
	1.5	45 (7)	10	35 (7)

1 Means (x+sd) based on two replicates

Table 4. Effect of cryoprotectant its concentration and larval stages of oysters on mean[1] post-thaw recovery (%)

CRYOPROTECTANT		LARVAL STAGE[1] (x+sd)	
Type	Conc(M)	Trochophore	D-Larvae
GLYCEROL	1.0	26 (6)	26 (9)
	1.5	35 (4)	21 (11)
DMSO	1.0	63 (6)	49 (11)
	1.5	52 (10)	56 (13)

1 Means based on three replicates

During these trials post-thaw D-larvae were kept alive for 5 days and no difference between the survival rates of the controls and frozen treatments were

observed, both were about 1.0%. Technical problems associated with the laboratory culture of shellfish resulted in very low survivals for both cryopreserved and unfrozen controls.

Cryotrials on gamete quality have suggested that embryos from larger eggs (> 25 µm sieve) were more uniform than those from small eggs (< 25µm sieve). Overall, post-thaw viability was increased by 15% by the use of the larger embryos.

Post-thaw fertilized and unfertilized eggs ruptured on thawing. In the post-thawed trochophores samples most larvae developed to the D-stage, but, many of these were abnormal, showing gaping shells and non-directional movement. The normal larvae were able to close their shells and change direction and there was evidence of ingested food in their guts.

This study is the first to show the successful and reproducible cryopreservation of any commercially important aquatic organism. Further development of this system would have resulted in methods for the freezing of large quantities of oyster larvae for both commercial and scientific purposes.

3.2.1:2 Cryopreservation of cysted and decapsulated *Artemia* embryos : A model to assess the importance of intracellular water.

Artemia embryos are able to lie dormant and survive long periods in the

dehydrated state (5-10% moisture) in an encysted form (200-400µm). In this form the embryos are composed of a partial syncytium of about 4000 nuclei (gastrula stage) surrounded by complex shells (7-8 µm thick). The dehydrated cysts need to be hydrated in brackish water for successful hatching. In addition, the dormant encysted embryos can be put through a decysting process (termed decapsulation) by immersing the embryos in a hypochlorite solution which oxidises and dissolves the outer alveolar (chorion) layer. The denuded embryos can also be rehydrated in a controlled manner and this makes them a useful model for assessing the effects of intracellular water within the embryo on cryosurvival. This would enable us to evaluate the effect of intracellular water on the successful cryopreservation of larger eggs and embryos.

Two trials were initiated.

1. Effect of freezing conditions on the viability of post- thawed decapsulated dehydrated and rehydrated *Artemia* embryos.

Dehydrated and fully hydrated cysted and decapsulated *Artemia* were suspended in fish Ringer containing 1M MeOH, DMSO or glycerol and stored in 1.5ml vials. All samples were cooled and stored for 3 weeks in -20 and -70°C freezers. In addition, samples in cryovials were plunged and stored in liquid nitrogen. Samples were thawed to room temperature, rinsed three times with 12% sea water and incubated alongside unfrozen controls from the same *Artemia* stock until hatching. The hatch rates were noted in all treatments.

2. Effects of controlled rehydration on the viability of post-thawed cysted and decapsulated *Artemia* embryos cooled at a slow rate of 2°C/min.

One of the major factors inhibiting successful cryopreservation of fish eggs and embryos is considered to be the relatively large amounts of unbound water found in fish eggs and embryos. If the amount of unbound water within a egg is critical for successful cryopreservation then this can be conveniently evaluated by freezing *Artemia* embryos which have been rehydrated to a known level prior to cryopreservation. In addition, since the relatively thick outer water permeable cyst can be chemically removed it's importance in cryosurvival can be evaluated.

To test the above hypothesis, cysted and decapsulated *Artemia* were rehydrated in fresh water for up to 70 min. Following the results of these trials a second series of experiments were initiated in which the rehydration time was extended to 180 min to ensure complete hydration.

Tritiated water was used to determine the rate of uptake of water after varying periods of rehydration in the cysted and decapsulated *Artemia*. The *Artemia* in the trials were then digested in Soluene and a scintillation fluid was then added so the amount of radiolabelled uptake could be measured in a scintillation counter.

Results for experiment 1.

Results suggested that only dehydrated decapsulated *Artemia* embryos cooled to -20 and -70°C in freezers and plunged in liquid nitrogen could be successfully stored. Measured cooling profiles in the freezer samples showed evidence of latent heat of crystallization indicating that samples were not just undercooled but were completely frozen.

In the -20°C freezer samples cooled at approximately -14°C/min prior to freezing and -26°C/min after ice crystal formation. In the -70°C freezer, samples cooled at -45°C/min and -16°C/min, respectively. Plunging samples directly into liquid nitrogen resulted in cooling rates of approximately 2800°C/min.

Despite the wide cooling rates tested decapsulated dehydrated *Artemia* embryos were able to survive the freezing procedure when suspended in a saline solution containing 1M glycerol, DMSO and MeOH (Fig. 1). Post-thawed embryos were allowed to develop to either the prehatched eyed stage or to hatch. In all except one of the above cooling protocols the survival rates were between 60-100 % of control values (Fig. 1). No survival was observed in any of the hydrated embryos. These results suggest the internal water must be reduced for successful cryopreservation when rapid cooling rates (plunging into liquid nitrogen) are applied and that *Artemia* containing 5-20% moisture can tolerate internal freezing.

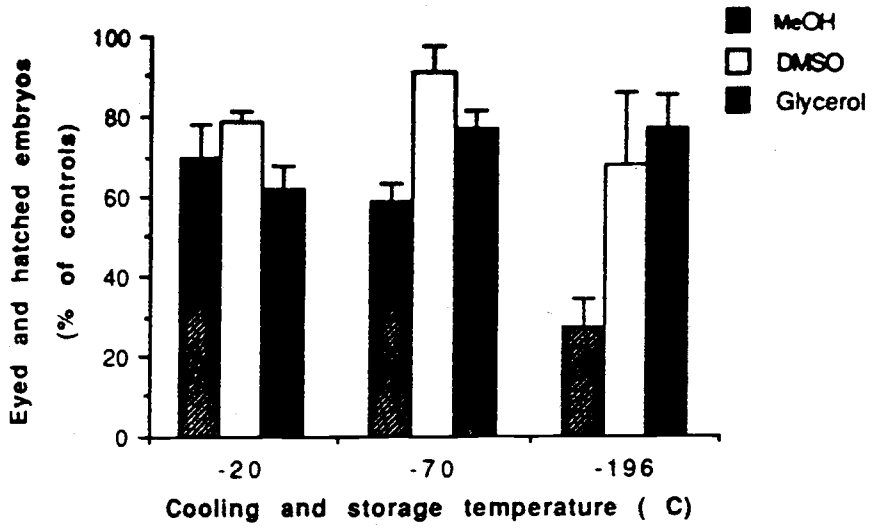

Figure 1 Effect of cooling temperatures and cryoprotectants on the viability of *Artemia* Embryos.

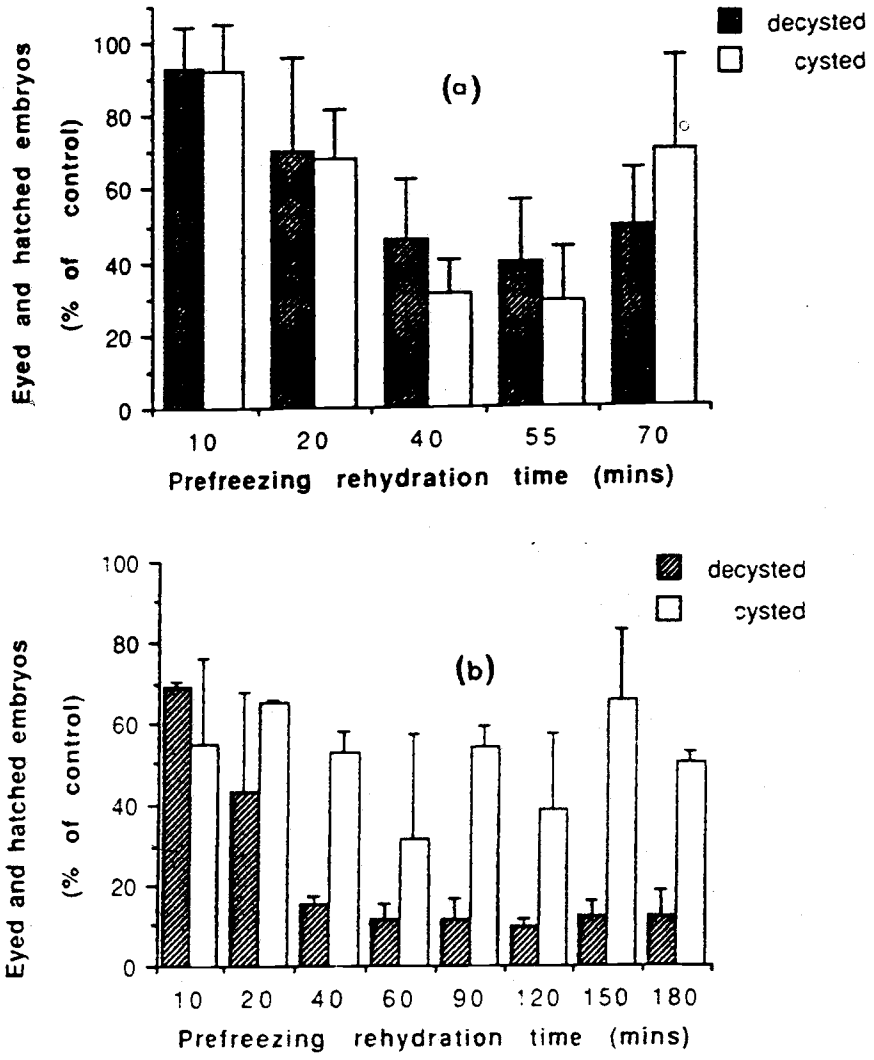

Figure 2 Effect of rehydration times on the viability of *Atremia* Embryos. Hydration up to a) 70 and b) 180 min.

Results for experiment 2

In the second set of experiments, decapsulated and cysted *Artemia* embryos were rehydrated for up to 70 minutes and were then cooled slowly at -2°C/min in the programmable cooler. Cysted and decapsulated *Artemia* embryos were able to survive slow freezing despite being fully hydrated (Fig. 2). Rehydration of up to 20 minutes did not appear to affect the viability of post-thaw cysted and decapsulated embryos and over 70 % of embryos developed to either the eyed or hatching stage (Fig. 2a). To ensure that both types of embryos had been fully rehydrated the rehydration period was increased to 180 minutes and the experiment repeated. The results for the extended period of rehydration were similar to the previous series (Fig. 2b). The viability of cysted embryos rehydrated for up to 180 min was about 50%. Viability of the decapsulated embryos was low but consistent for rehydration times in excess of 20 minutes. The generally poorer results for the decapsulated embryos in both sets of trials may be a result of the decysting procedure reducing overall viability.

One possibility that was investigated was that if the cysts could readily rehydrate they may also readily dehydrate. During ice crystal formation water is frozen out of the freezing medium thus increasing its osmotic pressure. The increase in osmotic pressure may result in the rapid dehydration of cysts prior to freezing which could be readily viewed as the asymmetrical contraction of the cyst. The cooling protocol used to cryopreserve embryos was repeated on the cryomicroscope cooling stage. No measurements were taken but it was clear that there was no noticeable change in cyst shape or volume during the freezing or

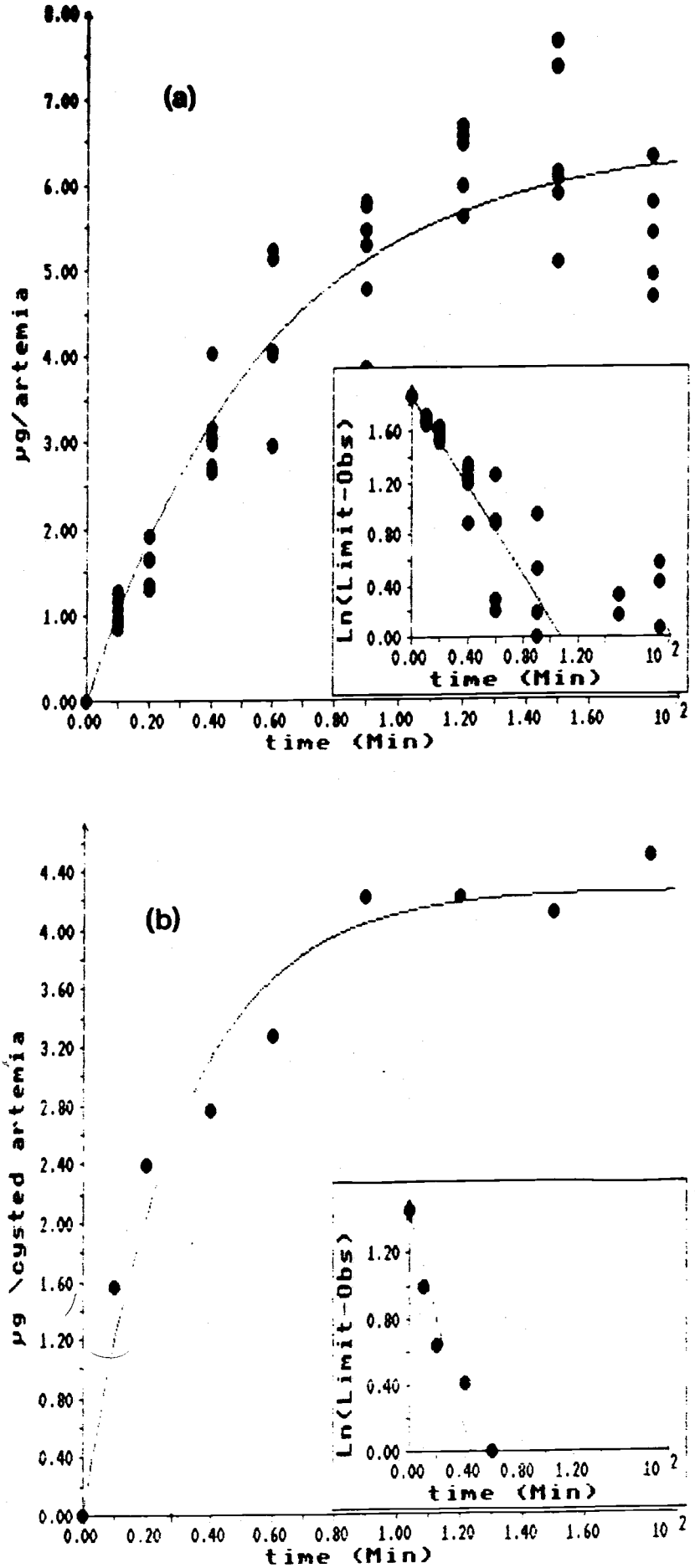

Figure 3 Uptake of tritiated water by Artemia embryos. a) Decapsulated and b) Cysted embryos

rapid thawing process, suggesting that there was no significant dehydration during freezing.

Radio labelled uptake studies using tritiated water (Fig. 3) showed that rate of hydration in the cysted and decapsulated *Artemia* embryos was rapid and that the decapsulated embryos reached equilibrium at 6.4ul ^3H_2O/embryo (Fig. 3a) compared with 4.6 ul ^3H_2O/embryo (Fig. 3b) for the cysted embryos. The cysted embryos reached 50% of maximum rehydration about 30 min and 100% within 40 min. In the decapsulated embryos these values were longer at 40 and 80 min respectively. The reasons for these higher values are unclear at present. Overall, these studies suggest that even though the embryos contain in excess of 45% moisture they can be successfully frozen and recovered from liquid nitrogen. This observation is contrary to current opinion that maximal dehydration is a prerequisite for successful cryopreservation. Clearly other factors are implicated and these will require further studies. It is known that the trehalose (a sugar) content of *Artemia* cysts is high (15% dry body weight) and may offer some protection during dehydration but its role in cryoprotection is not clearly understood.

3.2.2 Fish Eggs and Embryos (1-3mm)

This work was split into two distinct periods. The first three sections were initial studies using tilapia and catfish which showed that the eggs of these species were not suitable models for trying to elucidate the critical parameters for the successful

TABLE 5 **Effect of sub-zero temperatures on the of 48hr old O. niloticus embryos cooled in fish Ringer.**

Cryoprotectant 12.5%	Temperature (°)	Survival in thawed sample (%)	Comments
DMSO	- 4	100	Fluid not frozen
	-10	86	Fluid partially frozen
	-20	11	Fluid frozen
	-30	0	Fluid forzen
	-60	0	Fluid frozen
MeOH	- 4	100	Fluid not frozen
	-10	88	Fluid partially frozen
	-20	12	Fluid frozen
	-30	0	Fluid frozen
	-60	0	Fluid frozen

1 cooling protocol: 1°C min to - 4°C hold 2 min
0.3°C min to -10°C hold 2 min
0.3°C min to -20°C hold 2 min
0.3°C min to -30°C hold 2 min
0.3°C min to -60°C hold 2 min

TABLE 6 **Effect of sub-zero temperatures on the viability of catfish (Clarias gariepinus) embryos[2] suspended in fish Ringer.**

Cryoprotectant	Temperature	% Survival	Comments
DMSO	- 4	100	Fluid not forzen
	-10	93	Fluid partially frozen
	-20	0	Fluid frozen
	-30	0	Fluid frozen
	-60	0	Fluid frozen
MeOH	- 4	85	Fluid not frozen
	-10	92	Fluid partially frozen
	-20	0	Fluid frozen
	-30	0	Fluid frozen
	-60	0	Fluid frozen

1 Cooling protocol used as in Table5

2 No survival of 16 cell embryos in all treatments.

cryopreservation of the eggs of aquatic organisms. Because of this the main thrust of the research was then redirected to the study of the smaller invertebrate egg models. The other sections on the finfish began after the invertebrate work was abandoned.

3.2.2:1 Low temperature tolerance (egg diameters, 1-3mm)

Initial freezing trials were set up using two commercially important tropical fish species *Oreochromis niloticus* and *Clarias gariepinus* to assess two different cryoprotectants and a range of cooling protocols on the survival of eggs at a number of different developmental stages.

The chill tolerance of *O. niloticus* (egg diameter, 2-3mm) and *C. gariepinus* (egg diameter, 1-1.5mm) eggs was investigated initially . Gametes were manually stripped and incubated to the appropriate embryonic stages. Ten samples of *O. niloticus* embryos and 40 of *C. gariepinus* each containing 20 embryos were placed in Petri dishes (30mm) containing 12.5% solutions of either DMSO or MeOH. All samples were cooled according to the protocols given in Tables 5 and 6.

At each temperature hold (Table 5 & 6) in the cooling programme, duplicate samples were removed, thawed in a 38°C water bath, rinsed and incubated overnight and the survival noted. In both species the earlier embryonic stages

(cleavage and blastula) were found to be more susceptible to chill and freeze damage. Eyed embryos could survive down to -10 to -20°C for a maximum of an hour. At this temperature, however, the extenders were only partially frozen (Table 5 & 6). At lower temperatures internal freezing resulted in total destruction of eggs and embryos. Using similar protocols on the stage of the cryomicroscope confirmed internal freezing seen as " flashing" (Intra-egg blacking due to ice crystals) occurred at -18°C.

In view of these results it was clear that the eggs had either not taken up sufficient cryoprotectant or had not been sufficiently dehydrated to avoid intracellular freezing which had resulted in the poor survival of the frozen embryos. Experiments which looked at the permeability of egg and embryos membranes were instigated.

3.2.2:2 Membrane permeability at differing developmental stages

Membrane permeability of *O. niloticus* eggs was evaluated. The permeability of the egg chorion membrane may undergo a change after water hardening. Any significant difference in the uptake of water would suggest a mechanism by which we could increase the uptake of cryoprotectant.

Unfertilized and water hardened eggs were incubated at 25°C in tritiated water (^3H_2O) for 0.25, 0.50. 0.75, 1.0. 2.0. 3.0 and 4.0 hours. The uptake

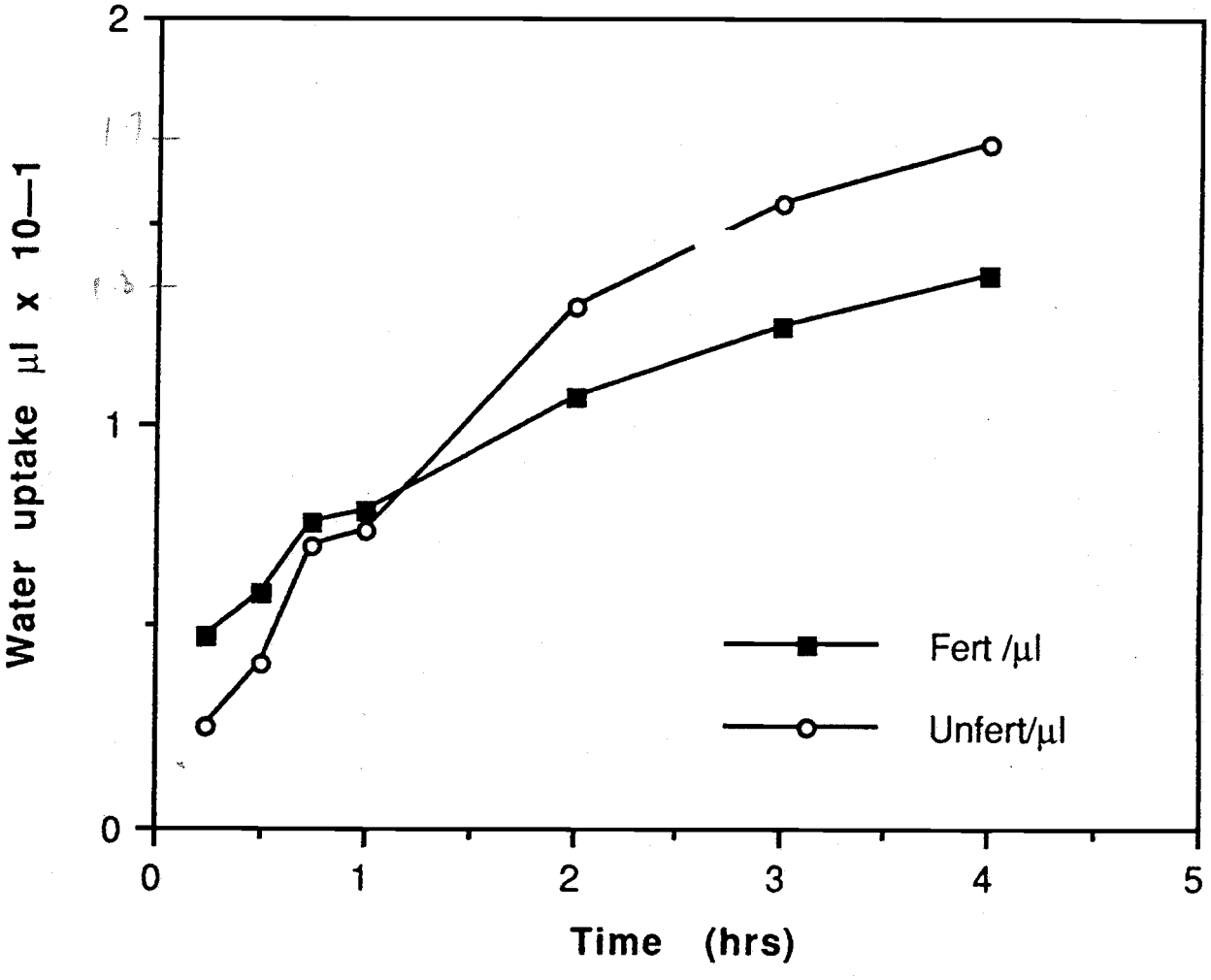

Figure 4 Water uptake of unfertilized and fertilized O. niloticus eggs

of tritiated water was measured using a scintillation counter.

The summary data on the uptake of tritiated water by fertilized and water hardened eggs are given in Figure 4. The amount of tritiated water gave an estimate of water uptake .

These studies showed that the permeability of tilapia egg membranes does change with embryonic development and that unfertilized eggs have a higher rate of water uptake than water hardened ones. The uptake of water occurs at two different rates, an initial rapid phase followed by a progressively slower rate of absorption. The higher permeability of the egg membranes in the earlier embryonic stages was still insufficient for the necessary uptake of cryoprotectant and the simultaneous dehydration of the eggs required for cyopresevation. Use of unfertilized *O. niloticus* eggs in cryopreservation trials resulted in no post-thaw survival. In view of the large volume and the composition of the egg yolk the proportion of osmotically active water may be low. An estimate of the amount of bound or osmotically inactive water would be important to establish the maximum possible dehydration that may be possible.

3.2.2:3 Measurement of osmotically inactive or bound water

The classical method of estimating the level of bound water involves the dehydration (removal of osmotically active water) of eggs and embryos in a range

of varying osmotic solutions and measuring the change in relative volume. The proportion of bound water is then extrapolated from a van Hoff plot. This method is based on the assumption that the egg and yolk are spheres and contracts uniformly thereby enabling volume changes based on a sphere to be calculated. When such studies were conducted on fish and *Artemia* embryos it was observed that the egg and yolk volume did not dehydrate uniformly and it was impossible to accurately calculate the volume changes. Therefore, new methods will have to be developed, validated and then used to calculate the % of osmotically inactive water. Similar recent studies on abalone eggs (which contract uniformly) conducted elsewhere suggest the proportion of osmotically inactive water in this species is three fold higher (0.54) when compared with mammalian (0.18) eggs. Whether this is a general phenomenon of aquatic species eggs needs to be determined.

Different methods will need to be explored. Changes in ratios of non-permeable ^{14}C: $^{3}H_2O$ in the extracellular medium, the use of Nuclear Magnetic Resonance (NMR) and the use of confocal microscopy could be developed to determine the amount of water in the gametes. The facilities mentioned are not available at Institute of Aquaculture ,if such studies are to be undertaken they will need to be in collaboration with other laboratories.

3.2.2:4 Development of in vitro culture techniques for fish eggs and embryos of the new model species.

Many fish species release their eggs into the environment in small batches over a period of time and hence the rate of development among the embryos of these different batches varies. Therefore, to obtain large numbers of embryos in a similar stage of embryonic development, our objective, at the beginning of these studies, was to develop artificial techniques to obtain spawns containing synchronised embryos. The species *Barbus conchonius* and *Brachydanio rerio* which produces small eggs (egg diameter, 0.7-1.0mm) were investigated. Techniques for anaesthetization, stripping, gamete fertilization and artificial incubation of the larvae had to be developed before any cryobiological aspects of gametes and embryos could be investigated.

3.2.2:5 Procurement of gametes and embryos

The broodstock of *B. conchonius*, and *B. rerio* were purchased from a local aquarist supplier and held at the Tropical Aquarium, Institute of Aquaculture, University of Stirling. Males and females were held in separate plastic tanks.

To obtain embryos from natural spawning, a mature female ready for spawning (distended belly) and three males were transferred to a spawning tank with grille plates to allow fertilized eggs to fall through and avoid being eaten by the adults. The fishes were left overnight and spawning usually took place the following

morning. Spawning times were noted and embryos were then removed and incubated in sterile Petri dishes and development and hatch rates noted.

To obtain embryos by artificial fertilization, males were anaesthetized in 0.5 ml benzocaine , rinsed in water and held upside down against the lip of a Petri dish. The fish were gently squeezed and the milt collected in 5ul microtubes which were then emptied into a 1.5 ml Eppendorf vial containing 0.5ml of a suitable extender.

Non-anaesthetized females were squeezed directly into salt solutions and milt pre-tested for motility was added to the eggs and activated with water. The eggs were then left for 5 minute before they were incubated in sterile Petri dishes.

Artificial stripping, fertilization and incubation resulted in up to 95 % hatch rates. Low success was noted in females with overripe eggs and the supply and quality of eggs depended on timing of stripping of eggs

3.2.2:6 Dechorionation of eggs and embryos

The presence of the chorion around fish eggs, constitutes a formidable barrier to permeation of cryoprotectant and removal of water from their eggs and embryos. A comparison between the rate of uptake of dechorionated and normal eggs will give us an insight into the relative importance of chorionic and perivitelline

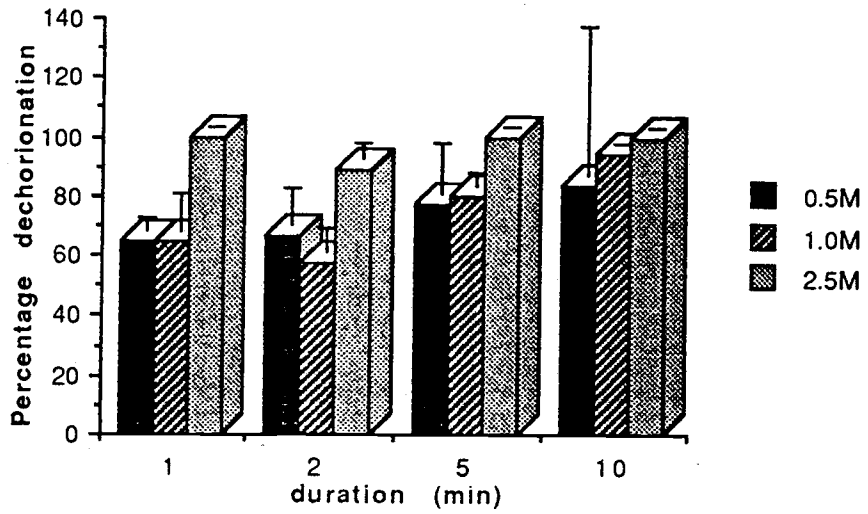

Fig.5(a) Effect of trypsin concentration on the dechorionation of *Brachydanio rerio* (cleavage stage), at 30 C.

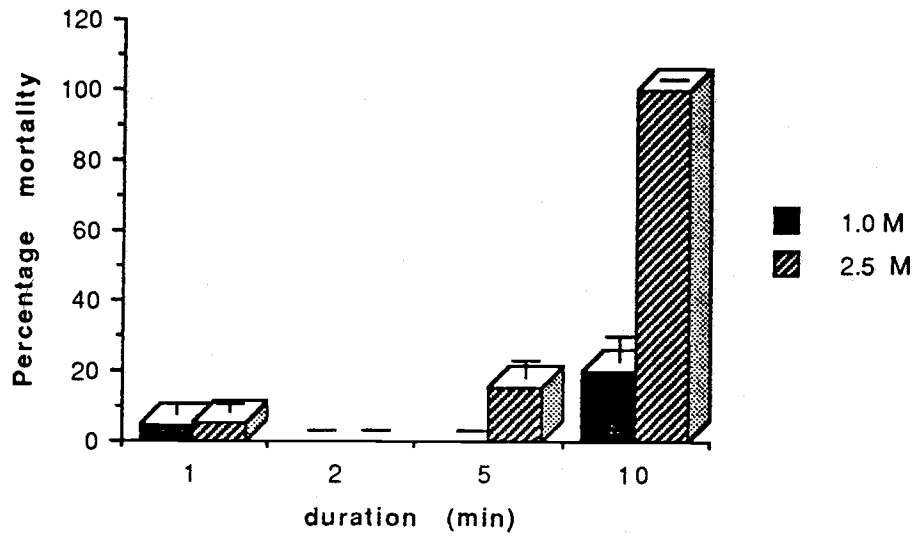

Fig.5(b) Effect of trypsin concentration on the viability of *Brachydanio rerio* embryos (cleavage stage).

membranes in the movement of water and molecules.

In the present study enzymatic digestion (Wallerman et al., 1988) has been favoured over mechanical methods of dechorionation (Harvey, 1983), because of its ease of application and its ability to produce a large number of denuded embryos.

Trypsin was used to digest the chorion of fish embryos adopting the technique used by Wallerman et al.(1988). The different embryonic stages were equilibrated in enzyme solutions (0.5, 1.0 and 2.5mg/l) and changes in chorion structure were monitored under a dissecting microscope. When the chorion began to peel off, the enzyme digestion was stopped by adding 5% foetal calf serum. Embryos were washed several times and then incubated in Holt's solution. Development and hatch rates were recorded.

The success of enzyme digestion of the chorion depended on the species and embryonic stage. In general embryos later than the blastula stage were more difficult to dechorionate. Although 10 min digestion gave the greatest percentage dechorionation (Fig. 5a) the embryos did not survive the digestion process (Fig. 5b).

3.2.2:7 Determination of the permeability of egg membranes to water

All the studies on the permeability of egg barriers using radio labelled water

(^3H_2O) and cryoprotectants were delayed by 2-3 months. This had arisen because the legal permissable amount for the tritiated products within the IOA was exceeded and no new labelled compounds could be purchased until this limit was increased. A new licence for the increased amounts of radiolabelled chemicals was delayed due an administrative error outside our control and consequently studies requiring tritiated water and cryoprotectants were postponed.

An added difficulty with the new fish model species was that they only produce a few hundred eggs/spawn and several different trials have to be conducted to obtain the required replicates and embryonic stages compared with the invertebrate models.

General procedure

About 50 embryos of known developmental stage were placed into 4 sealable specimen jars containing filtered and autoclaved aquarium water. A sample of 5 embryos was removed from each jar with a bulb pipette and drained in a plastic tea strainer and blotted on tissue paper to remove excess water. These eggs were then transferred into scintillation vials (Packard), containing 0.5 ml Soluene (Packard). These samples served as untreated controls.

Fifty microlitres of tritiated water were then added to each jar. The jars were then placed in a water bath set at 27°C. The ova were then sampled after 5, 15, 30, 45, 60, 180, 240, min. as described above. The ova were washed three times in fish

Ringers, before they were transferred to the scintillation vials. At each sampling time, a standard volume (25µl) of radiolabelled water was removed from each treatment so the radioactivity could be monitored throughout the experiment. At the end of the treatment, the ova were kept in the dark for 12 hours before 4.5 ml of scintillation fluid (Packard) was added to each vial and the radiation monitored in a scintillation counter (Packard, 2000). The permeability coefficient, the rate of uptake (K) and the limit (Q) were determined using the Enzfitter software package, and ANOVA was used to assess difference between the treatments.

Data on the permeability of the full range of embryonic stages are incomplete. To date, the results suggest that the permeability and rate of uptake of water decreases with embryonic development and that there appear to be species differences (Table 7).

Table 7 Permeability *Oreochromis niloticus* and *Brachydanio rerio* embryos acclimated at 27°C in tritiated water

Fish spp.	Embryonic stage	Limit(ug)	K(rate of uptake)	Pdiff µm/sec
a	Zygote	2250±51	0.03±1.6	$3.0x10^{-4}$
a	4-cell	2251±40	0.04±4.0	$2.2x10^{-4}$
b	High blastula	247.3±20	0.048	$2.5x10^{-4}$
b	Flat blastula	270±20	0.011	$6.5x10^{-4}$
b	late blastula	159±13	0.029	$1.0x10^{-4}$
b	1/2 epiboly	228.4±10	0.057	$2.8x10^{-4}$

N.B. a= *Oreochromis niloticus* b= *Brachydanio rerio*

Published data on other species suggest that the chorion is in fact relatively permeable but the permeability of the perivitelline membrane is very low and it is this that may need to be investigated more closely.

The effect of the chorionic and the perivitelline membranes on permeability was investigated. The aim of this study was to determine the effect of the presence or absence of the chorion on the permeation of ^3H_2O. In this experiment intact and dechorionated embryos were subjected to the permeability study procedure given above. These studies were abandoned after three attempts. The denuded embryos were extremely fragile and the washing procedure required prior to counting could not be performed. Removing the embryos through the water meniscus was sufficient to rupture the embryos. A new approach would need to be considered.

The above trials are incomplete but if this programme is extended we plan to investigate if higher equilibration temperatures can be exploited to increase the permeability of egg barriers.

3.2.2:8 Effect of electroporation on the viability and permeability of eggs and embryos

The principles of electroporation:

When a current is discharged through a conducting medium containing cells, the

high voltage causes the cell membranes to become porous for the duration of the charge. This process known as electroporation is used to introduce macromolecules into eukaryotic cells suspended in buffered saline or culture media.

Electroporation, is examined in the present study, to manipulate the perivitelline membrane of the fish embryo to see whether it will enhance the permeability of ^3H_2O and cryoprotectants. Since no information was available on the use of this technique on fish eggs initial studies had to be conducted to determine the viability of eggs and embryos subjected to electroporation.

The effect of electroporation on the survival of the embryos

Twenty embryos were placed in 0.4ml electroporation cuvettes and filled to the desired level with 2M DMSO in fish Ringers. The gene pulser was set at voltages ranging between 0.1-0.2 Kv at a capacitance range of 0 to 125 uF. The embryos were shocked for 0.1 msec and then incubated as described above. The viability was determined and percentage survival was related to control. A window for successful application was identified at a voltage of 0.2Kv and a capacitance of up to 0.25uF (Table 8).

Table 8. Effect of electroporation on the viability of *Barbus conchonius* embryos (blastula)

SAMPLE NO.	VOLTAGE (KV)	CAPACITANCE (μF)	SURVIVAL(%) X
1	0	0	100
2	0.1	0.25	100
3	0.15	0.25	100
4	0.20	0.25	74.5(2.5)

The studies on the effect of electroporation on the uptake of radiolabelled water and cryoprotectants into fish embryos could not be started because of a delay in licensing the laboratory. This approach shows some promise and should be continued.

3.2.2:9 Prefreezing toxicity of cryoprotectants to fish embryos

The use of slow cooling rates (0.3°C/min) which appear to be necessary from the *Artemia* work will result in an increased contact time between eggs and the cryoprotectants during cryopreservation procedures. This in itself may be toxic to eggs and embryo and reduce survival. Therefore, to separate the confounding effects of cryoprotectant toxicity and freezing damage it was thought important to initiate studies on the prefreezing toxicity of cryoprotectants to eggs and embryos. Two different protocols were used to assess the effects of the prefreezing toxicity of DMSO on the viability of fish embryos.

1. One step addition and removal of cryprotectant.

2. Stepwise addition and removal of cryoprotectant.

Objectives:

To determine the effect of osmotic stress caused by the mode of equilibration and removal of the cryprotectant.

To examine whether the cryoprotectant induced injury has any measurable effect on the biochemical nature of the fish embryos.

1) One step addition and removal of cryoprotectant

Twenty fish eggs at various developmental stages were immersed for varying periods of time (15, 30, 60, 120, and 180 min) in various concentrations of cryoprotectant (0.5 - 3.0 M in 0,5 M steps). After each equilibration period samples were simultaneously removed, washed three times in Ringers solution before being finally incubated in vials containing autoclaved water. The water was changed every day until the embryos hatched. Percentage hatch was related to the control.

2) Stepwise addition and removal of cryoprotectant

In this procedure, the embryos were equilibrated in a given concentration of cryoprotectant for equal periods until the final desired concentration was reached

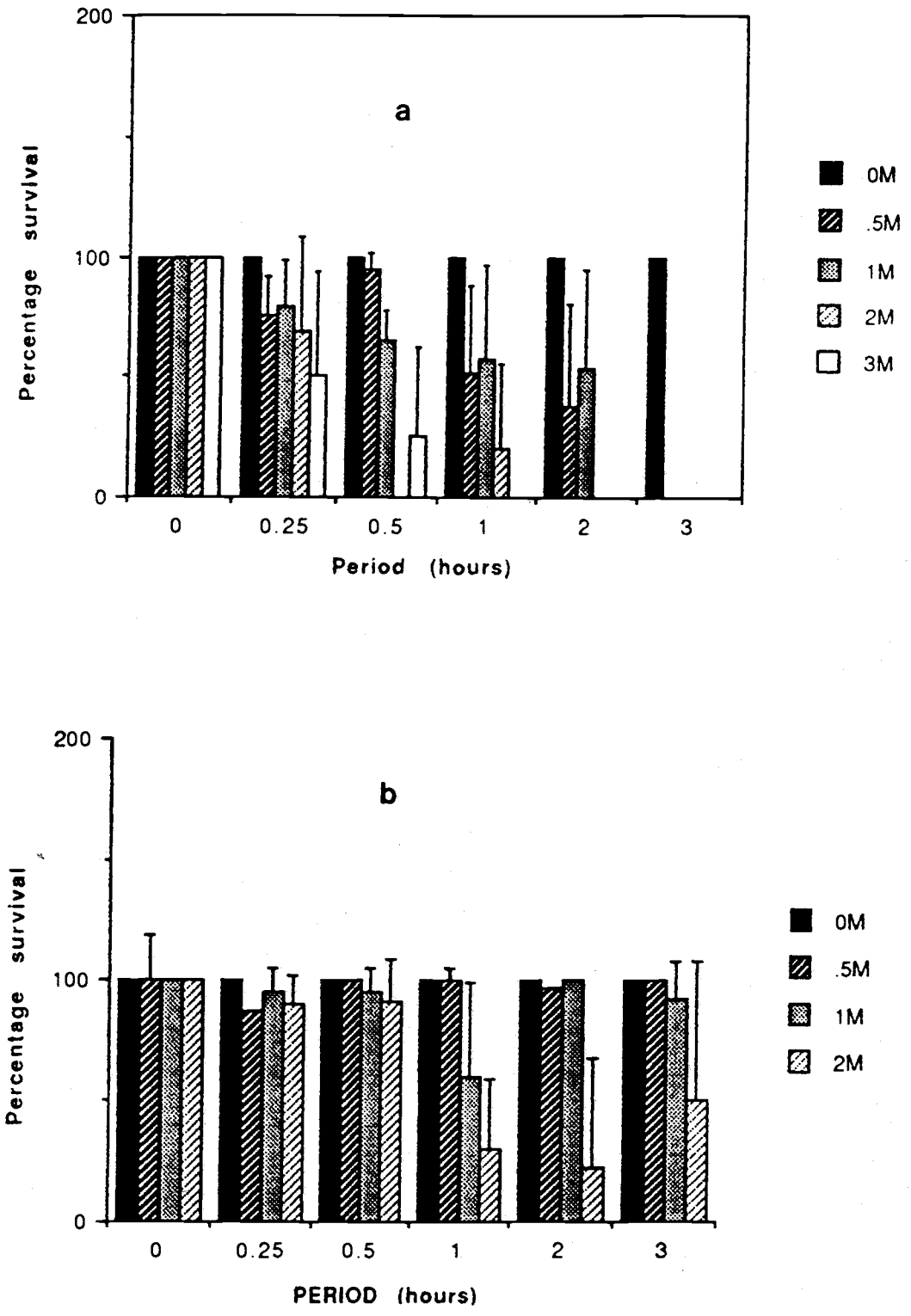

Figure 6 Effect of equilibration time and DMSO concentration on the viability of blastula stage *Brachydanio rerio* embryos. a) One step addition and removal technique and b) Several steps addition and removal technique

(eg. the sample to be equilibrated in one molar cryoprotectant for one hour, was incubated for 15 min intervals in 25% increments). The cryoprotectant was gradually diluted in the same manner by adding 2ml of Ringer solution every 15 min, for one hour, before being finally incubated in autoclaved and UV irradiated aquarium water. The hatching and abnormality rates were determined.

Results

The viability of embryos was affected by the cryoprotectant concentration, equilibration time and the mode of administration (Fig. 6). At all concentrations even a short exposure of 30 min using a one step dilution was sufficient to reduce the viability of eggs (Fig. 6a). This effect, however, could be moderated by the use of the stepwise procedure and good survival was achieved even after 2-3 h of equilibration in 2M DMSO (Fig. 6b).

Effect of cryoprotectant toxicity on the enzyme Lactate dehydrogenase (LDH)

Fish embryos previously equilibrated in different concentrations of cryoprotectants for varying periods were used for enzymatic determination. The enzyme activity was measured as the production level of NADH.

Fifty embryos were homogenised and then centrifuged. The supernatant was used

Table 9 LDH activity of *Brachydanio rerio* embryos (1/2 epiboly) equilibrated in various concentration of DMSO for different periods.

Equilibration period (min)	LDH activity (IU X 10^{-3})[a] at various molar concentration of DMSO.			
	1	2	3	4
0	8.69 ± 0.54	8.69 ± 0.54	8.69 ± 0.54	8.69 ± 0.54
15	3.99 ± 0.00	2.67 ± 0.24	2.59 ± 0.43	2.25 ± 0.24
30	1.93 ± 0.03	1.74 ± 0.10	1.22 ± 0.12	1.34 ± 0.13
60	2.53 ± 0.12	1.55 ± 0.20	1.25 ± 0.15	0.66 ± 0.10
120	0.99 ± 0.16	0.38 ± 0.70	0.48 ± 0.00	0.78 ± 0.36
180	0.98 ± 3.80	0.62 ± 0.92	0.54 ± 0.28	0.49 ± 0.14

a) Means given with sd based on 3 replicates.
N.B. Means between all concentrations are significantly different. ($P < 0.0001$). Similarly equilibration periods except 60 and180 min are also significantly different ($P < 0.001$).

for enzymatic determination. The enzyme levels were expressed in terms of international units.

Results to date suggest that the commonly used cryoprotectant, DMSO can have a denaturing effect on the enzymes of intact embryos (Table 9) and this was positively correlated with exposure time and concentration. This may cast into doubt the results of many previous studies from a number of laboratories which have unknowingly used detrimental cryprotectant exposures in their trials. Therefore, in addition to freeze damage the viability of eggs and embryos may also be influenced by the prefreezing toxicity of the cryprotectant. The cryoprotectants and the procedures used to equilibrate gametes and embryos will have to be better evaluated to consider the prefreezing factors such as chill damage and biochemical changes associated with long cooling programmes.

3.2.2:10 Short term preservation of eggs

Asynchrony in spawning times and the need to transport eggs and hold eggs during cryopreservation and in vitro fertilization studies means that some form of short term storage is always necessary. Fish species like the tilapia produce small volumes of ovarian fluid which can lead to rapid desiccation even if the unfertilized eggs are stored for relatively short periods. It is therefore important to determine how the short term viability of eggs can be prolonged.

The storage capability of three potential ovarian fluid extenders (salmon coelomic

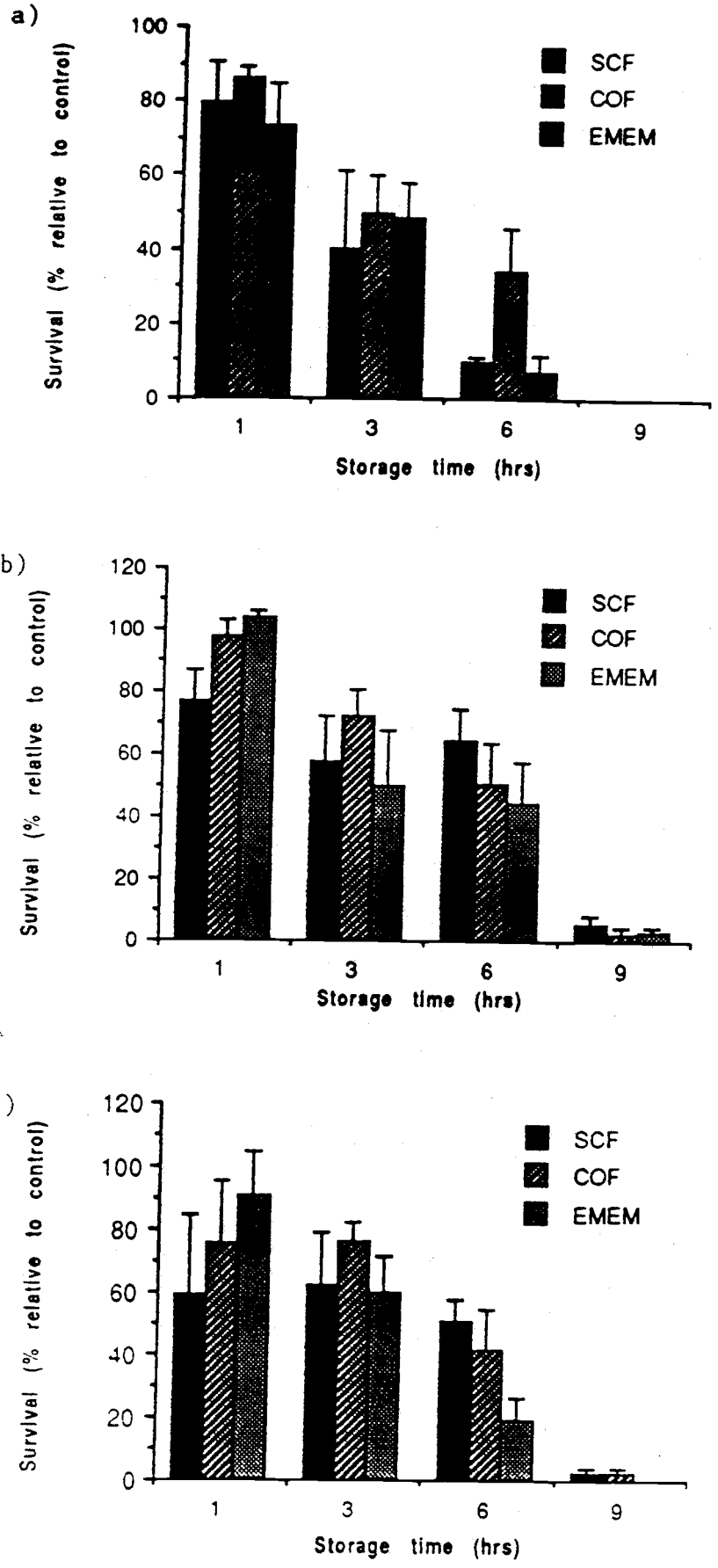

FIGURE 7 Comparison of mean viability of O. niloticus eggs incubated for different periods of time in three different storage media

a) 15°C; b) 22°C; c) 28°C

fluid (SCF), synthetic carp ovarian fluid (COF) and Eagle's medium (EMEM) was evaluated at three temperatures. Unfertilized eggs could only be maintained for one hour in all three media but the best storage temperature was 22°C (Fig. 7). Further studies showed that tap water performed equally well and after 4 h yielded 85% fertilization rates (Fig. 8).

3.3 Studies on Fish Spermatozoa

The steps involved in the developing and evaluating cryopreservation protocols involve a number of factors (Fig. 9). These various factors may have to be separately investigated and this may give the appearance of a series of disjointed studies. These studies therefore should be put into the context of Figure 9.

3.3:1 Assessment of cryopreservation protocols for milt.

In fish, some 55 marine and freshwater species have been cryopreserved to date. The consistency of the post-thaw motility and fertility of milt even of the same species using identical protocols in different laboratories is highly variable. Despite the many publications since the 1960's on the cryopreservation of salmonid milt no single reproducible and reliable protocol has emerged. This is largely due to the vast amount of variation which can be generated when dealing with each of the components of cryopreservation outlined in Figure 9 and that

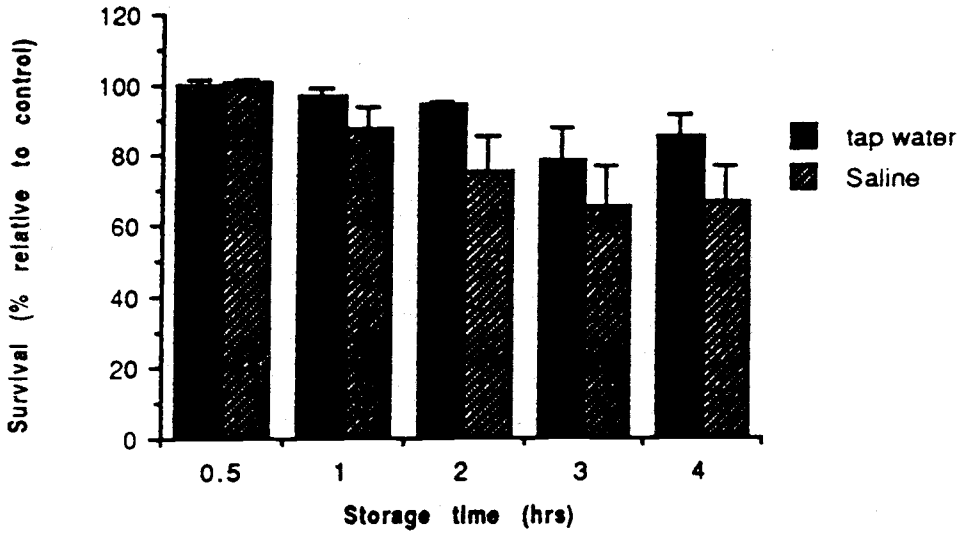

FIGURE 8 Viability of O. niloticus eggs stored in tap water and physiological saline at room temperature

COMPONENTS OF CRYOPRESERVATION	
A) Pre-freezing stage	Gamete quality
	Collection and transport techniques
	Extenders and cryoprotectants
	Equilibration time and temperature
B) Cooling stage	Cooling methods and rates
C) Storage stage	Configuration of storage system
D) Thawing stage	Warming rate and thawing solution
E) Motility and fertility stage	Method of motility assessment
	Volume of milt and cell concentration and no. of eggs

Figure 9 The various interrelated components of cryopreservation

many of the procedures are species-specific. If valuable and irreplaceable material is to be cryopreserved the reliability of the techniques is crucial. Therefore, the second major objective of this programme was to evaluate and assess post-thaw viability and understand the possible reasons for its variability.

It has been argued that even low (<30%) fertilization rates from cryopreserved milt are acceptable and therefore efforts to improve methods are unnecessary. This attitude will depend on the intended use and management of the cryopreserved frozen gene bank. If all the post-thawed milt is to be used at once in a " Big bang" fashion then at least some progeny will be possible by the use of all the post-thawed milt. If, however, the intention is to use the milt over a period of time as part of a rational genetic management programme then it will be imperative to maximise the use of post-thawed milt to achieve the minimum sperm: egg ratio.

3.3:2) Cooling rates and development of a multichannel temperature logger

The cooling rate of milt is regarded as one of the main factors governing successful cryopreservation. It would appear that all methods of cooling have to be carefully evaluated to avoid the problems associated with the occurrence of random undercooling and variable rates of cooling within the stored sample. Both these factors are known to affect the post-thaw viability of spermatozoa. Despite using a programmable cooler, which is the best equipment currently available, we felt that the cooling rate within the cooling chamber was a significant variable. To test the extent of this problem a multichannel temperature data logger had to be

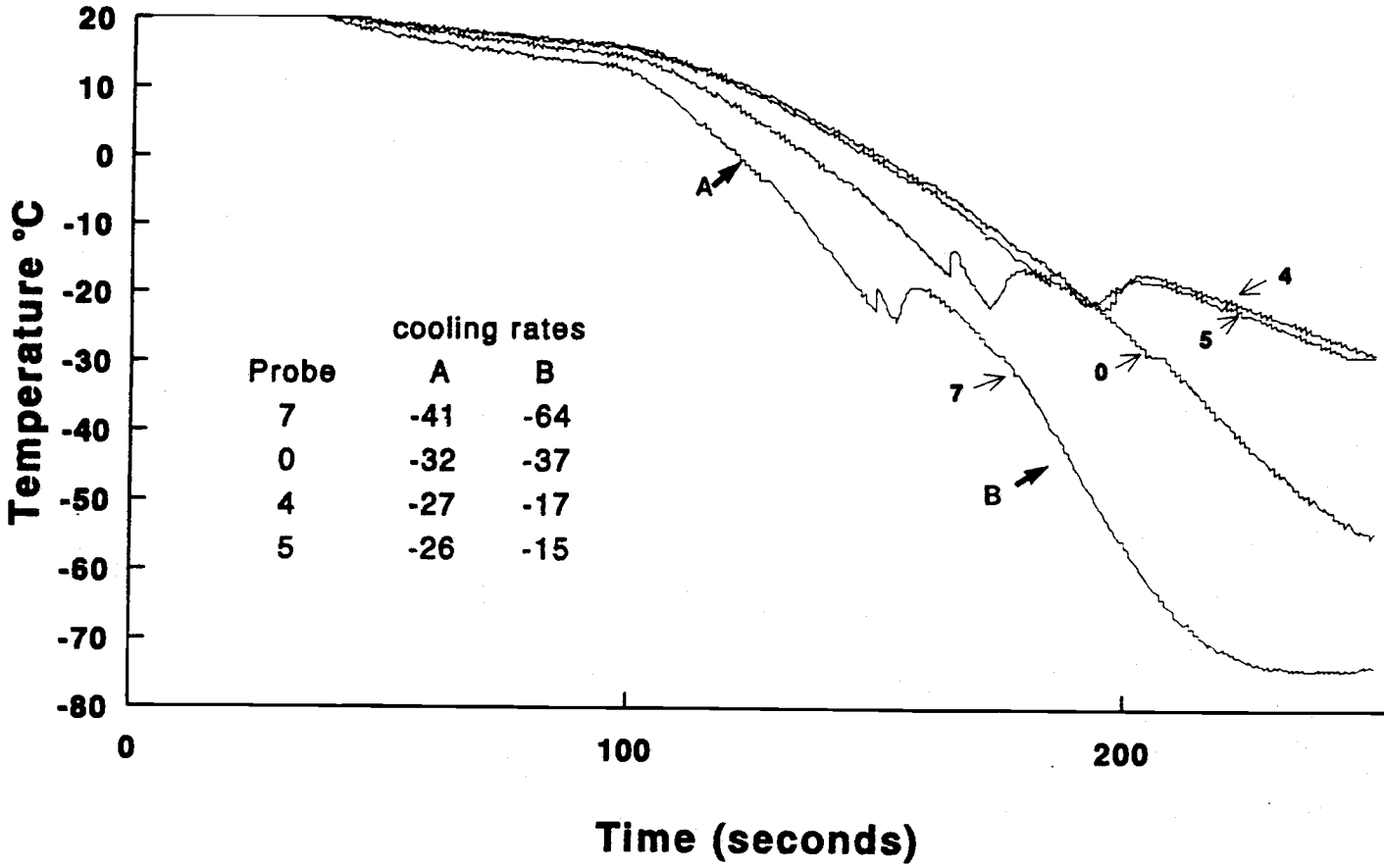

Figure 10 Effect of position on the cooling profiles within straws. Probes 7,0 4 and 5 were positioned in four corners of the chamber with 7 nearest the nitrogen stream and 5 furthest away. Note degree of cooling also variable as is the occurrence of undercooling.

developed to measure directly the cooling rates of milt within the straws. The computerised temperature logger was developed with the co-operation of the Microprocessor Unit at Stirling University and enabled the precise cooling profiles in up to 8 containers to be followed simultaneously and the relevant information on cooling rates in different parts of the chamber and the storage straws could be monitored to determine the extent of any variations.

Three main factors were considered; the influence of straw size, the position of straws and the loading of the freezing chamber. An example of profiles of such trials is given in Figure 10 which clearly shows that the actual cooling rates within the straws vary considerably between samples. More importantly the samples undercool at random and the extent of this undercooling also varies between samples (Fig. 10). In addition, there were clear differences in the cooling rates of the two phases of the cooling process (A&B) and consequently the timing of freezing in the same cooling run was very different. The current method to avoid undercooling in samples is to touch the straws with a pair of forceps precooled in liquid nitrogen. This method is adequate if only a few samples are being frozen and applies mainly to mammalian eggs and embryos. A system for avoiding undercooling during mass freezing will need to be developed.

A commercially available product formerly produced by Cell Systems Ltd. called "Zygon" was tested at the recommended concentration to prevent undercooling during large scale freezing runs. The cooling profiles produced by the multichannel data logger during the test showed that in all our trials this product

developed to measure directly the cooling rates of milt within the straws. The computerised temperature logger was developed with the co-operation of the Microprocessor Unit at Stirling University and enabled the precise cooling profiles in up to 8 containers to be followed simultaneously and the relevant information on cooling rates in different parts of the chamber and the storage straws could be monitored to determine the extent of any variations.

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A commercially available product formerly produced by Cell Systems Ltd. called "Zygon" was tested at the recommended concentration to prevent undercooling during large scale freezing runs. The cooling profiles produced by the multichannel data logger during the test showed that in all our trials this product

did not prevent undercooling. Preliminary studies on other possible systems such as specially designed clamps have been investigated and results are encouraging but the reproducibility of such a system will need to be evaluated. Automated systems for ice seeding are available but they are costly and are designed for handling a small number of containers of very specific configuration. Overall, it appears that random undercooling to unpredictable temperatures and variable cooling rates may be contributing to variation of post-thaw viability and this area will require more careful examination to ensure the high levels of reproducibility that will be required in future gene banks.

Having identified an acceptable cooling profile and rate for freezing tilapia and carp spermatozoa in the programmable tissue cooler, the data logger will help us to develop a suitable portable freezer with the desired cooling characteristics for field use. The temperature logger is being used to ensure that optimum cooling rates and profiles are achieved during our normal freezing protocols. The cooling rates obtained by using freezing methods such as cooler boxes and necks of liquid nitrogen Dewars are being assessed. Measurements so far have shown that temperature changes at fixed levels in the vapour phase above liquid nitrogen are not linear and will vary with levels of liquid nitrogen, ambient temperature and pressure. Therefore, the cooling rates will need to be defined to introduce some measure of reliability into these techniques.

At present all the equipment is dedicated to the cryobiology laboratory but we plan to develop portable hardware for field applications. The intention is to use

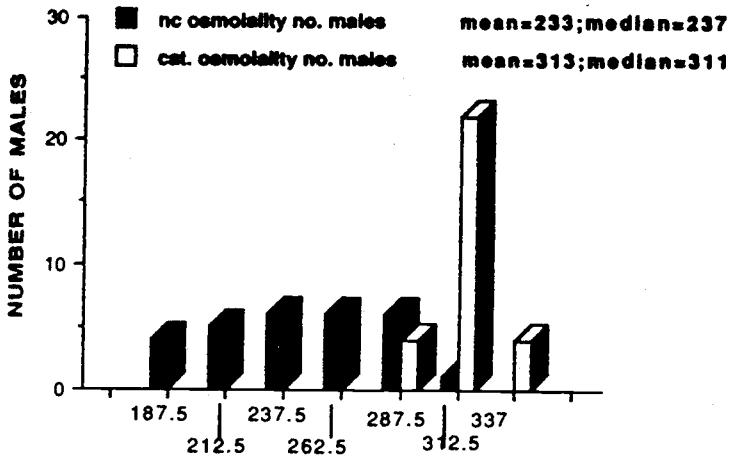
the equipment to develop field based cryopreservation protocols for spermatozoa which will allow field work in Africa and other areas where cryopreservation technology may have a role in conservation of commercially valuable and endangered wild species.

3.3:3 Urine contamination of milt

In most species milt is collected via the sperm duct by abdominal pressure after clearing the bladder of urine. This procedure, however, does not completely clear the bladder and varying degrees of contamination are possible. The resultant variation in the seminal plasma concentration may affect the freezing characteristics of the milt and add to the variability in freezing success observed. Urine is also a sperm activator and since freshwater fish sperm remain motile for a few minutes at best, any contamination will reduce the quality of the milt. Attempts to investigate the extent of urine contamination in tilapia was not possible due to their small milt and urine volume. The importance of this variable was therefore examined using salmon which produce large volumes of both milt and urine. A sample of milt was collected externally from individual males by abdominal pressure after clearing the bladder of urine and then by catheterization of the sperm duct of the fish. The milt characteristics were then evaluated. The order of this procedure was reversed for half the males investigated to minimise any bias associated with milt production.

Despite clearing the bladder, urine contamination was observed and ranged

Figure 11 Variation in osmolality of catheterised and non-catheterised milt



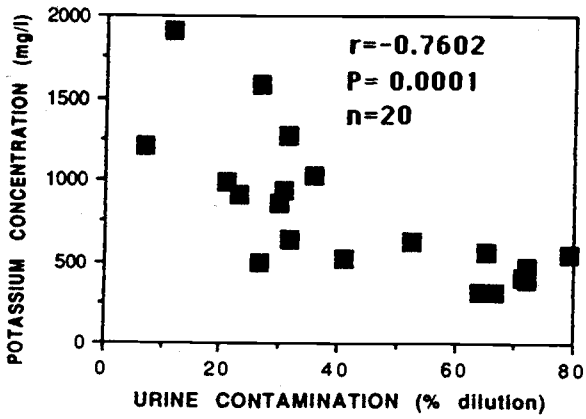
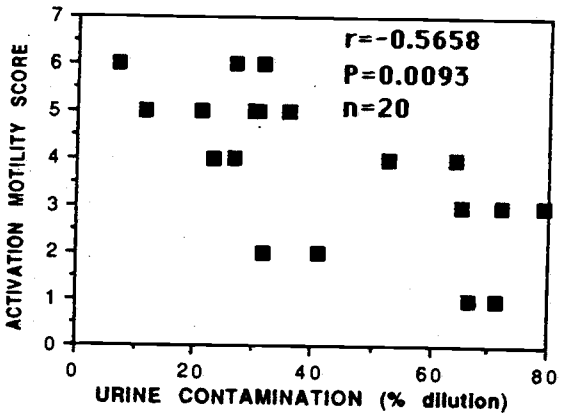


Figure 12 Effect of urine contamination on milt potassium levels

Figure 13 Effect of urine contamination on milt Activation



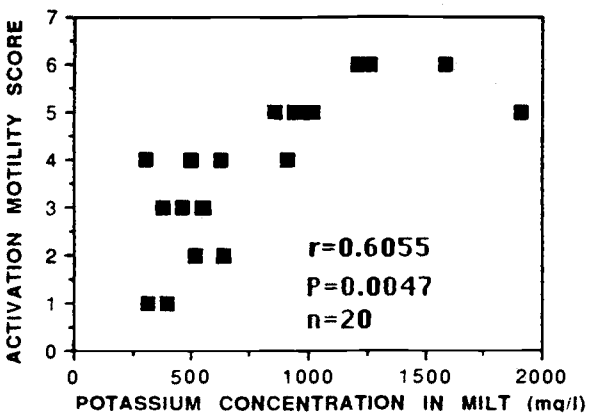


Figure 14 Effect of potassium levels on milt activation

between 5-79 % in uncatheterized samples. This significantly ($P<0.05$) reduced the level of all the seminal plasma constituents measured compared to catheterized samples. Osmolality of the seminal plasma varied markedly (Fig. 11), potassium ion (reduction triggers activation) levels were reduced (Fig. 12), activation of contaminated milt was negatively correlated with urine contamination (Fig. 13) and positively correlated with potassium levels (Fig. 14). Clearly these results, which will need to be verified for other species, show that the presence of urine is an important variable in spermatozoa cryopreservation especially if milt is frozen any later than 15 min after collection.

3.3:4 Cryopreservation of carp milt

As indicated in the project memorandum we planned to integrate these studies with other programmes such as that of the BAFRU Carp unit to obtain gametes for the cryopreservation studies. Due to technical difficulties these facilities were not functional during this study. Therefore, milt from another carp, *Carassius auratus* ,was used as a model.

Four extenders and two cryoprotectants were evaluated (Table 10).

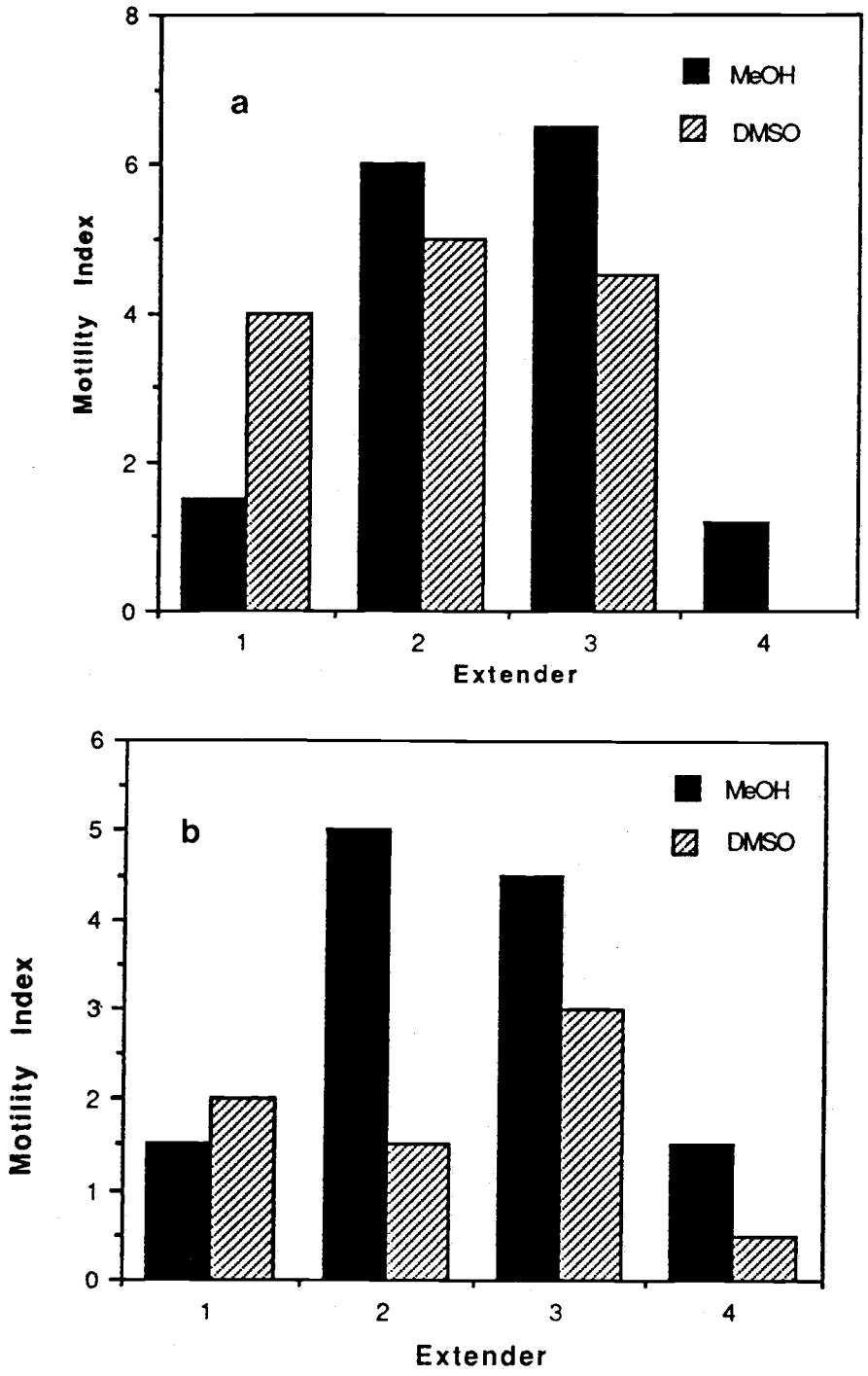


Figure 15 Post-thaw activation of carp milt using various extenders. a) milt activated with sodium citrate and b) tap water

Table 10 Chemical composition of extenders evaluated for carp milt cryopreservation

CHEMICALS (g/100ml)	EXTENDER			
	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

The milt from 4 males was pooled and diluted 20 fold with the above extenders containing either 10% MeOH or DMSO. Samples were equilibrated for 15 minutes and then cooled at -5°C/min to -60°C and stored under liquid nitrogen. Milt was thawed in a 40°C water bath for 8 sec., activated and motility scored on a scale of 0-10.

The post-thaw motility of spermatozoa cryopreserved in various extenders is shown in Figure 15. Higher post-thaw activation scores were noted when MeOH was the cryoprotectant. The highest post-thaw motility was obtained with extenders 2 and 3 containing 10% MeOH and better post-thaw activation was achieved with Sodium citrate [fig 15a] when compared with tap water [fig 15b]

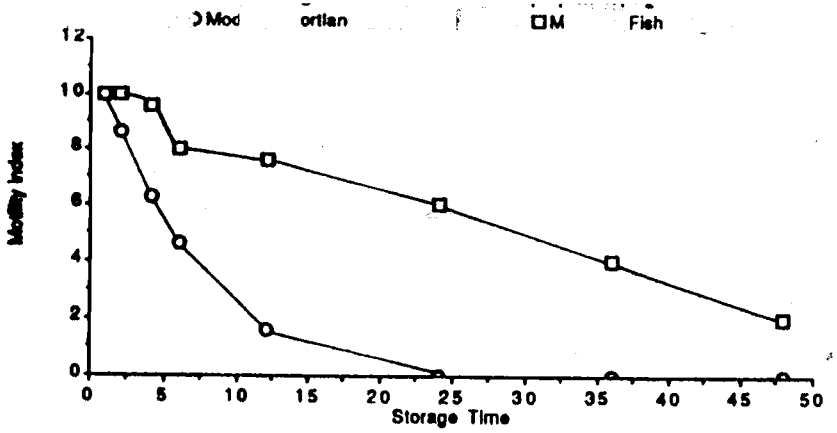


Fig.16 Motility index of UV irradiated milt in 2 diluents (MC, 2 and MFR, 2).

Table10 Mean percentage motility of UV irradiated sperm just after irradiation and after cryopreservation in liquid nitrogen for 8 days.

Tag No.	Mean % Motility (After Irradiation)*	Mean % Motility (After 8 days in LQN)
0904	91.0 (5.8)	46.4 (2.7)
0911	89.4 (1.5)	53.6 (6.4)
0913	96.5 (7.5)	58.3 (4.8)
0933	97.1 (4.2)	49.8 (3.6)

* numbers in brackets are SEM

LQN - Liquid Nitrogen

All observations were replica.ted 3 times using the Video Frame Analysis method.

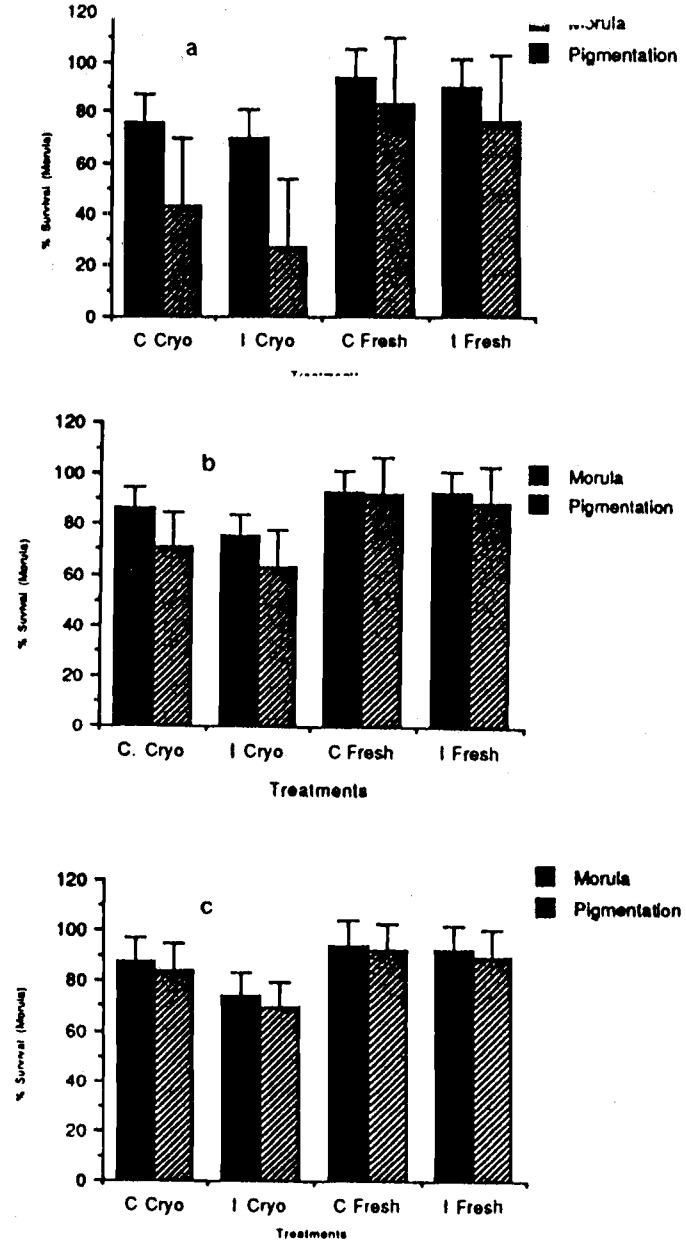


Figure17 Effect of post-thawed UV irradiated milt volume on the fertilization rates of *O. niloticus* eggs a) 200 b) 400 and c) 600ul.

We hope to extend these studies to other species of carp of commercial interest to tropical aquaculture.

3.3:5 Storage of UV-irradiated milt

The use of genetically manipulated milt is now widely used for the production clonal lines of fish, creating inbred stocks and giving a better understanding of the sex determining mechanism and other genetic traits of fish. The storage of milt from desired individuals and populations will therefore be of considerable value in fish genetics and a series of studies were initiated into the storage of UV irradiated milt.

The effect of different extender solutions on motility, deactivation and fertilization were investigated and the viability of cryopreserved UV irradiated milt was assessed.

Many of the extenders originally tested activated between 1-25% of spermatozoa. The levels of potassium concentration were adjusted but the storage properties of the two extenders tested varied (at 4°C). The storage of milt in modified fish Ringers was superior to modified Cortlands (Fig. 16). UV irradiation increased the fragility of sperm cells and the motility of post-thawed milt was reduced by half from between 90-97% to 50-58% (Table 10). Despite being subjected to UV irradiation milt could be successfully cryopreserved. The fertility rates achieved,

however depended on the volume of milt used for fertilization (Fig. 17) and up to 70 % of the eggs could be fertilized with cryopreserved UV irradiated milt.

3.3:6 Cryopreservation of milt extracted from testis

Generally, milt is collected by abdominal pressure via the spermduct. In some situations, for example in *T. zillii,* catfish and sex-reversed fishes milt cannot be expressed and the fish may need to be killed to collect the sperm cells. In these cases milt will need to be extracted in balanced physiological solutions, which may be species-specific, to obtain an acceptable quality of freezable milt. Gonads of *T. zillii* were excised and their surface scored under modified Cortlands to allow spermatozoa to disperse into the saline solution. The suspension containing the cells was then cryopreserved as described above. The post-thaw recovery was low and only about 20% of the cells retained their motility. This low recovery may be related to the method of collection as the extraction would have collected both mature and immature spermatozoa. This was also reflected in the prefreezing motility of milt where only 30-50% of the spermatozoa were motile.

3.3:7 The enhancement of the duration of sperm motility

Earlier observations suggested that the duration of sperm motility can be manipulated by the chemical composition and concentration of activators. Under

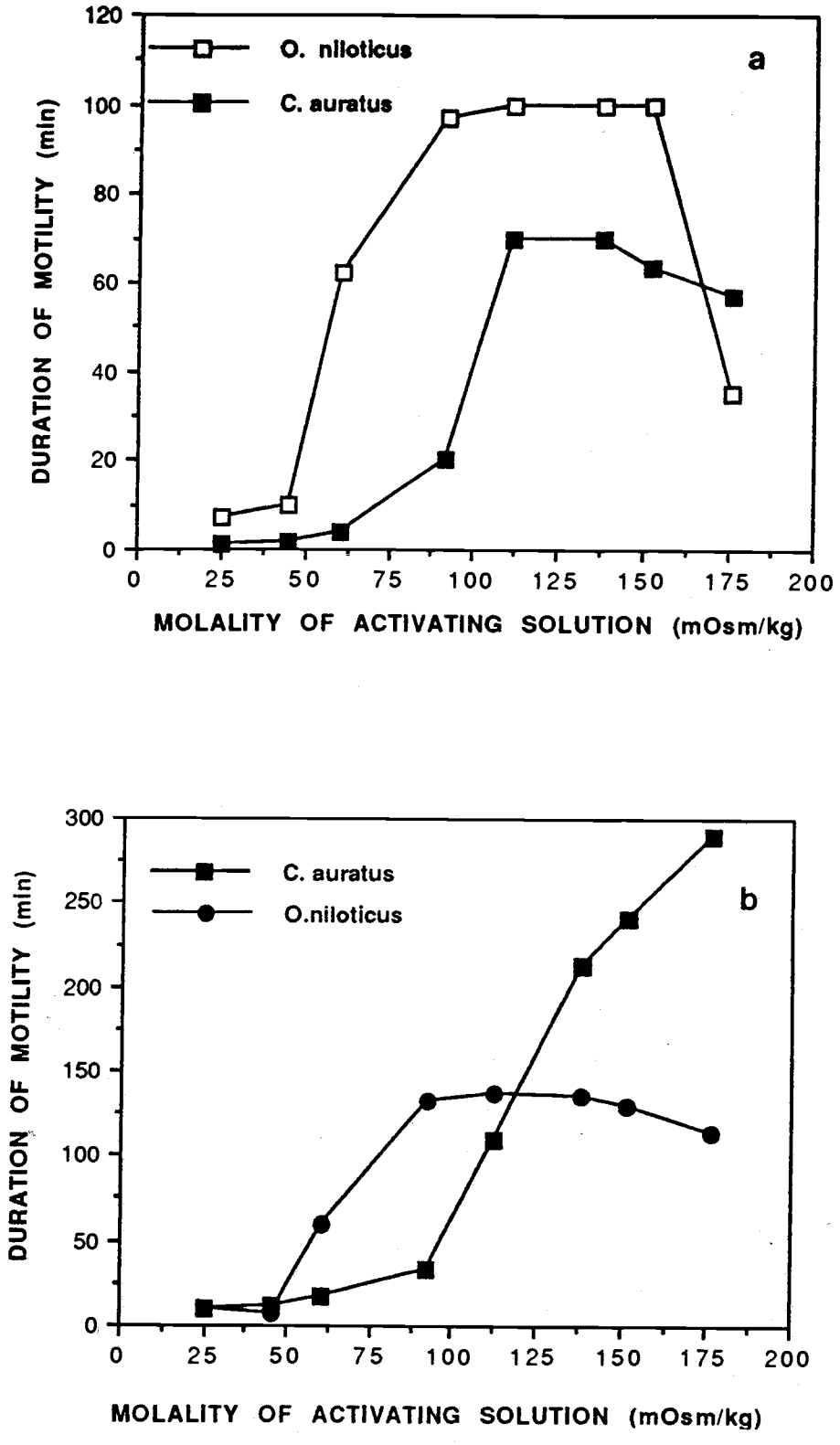


Figure 18 Effect of a) Sodium chloride and b) Sodium citrate on the duration of sperm motility. Time noted as duration to 10% of motile cells in a sample, milt diluted 1:200.

normal conditions tilapia spermatozoa remain motile for about 5 - 10 minutes when activated with water and the sperm : egg ratio required for high fertilization rates is a function of this short time. If the duration of motility of fresh and cryopreserved milt can be increased then the fertilization rates of batches of eggs with a given quality of post-thawed milt may be improved. The increased duration will provide a greater opportunity for spermatozoa to fertilize the eggs. Given that there is inevitable damage to spermatozoa during cryopreservation this will maximise the use of valuable marginal quality post-thawed milt.

Studies were conducted with varying concentration of sodium citrate and sodium chloride on milt collected from *O.niloticus* and *C. auratus* . The spermatozoa were activated and the duration of motility noted.

The influence of sodium citrate and sodium chloride on the duration of activated milt is given in Figure 18. In both species sodium citrate (Fig. 18b) was superior to sodium chloride (Fig. 18a) in maintaining motility of the activated sample. The duration of motility of the activated sample increased with osmolality to an optimal level at around 100 mOsm/kg in both species. The maximum duration, however, varied between the species and all were significantly ($P<0.05$) higher than when activated with fresh water.

In *C. auratus* fresh spermatozoa were motile for 290 and 60 min when diluted with 176mOsm/kg sodium citrate and sodium chloride, respectively. *O.niloticus* spermatozoa activated with sodium citrate remained motile for up to 135 minutes

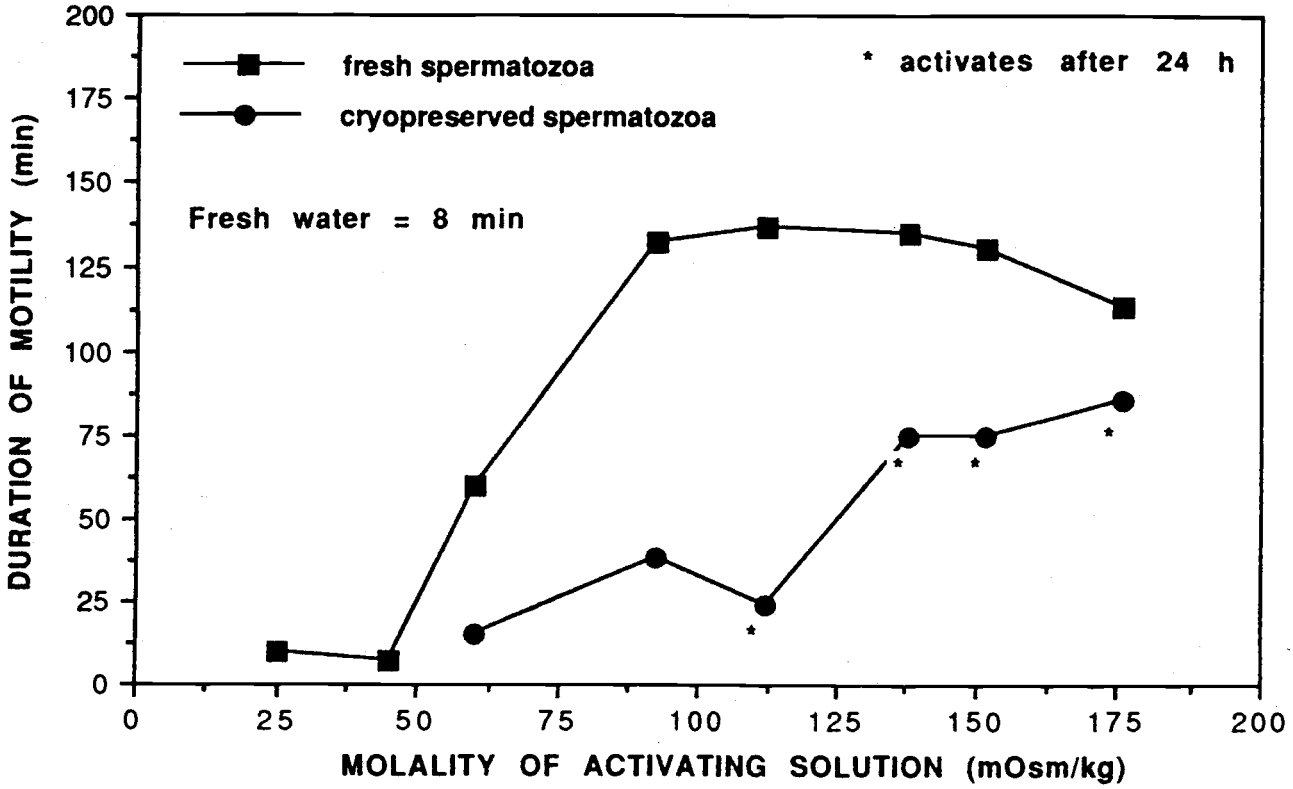


Figure 19 Duration (to 10% motile cells in a sample) of sperm motility of fresh and post-thawed *O. niloticus* milt activated with 2% sodium citrate

compared with only 4 minutes when activated with tap water. Similarly, the duration (to 10% motile cells) of post-thawed *O. niloticus* spermatozoa was increased from 3 minutes when activated with tap water to over 75 minutes using sodium citrate (Fig. 19).

In *O. niloticus* and *C.auratus* motility of activated fresh milt ceased by 135 and 290 min respectively, but between 10-20% of these cells could be reactivated with water after being stored at 4°C for 24 hours. Activation of post-thawed *O. niloticus* milt produced similar results in that 10% of the cells could again be reactivated after 24h of storage at 4°C.

3.3:8 Preliminary studies on the activation of immotile spermatozoa

Investigations into the viability of post-thawed milt suggested that many of the cells were in fact intact but were not motile after activation. It is likely that the activation mechanism of the membrane had been damaged and therefore motility could not be initiated. If this was the case it may be possible to demembranate the cells and activate the cells using an appropriate external energy source. Activation of demembranated cells has been demonstrated for salmonid sperms (Moriswa, 1983).

The experiments reported for salmonids were repeated using unfrozen tilapia milt.

Spermatozoa were demembranated with Triton and treated with varying concentrations of ATP and cyclic AMP and changes in the activation noted.

Numerous attempts in this study to repeat the reported results of progressive movement of demembranated salmonid spermatozoa on tilapia spermatozoa were unsuccessful. Cells could be successfully demembranated but at best these cells would only vibrate when treated with a external energy source such as ATP and cAMP.

4 IMPLICATIONS OF RESULTS

a) Trials to date suggest that embryos but not eggs of aquatic organisms can be successfully recovered from cryostorage. The range of organisms that can be cryopreserved, however, has not been established. Earlier studies using tilapia and catfish as models suggest that they are prone to chill damage and cooling rates as low as 0.3°C/min were insufficient to dehydrate the eggs.

The success with oyster and *Artemia* embryos suggest that complex multicellular organisms and unhatched embryos containing as much as 60 % moisture can be successfully cryopreserved. Both these principal observations will need further investigation if we are to understand the mechanisms that enable them to be cryopreserved.

Cryopreservation studies on oysters demonstrated that eggs as small as 30-40 µm cannot be successfully cryopreserved whereas larvae of 60-100 µm can. When compared to mammalian eggs, oyster eggs are smaller but the bound water may be considerably higher and therefore may have been more difficult to cryopreserve with the methods used.

The successful cryopreservation of *Artemia* embryos (200-400µm) in the decapsulated and fully hydrated forms poses new questions. How can a macroscopic (200-400µm) embryo encysted with a thick outer coat (8µm) containing some 4000 cells survive cryopreservation even though it contains over 45% water? These results do suggest that the presence of water in the egg can be tolerated under some as yet unidentified conditions. *Artemia* can tolerate desiccation and this is attributed to the high level of the sugar trehalose, but its role in cryopreservation is unclear.

b) Differences in permeability of unfertilized and developing tilapia eggs may be useful in increasing cryoprotectant uptake. Despite the higher permeability of unfertilized eggs, dehydration was insufficient to prevent lethal intracellular freezing. Evidence to date suggests that the chorion is permeable, but the perivitelline membrane is impervious under the conditions so far studied. Studies on manipulating the permeability of this membrane to allow dehydration of the yolk will need to be pursued more fully in further work.

c) High concentrations of cryoprotectants and long cooling protocols have been commonly used in attempts to cryopreserve fish eggs. This has now been shown to be toxic to the gametes. Cryoprotectants such as the widely used DMSO effect the biochemical functioning of enzymes. These effects will need to be separated from chill and freeze damage. Cryoprotectants may cause irreversible damage to the metabolic and osmotic capabilities of the embryos, but the extent of the damage can be reduced by the gradual increase of cryoprotectant concentration to the desired level in several steps. The use of the stepwise equilibration and dilution procedures will help higher concentrations of cryoprotectants to be tested. The permanency of the effect of DMSO on enzymes will need to be evaluated.

d) The high viability of unfertilized tilapia eggs held in tap water for up to four hours will be useful in time consuming egg manipulation studies eg. evaluating chill damage of tilapia eggs (in some fish species viability is lost within a few minutes of contact with water). This will have important commercial implications as it will enable tilapia farmers to strip and collect eggs over several hours and therefore fertilize and manipulate relatively large numbers compared to single fish batches.

e) Still frame video analyses (STFA) has enabled fish sperm motility to be accurately quantified. To enable information on sperm fitness characteristics such as speed, tail beat frequency and numbers of immotile spermatozoa to be statistically assessed a computerised system capable of rapid handling of

data will need to be developed. Such a system will enable large numbers of samples to be analyzed in a short time and should be a priority for any future study.

f) The successful development of a multichannel temperature logger has enabled the cooling configuration within straws to be evaluated. This system can be developed further for similar commercial applications. The temperature logger should also be developed for field applications to evaluate the importance of unequal freezing rates in straws on the variability of post-thawed milt and to assist in the development of a reliable field based cryopreservation protocol which can be used in developing countries.

g) The variability of cooling rates within straws and vials placed in the programmable cooling chamber and the varying degree of undercooling may be the underlying reason for previously unexplained variation in post-thaw spermatozoa motility. These problems are likely to be even greater in an uncontrolled cooling system such as on dry ice or liquid nitrogen vapour.

h) During manual stripping milt may be contaminated with varying amounts of urine. Urine significantly changes the characteristics of salmon milt and may affect the cooling profiles during freezing. Urine contamination is an important consideration for short term storage and when milt is

cryopreserved any later than 15 min after collection. Its importance for other species will need to be evaluated.

i) The successful cryopreservation of milt from the carp, *C. auratus*, suggests that these techniques could be applied to the cryopreservation of milt from other tropical carp species, perhaps with minor modification of the protocol.

j) Milt can be extracted directly from the testis for cryopreservation, but this technique is indiscriminate and many of the cells collected are immature. This results in low levels of overall motility in fresh and post-thaw milt. Such a procedure will be invaluable for the cryopreservation of milt from species where it is difficult to manually strip from the sperm duct as in the case of sex inverted fishes and catfish. It is clear that methods for in vitro extraction need to be improved in future investigations.

k) Successful cryopreservation of UV treated milt will enable genetic studies on sex determination and selection studies to be conducted more easily in many developed and developing countries.

l) Since the duration of motility of activated spermatozoa can be manipulated the overall fertility rates using cryopreserved milt should be improved. These studies should be repeated and tested in fertilization trials.

m) Studies on the reactivation of demembranated spermatozoa were inconclusive. These studies should be pursued. There is evidence from dye exclusion tests to suggest that most post-thawed cells are not dead and that the axoneme system may be functional. If this is the case the use of extracellular energy may help improve fertilization success of post-thaw milt.

5 PRIORITY TASKS AND FUTURE DIRECTIONS

The priority tasks to follow up are divided below into eggs and embryos and spermatozoa.

5.1 Eggs and embryos

The mechanisms enabling the two invertebrate models to be cryopreserved should be investigated to determine if they can be applied to fish eggs and embryos. Other models which can tolerate some degree of dehydration under natural conditions, eg. Killifishes should also be investigated to determine wether these adaptions enable them to be cryopreseved.

Studies on the prefreezing toxicity of freezing medium should continue to define to what extent they are responsible for post-thaw mortality.

The methods that are used to determine the levels of osmotically inactive water within mammalian embryos cannot be applied to many aquatic eggs and embryos because they do not shrink uniformly thus making it difficult to determine volume changes and consequently bound water measurements. Other methods will need to be developed. The use of NMR, Confocal microscopy or the ratio of ^{14}C : $^{3}H_2O$ are possibilities that should be pursued.

In addition to the methods currently used to improve membrane permeability sugars such as trehalose and vacuum equilibration should be tried to dehydrate gametes.

In view of the toxicity of cryoprotectants such as DMSO to gametes, new vitrification solutions that are being developed should be evaluated and less toxic cryoprotectants identified for the cryopreservation of eggs and embryos.

5.2 Spermatozoa

The techniques developed at Stirling for milt cryopreservation should be modified and tested under field conditions. Cryopreservation protocols will need to be modified for field applications and should be aimed at the conservation of genetic variation of commercially important and threatened fishes. This development should be paralleled by the development of policies on the genetic management of frozen gene banks. Issues such as ownership and assess to cryopreserved material,

disease screening, the numbers of straws, volume of milt, number of individuals to be used etc. will need to be addressed. Discussions are already underway on the feasibility of developing protocols for the endangered Haplochromid cichlids from Lake Victoria.

The importance of variable cooling rates and undercooling should be established to determine their role in the reported variability of post-thaw gamete survival. When known a reliable portable field based cooling chamber could be developed to ensure proper cooling regimes for cryopreservation of milt in the field. Since the cooling rates are species-specific, the method may need to be modified for each species.

The development of a transport system for chilled stored milt will be extremely useful as this will allow different laboratories to freeze the same milt for storage. This development will be crucial for the international management of genetic material and will help spread the risk against loss of cryopreserved genetic material in the event of an accident.

In view of the species-specific differeces in the cryopreservation protocols, work should continue on the freezing other species of commercial and conservational value such as the Indian and Chinese carps.

The developments in cryopreservation at Stirling should be integrated with other ODA programmes. During this project have been several requests from scientists

in the Philippines, India, Israel, Mexico, Chile and Bangladesh on possible collaboration or inputs into similar programmes they wish to establish.

5.3 Dissemination

The work has been published and disseminated through papers, conferences, seminars, the Institute's Master's course and training of PhD students and visiting overseas scientists through the British Council. The work has resulted in many requests for assistance from all over the world. The most significant requests in scientific terms have come from the groups involved in the selective improvement of farmed fish. These workers face immense difficulties in maintaining unselected control populations so that they can assess genetic gain in their selected lines. Cryopreservation means that they can freeze germ plasm from every generation and regenerate these at some point in the future under standard conditions to compare performance.

International organisations like ICLARM and NASCO have both viewed this project with great interest and now the cryopreservation of milt is considered to play a significant role in stock management of cultured and threatened stocks. Discussions are underway to look into the feasibility of establishing a cryobiology laboratory in the Philippines and transferring and applying the technology developed at Stirling for the cryopreservation of tilapias and other tropical species under field conditions.

A number of students have been trained in cryopreservation techniques, to date five masters projects have been completed and a PhD is in progress. Two visiting scientists from developing countries (India and Sri Lanka) have been trained at Stirling in cryopreservation and a further two (Indians) intend to apply for training at Stirling. In addition to direct training over 80 reprints have been requested for the studies disseminated to date.

Papers in peer review journals, monographs, theses and published presentations at conferences and meeting are listed below.

1) Rana, K.J., Muiruri, R.M. & McAndrew, B.J. (1990) The influence of diluents, equilibration time and storage conditions on the viability of cryopreserved *Oreochromis niloticus* (L) spermatozoa. Aquaculture and Fisheries Management 21: 25-30.

2) Carrilho, M.C. (1990) Influence of storage medium, temperature and storage time on the viability of unfertilised *Oreochromis niloticus* (L) eggs. MSc Thesis. Institute of Aquaculture, University of Stirling. 52 pp.

3) Pascual, A.B. (1990) Low temperature storage of UV irradiated sperm of *Oreochromis niloticus* (L). MSc Thesis. Institute of Aquaculture, University of Stirling. 64 pp.

4) Aboyewa, M. (1991) Effects of storage on *Oreochromis niloticus* ova. MSc

Thesis. Institute of Aquaculture, University of Stirling. 60pp

5) Pasqual, A.B., Penman, D.J., Rana, K.J., McAndrew, B.J. (1991). Cryopreservation and refrigerated storage of UV and inactivated milt for gynogenesis. 4th. International Symposium on Genetics in Aquaculture. April 29-May 3. Wuhan, China.

6) Rana, K.J., Gupta, S.D., McAndrew, B.J. (1991). The influence of collection techniques on the spermatology of Atlantic salmon *(Salmo salar)*. June 23rd-July 6th. Genetic Conservation of Salmonid Fishes-Nato Advanced Study Institute, Moscow/Pullman, U.S.A.

7) McAndrew, B.J., Rana, K.J. & Penman, D.J. (1992) Conservation of genetic variation in Aquatic organisms. In: Recent Advances in Aquaculture, Vol. 4: 63 pp. Eds. J.F. Muir & R.J. Roberts. Croom Helm, London.

8) Rana, K.J., Edwardes, S., Shields, R., McAndrew, B. J. & Bromage, N. (1992). Quality of chilled and post-thawed cryopreserved Atlantic halibut, *Hippoglossus hippoglossus,* milt. Workshop on gamete and embryo storage and cryopreservation in aquatic organisms. Marly le Roy, France, 30th March-2nd April.(to be submitted to Aquaculture)

9) Rana, K.J. and McAndrew, B.J. (1992) Effect of mannitol, sodium citrate and sodium chloride on the duration of fresh and cryopreserved spermatozoa.

Workshop on gamete and embryo storage and cryopreservation in aquatic organisms. Marly le Roy, France, 30th March-2nd April.

10) Rana, K.J., McAndrew, B.J. (1992) Cryopreservation of oyster, *Crassostrea gigas*, eggs and embryos. Workshop on gamete and embryo storage and cryopreservation in aquatic organisms. Marly le Roy, France, 30th March 2nd April.
(to be submitted to Aquaculture and Fisheries Management)

11) Rana, K.J., Gupta, S.D. & McAndrew, B.J. (1992) The relevance of collection techniques to the prefreezing quality of manually stripped milt. Workshop on gamete and embryo storage and cryopreservation in aquatic organisms. Marly le Roy, France, 30th March 2nd April. (to be submitted to Aquaculture)

12) Rana, K.J. & McAndrew, B.J. (1992) Cryopreservation of gametes and embryos of Aquatic organisms: role, problems and recent developments for the conservation of genomic information. Experimental aspects of aquaculture. The Society for Experimental Biology, Lancaster meeting, 5-10th April.

6 SUMMARY OF FINANCIAL EXPENDITURE

See Appendix

7 AUTHORS OF THIS REPORT

Dr K. J. Rana

Dr B. McAndrew

8 APPENDIX

UNIVERSITY OF STIRLING
CRYOPRESERVATION AND MANIPULATION OF OVA AND MILT COMMERCIALLY IMPORTANT TROPICAL SPECIES
STATEMENT OF ACTUAL EXPENDITURE FOR THE PERIOD 1 APRIL 1989 TO 31 MARCH 1992

	ACTUAL EXPENDITURE			TOTAL EXPENDITURE
	01.04.89 31.03.90	01.04.90 31.03.91	01.04.91 31.03.92	1989/ 1992
SALARIES				
Lecturer	18,116.43	20,038.70	22,274.00	60,429.13
Technician	8,572.60	9,559.18	10,712.00	28,843.98
EQUIPMENT	1,077.45	14,700.00	0.00	15,777.45
TRAVEL	0.00	0.00	0.00	0.00
CONSUMABLES	1,750.89	5,162.70	5,150.00	12,063.59
	29517.57	49460.58	38,136.00	117,114.15

I CERTIFY THAT THE ACTUAL EXPENDITURE DETAILED ABOVE HAS BEEN ACTUALLY AND NECESSARILY EXPENDED UNDER THE GRANT IN ACCORDANCE WITH THE TERMS AND CONDITIONS OF RESEARCH SCHEME R4523

J S GORDON
FINANCE OFFICER

Your Ref :R4523
Our Ref :AF152.411
Investigator :Dr B J McAndrew

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