Cryopreservation and aquaculture: A case study with penaeid shrimp larvae

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The need for long-term storage of aquatic invertebrate larval stages by cryopreservation is now more acutely realized. Hence, a study on the economically important penaeid species, tiger shrimp *Penaeus monodon*, was conducted. With combinations of ethane diol and dimethyl sulphoxide or high concentrations of ethane diol, we obtained higher survival rates (~ 80%) between −40°C and −70°C for stage I protozoea larva. But the long-term cryogenic storage of the shrimp larva has not yet been fully achieved. Our understanding of the cryobiology of the penaeid shrimps will pave way for the successful biological cryogenic storage for numerous aquatic metazoan larvae.

MARINE resources are virtually vast and relatively unlimited. The utility of some of the marine bioresources, as food, for the burgeoning human population is well acknowledged. Fish and shrimp resources are primarily targeted for supplementation of the dwindling protein supply in the underdeveloped and developing countries.

Penaeid shrimps have restricted distribution to the tropical countries with abundant backwaters and estuaries, which are obligate habitats for early stages of their life cycle. Their larval stages are marine, post-larval stages are estuarine and subadult and adult stages are strictly marine (with some exceptions). In the Indian subcontinent, the availability of any of these stages is seasonal and virtually diminishes with the onset of monsoon. Hatcheries collect impregnated brooders from the wild and let them spawn to obtain the larvae, which are then sold to the farms. The larval procurement per brooder of the tiger shrimp, *Penaeus monodon*, is approximately 4–5 million eggs and about 1–2 million nauplii. Unlike the cysts of *Artemia*, which can be stored dry, the shrimp eggs or larvae cannot be stored. Development of a stable storage system is a viable and a promising option for ameliorating the scarcity of seeds both during the prime and during off-seasons. Hence, a technique for cryopreservation of shrimp larvae is important for long-term storage and for conservation activities.

Cryopreservation

Numerous cell types, tissues and organs could be *stored in a totally inactivated state at subzero temperatures to be revived later to complete physiological activity by the technique of cryopreservation*[1]. The biophysical and biochemical principles involved in cryopreservation have been amply reviewed earlier[2–4]. The procedure requires that the biological material be treated with cryoprotectants and frozen at a controlled rate to liquid nitrogen temperature (– 196°C). The cryoprotectant plays the role of a dehydrant, freezing point depressant and extra- and intra-cellular stabilizing agent for the biological membrane and cytoplasmic molecules. Some of the widely-used cryoprotectants include dimethyl sulphoxide (DMSO), various monohydric and polyhydric alcohols such as glycerol, ethylene glycol (ethane-1,2-diol), propylene glycol (propane-1,2-diol), sugars (trehalose), amino acids (glycine and proline) and certain naturally occurring antifreeze molecules. Based on the criteria such as freeze and thaw rates employed, which depend on the specimen volume and its water content, the cryopreservation protocols can be classified as slow, rapid or vitrification (Figure 1). In all the procedures, the sample is initially equilibrated in the cryoprotectant, leading to permeation of the sample by the protectant and partial dehydration. In the slow freeze method, the freezing of the external medium occurs or is induced (seeding) leading to removal of water from the system (due to vapour pressure difference), causing further dehydration. This inactivated and dehydrated sample, containing high and viscous concentrations of solutes, including the cryoprotectant, is plunged into liquid nitrogen. Then, the crystallization of the residual water will not damage the sample; instead, the cytoplasm solidifies without meagre crystallization (vitrification). In the case of rapid freeze protocols, very little crystallization of intracellular water is permitted and greater vitrification is achieved. Theoretically, this is possible only in samples of smaller volume, which are often easily permeated by the cryoprotectants and dehydrates faster. This implies that depending on the volume of the specimen under study, the freeze rate has to be optimized. Optimization of freeze rate is a difficult task requiring a number of trials[5].

Vitrification is a process of instant molecular arrest of a high viscosity medium, without permitting ice crystal nucleation. This process alone may suffice to cryopreserve certain specimens, provided they are well permeated and exhibit tolerance to very high concentrations of cryoprotectants. The cryoprotectant concentration during vitrification has to be as high as 6–8 M ethane-1,2-diol or propane-1,2-diol or glycerol (propane-

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1,2,3-triol). In addition, 5–10% w/v of polymers as ficoll, percoll, polyvinyl pyrollidone or albumins of bovine and foetal calf serum are also added. A well-dehydrated and cryoprotectant-permeated specimen vitrifies with very little damage, even if the survival is low. This procedure is practically not feasible in large samples unless an extremely high freeze rate (~ 20,000°C/min at the core of the sample) is imposed, allowing the intracellular water to vitrify[6]. Some insects are reported to vitrify at natural freeze rates by production of large amounts of sugars (trehalose) to avoid winter freezing[7].

Cryopreservation of invertebrate embryos and larvae

On the basis of the above principles, a great extent of work has been carried out to cryopreserve various biological samples with the objective of long-term conservation both for utilization at a later date and for easy transportation. Almost all the mammalian embryonic stages have a common protocol. The human embryos have been stored for long periods, and utilized for *in vitro* fertilization, ever since the procedure was standardized[8]. On the other hand, cryopreservation of the larval stages of aquatic invertebrates has had very limited success due to various factors. Elucidation of these factors remains the primary objective of a cryobiologist studying these larval stages. Though the male gametes of many species, including crustaceans[9,10], are cryopreserved with ease, very few invertebrate embryos/larval stages have been extensively studied with the aim of cryogenic storage. They include rotifer[11–13], sea urchin[14], oyster[15], mussel[16], intertidal nerid[5] and penaeid shrimp[17,18] (Table 1).

Studies on the shrimp embryos

Cryobiological studies on the developing embryonic stages of the shrimps have not received much attention, but toxicological aspects have been studied. Simon *et al.*[17] reported an average loss of 20% of any stage of the embryo of *Penaeus indicus* after a 15 min exposure to 4% cryoprotectant as DMSO, ethane diol and propane diol. Subramoniam and Newton[19] reported toxic effects of 5% (v/v) glycerol, better tolerance to DMSO, methanol and propane diol, and least toxicity with ethane diol (15% v/v for 15 min) in the embryos and larvae of *P. indicus*. Based on the toxicity studies[20] and freeze experiments[19] on the shrimp morula and nauplius, respectively, Subramoniam and his associates suggested naupliar stage larvae for further freezing studies.

Cryobiology of the shrimp larvae

The larval organization of the penaeid shrimps is quite complex and the various organ systems are well-developed and differentiated. The cryobiological characteristics of different cells and tissues greatly vary. Hence, the cryobiology of shrimp larva is expected to be complex. The larva has a nearly impermeable chitinous exoskeleton with only the soft intersegmental membranes, permitting ingression of water and certain solutes. Further, the larvae contain numerous yolk granules, which will reduce the rate of cryoprotectant ingression, as well as its removal after thawing. Therefore, developing a cryogenic storage protocol for this specimen requires an in-depth understanding of the cryobiology of the larval stages. Our earlier studies had indicated

Table 1. Reported studies on the cryopreservation of a few aquatic invertebrate larvae

Animal	Data and comments	Ref.
Rotifer		
Brachionus plicatilis	23.8% of eyed-stage embryos	Toledo and Kurakura[11]
Philodina sp.	67% of adults survived from – 196°C; rotifer is a known stress biotic (anhydro- and cryptobiotic) as an adult	Koehler[12]
B. plicatilis	2% survival of adults	King *et al.*[13]
Sea urchin		
Hemicentrotus pulcherrimus and *Strongylocentrotus intermedius*		Asahina and Takahashi[14]
Oyster		
Crassostrea sp.	48.8% survival of well differentiated trochophore larvae	Toledo *et al.*[16]
Ragworm		
Nereis virens	86% of setiger larvae	Olive and Wang[5]
Shrimp		
Penaeus indicus	63% nauplius IV at – 40°C by slow freeze	Subramoniam and Newton[19]
P. monodon	85% protozoea I at – 70°C by rapid freeze	Arun and Subramoniam[21]

loss of viability, when the shrimp larvae were slow frozen beyond −40°C (refs. 19, 20). The yolk in *P. monodon* larvae not only reduced the rate of cryoprotectant permeation, but was also implicated in (i) reduction of the larval tolerance to freezing, and (ii) initiation of freezing by ice nucleation[21]. These aspects were analysed by conducting numerous freeze trials using different combinations of cryoprotectants. Thereafter, the equilibration time was increased to 30 min or more, and the freeze rate optimized at −2.5°C/min, using highly permeating and low toxic combinations of the cryoprotectants such as ethylene glycol and DMSO. Yet, these follow-up controlled cooling procedures did not yield significant results.

Cryoprotectant permeation and toxicity

Permeation: Among the role played by a cryoprotectant, its permeation and subsequent dehydration of the biological system is important for the success of a freeze procedure. Large-sized samples are less permeated and hence the possibility for internal ice formation is high. The size and structure of the sample used, therefore, affect the permeability. Consequently, the freeze dynamics of the sample is also influenced[3]. Permeation of a cryoprotectant into the embryos is often assessed by the analysis of volume changes associated with the equilibration of the embryo with the cryoprotectant. Either this may be done by timed microscopic observations[21] or using sophisticated volumetric assessment by image analysis systems[22]. Using radiolabelled cryoprotectants during equilibration, permeation is assessed on a scintillation counter[23]. Autoradiography of the treated materials also gives an estimate of the depth of permeation of the protectant as a function of equilibration time. An indirect but approximate estimate is obtained by any of the above methods. Analysis of the extract of the specimen on high performance liquid chromatography (HPLC) should yield precise quantitative data of the cryoprotectant permeated. Many studies have highlighted the importance of making embryonic systems permeable using permeabilization techniques. Studies on the *Drosophila* embryos had used permeabilization procedures, which included dewaxing and dechorionation using hexanes, heptanes and butanes and sodium hypochlorite, respectively[24,25]. Simon *et al.*[17] had used mechanical and chemical (protease) dissolution of the *P. indicus* egg membranes to improve the permeability. Table 2 shows the results for some of the cryoprotectants assayed by timed microscopic observation of the volume change in *P. monodon* protozoea I larvae. This permeation assay indicates that the permeability of a cryoprotectant is directly related to its molecular weight and density. Thus, methanol and dimethyl sulphoxide were rated to be the two best penetrants. Sucrose, on the other hand, is a better-known high molecular nonpermeating cryoprotectant. Virtually, glycerol was unable to permeate into the larva. However, its toxicity assay indicated otherwise[21] (Figures 2 and 3). The precise quantification of the entry of cryoprotectants in the penaeid shrimp larvae remains to be done using radiological methods.

Toxicity: The negative effects of the cryoprotectants on the biological materials are multipronged. In the shrimp nauplius, glycerol induced both osmotic and biochemical toxic effects, but methanol and DMSO showed biochemical toxicity alone[17]. The mechanism of toxic effect of various cryoprotectants has been reviewed by Fahy[26,27]. DMSO and propane diol, when used on developing systems, have been reported to induce developmental anomalies. Vincent and Johnson[28] have reviewed the mechanisms of action of these two cryoprotectants. A few studies have been conducted on shrimp embryo/larval toxicity tolerance[17–21,29]. Although in the permeation assays glycerol did not seem to permeate, its toxic effect on the larva was evident in the toxicity assays[21] (Table 2; Figures 2 and 3). The larvae exhibited 'delayed toxic' effect on equilibration in glycerol and later succumbed after a high percentage revival (Figure 3). The tolerance of the shrimp larvae to other cryoprotectants is shown in Figure 2.

Chill tolerance

Numerous studies have been conducted on the chill tolerance (or prefreeze tolerance) of the arctic and temperate species of insects[30] and fishes[31]. Although attempts to cryopreserve the species of the tropics are constantly made, their chill tolerance is rarely analysed[21,23,32]. Animal

Table 2. Permeation efficiency of a few cryoprotectants in *Penaeus monodon* stage V nauplius (from Arun and Subramoniam[21])

Cryoprotectant	Molecular weight	Density (g/ml)	Permeability*	Time for larval reexpansion (sec)
Glycerol	92.09	1.25	–	No reexpansion
Dimethyl sulphoxide	78.13	1.10	+++++	< 30
1,2-Ethane-diol	62.07	1.11	++++	*circa.* 60
1,2-Propane-diol	76.10	1.04	+++	*circa.* 60
Methanol	32.04	0.79	+++++	< 7
Sucrose	342.30	50% w/v	–	No reexpansion

'+' Represents ~ 20% recovery of the original morphology without damage.

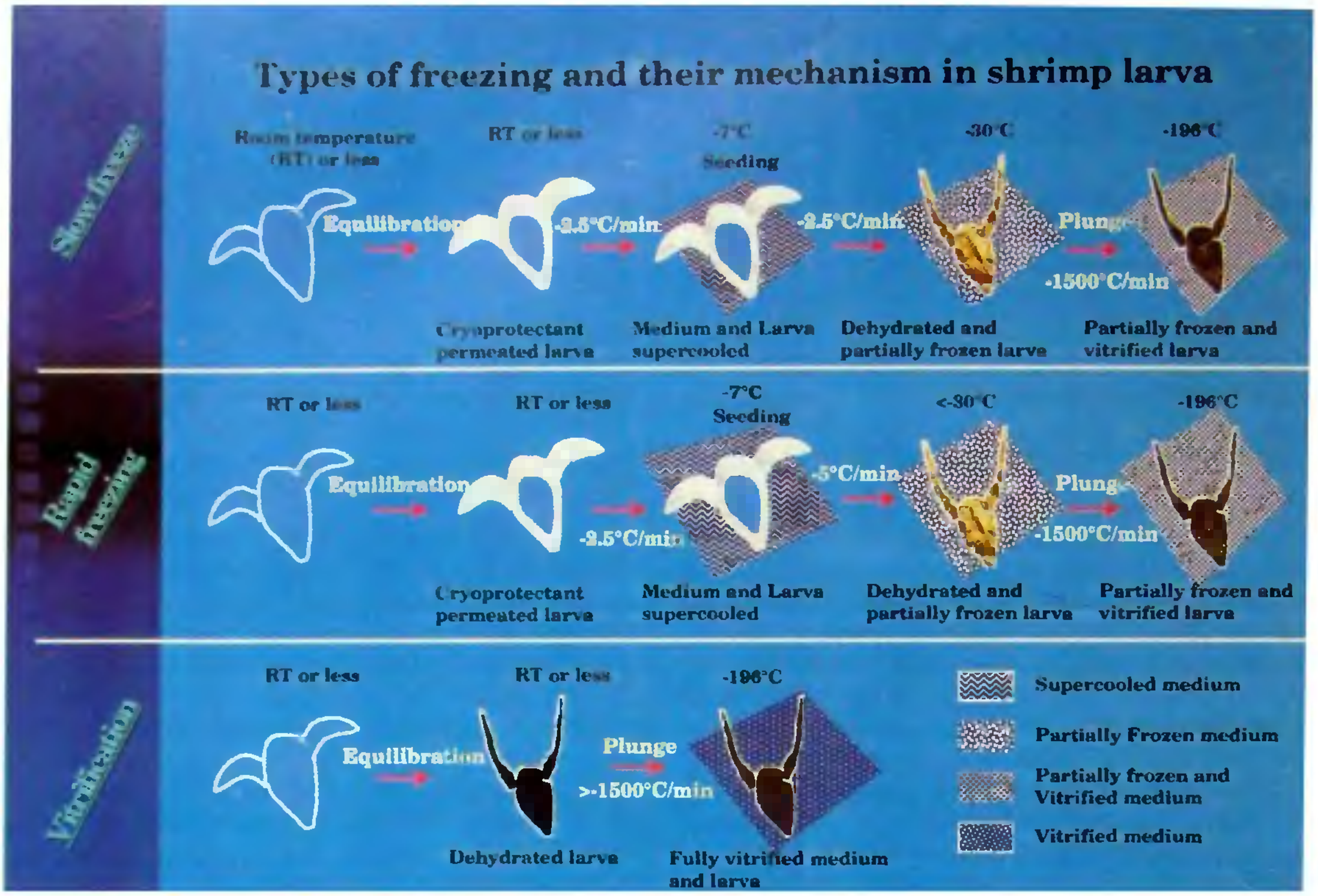

Figure 1. Three types of freezing and their effects as understood on a penaeid shrimp nauplius. The normal conventional slow freeze method has at least three ramps (segments). At the end of the first ramp (−5 to −7°C) seeding is done. At the end of the second ramp (−30 to −40°C), after a short hold, the sample is plunged into liquid nitrogen. In rapid freeze protocols, after seeding, the sample is frozen rapidly to a final temperature and plunged. The vitrification procedure requires only two steps before freezing, namely equilibration and plunging. In all the cases, the equilibration processes may be carried out at a lower temperature (15°C to 10°C), which helps to reduce the toxicity impact. Diagrammatic representations have been made from direct cryomicroscopic (Planer CM3, Planer Biomed, UK) observations (unpublished).

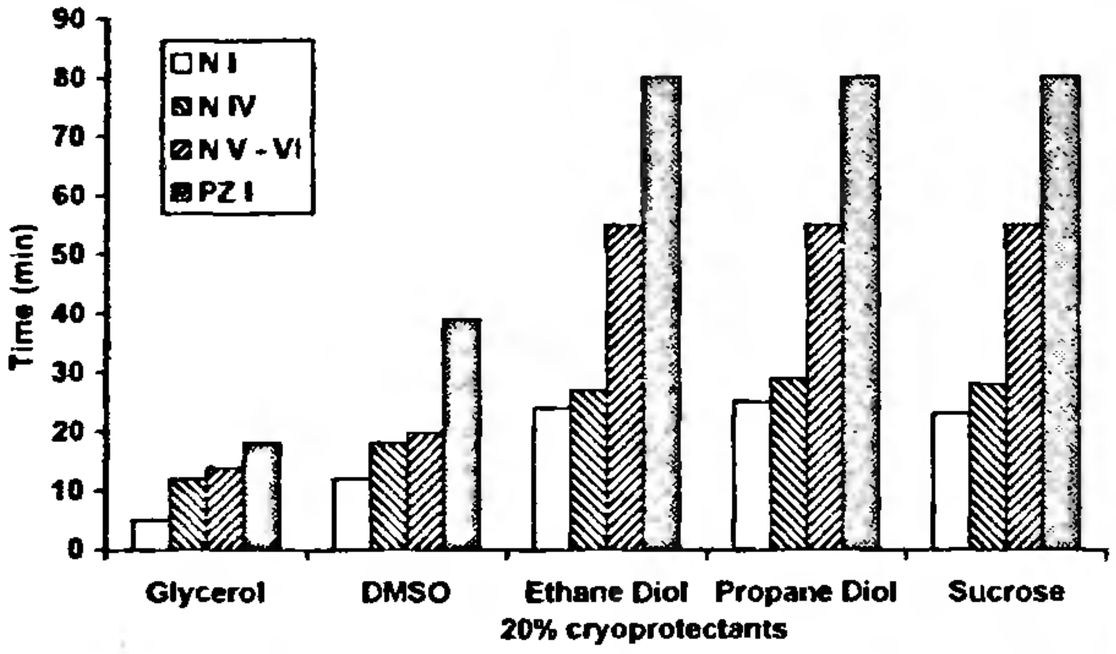

Figure 2. Tolerance to cryoprotectant toxicity in *P. monodon* larval stages expressed as exposure time causing 50% mortality. The cryoprotectant was mixed with the medium containing the sample by drop-wise addition through 15 min. The dilution was done serially. (From Arun and Subramoniam[21]). NI, nauplius stage I; N IV, nauplius stage IV; N V–VI, nauplius stage V to VI; PZI, protozoea stage I; DMSO, dimethyl sulphoxide.

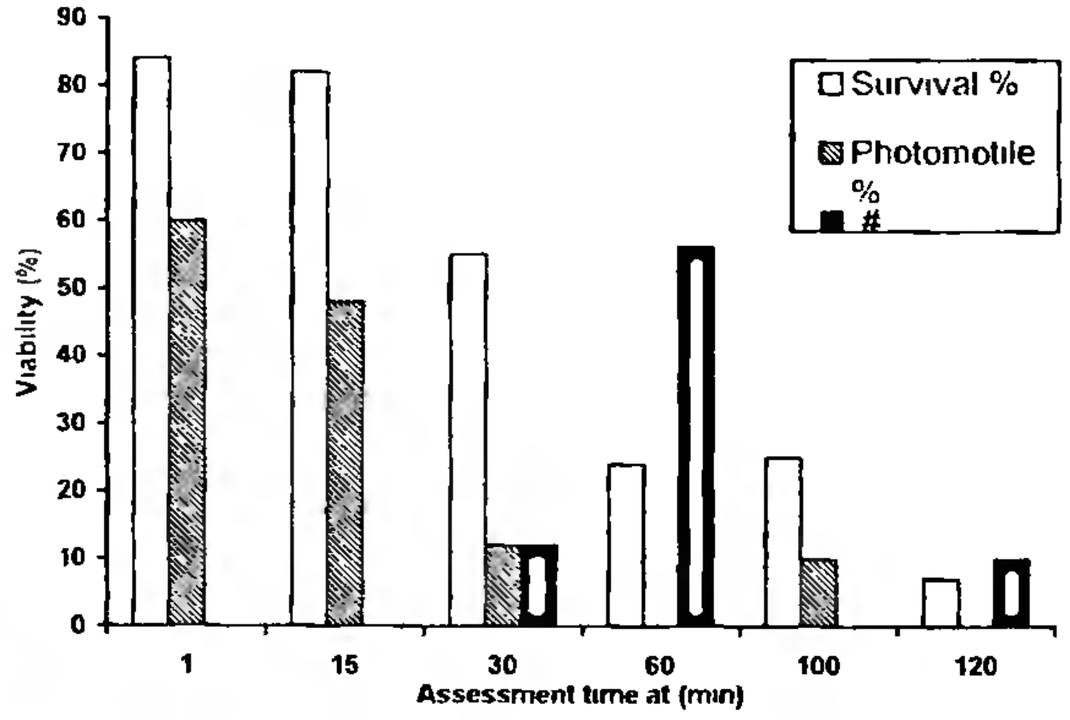

Figure 3. Delayed toxic effect of glycerol. Note the fall in survival of the shrimp nauplius equilibrated in glycerol after complete washing and revival. The glycerol-caused delayed toxicity was assessed from the first minute after the revival of the larvae. '#' Represents the effect of change of fresh medium after 30 min of observation. This replacement of the medium expresses as an increase in survival at the 45th minute. The survival, subsequently, declines with time (From Arun and Subramoniam[21]).

systems do possess a vaguely understood mechanism of chill hardening[30,33]. Nevertheless, it helps them overcome extreme chilliness, especially after they are acclimated by short-term chill exposures. Preliminary studies conducted in our laboratory on *P. monodon* larvae indicate susceptibility of the shrimp larvae to chilliness[21]. Protocols with initial slow ramps have always yielded lower survival rates (Figure 4). This is illustrative of the lack of prefreeze chill tolerance in *P. monodon* larvae. A major drawback of this deficit is loss of viability in the initial segments of the freeze protocol, which is designed to freeze the external medium and dehydrate the larvae. Studies on the effect of chilliness at cellular level have been reported to cause membrane damages by phase changes in the lipid bilayer, which renders the cell susceptible to osmotic stress[34].

Rate of freezing

Selection of a freeze rate for the nauplius larvae depends on the naupliar size and its water content together with its permeability properties to the cryoprotectant and solvent. Mazur[3] had reviewed the importance of size and geometry of the biological system being cryopreserved. Smaller samples such as single cells may be frozen at extremely high velocities. Larger specimens will require slower protocols to accommodate the slower replacement of the water by the cryoprotectant. The series of studies culminating in the development of a protocol for cryopreserving the *Drosophila* embryos clearly showed the importance of permeability of the cryoprotectant and the role of water content during freezing[24,25]. Slow freeze protocols on permeabilized embryos caused chill damages; while rapidly frozen embryos showed freeze damages. Hence, highly dehydrated embryos were vitrified at high thermal velocities of – 20,000°C/min to nitrogen slush temperature (– 205°C) resulting in 60–70% hatching.

Studies were conducted to select an optimized freeze rate for the cryopreservation of shrimp larvae (Figure 5). When frozen at – 0.75°C/min the nauplii showed lower viability. Through – 1°C/min to – 3°C/min, the survival percentage attains a plateau and this range of freeze rate is considered ideal. Rates beyond – 3°C/min resulted in higher survival due to incomplete freezing of the nauplius because at higher rates and a constant final temperature, the sample remains more supercooled than frozen[21]. In contrast to slow freezing, when the larva and the medium freeze nearly at the same time, during rapid freezing, the larva remains more supercooled and continues to dehydrate before the final temperature is reached. Trials conducted in these studies using various freeze rates reveal more on the cryobiological characteristics of the shrimp (Figure 5).

Vitrification as another possibility was also studied. Based on the above-mentioned toxicity and permeability assays, two of the best cryoprotectants were chosen for vitrifying and permeating characteristics namely ethane diol and DMSO, respectively. Ethane diol : DMSO (20 : 15 v/v) gave higher survival in toxicity studies (see Figure 6). We have observed certain combinations of these cryoprotectants to vitrify at extremely low rates of freezing (— 50°C/min), when plunged into liquid nitrogen vapour (unpublished). Further work needs to be done to stabilize a strategy for obtaining vitrified shrimp larvae.

Final temperature

The final temperature is that temperature from which the sample is plunged into the liquefied gas for long-term storage. Selection of this temperature is made by careful trials. At this temperature, the cellular constituents should not be stressed beyond the predictions of damage by

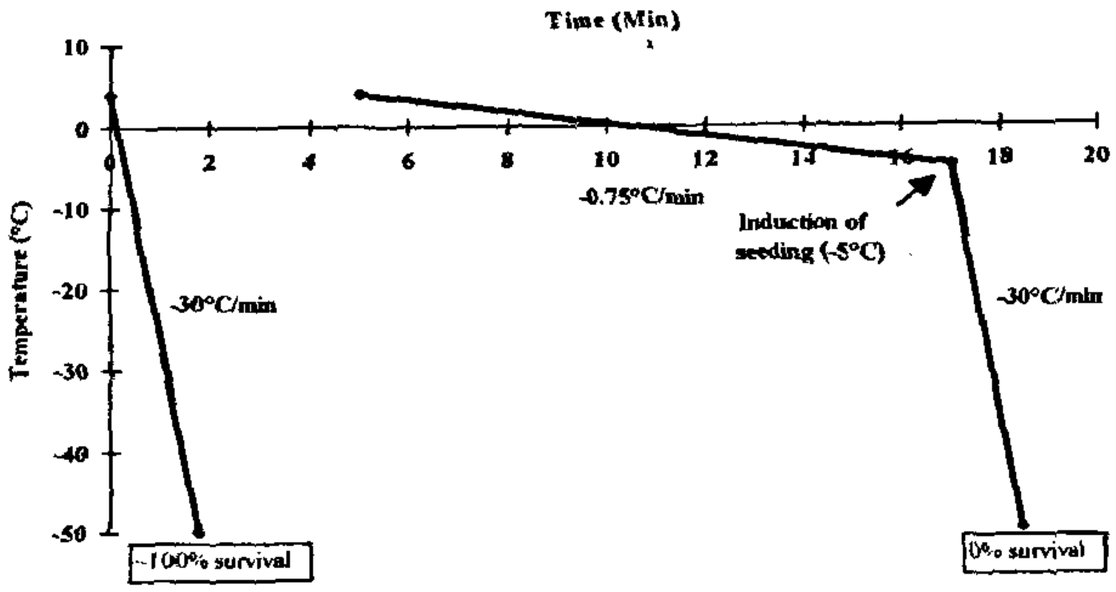

Figure 4. Experimental protocols to illustrate intolerance of *Penaeus monodon* to chilliness during freezing. In both the protocols, the samples were frozen (Kryo 10/1.7. Planer, UK) at – 30°C/min, but in the second protocol, an initial slow ramp of – 0.75°C/min was imposed at prefreezing temperatures. The samples recovered any time after the slow ramp showed total mortality. Compare with Figure 5, where rates less than – 1°C/min cause lower survival. Similarly, in Figure 6 equilibration at 0°C and – 4°C cause lowered survival. (From Arun and Subramoniam[21]).

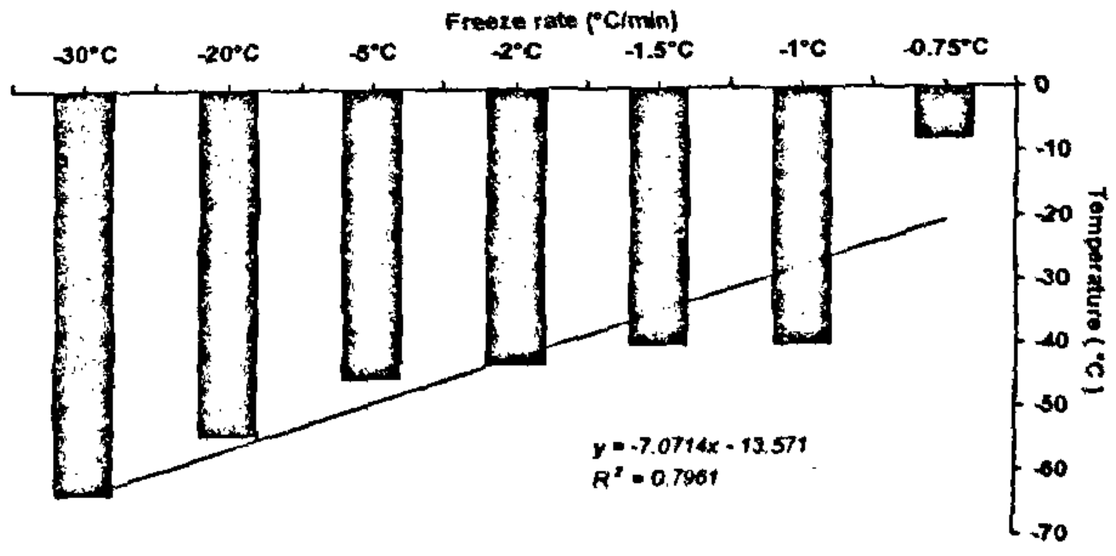

Figure 5. Effect of freeze rate and the final temperatures causing 50% mortality. Freeze rates between – 1°C/min and – 3°C/min give almost similar survival, while faster rates give higher survival. The larvae were frozen on Kryo 10/1.7. Planer, UK (From Arun and Subramoniam[21]).

'minimum cell volume theory' or 'salt concentration' leading to osmolarity damages due to higher solute concentration[35]. Viscous solutes may interact with biomolecules causing irreversible damages[36,37]. Experiments on freeze rate optimization for shrimp nauplius showed that the larvae, exposed between −20°C and −30°C, by freezing at rates between −1°C/min and −2.5°C/min are either incompletely frozen or remain supercooled. When the samples were held at −40°C for more than 25 min or frozen beyond −40°C, progressive loss of viability was observed. Understandably, the suitable plunge point lies between −30 and −40°C.

Thaw rate

The thawing process is greatly affected by the sample volume which was frozen. Meryman[2] had thus summarized the importance of thawing: 'whenever rapid freezing is necessary for survival, rapid thawing is also almost universally necessary'. Slower thaw rates cause extensive structural damage to well-developed multicellular structures by formation and fusion (recrystallization and devitrification) of ice crystals during thawing. Newton[38] refers to the requirement of rapid thawing for *P. indicus* larvae after exposure to −30°C, rather than when exposed to −20°C. This clearly showed that at −30°C the larvae have more internal ice formations. Hence, it is more susceptible to ice crystal fusion and freeze damage at slower thaw rates.

Larval dehydration

Optimal dehydration is necessary during any freeze procedure including vitrification. In the slow and rapid procedures, dehydration takes place during equilibration and during extracellular ice formation. In vitrification, however, the dehydration is completed to the optimum during the equilibration. This is accomplished by large volumes of proteins (bovine serum albumin and foetal calf serum) and polymers (ficoll and percoll) in the cryoprotectant medium. When conventional procedures are adopted, and the system dehydrates with the progress of the protocol, the dehydration stress may play a crucial role affecting the viability. Tolerance to dehydration is a seldom-studied aspect on any animal system before being taken up for cryopreservation. Figure 7 depicts the results of comparative studies conducted on the nauplius stage V and protozoea stage I of *P. monodon*[21]. Various stages of the larva were exposed up to 50% (v/v) sucrose in seawater, and the survival was assessed. The lower stage larva was found more susceptible to dehydration damage, resulting in abdominal fracture.

Recent developments in cryopreservation of larval stages

The development of freeze protocols for metazoan systems is more complex and involves in-depth analysis of the cryobiological characteristics of the system. Examples of such studies include, development of a cryopreservation protocol for (i) *Drosophila* by Steponkus *et al.*[24] and Mazur *et al.*[25], (ii) zebra fish embryos by *Harvey et al.*[39], *Hagedorn et al.*[40] *and Zhang et al.*[22] and (iii) the larva of *Nereis virens* by Olive and Wang[5]. These studies have improvized on modern techniques to solve the complex problem of embryo cryopreservation. Hagedorn *et al.*[40] have made use of magnetic resonance imaging (MRI) technique, modified for use on small fish embryos. MRI generates data on the distribution as well as the quantity of the permeated cryoprotectant.

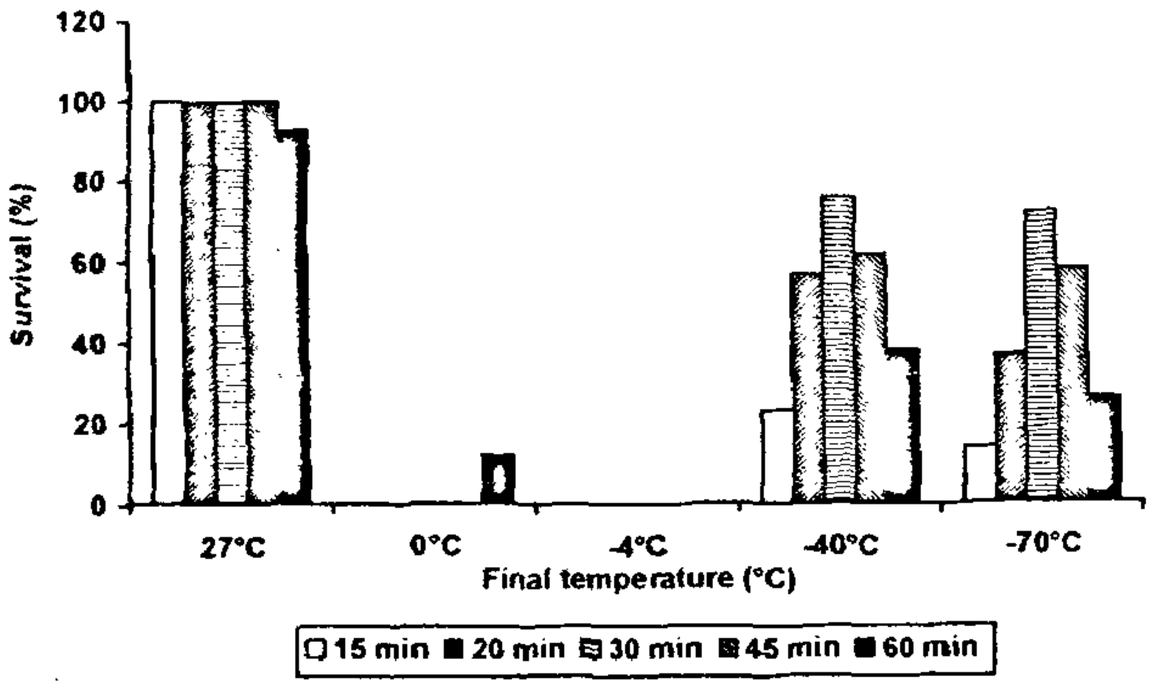

Figure 6. Effect of equilibration in DMSO : ethane diol (15 : 25 by volume in sea water) on the shrimp *Penaeus monodon* protozoea I. After 15, 20, 30, 45 and 60 min of equilibration in the mixture, the samples were frozen (Kryo 10/1.7. Planer, UK) to various final temperatures at −2.5°C/min to −40°C and at −5°C/min beyond −40°C. Thereafter, it was held at that temperature for 5 min (Modified from Arun and Subramoniam[21]).

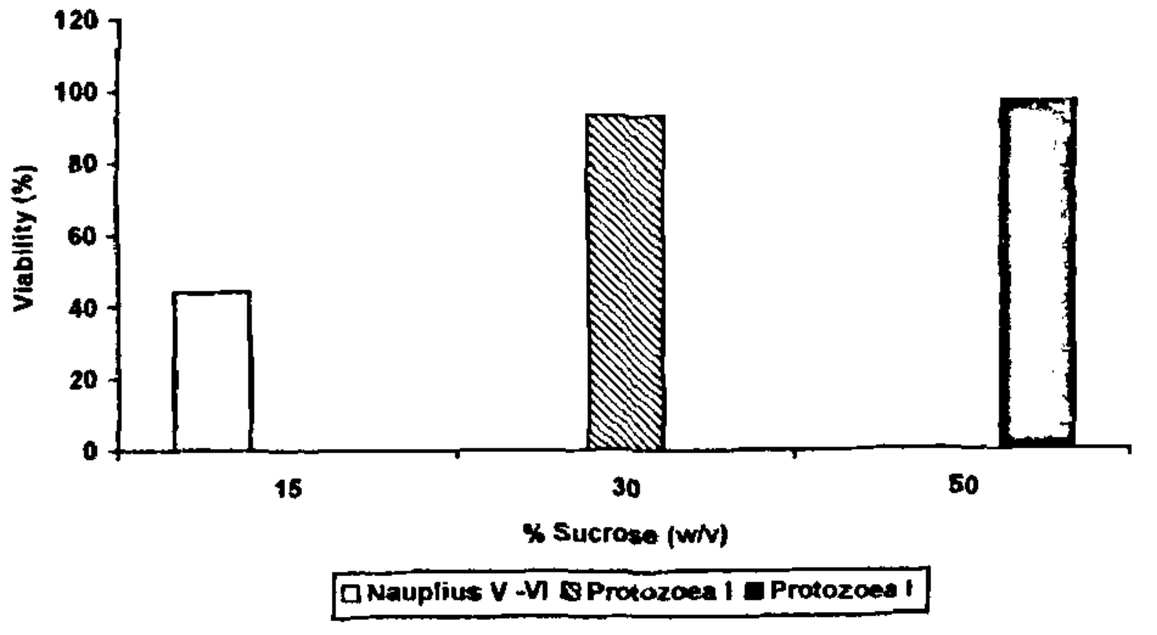

Figure 7. Assessment of tolerance to dehydration vector, using the nonpermeating cryoprotectant sucrose as dehydrant. The cryoprotectant was added drop-wise over a period of 10 min, equilibrated for 20 min, serially diluted, and later washed repeatedly in fresh medium. Note the lower tolerance of nauplius to 15% sucrose (~ 0.5 M) and higher tolerance of the protozoea to 30% (< 1.0 M) and 50% (~ 1.5 M) sucrose (From Arun and Subramoniam[21]).

The importance of these techniques lies in eliciting a better understanding of cryobiology to help the cryobiologist to solve the difficulties that have hitherto prohibited successful development of a freeze protocol for certain biological systems. A major concern in the case of the invertebrate embryos and certain vertebrate embryos is their large amount of yolk which makes the cells susceptible to intracellular freezing by obstructing both the permeation and removal of the cryoprotectant[5,20,40]. Nakashima *et al.*[41] studying the developing porcine embryos obtained a greater percentage of survival, after the removal of internal yolk by microsuction. In a recent study, on the honeybee embryo cryopreservation, nucleii were isolated from the embryos and frozen. This nucleii may later be used by micropylar injection into the bee eggs of any related species. Another strategy of yolk avoidance was adopted by Harvey[39] who isolated the blastoderm of the zebrafish embryos and attempted to cryopreserve it. Calvi and Maisse[42] have reported on blastomere freezing of rainbow trout, *Onychorhynchus mykiss* at various stages of development. The nuclear and blastoderm cryopreservations should have great implications in interspecific cloning.

Importance of the use of cryomicroscope has amply been reviewed[43,44]. Despite numerous cryomicroscopic studies on the various single-celled structures such as spermatozoa[45], very few studies have been conducted on the meso- and metazoan systems[46]. Cryomicroscopic identification of locations that may be under cryogenic stress reveals details of cryoprotectant permeation, apart from freeze and dehydration induced morphological strains on the nauplius larva (Figure 8). Modifications to the protocol will be required according to these cryomicroscopic evidences. In brief, cryomicroscopy offers an excellent tool to optimise freeze rates and visualise freeze effects on a biological sample.

There are many instances of efficient use of vitrification, as a procedure for cryopreservation[25,26]. Indeed, vitrification has been suggested as a better alternative to conventional slow cooling protocols in the case of zebra fish embryos and penaeid shrimp larvae[6,20,21]. Vitrification has improved post-freeze survival of mammalian embryos[47] and has simplified the cryopreservation procedure. However, when dealing with complex structures, the highest possible freeze rates will be required. Vitrification of the *Drosophila* embryos was done by rapid exposure to nitrogen slush (– 205°C), of the equilibrated sample held on a copper electron microscope grid[25]. Vitrification has limitations with respect to sample volume being frozen. Less than 10 µl is often the recommended volume for efficient vitrification, ruling out larger structures from being vitrified. Further, well-differentiated structures have seldom shown promise in vitrification studies[46]. Conventional straws and cryovials are not always used with best results. Vajta and associates[49] reported successful vitrification of bovine embryos by open pulled straw method. This involves use of thin heat-pulled soft glass capillaries. The samples are loaded by the capillary action and the ends are not sealed.

Conclusion

Cryobiological studies at single and multicellular levels of living organisms have been carried out with the primary objective of long-term cryogenic storage. Successful cryopreservation techniques have been developed for a few gametes, mammalian embryos and microbes. However, extension of these techniques to organ systems and larvae is often found difficult. Many factors seem to influence this, of which incomplete permeation and high differentiation (and complexity) of the larva are primary. Nevertheless, a few marine invertebrate larvae, such as those of the sea urchins, molluscs and ragworms, have been reported to be cryopreserved[5,14–16]. They are essentially soft bodied and hence are easily permeated by the cryoprotectants. Further, some of the bivalve molluscs like mussels and oysters have the capacity to

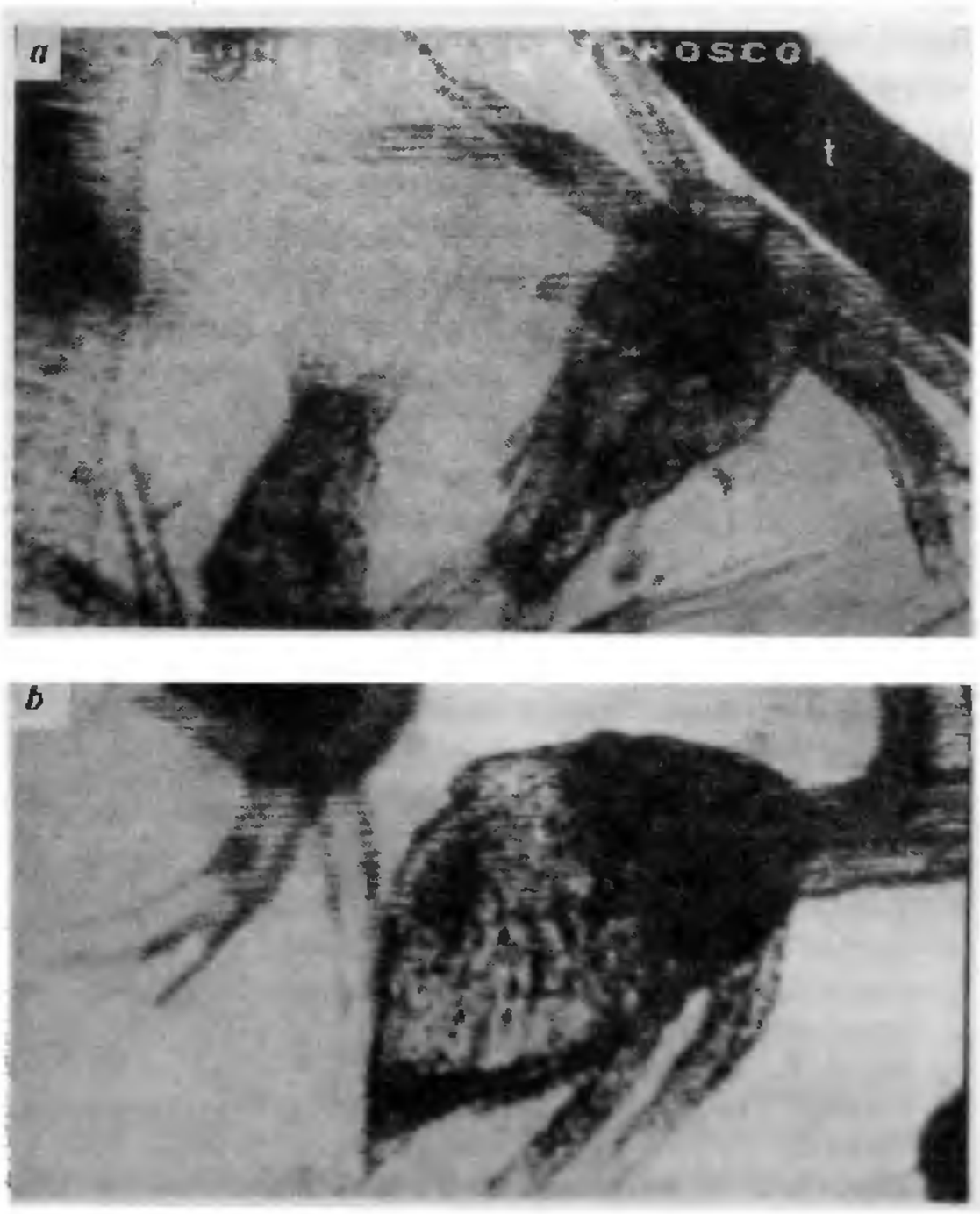

Figure 8. Cryomicroscopic (Planer CM3, Planer Biomed, UK) observations of the effect of freezing on the nauplius. A dehydrated nauplius (*b*) after freezing to – 30°C and at 2°C during the thaw phase is compared with a normal nauplius VI of *Penaeus monodon* (*a*) at 4°C during the freeze phase. The larva was frozen on a cooling stage at a rate of – 1°C/min to – 7°C, held there for 15 min and then frozen to – 30°C at – 2°C/min. t, In-built copper–constantan thermocouple for measuring the sample temperature on the heater slide (unpublished).

survive cold and anoxic conditions by production of certain natural protectants in their body tissue[50]. Such naturally protected systems have seldom posed serious problems for freeze preservation compared to animals of the same group that have lower tolerance[12,13]. In this context, the larvae of the shrimp have been recalcitrant to our consistent efforts to modify the existing protocols to suit them or to develop a new protocol[19–21].

Cryobiologically, the shrimp larva is unique in many ways. During freezing, the incompletely permeated larva will either exhibit random internal ice nucleation beyond the supercooling point or it may succumb to prefreeze chill-induced damages. Hence, the immediate problem that needs to be overcome is permeation. Thrusting in of the cryoprotectant will require either the dissolution of the exoskeleton or use of very high concentrations of low molecular weight cryoprotectants. Other factors include determination of the optimal freeze point from which the samples may be plunged, and an optimized thaw rate to avoid recrystallization. Recrystallization will occur in large-sized samples due to differential thermal fluctuations in different regions of the body. Despite these drawbacks, our studies have used DMSO–ethane diol combinations and effectively augmented the naupliar survival to ~80% at −70°C. They were frozen at rates of −2.5°C/min to −40°C and −5°C/min beyond −40°C. Similarly, near total revival of protozoa 1 was obtained after 30 min of direct storage at −40°C, using 15% methanol as a cryoprotectant without an equilibration (unpublished data). Further studies are being directed towards the possibilities of vitrifying the shrimp nauplius larva. Use of recently adopted diagnostic techniques as differential scanning calorimetry (DSC) and magnetic resonance imaging (MRI) would help in thermal studies and imaging of the solvent. However, the less sophisticated cryomicroscopic analysis of the freeze process should produce greater understanding. Only long-term trials and in-depth understanding of the cryobiology of the larva would yield a working protocol for the cryogenic storage of penaeid shrimps.

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ACKNOWLEDGEMENTS. We thank Department of Biotechnology for financial support (BT/AA/03/53/93). R.A. thanks CSIR for the award of a SRF.