

## Cryopreservation of monogonont rotifers

Charles E. King, H. Berkeley Bayne, Todd K. Cannon & Andrew E. King  
*Department of Zoology, Oregon State University, Corvallis, OR 97331, USA*

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### Abstract

Development of techniques to maintain viable rotifer clones in a frozen state would preserve the genotype and reduce routine maintenance for those clones not being actively studied. To this end we have frozen *Brachionus plicatilis* in dimethyl sulfoxide at concentrations ranging from 6% to 18%. Survival rates decreased as the endpoint temperature was reduced from  $-20^{\circ}\text{C}$  to  $-45^{\circ}\text{C}$ , but did not decrease when the temperature was further reduced to  $-196^{\circ}\text{C}$  (liquid nitrogen). Only 2% of the individuals survived freezing in liquid nitrogen.

### Introduction

Monogonont rotifers have been extensively used in laboratory investigations. Species in this group reproduce by both sexual and parthenogenetic modes and most studies have been conducted using clones to avoid variability derived from genetic sources. It is quite likely, however, that through time, spontaneous mutation, somatic recombination, and subsequent selection for adaptation to the laboratory environment do occur. Thus even in lines derived from a single female and maintained exclusively by parthenogenesis it may not be valid to assume constancy of genotype among individuals through time.

To minimize the probability of genetic change it would be highly beneficial if it were possible to freeze either the rotifers or their parthenogenetic eggs at very low temperatures so that they could be stored for extended periods and thawed for use when viable individuals of the clone were desired. In addition, most monogonont cultures require a substantial amount of maintenance including culture of algae for food. Cryopreservation would greatly reduce this laboratory routine.

We report herein our attempts to maintain viable clones of *Brachionus plicatilis* by freezing in liquid nitrogen. The only previous report of rotifer cryopreservation is that of Koehler (1967) with the bdelloid *Philodina*. That obligately asexual bdelloids can be frozen and retain viability is not remarkable since the adults in nature are frequently exposed to and survive conditions of desiccation and freezing. In contrast, monogonont adults in nature do not enter an anhydrobiotic state. During periods of extreme environmental harshness, monogonont rotifers depend upon sexual reproduction to produce highly resistant resting eggs that serve to maintain continuity of the gene pool.

### Methods

A general review of the cryopreservation literature has recently been published by Ashwood-Smith & Farrant (1980). Organisms that retain viability after freezing range from bacteria to insects. Critical variables associated with successful cryopreservation include: (1) health of the culture at time of freezing, (2) cryoprotectant, (3) cooling rate, and (4) warming rate following freezing.

Our experiments were conducted using clones of *Brachionus plicatilis* from Spain (SP), Israel (IS), China (CH), and the USA (SO). No significant differences in viability among clones were identified by preliminary experiments. All rotifer cultures were maintained on Colorado Medium (King 1982) and fed with *Dunaliella tertiolecta* (UTEX culture collection 999) reared on BB medium (King 1982). Both rotifers and algae were cultured at 25 °C.

The cryoprotectant used in this study was dimethyl sulfoxide (DMSO) at concentrations ranging from 6% (and 94% Colorado medium) to 18%. Toxicity of DMSO to the rotifers will be discussed in the following section.

In preliminary freezing experiments, the highest survival rates were obtained from rapidly growing cultures in which there were large proportions of neonates, juveniles, and ovigerous females. These conditions were maintained by feeding and transferring rotifers to fresh medium at two-day intervals. To prepare the replicates for freezing, a culture was filtered through 44  $\mu\text{m}$  nitex screen and washed from the filter with a DMSO/Colorado solution of the appropriate concentration. This suspension was then further diluted to obtain a density of approximately 300–350 individuals  $\text{ml}^{-1}$ . A 0.75 ml aliquot of the rotifer suspension was placed in each of five replicate 6  $\times$  50 mm glass culture tubes which were capped with rubber stoppers and placed in the freezing chamber in a horizontal position (using a plastic Falcon 3042 microtest plate to hold and separate the replicate tubes).

Freezing was accomplished using a Linde Biological Freezing System to inject liquid nitrogen to the freezing chamber at the desired rate. Freezing rates were continuously monitored on a strip chart recorder connected to a thermocouple immersed in another tube inside the chamber.

Figure 1 presents the freezing rate profile used to obtain the data reported in this paper. The phase change from liquid to solid at freezing occurred approximately 35 min after introduction of the rotifer suspension aliquots to the culture tubes (which was approximately 15 min after initial suspension of the rotifers in the DMSO/Colorado solution). The phase change occurred between  $-10$  and  $-20$  °C; during this interval the cooling rate was adjusted to average 2 °C  $\text{minute}^{-1}$ . Otherwise, a cooling rate of approximately 0.7°  $\text{minute}^{-1}$  was used.

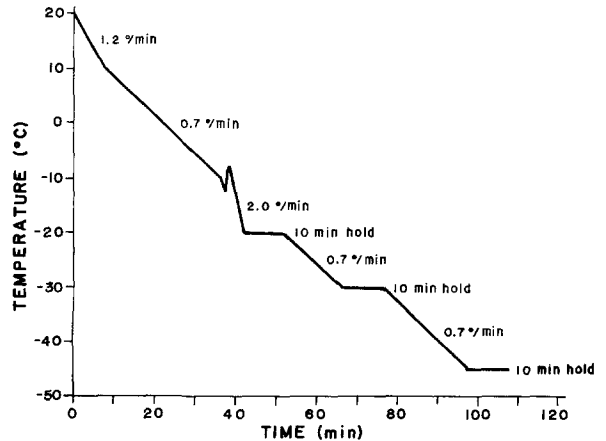


Fig. 1. Freezing rate profile for *Brachionus plicatilis* samples treated as described in the reported experiments.

Replicates were removed at three endpoint temperatures,  $-20$  °C,  $-30$  °C, and  $-45$  °C. Prior to removal, the temperature was held at the desired level for 10 min to insure that all replicates were at the same temperature.

The thawing procedure is as critical as the freezing procedure since recrystallization damage can greatly reduce survival. Substantially lower rates of survival were noted in preliminary experiments when thawing took place in 10° C and 20° C waterbaths than when thawing was accomplished at 37° C. Accordingly, all data reported herein were obtained by plunging the replicate tubes at their endpoint temperature directly into a 2-liter waterbath at 37° C.

Following thawing, survival was scored in the DMSO/Colorado solution. To test for long-term survival, the 0.75 ml replicates were slowly diluted to a DMSO concentration of 2% by adding 50  $\mu\text{l}$  Colorado medium to each sample at 10 min intervals for the first hour, and 100  $\mu\text{l}$  at 10 min intervals thereafter. Surviving individuals were then isolated and transferred directly from the 2% DMSO solution to 100% Colorado medium.

## Results

After freezing and thawing, a large proportion of individuals commonly display some movement. These movements can be placed in three categories

indicating progressively less damage from the treatment. First, individuals may exhibit a 'twitching' response cause by strong contractions of the foot and coronal retraction muscles. These individuals always die within a 12-h period – usually within 2–4 h. Second, individuals may lack the twitching response, but do have coronal ciliary activity. These individuals typically swim slowly in tight circles at the bottom of the dish. They do not ingest algae and die within 24 hr – typically within 6–8 h of thawing. A third group exhibits normal swimming and feeding activity within 30 min of thawing. These individuals, when returned to 100% Colorado medium, have approximately 90–100% survival rates and reproduce by normal parthenogenesis. Only individuals in this third category were scored as survivors.

DMSO functions as a cryoprotectant by replacing cellular water and changing the pattern of ice crystal formation. To determine the effects of DMSO concentration, the freezing protocol displayed in Fig. 1 was used. Five replicates at each of four DMSO concentrations (6%, 9%, 12%, and 18%) were prepared for each of three endpoint temperatures (–20, –30, and –45 °C). The highest survival rates were obtained for 6% DMSO at –20 °C. At this temperature, survival progressively decreased with increased DMSO concentration (Fig. 2). Although unfrozen *B. plicatilis* survive and swim normally in concentrations of DMSO as high

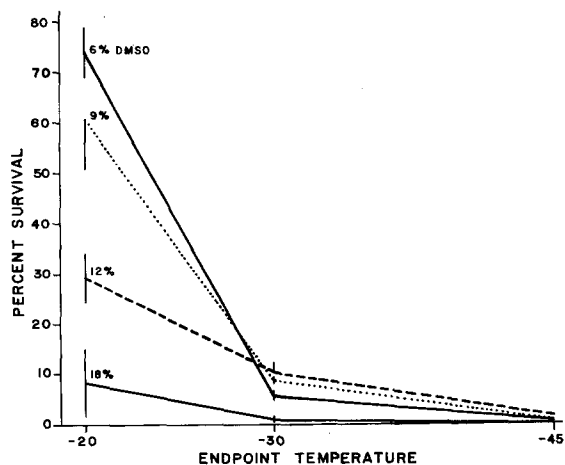


Fig. 2. Survival, measured as percent swimming ( $\pm$  S.E.), for clone 1S of *Brachionus plicatilis* at three endpoint temperatures for each of four dimethyl sulfoxide (DMSO) concentrations.

as 12% for periods in excess of two hours, our clones appeared to be unable to acclimate to the 18% concentration. Consequently, the decreased survival rates after freezing to –20 °C suggest an interaction between injury caused by freezing and the toxic effect of increased DMSO concentrations. That 18% DMSO is toxic to *Brachionus plicatilis* is further indicated by the low survival rates at –30 °C (less than 2%) and –45 °C (no survival). With the exception of the 18% DMSO replicates, it is interesting that the observed rates of survival were directly related to DMSO concentrations at –30 °C. There was no significant difference between the three groups of replicates at –45 °C. We interpret this observation to indicate that the cryoprotectant effect of DMSO increases faster than the toxic effect as the concentration changes from 6% to 12%. We therefore suggest that a DMSO concentration of between 9% and 12% is optimal under these conditions.

For long-term storage of cultures, it is desirable to achieve an endpoint that is considerably colder than –20 °C. Although we have not measured survival as a joint function of endpoint temperature and time, an analysis of variance of the data in Table 1, which are based on only four replicates in each cell, indicates that there is no difference in short-term survival rates at endpoints of –40° and –196° C (liquid nitrogen).

Table 1. Survival percentages ( $\pm$  S.E.) at two endpoint temperatures (–40 °C and –196 °C) for clone SP of *Brachionus plicatilis* in 6% and 15% DMSO/Colorado solutions.

DMSO	Endpoint temperature	
	–40 °C	–196 °C
6%	1.3 $\pm$ 0.52	2.1 $\pm$ 0.83
15%	2.1 $\pm$ 0.58	0.5 $\pm$ 0.35

## Discussion

The results of this study show a survival (swimming after freezing) rate of approximately 2% at temperatures below –40 °C. Several papers reviewed by Ashwood-Smith & Farrant (1980) involve low-temperature preservation and discuss aspects of protozoan and insect cryopreservation. It is fre-

quently found that different life stages of a single species may require different cooling rates for survival. When comparing Koehler's (1967) results with *Philodina* against our study of *Brachionus plicatilis*, a difference is observed in survival rates (50–60% versus 2%) even though similar freezing techniques were used. This difference is probably due to differences in the normal environmental stresses to which the two species are adapted.

The two major conclusions of this study are that (1) cryopreservation of *Brachionus plicatilis* at temperatures below  $-40^{\circ}\text{C}$  is possible, but (2) the observed survival rates are very low. Before these techniques can be used and relied upon, survival rates must be substantially increased. Other cryoprotectants, for instance glycerol, should be tried. Age of the individual may be a critical variable; we have observed that neonates and juveniles survive at higher rates than ovigerous or old females. On the other hand, frozen amictic eggs seldom hatched, although movement of the embryos was frequently observed. Males survive freezing to  $-45^{\circ}\text{C}$  at very high rates (greater than 50%).

It is likely that substantial increases in survival may be accomplished with subtle changes in method. For instance, in preliminary work freezing was accomplished in vertically oriented tubes and survival at  $-45^{\circ}\text{C}$  was quite rare. Repositioning the tubes to a horizontal orientation so that all individuals would not be concentrated at the bottom sub-

stantially increased success. We have also employed straws and Eppendorf vials with results not appreciably different than those reported here.

It is likely that other monogonont species can also be frozen successfully. *B. plicatilis* is a marine and alkaline lake rotifer and is capable of surviving in much higher DMSO concentrations than freshwater species. We have obtained survival of *Brachionus calyciflorus*, *Euchlanis dilatata* and *Asplanchna girodi* in 2% DMSO, but these results have not been quantified or pursued. The potential benefits of this technology clearly warrant further study.

### Acknowledgement

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