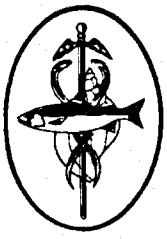

Project number 4914

Investigations into the problems of cryostorage of eggs and embryos of commercially important tropical species

Final Report
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CONTENTS

1. EXECUTIVE SUMMARY

i) This project aims to investigate the fundamental constraints associated with the cryostorage of fish eggs and embryos. The tasks of the project were broadly segregated into the precooling and post-cooling phases and the cryobiological factors within each phase evaluated. In the prefreezing phase suitable tropical fish models were identified and the permeability of embryos to water and the proportion of intracellular water was established. The cooling phase was segmented to establish the significance of prefreezing toxicity of cryoprotectants, chill tolerance and freezing damage to overall cryosuccess.

ii) Techniques for maintaining, breeding and procuring gametes were successfully developed for the two selected model species; the zebra fish (*Brachydanio rerio*) and rosy barb (*Puntius conchonius*). The precleavage, cleavage, epiboly, closure of blastopore stage and heartbeat stages of zebra fish and rosy barb embryos were used. Following each treatment embryos were incubated in Petri dishes at 28°C.

iii) The precooling toxic effects of cryoprotectants were ascertained using endpoints such as osmotic damage, enzymatic activity, hatch rates and morphological deformities. The mode of cryoprotectant administration and concentrations that were shown to be least harmful were used in subsequent trials. Cleavage, epiboly, and closure of the blastopore stage embryos were exposed to final a concentration of either 1, 2 or 3M Me_2SO, methanol, EG and glycerol. Cryoprotectant additions and removal were made in up to 4 equal steps. The mode of administration, type and concentration of cryoprotectant and embryonic stage had a significant effect on the viability of embryos. Gradual step-wise equilibration significantly ($P<0.05$) increased the tolerance of embryos to cryoprotectants, particularly at high concentrations. For all treatments and species the cleavage stage was the least tolerant to the traumas of cryoprotectants. Glycerol was the most toxic and embryos of all stages could not be revived from concentrations greater that 1M.

iv) The effect of the exposure of cleavage, epiboly and closure of the blastopore stages of zebra fish and rosy barb embryos to up to 4M Me_2SO and EG on two metabolic enzymes, LDH and G-6-PDH were investigated. Cryoprotectant concentration, equilibration period and developmental stage had a significant ($P<0.05$) effect on the total activity of both enzymes. In both species the decline in enzymatic activity was more pronounced for G-6-PDH and EG was more toxic than Me_2SO. For both

cryoprotectants the cleavage stage was the most sensitive.

v) The effects of the type and concentration of cryoprotectant on morphological characteristics were investigated. Three stages of embryos were subjected to 1M Me_2SO, EG, methanol and glycerol for up to of 3h. Six body parameters were measured at hatching. Type and concentration of cryoprotectant and embryonic stage, had a significant ($P < 0.05$) effect on body measurements but this varied between the two model species. Most cryoprotectants had an adverse affect on at least one morphological parameter. Methanol was least teratogenic and greatest effects were observed in hatchlings originating from cleavage stage embryos subjected to all cryoprotectants. The hatchlings originating from the embryos that were equilibrated for the longest period were grossly deformed. The pericardial cavity was enlarged and the spine severely deformed.

vi) The permeability parameters of embryos to water were measured and potential methods to manipulate them investigated. The effects of temperature (20, 25 & 30°C), dechorionation and electroporation on the rate constants, permeability coefficient, flux, Q_{10} and activation energy were determined. Higher temperatures significantly ($P < 0.05$) increased all permeability parameters in all embryonic stages. Permeability coefficient was highest for the heartbeat stage at 30°C. All subsequent trials were conducted at 30°C.

vii) Dechorionation significantly ($P < 0.05$) reduced the permeability parameters of all embryonic stages in both species except for the rate constant for the closure of blastopore stage of both species. In contrast electroporation significantly ($P < 0.05$) increased the permeability coefficient and flux of all embryonic stages of both species. Unfortunately, dechorionated embryos could not withstand electroporation and permeability characteristics could not be established.

viii) The influence of vacuum equilibration on the permeability of embryos was investigated. Equilibration of 4-cell stage rosy barb embryos under either normal, half and full vacuum conditions had no significant ($P > 0.05$) effect on either the permeability coefficient, flux or rate constant and was therefore of little value in facilitating successful cryopreservation.

ix) The osmotically active fraction of intracellular water in embryos was estimated using three methods; volume, mass and nuclear magnetic resonance spectroscopy (NMR).

The volume method could only be successfully used for the precleavage stage embryos. Embryos were dehydrated in up to 3M sucrose solutions for 30min and the total egg and yolk volume and mass changes estimated. At best the yolk volume of precleavage zebra fish and rosy barb could be reduced by 38 and 41 %, respectively. Dehydration of zebra fish and rosy barb precleavage embryos in 3M sucrose for 30 min removed 47.0 and 51.0 % of the internal mass of water, respectively. The volume of water that could be removed decreased with embryonic development. The non-active volumes of precleavage fish embryos determined in this study did not deviate noticeably from other successfully frozen animal embryos.

x) The effect of sucrose dehydration on pre-cleavage rosy barb embryos and their eventual hatch rate was investigated. The hatch rate of precleavage embryos dehydrated in 0.5 to 2.5M sucrose ranged between 80-99%. Dehydration in 3M sucrose reduced the hatch rate to 69%. Overall, sucrose dehydration did not noticeably reduce the viability of inseminated eggs.

xi) The survival rates of cleavage, epiboly and closure of the blastopore zebra fish embryos cooled from ambient to between 15 to 0°C and held at these target temperatures for up to 5 min were investigated. Survival ranged between 35-83% and varied with type of cryoprotectant and cooling rate. The order of cryoprotection offered by the cryoprotectants was Me_2SO > methanol > EG > glycerol. Poorest hatch rates were obtained for embryos equilibrated in EG and glycerol. Cooling pre heartbeat stage embryos below -5°C was fatal. Overall, these data suggest that depending on the type of cryoprotectant, cooling rate and embryonic stage, between 20-80% of mortality can be accounted for by cold shock and not freeze damage.

xii) Heartbeat stage embryos were most permeable to water and therefore this stage was selected to establish freeze tolerance of embryos. For both species there was a rapid decline in the survival of embryos cooled to below -10°C. Overall, zebra fish embryos were more resistant to damage than rosy barb. The degree of cryoprotection offered varied; the order of protection was Me_2SO> methanol> EG> glycerol. A reduction in temperature from -10 to -30°C significantly ($P<0.05$) reduced the survival of zebra fish and rosy barb embryos, cooling below -30°C was lethal to embryos of both species.

xiii) The incidence of intracellular ice formation (IIF) in epiboly rosy barb embryos cooled at 0.1, 0.5 and 1.0°C/min was observed using the cryomicroscope. The incidence

of IIF was dependant on the cooling rate between 0 and -4°C. The onset of IIF, however, was related to the type of cryoprotectant but none of the cryoprotectants prevented IIF below -20°C.

xiv) Isolated blastomeres from both species were successfully cryopreserved. Survival rate of isolated rosy barb blastomeres decreased significantly ($P < 0.05$) as the cooling rate was increased from 0.1 to 1.0°C/ min irrespective of the type of the cryoprotectant used. At the minimum and optimal cooling rate of 0.1°C/min rosy barb blastomeres cooled in 1M Me_2SO, methanol, EG, and glycerol showed survival rates of 65, 32, 23 and 16%, respectively.

xv) Heartbeat stage zebra fish and rosy barb embryos were vitrified. Vitrified embryos were morphologically intact on thawing but were non viable. Further studies are required to develop new vitrification solutions. Several trials were conducted to ascertain if vacuum equilibration could be applied to cryopreserve fish eggs. Unfortunately, although post-thaw embryos were intact none were viable.

xvi) Two papers were presented at the International Low Temperature Biology conference on embryo permeability and the determination of osmotically inactive water fraction. In addition, a paper on the effects of cryoprotectants on embryonic enzymes has now been published in Cryobiology.

xvii) The amount of water removed by sucrose dehydration was unexpectedly high and its implication for cryopreservations merits further investigation.

2. OBJECTIVES AS SET OUT IN THE PROJECT MEMORANDUM

i) Procurement of broodstock and gametes from selected model finfish species.

ii) Complete work started in previous project (R4523) on defining the importance of various parameters believed to be constraining the successful cryopreservation of fish eggs and embryos.

iii) Develop techniques to assess the proportion of bound water in the eggs of aquatic organisms.

iv) Assess prefreezing toxicity of a range of cryoprotectants to find less harmful materials and methods of administration of cryoprotectants.

v) Evaluate whether techniques, such as vacuum equilibration and vitrification, have potential for the cryopreservation of fish eggs.

vi) Conclude, definitatively, whether the technical limitations indicated above will allow fish egg conservation by cryopreservation to ultimately succeed in the foreseeable future or whether it will require further long term strategic research.

vii) Disseminate information about all aspects of the project to interested parties through a wide range of scientific and popular literature.

3. BACKGROUND AND RATIONALE

Gene banks for mammalian eggs and embryos and plant genetic resources are well established with clear roles in conservation and/or improvements in agricultural production. The conservation of aquatic genetic resources is less well defined but no less important. There is growing international concern over the rate of extinction of freshwater fish species. The limited geographic range of many species means that human developments even within a single drainage basin or lake may result in the extinction of a number of indigenous species.

The current technology used for the conservation of fishes is based on the cryopreservation of milt and still relies on the use of live fish banks to maintain the female genome with all of the concomitant problems of expense and inbreeding. The ability to freeze and store female gametes and diploid embryos as well as milt would provide an immense impetus for the establishment of the much needed fish gene banks in the developing countries in which many of these fish resources are threatened. This project therefore aims to investigate the fundamental constraints associated with the cryostorage of eggs and embryos.

Mammalian eggs and embryos are very small (20-80μm), lack any significant yolk reserves and have proved relatively easy to freeze. In contrast all attempts to date to cryopreserve fish eggs have been unsuccessful. The major obstacles are considered to be the relatively low surface area to volume ratio and low permeability of fish eggs and embryos to water and

cryoprotectants. The basic data required to evaluate the significance of each of these and other factors are limited and often available data on embryo permeability relate either to non-cryobiological studies or relate mainly to temperate species, notably the salmonids.

4. WORK CARRIED OUT DURING THIS PERIOD

4.1 Organisation of cryobiological studies

An understanding of the cryobiological problems associated with tropical fish eggs and embryos demands the segmentation of the entire cryopreservation protocol to establish the significance and constraints of each interrelated stage towards cryosuccess. Therefore, for this project the cryopreservation procedures were broadly segregated into two phases; precooling and post-cooling. The cryobiological factors and constraints influencing the preservation of embryos within each phase were evaluated. In the prefreezing phase suitable tropical fish models were identified, permeability of selected stages of embryos to water and the proportion of intracellular water established and possible methods that could influence water removal were investigated. The cooling phase was segmented to establish the significance and contribution of precooling toxicity of cryoprotectants, chill tolerance and freezing damage to overall cryosuccess.

4.2 Procurement of broodstock and gametes of selected model species

Model species with ideal biological and managerial characteristics were selected to ensure easy maintenance of the broodstock and continuous supply of relatively small sized eggs suitable for cryopreservation investigations. Earlier studies (R 4523) suggested that tilapias were a poor finfish model. Therefore in the present study, models with more suitable cryobiological characteristics were considered. Two smaller non-seasonal tropical fish species, zebra fish (*Brachydanio rerio*) and rosy barb (*Puntius conchonius*) were selected as model species. The quality and quantity of eggs from the short and long fin varieties of each species were compared and the best variety used as model species. The eggs of both species are relatively small (0.8-1.2 mm) compared with the tilapias (2-3mm) and salmonids (7-9mm) and their embryology is well documented.

4.2.1 Methods of procurement of gametes

For both species, techniques were successfully developed at Institute of Aquaculture(IOA) to procure gametes and embryos either by natural spawning (zebra fish) or manual stripping (rosy barb) .

To procure zebra fish embryos a prespawning female (extended belly) and three males were held overnight in a spawning tank with plastic arena plates. Following spawning the eggs and embryos were syphoned and incubated in filtered water in a sterile Petri dish at 28°C until required.

Available information on the artificial propagation of rosy barbs was inadequate and therefore *in vitro* techniques for manual stripping and fertilization were initially developed. Preliminary trials were successfully conducted on suitable sedation methods, milt collection techniques, identification and evaluation of suitable extenders for the short term storage of gametes and optimal *in vitro* fertilization and incubation techniques. The low fecundity of these species, however, necessitated the trials to be prolonged to complete all treatments within experiments.

4.3 Precooling toxicity of cryoprotectants to embryos

The use of cryoprotectants to ameliorate cryoinjuries is essential. These compounds, however, are toxic to biological systems and therefore the concentration finally used will be a compromise between their toxicity and the cryoprotection offered during cryopreservation. Moreover, in the case of fish eggs and embryos the use of slow cooling rates (0.01 - 2°C/min) increases the contact time between embryos and cryoprotectants. This in itself may be toxic and may reduce post-treatment survival. Therefore, to separate the effect of cryoprotectant toxicity and cooling injury the precooling toxicity of cryoprotectants to eggs and embryos was initially established. The effects of the mode of administration of dimethyl sulphoxide (Me_2SO), methanol, ethylene glycol (EG) and glycerol on endpoints such as osmotic damage, enzymatic activity, hatch rates and morphological deformities were successfully studied. The concentrations that were shown to be least harmful were used in subsequent trials.

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4.3.1 The effect of step-wise equilibration of cryoprotectant on the viability of embryos

introduction and removal of cryoprotectant and embryonic stage. In this section of the study, the effects of these factors on the survival of three stages of fish embryos were investigated. For both species, replicated samples of embryos at the cleavage, epiboly, and closure of the blastopore stages were exposed to final concentrations of either 1, 2 or 3M Me_2SO, methanol, EG and glycerol for 60min. Cryoprotectant addition and removal was made in up to 4 steps, each of equal cryoprotectant concentration and time. The scheme used throughout the study for the four step addition and removal is shown in Fig. 1. Following final rinsing the embryos were incubated and survival noted.

4.3.2 The effect of cryoprotectant on the activity of selected enzymes in embryos

In cryopreservation studies using fish embryos, the toxicity of cryoprotectants is often ascribed as mortality and therefore the nature of such toxicity is unknown (Fahy, 1984). Cryoprotectant induced-injury to biological systems could be caused either by osmotic, biochemical or physio-chemical (Fahy, 1983) effects. The separation of osmotic and biochemical components of toxicity will help to elucidate the nature of cryoprotectant toxicity. In the previous section it was demonstrated that by slowing the rate of cryoprotectant introduction and removal the osmotic stress can be significantly reduced and survival increased. In the present study the least and most toxic cryoprotectants were selected to evaluate the biochemical effects by monitoring intracellular enzymes and survival. Three stages of embryos were exposed to 1, 2, 3 and 4M Me_2SO and EG for up to 180 min and the levels of two enzymes, lactate dehydrogenase (LDH) and glucose-6-phosphate (G-6-PDH) were investigated. The LDH and G-6-PDH levels in replicate homogenate samples of 50 embryos/treatment were determined according to the method of Shaklee *et al.* (1974). Statistical interpretation of the effect of cryoprotectant concentration, equilibration time and embryonic stage on the total activity of the enzymes was conducted using a three way ANOVAR. This study has now been published as a full paper in the journal of Cryobiology.

4.3.3 The effect of cryoprotectant on the morphology of hatchlings

The cumulative osmotic and biochemical effect investigated in the preceding sections was extended to investigate the longer term effect of sublethal levels of cryoprotectant on the morphological characteristics of hatchlings using the least toxic cryprotectant concentration. The morphological characteristics of twenty zebra fish and rosy barb hatchlings which were equilibrated in a stepwise manner in up to 1M of either Me_2SO, EG, glycerol, or methanol

for 1, 2 and 3 h at the cleavage, epiboly and closure of blastopore stage were estimated. Immediately after hatching, the fry were preserved in buffered formalin and their standard length, yolk-sac width and depth, head width, eye diameter, body depth at the anus and pectoral fin measured and typical morphological abberations photographed. The effect of equilibration period with each cryoprotectant on each morphological character was evaluated using one way ANOVAR and multiple range test.

4.4 The effect of temperature and chorion manipulation (dechorionation, electroporation and vacuum equilibration) on the survival and permeability of embryos

The failure to cryopreserve fish eggs and embryos to date is attributed to the extremely low water permeability coefficient of fish embryos and their low surface to volume ratio. Therefore, one objective of this project was to determine the permeability characteristics of the model species and establish the extent to which these characteristics can be manipulated.

In the present study the potential for either manipulating temperature (20, 25 and 30°C) or using electroporation, dechorionation and vacuum equilibration to improve the permeability of three stages of zebra fish and rosy barb embryos was investigated. All replicated studies were conducted using tritiated water according to Motais and Isaia (1972) and permeability parameters such as the rate constant (K), permeability coefficient (P_{diff}), flux (k) and activation energy (E_a), were calculated using a computer software package (Enzfitter, Elsevier). Since the application of several of these techniques to alter the permeability of fish eggs and embryos to water were novel their effect on the tolerance of zebra fish and rosy barb embryos were initially established prior to using the defined sublethal conditions for determining their potential influence on permeability parameters of fish embryos. Where possible the permeability of intact and dechorionated embryos were estimated to help establish the significance of the different membrane barriers for successful cryopreservation.

Techniques for dechorionating embryos of the appropriate stages were successfully developed using trypsin and the permeability of intact and dechorionated embryos compared. Permeability measurements of dechorionated electroporated embryos were not possible. The severity of the high intensity electric pulse at the lowest possible setting of the electroporator (Bio-rad) was lethal.

There is anecdotal evidence to suggest that equilibration of fish eggs in cryoprotectants under

full vacuum (-760mm Hg) prior to cooling, facilitated successful cryopreservation of fish eggs (Leung and Jamieson, 1991) possibly by improving the permeability of the embryos to water and cryoprotectants. This hypothesis was tested for rosy barb 4-cell embryos using tritiated water. Embryos were equilibrated under normal, half and full vacuum conditions for up to 180min and permeability estimated together with parallel trials to establish the outcome of embryos cryopreserved following the above treatments.

4.5 Estimation of osmotically active and non-active water fractions in embryos

One of the major obstacles to successful fish egg and embryo cryopreservation is the inability to remove the high internal volume of water. It is suggested that most of the intracellular water (90% in salmonids) is in the bound or osmotically inactive form and therefore cannot be removed. This, however, may vary with embryonic development. To establish if this is the case for the model species used in this project, three aspects of the problem were investigated; suitable embryonic stages were identified, appropriate techniques for accurate estimation and comparison of internal water in fish embryos were evaluated and the proportion of osmotically inactive and active water was calculated.

The classical application of the Boyle van't Hoff relationship to estimate the osmotically active water in cleavage, epiboly and closure of the blastopore stage embryos was not feasible. The estimation of yolk volume from diameter measurements in these stages of embryos subjected to sucrose solutions (0-3M) was not possible due to the irregular shape of the dehydrated embryos. Therefore two other approaches were considered to measure internal water in post cleavage embryos; air drying to a constant weight and the development and use of NMR (nuclear magnetic resonance spectroscopy). The feasibility of measuring volume changes in earlier stages of embryos was investigated and the osmotically active fraction of intracellular water was estimated from volume changes in precleavage stage embryos.

To estimate the proportion of osmotically active water precleavage embryos were dehydrated in six concentrations of sucrose (0.5-3M) for up to 30min and either their diameters (total egg and yolk diameter) or dry weight estimated. For NMR studies, the pretreated (3M sucrose for 30min) precleavage, cleavage, epiboly and closure of the blastopore stage embryos were introduced into a 5mm diameter NMR reference tube and water content estimated using a Bruker WP80.

Internal water estimates from all three techniques were statistically compared using one way ANOVAR. The Boyle van't Hoff relationship for precleavage embryos was established by plotting the minimum relative changes in precleavage yolk volumes against the reciprocal of sucrose concentration. The proportion of osmotically inactive internal water was then extrapolated from the Y intercept. Data from the air drying and volume methods were used to calculate and compartmentalise the dry matter, removable and non-removal water in zebra fish and rosy barb precleavage embryos.

4.6 The effect of cold shock and subzero cooling on the survival of embryos

During cooling, eggs and embryos, notably of tropical species, may be susceptible to chill as well as freeze damage. Therefore to separate these effects the tolerance of three stages of embryos from both model species to pre and post-freezing temperatures were investigated. Embryos at the cleavage, epiboly and closure of the blastopore stages were equilibrated in 1M Me_2SO, methanol, EG and glycerol for 1 h and cooled at 1, 2 and 5°C/min to either 15, 10, 5, or 0°C and held at these target temperatures for 30min and then removed, warmed and incubated and hatch rate noted.

For post-freeze studies the heartbeat stage was selected because of its relatively higher permeability coefficient compared with cleavage, epiboly and closure of the blastopore. It was assumed that the higher permeability may result in greater dehydration during freezing. Heartbeat stage embryos were equilibrated in 1M Me_2SO, EG, methanol and glycerol and cooled at the lower rates of 0.1, 0.5 and 1.0°C/min to -6, -10, -15, -20 and -30 °C. The embryos were held for 5 min at these target temperatures and then warmed to room temperature at 25°C/min and incubated.

Cooling of heartbeat stage embryos to below -30°C was lethal. The target temperature and cooling rate at which freezing damage occurred could not be established using the conventional empirical approach of cooling in the programmable cooler. Therefore embryos were cooled on the cryomicroscope to enable freezing events to be monitored. Half epiboly stage of rosy barb and zebra fish embryos were cooled at 0.1, 0.5 and 1.0°C/min from 0 to -20°C and freezing events monitored. Incidence of intracellular ice formation (IIF), in the presence of 1M Me_2SO, EG, methanol and glycerol was noted.

4.7 The effect of cooling rate and subzero temperature on the survival of isolated blastomeres

Attempts to cryopreserve fish embryos during the past few years and in the present study have been unsuccessful. An alternative approach may be to cryopreserve embryonic cells. Chimeras have been produced in rainbow trout by transplanting post-thawed blastomeres into recipient blastulae in rainbow trout (Nilsson and Cloud, 1989, 1993) and zebra fish (Lin et al, 1992). In this study blastomeres of both model species were successfully isolated from half epiboly embryos and the effects of cooling rates (0.1, 0.5 and 1°C/min) and subzero target temperatures (-15, -20,- 30, -40 and -50°C) on the survival of blastomeres were investigated. In addition, zebra fish and rosy barb blastomeres were cooled at 0.1, 0.5 and 1.0 °C/min to -50°C in 1 M Me_2SO, EG, MET and glycerol, stored for 24h in LN, thawed and viability estimated. For all trials viability was estimated using the vital dye exclusion test.

4.8 Vitrification of heartbeat stage embryos

The failure of fish embryos to tolerate the traumas of cryopreservation is thought to be caused by long equilibration periods, low permeability, high surface to volume ratio, cold shock and freezing damage. An alternative approach used for cryopreserving mammalian embryos involves the equilibration and rapid cooling of embryos in highly concentrated aqueous solutions of cryoprotectant (up to 6M). The high viscosity causes the solidification of the aqueous medium without passing through the stage of ice formation, a process called vitrification.

In the present study, zebra fish and rosy barb embryos at heartbeat stage were vitrified in vitrification solutions (VSI) described by Rall and Fahy (1985) and post-thaw viability noted.

5. RESULTS OF FINDINGS OBTAINED BY THE PROJECT

5.1 Choice of model species and procurement of gametes and embryos

The shortfin varieties of zebra fish and rosy barb were eventually selected as model species.

Both species were relatively easy to maintain and relatively small size of their eggs and embryos made them very suitable for cryobiological observations. Gametes were obtained on demand although the small quantities of eggs limited the number of treatments that could be conducted at any one time. Long fin zebra fish embryos were easily obtained from natural spawning but in common with long fin rosy barbs these inbred varieties and stocks resulted in a high incidence of deformed fry. Therefore, for both species the short fin varieties were used.

Rosy barb embryos could be routinely obtained from manual stripping and fertilization. Physiological saline proved an effective extender and sperm could be held at 4°C and could be used for up to 4 days. Eggs, however, could only be stored for up to one hour. Fertilization rates of manually stripped and fertilized eggs were typically in excess of 90%.

5.2 Precooling toxicity of cryoprotectants to embryos

5.2.1 The effect of step-wise equilibration of cryoprotectants on the viability of embryos

The mode of administration, type and concentration of cryoprotectant and embryonic stage had a marked effect on the viability of embryos. Gradual step wise equilibration and gradual dilution significantly ($P<0.05$) increased the tolerance of embryos to cryoprotectants, particularly at high concentrations. For most treatments and species the cleavage stage was the least tolerant to the traumas of cryoprotectants. Glycerol was the most toxic cryoprotectant and embryos of all stages could not be revived from concentrations greater than 1M. Ethylene glycol was the second most toxic cryoprotectant and overall, rosy barb was the least tolerant of the two species. The hatch rate of embryos equlibrated in step-wise mode to a final concentration of 1, 2 and 3 M Me_2SO, methanol, EG and glycerol at the cleavage, epiboly and closure of the blastopore stages are presented in Fig. 2 A-D. Increasing the equilibration steps from 1 to 4 significantly ($P<0.05$) increased the hatch rate but the impact on survival varied between the cryoprotectants.

Comparing Me_2SO (Fig. 2A) and methanol (Fig. 2B) the former was more toxic at all concentrations but this was most noticeable at 3M concentration where no survival was recorded for Me_2SO except for 3-4 step equilibration. Increasing the number of steps from 1 to 3-4 Me_2SO significantly ($P<0.05$) increased the hatch rate (Fig. 2A). The improvement in hatch rate was correlated with developmental stage. The hatch rates of zebra fish embryos

fish embryos equilibrated in one 60 min step in 1, 2 and 3M Me_2SO were 80.0, 50.0 and 0.0% for cleavage and 82.0, 83.0 and 0.0% for epiboly stage and 93.0, 81.0 and 0.0% for the closure of the blastopore stages, respectively. Increasing the equilibration steps to four increased the hatch rates to 82.0, 73.0 and 13.8% for cleavage, and 90.0, 86.3 and 45.0% for epiboly stage and 92.5, 86.3 and 80.0 % for closure of the blastopore stages, respectively. For methanol, step-wise equilibration in 3M conferred an advantage only for the rosy barbs (Fig. 2B).

Ethylene glycol was highly toxic to cleavage embryos of both species (Fig 2C). In both species, with the exception of zebra epiboly stages, cleavage and epiboly stage embryos equilibrated in 3M EG failed to survive even after four 15 min step additions. In both species the resistance to EG toxicity increased with embryonic development. Rosy barb embryos showed higher resistance to EG toxicity than those of zebra fish.

The effect of step-wise addition of glycerol on the hatch rates of embryos of both species are presented in Fig. 2D. The concentration of glycerol used ranged between 0.25 and 1.0 M. In both species an increase in the number of equilibration steps for 1M glycerol from one 60 min step to two 30min steps did not reduce the mortality of fish embryos irrespective of stages of development. Embryonic stages from both species were equally sensitive to equilibration in glycerol.

5.2.2 The effect of cryoprotectant on the activity of selected enzymes in embryos

Slowing the rate of cryoprotectant introduction and removal, can significantly ameliorate cryoprotectant toxicity and increase hatch rate of embryos. In the present study, the effects of the exposure of cleavage, epiboly and closure of the blastopore stages of zebra fish and rosy barb embryos to up to 4M Me_2SO and EG on two metabolic enzymes, LDH and G-6-PDH, were investigated.

The results of this study are shown in Fig. 3A-D. Cryoprotectant concentration, equilibration period and developmental stage had a significant ($P < 0.05$) effect on the total activity of the metabolic enzymes. In both species the decline in enzymatic activity was more pronounced for G-6-PDH and EG was more toxic than Me_2SO. For both species the total LDH activity measured after 3.0h equilibration in 4.0M Me_2SO and EG declined sharply. Total LDH activity in zebra fish embryos at cleavage, epiboly and closure of the blastopore stages, following exposure to Me_2SO and EG, was reduced by 70.0 and 86.1%, 79.2 and 83.0% and

57.0 and 75.0% for the three embryonic stages, respectively. Under similar conditions the G-6-PDH (Fig. 3B) activity was reduced by 100 and 100%, 88.3 and 100% and 69.0 and 100%, respectively. Rosy barb embryos equilibrated in Me_2SO and EG under similar conditions showed a reduction in total activity of LDH by 89.2 and 98.8%, 85.0 and 87.1%, 80.6 and 95.4% (Fig. 3C) for the three embryonic stages, respectively. No G-6-PDH activity was recovered following equilibration of rosy barb embryos at the cleavage and epiboly stages. Closure of the blastopore stage embryos were most resistant and 29.8% of the total enzymatic activity was retained under the above conditions (Fig. 3D).

In a parallel experiment the longer term effect of enzymatic denaturation on the survival of zebra fish and rosy barb embryos was determined under similar conditions (Fig. 4A&B). The recovery of enzymatic activity was not co-related with subsequent survival (% hatch rate). Equilibration in 3 or 4M Me_2SO and EG for 2-3 h was lethal for all stages of embryos even though enzymatic activity was evident (see Fig. 3A-F).

All the embryonic stages were more tolerant to Me_2SO when compared with EG and this was most noticeable at the highest concentration. The hatch rates of zebra fish embryos which were equilibrated in 1, 2 and 3M Me_2SO at the cleavage, epiboly and closure of the blastopore stages were 82, 75 and 55% for cleavage and 88, 84 and 60 % for epiboly and 88, 90 and 62% for closure of blastopore. Equilibration in ethylene glycol under similar conditions resulted in a decline in hatch rate to 84, 58 and 0% for cleavage and 78, 59 and 0% for epiboly and 86, 60 and 0% for closure of the blastopore embryos. The tolerance of rosy barb cleavage embryos to both cryoprotectants was lower when compared with zebra fish. Ethylene glycol was lethal to cleavage stage embryos from both species following equilibration for more than 30 min at any concentration (Fig. 4A&B).

5.2.3 The effect of cryoprotectant on the morphology of hatchlings

The cumulative osmotic and biochemical effects investigated in the preceding sections were extended to investigate the longer term sublethal effect of cryoprotectants on the morphological characteristics of hatchlings.

Body measurements of zebra fish and rosy barb hatchlings originating from embryos subjected to 1M Me_2SO, EG, methanol and glycerol at the cleavage, epiboly and closure of the blastopore stage for up to 3 h are presented in Table 1 A-F. Methanol was the least teratogenic cryoprotectant and greatest abnormalities were observed in hatchlings originating

from embryos subjected to all cryoprotectants at the cleavage stage.

Body measurements of zebra fish hatchlings subjected to cryoprotectants at the cleavage stage embryos are presented in Table 1A. Equilibration of cleavage stage embryos in 1M Me_2SO for 3h, reduced the mean standard length, yolk-sac width and depth, head width and body depth at the anus and pectoral fin at hatching by 5.5, 12.8, 26.2, 31.7, 26.0 and 15.8%, respectively, whereas methanol reduced only the yolk-sac depth by 4.7%. The body measurements of zebra fish hatchlings subjected to cryoprotectants at the epiboly stage are presented in Table 1B. Equilibration in Me_2SO significantly ($P<0.05$) reduced the head width and body depth at the anus by 40.0 and 33.0%, respectively. Both methanol and glycerol had no significant ($P>0.05$) bearing on all body measurements. Ethylene glycol, however, significantly reduced head width by 26%. Equilibrating closure of the blastopore stage embryos in each cryoprotectant for 3h had no detrimental effect ($P>0.05$) on any of the hatchling body measurements (Table 1C).

Body measurements of rosy barb hatchlings originating from embryos exposed to 1M Me_2SO for three hours at the cleavage stage are presented in Table 1D. In common with zebra fish, cleavage stage rosy barb embryos were the most vulnerable developmental stage to any teratogenic effect of cryoprotectants. Dimethyl sulphoxide significantly ($P<0.05$) reduced the standard length, yolk-sac width and depth and body depth at the pectoral fin by 11.5, 20.0, 19.0 and 31.0%, respectively. Methanol equilibration for the same embryonic stage significantly ($P<0.05$) reduced standard length, yolk-sac width and depth, head width and body depth the at anus by 12.0, 26.0, 24.0, 28.0 and 23.0 %, respectively. Glycerol on the other hand, significantly ($P<0.05$) reduced standard length, yolk-sac width and depth, head width and body depth at the anus and pectoral fins by 14.4 , 24.0, 34.0, 30.0, 33.3 and 13.9%, respectively. The morphological characteristics of rosy barb hatchlings subjected to cryoprotectants at the epiboly stage are presented in Table 1E. Following an equilibration period of three hours in 1M Me_2SO the standard length, head width and body depth at the pectoral fin was significantly ($P<0.05$) reduced by 14.0, 6.4 and 24.0 %, respectively. Methanol, however, significantly ($P<0.05$) reduced only the head width by 51.0 % while ethylene glycol significantly ($P<0.05$) reduced the standard length, yolk-sac width and depth, head width and body depth at the anus and pectoral fin by 20.5, 11.1, 16.0, 34.0, 15.4 and 30.0 %, respectively. Glycerol on the other hand significantly ($P<0.05$) reduced yolk width and head width by 5.5 and 32.0 %, respectively. Body measurements of rosy barb hatchlings from embryos treated with the four cryoprotectants at the closure of the blastopore stage are shown in Table 1F. The body measurement of hatchlings originating from embryos treated with 1M Me_2SO at the closure of the blastopore stage showed a significant ($P<0.05$)

reduction in head width, body depth at the anus and body depth at the pectoral fins by 30.0, 29.0 and 21.0 %, respectively. Methanol significantly ($P<0.05$) reduced head width and body depth at the anus by 20.5 and 25.0%, respectively. Ethylene glycol, significantly ($P<0.05$) reduced head width and body depth at the anus by 30.0 and 36.0 %, respectively. Glycerol on the other hand significantly ($P<0.05$) reduced standard length, head width and body depth at the anus by 11.7, 22.7 and 32.0%, respectively.

Morphological abberations are also presented photographically (Fig. 5 i-v). Hatchlings from cleavage embryos exposed to both cryoprotectants showed a clear reduction of the standard length when compared with the control (Fig. 5i). In addition, the pericardial cavity of hatchlings was abnormally enlarged by 25% when equilibrated at the cleavage stage for 2h in Me_2SO (Fig. 5ii) and the extent of enlargement of the pericardial cavity doubled when the equilibration period was increased by an hour (Fig. 5iii). Deformation of the spinal cord was also evident in many hatchlings. The effect of 0.25-1.0M EG on the gross morphology of hatchlings under comparable conditions were more dramatic (Fig. 5iv-v). The majority of embryos equilibrated in EG for 2h failed to hatch. In those specimens that did hatch the pericardial cavity was grossly enlarged and the spinal cord severely deformed (Fig. 5iv). Although most of the embryos were alive, the deformations were so extensive that their normal shape was beyond recognition (Fig. 5v).

5.3 The effect of temperature and chorion manipulation (dechorionation, electroporation and vacuum equilibration) on the survival and permeability of embryos

5.3.1 The effect of dechorionation and electroporation on the survival of embryos

Various stages of embryos were dechorionated using either trypsin, pancreatin or protease at up to 2.5 mg/ml for up to 5 min. Due to rapid polymerisation of the chorion in water only embryos up to the four cell stage could be successfully dechorionated. The average yield of dechorionated four cell embryos using trypsin digestion was significantly ($P<0.05$) higher than those from either pancreatin or protease digestion. The digestion process had no obvious detrimental effect on the embryos. At the maximum concentration a 5 min trypsin digestion yielded over 90% hatch rate (Fig. 6a&b). Trypsin digestion was therefore used in subsequent treatments.

The viability of embryos electroporated (embryos subjected to a momentary electric pulse) at the three developmental stages is presented in Fig. 7. The hatch rate of three embryonic stages in both fish species were not significantly ($P>0.05$) different at all field strengths. Maximum hatch rate of embryos subjected to a field strength of 500 v/cm and capacitance of 0.25μF at the cleavage, epiboly at the closure of blastopore stage were 47.0, 56.0, 60.0% and 56.0, 55.0, 66.0% for zebra fish and rosy barb, respectively (Fig. 7a&b). Several attempts to electroporate dechorionated embryos at all developmental stages were unsuccessful.

5.3.2 The effect of temperature on water permeability parameters of intact embryos

The effects of temperature (20, 25 and 30°C) on mean rate constant of influx (K), permeability coefficient (P_{diff}) and the flux for cleavage, epiboly, closure of blastopore and heartbeat stages of zebra fish and rosy barb are shown in Table 2.

For all embryonic stages the permeability parameters increased significantly ($P<0.05$) with temperature. Overall, for both species all the permeability parameters decreased from the cleavage to the closure of the blastopore stage and then increased dramatically at the heartbeat stage. At 30°C for example, the permeability coefficients for zebra fish and rosy barb embryos at the cleavage, epiboly, closure of the blastopore and heartbeat stage, respectively, were 24.5, 11.1, 5.0 and 17.6μm/sec and 12.5, 11.6, 5.4 and 12.2 μm/sec.

The activation energy (E_a) of the cleavage, epiboly, closure of the blastopore and heartbeat stage rosy barb and zebra fish embryos were, respectively, 11.0 and 27.4, 15.3 and 14.8, 9.0 and 9.1 and 10.0 and 13.6 Kcal/mol. The average activation energy for the developmental period from cleavage to heartbeat stage was 16.3 ± 3.9 and 11.3 ± 1.4 Kcal/mol for rosy barb and zebra fish, respectively (Fig. 8a&b).

The permeability of fish embryos was very low when compared with mammalian embryos. Overall, highest permeability was achieved at 30°C. This equilibration temperature was therefore used in all further attempts at manipulating the membrane permeability of embryos.

5.3.3 The effect of dechorionation and electroporation on the permeability of embryos

The effects of dechorionation and electroporation on the permeability parameters were

investigated for the cleavage, epiboly and closure of blastopore stages.

Mean permeability parameters for the different stages of embryos from both species are presented in Table 3. Dechorionation significantly ($P<0.05$) reduced all the permeability parameters of all embryonic stages in both species except for the rate constant for the closure of the blastopore stages of both species. In contrast electroporation significantly ($P<0.05$) increased the permeability coefficient and flux of all embryonic stages of both species. The impact of both these manipulation techniques can be more easily seen in Fig. 9. The higher permeability of intact electroporated eggs was probably due to improved permeability of the chorion rather than any significant increase in the permeability of the vitelline membrane. Unfortunately, dechorionated embryos could not withstand electroporation and permeability characteristics could not be established.

5.3.4 The effect of vacuum equilibration on the permeability parameters of 4-cell stage rosy barb embryos

Equilibration of 4-cell stage embryos under normal, half and full vacuum conditions had no significant ($P>0.05$) effect on either the permeability coefficient, flux or rate constant. The volumes of tritiated water imbibed over the time course of 120 min are shown in Fig. 10a and the logarithmic transformation for these time courses is shown in Fig. 10b. Equilibration period had a highly significant ($P<0.001$) effect on the volume of imbibed water during the time course of the experiment.

5.4 Estimation of osmotically active and non-active water in precleavage embryos using various techniques

Three methods were investigated for quantifying intracellular water of fish embryos; volumetric method, air drying and NMR.

Due to the irregular shape of advanced fish embryos during dehydration the volume technique could only be applied to precleavage embryos. There were no significant ($P>0.05$) differences between the yolk volume estimated from direct microscopic measurements of egg diameters and those estimated from video taped images of embryos. Since the video technique was easier to use this method was adopted throughout the investigation.

The volume of the yolk sphere, whole embryo and perivitelline space changed to a constant magnitude within 30 min of dehydration (Fig. 11a&b). The minimum relative yolk volume attained in embryos of both species were regressed with the reciprocal of the measured osmolality to calculate osmotically inactive yolk volume. Embryos from both species obeyed ($r = 0.922$ and 0.95 for rosy barb and zebra fish precleavage embryos, respectively) the Boyle van't Hoff relationship (Fig. 12a&b). The Ponder's R value for zebra fish and rosy barb precleavage embryos were 0.38 and 0.41, respectively. The above data suggest that at best the yolk volume of zebra fish and rosy barb embryos could be reduced by a maximum of 38 and 41%, respectively. It is generally assumed that this reduction in volume is attributable to water loss. To estimate if such volume changes represented water loss, volume changes were compared with those estimated by the air drying mass technique.

The mass and NMR techniques were applied to estimate the osmotically inactive fraction of intracellular water of advanced embryos. The mass of water remaining in precleavage embryos following dehydration in up to 3M sucrose is shown in Fig. 13a&b. The volume changes in both species responded in a similar manner. Increasing the concentration of sucrose significantly ($P < 0.05$) increased the mass of water removed. Dehydration of zebra fish and rosy barb precleavage embryos in 3M sucrose for 30 min removed 47.0 and 51.0% of the internal water, respectively.

The NMR technique was used to determine the water content of sucrose dehydrated precleavage, cleavage, epiboly and closure of blastopore stage embryos of both species. A comparison of all three techniques for estimating internal water volumes of precleavage embryos is presented in Fig. 14. For both species the trends in the relative changes in volume and mass estimated from the volumetric and air drying techniques were similar.

The maximum water removed as estimated by the volume, mass and NMR techniques was 36, 47 and 64% for zebra fish and 42, 51 and 50% for rosy barb precleavage embryos, respectively.

The amount of water that could be removed from more advanced embryos was lower. Water content in zebra fish and rosy barb embryos subjected to sucrose dehydration at cleavage, epiboly and closure of blastopore stage determined by either air drying or NMR are shown in Fig. 15. Water content of zebra fish embryos determined by NMR and air drying techniques were, respectively, 36 and 33% for the cleavage, 33 and 33% for the epiboly and 32 and 32 % for the closure of the blastopore stages. Similarly, for rosy barb embryos the volume removed by the two techniques were, respectively, 24 and 39% for the cleavage, 24

and 37% for the epiboly and 22 and 36.0 % for the closure of the blastopore stages.

In the present study the air drying technique was used to estimate the amount of dry matter, removable and unremovable water in precleavage embryos of zebra fish and rosy barb embryos. In zebra fish precleavage embryos the fraction of removable, unremovable water and the dry matter represented 34.3, 38.6 and 27.1% of the total mass of the embryo, respectively. While in rosy barb precleavage embryos the amount of removable, unremovable water and dry matter were 37.4, 35.9 and 26.7%, respectively (Fig. 16a&b).

In this study 47 and 51% of the total internal water mass was successfully removed from zebra fish and rosy barb precleavage embryos, respectively, and the survival of such embryos was in excess of 68%. These values are considerably higher than those reported for other species. In salmonid embryos the whole embryo was reported to represent the osmotically non-active volume (Zotin, 1965). Similarly, in neural stage sturgeon embryos this was 92.2% (Zotin, 1965). Interestingly, the osmotically non-active water volumes of precleavage embryos from the present study did not deviate noticeably from other systems that have been successfully frozen. Drosophila (Steponkus et al., 1990) and sea urchin (Asahina and Takahashi, 1978) embryos and marine rotifers (Lubzens et al.,1992) which have been successfully cryopreserved have similar non-osmotic volumes to precleavage zebra fish and rosy barb embryos, yet these could not be successfully cryopreserved.

Following the above trials on precooling aspects studies were focused on establishing the response and behaviour of embryos during cooling.

5.5 The effect of cold shock and subzero cooling on the survival of embryos

5.5.1 Cold shock

The chill tolerance of the two tropical model species was studied using sublethal precooling conditions established in the above trials. The hatch rates of embryos cooled at various rates and to different prefreezing temperatures at the cleavage, epiboly and closure of the blastopore stage are summarised in Table 4 A&B. The cryoprotectant type, embryonic stage and cooling rate influenced the survival of embryos. Embryos from both species were less sensitive to cold shock injury when cooled in the presence methanol.

Hatch rates of zebra fish embryos cooled in methanol from room temperature to 0°C at a

maximum cooling rate of 5.0°C/min at the cleavage, epiboly and closure of blastopore stages were reduced to 60, 45 and 60%, respectively. Glycerol offered the least cryoprotection. In the presence of glycerol survival was reduced to 35, 38 and 40 %, respectively (Fig. 17).

For rosy barb the hatch rates of embryos cooled in cryoprotectants at the cleavage, epiboly and closure of the blastopore stages were reduced to 58, 64 and 67% with methanol; 52, 59 ; 59% with Me_2SO; 45, 48 and 42% in EG and 38, 31 and 32% in glycerol, respectively (Table 4B).

Overall, these data suggest that depending on the type of cryoprotectant, cooling rate and embryonic stage used between 20-80% of mortality can be accounted for by cold shock and not freeze damage.

5.5.2 Freeze damage

The permeability coefficient of the heartbeat stage embryos was the highest when compared with cleavage, epiboly and closure of the blastopore stages (see Table 2). Therefore this stage was selected for subzero cooling trails.

Survival rates of post-thaw heartbeat stage embryos cooled to various target temperatures are presented in Fig. 16A&B. For both species there was a rapid decline in survival of embryos cooled below -10°C and overall, zebra fish embryos (Fig. 17A) were more resistant to damage than rosy barb (Fig. 17B) at similar subzero temperatures. The degree of cryoprotection conferred varied between the cryoprotectants; the order of protection was Me_2SO > methanol > EG > glycerol. Zebra fish heartbeat stage embryos cooled at 0.1, 0.5 and 1.0°C/min to -20°C in the presence of 1M Me_2SO exhibited survival rates of 27.0, 18.0 and 14.0 %, respectively. Rosy barb heartbeat stage embryos cooled at 0.1 and 0.5° C/min in 1M glycerol failed to survive below -15°C and at 1.0°C/min embryos failed to survive below -10°C. .

The reduction of temperature from -10 to -30°C significantly ($P < 0.05$) reduced the survival of zebra fish and rosy barb embryos, cooling below -30°C, was lethal for embryos of both species.

5.5.3 Cryomicroscopical observation of intracellular ice formation (IIF) in epiboly stage embryos

Cryomicroscopy was used to observe the freezing events of half epiboly stage embryos cooled at various rates in 4 cryoprotectants (Table 5 A&B). Increasing the cooling rate from 0.1 to 1.0 °C/min and reducing the subzero temperature from 0 to -20°C significantly ($P < 0.05$) increased the number of zebra fish and rosy barb embryos that froze. Between 0 to -4°C the number of frozen embryos depended on the cooling rate. None of the cryoprotectants prevented intracellular ice formation beyond -20°C.

5.6 The effect of cooling rate and subzero temperature on the survival of isolated blastomeres

5.6.1 Subzero cooling

Attempts to cryopreserve fish embryos in the present study were unsuccessful. An alternative approach may be to preserve isolated blastomeres.

The survival rates of post-thaw zebra fish and rosy barb blastomeres cooled in the presence of 1M Me_2SO, methanol, ethylene glycol and glycerol and cooled to -15, -20, -30, -40 and -50°C are presented in Fig. 18A&B. Blastomeres isolated from rosy barb embryos were more sensitive to cooling than those from zebra fish but cryoviability varied with cryoprotectants. In common with whole embryos glycerol afforded the least protection against freeze injury. There was an inverse relationship between the cooling rate and survival of blastomeres. Increasing the cooling rate from 0.1 to 1.0 °C/min significantly ($P < 0.05$) reduced the survival of the blastomeres. One-way analysis of variance showed that the survival rates of zebra fish blastomeres cooled at 0.1 C°/min in the presence of Me_2SO were not significantly ($P < 0.05$) different at all the subzero temperatures.

5.6.2 Post-thaw viability of blastomeres stored in LN

Post-thaw viabilities of zebra fish and rosy barb blastomeres cooled at 0.1, 0.5 and 1.0°C/min in 1M Me_2SO, methanol, EG, and glycerol and stored in LN are shown in Fig. 19A&B. Survival rates of blastomeres from both species were significantly ($P < 0.05$) reduced as the cooling rate was increased from 0.1 to 1.0°C/ min irrespective of the type of cryoprotectant used. The type of cryoprotectant significantly ($P < 0.05$) influenced the survival rate of the blastomeres from both species. Blastomeres cooled in the presence of

Me_2SO, gave a higher survival rate than those cooled in the presence of either methanol, EG or glycerol. At the minimum cooling rate of 0.1°C/min, 82 and 65, 42 and 32, 24 and 23 and 20 and 16 % of the post-thaw zebra fish and rosy barb blastomeres cooled in 1M Me_2SO, methanol, EG and glycerol, respectively, were viable.

5.7 Vitrification of heartbeat stage embryos

In the present study heartbeat stage zebra fish and rosy barb embryos were vitrified in vitrification solutions (VSI) described by Rall and Fahy (1985). The results to date have shown that vitrified embryos were recovered intact morphologically although they failed to survive. Further studies are required to develop new vitrification solutions.

5.8 Post-thaw viability of vacuum equilibrated embryos

Several trials were conducted to ascertain if vacuum equilibration could be applied to cryopreserve fish eggs. Unfortunately, although post-thaw embryos were intact none were viable.

5.9 Dissemination of information

Two papers were presented at the International Low Temperature Biology meeting on embryo permeability and the determination of osmotically inactive water fraction (Appendix 1). In addition the paper on the effects of cryoprotectants on embryonic enzymes has now been published in journal of Cryobiology (Appendix 1).

During the term of this project 2 postgradute (1 MSc and 1 PhD) students researched aspects of preservation technology of fish eggs.

6. IMPLICATION OF RESULTS

The toxicity of cryoprotectants to eggs and embryos varied with the type and concentration of cryoprotectant, species and their embryonic stage. Given the long equilibration times necessary for fish embryos the appropriate selection and use of cryoprotectant needs to be

considered to eliminate or minimise this variable when evaluating problems related to cooling of eggs and embryos.

Overall, the results of the present studies suggest that each stage of the cryopreservation procedure contributes to the gradual decline in embryo viability. It is often suggested that the combination of minimal dehydration and poor penetration of cryoprotectants are the key causes of embryo death during cryopreservation. Our studies suggest that whilst this contributes to the fatality of post-thaw embryos, there is a gradual loss in viability during the entire cooling protocol and depending on embryonic stage and cryoprotectant type and concentration used between 20-80% the embryo mortality occurred before freezing. Therefore loss in post-thaw embryo viability due to chill shock is an equally important consideration and emphasis should also be placed on methods for ameliorating chill damage. In this study, earlier embryonic stages were shown to be more sensitive to subzero temperatures. For the closure of the blastopore stage embryos, -5°C was fatal suggesting that total damage had occurred just prior to freezing.

The permeability characteristics of the most permeable embryonic stage suggests that vacuum equilibration has no effect on the permeability of embryos to water and consequently offers no advantage in manipulating the dehydration and penetration of cryoprotectants.

The amount of internal water of fish embryos suggest that, contrary to several published reports, a significant proportion of the internal water is osmotically active and can be removed. To date we have demonstrated that around 50% of the internal water is osmotically active and can be removed. The comparison of air drying and volumetric techniques confirm that changes in yolk volume were in fact due to water removal. The inability to demonstrate such high levels of osmotically active water in the past by several researchers may be related to the embryonic stage used for such studies.

Although the freezing point of the dehydrated embryos in the present study were lowered, the embryos still froze internally. Nevertheless, it is emphasised that the non-active volumes of precleavage fish embryos determined in this study did not deviate noticeably from other systems that have been successfully frozen. Drosophila (Steponkus et al., 1990) and sea urchin (Asahina and Takahashi, 1978) embryos and marine rotifers (Lubzens et al., 1992) which have been successfully cryopreserved have similar non-osmotic volumes to precleavage zebra fish and rosy barb embryos. Given these results we conclude that although we have not successfully cryopreserved embryos during this project the removal of about half the water from precleavage embryos and the recovery of intact embryos following unsuccessful vitrification increases the probability of successful cryopreservation in the near future. The

rate at which this goal will be reached will depend on the availability of longer term funding for such studies.

APPENDICES

Appendix 1. Meetings attended and papers presented and published

Meetings attended:

Dr Rana attended the International Low Temperature Biology Meeting in Leuven, Belgium, July 1994 and The Society of Low Temperature Biology meeting in London, November 1994.

Papers presented and published:

Rana, K. J. (1994) The cryopreservation of pre-and post fertilised non-mammalian oocytes SLTB symposium- Cryopreservation of oocytes.The Royal Veterinary College. November, London.

Adam. M., Rana, K. J. and McAndrew, B. J. (1995) The effect and temperature chorion manipulation on the permeability of rosy barb and zebra fish embryos. Cryo-letters 16, 65.

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Appendix 3 Tables

Table 1A The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of zebra fish (*Brachydanio rerio)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the cleavage stage.

Cryo*.	Period (h)	Standard Length length	Yolk-sac		Head width	Eye diameter	Body depth at	
			Width	Depth			Anus	Pectoral fin
Control		3.45 ± 0.09[a]	1.41 ± 0.60[a]	0.42 ± 0.05[a]	0.41 ± 0.04[a]	0.25 ± 0.02[a]	0.23 ± 0.03[a]	0.57 ± 0.06[a]
D	1	3.37 ± 0.06[ab]	1.35 ± 0.05[ab]	0.37 ± 0.04[ab]	0.36 ± 0.03[ab]	0.24 ± 0.03[a]	0.19 ± 0.02[ab]	0.53 ± 0.03[ab]
D	2	3.33 ± 0.07[ab]	1.27 ± 0.12[bc]	0.34 ± 0.04[b]	0.33 ± 0.06[bc]	0.23 ± 0.03[a]	0.18 ± 0.03[ab]	0.50 ± 0.07[ab]
D	3	3.26 ± 0.14[c]	1.23 ± 0.08[c]	0.31 ± 0.07[b]	0.28 ± 0.05[c]	0.23 ± 0.03[a]	0.17 ± 0.03[c]	0.48 ± 0.10[c]
M	1	3.44 ± 0.18[a]	1.42 ± 0.17[a]	0.44 ± 0.07[ab]	0.45 ± 0.19[a]	0.25 ± 0.04[a]	0.24 ± 0.06[a]	0.57 ± 0.10[a]
M	2	3.39 ± 0.20[a]	1.39 ± 0.18[a]	0.42 ± 0.05[ab]	0.42 ± 0.10[a]	0.25 ± 0.04[a]	0.22 ± 0.08[a]	0.57 ± 0.14[a]
M	3	3.38 ± 0.33[a]	1.38 ± 0.05[a]	0.40 ± 0.10[c]	0.14 ± 0.12[a]	0.25 ± 0.03[a]	0.24 ± 0.06[a]	0.56 ± 0.13[a]
E	1	3.39 ± 0.12[a]	1.37 ± 0.06[a]	0.38 ± 0.04[ab]	0.40 ± 0.04[a]	0.24 ± 0.03[a]	0.22 ± 0.04[a]	0.52 ± 0.06[a]
E	2	3.36 ± 0.10[a]	1.34 ± 0.07[a]	0.35 ± 0.05[c]	0.36 ± 0.05[a]	0.24 ± 0.02[a]	0.18 ± 0.02[b]	0.47 ± 0.02[ab]
E	3	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
G	1	3.41 ± 0.75[a]	1.40 ± 0.31[a]	0.42 ± 0.10[a]	0.38 ± 0.11[a]	0.23 ± 0.06[a]	0.23 ± 0.06[a]	0.60 ± 0.14[a]
G	2	3.38 ± 0.79[a]	1.40 ± 0.32[a]	0.41 ± 0.10[a]	0.37 ± 0.08[a]	0.24 ± 0.06[a]	0.23 ± 0.05[a]	0.59 ± 0.13[a]
G	3	3.31 ± 0.77[a]	1.37 ± 0.32[a]	0.38 ± 0.10[a]	0.35 ± 0.08[a]	0.24 ± 0.06[a]	0.23 ± 0.05[a]	0.57 ± 0.13[a]

[1]Means based on 10 individual hatchlings.

Means with similar letters within each column for each cryoprotectant are not significantly different(P>0.05)

*Cryoprotectants: D=Dimethyl sulphoxide, M=Methanol, E= Ethylene glycol, G= Glycerol

Table 1B The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of zebra fish (*Brachydanio rerio)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the epiboly stage.

Cryo*.	Period (h)	Standard Length length	Yolk-sac		Head width	Eye diametr	Body depth at	
			Width	Depth			Anus	Pectoral fin
Control		3.47 ± 0.21[a]	1.41 ± 0.05[a]	0.40 ± 0.09[a]	0.43 ± 0.07[a]	0.23 ± 0.03[a]	0.24 ± 0.07[a]	0.56 ± 0.09[a]
D	1	3.36 ± 0.32[a]	1.41 ± 0.19[a]	0.39 ± 0.05[a]	0.37 ± 0.06[a]	0.25 ± 0.04[a]	0.23 ± 0.03[a]	0.57 ± 0.09[a]
D	2	3.34 ± 0.32[a]	1.42 ± 0.20[a]	0.40 ± 0.05[a]	0.36 ± 0.07[a]	0.24 ± 0.04[a]	0.22 ± 0.03[ab]	0.55 ± 0.08[a]
D	3	3.31 ± 0.21[a]	1.38 ± 0.17[a]	0.28 ± 0.06[a]	0.26 ± 0.04[b]	0.24 ± 0.02[a]	0.16 ± 0.03[c]	0.45 ± 0.05[a]
M	1	3.47 ± 0.17[a]	1.40 ± 0.09[a]	0.38 ± 0.05[a]	0.40 ± 0.04[a]	0.23 ± 0.02[a]	0.24 ± 0.08[a]	0.56 ± 0.07[a]
M	2	3.46 ± 0.21[a]	1.38 ± 0.28[a]	0.40 ± 0.13[a]	0.38 ± 0.07[a]	0.24 ± 0.03[a]	0.24 ± 0.10[a]	0.54 ± 0.10[a]
M	3	3.45 ± 0.28[a]	1.37 ± 0.09[a]	0.40 ± 0.06[a]	0.38 ± 0.07[a]	0.23 ± 0.02[a]	0.22 ± 0.06[a]	0.54 ± 0.13[a]
E	1	3.35 ± 0.27[a]	1.38 ± 0.07[a]	0.40 ± 0.07[a]	0.37 ± 0.04[ab]	0.23 ± 0.03[a]	0.23 ± 0.03[a]	0.52 ± 0.08[a]
E	2	3.30 ± 0.32[a]	1.35 ± 0.08[a]	0.36 ± 0.10[a]	0.35 ± 0.05[b]	0.23 ± 0.04[a]	0.23 ± 0.03[a]	0.50 ± 0.07[a]
E	3	3.19 ± 0.38[a]	1.33 ± 0.07[a]	0.34 ± 0.10[a]	0.32 ± 0.06[b]	0.23 ± 0.04[a]	0.20 ± 0.04[a]	0.46 ± 0.09[a]
G	1	3.38 ± 0.29[a]	1.41 ± 0.12[a]	0.43 ± 0.07[a]	0.40 ± 0.07[a]	0.24 ± 0.03[a]	0.23 ± 0.04[a]	0.53 ± 0.07[a]
G	2	3.35 ± 0.29[a]	1.39 ± 0.12[a]	0.43 ± 0.13[a]	0.39 ± 0.06[a]	0.24 ± 0.02[a]	0.22 ± 0.06[a]	0.54 ± 0.09[a]
G	3	3.30 ± 0.29[a]	1.35 ± 0.07[a]	0.39 ± 0.10[a]	0.37 ± 0.08[a]	0.24 ± 0.02[a]	0.21 ± 0.04[a]	0.51 ± 0.13[a]

[1]Means based on 10 individual hatchlings.
Means with similar letters within each column for each cryoprotectant are not significantly different($P>0.05$).
*Cryoprotectants: D=Dimethyl sulphoxide M=Methanol, E= Ethylene glycol, G= Glycerol

Table 1C The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of zebra fish (*Brachydanio rerio)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the closure of the blastopore stage.

Cryo*.	Period (h)	Standard Length	Yolk-sac		Head width	Eye diameter	Body depth at	
		length	Width	Depth			Anus	Pectoral fin
Control		3.46 ± 0.76[a]	1.37 ± 0.30[a]	0.38 ± 0.10[a]	0.37 ± 0.09[a]	0.23 ± 0.05[a]	0.24 ± 0.07[a]	0.55 ± 0.09[a]
D	1	3.44 ± 0.20[a]	1.37 ± 0.16[a]	0.40 ± 0.06[a]	0.38 ± 0.08[a]	0.25 ± 0.05[a]	0.23 ± 0.06[a]	0.55 ± 0.09[a]
D	2	3.41 ± 0.22[a]	1.35 ± 0.08[a]	0.39 ± 0.09[a]	0.37 ± 0.08[a]	0.25 ± 0.05[a]	0.22 ± 0.04[a]	0.54 ± 0.07[a]
D	3	3.39 ± 0.23[a]	1.31 ± 0.12[a]	0.37 ± 0.07[a]	0.36 ± 0.07[a]	0.25 ± 0.07[a]	0.21 ± 0.02[a]	0.53 ± 0.09[a]
M	1	3.47 ± 0.76[a]	1.37 ± 0.34[a]	0.38 ± 0.12[a]	0.24 ± 0.07[a]	0.24 ± 0.07[a]	0.23 ± 0.06[a]	0.55 ± 0.12[a]
M	2	3.46 ± 0.75[a]	1.35 ± 0.29[a]	0.34 ± 0.11[a]	0.33 ± 0.09[a]	0.23 ± 0.05[a]	0.24 ± 0.07[a]	0.53 ± 0.12[a]
M	3	3.38 ± 0.11[a]	1.34 ± 0.19[a]	0.34 ± 0.11[a]	0.34 ± 0.08[a]	0.24 ± 0.02[a]	0.23 ± 0.06[a]	0.53 ± 0.13[a]
E	1	3.36 ± 0.21[a]	1.37 ± 0.17[a]	0.36 ± 0.05[a]	0.38 ± 0.05[a]	0.26 ± 0.04[a]	0.22 ± 0.03[a]	0.52 ± 0.14[a]
E	2	3.34 ± 0.35[a]	1.35 ± 0.13[a]	0.35 ± 0.05[a]	0.36 ± 0.04[a]	0.26 ± 0.05[a]	0.17 ± 0.06[a]	0.49 ± 0.13[a]
E	3	3.19 ± 0.35[a]	1.31 ± 0.09[a]	0.32 ± 0.06[a]	0.33 ± 0.07[a]	0.26 ± 0.05[a]	0.19 ± 0.02[a]	0.47 ± 0.12[a]
G	1	3.38 ± 0.75[a]	1.37 ± 0.30[a]	0.40 ± 0.10[a]	0.38 ± 0.09[a]	0.23 ± 0.06[a]	0.20 ± 0.05[a]	0.51 ± 0.13[a]
G	2	3.38 ± 0.75[a]	1.37 ± 0.30[a]	0.40 ± 0.10[a]	0.38 ± 0.09[a]	0.23 ± 0.06[a]	0.20 ± 0.05[a]	0.51 ± 0.13[a]
G	3	3.35 ± 0.25[a]	1.34 ± 0.11[a]	0.37 ± 0.07[a]	0.36 ± 0.06[a]	0.24 ± 0.02[a]	0.23 ± 0.03[a]	0.51 ± 0.05[a]

[1]Means based on 10 individual hatchlings.
Means with similar letters within each column for each cryoprotectant are not significantly different(P>0.05).
*Cryoprotectants: D=Dimethyl sulphoxide, M=Methanol, E= Ethylene glycol, G= Glycerol

Table 1D The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of rosy barb (*Puntius conchonius)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the cleavage stage.

Cryo*.	Period (h)	Standard length	Yolk-sac		Head width	Eye diameter	Body depth at	
			Width	Depth			Anus	Pectoral fin
Control		3.40 ± 0.06[a]	1.70 ± 0.10[a]	0.58 ± 0.04[a]	0.46 ± 0.04[a]	0.22 ± 0.02[a]	0.30 ± 0.05[a]	0.72 ± 0.08[a]
D	1	3.19 ± 0.33[ab]	1.57 ± 0.06[ab]	0.55 ± 0.09[ab]	0.42 ± 0.06[a]	0.20 ± 0.02[a]	0.29 ± 0.07[a]	0.61 ± 0.06[b]
D	2	3.09 ± 0.31[ab]	0.43 ± 0.27[b]	0.53 ± 0.13a[b]	0.39 ± 0.08[a]	0.19 ± 0.02[a]	0.27 ± 0.05[a]	0.58 ± 0.06[bc]
D	3	3.01 ± 0.38[c]	1.36 ± 0.18[b]	0.47 ± 0.04[b]	0.37 ± 0.06[a]	0.19 ± 0.02[a]	0.27 ± 0.05[a]	0.50 ± 0.09[c]
M	1	3.02 ± 0.71[ab]	1.27 ± 0.32[b]	0.45 ± 0.11[b]	0.36 ± 0.09[b]	0.21 ± 0.05[a]	0.22 ± 0.05[b]	0.68 ± 0.16[a]
M	2	3.00 ± 0.73[b]	1.26 ± 0.31[b]	0.44 ± 0.11[b]	0.36 ± 0.09[b]	0.21 ± 0.05[a]	0.22 ± 0.05[b]	0.68 ± 0.16[a]
M	3	2.99 ± 0.70[b]	1.26 ± 0.29[b]	0.44 ± 0.11[b]	0.33 ± 0.09[b]	0.21 ± 0.05[a]	0.23 ± 0.06[b]	0.66 ± 0.16[a]
E	1	2.59 ± 0.60[b]	1.27 ± 0.32[b]	0.45 ± 0.11[b]	0.34 ± 0.09[b]	0.20 ± 0.05[b]	0.22 ± 0.05[a]	0.64 ± 0.16[ab]
E	2	2.65 ± 0.61[b]	1.22 ± 0.27[b]	0.42 ± 0.10[b]	0.29 ± 0.08[b]	0.21 ± 0.05[b]	0.19 ± 0.05[a]	0.57 ± 0.15[b]
E	3	-	-	-	-	-	-	-
G	1	3.14 ± 0.75[ab]	1.36 ± 0.32[b]	0.45 ± 0.11[b]	0.37 ± 0.09[b]	0.22 ± 0.05[a]	0.22 ± 0.05[b]	0.69 ± 0.13[b]
G	2	3.09 ± 0.73[ab]	1.29 ± 0.31[b]	0.42 ± 0.12[b]	0.35 ± 0.10[b]	0.22 ± 0.06[a]	0.20 ± 0.05[b]	0.64 ± 0.16[b]
G	3	2.91 ± 0.68[c]	1.29 ± 0.31[a]	0.38 ± 0.11[b]	0.32 ± 0.08[b]	0.21 ± 0.05[a]	0.20 ± 0.06[b]	0.62 ± 0.16[b]

[1]Means based on 10 individual hatchlings.
Means with similar letters wihin each column for each cryoprotectant are not significantly different(P>0.05).
*Cryoprotectants: D=Dimethyl sulphoxide, M=Methanol, E= Ethylene glycol, G= Glycerol

Table 1E The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of rosy barb (*Puntius conchonius)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the epiboly stage.

Cryo*.	Period (h)	Standard Length length	Yolk-sac		Head width	Eye diameter	Body depth at	
			Width	Depth			Anus	Pectoral fin
Control		3.37 ± 0.77[a]	1.43 ± 0.33[a]	0.44 ± 0.14[a]	0.47 ± 0.12[a]	0.22 ± 0.05[a]	0.26 ± 0.06[a]	0.67 ± 0.16[a]
D	1	3.30 ± 0.82[ab]	1.57 ± 0.06[ab]	0.42 ± 0.13[a]	0.45 ± 0.12[a]	0.20 ± 0.02[a]	0.29 ± 0.07[a]	0.59 ± 0.16[ab]
D	2	3.10 ± 0.70[ab]	1.36 ± 0.31[a]	0.41 ± 0.11[a]	0.44 ± 0.12[a]	0.23 ± 0.06[a]	0.25 ± 0.07[a]	0.53 ± 0.14[b]
D	3	2.90 ± 0.69[b]	1.35 ± 0.31[a]	0.40 ± 0.12[a]	0.44 ± 0.12[b]	0.23 ± 0.06[a]	0.25 ± 0.07[a]	0.51 ± 0.14[b]
M	1	3.09 ± 0.70[a]	1.41 ± 0.31[a]	0.45 ± 0.14[a]	0.36 ± 0.10[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.67 ± 0.15[a]
M	2	3.08 ± 0.75[a]	1.38 ± 0.31[a]	0.43 ± 0.13[a]	0.34 ± 0.09[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.64 ± 0.14[a]
M	3	3.07 ± 0.71[a]	1.33 ± 0.30[a]	0.41 ± 0.11[a]	0.23 ± 0.06[b]	0.33 ± 0.09[a]	0.21 ± 0.06[a]	0.62 ± 0.14[a]
E	1	2.88 ± 0.65[b]	1.38 ± 0.31[ab]	0.43 ± 0.13[a]	0.36 ± 0.10[b]	0.23 ± 0.06[a]	0.22 ± 0.06[a]	0.59 ± 0.14[ab]
E	2	2.82 ± 0.64[b]	1.32 ± 0.30[ab]	0.41 ± 0.13[a]	0.34 ± 0.09[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.55 ± 0.13[ab]
E	3	2.68 ± 0.65[b]	1.27 ± 0.29[c]	0.37 ± 0.11[a]	0.31 ± 0.08[b]	0.23 ± 0.06[a]	0.22 ± 0.06[a]	0.47 ± 0.13[c]
G	1	3.14 ± 0.75[a]	1.41 ± 0.31[ab]	0.45 ± 0.14[a]	0.36 ± 0.10[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.59 ± 0.14[a]
G	2	3.12 ± 0.76[a]	1.38 ± 0.31[ab]	0.44 ± 0.13[a]	0.35 ± 0.10[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.58 ± 0.14[a]
G	3	3.09 ± 0.69[a]	1.35 ± 0.30[c]	0.40 ± 0.13[a]	0.32 ± 0.08[b]	0.23 ± 0.06[a]	0.21 ± 0.06[a]	0.57 ± 0.15[a]

[1]Means based on 10 individual hatchlings.
Means with similar letters wihin each column for each cryoprotectant are not significantly different(P>0.05).
*Cryoprotectants: D=Dimethyl sulphoxide, M=Methanol, E= Ethylene glycol, G= Glycerol

Table 1F The effect of type of cryoprotectant* and equilibration time on the body measurements (mm) of rosy barb (*Puntius conchonius)* hatchlings exposed to 1 molar solution of cryoprotectants for various periods (h) during the closure of the blastopore stage.

Cryo*.	Period (h)	Standard Length length	Yolk-sac Width	Yolk-sac Depth	Head width	Eye diameter	Body depth at Anus	Body depth at Pectoral fin
Control		3.26 ± 0.72[a]	1.38 ± 0.34[a]	0.41 ± 0.10[a]	0.44 ± 0.10[a]	0.25 ± 0.06[a]	0.28 ± 0.06[a]	0.61 ± 0.16[a]
D	1	3.23 ± 0.71[a]	0.34 ± 0.30[a]	0.36 ± 0.09a	0.39 ± 0.09[ab]	0.25 ± 0.06[a]	0.23 ± 0.07[ab]	0.57 ± 0.15[ab]
D	2	3.19 ± 0.70[a]	1.31 ± 0.29[a]	0.35 ± 0.10[a]	0.34 ± 0.09[bc]	0.24 ± 0.06[a]	0.22 ± 0.05[b]	0.53 ± 0.14[ab]
D	3	3.09 ± 0.68[a]	1.27 ± 0.28[a]	0.30 ± 0.09[a]	0.31 ± 0.08[c]	0.24 ± 0.06[a]	0.20 ± 0.06[b]	0.48 ± 0.12[c]
M	1	3.02 ± 0.72[a]	1.41 ± 0.31[a]	0.59 ± 0.13[b]	0.36 ± 0.10[ab]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.63 ± 0.14[a]
M	2	3.02 ± 0.72[a]	1.38 ± 0.31[a]	0.57 ± 0.13[b]	0.36 ± 0.10[ab]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.62 ± 0.14[a]
M	3	2.99 ± 0.73[a]	1.36 ± 0.32[a]	0.36 ± 0.13[b]	0.35 ± 0.10[c]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.60 ± 0.14[a]
E	1	3.19 ± 0.75[a]	1.27 ± 0.29[a]	0.44 ± 0.13[a]	0.36 ± 0.10[ab]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.62 ± 0.14[a]
E	2	3.16 ± 0.77[a]	1.25 ± 0.29[a]	0.43 ± 0.13[a]	0.35 ± 0.09[b]	0.23 ± 0.06[a]	0.20 ± 0.05[bc]	0.61 ± 0.14[a]
E	3	3.09 ± 0.74[a]	1.22 ± 0.28[a]	0.37 ± 0.10[a]	0.31 ± 0.09[b]	0.23 ± 0.06[a]	0.18 ± 0.05[c]	0.59 ± 0.14[a]
G	1	3.11 ± 0.72[ab]	1.39 ± 0.31[a]	0.45 ± 0.14[a]	0.36 ± 0.10[ab]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.69 ± 0.15[a]
G	2	3.09 ± 0.73[ab]	1.38 ± 0.31[a]	0.44 ± 0.13[a]	0.36 ± 0.10[ab]	0.23 ± 0.06[a]	0.21 ± 0.06[b]	0.63 ± 0.14[a]
G	3	2.88 ± 0.65[c]	1.36 ± 0.31[a]	0.41 ± 0.11[a]	0.34 ± 0.09[c]	0.23 ± 0.06[a]	0.19 ± 0.05[b]	0.59 ± 0.14[a]

[1]Means based on 10 individual hatchlings.
Means with similar letters wihin each column for each cryoprotectant are not significantly different(P>0.05).
*Cryoprotectants: D=Dimethyl sulphoxide, M=Methanol, E= Ethylene glycol, G= Glycerol

Table 2 The effect of temperature (°C), on the mean[1] rate constant (K), diffusional permeability (P_{diff}), and flux ($nlmin^{-1}$) in three embryonic stages of rosy barb (*Puntius conchonius*) and zebra fish (*Brachydanio rerio*).

Species	Embryonic Stage	K (h^{-1})			P_{diff} x e^{-03} ($um.s^{-1}$)			Flux ($nlmin^{-1}$)		
		20°C	25°C	30°C	20°C	25°C	30°C	20°C	25°C	30°C
Zebra fish	Cleavage	0.26 ± 0.01^{a}	0.54 ± 0.04^{b}	1.77 ± 0.04^{c}	5.48 ± 0.11^{a}	8.68 ± 0.25^{b}	24.5 ± 1.10^{c}	1.02 ± 0.02^{a}	1.62 ± 0.05^{b}	4.58 ± 0.20^{c}
	Epiboly	0.35 ± 0.00^{a}	0.48 ± 0.01^{b}	1.02 ± 0.03^{c}	4.96 ± 0.06^{a}	6.01 ± 0.16^{b}	11.11 ± 0.44^{c}	0.93 ± 0.01^{a}	1.12 ± 0.03^{b}	2.10 ± 0.09^{c}
	*Closure	0.29 ± 0.11^{a}	0.74 ± 0.02^{b}	0.81 ± 0.04^{c}	2.96 ± 0.69^{a}	4.23 ± 0.21^{ab}	04.96 ± 0.41^{b}	0.45 ± 0.04^{a}	0.79 ± 0.04^{b}	0.93 ± 0.07^{b}
	**H.beat	0.52 ± 0.03^{a}	0.75 ± 0.04^{b}	1.18 ± 0.10^{c}	8.32 ± 0.45^{a}	11.52 ± 0.63^{a}	17.56 ± 1.62^{b}	1.55 ± 0.08^{a}	2.15 ± 0.12^{a}	3.29 ± 0.30^{b}
Rosy barb	Cleavage	0.36 ± 0.02^{a}	0.45 ± 0.01^{b}	0.82 ± 0.02^{c}	6.73 ± 0.22^{a}	7.87 ± 0.20^{b}	12.47 ± 0.39^{c}	1.26 ± 0.04^{a}	1.48 ± 0.04^{b}	2.34 ± 0.48^{c}
	Epiboly	0.25 ± 0.05^{a}	0.51 ± 0.00^{b}	0.89 ± 0.02^{c}	5.02 ± 0.29^{a}	7.34 ± 0.10^{b}	11.64 ± 0.34^{c}	0.94 ± 0.05^{a}	1.36 ± 0.02^{b}	2.17 ± 0.06^{c}
	*Closure	0.80 ± 0.20^{a}	0.96 ± 0.13^{a}	1.07 ± 0.08^{a}	3.29 ± 0.56^{a}	4.27 ± 0.45^{ab}	05.41 ± 0.32^{b}	0.61 ± 0.10^{a}	0.791 ± 0.09^{ab}	1.01 ± 0.72^{b}
	**H.beat	0.64 ± 0.06^{a}	0.78 ± 0.03^{b}	1.04 ± 0.05^{c}	7.07 ± 0.51^{a}	8.82 ± 0.49^{b}	12.24 ± 0.95^{c}	1.32 ± 0.10^{a}	1.65 ± 0.09^{b}	2.29 ± 0.18^{c}

[1]Means (±SEM, n=4) showing similar superscripts for each parameter at different temperature and within each embryonic stage are not significantly different (P>0.05).
* closure of blastopore ** heart beat stage .

Table 3 The effect of chorion manipulation on the mean[1] rate constant (K) , diffusional permeability coefficient (P_{diff}) and flux ($nlmin^{-1}$), in three embryonic stages of rosy barb (*Puntius conchonius)* and zebra fish (*Brachydanio rerio).*

Species	Treatment	Cleavage			Epiboly			Closure of blastopore		
		$K(h^{-1})$	P_{diff} x 10^{-03} ($um.s^{-1}$)	Flux ($nlmin^{-1}$)	$K(h^{-1})$	P_{diff} x 10^{-03} ($um.s^{-1}$)	Flux ($nlmin^{-1}$)	$K(h^{-1})$	P_{diff} x 10^{-03} ($um.s^{-1}$)	Flux ($nlmin^{-1}$)
Zebra fish	Control	1.77 ± 0.04^{a}	24.50 ± 1.1^{a}	4.58 ± 0.20^{a}	1.02 ± 0.02^{a}	11.11 ± 0.44^{a}	2.10 ± 0.09^{a}	0.81 ± 0.04^{a}	04.96 ± 0.41^{a}	0.93 ± 0.07^{a}
	Electroporated	02.28 ± 0.44^{ab}	30.83 ± 0.95^{b}	5.77 ± 0.19^{b}	1.19 ± 0.11^{ab}	16.87 ± 0.42^{b}	3.16 ± 0.08^{b}	1.19 ± 0.11^{a}	06.80 ± 0.67^{b}	1.27 ± 0.13^{b}
	Dechorionated	1.07 ± 0.11^{c}	1.33 ± 0.49^{c}	0.18 ± 0.02^{c}	0.89 ± 0.14^{c}	1.02 ± 0.43^{c}	0.14 ± 0.02^{c}	0.86 ± 0.27^{a}	00.74 ± 0.29^{c}	0.11 ± 0.02^{c}
Rosy barb	Control	0.82 ± 0.02^{a}	12.47 ± 0.39^{a}	2.34 ± 0.48^{a}	0.89 ± 0.02^{a}	11.64 ± 0.34^{a}	2.17 ± 0.06^{a}	1.07 ± 0.08^{a}	05.41 ± 032^{c}	1.01 ± 0.72^{a}
	Electroporated	1.51 ± 0.13^{b}	22.88 ± 2.26^{b}	4.31 ± 0.38^{b}	1.36 ± 0.06^{ab}	15.69 ± 0.13^{b}	$2.95\pm.64^{b}$	1.22 ± 0.14^{a}	08.43 ± 0.61^{c}	1.58 ± 0.11^{b}
	Dechorionated	1.52 ± 0.25^{b}	1.78 ± 0.73^{c}	0.24 ± 0.03^{c}	1.18 ± 0.19^{c}	1.36 ± 0.66^{c}	0.18 ± 0.02^{c}	1.28 ± 0.28^{a}	01.32 ± 0.70^{b}	0.17 ± 0.04^{c}

[1]Means (±SEM; n=4) with similar superscripts, within each row, are not significantly different (P> 0.05).

Table 4A The effect of cooling rate and near zero target temperature (°C) on the mean (±SEM[@]) hatch rate (%) of three embryonic stages of zebra fish (*Brachydanio rerio*), following stepwise equilibration for one hour in four cryoprotectants (1 M).

Cryo.[*]	C.R>	Cleavage				Epiboly				Closure of blastopore			
		15.0	10.0	5.0	0.0	15.0	10.0	5.0	0.0	15.0	10.0	5.0	0.0
DMSO[1]	1.0	69.0±04.0^{a}	74.0±05.0^{a}	60.0±12.0^{a}	63.0±13.0^{a}	83.0±05.0^{a}	73.0±10.0^{a}	60.0±03.0^{a}	61.0±03.0^{a}	79.0±07.0^{a}	64.0±10.0^{a}	62.0±09.0^{a}	61.0±08.0^{a}
	2.0	78.0±07.0^{a}	68.0±03.0^{ab}	66.0±01.0^{ab}	56.0±03.0^{b}	80.0±05.0^{a}	59.0±10.0^{a}	60.0±11.0^{a}	57.0±06.0^{a}	80.0±08.0^{a}	62.0±08.0^{a}	60.0±08.0^{a}	64.0±07.0^{a}
	5.0	63.0±07.0^{a}	57.0±05.0^{a}	55.0±02.0^{a}	52.0±07.0^{a}	70.0±07.0^{a}	67.0±05.0^{a}	62.0±05.0^{a}	66.0±08.0^{a}	65.0±08.0^{a}	70.0±07.0^{a}	71.0±10.0^{a}	57.0±05.0^{a}
METH.[2]	1.0	79.0±04.0^{a}	71.0±13.0^{a}	63.0±15.0^{a}	63.0±13.0^{a}	82.0±05.0^{a}	70.0±08.0^{a}	77.0±03.0^{a}	68.0±06.0^{a}	67.0±09.0^{a}	59.0±07.0^{a}	58.0±06.0^{a}	61.0±10.0^{a}
	2.0	80.0±03.0^{a}	73.0±04.0^{ab}	67.0±07.0^{ab}	57.0±03.0^{b}	73.0±06.0^{a}	57.0±03.0^{a}	75.0±05.0^{a}	57.0±05.0^{a}	61.0±06.0^{a}	49.0±03.0^{a}	57.0±08.0^{a}	52.0±10.0^{a}
	5.0	61.0±01.0^{a}	55.0±03.0^{a}	60.0±08.0^{a}	60.0±03.0^{a}	69.0±06.0^{a}	66.0±03.0^{a}	57.0±04.0^{ab}	45.0±01.0^{b}	67.0±08.0^{a}	58.0±06.0^{a}	60.0±05.0^{a}	60.0±10.0^{a}
E.GLY[3]	1.0	76.0±04.0^{a}	65.0±14.0^{a}	59.0±12.0^{a}	54.0±12.0^{a}	75.0±04.0^{a}	70.0±08.0^{a}	58.0±08.0^{a}	50.0±06.0^{a}	65.0±16.0^{a}	64.0±14.0^{a}	53.0±07.0^{a}	52.0±15.0^{a}
	2.0	69.0±06.0^{a}	55.0±02.0^{a}	54.0±04.0^{a}	40.0±06.0^{b}	70.0±10.0^{a}	51.0±05.0^{a}	50.0±04.0^{a}	46.0±06.0^{a}	70.0±10.0^{a}	51.0±05.0^{a}	50.0±04.0^{a}	46.0±06.0^{a}
	5.0	40.0±05.0^{a}	51.0±11.0^{a}	36.0±03.0^{a}	47.0±08.0^{a}	54.0±09.0^{a}	59.0±05.0^{a}	46.0±02.0^{a}	60.0±11.0^{a}	53.0±02.0^{a}	57.0±09.0^{a}	46.0±04.0^{a}	39.0±04.0^{a}
GLYC.[4]	1.0	56.0±11.0^{a}	53.0±06.0^{a}	50.0±06.0^{a}	44.0±05.0^{a}	69.0±07.0^{a}	59.0±03.0^{ab}	58.0±09.0^{ab}	38.0±04.0^{b}	57.0±06.0^{a}	47.0±10.0^{a}	39.0±10.0^{a}	39.0±17.0^{a}
	2.0	54.0±04.0^{a}	48.0±05.0^{a}	46.0±05.0^{a}	41.0±04.0^{a}	59.0±07.0^{a}	49.0±04.0^{a}	36.0±05.0^{a}	44.0±04.0^{a}	47.0±06.0^{a}	46.0±07.0^{a}	37.0±12.0^{a}	45.0±08.0^{a}
	5.0	37.0±04.0^{a}	49.0±06.0^{a}	32.0±01.0^{a}	35.0±04.0^{a}	34.0±03.0^{a}	43.0±05.0^{a}	45.0±05.0^{a}	38.0±06.0^{a}	35.0±05.0^{a}	39.0±08.0^{a}	37.0±05.0^{a}	40.0±02.0^{a}

[*] Cryo 1=dimethyl sulphoxide, 2=methanol, 3=ethylene glycol, 4=glycerol

Means[@] in the same row for each stage with similar superscripts are not significantly different (P>0.05)

C.R. = cooling rate (°C/min)

Table 4B The effect of cooling rate and near zero target temperature (°C) on the mean (±SEM[@]) hatch rate(%) of three embryonic stages of rosy barb (*Puntius conchonius)* following stepwise equilibration for one hour in four cryoprotectants (1 M).

Cryo.*	C.R.	Cleavage				Epiboly				Closure of blastopore			
		15.0	10.0	5.0	0.0	15.0	10.0	5.0	0.0	15.0	10.0	5.0	0.0
DMSO[1]	1.0	68.0 ± 8.7[a]	69.0 ± 3.9[a]	68.0 ± 7.6[a]	66.0 ± 3.1[a]	69.0 ± 7.0[a]	55.0 ± 6.6[a]	72.0 ± 4.4[a]	68.2 ± 5.8[a]	66.0 ± 5.0[a]	68.0 ± 8.0[a]	68.0 ± 8.6[a]	66.0 ± 5.0[a]
	2.0	70.0 ± 5.0[a]	73.0 ± 6.0[a]	73.0 ± 2.0[a]	56.0 ± 7.0[a]	68.0 ± 8.0[a]	67.0 ± 7.0[a]	60.0 ± 8.0[a]	64.0 ± 5.0[a]	78.0 ± 2.0[a]	65.0 ± 6.0[a]	59.0 ± 7.0[a]	62.0 ± 8.0[a]
	5.0	68.0 ± 7.0[a]	64.0 ± 3.0[a]	63.0 ± 4.0[a]	52.0 ± 4.0[a]	63.0 ± 3.0[a]	68.0 ± 5.0[a]	72.0 ± 2.0[a]	59.0 ± 4.0[a]	63.0 ± 3.0[a]	68.0 ± 5.0[a]	72.0 ± 2.0[a]	59.0 ± 4.0[a]
METH.[2]	1.0	70.0 ± 6.9[a]	53.0 ± 2.7[a]	63.0 ± 5.9[a]	62.0 ± 6.8[a]	66.0 ± 7.3[a]	68.0 ± 3.3[a]	66.0 ± 0.8[a]	70.0 ± 7.1[a]	85.0 ± 6.0[a]	71.0 ± 5.0[ab]	65.0 ± 2.0[a]	63.0 ± 5.0[a]
	2.0	63.0 ± 3.0[a]	67.0 ± 1.0[a]	58.0 ± 5.0[a]	59.0 ± 5.0[a]	77.0 ± 7.0[a]	56.0 ± 3.0[a]	61.0 ± 4.0[a]	58.0 ± 4.0[a]	75.0 ± 2.0[a]	55.0 ± 8.0[a]	59.0 ± 4.0[a]	58.0 ± 4.0[a]
	5.0	61.0 ± 4.0[a]	56.0 ± 2.0[a]	59.0 ± 1.0[a]	58.0 ± 4.0[a]	73.0 ± 5.0[a]	59.0 ± 5.0[a]	62.0 ± 3.0[a]	64.0 ± 5.0[a]	65.0 ± 5.0[a]	67.0 ± 3.0[a]	67.0 ± 3.0[a]	67.0 ± 1.0[a]
E.GLY[3]	1.0	50.0 ± 6.7[a]	38.0 ± 6.7[a]	34.0 ± 5.8[a]	43.0 ± 5.0[a]	52.0 ± 4.5[a]	52.0 ± 4.5[a]	43.0 ± 5.9[a]	42.0 ± 1.0[a]	57.0 ± 4.0[a]	56.0 ± 6.0[a]	48.0 ± 2.0[a]	60.0 ± 4.0[a]
	2.0	45.0 ± 2.0[a]	48.0 ± 3.0[a]	46.0 ± 4.0[a]	44.0 ± 1.0[a]	52.0 ± 7.0[a]	48.0 ± 4.0[a]	44.0 ± 4.0[a]	41.0 ± 6.0[a]	53.0 ± 2.0[a]	52.0 ± 6.0[a]	57.0 ± 9.0[a]	45.0 ± 1.0[a]
	5.0	44.0 ± 9.0[a]	44.0 ± 7.0[a]	38.0 ± 4.0[a]	45.0 ± 5.0[a]	42.0 ± 3.0[a]	50.0 ± 3.0[a]	54.0 ± 9.9[a]	48.0 ± 3.0[a]	51.0 ± 3.0[a]	52.0 ± 3.0[a]	48.0 ± 5.0[a]	42.0 ± 3.0[a]
GLYC.[4]	1.0	33.0 ± 8.3[a]	31.0 ± 2.0[a]	19.0 ± 4.4[a]	24.0 ± 5.7[a]	41.0 ± 7.1[a]	43.0 ± 7.4[a]	37.0 ± 2.5[a]	36.0 ± 4.6[a]	44.0 ± 1.0[a]	40.0 ± 5.0[a]	38.0 ± 3.0[a]	36.0 ± 2.0[a]
	2.0	32.0 ± 6.0[a]	31.0 ± 5.0[a]	33.0 ± 3.0[a]	25.0 ± 4.0[a]	41.0 ± 6.0[a]	41.0 ± 4.0[a]	33.0 ± 2.0[a]	33.0 ± 4.0[a]	41.0 ± 3.0[a]	45.0 ± 5.0[a]	41.0 ± 5.0[a]	36.0 ± 2.0[a]
	5.0	31.0 ± 3.0[a]	28.0 ± 2.0[a]	34.0 ± 3.0[a]	38.0 ± 4.0[a]	37.0 ± 4.0[a]	29.0 ± 8.0[a]	44.0 ± 5.0[a]	31.0 ± 4.0[a]	27.0 ± 6.0[a]	38.0 ± 4.0[a]	36.0 ± 6.0[a]	32.0 ± 5.0[a]

* Cryo 1=dimethyl sulphoxide, 2=methanol 3=Ethylene glycol 4=glycerol

Means[@] in the same row for each stage with similar superscripts are not significantly different (P>0.05)

C.R. = Cooling rate (°C/min).

Table 5A The effect of cooling rate (°C/min) and target temperature (°C) on incidence [1](%) of intracellular ice formation in epiboly stage of zebra fish (*Brachydanio rerio*) cooled in the presence of 1 M of various cryoprotectants.

[2]Cryo	[3]C.R.	Target Temperature (°C)					
		0	-4	-8	-12	-16	-20
[4]Me_2SO	0.1	20.0 ± 11.6^{a}	33.3 ± 6.7^{ab}	53.3 ± 13.4^{ab}	$86.7\pm13.4b^{c}$	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
	0.5	33.3 ± 6.7^{a}	40.0 ± 0.0^{a}	60.3 ± 11.6^{ab}	80.0 ± 11.6^{bc}	93.3 ± 6.7^{bc}	100.0 ± 0.0^{c}
	1.0	33.0 ± 6.6^{a}	46.7 ± 6.7^{a}	53.3 ± 13.4^{ab}	73.3 ± 17.7^{b}	86.7 ± 6.5^{b}	100.0 ± 0.0^{b}
[5]EG	0.1	33.3 ± 6.6^{a}	33.3 ± 17.0^{a}	66.7 ± 6.7^{a}	93.3 ± 6.7^{b}	100.0 ± 0.0^{b}	100.0 ± 0.0^{b}
	0.5	40.0 ± 11.5^{a}	46.7 ± 6.7^{ab}	66.7 ± 6.7^{abc}	80.0 ± 11.6^{bc}	93.3 ± 6.7^{c}	100.0 ± 0.0^{c}
	1.0	46.7 ± 17.7^{a}	47.7 ± 6.7^{a}	60.0 ± 11.6^{ab}	80.0 ± 11.6^{ab}	86.7 ± 7.0^{ab}	100.0 ± 0.0^{c}
[6]MET	0.1	40.0 ± 0.0^{a}	40.0 ± 11.6^{a}	86.7 ± 13.4^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{a}
	0.5	40.0 ± 0.0^{a}	40.0 ± 0.0^{a}	66.65±6.7ab	80.0 ± 11.6^{bc}	93.33 ± 6.7^{bc}	100.0 ± 0.0^{c}
	1.0	66.7 ± 17.0^{a}	80.0 ± 11.6^{a}	53.3 ± 13.4^{ab}	73.3 ± 17.7^{abc}	86.7 ± 6.7^{bc}	100.0 ± 0.0^{c}
[7]GLYC	0.1	00.0 ± 0.0^{a}	20 ± 13.4^{ab}	53.3 ± 13.4^{abc}	86.7 ± 13.4^{bc}	100 ± 0.0^{c}	100.0 ± 0.0^{c}
	0.5	13.3 ± 6.7^{a}	26.7 ± 6.7^{ab}	60.0 ± 11.6^{bc}	66.7 ± 6.7^{cd}	80.0 ± 11.6^{cd}	100.0 ± 0.0^{d}
	1.0	20.0 ± 11.0^{a}	26.7 ± 0.0^{a}	33.3 ± 6.7^{ab}	66.7 ± 13.4^{bc}	86.7 ± 6.7^{c}	100.0 ± 0.0^{c}

1= numbers of embryos showing evidence of intracelluar ice formation 2 = Cryoprotectants, 3 = Cooling rate, 4 = Dimethyl sulphoxide, 5 = Ethylene glycol, 6 = Methanol, 7 = Glycerol.
Means with similar superscript in each row are not significantly different ($P>0.05$).

Table 5B The effect of cooling rate (°C/min) and target temperature (°C) on incidence [1](%) of intracellular ice formation (IIF) in epiboly stage of rosy barb (*Puntius conchonius*) cooled in the presence of 1 M of various cryoprotectants.

[2]Cryo	[3]C.R.	Target Temperature (°C)					
		0	-4	-8	-12	-16	-20
[4]Me_2SO	0.1	13.3 ± 6.7^{a}	20.0 ± 0.0^{a}	53.3 ± 13.4^{ab}	86.7 ± 13.4^{bc}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
	0.5	26.7 ± 6.7^{a}	33.3 ± 6.7^{a}	60.3 ± 11.6^{ab}	80.0 ± 11.6^{bc}	93.3 ± 6.7^{bc}	100.0 ± 0.0^{c}
	1.0	26.7 ± 6.7^{a}	40.0 ± 11.6^{a}	53.3 ± 13.4^{ab}	73.3 ± 17.7^{bc}	86.7 ± 6.5^{bc}	100.0 ± 0.0^{c}
[5]EG	0.1	33.3 ± 6.6^{a}	40.0 ± 11.5^{ab}	60.0 ± 11.6^{a}	93.3 ± 6.7^{c}	100.0 ± 0.0^{c}	100.0 ± 0.0^{c}
	0.5	40.0 ± 11.5^{a}	40.0 ± 11.5^{a}	66.7 ± 6.7^{ab}	80.0 ± 11.6^{ab}	93.3 ± 6.7^{c}	100.0 ± 0.0^{c}
	1.0	40.0 ± 11.6^{a}	46.7 ± 6.8^{a}	66.0 ± 6.7^{ab}	73.3 ± 17.7^{ab}	86.7 ± 7.0^{ab}	100.0 ± 0.0^{c}
[6]MET	0.1	26.7 ± 6.5^{a}	26.7 ± 6.5^{a}	53.3 ± 13.4^{a}	73.3 ± 17.7^{a}	100.0 ± 0.0^{a}	100.0 ± 0.0^{c}
	0.5	33.3 ± 6.7^{a}	33.3 ± 6.7^{a}	66.65 ± 6.7^{ab}	80.0 ± 11.6^{b}	93.33 ± 6.7^{b}	100.0 ± 0.0^{c}
	1.0	60.7 ± 6.5^{a}	73.3 ± 13.4^{a}	86.7 ± 13.4^{ab}	100.0 ± 0.0^{ab}	86.7 ± 6.7^{b}	100.0 ± 0.0^{c}
[7]GLYC	0.1	13.3 ± 6.7^{a}	13.3 ± 6.7^{a}	33.3 ± 6.7^{b}	66.7 ± 6.7^{c}	100 ± 00.0^{c}	100.0 ± 0.0^{c}
		20.0 ± 0.0^{a}	33.3 ± 6.7^{ab}	60.0 ± 11.6^{bc}	66.7 ± 6.7^{abc}	80.0 ± 11.6^{bc}	100.0 ± 0.0^{c}
		20.0 ± 0.0^{a}	20.0 ± 0.0^{a}	40.0 ± 0.0^{ab}	86.7 ± 13.4^{bc}	86.7 ± 6.7^{c}	100.0 ± 0.0^{c}

1 = numbers of embryos showing evidence of intracelluar ice formation 2 = Cryoprotectants, 3 = Cooling rate, 4 = Dimethyl sulphoxide, 5 = Ethylene glycol, 6 = Methanol, 7 = Glycerol.
Means with similar superscript in each row are not significantly different ($P>0.05$).

Appendix 4 Figures

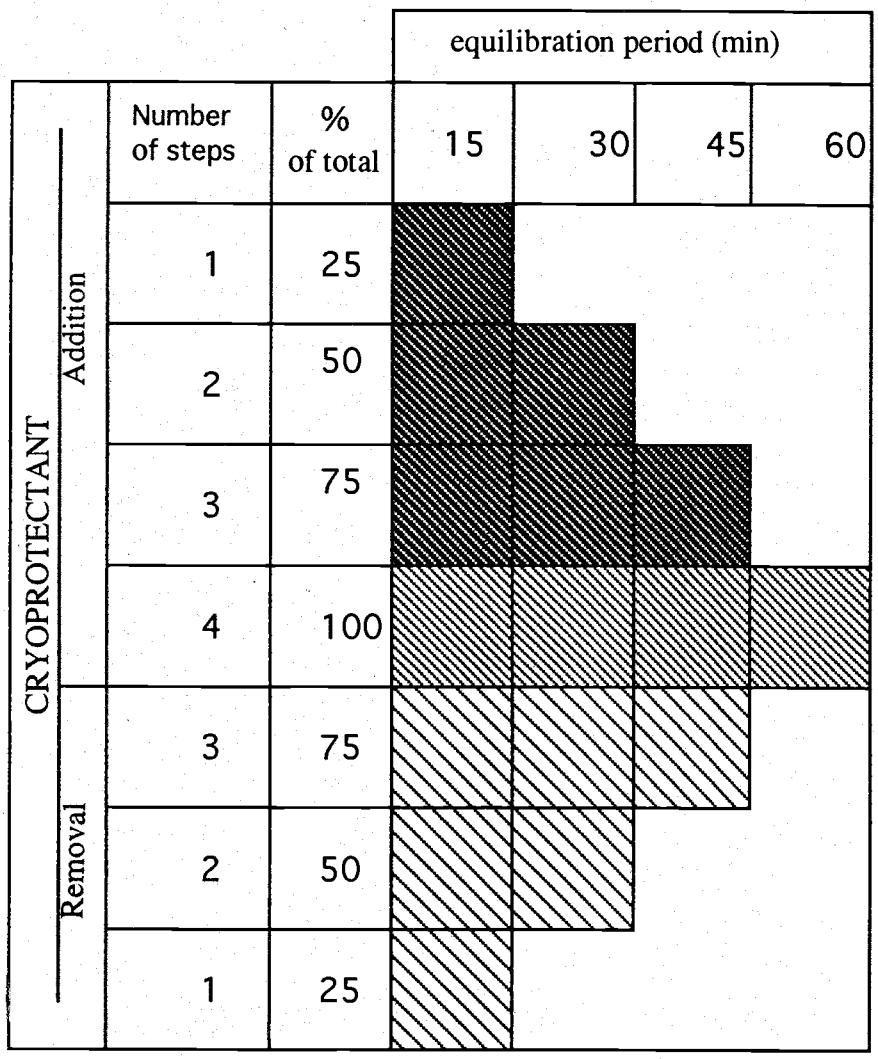


Fig.1 General method used for the 4 step equilibration procedure of cryoprotectants. Embryos were equilibrated in four 15 min steps each of equal concentration and the cryoprotectants were removed using the reverse procedure.

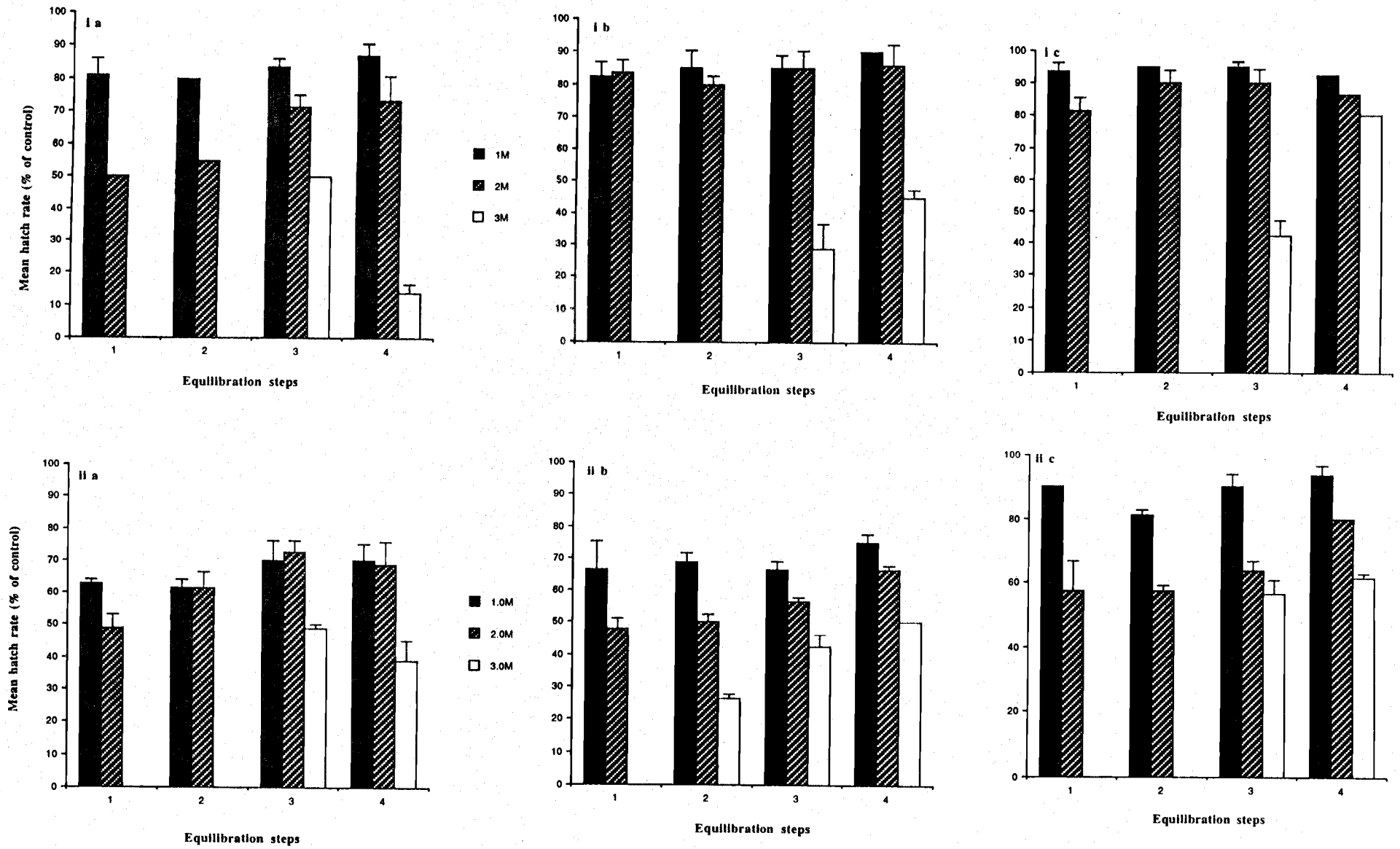


Fig. 2A Hatching rate (x±SE) of (i) zebra fish (*Brachydanio rerio*), and (ii) rosy barb (*Puntius conchonius*) embryos equilibrated at the (a) cleavage (b) epiboly and (c) closure of the blastopore after 4 step equilibration for 1 h (4 x 15 min) in 1, 2, and 3 M Me_2SO in fish Ringer solution. Means hatch rate (±SE) based on four replicates.

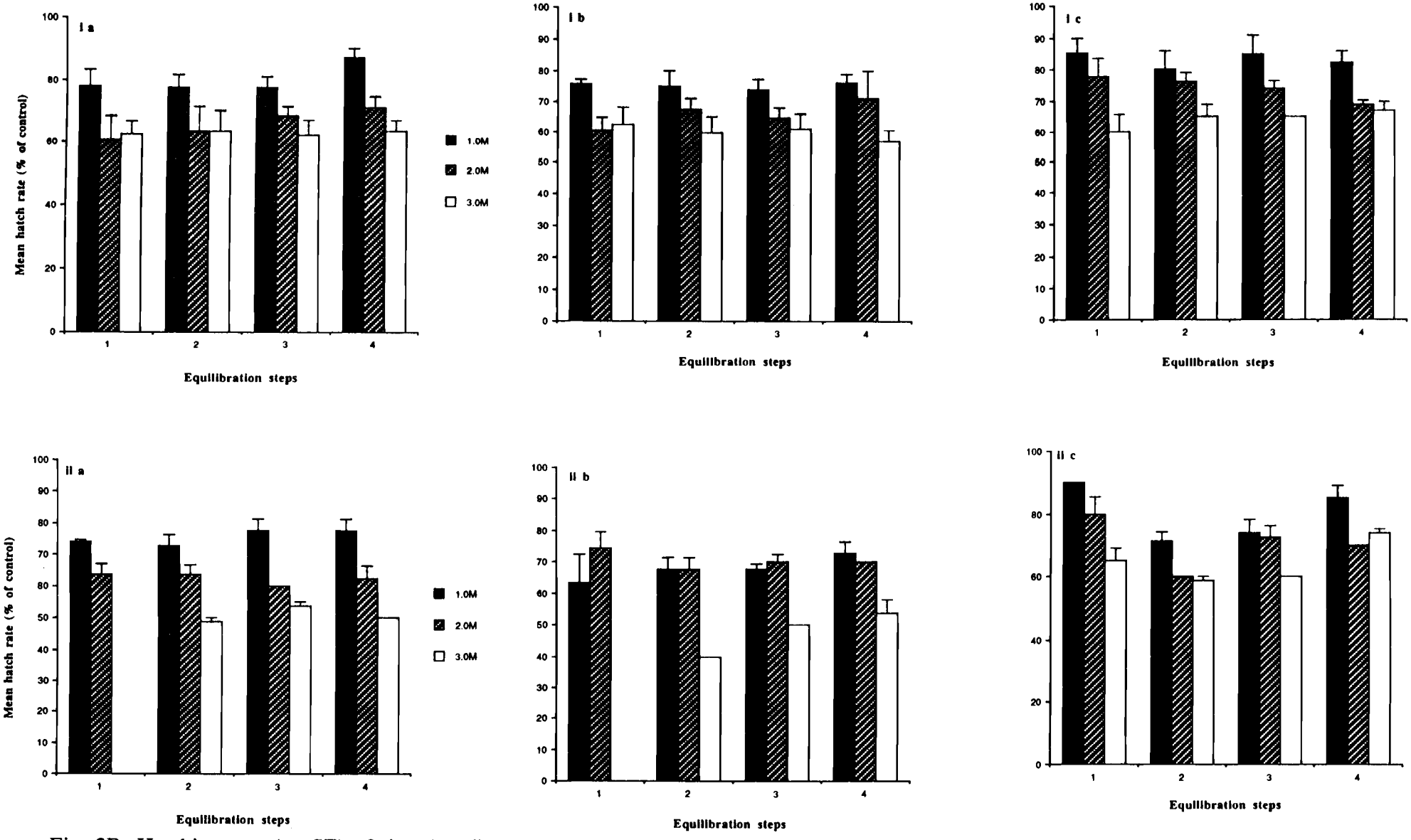


Fig. 2B Hatching rate (x±SE) of (i) zebra fish (*Brachydanio rerio*) and (ii) rosy barb (*Puntius conchonius*) embryos equilibrated at the (a) cleavage (b) epiboly, and (c) closure of the blastopore after 4 step equilibration for 1 h (4 x 15 min) in 1, 2, and 3 M methanol in fish Ringer solution. Means hatch rate (±SE) based on four replicates.

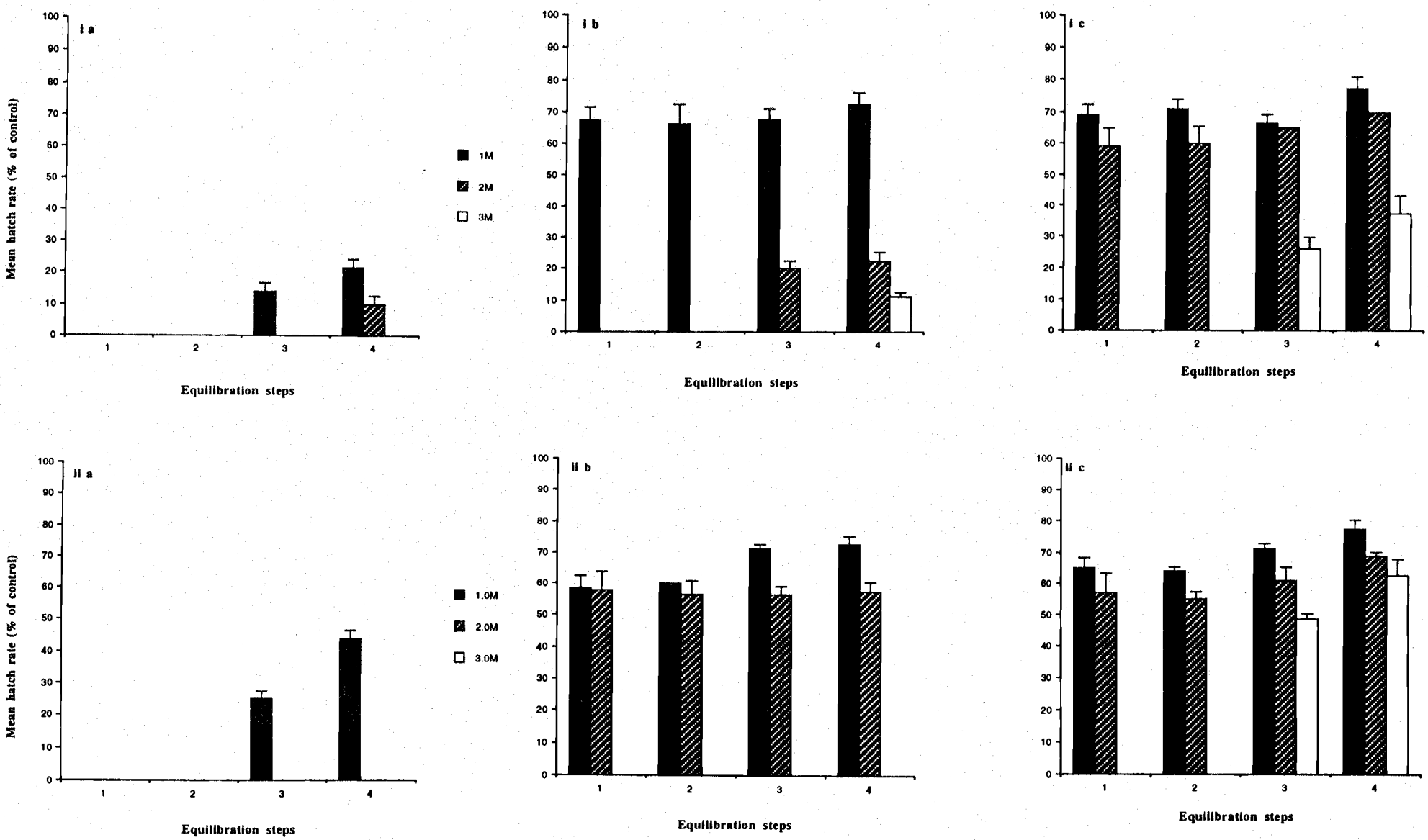


Fig. 2C Hatching rate (x±SE) of (i) zebra fish (*Brachydanio rerio*) and (ii) rosy barb (*Puntius conchonius*) embryos equilibrated at the (a) cleavage (b) epiboly and (c) closure of the blastopore after 4 step equilibration for 1 h (4 x 15 min) in 1, 2, and 3 M ethylene glycol in fish Ringer solution. Means hatch rate (±SE) based on four replicates.

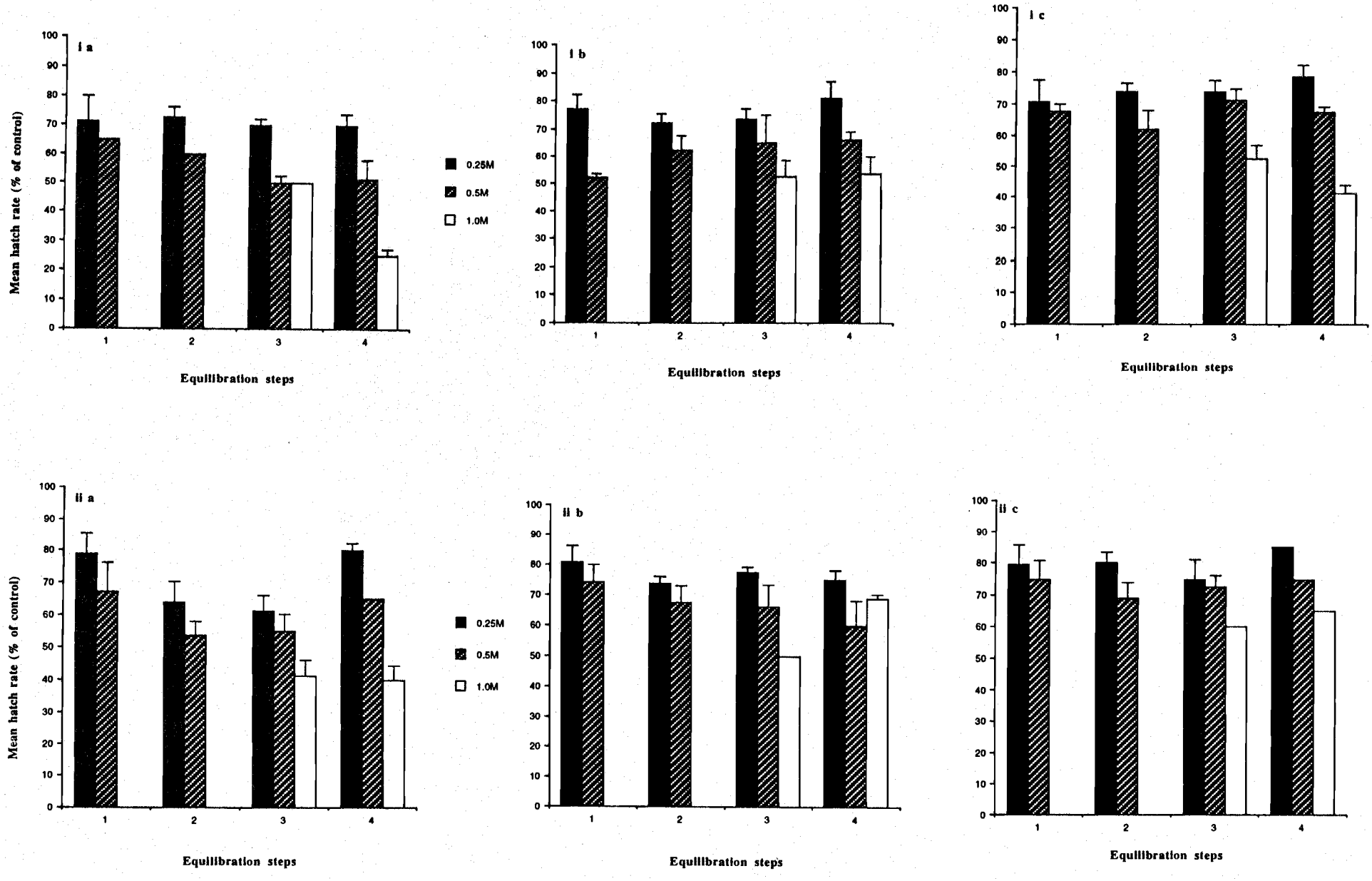


Fig. 2D Hatching rate (x±SE) of (i) zebra fish (*Brachydanio rerio*) and (ii) rosy barb (*Puntius conchonius*) embryos equilibrated at the (a) cleavage (b) epiboly and (c) the closure of blastopore after 4 step equilibration for 1 h (15 x 4 min) in 0.25, 0.5, and 1 M glycerol in fish Ringer solution. Means hatch (±SE) rate based on four replicates.

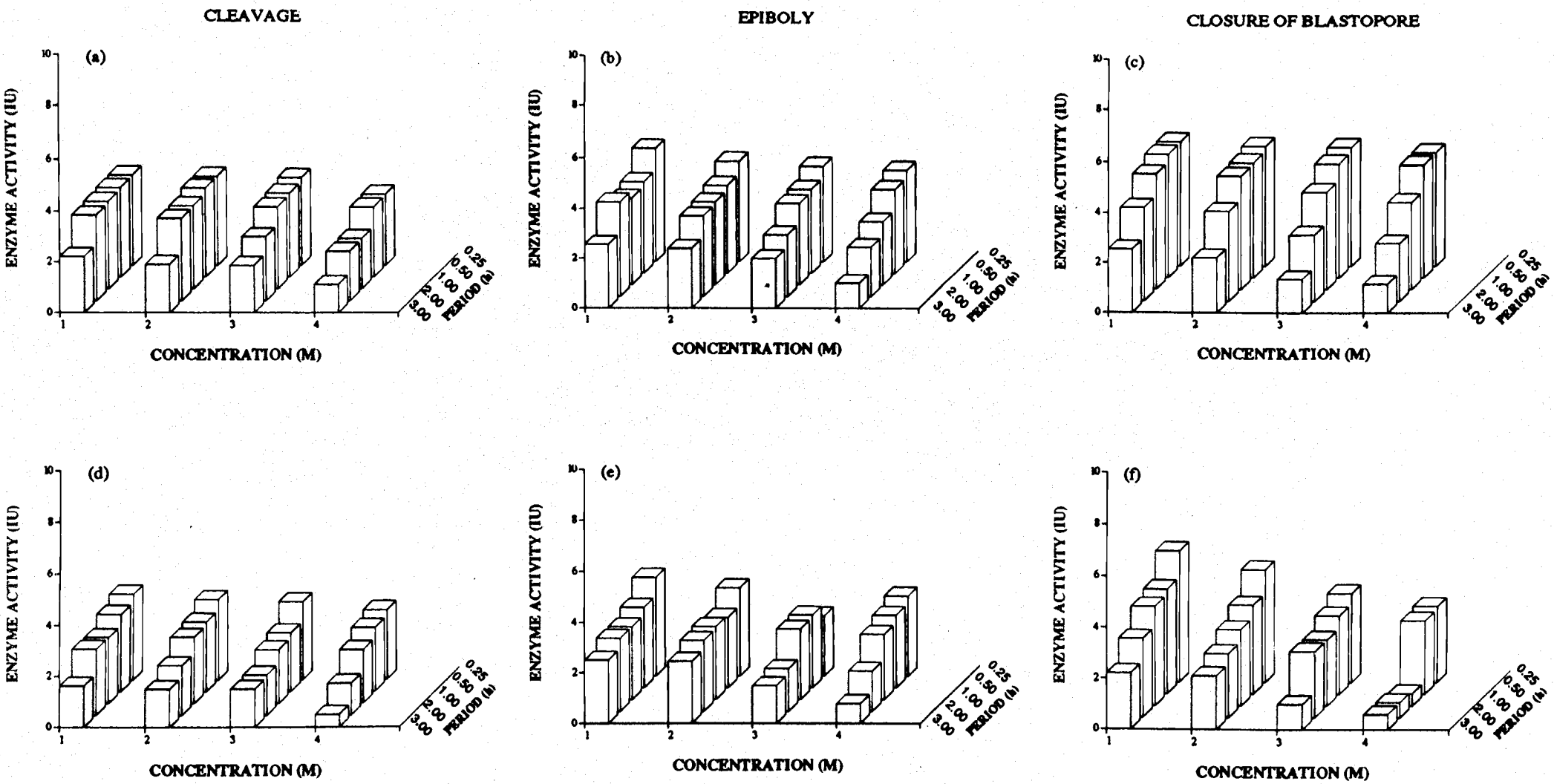


Figure 3A The effect of concentration and equilibration period (h) of dimethyl sulphoxide (a-c), ethylene glycol (d-f) on the mean enzymatic activity of lactate dehydrogenase in three stages of zebra fish (*Brachydanio rerio*) embryos. Means IU/embryo (±sem) based on three replicates.

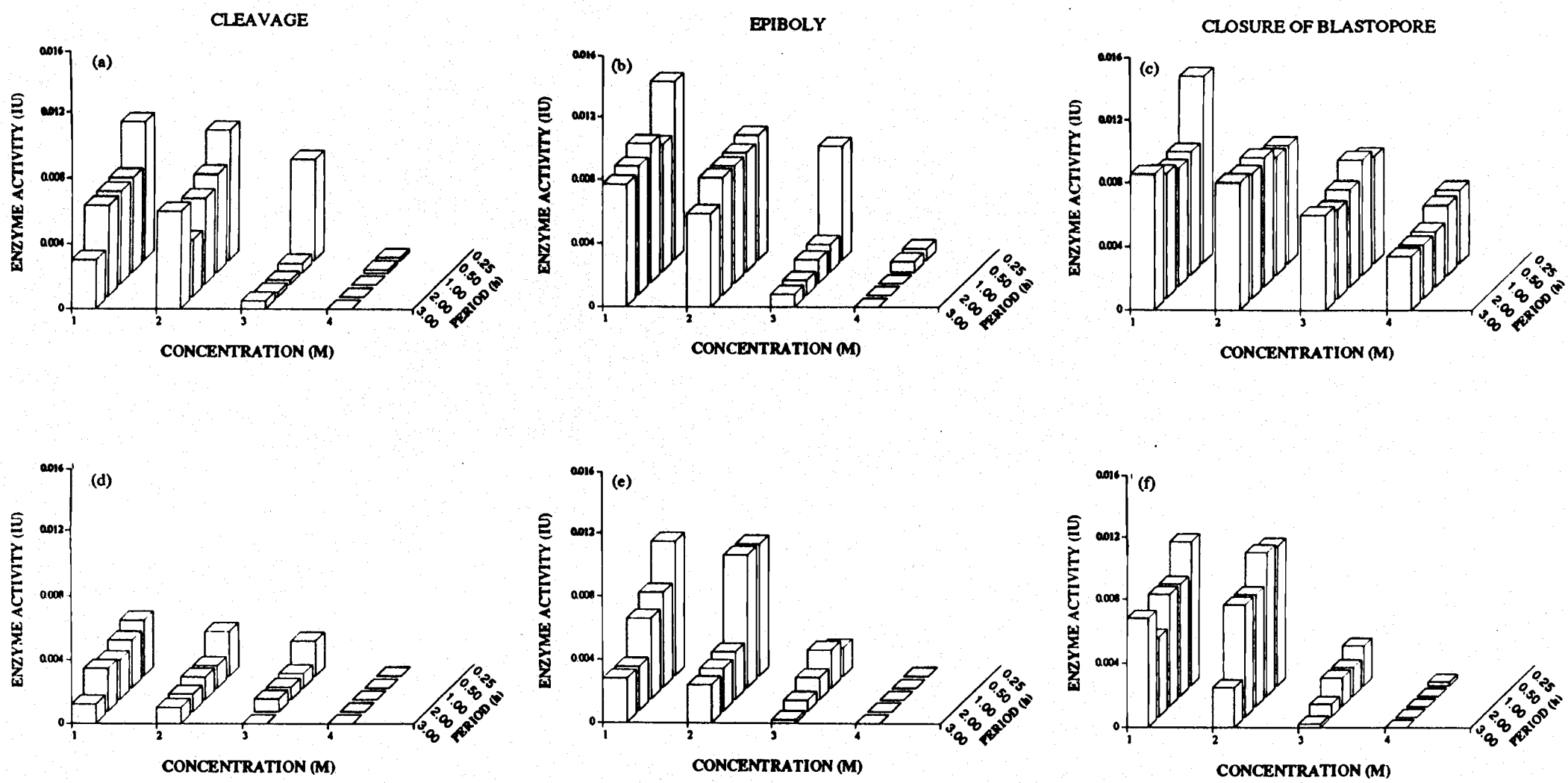


Figure 3B The effect of concentration and equilibration period (h) of dimethyl sulphoxide (a-c), ethylene glycol (d-f) on the mean enzymatic activity of glucose-6-phosphate dehydrogenase in three stages of zebra fish (*Brachydanio rerio*) embryo. Means IU/embryo based on three replicates.

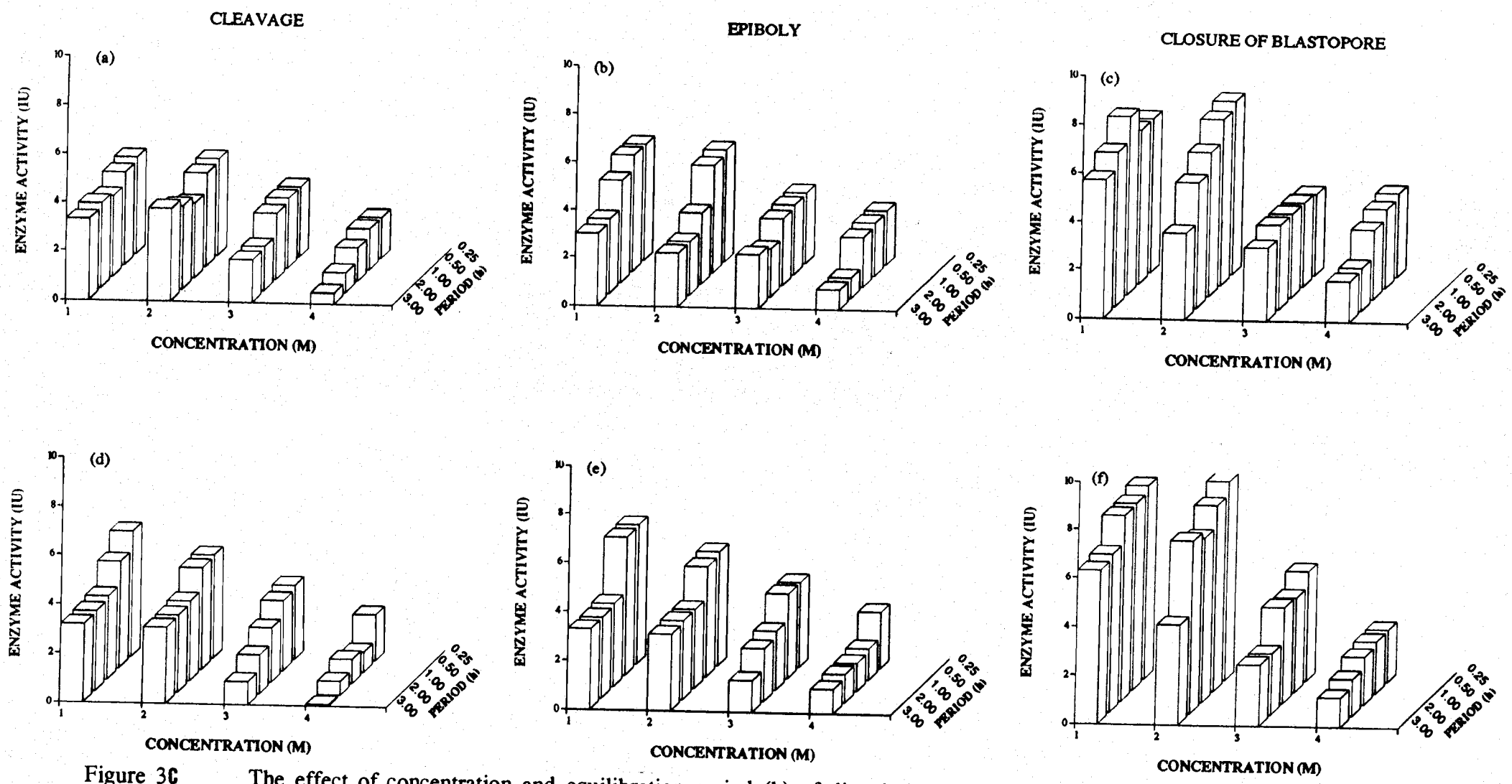


Figure 3C The effect of concentration and equilibration period (h) of dimethyl sulphoxide (a-c) ethylene glycol (d-f) on the mean enzymatic activity of lactate dehydrogenase in three stages of rosy barb (*Puntius conchonius*) embryos. Means (±sem) IU/embryo based on three replicates.

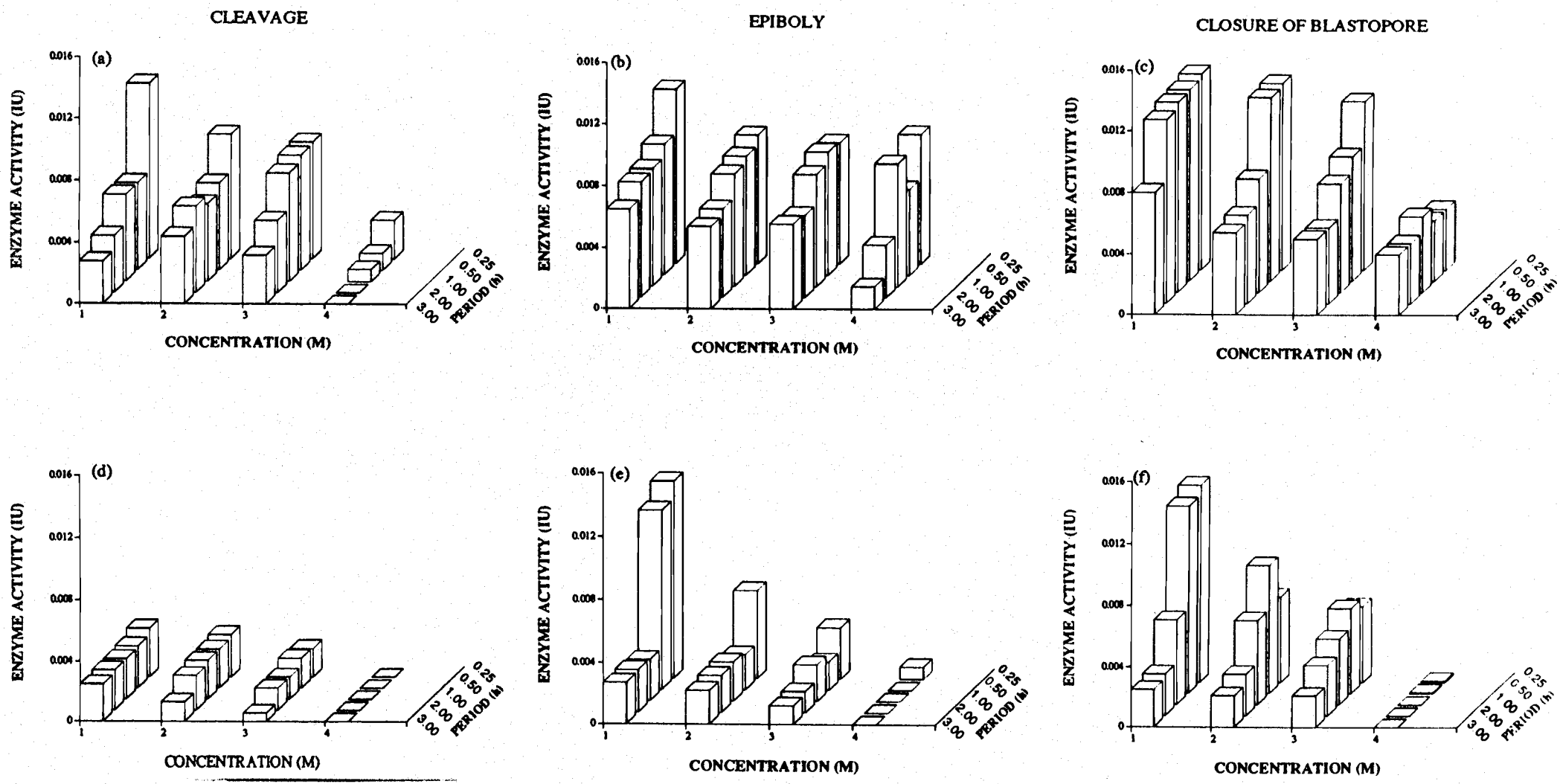


Figure 3D The effect of concentration and equilibration period (h) of dimethyl sulphoxide (a-c), ethylene glycol (d-f) on the mean enzymatic activity of glucose-6-phosphate dehydrogenase in three stages of rosy barb (*Puntius conchonius*) embryos. Means IU/embryo (±sem) based on 3 replicates.

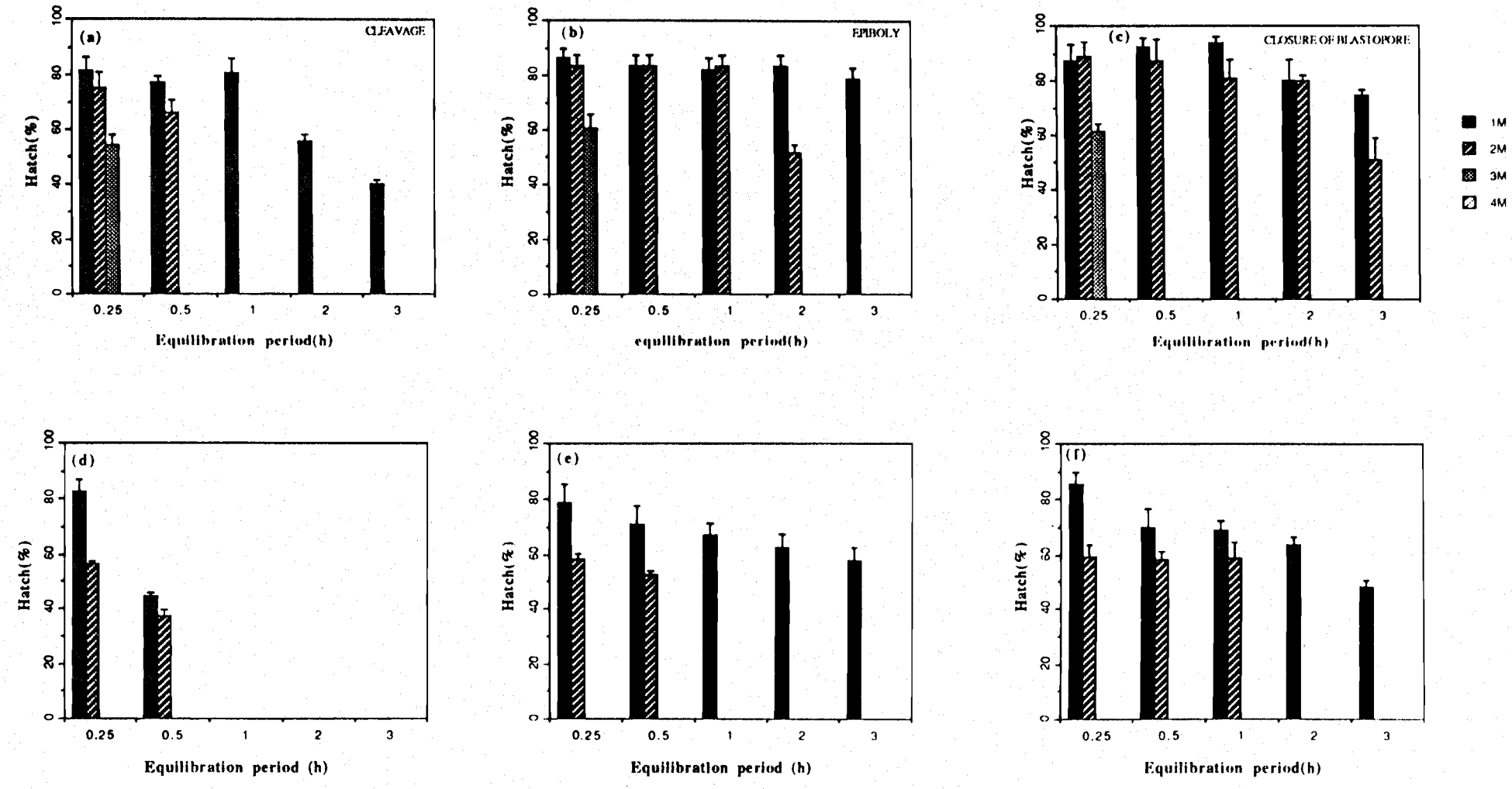


Figure 4A. The effect of dimethyl sulphoxide (a-c), ethylene glycol (d-f) concentration and equilibration period (h) on the hatch rate of three embryonic stages of zebra fish (*Brachydanio rerio*). Means based on four replicates.

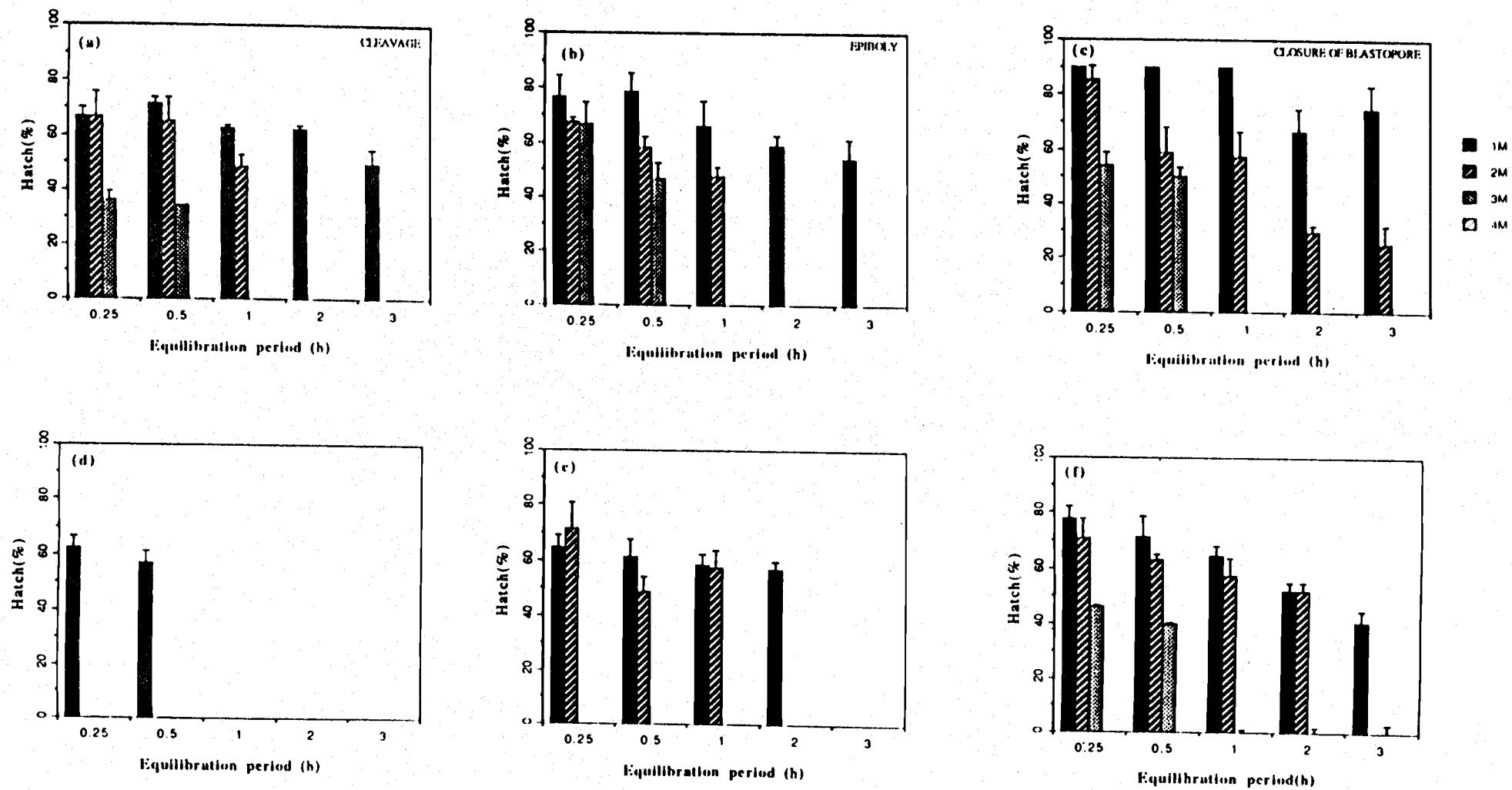


Figure 4B The effect of dimethyl sulphoxide (a-c), ethylene glycol (d-f) concentration and equilibration period (h) on the hatch rate of three embryonic stages of rosy barb (*Puntius conchonius*). Means based on four replicates.

Figure 5

Morphological abnormality of rosy barb hatchlings (Magnification x 40) equilibrated in 4 equal steps.

i) Control

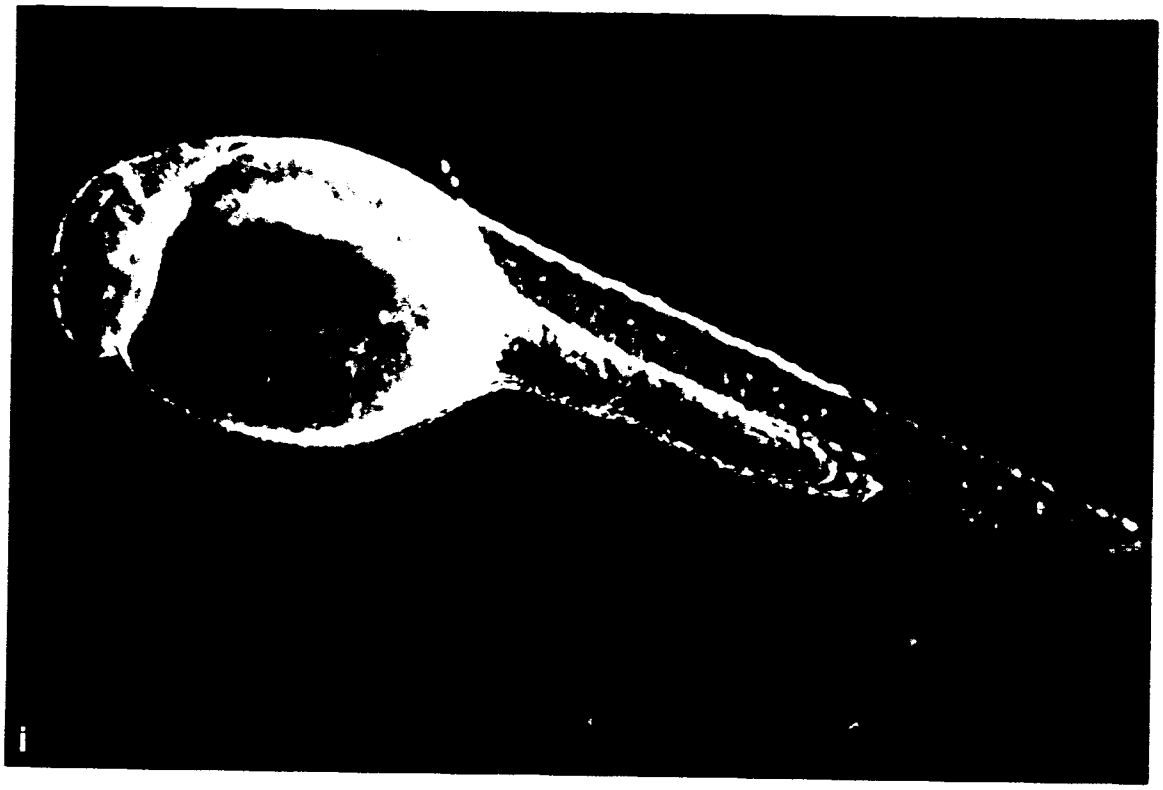
ii) 1 h in 2 M Me_2SO

iii) 3 h in 2 M Me_2SO

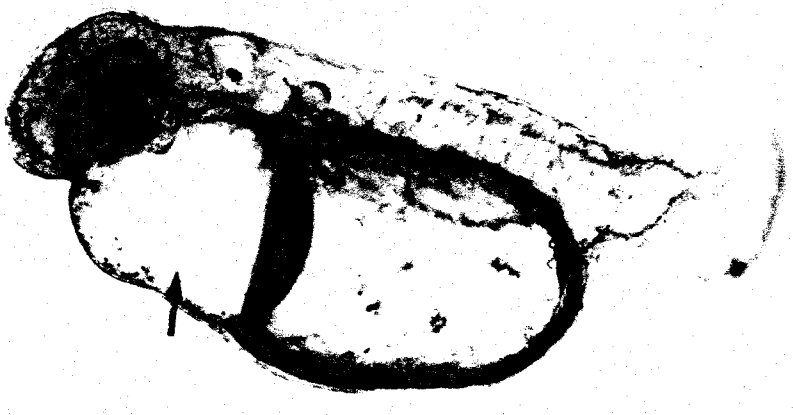
iv) 1 h in 2 M EG

v) 3 h in 2 M EG

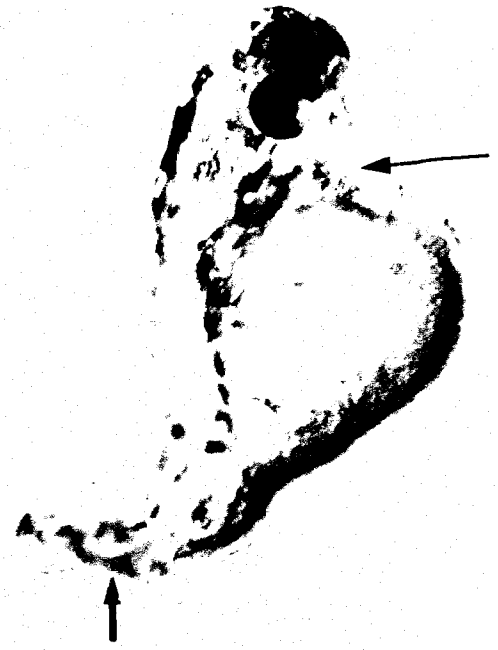
Arrows show enlargement of pericardial cavity (ii-iv), and deformation of the spinal cord (iv).


i

ii



iii



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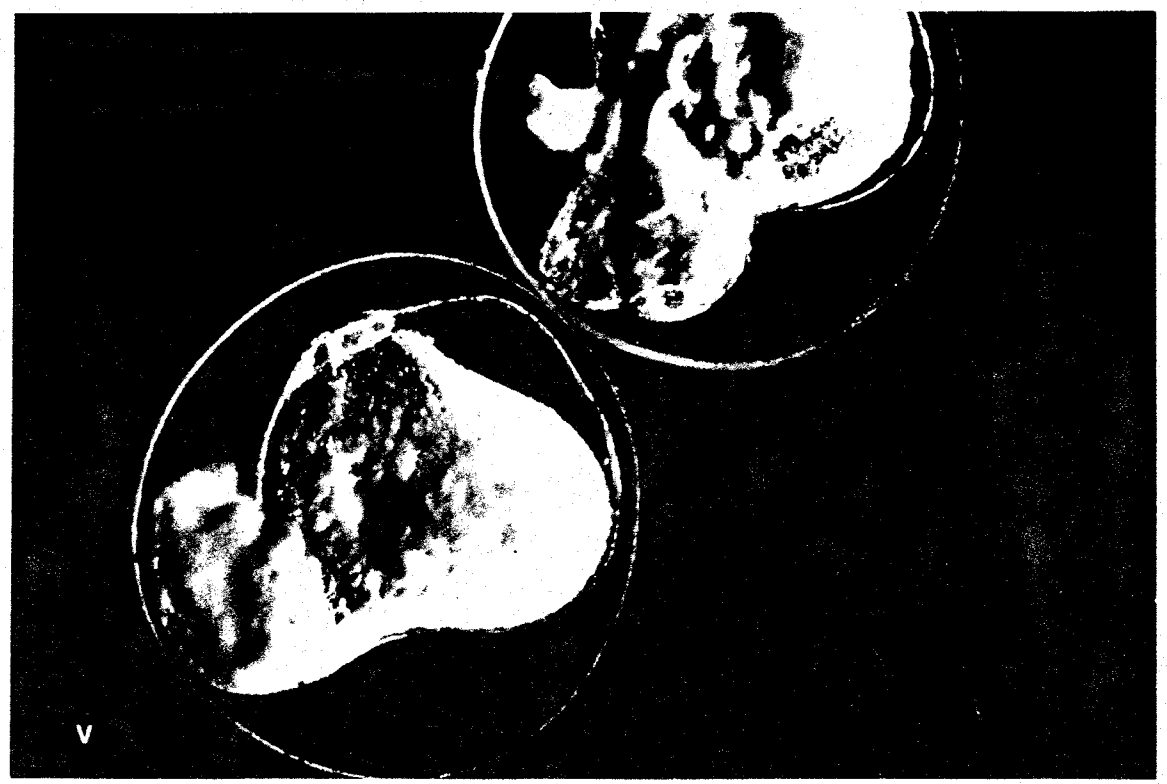

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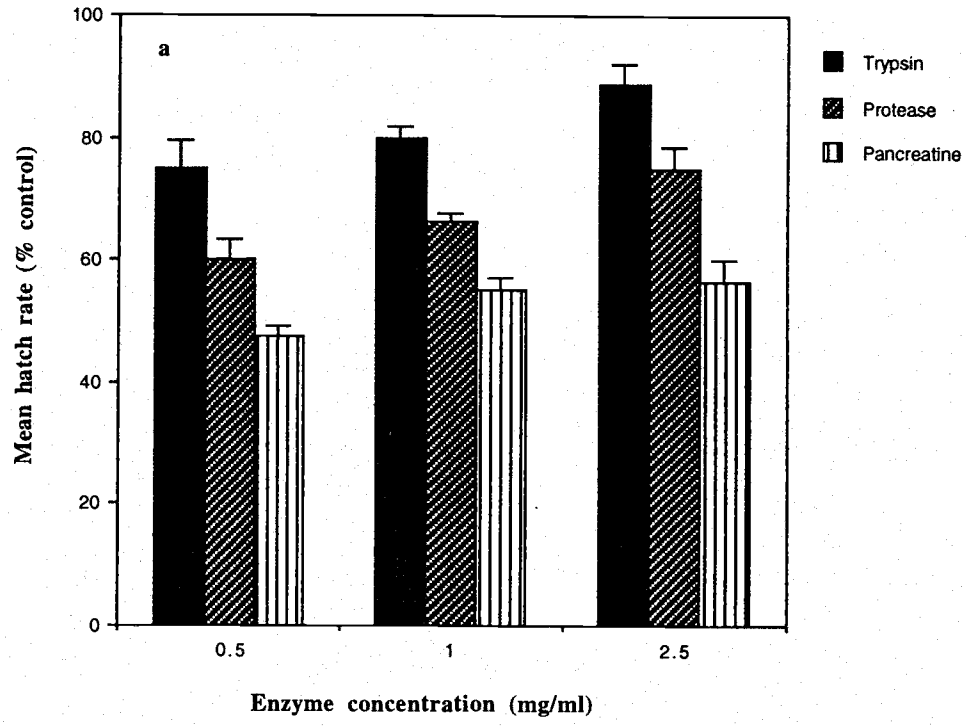


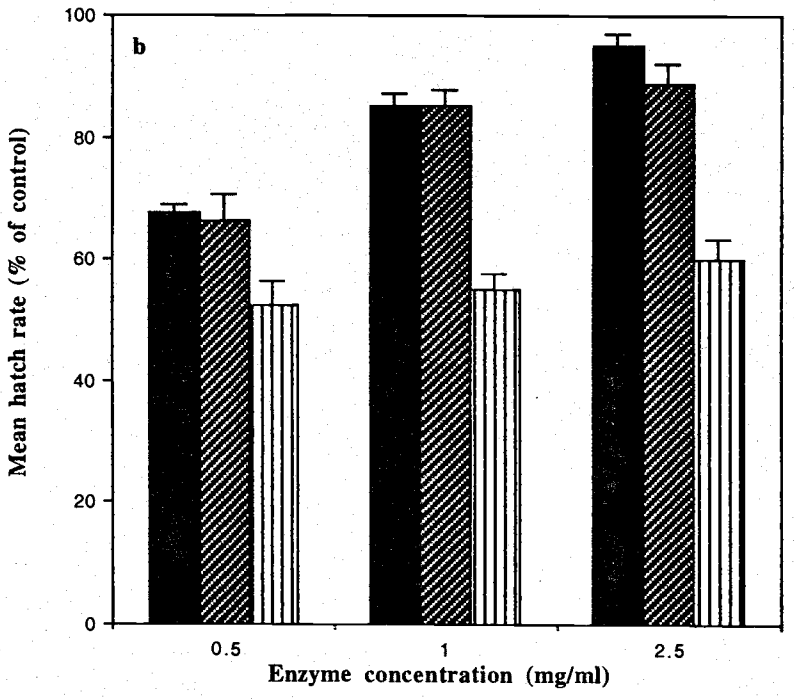
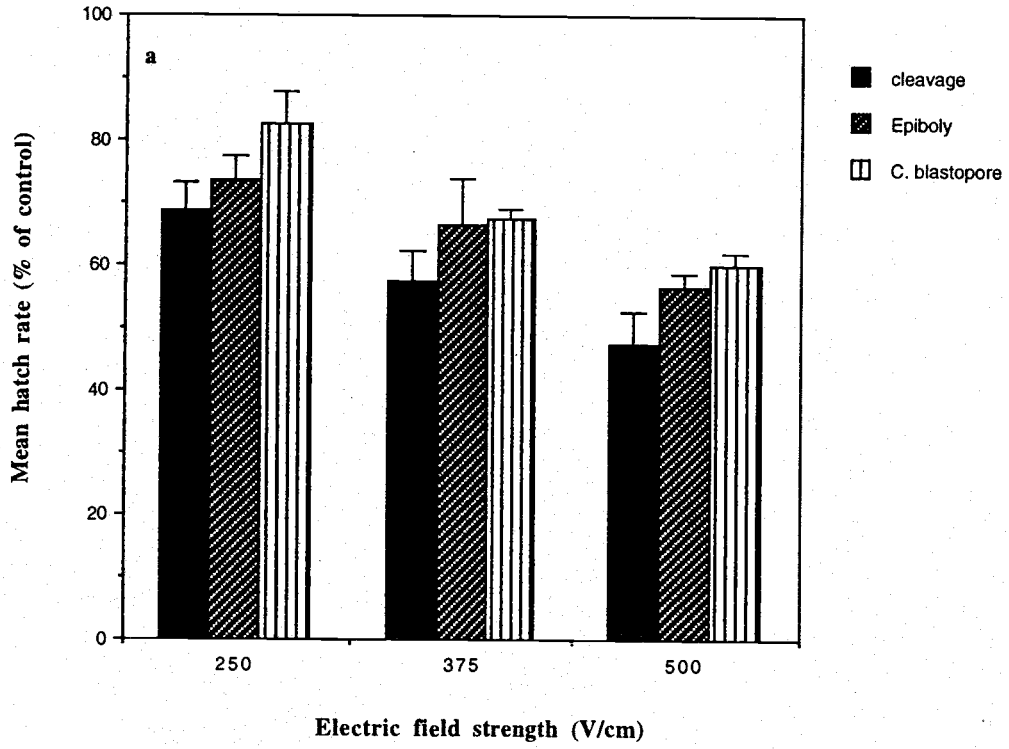


Fig. 6 The effect of five minute enzymatic digestion of 4-cell embryos on hatch rate of (a) zebra fish and (b) rosy barb. All means based on four replicates and they were relative to a control of 100 %.



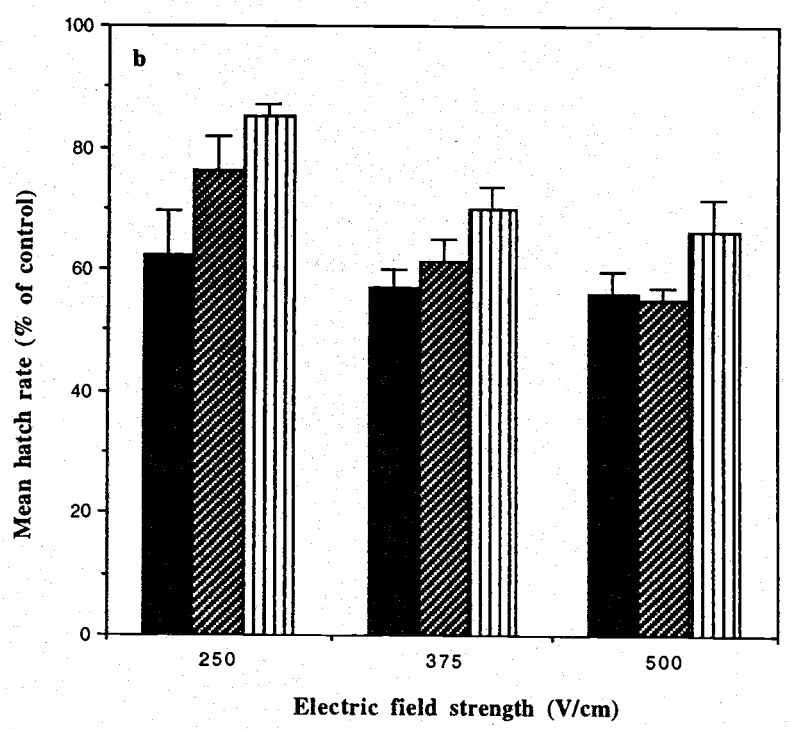
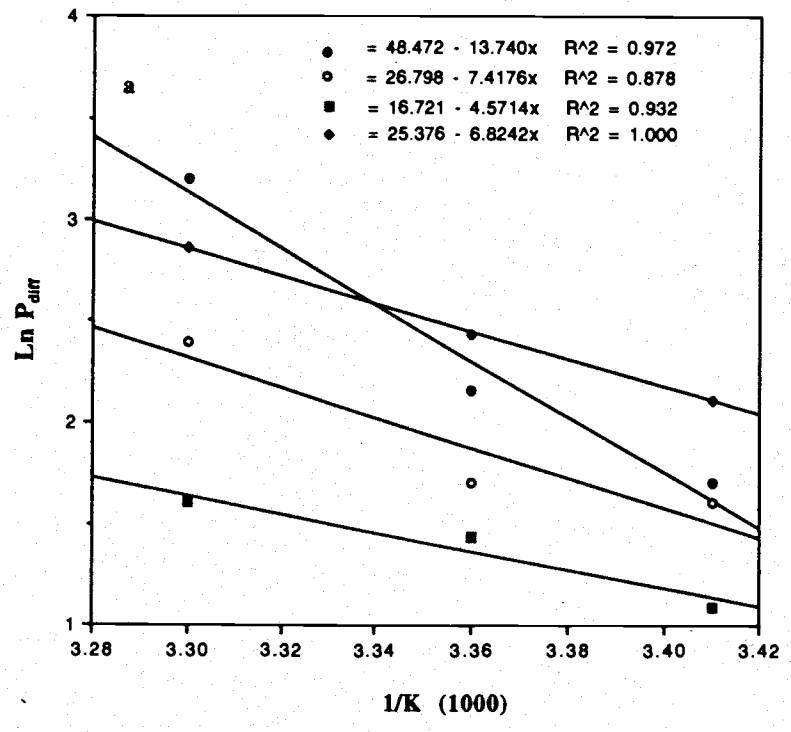


Fig. 7 Effect of electroporation at various field strengths and 0.25 u F capacitance on mean hatch rate of three embryonic stages: (a) zebra fish and (b) rosy barb. All Means based on four replicates and they were relative to a control of 100 %.



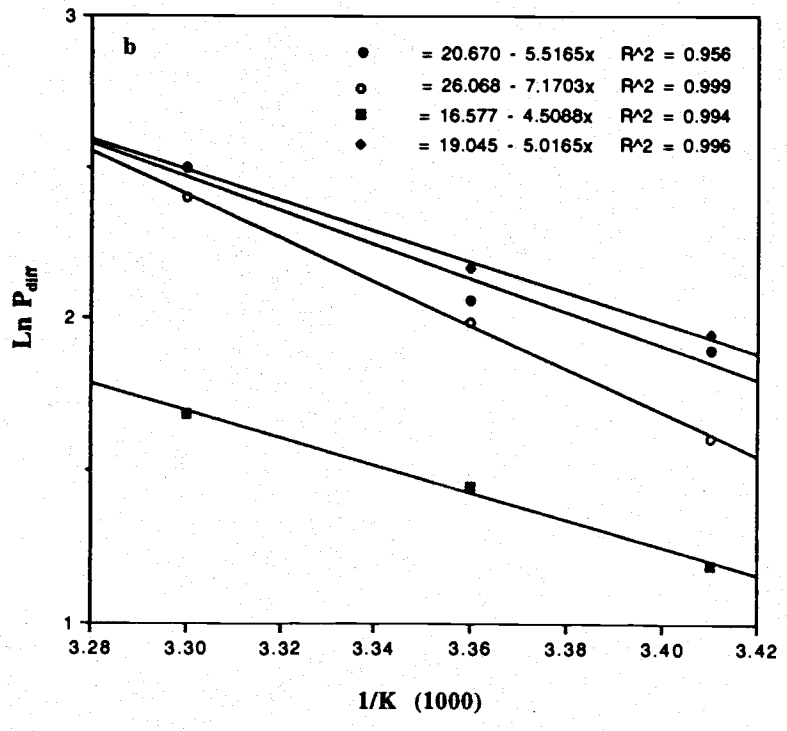


Fig. 8 Arrhenius plot of Ln permeability coefficient (Pdiff) versus the inverse absolute temperature at (●) cleavage, (○) epiboly, (■) closure of blastopore, and (◆) heart beat stage in (a) zebra fish and (b) rosy barb.

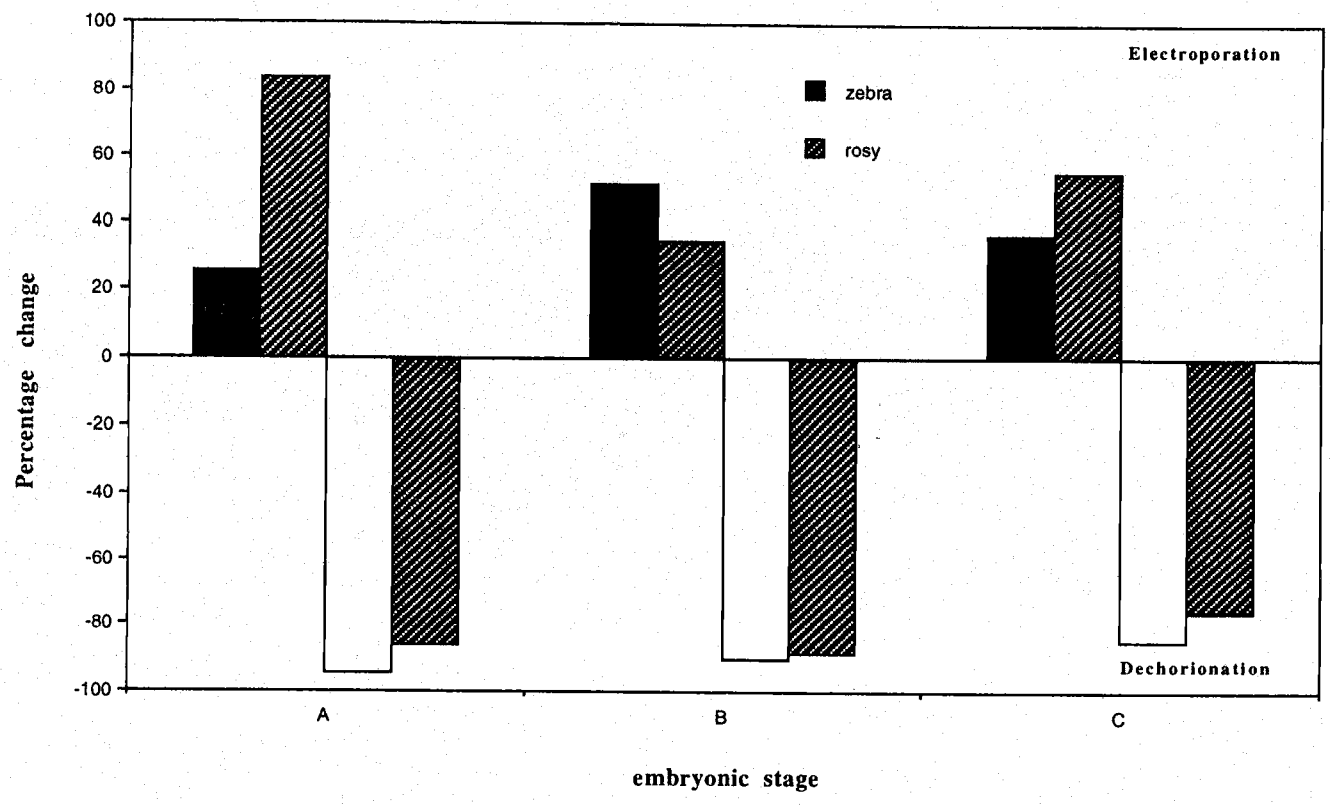


Fig.9 The effect of electroporation and dechorionation on the permeability of zebra fish and rosy barb embryos at (A) cleavage (B) epiboly , and (C) closure of the blastopore stages.

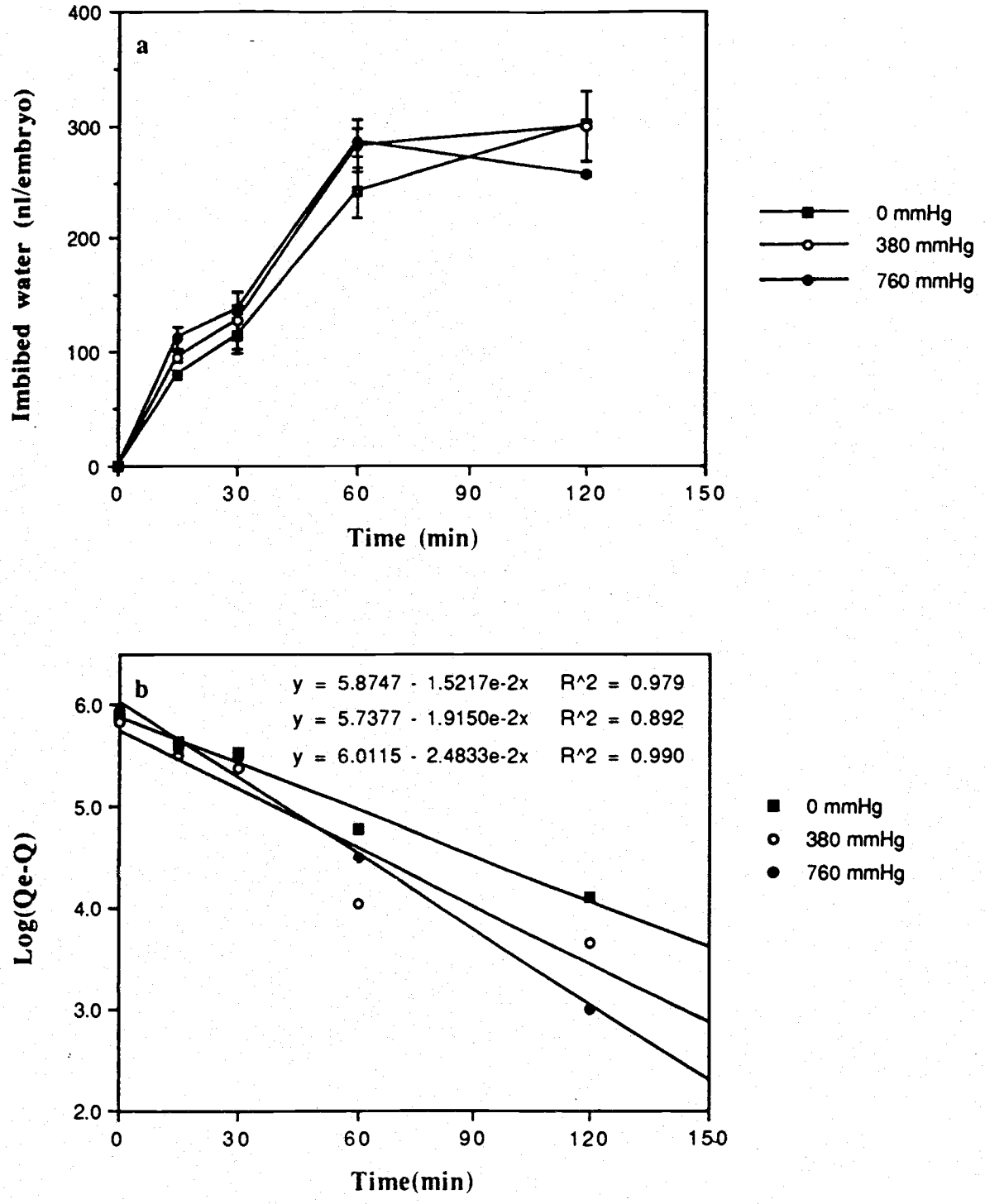


Fig 10 The effect of vacuum equilibration on (a) the uptake and (b) the rate constant of tritiated water in rosy barb embryos (precleavage) under the respective vacuum conditions.

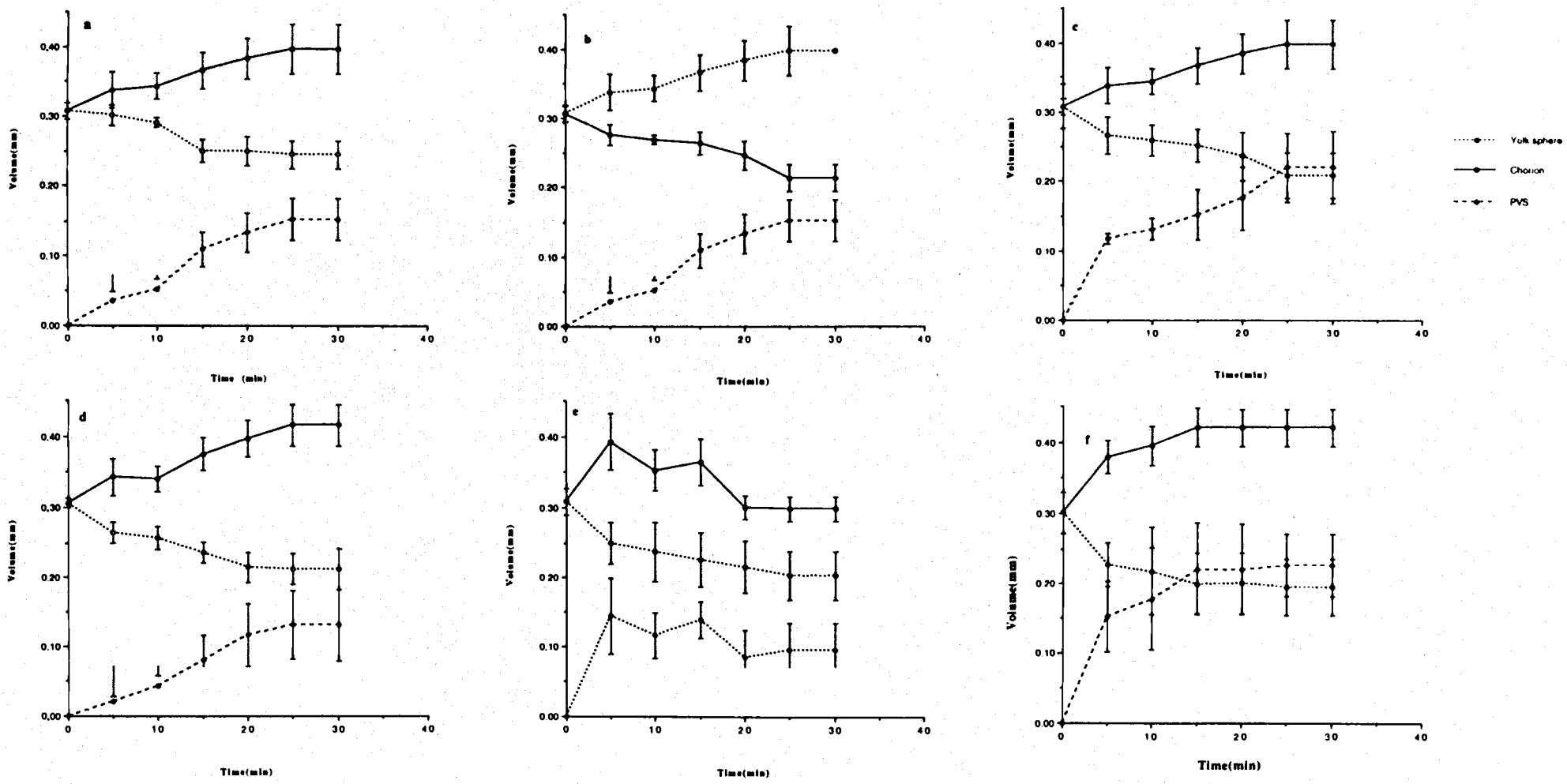


Fig11A Volumetric changes of yolk, chorion, and perivitelline space in zebra fish (*Brachydanio rerio*) precleavage embryos. Embryos equilibrated in (a) 0.5, (b) 1.0, (c) 1.5, (d) 2.0, (e) 2.5, and (f) 3.0 M sucrose solutions. Means (±sem) based on 5 eggs.

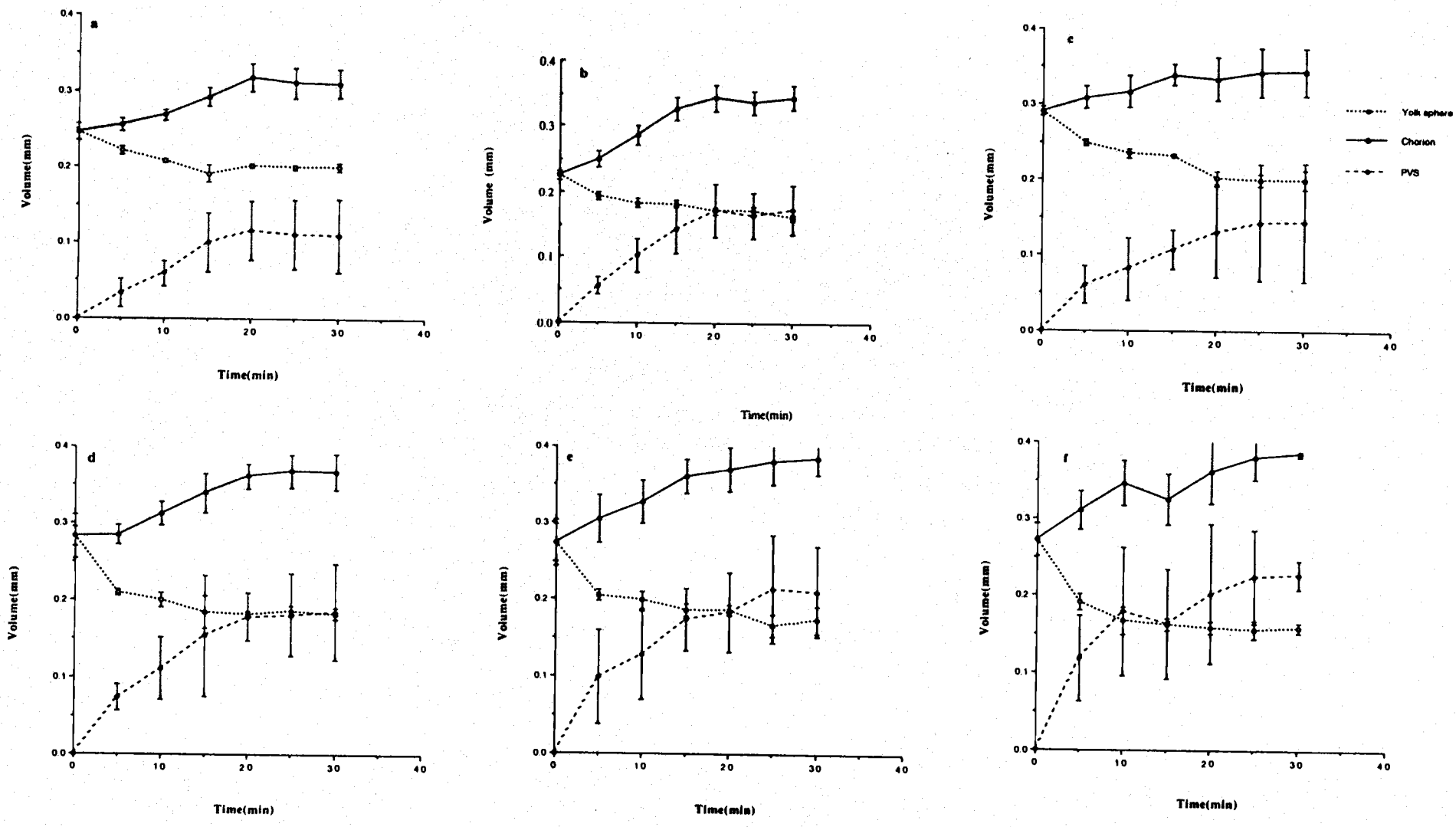
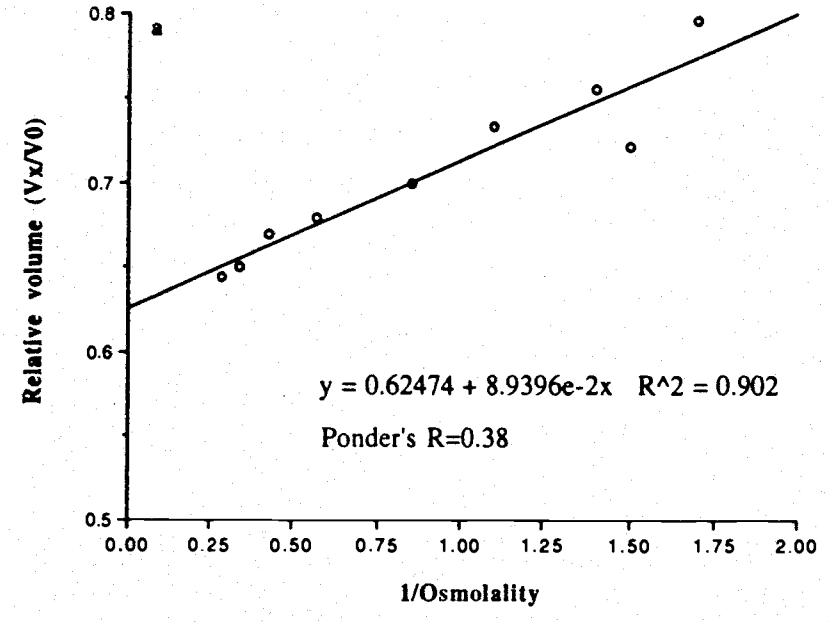


Fig 11B Volumetric changes of yolk, chorion, and perivitelline space in rosy barb (*Puntius conchonius*) precleavage embryos. Embryos equilibrated in (a) 0.5, (b) 1.0, (c) 1.5, (d) 2.0, (e) 2.5, and (f) 3.0 M sucrose solutions. Means (±sem) based on 5 eggs.



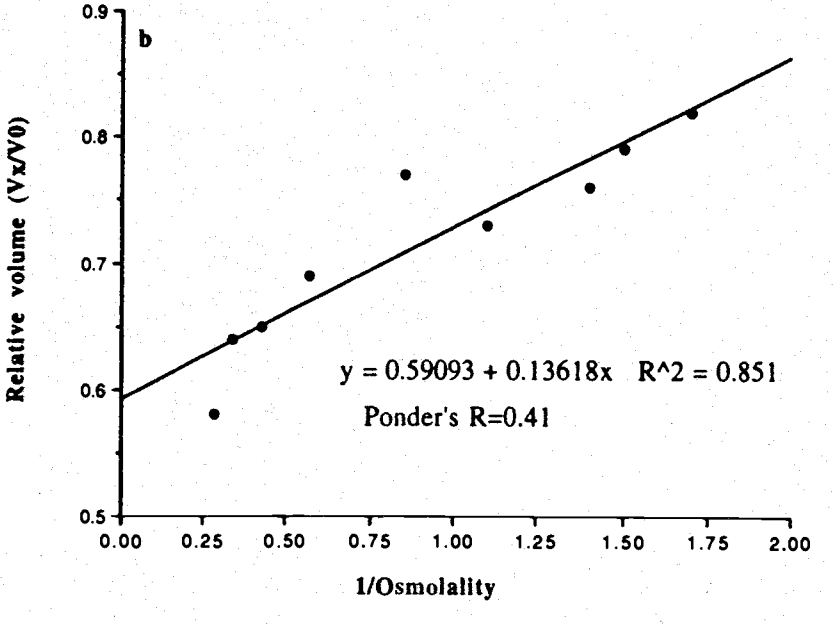
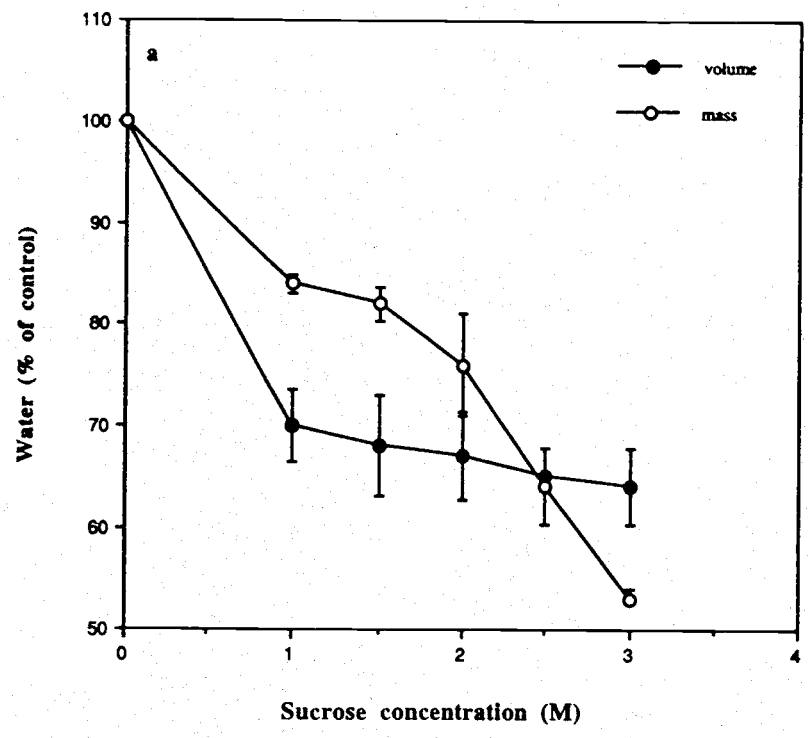


Fig 12 The effect of osmolality on the relative yolk sphere volume in (a) zebra fish and (b) rosy barb embryos after 30 min dehydration in various sucrose solutions.



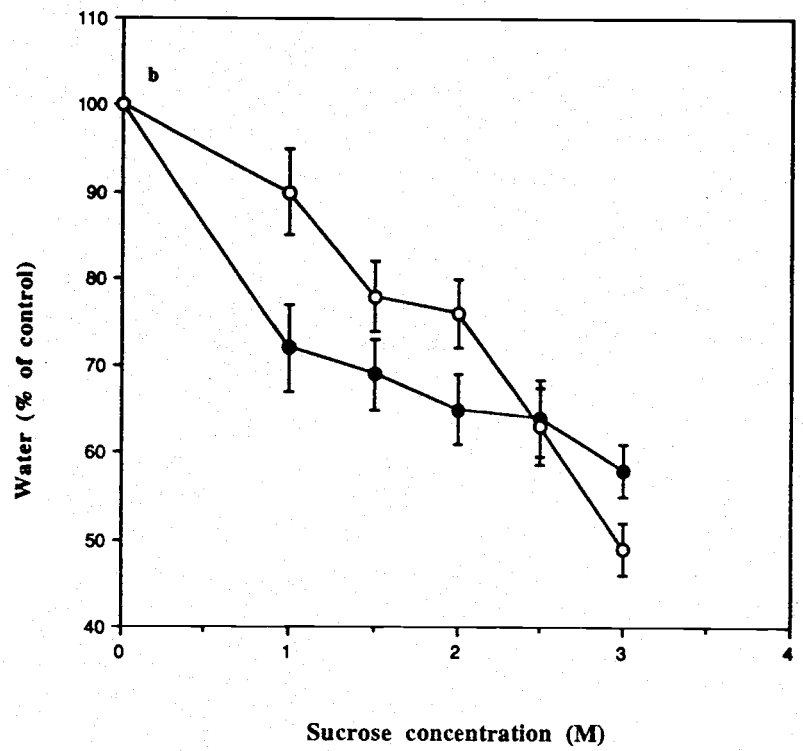


Fig.13 Water content in precleavage embryos of (a) zebra fish and (b) rosy barb determind by mass and volume methods.

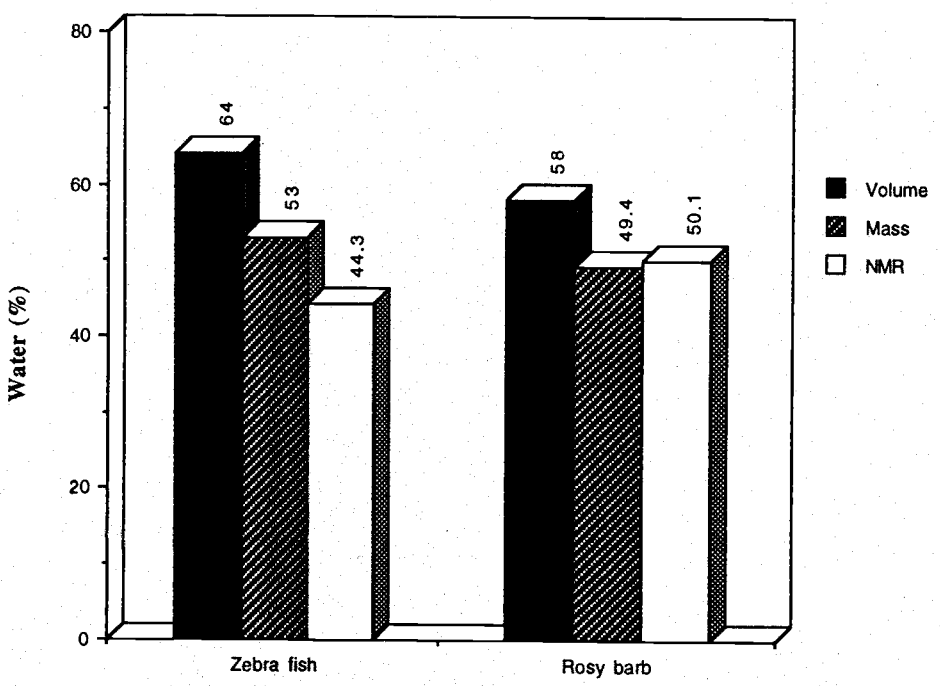


Fig.14 Comparison of water content between volumetric, mass and NMR methods following 30 min equilibration of precleavage rosy barb and zebra fish embryos in 3 M sucrose solution.

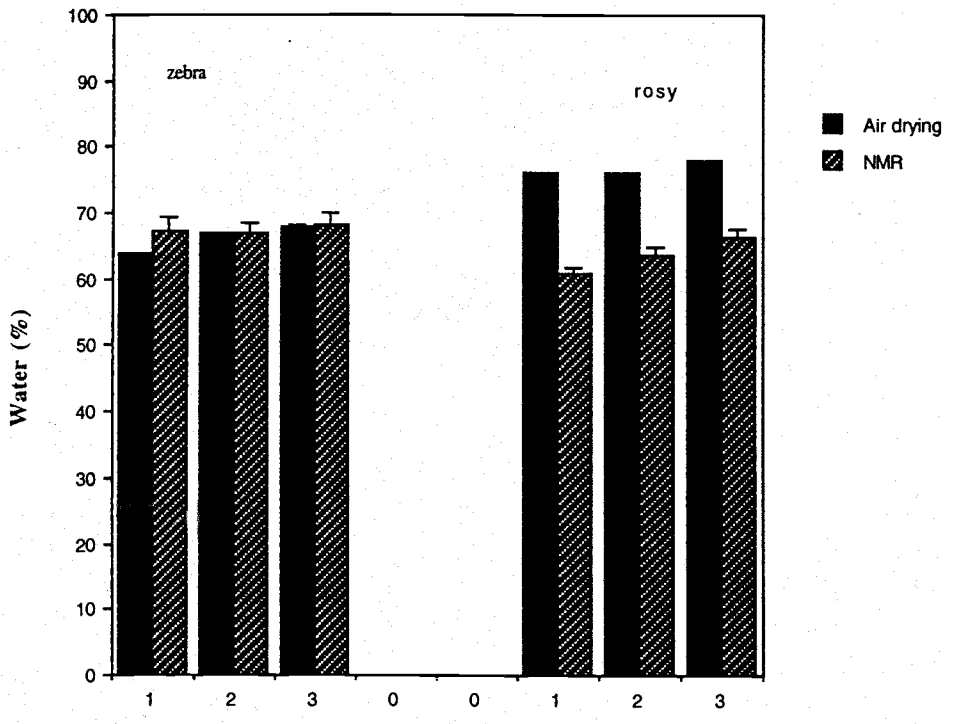
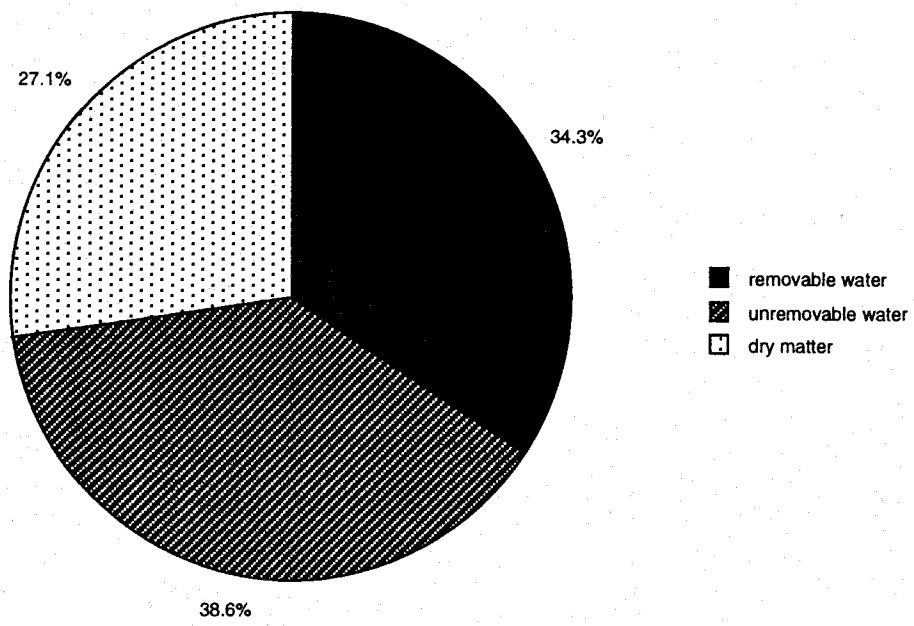


Fig.15 A comparative study of water content in three embryonic stages of zebra fish and rosy barb at (1) cleavage (2) epiboly (3) closure of blastopore using NMR and conventional air drying techniques . Embryos were either dehydrated for 30 min in 3 M sucrose solution or air dried for 24 h. Means + SE based on three replicates.

a



b

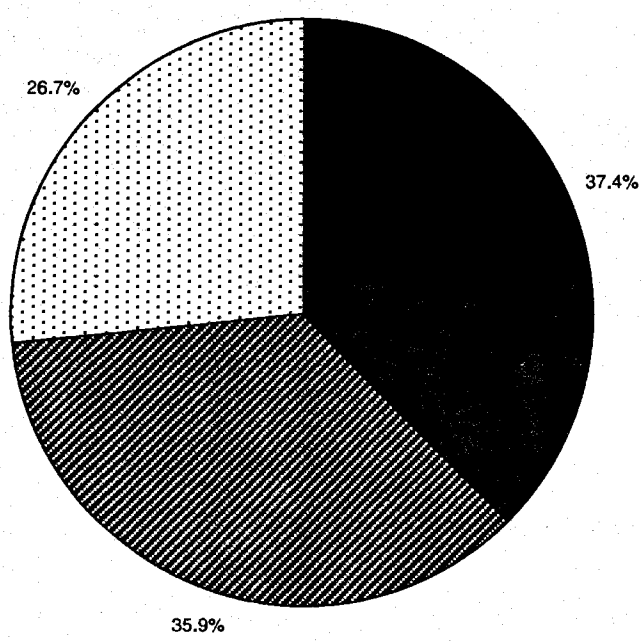


Fig.16 Percentage of removable and unremovable water and dry matter in (a) zebra fish and (b) rosy barb precleavage embryo.

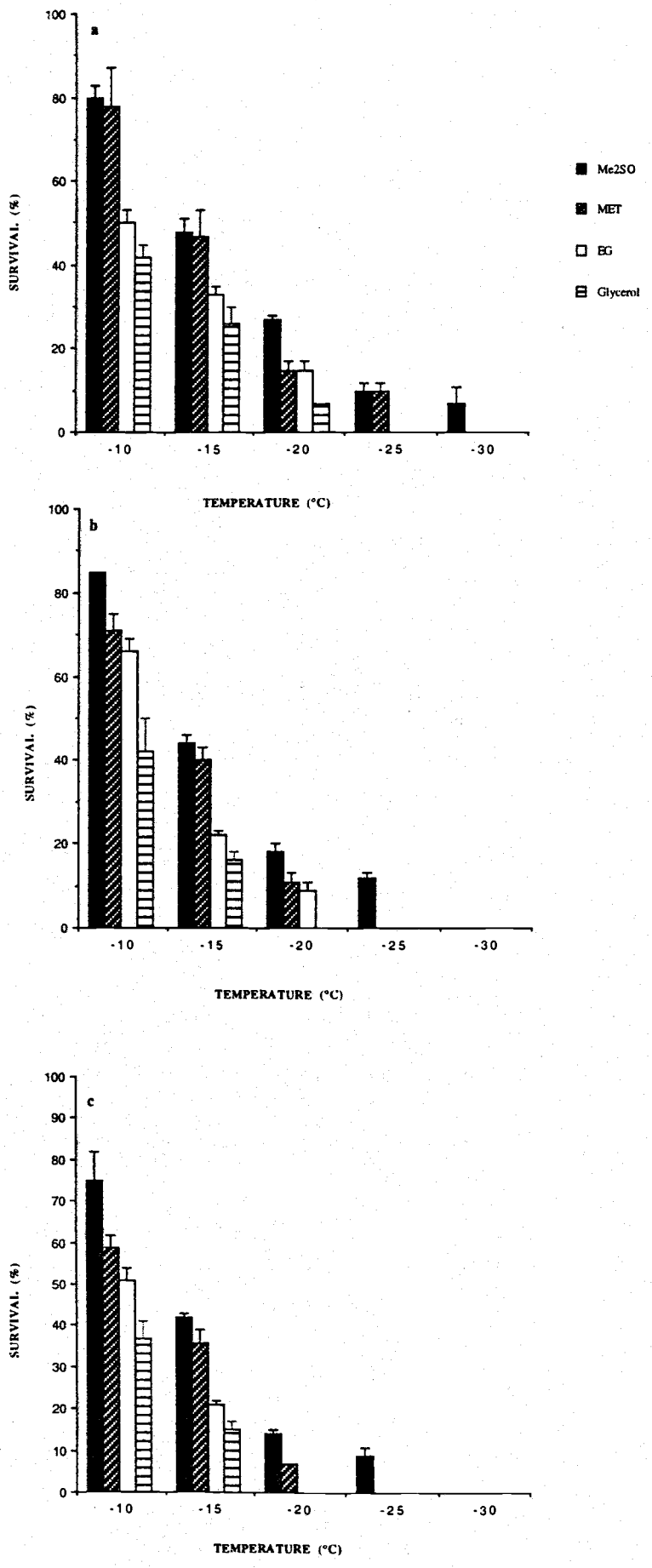


Fig17A The effect of cooling rates and target temperature on the survival of heart beat stage of zebra fish embryos after 1 h step wise equilibration in 1 molar dimethylsulphoxide (Me2SO), methanol (MET), ethylene glycol (EG) and glycerol cooled at (a) 0.1, (b) 0.5, (c) 1.0 C/min. Means + SE based on three replicates.

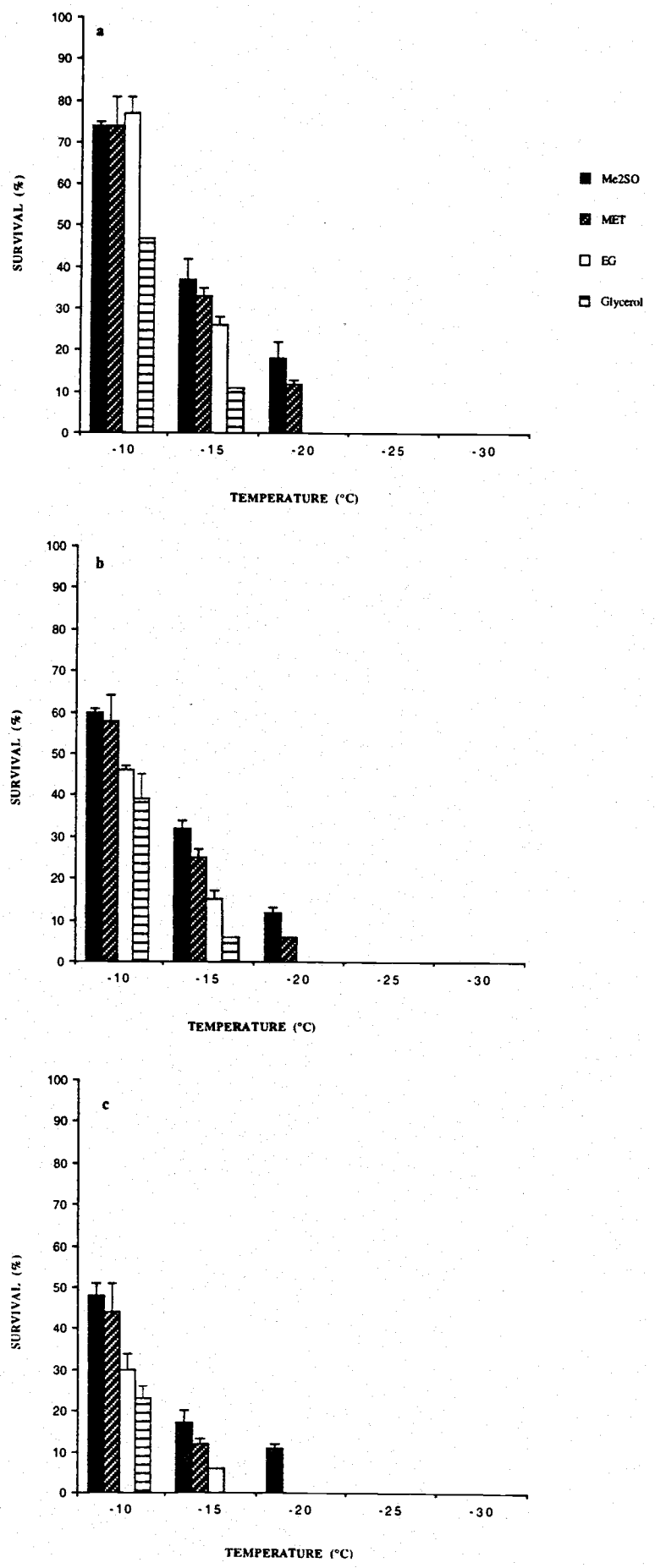


Fig.17B The effect of cooling rates and target temperature on the survival of heart beat stage of rosy barb embryos after 1 h step wise equilibration in 1 mol dimethylsulphoxide (Me2SO), methanol (MET), ethylene glycol (EG) and glycer cooled at (a) 0.1, (b) 0.5, (c) 1.0 C/min. Means + SE based on three replicates.

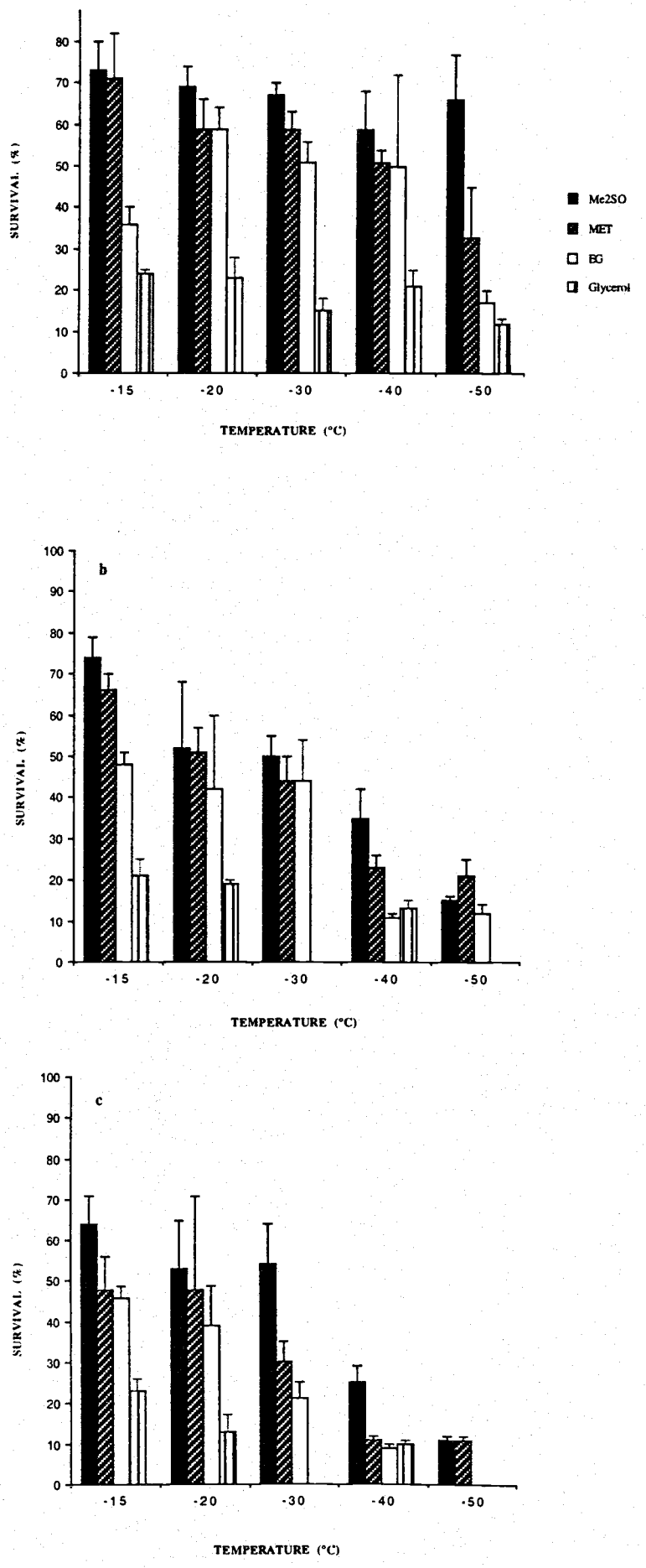


Fig.18A The effect of cooling rates and target temperature on the survival of isolated zebra fish blastomers after 30 min step wise equilibration in 1 molar dimethylsulphoxide (Me2SO), methanol (MET), ethylene glycol (EG) and glycerol cooled at (a) 0.1, (b) 0.5, (c) 1.0 C/min. Means + SE based on three replicates.

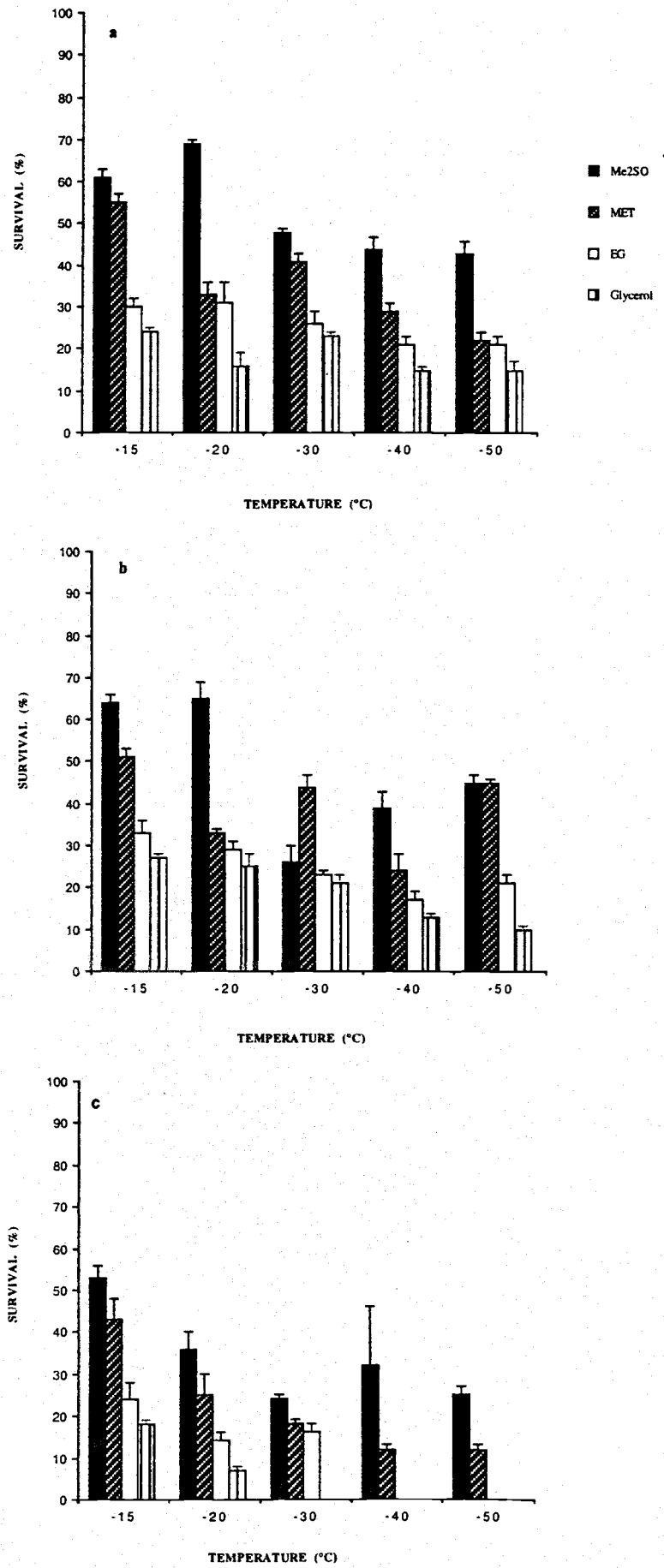
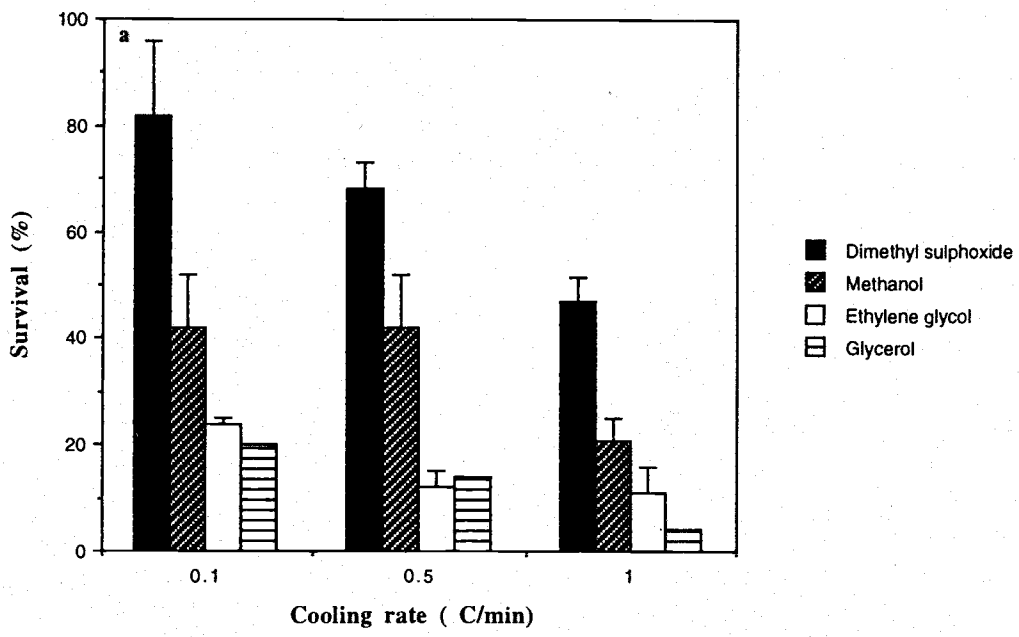


Fig.18B The effect of cooling rates and target temperature on the survival of isolate rosy barb blastomers after 30 min step wise equilibration in 1 molar dimethylsulphoxid (Me2SO), methanol (MET), ethylene glycol (EG) and glycerol cooled at (a) 0.1, (b) 0. (c) 1.0 C/min. Means + SE based on three replicates.



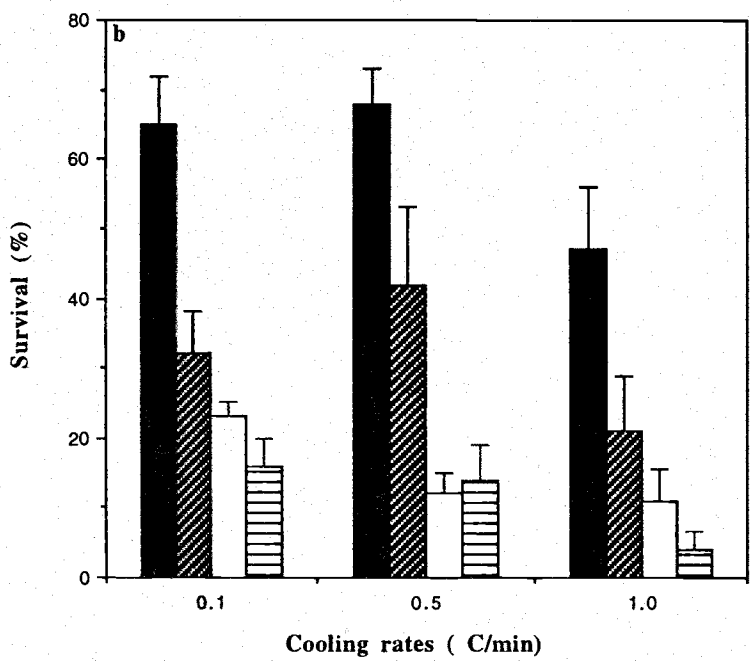


Fig.19 The effect of cooling rate on the survival of (a) zebra fish and (b) rosy barb blastomeres, cooled to -50 C at different rates in various cryoprotectants before plunging into liquid nitrogen. Means + SE based on three replicates.

FINAL FINANCIAL STATEMENT

Investigations into the problems of eggs and embryos of commercially important tropical species.
R4914

GRANT AWARDED	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
PERSONAL EMOLUMENTS					
Research Lecturer	£90,395.00	£23,148.99	£25,903.42	£27,623.49	£76,675.90
CAPITAL EQUIPMENT	£7,250.00	£7,250.00	£0.00	£0.00	£7,250.00
OTHER COSTS					
Broodfish, feed etc.	£1,200.00	£558.22	£555.94	£197.98	£1,312.14
Liquid nitrogen	£800.00	£296.24	£303.15	£205.15	£804.54
Disposables	£2,700.00	£1,014.58	£1,396.09	£784.82	£3,195.49
Reagents	£5,500.00	£2,846.39	£1,973.20	£619.85	£5,439.44
Overhead	£36,158.00	£9,259.97	£10,361.37	£11,049.40	£30,670.74
	£144,003.00	**£44,374.39**	**£40,493.17**	**£40,480.69**	**£125,348.25**