

## CRYOPRESERVATION OF MEDITERRANEAN FRUIT FLY EMBRYOS

A. Rajamohan<sup>1</sup>, R.A. Leopold<sup>2\*</sup>, W.B. Wang<sup>3</sup>, M. Harris<sup>1</sup>, S.D. McCombs<sup>4</sup>, N. C. Peabody<sup>4</sup>  
and K. Fisher<sup>4</sup>

<sup>1</sup>Entomology Department, North Dakota State University, Fargo, ND 58105, USA

<sup>2</sup>USDA/ARS Biosciences Research Laboratory, Fargo, ND 58105, USA

<sup>3</sup>Via-Cell, Worcester, MA 01605, USA

<sup>4</sup>USDA/APHIS Plant Protection Laboratory, Waimanalo, HI 96795, USA

### Abstract

In this paper we present a procedure to cryopreserve the embryos of a tephritid, the Mediterranean fruit fly (*Ceratitis capitata*), by vitrification. Developmental stages between 24 and 32 hours after oviposition were examined for tolerance to cryopreservation. Embryos, 27-hr-old and incubated at 29°C, were found to be at the most suitable stage for treatment. Effects of the previtrification steps of our protocol, dechoriation, permeabilization, cryoprotectant loading, and dehydration, on survival to hatching were also assessed. Dechoriation did not affect viability, while isopropanol and a hexane treatment used in the permeabilization step of the protocol reduced hatching by about 15%. This reduction was dependant on the amount of isopropyl alcohol carried over into the hexane rinse. The remaining previtrification steps reduced hatching by an additional 10%. After optimization of the procedure, normalized hatching was 44% after vitrification in liquid nitrogen vapor followed by storage under liquid nitrogen for a test period of 7 days. Post cryopreservation larval diets containing wheat bran, corncob grits, or agar as the base were examined for survival to pupation and emergence. A yield of 34% egg to adult emergence was obtained when the agar-based diet was used for rearing larvae that had experienced cryopreservation during the embryonic stage.

**Keywords:** vitrification, Tephritidae, *Ceratitis capitata*, medfly

### INTRODUCTION

The Mediterranean fruit fly or medfly, *Ceratitis capitata*, is one of the most serious pests of fruit crops world-wide (1). Control programs involve the use of pesticide sprays, poisoned baits and area-wide sterile-male releases. The sterile insect technique (SIT) involves mass-rearing large numbers of flies, which are sexually sterilized and released to reproductively compete with the wild population (2, 3). Even though the technique has been very successful, the rearing facilities may experience reduced production, which can be caused by various factors such as diseases, mechanical failures and inbreeding. An approach to alleviate potential production problems is to maintain a cryopreserved back-up store of insects, which could be revived to renew or revitalize the current production strain that is under duress.

Another potential use of insect cryopreservation is in research programs where the economics of maintaining large numbers of genetic strains becomes burdensome. To this end,

a number of dipteran species have been successfully cryopreserved, and they include, *Drosophila melanogaster* (4, 5), the sheep blowfly- *Lucilia cuprina* (6), the house fly - *Musca domestica* (7), the New World screwworm - *Cochliomyia hominivorax* (8) and the blue tongue virus vector - *Culicoides sonorensis* (9).

A major influence on the successful transfer of cryogenic technology to dipteran embryos was the development of a vitrification technique as a strategy to store numerous animal and plant tissues that were difficult or impossible to cryopreserve using conventional methodology (10, 11). Dipteran embryos generally have a low tolerance to chilling, especially during early stages of development, which requires that special accommodations be made when formulating a cryopreservation procedure (11, 12). *D. melanogaster* was the first insect species to be cryopreserved by using an embryo vitrification technique (4, 5). However, Leopold and Atkinson (6) in their studies on sheep blowfly, Wang *et al.* (7) with the house fly and Leopold *et al.* (8) with screwworm embryos found that the *Drosophila* technique was not readily transferable to these other insects.

Preliminary studies in our laboratory on three species of tephritid fruit flies revealed that certain steps of the procedures that were suitable for *Drosophila*, housefly, and screwworm cryopreservation were not optimal for tephritids. In this paper, besides reporting a procedure for vitrification and long-term storage of medfly embryos, we also point out various pitfalls and common features that generally need to be addressed when devising vitrification procedures for insect embryos.

## MATERIALS AND METHODS

Mediterranean fruit fly embryos were obtained from laboratory cages of flies (ca. 1000) or from the production colony both of which were maintained at the USDA-APHIS mass-rearing facility at Waimanalo, HI. Eggs were collected by allowing 4 to 8-day-old adult flies to oviposit for a period of 30 minutes. To determine the optimum incubation time and temperature, embryos were incubated at 27, 28, 29, and 30°C ( $\pm 1^\circ\text{C}$ ) for 25, 27, 29, 30, and 32 hours after oviposition before treatment began.

A detailed protocol of the pre-vitrification, vitrification and post-vitrification treatments is given in a stepwise order below. Basically, the procedure consists of dechoriation and permeabilization of the embryonated eggs, loading with a cryoprotectant (CPA), dehydration of the embryos and concentration of the CPA by placing in a vitrification medium, vitrification and annealing of the CPA in nitrogen vapor and then storage in liquid nitrogen. Recovery of the embryos consisted of a return to nitrogen vapor and rapid warming in a 0.5M trehalose solution at room temperature, then allowing the embryos to hatch in sterile Schneider's insect cell culture medium. All chemicals used in this procedure were analytical grade compounds obtained from Sigma-Aldrich, USA unless otherwise noted.

### *Medfly Embryo Cryopreservation Procedure*

1. Dechorionate approximately 100 embryos that have been incubated at 29°C for 27 hr and place into a PVC container with a stainless steel mesh bottom (6) by agitating 2 min in fresh aqueous household bleach diluted 1:1. Then wash the embryos thoroughly with running tap water for 3 min to remove any traces of the chlorine bleach on the embryos. Blot adhering water from the container with an absorbent tissue (e.g., Kimwipes<sup>®</sup>).

2. Transfer the embryo container to isopropyl alcohol (IPA) and rinse thoroughly for 20 sec. Blot container immediately and then place the embryos into a stream of humidified air until the surface of the embryos are dry (ca. 30-60 sec). **Note:** Data are presented in Figure 1, which shows the effects of leaving embryos slightly moist with IPA before transferring to hexane and also adding 0.5 or 1% IPA to the hexane before treating the surface-dry embryos.

3. Transfer surface-dry embryos to hexane (Burdick Johnson, USA) and rinse for 40 sec to remove the lipid layer associated with the vitelline membrane. Again, rapidly air-dry embryos for 30-60 sec.
4. Place the embryos into *Drosophila* Ringer's solution (5). After 2 minutes of equilibration in Ringer's, place embryos into 10% ethylene glycol in Schneider's insect cell medium and allow them to equilibrate for 20 minutes. Most of the embryos within the container float on the surface of the CPA solution. First, they will shrink and then the majority will return to their original dimensions as equilibration occurs.
5. Blot the container to remove excess CPA and place into vitrification solution for exactly 15 minutes at ice temperature. **Note:** The vitrification solution tested in this study contained either 40% (V/V) ethylene glycol in Schneider's medium or 40% (V/V) ethylene glycol, with 0.5M trehalose and 5% (W/V) polyethylene glycol in Schneider's medium. All solutions using Schneider's medium as a diluent were filtered after mixing (syringe filter – 25 mm, 0.2 µm pore size, GD/X, Whatman®).
6. After the dehydration and concentration of the CPA, remove the embryos from the surface of vitrification solution by placing a 25 mm diameter, 8.0 µm pore size, polycarbonate membrane (Nucleopore®, Whatman) over the floating embryos and carefully lift the membrane with the adhering eggs off the solution. Blot the reverse side of the membrane with absorbent tissue to remove excess CPA. **Note:** Removing 3-4 mm from two edges of the membrane by making two parallel cuts allows an easier placement of the membrane into the embryo container and minimizes clumping of the embryos on the membrane (clumping is to be avoided).
7. The embryos are vitrified and the CPA annealed by placing the membrane holding the embryos into nitrogen vapor approximately 1-2 cm above the liquid nitrogen for 1 minute. **Note:** To reduce the boiling of liquid nitrogen, the nitrogen container (e.g. styrofoam coffee cup) can be cooled by placing it into a larger container also holding liquid nitrogen.
8. While in the vapor, plunge the membrane into liquid nitrogen. Our storage was in a 30 mm stainless steel histology tissue cassette with a clamping lid (Lipshaw®). Normally, the tissue holders with the membrane(s) were stored in a liquid nitrogen-containing Dewar for 24 hours or more before assessing viability. In some of the experiments, membranes were held in liquid nitrogen for only a few minutes before being removed to permit the assessment of survival.
9. To recover the embryos, remove the membranes from the storage container under the liquid nitrogen and hold in the vapor for 1 minute. Then quickly plunge the membrane into room temperature Schneider's medium containing 0.5M trehalose. Dislodge the embryos from the membrane by gentle agitation. Hold embryos in this solution for 2 min to facilitate removal of the CPA. Holding longer than 2 min reduces hatching.
10. Wash embryos for 10 min a minimum of 3 times in Schneider's medium and then incubate in fresh Schneider's medium at 24-25°C until hatching. Elimination of the ethylene glycol from the embryos can be monitored during the washes in Schneider's medium and it appears as small bubbles on the surface of the vitelline membranes. **Note:** The removal of the CPA and the washes in Schneider's medium are preferably done in a sterile laminar flow hood to suppress contamination of the embryos and cell culture medium. We use sterile

70mm plastic petri dishes to hold the embryos. We also add gentamycin at a concentration of 0.01% to the Schneider's medium to suppress growth of microorganisms.

Larval hatching occurs approximately 48 hours after embryo recovery when they are held at 25°C. Survival is enhanced if the larvae are removed from the culture medium and transferred to the larval diet shortly after hatching occurs. The pupation and emergence success of the cryopreserved flies using chemically-defined larval diets formulated with 3 different bulking agents, wheat bran, corncob grit and agar was also assessed. The diet plates were incubated at ~25°C until the larvae reached the typical size to pupate and tended to move out of the diet containers. As the larvae stop feeding and migrate out of the containers, the containers were transferred to boxes containing vermiculite, where pupation occurred. The number of cryopreserved larvae that pupated was recorded. On emergence, the number of eclosed adult flies was also recorded. Where measurements are cited numerically, the standard error of the mean (SEM) is indicated by  $\pm$  values and on the graphs it is indicated by an error bar.

## RESULTS

Figure 1 shows the hatching rates for control, dechorionated, and permeabilized embryos, which were allowed to hatch in Schneider's insect cell culture medium. The difference in hatching between the untreated embryos placed directly into Schneider's medium and the dechorionated embryos was not significant. In fact, the hatching of dechorionated embryos was slightly better than the control samples (90.8 vs. 86.5 %).

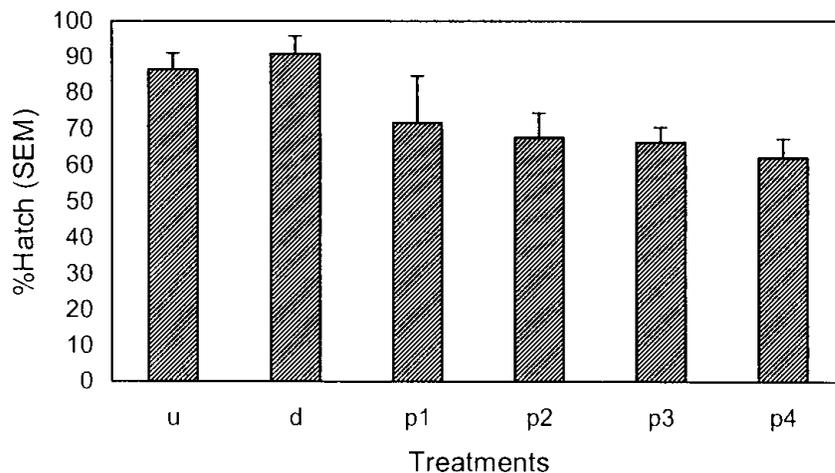


Figure 1. Effect of variation in the permeabilization treatment on hatching of Mediterranean fruit fly embryos. U= untreated; d = dechorionated; p1 = surface dried after IPA rinse; p2 = hexane with 0.5% IPA; p3 = hexane with 1% IPA; p4 = hexane with no drying of embryos after rinsing in IPA.

The permeabilization procedures that we tested, involving removal of surface water after dechorionation with isopropyl alcohol and lipid extraction from the vitelline membrane with hexane or hexane - IPA mixtures, all reduced hatching of the embryos. The data in Figure 1 shows the effect of IPA present in hexane both when added intentionally and also when carried over to the hexane rinse by incomplete removal of IPA from the embryos. Treatments

in pure hexane after removal of IPA by air-drying the embryos resulted in about 78% hatching, whereas the hatching was between 67-72%, when hexane contained either 0.5 or 1.0% IPA. Hatching was lowest (ca. 62%) when the embryos were rinsed in hexane without completely removing the IPA. Consequently, we used air-dried embryos in our protocol.

The CPA loading procedure using 10% ethylene glycol produces about a 20% reduction in hatching in comparison with the permeabilized samples. Hatching was about 63% following loading and unloading of the embryos with the CPA (data not shown). The dehydration and CPA concentration in either 40% ethylene glycol or the vitrification solution caused only a slight decrease (<5%) in hatching in addition to that caused by the CPA loading step.

Embryo survival following the sequence of vitrification, annealing, liquid nitrogen storage, and recovery steps was dependant on the incubation time and temperature following collection of the eggs. The mean hatching rate for cryopreserved embryos incubated at 29° C for 27 hr was 43.7% and ranged as high as 61% (Fig. 2). The other incubation times and temperatures tested yielded lower survival rates. Microscopic examination of embryos incubated at the optimum time and temperature revealed that the embryonic gut displayed a characteristic helical shape with a minimum amount of yolk present within (Fig. 3). Normally, we visually checked embryos about 30 min before the target treatment time to affirm that they were at the correct stage of development. Small variations in the egg collection protocol and incubation temperature were found to affect the developmental rate.

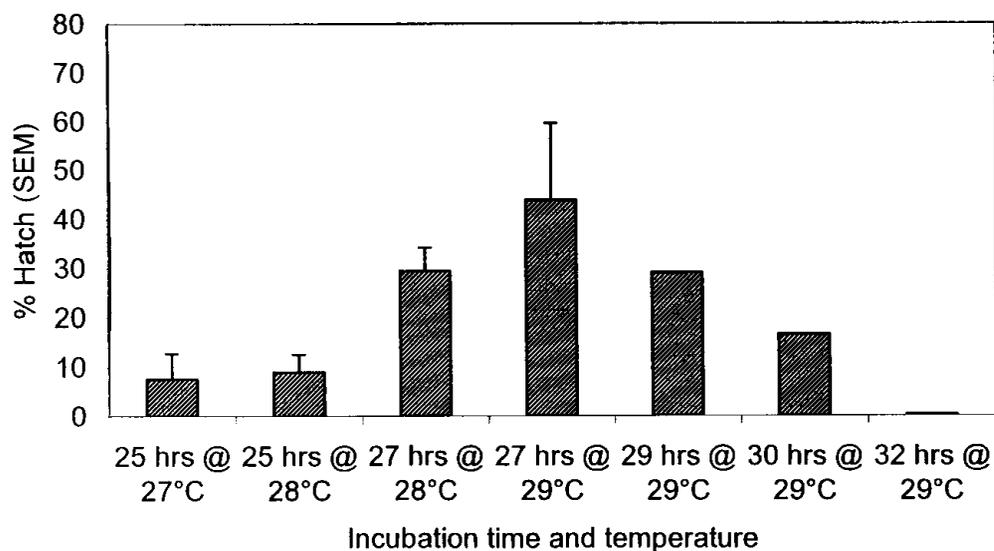


Figure 2. Effect of pretreatment incubation time and temperature on hatching of embryos after cryopreservation. Incubation times 29 through 32 hrs had only 1 replicate, all others had 3 or 4.

Survival of larvae cryopreserved as embryos and their pupation rates were found to be dependent on the type of bulking agent used in the larval diet. Table 1 shows a tabulation of the effect of the various diets tested for percentage yield of pupae and adult eclosion following recovery from cryopreservation. The agar-based diet yielded the highest pupation percentage as well as adult emergence from the pupae. The overall normalized yield, from hatching to adult emergence, of the cryopreservation procedure using the agar diet was 33.8% whereas the other two diets did not support development to the adult stage.

Table 1. Normalized hatching to emergence of cryopreserved Mediterranean fruit flies fed larval diets formulated with 3 different bulking agents.

| Diet Base     | % Hatch              | % Pupation           | % Emergence          | % Yield |
|---------------|----------------------|----------------------|----------------------|---------|
| Wheat bran    | 40.4 ± 11.6<br>(n=3) | 0                    | 0                    | 0       |
| Corn cob grit | 54.0 ± 9.48<br>(n=3) | 23.7 ± 11.8<br>(n=3) | 0                    | 0       |
| Agar          | 47.3 ± 0.7%<br>(n=3) | 80.3 ± 7.9<br>(n=4)  | 92.9 ± 7.96<br>(n=4) | 35.4    |

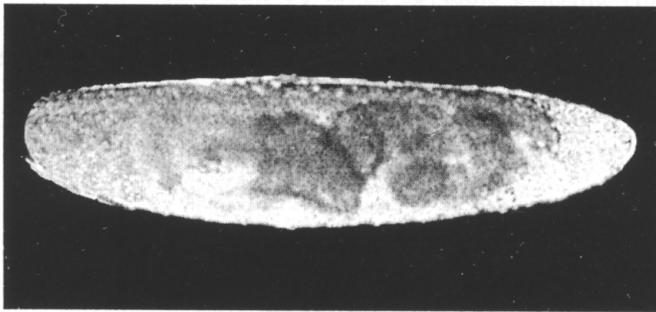


Figure 3. Photomicrograph of a dechorionated, 27-hr-old Mediterranean fruit fly embryo that had been incubated at 29°C after oviposition. Note the presence of coiled intestine and absence of yolk. Both the features are crucial in the determination of the correct embryonic stage for cryopreservation.

## DISCUSSION

The protocol developed here for the Mediterranean fruit fly embryos has many of the basic features of the vitrification procedures developed in our previous studies for the embryos of the blowfly (6), house fly (7), and the New World screwworm (8). However, as with each of the previous protocols, important modifications of the vitrification procedure originally designed for *Drosophila* embryo cryopreservation (4, 5) were required.

For example, the inclusion of IPA in the permeabilization procedure for the medfly, although necessary to remove the surface water from the embryos before exposure to hexane, is harmful when present in the alkane in concentrations  $\geq 0.5\%$ . Wang *et al.* (7) in their studies on housefly permeabilization also showed that while adding IPA to hexane caused a marked reduction in hatch percentage, it had a beneficial effect by increasing permeability. An increase in vitelline membrane permeability was first recognized by Mazur *et al.* (13) when using hexane and butanol to permeabilize *Drosophila* embryos. Further, we observed that when IPA was present in hexane at levels  $> 0.5\%$  it caused necrosis, which showed up as black patches within the tissues of the moribund embryos (not shown in this study). Thus, the presence of necrotic tissue is a good indicator that IPA toxicity is the source of the problem

when hatching is adversely affected after permeabilization.

Determination of the embryonic stage that is most tolerant to the rigors of CPA loading, cooling, vitrification, devitrification, removal of CPA, and recovery after cold storage is the main criterion for developing a successful cryopreservation protocol. The commonality shared by all dipteran species for which a vitrification protocol has been developed thus far, is that only relatively late-stage embryos have the capacity to survive the stressful conditions encountered during the cryopreservation procedure. Like the previous studies where insect cryopreservation has been successful (4,5,6,7,8,9), the medfly embryos are most tolerant to the process of vitrification at a stage of development where the yolk is nearly depleted, the gut displays coiling, and there is evidence that cuticle formation is just beginning. Leopold (11) pointed out that the absence of yolk lipid may be the critical factor in stage selection for cryopreservation in dipterans. Mammalian embryos with no or minimal amounts of lipid are often easily cryogenically frozen at one of the pre-blastula stages while porcine embryos equipped with an abundance of lipid droplets have been frozen successfully only after delipidation (14). Further, in fishes where the yolk is an integral part of the embryo even after hatching, cryopreservation has thus far has been virtually impossible (15).

The "window of opportunity" for cryopreservation of the medfly embryos was found to be nearly 2 hrs. Thus, the embryos can be treated  $27 \pm 1$  hr following egg collection and incubation at  $29^{\circ}\text{C}$  with about a 10-20% loss of survival when treatment is not at the optimum stage. This window for treatment was found to be considerably shorter in our earlier studies with both the housefly and screwworm. With the screwworm, the time period was approximately 15 minutes and with the housefly, it was approximately 20 min (7, 8). Obviously, the optimum time for treatment depends on the rate of development of a particular species at a particular temperature and hence it is possible to slightly increase or decrease this permissible window by adjusting the incubation temperature.

Our current procedure for vitrifying the medfly embryos in the vapor phase of liquid nitrogen before plunging into the liquid nitrogen differs from the embryo cryopreservation techniques of Mazur *et al.* (4); Steponkus & Caldwell (5); Leopold & Atkinson (6) and Nunamaker & Lockwood (9). It is similar to the protocols of Wang *et al.* (7) and Leopold *et al.* (8). Except for these latter two procedures, the aim of the other workers was to cool the insect embryos as rapidly as possible and vitrify the embryos at, or close to the temperature of liquid nitrogen or nitrogen slush. With the embryos of the medfly, house fly, screwworm, and two other tephritids we have been studying, this type of strategy yields low or no survival after recovery from liquid nitrogen storage. Vitrifying and annealing CPA-loaded embryos at the higher temperature of nitrogen vapor before exposure to the temperature of liquid nitrogen has been suggested by Rall & Meyer (16) and Wang *et al.* (7) as a means to reduce fracture damage to embryos during vitrification. Our preliminary studies do indeed indicate that fracturing of the vitrification solution used in this study can be significantly reduced by prior annealing in nitrogen vapor before exposure to liquid nitrogen (17).

Embryo size may be the decisive factor, which dictates whether or not fracture damage to embryos occurs during vitrification. Wang *et al.* (7) pointed out that the smaller size of the *Drosophila* embryos, relative to that of the house fly, was perhaps the reason that fracture damage was not occurring upon vitrification and why the annealing step was unnecessary for that insect. *Drosophila* embryos measure  $150 \times 420 \mu\text{m}$  while the house fly, medfly and screwworm range from 3.5 - 13 times larger and from 7 - 25 times larger than that of *Culicoides sonorensis*. Further, the sheep blow fly embryos are large as or larger than those of the screwworm and this may be the reason why Leopold & Atkinson (6) obtained a low larval hatch after vitrifying the embryos of this species without an annealing step.

It is becoming more apparent that the recovery of the cryopreserved insects after hatching and the subsequent development to adulthood is also a critical stage of the protocol. Leopold *et al.* (8) found that the screwworm required a recovery generation following

cryopreservation before the insects cryopreserved as embryos again had normal body weights and survival rates throughout the developmental stages to adulthood. In this study, we found that the type of bulking agent included within the larval diet was critical to the survival of the larvae to the pupal stage. The coarse-textured wheat bran and, to a lesser extent, the corncob grits were not favorable for development after cryopreservation, even though these materials are used in large scale Mediterranean fruit fly rearing facilities. Our recent studies on Mexican fruit fly cryopreservation indicate that using a diet containing carrot powder as modified from Awadallah & Fares (18) yields even a better recovery to the adult stage after embryo cryopreservation than the corncob grit diet used to produce this insect in the USDA/APHIS mass-rearing facility in Edinburg, TX. Generally, diets formulated for insects produced under factory-like conditions are created with materials which accommodate and support growth of thousands of insects and not small batches of 100-200 insects that have been submitted to dechoriation, permeabilization, and an ultra-low temperature plus loading and unloading of a relatively toxic chemical by rigorous osmotic manipulation. Essential nutrients and salts are undoubtedly leached from the insect embryo during treatment with this protocol and are only regained by incubation in a nutrient-rich insect cell culture medium and a fortified larval diet.

**Acknowledgements:** The authors wish to thank Jon Nishimoto for his assistance in this project and Dr. Rod Mahon for his helpful suggestions during preparation of this manuscript.

### REFERENCES

1. Christenson LD & Foote RH (1960) *Ann Rev Entomol* **5**, 171-192.
2. Rhode RH, Simon J, Perdomo A, Gutierrez J, Dowling CF, Jr., & Lindquist DA, (1971) *J Econ Entomol* **64**, 708-13.
3. Knipling EF (1979) *The Basic Principles of Insect Population Suppression and Management*. USDA Agriculture Handbook 512, 623 pp.
4. Mazur P, Cole KW, Hall JW, Schreuders PD & Mahowald AP (1992) *Science* **258**, 1932-1935.
5. Steponkus PL & Caldwell S (1993) *CryoLetters* **14**, 375-380.
6. Leopold RA & Atkinson PA (1999) *CryoLetters* **20**, 37-44.
7. Wang WB, Leopold RA, Nelson DR & Freeman TP (2000) *Cryobiology* **41**, 153-166.
8. Leopold RA, Wang WB, Berkebile DR & Freeman TP (2001) *Ann Entomol Soc Amer* **94**, 695-701.
9. Nunamaker RA & Lockwood JA (2001) *J Med Entomol* **38**, 55-58.
10. Fahy GM, McFarlane DR, Angel CA & Meryman HT (1984) *Cryobiology* **21**, 407-426.
11. Leopold RA (1991) in *Insects at low temperature*, (ed) RE Lee Jr. & DL Denlinger, Chapman and Hall, New York, pp 379-407.
12. Mazur P, Schneider U & Mahowald AP (1992) *Cryobiology* **29**, 39-68.
13. Mazur P, Cole KW & Mahowald AP (1992) *Cryobiology* **29**, 210-239.
14. Nagashima H, Kashiwazaki N, Ashman RJ, Grupen CG & Nottle MB (1995) *Nature, Lond.* **374**, 416.
15. Hagedorn M, Kleinhans FW, Freitas R, Liu J, Hsu EW, Wildt DE & Rall WF (1997) *J Exp Zool* **278**, 356-371.
16. Rall WF & Meyer TK (1989) *Theriogenology* **31**, 683-692.
17. Rajamohan A & Leopold RA (2003) *Cryobiology* **45**, 247.
18. Awadallah A & Fares F (1973) *Agric Res Rev* **51**, 63-67.

Accepted for publication 11/4/03