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Viability of subitaneous eggs of the copepod, *Acartia tonsa* (Dana), following exposure to various cryoprotectants and hypersaline water

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ABSTRACT

Subitaneous eggs were obtained from monocultures of the calanoid copepod *Acartia tonsa* (Dana), Gulf of Mexico strain. Eggs were exposed to methanol, ethylene glycol, propylene glycol, glycerine, and DMSO at 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 M and hypersaline water at 50, 75, 100, 150, and 200 g/L. Treatments were evaluated after 10 and 20 min of exposure and at 4 and 26 °C. Viability (percent hatched) was determined after 24 h of incubation in 35 g/L saltwater at 26 °C.

Methanol, ethylene glycol, and glycerine had high viability up to 2M, and all experienced large decreases at 5M when the exposure temperature was 26 °C compared to 4 °C. Eggs exposed to propylene glycol had lower mean viability with greater variability at the lower concentrations although viability was greater than 81.4% at 2 M. Significant decreases in viability were observed at 5 M, and the decreases were much greater at an exposure temperature of 26 °C versus 4 °C. DMSO exposed at 26 °C produced high viability up to 1 M before significant decreases occurred, while an exposure temperature of 4 °C produced high viability up to 2 M. Viability of eggs exposed to hypersaline water of 50, 75, and 100 g/L were not significantly different from controls for all treatment combinations except the 26 °C temperature exposed for 20 min, which was significantly lower at 100 g/L. Concentrations of 150 and 200 g/L produced very few to no viable eggs. These results indicate further research is justified to investigate if viability of *A. tonsa* eggs can be protected by these cryoprotectants and hypersaline water after exposure to cryopreservation conditions.

1. Introduction

Copepods are the dominant natural prey of most marine fishes during the larval stage. Due to their small mouth gape, many newly hatched marine fish larvae have difficulty ingesting commonly cultured rotifers, *Brachionus* sp., and brine shrimp nauplii, *Artemia* sp. Additionally, there are behavioral differences between these live food organisms which can affect preference and feeding behavior by larval fish. Thus, there is a great need to develop culture techniques for various copepod species for use in aquaculture and to identify methods to store and market copepods eggs to hatcheries.

The addition of copepods to the diet of various cultured larval fishes has been reported to have beneficial effects on survival and growth of larvae when fed in combination with *Artemia* and/or rotifers to golden snapper, *Lutjanus johnii* (Schipp et al., 1999), flounder, *Paralichthys lethostigma* (Wilcox et al., 2006), and when fed exclusively to Asian seabass, *Lates calcarifer* (Rajkumar and Kumaraguru vasagam, 2006), grouper, *Epinephelus coioides* (Toledo et al., 1999), West Australian dhufish, *Glaucosoma hebraicum*, and pink snapper, *Pagrus auratus* larvae (Payne et al., 2001). Growth and survival of larvae fed copepods is increased because of their size and nutritional composition, particularly their high concentrations of the essential fatty acids including DHA and EPA. The lowest level of DHA reported in copepods exceeds the levels found in enriched rotifers and *Artemia* (Støttrup et al., 1999). Calanoid copepod nauplii are the ideal size, induce larval feeding behavior, and have a complete nutrient profile necessary for growth and survival of larval marine fish (Støttrup, 2000; Drillet et al., 2007).

Currently, the technology for mass-scale production of copepods is in the research stage. However, methods to consistently produce small batches of copepods have been reported for several species, including *A. tonsa* (Støttrup et al., 1986). Culture conditions for maximum production of *A. tonsa* eggs and hatching success in intensive batch systems occur at a salinity between 14 and 20 g/L, photoperiod between 16 and 20 h, a low stocking density of 50 individuals per liter, and feed >50,000 cells/mL of *Rhodomonas* sp. algae (Peck and Holste, 2006). In order to meet the demand of hatcheries for large quantities of copepods at one time, mass scale production techniques and viable methods for long term cold storage of copepod eggs must be defined.

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Both subitaneous and diapause eggs have been investigated for storage and use in aquaculture. Diapause eggs have been induced in *Centropages hamatus* (Marcus and Murray, 2001) and *Labidocera aestiva* (Marcus, 1982). Diapause eggs are produced when environmental conditions signal the possibility of long term changes thus they have hardened shells. While it appears feasible to store diapause eggs for a long period of time at 25 °C (up to 17 months) and still have high viability (>80%) (Marcus and Murray, 2001), the successful induction of mass quantities of diapause eggs has never been reported. This may limit their application for aquaculture.

Subitaneous eggs are easily produced, occur during normal reproduction, do not have hardened shells, and do not need to remain in a refractory phase before hatching. Cold storage of subitaneous eggs of *A. tonsa* has been recently investigated. Drillet et al. (2006) reported that 70% of eggs remained viable after being stored at 2–3 °C for up to 11 months but viability dropped significantly by 12 months of cold storage and there were no viable eggs after 20 months. Differences in regional strains of *A. tonsa* have been reported and this may affect their long term storage capability and may contribute to variability in the viability of eggs stored at different stages of development (Drillet et al., 2007). Peck and Holste (2006) reported that the viability of *A. tonsa* subitaneous eggs stored at 4 °C decreased linearly by 4% for every 20 days of storage. Holmstrup et al. (2006) evaluated effects of temperature, salinity, and anoxia on survival of *A. tonsa* quiescent eggs following cold storage. The best results were obtained when the temperature was below 5 °C, salinity was between 10–20 g/L, and there was no oxygen added to capped vials. This information clearly shows that subitaneous eggs can be stored for periods of time which could prove useful for feeding larval fish. However, this does not provide a method for long term storage with high viability. In order to achieve this, it is likely that cryopreservation methods will need to be used.

There are many advantages to cryopreservation of copepod eggs for aquaculture including increasing the availability of eggs as a source of nauplii for feeding large quantities of larvae, to preserve genetically superior lines of cultured copepods long term, and to bank or store quantities of eggs over time to insure a hatchery has sufficient quantities of eggs available when they are needed as either a primary or backup source of nauplii. To effectively store or "bank" eggs long term, protocols for cryopreservation must be defined. The first step is to determine the effects of various commonly used cryopreservation compounds, at different concentrations, on the viability of the eggs after exposure.

There is a great desire to cryopreserve penaeid shrimp embryos for use when supplies are scarce and to maintain specific genetic lines. Thus, there has been substantial research on cryopreservation methods for shrimp embryos. The recommended methods for cryopreservation of shrimp may be similar for copepod eggs. Initial studies with *Penaeus indicus* evaluated toxicities of methanol, methanol plus DMSO, methanol plus ethylene glycol, and methanol plus propylene glycol on embryos at various stages of development and found that these lower molecular weight compounds penetrated the embryos well and may allow for successful cryopreservation (Newton and Subramoniam, 1996; Simon et al., 1994). Cryopreservation of penaeid shrimp, *Penaeus japonicus*, embryos, nauplii, and zoea has been attempted and methanol, glycerine, DMSO, ethylene glycol, and propylene glycol were shown to not be toxic to embryos, and the embryos remained viable following exposure to 0 °C (Gwo and Lin, 1998). Vuthiphandchai et al. (2005) evaluated the toxicity of the same cryoprotectants and ethanol, acetamide, formamide, glycerol, and sucrose on three stages of embryos of *P. monodon* at four concentrations for 10 and 20 min of exposure. Their results showed variability in survival of embryos at high concentrations but showed viable embryos remained after exposure, although the 20 min exposure and higher concentrations experienced decreased survival. Additionally, Dong et al. (2004) evaluated the effects of exposure of embryos and larvae of *Litopenaeus vannamei* to methanol, ethylene glycol, propylene glycol, and DMSO for seven time periods up to 60 min. They reported that the toxicity to the cryoprotectant exposure was concentration and exposure time dependant, thus the longest exposure and highest concentration produced the lowest survival.

Currently, there is no reported research investigating the effects of exposure to various cryopreservation compounds on the viability of *A. tonsa* subitaneous eggs. The objectives of this study are to determine if several commonly used cryopreservation compounds and hypersaline water at various concentrations are toxic to *A. tonsa* subitaneous eggs and affect viability. This will define the best cryopreservation compounds and exposure protocol to achieve the highest viability and will evaluate if eggs remain viable following exposure to hypersaline water. The results will serve as the foundation for continued research to evaluate and define methods for long-term cryopreservation and storage of copepod subitaneous eggs.

2. Methods

2.1. Eggs

Subitaneous eggs were obtained from a Gulf of Mexico strain of *A. tonsa* cultured in 100 L polyethylene tanks at AlgaGen LLC following a similar culture protocol as Støttrup et al. (1986). Subitaneous eggs were siphoned from the culture tanks and sieved through a 105 μm nylon screen and collected on a 55 μm nylon screen. Eggs were gently rinsed with filtered seawater (35 g/L and 26 °C), concentrated, and placed into capped test tubes containing the rinse water. Eggs were then placed in a cooler (4 °C) to prevent hatching during transport to the laboratory (approximately 30 min). To complete this study, two batches of eggs were collected in consecutive days and all the eggs used were <18 h old.

Upon arrival at the laboratory, eggs were volumetrically sampled and quantified. The total number of eggs in a 200 mL test tube was calculated by pipetting five random samples of 0.25 mL of seawater and suspended eggs and quantifying the eggs. Before each sample was taken, the eggs were suspended with gentle aeration through a pipette and the sampled eggs were quantified with a dissecting microscope. Using this volumetric mean, it was calculated that approximately 100 eggs was contained in 0.5 mL of seawater. Thus, 0.5 mL of seawater and 100 suspended eggs was collected and transferred to each labeled microcentrifuge tube.

2.2. Cryopreservation chemicals and procedures

Methanol (Scientific Grade 99%+Sigma M3641), ethylene glycol (Spectrophotometer Grade 99%+Sigma 293237), propylene glycol (1,2 Propanediol Reagent Plus 99% Sigma 134368), glycerine (Glycerol anhydrous >99.5% Fluka Biochemica 49767), and DMSO (Dimethyl Sulphoxide Biotech Performance Certified Sigma D2438) were evaluated at 0.0, 0.1, 0.5, 1.0, 2.0, and 5.0 M. Additionally, hypersaline water was evaluated at 50, 75, 100, 150, and 200 g/L. Each of these chemical concentrations was evaluated after 10 and 20 min of exposure and at both 4 °C and 26 °C. Each of these treatment combinations were replicated four times. There were four control treatments, one for each of the temperature and exposure time combinations so the potential effects of exposure temperature and time period could be controlled for. Each control treatment was replicated 12 times. To eliminate potential bias if all control replicates were exposed at one time, two replicates were taken at set times during the two days required to conduct the chemical exposures. There were a total of 528 replicate microcentrifuge tubes used in this experiment ((5 concentrations×4 temperature/times×4 replicates each=80×6 cryoprotectants=480)+(4 controls×12 replicates=48)=528 total)).

To achieve the desired chemical concentrations in a 1 mL total volume in microcentrifuge tubes, suspended eggs and chemical were mixed in a 1:1 ratio. This procedure consisted of mixing the chemical to double the desired final concentration in 35 g/L saltwater to a volume of 0.5 mL, and then combining this solution with the desired total quantity of eggs suspended in 0.5 mL of 35 g/L saltwater. When combined, the resulting solution was thoroughly mixed with a micro

pipette by drawing into and expelling three times. All 35 g/L saltwater used was obtained from the Atlantic Ocean and was filtered through a 5 μm carbon filter, a 0.35 μm cartridge filter, and passed though a UV sterilizer before being used. For hypersaline solutions a salt mixture (HW Marine Mix Professional, Hawaiian Marine Imports Inc. Houston, TX) was added to 35 g/L saltwater and heated to dissolve the salt into solution and achieve the desired salinities.

The pipetting of cryoprotectants into the microcentrifuge tubes containing eggs was timed to precisely expose the eggs for either 10 or 20 min. Then the eggs were poured onto a 50 μm nylon screen and rinsed with 35 g/L saltwater at 26 °C. Additionally, all replicate combinations were either conducted at 26 °C or 4 °C. After rinsing the eggs, they were placed into labeled 60×15 mm petri dishes containing 10 mL of 35 g/L saltwater. Eggs were allowed to hatch for 24 h at room temperature (26 °C) in static conditions and a 12:12 h light:dark cycle with ambient fluorescent room lights.

After 24 h of incubation, all eggs and nauplii were quantified. From these data, viability (percent hatched) was calculated.

2.3. Statistical analysis

The viability (percentage hatch) data was arc sin square root transformed and was analyzed by an analysis of variance using the general linear model (PROC GLM) of SAS (SAS, 1999). The independent variables were chemical concentration, temperature, and time of exposure and the dependant variable was viability. Within a treatment combination of chemical, temperature, and time, viability was compared at the various concentrations and to the appropriate control treatment means ($n=12$). The means were separated by the Ryan–Einot–Welsh multiple range test by using the REGWQ procedure of SAS (SAS, 1999). This test was used because it decreases experiment wise error when a large number of treatments are compared. Statistical significance occurred in all analyses when the calculated p-value was ≤0.05.

3. Results

The viability of the control treatment eggs following the physical handling (4 °C and 26 °C exposure temperature for 10 and 20 min) and hatching procedures ranged from 97.4% to 92.9%. There was a general trend of slightly reduced viability at the longer 20 min exposure time at 4 °C. The observed high viabilities of the control treatments confirm that our handling and hatching procedures were effective. The viability of eggs for each treatment is summarized in Table 1 and is graphically represented in Fig. 1 to show the trend in the data.

Table 1
Viability (percent hatch)±SE using five cryopreservation compounds and hypersaline water, each at six concentrations for two exposure times and at two temperatures

Chemical	Conc. (M)	26 °C		4 °C	
		10 min	20 min	10 min	20 min
Control methanol	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13$^{a, b}$
	0.1	93.7±3.43^{a}	96.3±1.55^{a}	96.6±1.37^{a}	97.2±1.47$^{a, b}$
	0.5	94.7±0.67^{a}	97.3±0.34^{a}	96.2±2.28^{a}	99.6±0.43^{a}
	1	93.7±1.86^{a}	97.3±1.14^{a}	97.9±1.58^{a}	96.7±2.72$^{a, b}$
	2	97.2±1.21^{a}	98.0±0.82^{a}	96.5±1.54^{a}	91.0±2.62$^{a, b}$
	5	15.1±2.08^{b}	3.57±3.57^{b}	95.4±1.57^{a}	82.2±3.83^{b}
		<0.0001	<0.0001	0.9140	0.0458
Control ethanol glycol	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13^{a}
	0.1	99.1±0.50^{a}	94.7±2.20^{a}	99.6±0.44^{a}	99.5±0.48^{a}
	0.5	98.4±0.62^{a}	97.2±1.23^{a}	97.8±1.06^{a}	98.4±0.56^{a}
	1	98.7±0.85^{a}	97.8±1.35^{a}	97.6±1.00^{a}	97.8±0.89^{a}
	2	97.3±0.85^{a}	84.9±3.88^{b}	96.4±0.10^{a}	96.1±0.81^{a}
	5	0.0±0.00^{b}	0.0±0.00^{c}	98.5±0.95^{a}	85.6±2.57^{a}
		<0.0001	<0.0001	0.2267	0.0684
Control pro. glycol	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13^{a}
	0.1	72.1±9.31^{b}	89.2±3.59$^{a, b}$	90.2±2.88$^{a, b}$	79.9±5.74^{a}
	0.5	69.2±5.31^{b}	89.5±2.17$^{a, b}$	92.9±3.65^{a}	88.8±3.41^{a}
	1	75.3±6.71^{b}	89.8±3.43$^{a, b}$	85.9±3.00$^{a, b, c}$	90.8±2.18^{a}
	2	81.4±1.54$^{a, b}$	85.3±2.8^{b}	82.1±0.76$^{b, c}$	91.6±2.49^{a}
	5	21.1±12.2^{c}	10.9±4.12^{c}	77.9±3.38^{c}	63.0±1.00^{b}
		<0.0001	<0.0001	<0.0001	<0.0001
Control DMSO	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13^{a}
	0.1	84.6±1.80^{b}	86.3±2.15^{a}	77.0±2.31^{b}	81.5±1.97^{a}
	0.5	87.3±4.39^{b}	90.1±3.19^{a}	85.1±3.10$^{a, b}$	89.8±1.85^{a}
	1	91.3±1.32$^{a, b}$	83.9±1.35^{a}	85.1±2.42$^{a, b}$	83.9±2.48^{a}
	2	72.9±2.63^{c}	52.9±9.80^{b}	87.4±1.84$^{a, b}$	87.8±2.40^{a}
	5	11.6±3.44^{d}	7.66±3.28^{c}	46.8±11.14^{c}	14.7±4.01^{b}
		<0.0001	<0.0001	<0.0001	<0.0001
Control glycerine	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13^{a}
	0.1	93.6±0.75^{a}	93.7±1.83^{a}	93.3±2.60$^{a, b}$	96.6±1.76^{a}
	0.5	92.9±1.90^{a}	90.2±2.45^{a}	93.4±0.95$^{a, b}$	95.1±2.10^{a}
	1	95.0±2.45^{a}	97.6±0.61^{a}	95.2±0.83^{a}	95.9±2.53^{a}
	2	91.7±2.69^{a}	72.0±6.40^{b}	97.4±0.88^{a}	94.6±0.15^{a}
	5	0.6±0.60^{b}	2.0±1.26^{c}	87.0±3.58^{b}	87.3±9.68^{a}
		<0.0001	<0.0001	0.0180	0.7883
Control saltwater	0	97.4±0.60^{a}	96.5±0.56^{a}	95.6±1.15^{a}	92.9±3.13^{a}
	50	96.2±1.23^{a}	98.5±0.5^{a}	98.7±0.82^{a}	97.0±1.38^{a}
	75	96.4±1.43^{a}	97.3±1.69^{a}	98.3±0.65^{a}	97.7±0.55^{a}
	100	99.2±0.49^{a}	80.9±3.62^{b}	97.9±0.55^{a}	97.8±0.81^{a}
	150	29.7±10.09^{b}	0.0±0.00^{d}	0.0±0.00^{b}	0.0±0.00^{b}
	200	0.0±0.00^{c}	6.2±3.02^{c}	0.0±0.00^{b}	0.0±0.00^{b}
		<0.0001	<0.0001	<0.0001	<0.0001

Each column represents an exposure time and a temperature and has its own control treatment which was used for statistical analysis of each chemical and the various concentrations. Statistically significant differences ($p\leq0.05$) between the control and the chemical's concentrations is indicated by different superscript letters within a section of each column. The calculated p-value from the ANOVA is listed following each section of a column.

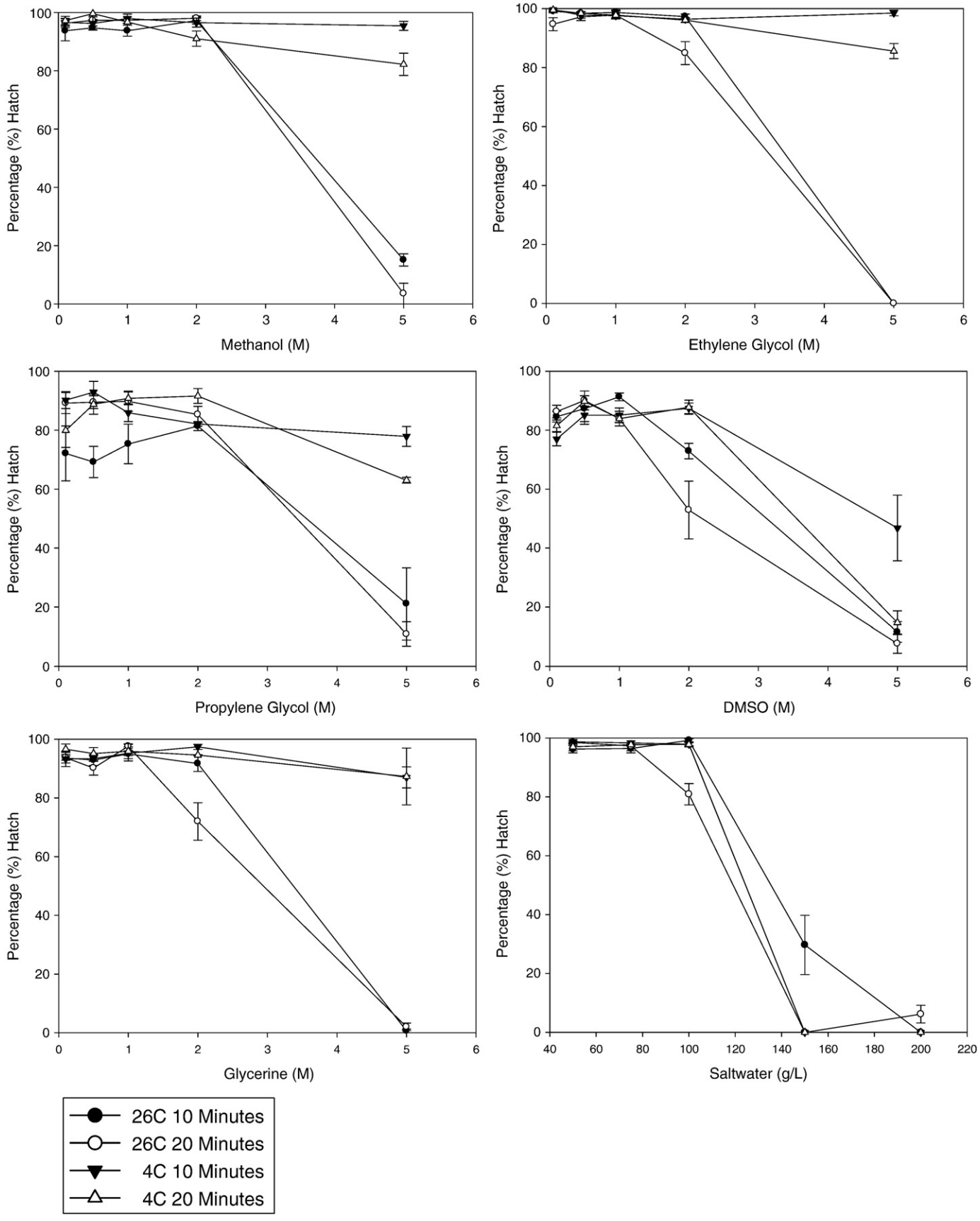

Fig. 1. Viability (percent hatch) results using five cryopreservation compounds and hypersaline water, each at six concentrations for two periods of exposure time and at two temperatures. Error bars represent ± SE.

3.1. Methanol

The viability of eggs exposed to methanol at 26 °C for 10 min and 20 min was not significantly different until 5 M was used. In the 4 °C and 10 min exposure treatment there were no significant differences between any concentration of methanol and the control. In the 4 °C and 20 min exposure treatment, 0.5 M had the highest viability and was significantly higher than 5 M, and although greater than the control and other concentrations, the 0.5 M treatment did not statistically differ.

3.2. Ethylene glycol

The viability following exposure to ethylene glycol at 26 °C for 10 min were not significantly different until 5 M was used. For eggs exposed at 26 °C for 20 min statistical difference was achieved when 2 M was used, although viability of 84.9% was attained. In the 26 °C treatments at both 10 and 20 min, there was no viability at 5 M. However, in the treatments where the eggs were incubated and exposed at 4 °C for 10 and 20 min, there were no significant differences in any of the concentrations and a mean viability of 98.5% and 85.6%, respectively, was attained at 5 M.

3.3. Propylene glycol

Viability of eggs exposed to propylene glycol was lower than any other chemical at the lower concentrations and there was more variation in the replicates as indicated by the standard errors of the means (Table 1). The mean viability at the lowest treatment concentration (0.1 M) ranged from 72.1 to 90.2% in the various time and temperature exposures. Viability of the controls were the highest for the 26 °C exposure at both 10 and 20 min, however, statistical significance was not observed at any concentration. Viability at 5 M at 26 °C exposed for 10 and 20 min were very low and were significantly lower than all other concentrations. Viability of the control at the 4 °C and 10 min exposure treatment combination was significantly different than 2 M and 5 M treatments but not from the others. There were no significant differences in viability exposed at 4 °C for 20 min until 5 M was used. The viability at 5 M was much higher at 4 °C than those means attained at 26 °C.

3.4. DMSO

The mean viability of eggs exposed to DMSO at 26 °C in both 10 and 20 min exposure treatments experienced significant decreases at 2 M and again at 5 M which experienced very low viability. The 4 °C and 10 min exposure treatments experienced a significant decrease from the control mean at 0.1 M while the other means were not significantly differ from each other and the control until 5 M. For the 4 °C and 20 min exposure treatments, the only significant difference between occurred at 5 M with a large decrease in viability.

3.5. Glycerine

In the 26 °C, 10 and 20 min treatments, the mean viability of eggs was not significantly different until 5 M and 2 M, respectively. Viability was near zero at 5 M at 26 °C for both 10 and 20 min. There were significant differences in mean viability of eggs exposed at 4 °C for 10 min; however, the viability remained high with the lowest mean of 87%. For the 4 °C and 20 min treatment, there were no significant differences in viability at any concentration.

3.6. Hypersaline water

In all treatment combinations, the mean viability of eggs exposed up to 100 g/L was not significantly different than the appropriate control means except in the 26 °C and 20 min treatment where viability at 100 g/L was significantly lower than the control, 50, and 75 g/L treatments. At 26 °C and 4 °C for both 10 and 20 min exposures, viability significantly decreased from the lower concentrations at 150 and 200 g/L and very low viability was attained. Therefore, it appears that hypersaline water up to a concentration of at least 100 g/L may have potential for short and long term storage of subitaneous *A. tonsa* eggs.

4. Discussion

Results identify numerous cryopreservation compounds and several concentrations which did not significantly decrease viability of *A. tonsa* subitaneous eggs following exposure at 4 °C and 26 °C for 10 and 20 min. These data suggest further research is warranted to determine if these compounds will maintain copepod egg viability during exposure to cryopreservation conditions.

The goal of a chemical toxicity experiment is to determine the upper limit of tolerance by increasing the concentration to a level where an effect is evident. This was the case with all of the cryopreservation chemicals when the exposures were conducted at 26 °C. In general, the trends in viability of copepod eggs exposed to the various cryoprotectants show a difference between 4 °C and 26 °C; with all cryoprotectant chemicals, an exposure temperature of 4 °C produced higher viability than 26 °C at all the 5 M concentrations and at some of the 2 M concentrations.

The necessary concentration of each cryopreservation compound to attain high survival after freezing for extended periods is unknown. Further research is needed to define this; however, the results of the present study provide important data to identify which compounds should be evaluated with cryopreservation studies. Methanol, ethylene glycol, and glycerine at 5 M produced high viability at an exposure temperature of 4 °C. Propylene glycol also produced high viability at an exposure temperature of 4 °C even though viability was significantly lower than the other concentrations. DMSO at an exposure temperature of 4 °C up to 2 M did not greatly decrease the viability, but at 5 M, viability declined greatly. Therefore, all of these cryopreservation chemicals have potential. If 5 M is required to protect eggs during exposure to cryopreservation conditions, it is recommended that the temperature of the cryoprotectants and water used for storage be reduced to 4 °C. At 26 °C, the viability of the eggs was not decreased up to 2 M with most chemicals. Therefore, if 2 M or less is enough to protect eggs during cryopreservation conditions, the procedure can be conducted at room temperature.

Several studies have manipulated the salinity of the water containing eggs of *A. tonsa* and shown eggs can survive (Holmstrup et al., 2006; Højgaard et al., 2008). Højgaard et al. (2008) had success inducing quiescence in eggs after an abrupt salinity change and high viability was achieved at salinities from 5 and 30 g/L, with low viability at 0 g/L. No salinity above 30 g/L was investigated. In the present study, hypersaline water (50, 75, and 100 g/L) produced similar results as Højgaard et al. (2008) and to the cryoprotectants tested. High salinity storage of eggs may facilitate short or long term storage with high viability of eggs. This could be useful for distribution and marketing or for banking of eggs. Long term storage may be possible while maintaining high viability of eggs using 100 g/L hypersaline water, and the storage temperature may not need to be below 0 °C. Current research in our laboratory is investigating hypersaline water for short and long term storage at various temperatures.

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