Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 66 (2006) 964–973
Theriogenology
www.journals.elsevierhealth.com/periodicals/the

Freezing response and optimal cooling rates for cryopreserving sperm cells of striped bass, *Morone saxatilis*

S. Thirumala [a], W.T. Campbell [b], M.R. Vicknair [c], T.R. Tiersch [b], R.V. Devireddy [a,*]

[a] *Bioengineering Laboratory, Department of Mechanical Engineering, Louisiana State University, Baton Rouge, LA, USA*
[b] *Aquaculture Research Station, Louisiana Agricultural Experiment Station, Louisiana State University Agricultural Center, Baton Rouge, LA, USA*
[c] *Kent SeaTech Corporation, 11125 Flintkote Avenue, Suite J, San Diego, CA, USA*

Received 31 October 2005; accepted 7 February 2006

Abstract

This study explored the optimization of techniques for sperm cryopreservation of an economically important fish species, the striped bass *Morone saxatilis*. The volumetric shrinkage or the water transport response during freezing of sperm cells was obtained using a differential scanning calorimeter (DSC) technique. Water transport was obtained in the presence of extracellular ice at a cooling rate of 20 °C/min in two different media: (1) without cryoprotective agents (CPAs), and (2) with 5% (v/v) dimethyl sulfoxide (DMSO). The sperm cell was modeled as a cylinder of length of 22.8 μm and diameter 0.288 μm and was assumed to have an osmotically inactive cell volume (V_b) of $0.6V_0$, where V_0 is the isotonic or initial cell volume. By fitting a model of water transport to the experimentally determined water transport data, the best fit membrane permeability parameters (reference membrane permeability to water, L_{pg} or L_{pg}[cpa] and the activation energy, E_{Lp} or E_{Lp}[cpa]) were determined and ranged from L_{pg} = 0.011–0.001 μm/min-atm, and E_{Lp} = 40.2–9.2 kcal/mol). The parameters obtained in this study suggested that the optimal rate of cooling for striped bass sperm cells in the presence and absence of DMSO range from 14 to 20 °C/min. These theoretically predicted rates of optimally freezing *M. saxatilis* sperm compared quite closely with independent and experimentally determined optimal rates of cooling striped bass sperm.

Keywords: Differential scanning calorimetry; Water transport; Reference membrane permeability and activation energy

1. Introduction

Four economically important species of the genus *Morone* are cultured in the United States. White perch (*Morone americana*) is a brackish water species but also inhabits fresh water lakes and reservoirs. White bass (*M. chrysops*) and yellow bass (*M. mississippiensis*) are restricted to freshwater environments, whereas striped bass (*M. saxatilis*) is anadromous. Also, the hybrid (HB) of *M. chrysops* × *M. saxatilis*, although generally stocked into freshwater systems, does well in a wide range of salinities from 0 to 25 ppt. The genus *Morone* provides a highly desired sport fish in freshwater and brackish impoundments, and several hatcheries for artificial propagation of these species and hybrids provide seedstock for fisheries and for commercial aquaculture. The impetus for the research reported here, directed toward cryopreservation of striped bass spermatozoa, is aimed at enhancing our predictive

* Corresponding author. Tel.: +1 225 578 5891; fax: +1 225 578 5924.
E-mail address: devireddy@me.lsu.edu (R.V. Devireddy).

0093-691X/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2006.02.035

ability and towards a rational design of optimally cryopreserving sperm cells of *Morone*, and other aquatic species.

Preservation of gametes of fishes has been of interest to scientists since de Quatrefages ([1]; as cited in [2]) began investigations in the 1800s. Adequate methods of cryopreserving fish sperm would facilitate animal husbandry techniques that are now difficult or impossible. For example, cryopreserved sperm would allow hybridization of species with temporal spawning differences, provide greater ease in carrying on selective breeding programs for disease resistance and stock improvement, expedite sperm transfer from one hatchery to another, allow sperm from desirable individuals to be used after their death, enhance propagation where there is a deficiency of males, and reduce the number of males maintained by hatcheries [2]. Blaxter [3] was the first to successfully store fish sperm in a frozen condition; slices of the testes of spring-spawning Atlantic herring *Clupea harengus*, frozen in glycerol and seawater and stored for 6 mo in solid carbon dioxide (−79 °C), yielded 80 and 85% fertilization when mixed with fresh ova. Spermatozoa of striped bass *M. saxatilis* were also successfully cryopreserved, and when frozen in the presence of 5% dimethyl sulfoxide (DMSO) retained fertilizing capacity for 2 yr [4]. However, no fertilization was obtained when striped bass sperm were frozen in the presence of glycerol, ethylene glycol, or propylene glycol as the cryoprotectants [3]. The harvest and size of young striped bass grown in culture ponds from ''natural'' (derived from eggs fertilized with fresh sperm) and ''cryogenic'' larvae (derived from eggs fertilized by thawed sperm) were compared and no significant differences were found between them [5]. Cryopreservation protocols for spermatozoa of more than 200 species of freshwater and marine fish have been experimentally determined and many of these studies have been reviewed in several publications [6–13]. Despite this work, a firm theoretical basis for cryobiology of aquatic species gametes has yet to be developed.

The largely experimental nature of determining the optimal rate of cooling for sperm cells (mammalian or aquatic) is due to their small size and unique morphology; this limits the application of traditional microscopy-based low temperature measurement techniques to measure the freezing response in sperm cells. To circumvent this problem, we used a shape-independent differential scanning calorimetry (DSC) technique to measure the freezing response (water transport) of *M. saxatilis* sperm cells [14–20]. Water transport during freezing (and consequently the membrane permeability parameters) of striped bass sperm cells was obtained at 20 °C/min in the absence and presence of 5% (v/v) ratio of DMSO. The experimentally determined membrane permeability parameters were then incorporated into a recently developed ''generic optimal cooling rate equation'' to predict a priori the optimal rates of cooling striped bass sperm [14–18].

2. Materials and methods

2.1. Sperm collection and isolation

Five-year-old striped bass males were maintained in tanks in a recirculating system at Kent SeaTech Corp (San Diego, CA, USA). Males were anesthetized by the use of a standard fish anesthetic, tricaine methanesulfonate (Argent Laboratories, Inc., Redmond, WA, USA) prior to collection of milt. The males were dried with towels and milt was collected into sterile 2 mL vials by applying gentle pressure to the abdominal cavity. Care was taken to not contaminate the samples with urine, feces, or water. Sperm motility was evaluated qualitatively using 200× magnification by diluting 0.5 μL of undiluted sample with 50 μL of system water to screen samples to ensure >90% motility. The samples (∼1 mL) were diluted 1:4 (v/v) with calcium-free Hanks' balanced salt solution (Ca-free HBSS) prepared at 350 mOsmol/kg (measured with a vapor pressure osmometer [Model 550, Wescor Inc., Logan, UT, USA]), and were placed in 15 mL conical tubes. The samples were secured in a ZipLoc® bag and shipped to the Aquaculture Research Station (ARS) of the Louisiana State University Agricultural Center in a Styrofoam shipping container packed with Styrofoam packing material and two foam refrigerant blocks [21,22].

Upon arrival, samples were inverted to mix, and motility was estimated using dark-field microscopy (Optiphot-2, Nikon Inc., Garden City, NY, USA) at 200× magnification with and without activation (2 μL of sperm sample diluted with 20 μL of tank system water). Therefore, percent motility was estimated after the samples were received at the ARS before and after dilution with system water. Sperm cells that vibrated in place were not considered to be motile. Sperm cell concentrations were estimated using quadruplicate hemacytometer counts and samples were diluted to 1.1×10^9 cells/mL in preparation for DSC and cryopreservation experiments.

2.2. Cryopreservation experiment

Three cooling rates (4, 16, and 40 °C/min) were used for cryopreservation of the samples independent of the

DSC experiments, described below. The 4 and 40 °C/min rates were produced by the use of a computer-controlled freezer. The 16 °C/min rate was performed using methods developed for cryopreservation of dairy bull semen [23]. The samples were mixed with a 1:9 ratio of sperm solution to a solution of 50% DMSO and Ca-free HBSS resulting in a final sperm cell concentration of 1×10^9 and a DMSO concentration of 5%. The samples were loaded into 0.5-mL French straws (IMV International Corp., Minneapolis, MN, USA) and allowed to equilibrate for 15 min at +5 °C prior to freezing at one of three cooling rates (4, 16 and 40 °C/min).

Cryopreserved samples were evaluated for post-thaw motility at 7–30 d after freezing. Individual straws were thawed at 40 °C for 10 s. Immediately after thawing the samples were transferred from the straws into 1.8 mL vials. Percent motility was estimated as described above at <30 s after thawing.

2.3. DSC experiments

The DSC experiments were carried out in the absence and presence of a permeating cryoprotective agent, DMSO (5% (v/v)). These protocols have been described extensively in earlier publications [14–20] and will only be briefly described here. Approximately, 10 μL of the sperm suspension was placed in a DSC standard aluminum sample pan (Perkin-Elmer Corporation, Norwalk, CT, USA) with ~0.1 mg of *Pseudomonas syringae* (American Type Culture Collection, Rockville, MD, USA). The sample pan was then sealed and placed in a DSC-7 (Perkin-Elmer Corporation, Norwalk, CT, USA), to measure two heat releases: (i) during freezing of osmotically active (live) cells in suspension; and (ii) during freezing of osmotically inactive (dead) cells in the same suspension. The measured difference between these two heat releases from the same sample/experiment was correlated to water transport during freezing [14–20]. All DSC experiments were completed within 3–6 h after the sperm were delivered to the LSU bioengineering laboratory.

2.4. Translation of heat release to cell volume data for dynamic cooling

As stated elsewhere [14–20], the heat release measurements of interest are Δq_{dsc} and $\Delta q(T)_{dsc}$ which are the total and fractional difference between the heat releases measured by integration of the heat flows during freezing of osmotically active (live) cells in media, and during freezing of osmotically inactive (dead) cells in media. This difference in heat release has been shown to be related to cell volume changes; as demonstrated in a variety of sperm cells suspensions including canine [14], porcine [15], and recently in aquatic species, the Pacific oyster *Crassostrea gigas* [16], the green swordtail *Xiphophorus helleri* [17], the southern platyfish *Xiphophorus maculatus* [18] and independently verified by Yuan and Diller [19] and Diller [20] as:

$$V(T) = V_0 - \frac{\Delta q(T)_{dsc}}{\Delta q_{dsc}}(V_0 - V_b) \quad (1)$$

The two unknowns required in Eq. (1) apart from the DSC heat release readings (Δq_{dsc} and $\Delta q(T)_{dsc}$) are V_0 (the initial or isotonic cell volume) and V_b (the final or osmotically inactive cell volume). By analyzing microscopic images of the *M. saxatilis* sperm cells the initial volumes were obtained (Table 1). The final volumes for *M. saxatilis* sperm cells were assumed to be $0.6V_0$ (or $V_b = 0.6V_0$). To account for any variations in the assumed values of V_0 and V_b, a detailed parametric analysis is presented in the Results section. To ensure the accuracy and repeatability of the experimental data, a set of calibration and control experiments were performed as detailed previously for the DSC-7 [24].

2.5. Water transport model

During freezing in the presence of extracellular ice, the difference in the chemical potential between the unfrozen intracellular fluid and partially frozen extracellular space results in a transport of fluid/water from

Table 1
Measurements, and calculated volumes, surface areas, and ratios of surface area to volume (A_c:V_0) for head, tail and combined total of sperm cells from *M. saxatilis*

Cell component	Radius (μm)	Length (μm)	Volume (V_0) (μm^3)	Surface area (A_c) (μm^2)	(A_c:V_0) (μm^{-1})
Head[a]	0.80	2.2	4.43	11.06	2.51
Tail[a]	0.10	48.0	1.51	30.17	20.00
Total[b]	0.29	22.8	5.94	41.27	6.94

[a] The volumes of the head and the tail were all assumed to be cylinders.
[b] Sum of the volumes and surface areas of the head and the tail.

the intracellular space to the extracellular milieu. This process can be modeled using irreversible thermodynamics as shown by Mazur [25] as:

$$\frac{dV}{dT} = -\frac{L_p A_c RT}{B}[C_i - C_0] \quad (2)$$

with L_p, the sperm cell membrane permeability to water defined by Levin et al. [26] as

$$L_p = L_{pg}[cpa]\exp\left(-\frac{E_{Lp}[cpa]}{R}\left(\frac{1}{T} - \frac{1}{T_R}\right)\right) \quad (3)$$

where, L_{pg} or $L_{pg}[cpa]$ is the reference membrane permeability (μm/min-atm) at a reference temperature, T_R (=273.15 K) in the absence and presence of a CPA, E_{Lp} or $E_{Lp}[cpa]$ the apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability in the absence and presence of a CPA, A_c is the effective membrane surface area for water transport, assumed to be constant during the freezing process, R the universal gas constant, B the constant cooling rate (K/min), finally C_i and C_0 represent the concentrations of the intracellular and extracellular (unfrozen) solutions. In this study, the *M. saxatilis* sperm cells were modeled as a cylinder with length ($L = 22.8$ μm) and a radius ($r_0 = 0.288$ μm), which translated to an initial isotonic volume, V_0 of ~5.94 μm^3 and the initial surface area available for water transport, A_c of ~41.27 μm^2 (Table 1). Note that when no CPA is present, L_p is an Arrhenius function of L_{pg} and E_{Lp}. The two unknown water transport parameters of the model, either $L_{pg}[cpa]$ and $E_{Lp}[cpa]$ in the presence of CPA or L_{pg} and E_{Lp} in the absence of CPA, are determined by curve-fitting the water transport model to experimentally obtained volumetric shrinkage data (dV/dT) during freezing at a prescribed cooling rate. The various assumptions made in the development of Mazur's model of water transport are discussed in detail elsewhere [24–29] and are beyond the scope of the present report.

2.6. Numerical methods

A nonlinear least squares curve fitting technique was implemented using a computer program to calculate the membrane permeability parameters that best fit the volumetric shrinkage data [30]. The optimal fit of Eq. (2) to the experimental data was obtained by selecting a set of parameters that minimized the residual variance, χ^2, and maximized a goodness of fit parameter, R^2 [31]. All of the curve fitting results presented have an R^2-value greater than or equal to 0.99, indicating that there was good agreement between the experimental data and the fit calculated using the estimated membrane permeability parameters. To simulate the biophysical response of a sperm cell under a variety of cooling rates, the best fit parameters were substituted in the water transport equation. The water transport equation was then solved numerically using a custom written FORTRAN code (the code utilized a 4th order Runge-Kutta method) on a Mac Powerbook G4 (Apple Computer Inc., Cupertino, CA, USA) workstation [15–17].

2.7. Theoretical prediction of optimal cooling rates

Thirumala and Devireddy [32] reported that for a variety of biological systems a comparison of the published experimentally determined values of B_{opt} (in °C/min) agreed quite closely with the value obtained using a generic optimal cooling rate equation (GOCRE) that defines:

$$B_{opt} = 1009.5\exp^{(-0.0546E_{Lp})}(L_{pg})\left(\frac{A_c}{V_0 - V_b}\right) \quad (4)$$

In this equation, L_{pg} and E_{Lp} represent the membrane permeability parameters (in μm/min-atm and Kcal/mol, respectively), whereas the last term $A_c/(V_0 - V_b)$ (in μm^{-1}) represents the ratio of the available surface area for water transport (A_c) to the initial volume of intracellular water ($V_0 - V_b$). Based on the cell dimensions shown in Table 1, the ratio of $A_c/(V_0 - V_b)$ is 17.37 μm^{-1} (assuming $V_b = 0.6V_0$). Once the cell level parameters L_{pg} and E_{Lp} were determined using the curve-fitting procedure described above, we utilized Eq. (4) to predict the optimal rates of cooling striped bass sperm cells.

3. Results

3.1. Dynamic cooling response and membrane permeability parameters

The DSC water transport data obtained at a cooling rate of 20 °C/min for striped bass spermatozoa are shown in Fig. 1. The data obtained in the absence of DMSO are shown in Fig. 1A, whereas the corresponding data obtained in the presence of DMSO are shown in Fig. 1B. Water transport cessation was observed in the DSC heat release data as an overlap of the thermograms from the heat release signature obtained using

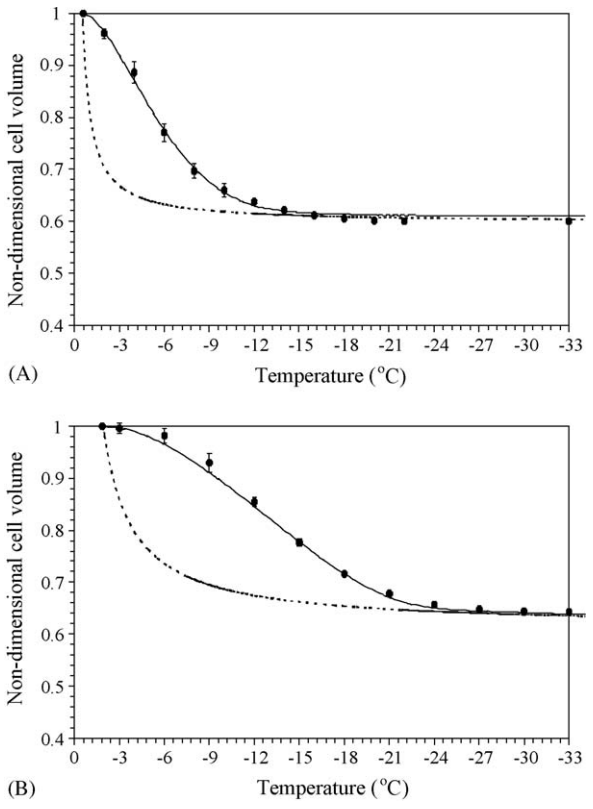

Fig. 1. Volumetric response of *M. saxatilis* (striped bass) sperm cells as a function of sub-zero temperatures obtained using the DSC technique in the presence of extracellular ice (A) and in the presence of extracellular ice and DMSO (B). The filled circles represent the experimentally obtained water transport (volumetric shrinkage) at a cooling rate of 20 °C/min. The dynamic cooling response at 20 °C/min is shown as a solid line and was obtained by using the "best fit" membrane permeability parameters (L_{pg} and E_{Lp} or L_{pg}[cpa] and E_{Lp}[cpa]) (Table 2) in the water transport equation (Eqs. (2) and (3)). The model-simulated equilibrium cooling response obtained is shown as a dotted line in both figures. The non-dimensional cell volume is plotted along the *y*-axis and the sub-zero temperatures are shown along the *x*-axis. The error bars represent the standard deviation for the mean values of nine separate DSC experiments ($n = 9$).

osmotically active (initial) and inactive (final) cells [14–18]. The dynamic portion of the cooling curve (where the thermograms were distinct) was between −0.6 and −15 °C in the absence of DMSO and between −1.8 and −24 °C in the presence of DMSO. The membrane permeability parameter values that best fit the 20 °C/min water transport data in the absence and presence of DMSO were calculated, as described earlier, using the nonlinear curve fitting technique (Table 2).

3.2. Membrane permeability parametric space

To further demarcate the range and combination of the membrane permeability parameters (either L_{pg} and E_{Lp}, or L_{pg}[cpa] and E_{Lp}[cpa]) that "best fit" the measured striped bass water transport data, we generated contour plots (Fig. 2) of the parametric space that "fits" the water transport with a goodness of fit parameter, R^2 ($\geq$0.98). Distinctive contours are shown (Fig. 2) and correspond to the two media investigated (with and without DMSO) with $V_b = 0.6V_0$. Any combination of L_{pg} and E_{Lp} (or L_{pg}[cpa] and E_{Lp}[cpa]) shown to be within the contour will "fit" the experimentally measured water transport data in that media with an R^2-value of >0.98. Note that the contours were distinct and separate, with no common region. Thus, there was not even a single combination of L_{pg} and E_{Lp} (or L_{pg}[cpa] and E_{Lp}[cpa]) that can concomitantly predict the water transport response in both the media studied.

3.3. Optimal rates of cooling

By incorporating the best fit parameters of water transport in Eq. (4), the theoretically predicted values for optimally cooling *M. saxatilis* spermatozoa were obtained (Table 2). To independently verify the

Table 2
Predicted sub-zero membrane permeability parameters and the optimal rates of freezing for *M. saxatilis* sperm cells in the absence and presence of DMSO, using cell dimensions shown in Table 1

Inactive cell volume (V_b)	Freezing media	L_{pg} or L_{pg}[cpa] (μm/min-atm)	E_{Lp} or E_{Lp}[cpa] (kcal/mole)	B_{opt} (°C/min)
$0.6V_0$[a]	Without DMSO	0.011	40.2	20
	5% (v/v) DMSO	0.001	9.2	14
$0.4V_0$[b]	Without DMSO	0.016	39.8	21
	5% (v/v) DMSO	0.002	8.8	14
$0.8V_0$[c]	Without DMSO	0.005	37.8	21
	5% (v/v) DMSO	0.001	8.9	14

[a] Obtained using Eq. (4) with $A_c/(V_0 - V_b) = 17.37\ \mu m^{-1}$.
[b] Obtained using Eq. (4) with $A_c/(V_0 - V_b) = 11.58\ \mu m^{-1}$.
[c] Obtained using Eq. (4) with $A_c/(V_0 - V_b) = 34.74\ \mu m^{-1}$.

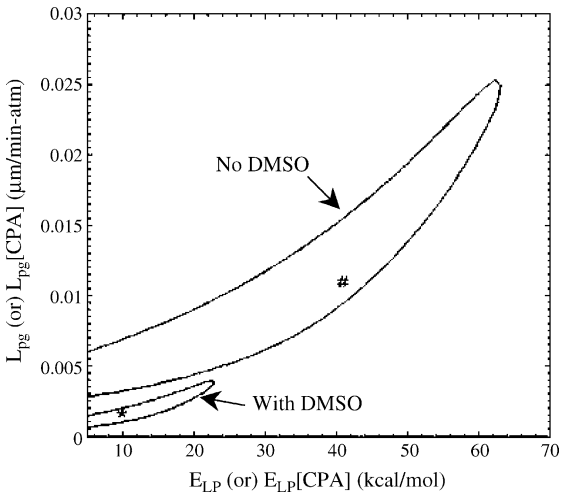

Fig. 2. Contour plots of the goodness of fit parameter R^2 (=0.98) for water transport response of *M. saxatilis* sperm cells assuming $V_b = 0.6V_0$. Two contours are shown corresponding to the two media studied, i.e., without DMSO and with DMSO. Note that the best fit parameters (Table 2; $V_b = 0.6V_0$) are represented within the contours by a "#" (without DMSO), and "*" (with DMSO). The membrane permeability at 0 °C, L_{pg} (or L_{pg}[cpa]) (μm/min-atm) is plotted on the *y*-axis, whereas the apparent activation energy of the membrane, E_{Lp} (or E_{Lp}[cpa]) (kcal/mol) is plotted on the *x*-axis.

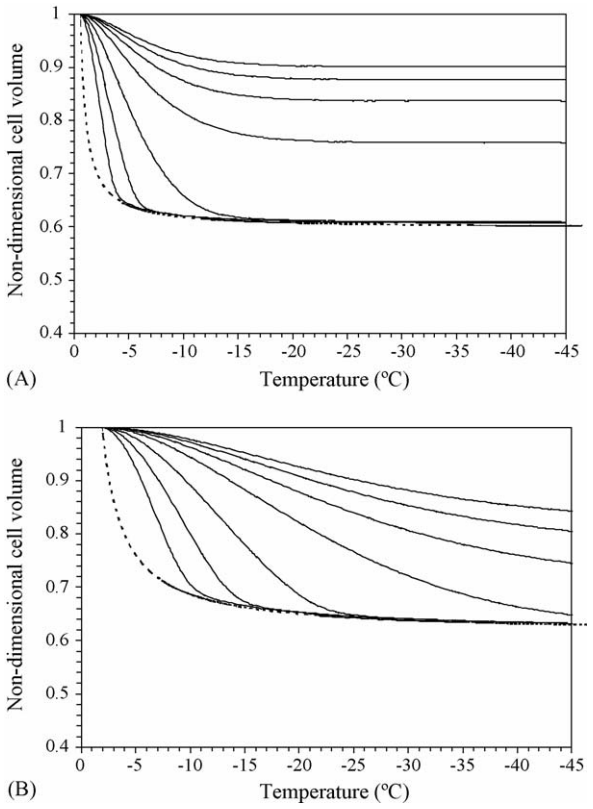

Fig. 3. Volumetric response of *M. saxatilis* sperm cells at various cooling rates as a function of sub-zero temperature using the best-fit membrane permeability parameters assuming $V_b = 0.6V_0$ (Table 2). The changes in the normalized cell volume (V/V_0) are shown as a function of temperature for different cooling rates in striped bass sperm cell suspensions without DMSO (A) and with DMSO (B). The water transport curves represent the model simulated response for different cooling rates (from left to right: 5, 10, 20, 40, 60, 80 and 100 °C/min). The model simulated equilibrium cooling response obtained is shown as a dotted line. The sub-zero temperatures are shown along the *x*-axis, whereas the nondimensional volume is plotted along the *y*-axis.

predicted rates of optimally cooling *M. saxatilis* spermatozoa, additional numerical simulations were also performed at various cooling rates (5–100 °C/min) using the water transport model (Eq. (2)) and the best fit parameters (Table 2; $V_b = 0.6V_0$) for both the conditions studied, i.e., (i) without DMSO (Fig. 3A); and (ii) with DMSO (Fig. 3B). A detailed analysis of these numerical simulations was performed, as described earlier for spermatozoa of canine [14], porcine [15], Pacific oyster [16] and green swordtail [17], to determine the optimal rates of cooling *M. saxatilis* spermatozoa. The predicted optimal rates of cooling from this analysis of the numerical simulations were within ±20% of the values obtained using Eq. (4), thus corroborating further the predicted rates of optimal cryopreservation of *M. saxatilis* spermatozoa shown in Table 2.

3.4. Effect of varying osmotically inactive cell volume

To study the effect of varying the osmotically inactive cell volume on the predicted membrane permeability parameters (L_{pg} and E_{Lp}), the value of V_b was varied from $0.8V_0$ to $0.4V_0$ (i.e. a 33% change from the initially assumed value of $0.6V_0$). The DSC water transport data were correspondingly modified (using Eq. (1)) and as previously described, the data were curve-fitted to the water transport model (Eqs. (2) and (3)) by the use of a nonlinear least squares curve-fitting technique. Note that the predicted values of the reference membrane permeability parameters (L_{pg}) with assumed values of $0.8V_0$ or $0.4V_0$ as the osmotically inactive cell volume were significantly different from the values obtained with an assumed osmotically inactive cell volume of $0.6V_0$ (Table 2). This variation in the value of L_{pg} was in direct contrast to the behavior of E_{Lp}, which remained essentially unchanged (±10%) when V_b was varied from $0.6V_0$ to either $0.8V_0$ or $0.4V_0$. However, the predicted rates of optimal freezing were essentially unchanged (±5%) when the assumed value of V_0 was varied by ±33% (Table 2). As an independent assessment of the predicted rates of optimal freezing

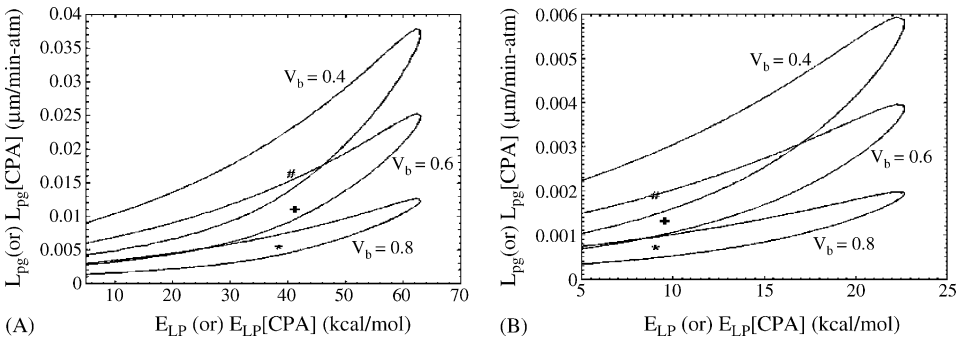

Fig. 4. A comparison of contour space for the three different values of V_b ($0.4V_0$, $0.6V_0$, and $0.8V_0$) are shown for *M. saxatilis* sperm cells in (A) (without DMSO) and in (B) (with DMSO). In each figure, there are three contours corresponding to the three assumed values of V_b, either $0.4V_0$ or $0.6V_0$ or $0.8V_0$. The best-fit parameters (Table 2) are represented within the contours by a "#" ($0.4V_0$), "+" ($0.6V_0$) and "*" ($0.8V_0$). The membrane permeability at 0 °C, L_{pg} (or L_{pg}[cpa]) (μm/min-atm) is plotted on the *y*-axis, whereas the apparent activation energy of the membrane, E_{Lp} (or E_{Lp}[cpa]) (kcal/mol) is plotted on the *x*-axis. Note the significantly smaller scale in both the *x*- and *y*-axes in (B), when compared with (A).

(Table 2), a separate set of numerical simulations was also performed with the predicted best fit permeability parameters obtained with the assumed values of 0.8 and $0.4V_0$ as V_b (data not shown). An analysis of these simulations showed that the striped bass sperm cells were essentially dehydrated (or trapped intracellular water) in a similar fashion, as observed earlier with an osmotically inactive cell volume of $0.6V_0$. Thus, errors in the assumed value of V_b do alter the model-predicted membrane permeability parameters, but the predicted optimal rates of freezing remain essentially unaltered. This insensitivity of the predicted optimal rate of freezing to the assumed value of V_b has also been previously noted in sperm cells of various species [14–18].

To further illustrate the effect of varying the osmotically inactive cell volume on the predicted membrane permeability parameters, contour plots (similar to those shown in Fig. 2) were generated for an assumed V_b values of 0.8 and $0.4V_0$. A comparison of contour space for the three different values of V_0 ($0.4V_0$, $0.6V_0$, and $0.8V_0$) are shown in Fig. 4A (for spermatozoa of *M. saxatilis* without DMSO) and Fig. 4B (for spermatozoa of *M. saxatilis* with DMSO). In each figure, there are three contours corresponding to the three assumed values of V_b. Examination shows that increasing the value of V_b decreases the predicted best fit value of the membrane permeability (Table 2), whereas the predicted best fit value of the activation energy remained essentially unchanged (Table 2).

3.5. Effect of varying assumed cell dimensions (or surface area to volume ratio)

To account for any errors and uncertainties in the measured *M. saxatilis* sperm cell dimensions, we also investigated the effect of varying the ratio of the surface area available for water transport to the initial cell volume (A_c:V_0) by ±50% on the predicted membrane permeability parameters (Table 3). The water transport model was appropriately modified (Eqs. (2) and (3)) and using the nonlinear least squares curve fitting technique described earlier, the corresponding best fit permeability parameters (L_{pg} or L_{pg}[cpa] and E_{Lp} or E_{Lp}[cpa]) were obtained. The membrane permeability parameters

Table 3
Predicted sub-zero membrane permeability parameters and the optimal rates of freezing for *M. saxatilis* sperm cells in the absence and presence of DMSO, with $V_b = 0.6V_0$

Surface area to volume ratio (A_c:V_0)	Freezing media	L_{pg} (or) L_{pg}[cpa] (μm/min-atm)	E_{Lp} (or) E_{Lp}[cpa] (kcal/mol)	B_{opt} (°C/min)
10.41	Without DMSO	0.007	39.5	20
	5% (v/v) DMSO	0.001	10.3	14
6.94[a]	Without DMSO	0.011	2	20
	5% (v/v) DMSO	0.001	9.2	14
3.46	Without DMSO	0.018	35.6	22
	5% (v/v) DMSO	0.003	8.8	14

[a] Values shown in Table 2.

Table 4
Percent sperm motility before freezing at three cooling rates (40, 16 and 4 °C/min) and after thawing of samples diluted or not diluted in activating solution (system water)

Male	Before freezing		After thawing					
	Not diluted	Diluted	40 °C/min[a]		16 °C/min[b]		4 °C/min[a]	
			Not diluted	Diluted	Not diluted	Diluted	Not diluted	Diluted
1	10	80	10	10	20	20	20	20
2	60	80	20	20	20	20	20	20
3	70	80	5	5	5	5	1	1
4	50	70	1	1	20	20	1	1
5	40	70	5	5	10	10	1	1
6	40	60	1	1	20	20	1	1
Mean ± S.D.	45 ± 8	73 ± 3	7 ± 7	7 ± 7	16 ± 3	16 ± 3	7 ± 10	7 ± 10

[a] Rate produced using a computer-controlled freezer.
[b] Rate produced using commercial methods developed for cryopreservation of dairy bull semen.

obtained by increasing or lowering the A_c:V_0 ratio are shown in Table 3. The observed inverse relationship between A_0:V_0 ratio and the predicted membrane permeability parameters (L_{pg} or L_{pg}[cpa] and E_{Lp} or E_{Lp}[cpa]) was not unexpected, as Eq. (2) shows that the variation in the cell volume as a function of temperature (dV/dT) was proportional to the product of L_p and A_c. Thus, for a predefined variation in the cell volume as a function of temperature (dV/dT), an increase in A_c (or A_c:V_0 ratio) will cause a corresponding decrease in the predicted value of L_p, where $L_p = fn(L_{pg}, E_{Lp})$ as shown in Eq. (3) and vice-versa. Also, note that as shown in Eq. (3), L_p is directly proportional to L_{pg}; thus a decrease in L_p will cause a decrease in L_{pg}. In essence, as the ratio between A_c:V_0 increases, L_{pg} will decrease (as seen in Table 3). However, as shown in Eq. (4), B_{opt} is directly proportional to ratio of the cell surface area to the intracellular water volume ratio ($A_c/(V_0 - V_b)$) and L_{pg}. Thus, varying the A_c:V_0 should not significantly affect the predicted B_{opt}, as any increase in A_c:V_0 ratio is compensated by a corresponding decrease in L_{pg}. This assertion was further verified by comparing the predicted optimal rates of freezing *M. saxatilis* sperm with a surface-area-to-volume ratio (A_c:V_0) of 10.41 and 3.46 to a ratio of 6.94 (Table 3). As expected, the predicted optimal rates of freezing were within ±10%, even when the A_c:V_0 was increased or lowered by 50%; therefore, errors in the measured dimensions did not significantly vary the theoretically predicted optimal rates of cooling striped bass spermatozoa.

3.6. Cryopreservation experiment

As stated earlier, percent motility was estimated for thawed *M. saxatilis* sperm before and after dilution with system water for three different cooling rates: 40, 16 and 4 °C/min (Table 4). The highest mean post-thaw motility was obtained from the 16 °C/min rate. However, there was no difference ($P \geq 0.05$) among the three cooling rates tested. The experimentally measured optimal rate of cooling *M. saxatilis* sperm compares quite closely with the theoretically predicted optimal rate of 14 °C/min (see Tables 2 and 3).

4. Discussion

The DSC technique was used to obtain water transport data and water permeability parameters (L_{pg} or L_{pg}[cpa] and E_{Lp} or E_{Lp}[cpa]) of *M. saxatilis* sperm cells in a freezing media with and without DMSO. The water permeability parameters of *M. saxatilis* spermatozoa ranged from 0.011 to 0.001 μm/min-atm and 40.2 to 9.2 kcal/mol. A comparison of these values with previously published values for aquatic sperm cells [16–18], revealed several similarities and differences. For example, when compared with *X. helleri* sperm cells, the L_{pg} and E_{Lp} obtained in the absence of CPAs for *M. saxatilis* sperm were significantly higher (0.011 versus 0.0046 μm/min-atm and 40.2 versus 11.97 kcal/mol) [17]. The parameters obtained in the presence of DMSO were comparable to those obtained earlier for haploid and diploid *Crassostrea gigas* sperm cells (0.001 versus 0.002 μm/min-atm and 9.2 versus ∼10 kcal/mol) [16]. However, the best-fit parameters obtained in this study using the DSC volumetric shrinkage data during freezing of *M. saxatilis* spermatozoa were significantly lower than the reported suprazero (above zero and in the absence of extracellular ice) permeability values for mammalian species [33]. A similar lowering of the sperm cell water permeability parameter values was also found for canine [14], boar [15], Pacific oyster [16] and *Xiphophorus* [17,18]. This discrepancy between the

membrane permeabilities may be associated with possible changes in the sperm cell plasma membrane during suprazero cooling, as described elsewhere [14–18] and include a lipid phase transition between 0 and 4 °C, the alterations in membranes due to the presence of extracellular ice, thermotropic (temperature dependent) phase phenomena, lyotropic (i.e., independent of cooling rate) membrane phase changes, and alterations in membrane fluidity.

Although the exact mechanism by which DMSO alters membrane transport is as yet unknown in aquatic species, previous studies on the sperm cells of *Xiphophorus* [17,18] and *C. gigas* [16] suggested that the addition of cryoprotective agents (CPAs) tends to lower the membrane transport parameters. This was also the case with the sperm cells of *M. saxatilis* (Tables 2 and 3), where the addition of DMSO lowered the measured values of the reference membrane permeability and the activation energy significantly; the former was lowered by a factor of 10, whereas the latter was lowered by a factor of four. It is interesting to note also that the range of parameters that best fit the measured water transport data was much smaller in the presence of DMSO than in its absence (Fig. 2). However, the theoretically predicted optimal rates of freezing were lower in the presence of DMSO than in its absence. This reduction in the predicted optimal rate of freezing in the presence of CPAs was similar to that observed earlier for spermatozoa of canine [14], *C. gigas* [16] and *X. helleri* [17], but was contrary to the observed behavior in boar sperm [15] and in the sperm cells of *X. maculatus* [18]. Clearly, further studies are needed to further delineate the effects of DMSO and other CPAs on membrane transport and predicted rates of optimal freezing.

As discussed earlier, three cooling rates (4, 16 and 40 °C/min) were used for freezing samples independent of the DSC experiments to compare theoretically predicted values with experimentally determined values for the cryopreservation of the *M. saxatilis* sperm. Although there was no significant difference in post-thaw motility among the rates tested, the 16 °C/min rate had the highest mean after thawing motility. The close agreement between the experimentally determined (16 °C/min) and the theoretically predicted (14 °C/min) optimal cooling rates was quite remarkable considering the various assumptions made not only in the development of the water transport model (Eqs. (2) and (3)) but also in the development of the generic optimal cooling rate equation (Eq. (4)). Moreover, a review of eight published studies that utilized DMSO as a cryoprotectant for *M. saxatilis* sperm revealed that cooling rates of 20 °C or faster were the most effective for cryopreservation [4,5,34–39]. When discussing the perceived success of cryopreservation determined by estimated motility, it is important to note the variability of the shipped sperm. The shipped samples all had motile sperm prior to dilution with the activating solution. This could be an indicator of hypersensitivity of the cells due to the physical stresses encountered during shipping, or the interplay of a variety of factors in sperm activation. Further research is needed to determine the causes of variability in cryopreserved *M. saxatilis* sperm samples.

In conclusion, water transport (volumetric shrinkage) data for *M. saxatilis* sperm cells in the presence of extracellular ice and DMSO during freezing were obtained using a DSC technique at a cooling rate of 20 °C/min. The measured water transport data in the presence and absence of DMSO were curve-fitted to a model of water transport, to predict the ''best fit'' membrane permeability parameters (L_{pg} and E_{Lp} or L_{pg}[cpa] and E_{Lp}[cpa]). The measured ''best fit'' permeability parameters ranged from $L_{pg} = 0.011$–0.001 μm/min-atm) and $E_{Lp} = 40.2$–9.2 kcal/mol for *M. saxatilis* sperm. The permeability parameters obtained in this study predicted an optimal rate of freezing for *M. saxatilis* sperm cells ranging from 14 to 20 °C/min; these values compared favorably with empirically and independently determined experimental rates of optimally freezing sperm from striped bass.

Acknowledgments

This work was supported in part by a grant from the Louisiana Board of Regents (LEQSF 2002–05-RD-A-03) to RD, and a grant from the National Institutes of Health to TRT. Kent SeaTech's role in the research was supported by the Small Business Innovation Research program of the U.S. Department of Agriculture (Grant No. 2004-33610-14314). We thank J. Buchanan and J. Stannard for assistance and advice in this study, and Guanglei Li for help with the DSC experiments. This manuscript was approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript number 06-11-0084.

References

[1] De Quatrafages MA. Recherches sur lavitalité des spermatozoides de quelques poisons d'eau douce. Ann Sci Nat 1853;19:341–69.

[2] Kerby JH. Cryogenic preservation of sperm from striped bass. Trans Am Fish Soc 1983;112:86–94.
[3] Blaxter JHS. Sperm storage and cross-fertilization of spring and autumn spawning herring. Nature (London) 1953;172:1189–90.
[4] Kerby JH. Cryogenic preservation of sperm from striped bass. Trans Am Fish Soc 1983;112:86–94.
[5] Kerby JH, Bayless JD, Harrell RM. Growth, survival, and harvest of striped bass produced with cryopreserved spermatozoa. Trans Am Fish Soc 1985;114:761–5.
[6] Mounib MS. Cryogenic preservation of fish and mammalian spermatozoa. J Reprod Fert 1978;53:13–8.
[7] Scott AP, Baynes SM. A review of the biology, handling and storage of salmonid spermatozoa. J Fish Biol 1980;17:707–39.
[8] Rana KJ. Cryopreservation of fish spermatozoa. Cryopreservation and freeze–drying protocols. Methods Mol Biol 1995;38: 151–65.
[9] Suquet M, Dreannol C, Fauvel C, Cosson J, Billard R. Cryopreservation of sperm in marine fish. Aquaculture Res 2000; 31:231–43.
[10] Gwo J-C. Cryopreservation of aquatic invertebrate semen: a review. Aquaculture Res 2000;31:259–72.
[11] Chao N-H, Chiu Liao I. Cryopreservation of finfish and shellfish gametes and embryos. Aquaculture 2001;197:161–89.
[12] Tiersch TR. Cryopreservation in aquarium fishes. Mar Biotech 2001;3:S212–23.
[13] Billard R, Cosson J, Noveiri SB, Pourkazemi M. Cryopreservation and short-term storage of sturgeon sperm, a review. Aquaculture 2004;236:1–9.
[14] Thirumala S, Ferrer MS, Al-Jarrah A, Eilts BE, Paccamonti DL, Devireddy RV. Cryopreservation of canine spermatozoa: theoretical prediction of optimal cooling rates in the presence and absence of cryoprotective agents. Cryobiology 2003;47:109–24.
[15] Devireddy RV, Fahrig B, Godke RA, Leibo SP. Subzero water transport characteristics of boar spermatozoa confirm observed optimal cooling rates. Mol Reprod Dev 2004;67:446–57.
[16] He Y, Dong Q, Tiersch TR, Devireddy RV. Variation in the membrane transport properties and predicted optimal rates of freezing for spermatozoa of diploid and tetraploid pacific oyster *Crassostrea gigas*. Biol Reprod 2004;70:1428–37.
[17] Thirumala S, Huang C, Dong Q, Tiersch TR, Devireddy RV. A theoretically estimated optimal cooling rate for the cryopreservation of sperm cells from a live-bearing fish, the green swordtail *Xiphophorus helleri*. Theriogenology 2005;63:2395–415.
[18] Pinisetty D, Huang C, Dong Q, Tiersch TR, Devireddy RV. Subzero water permeability parameters and optimal freezing rates for sperm cells of the southern platyfish, *Xiphophorus maculates*. Cryobiology 2005;50:250–63.
[19] Yuan S, Diller KR. Study of freezing biological systems using optical differential scanning calorimeter. ASME Summer BED Conf Proc 2001;50:117–8.
[20] Diller KR. Bioheat and mass transfer as viewed through a microscope. ASME J Biomech Eng 2005;127:67–84.
[21] Tiersch TR. Shipping of refrigerated samples. In: Tiersch TT, Maxik PM, editors. Cryopreservation in aquatic species. Baton Rouge, LA: World Aquaculture Society; 2000. p. 281.
[22] Tiersch TR, Wayman WR, Skapura DP, Neidig Cl, Grier HJ. Transport and cryopreservation of sperm of the common snook, *Centropomus undecimalis* (Bloch). Aquaculture Res 2004;35: 278–88.
[23] Chandler JE, Adkinson RW, Smith JW, Saxton AM. Use of linear semen quality score for classification and decision making in evaluation of individual ejaculates of Holestein bulls. J Dairy Sci 1987;70:1036–44.
[24] Devireddy RV, Raha D, Bischof JC. Measurement of water transport during freezing in cell suspensions using a differential scanning calorimeter. Cryobiology 1998;36:124–55.
[25] Mazur P. Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. J Gen Physiol 1963;47:347–69.
[26] Levin RL, Cravalho EG, Huggins CG. A membrane model describing the effect of temperature on the water conductivity of erythrocyte membranes at subzero temperatures. Cryobiology 1976;13:415–29.
[27] Mansoori GA. Kinetics of water loss from cells at subzero centigrade temperature. Cryobiology 1975;12:34–45.
[28] McGrath JJ. Membrane transport properties. In: McGrath JJ, Diller KR, editors. Low temperature biotechnology: emerging applications and engineering contributions. ASME Press; 1988. p. 273–330. BED-Vol. 10, HTD-Vol. 98.
[29] Toner M, Tompkins RG, Cravalho EG, Yarmush ML. Transport phenomena during freezing of isolated hepatocytes. AIChE J 1992;38:1512–22.
[30] Bevington PR, Robinson DK. Data reduction and error analysis for the physical sciences, 2nd ed., New York: McGraw-Hill; 1992.
[31] Montgomery DC, Runger GC. Applied statistics and probability for engineers New York: John Wiley & Sons, Inc.; 1994, 471–529.
[32] Thirumala S, Devireddy RV. A simplified procedure to determine the optimal rate of freezing biological systems. ASME J Biomech Eng 2005;127:295–300.
[33] Curry M. Cryopreservation of semen from domestic livestock. Rev Reprod 2000;5:46–52.
[34] Brown GG, Brown LD. Cryopreservation of sperm of striped and white bass. In: Tiersch TT, Mazik PM, editors. Cryopreservation of aquatic species. Baton Rouge, LA: World Aquaculture Society; 2000. p. 130–7.
[35] Jenkins-Keeran K, Woods LC. The cryopreservation of striped bass *Morone saxatilis* semen. J World Aquaculture Soc 2002; 33:70–7.
[36] He S, Woods LC. The effects of osmolality, cryoprotectant and equilibration time on striped bass (*Morone saxatilis*) sperm motility. J World Aquaculture Soc 2003;34:255–65.
[37] He S, Woods LC. Effects of glycine and alanine on short-term storage and cryopreservation of striped bass (*Morone saxalitis*) spermatozoa. Cryobiology 2003;46:17–25.
[38] He S, Curry LC. Changes in motility, ultrastructure, and fertilzation capacity of striped bass *Morone saxatilis* spermatozoa following cryopreservation. Aquaculture 2004;236: 677–86.
[39] He S, Woods LC. Effects of dimethylsulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) sperm. Cryobiology 2004;48:254–62.