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Brief communication

Freezing response of white bass (*Morone chrysops*) sperm cells ☆

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Abstract

The water transport response during freezing of sperm cells of *Morone chrysops* (white bass, WB) was obtained using a shape-independent differential scanning calorimeter (DSC) technique. Sperm cell suspensions were frozen at a cooling rate of 20 °C/min in two different media: (1) without cryoprotective agents (CPAs), or (2) with 5% (v/v) dimethyl sulfoxide (Me_2SO). For calculations, the sperm cell was modeled as a cylinder of length 24.8 μm and diameter of 0.305 μm, while the osmotically inactive cell volume (V_b) was assumed to be 0.6 V_o, where V_o was the isotonic or the 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 $L_{pg} = 0.51–1.7 \times 10^{-15}$ m^3/Ns (0.003–0.01 μm/min-atm), and $E_{Lp} = 83.6–131.3$ kJ/mol (20.0–31.4 kcal/mol). The parameters obtained in this study suggest that the optimal rate of cooling for *M. chrysops* sperm cells is ~22 °C/min, a value that compares closely with experimentally determined optimal rates of cooling (~16 °C/min).

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

Preservation of viable gametes of fishes has been of interest to fishery scientists, since de Quatrafages [6] began his investigations in the 1850s. Cryopreservation protocols for spermatozoa of more than 200 species of freshwater and marine fish have been experimentally determined [1,7]. Despite this large body of work, a firm theoretical basis for cryobiology of aquatic species gametes has yet to be developed. For example, although an important species in aquaculture, little information is available on the cryopreservation of sperm cells from *Morone chrysops* (white bass, WB) [1]. As the *M. chrysops* hybrid with *Morone saxatilis* (striped bass) is used extensively in aquaculture with artificial fertilization being used exclusively for fingerling production,

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there is a clear need for improvement in cryopreservation techniques. This work offers basic information on freezing of sperm from *M. chrysops* that is also relevant to commercial aquaculture and conservation efforts for wild fish species through cryopreservation of germ plasm resources.

During freezing of cells in suspension, electrolytes and proteins from the original extracellular solution are left within highly concentrated unfrozen liquid fractions. The cells, which remain unfrozen at the instant of ice formation within the extracellular space, respond to the increased concentration of the unfrozen fraction by either dehydrating (water transport), or by the formation of intracellular ice in the cytoplasm. Observations of these events can then be fit to mathematical models which can then be used to predict the cell's response to freezing under arbitrary cooling conditions, and hence help to develop better cryopreservation protocols. The highest rates of cellular survival are typically found for cooling rates which are fast enough to minimize dehydration (solute effects) injury while still slow enough to preclude large amounts of intracellular ice. In the present study, we measure the water transport response during freezing of sperm cells from *M. chrysops* using a shape-independent differential scanning calorimetry (DSC) technique [5,8]. Water transport during freezing (and consequently the membrane permeability parameters) of WB sperm cells was obtained at a cooling rate of 20 °C/min in the absence and presence of 5% v/v ratio of dimethyl sulfoxide (Me_2SO). The experimentally determined membrane permeability parameters were then used to predict a priori the optimal cooling rates[1] for WB sperm cryopreservation.

One-year-old WB males were maintained in tanks in a recirculating system at Kent SeaTech Corp. (San Diego, CA). Males were anesthetized by use of a standard fish anesthetic, tricaine methanesulfonate (Argent Laboratories, Inc., California), 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, Utah]), and were placed in 15 mL conical tubes. The samples were secured in a ZipLoc® bag and shipped overnight 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 [10]. Upon arrival, samples were inverted to mix, and motility was estimated using dark-field microscopy (Optiphot-2, Nikon Inc., Garden City, New York) at 200-× magnification. 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 experiments. All water transport (DSC) experiments were completed within 3–6 h after the sperm were delivered to the LSU Bioengineering Laboratory and within 24 h of sperm collection.

The DSC experiments were carried out in the absence and presence of a permeating cryoprotective agent (CPA), Me_2SO (5% v/v). The DSC dynamic cooling protocol used to measure the water transport out of the sperm cells was the same as reported in earlier studies on mammalian [8] and aquatic [5] sperm cells and will not be repeated here. The heat release measurements of interest were 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 recently for the sperm cells of the southern platyfish *Xiphophorus maculatus* [5] and canine [8] as:

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

The unknowns required in Eq. (1) apart from the DSC heat release readings are V_o (the initial or isotonic cell volume) and V_b (the final or osmotically inactive cell volume). By analyzing microscopic images of WB sperm cells the initial volumes were obtained (Table 1). Similarly, the final volumes for WB sperm were assumed to be 0.6 V_o (or $V_b = 0.6\ V_o$).

[1] Optimal cooling rate is defined as the cooling rate that minimizes damaging intracellular ice formation and concurrently limits the time of exposure of the sperm cell to highly concentrated extracellular salt solutions.

Table 1
Measurements and calculated volumes, surface areas and ratios of surface area to volume (A_c:V_o) for head, tail, and combined total of a sperm cell from *Morone chrysops*

Sperm cell geometry	Radius (μm)	Length (μm)	Volume (V_o) (μm^3)	Surface area (A_c) (μm^2)	(A_c:V_o) (μm^{-1})
Head[a]	0.9	2.0	5.09	11.31	2.22
Tail[a]	0.12	48.0	2.17	36.21	16.69
Total[b]	0.305	24.8	7.25	47.5	6.55

[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.

During freezing in the presence of extracellular ice, the difference in the chemical potential between the unfrozen intracellular water and partially frozen extracellular water results in a transport of water from the intracellular space to the extracellular milieu. This process can be modeled using irreversible thermodynamics as [2,4]

$$\frac{dV}{dT} = -\frac{L_p A_c R T}{B}[C_i - C_o] \tag{2}$$

with L_p, the sperm cell membrane permeability to water defined as [3]

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

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 cryoprotective agent (CPA), E_{Lp} or E_{Lp} [cpa] is 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 is the universal gas constant, B is the constant cooling rate (K/min), and finally C_i and C_o represent the concentrations of the intracellular and extracellular (unfrozen) solutions. 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 [5,8]. The various assumptions made in the development of Mazur's model of water transport are discussed in detail elsewhere and are beyond the scope of the present report [2–4].

Recently, Thirumala and Devireddy [9] 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

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

In this equation, L_{pg} and E_{Lp} represent the membrane permeability parameters (in μm/min-atm and kcal/mol, respectively), while the last term $\frac{A_c}{V_o - V_b}$ (in μm^{-1}) represents the ratio of the available surface area for water transport (A_c) to the initial volume of intracellular water ($V_o - V_b$). Based on the cell dimensions (Table 1) and assuming $V_b = 0.6\ V_o$, the ratio of $\frac{A_c}{V_o - V_b}$ is 16.375 μm^{-1} for WB sperm cells. Similarly, when the value of $V_b = 0.4\ V_o$ and $0.8\ V_o$, the ratio of $\frac{A_c}{V_o - V_b}$ is 10.92 and 32.75 μm^{-1}, respectively. Once the cell level parameters L_{pg} and E_{Lp} are determined using the curve-fitting procedure described elsewhere [5,8], we proposed to utilize Eq. (4) to predict the optimal rates of cooling WB sperm cells.

Fig. 1 shows the water transport data obtained using a cooling rate of 20 °C/min in the presence and absence of Me_2SO for WB spermatozoa. Water transport cessation was observed in the DSC heat release data as an overlap of the thermograms from the heat release signature obtained using osmotically active (initial) and inactive (final) cells. The dynamic portion of the cooling curve (where the thermograms were distinct) was found to be between −0.65 and −15 °C in the absence of CPAs, between −2.0 and −20 °C with 5% Me_2SO. The membrane permeability parameter values that best fit the 20 °C/min water transport data in the absence and presence of Me_2SO were calculated using a nonlinear curve-fitting technique (Table 2). By incorporating the best fit parameters of water transport in Eq. (4), the theoretically predicted values for optimal rate of cooling WB spermatozoa were obtained

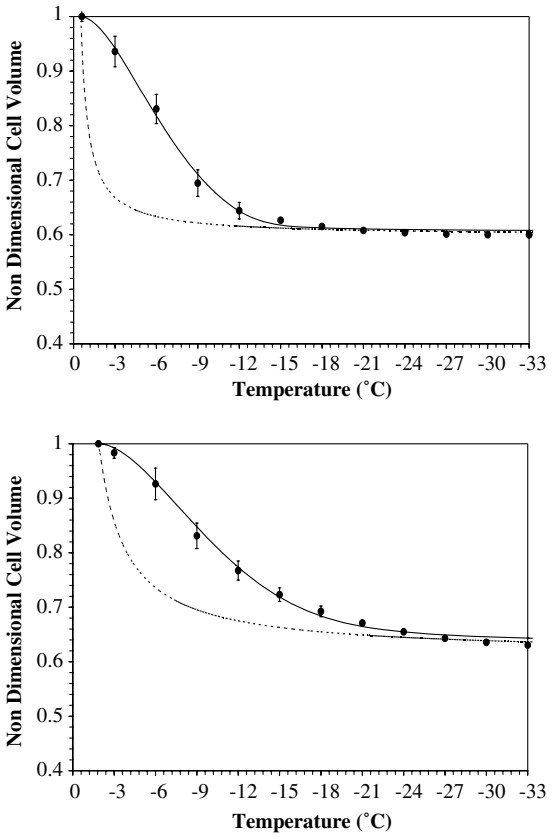

Fig. 1. Volumetric response of *Morone chrysops* (white bass, WB) sperm cells as a function of sub-zero temperatures obtained using the DSC technique in the presence of extracellular ice (top panel) and in the presence of extracellular ice and Me_2SO (bottom panel). The filled circles represent the experimentally 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 with $V_b = 0.6\ V_o$) in the water transport equation (Eqs. (2) and (3)). The model simulated equilibrium cooling response obtained is shown as a dotted line in all the figures. The non-dimensional cell volume is plotted along the y-axis and the sub-zero temperatures are shown along the x-axis ($n = 6$ separate DSC experiments).

(Table 2). The predicted optimal rates of cooling WB sperm from this analysis (~22 °C/min) are comparable with experimentally determined satisfactory rates (~16 °C/min), obtained in independent studies (1; unpublished results of Buchanan et al.).

To further delineate 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 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 ($\geqslant 0.98$). In Fig. 2, two distinctive contours are shown and correspond to the two media investigated (with and without Me_2SO). 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.

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 0.8 V_o to 0.4 V_o (i.e., a 33% variation in the assumed value of 0.6 V_o). The DSC data were correspondingly modified (using Eq. (1)) and the modified DSC water transport data were fitted to the water transport model (Eqs. (2) and (3)) by use of a non-linear least squares curve-fitting technique as previously described [5,8]. The parameters obtained with the new values of V_b are shown in Table 2. Note that the predicted values of the reference membrane permeability parameters (L_{pg}) with assumed values of 0.8 V_o or 0.4 V_o as the osmotically inactive cell volume were significantly different from the values obtained with an assumed osmotically inactive cell volume of 0.6 V_o. This variation in the value of L_{pg} is in direct contrast to the behavior of E_{Lp}, which remained essentially unchanged (±10%), when V_b was varied from 0.6 V_o to either 0.8 V_o or 0.4 V_o. However, the predicted rates of optimal cooling were essentially unchanged (±7%), when the assumed value of V_b was varied by ±33% (Table 2). Thus, errors in the assumed value of V_b did alter the model predicted membrane permeability parameters but the trends (and the predicted optimal rates of cooling) remained essentially unaltered. This insensitivity of the predicted optimal rate of cooling to the assumed value of V_b has also been previously noted in sperm cells of other species [5,8].

Although the exact mechanism by which Me_2SO alters water transport is as yet unknown, previous studies on the sperm cells of *X. maculatus* [5], an atypical teleost fish with internal fertilization, suggest 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. chrysops* (Table 2), a more typical teleost fish with external fertilization, where the addition of Me_2SO lowered the measured values of the reference membrane permeability and the activation energy by ~40%. Also the range of parameters that best fit the measured water transport data was much

Table 2
Predicted sub-zero membrane permeability parameters and the optimal rates of cooling for *M. chrysops* sperm cells in the presence of extracellular ice and Me_2SO, using cell dimensions shown in Table 1

Assumed inactive cell volume (V_b)	Freezing media	L_{pg} or $L_{pg}[cpa]$ (μm/min-atm)	E_{Lp} or $E_{Lp}[cpa]$ (kcal/mol)	B_{opt}[a] (°C/min)
0.6 V_o	No CPA	0.008	31.4	21.4
	with Me_2SO	0.004	20.6	22.0
0.4 V_o	No CPA	0.01	29.0	22.4
	with Me_2SO	0.007	21.3	22.0
0.8 V_o	No CPA	0.003	28.0	22.8
	with Me_2SO	0.002	20.0	21.6

Note that 1.0 μm/min-atm = 1.7×10^{-13} m^3/Ns and 1.0 kcal/mol = 4.18 kJ/mol. All values have a goodness of fit parameter of $R^2 > 0.995$.
[a] Obtained using Eq. (4).

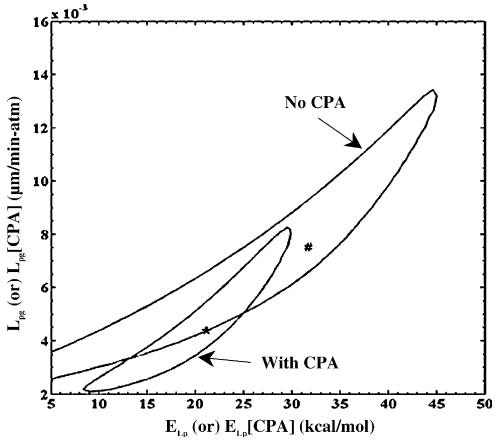

Fig. 2. Contour plots of the goodness of fit parameter R^2 (=0.98) for water transport response in WB sperm cells. The best fit parameters obtained in the absence of CPAs and in the presence 5% v/v Me_2SO are represented within the contours as "#" and "*", respectively (the parameter values are shown in Table 2 with $V_b = 0.6$ V_o). The membrane permeability at 0 °C, L_{pg} (or $L_{pg}[cpa]$) (μm/min-atm) is plotted on the *y*-axis while the apparent activation energy of the membrane, E_{Lp}(or $E_{Lp}[cpa]$) (kcal/mol) is plotted on the *x*-axis.

smaller in the presence of Me_2SO than in its absence (Fig. 2). Intriguingly, the theoretically predicted optimal rates of cooling WB sperm cells in the presence of Me_2SO were quite close (±5%) to those in its absence. Similar findings were obtained for striped bass (*M. saxatilis*) sperm cells (unpublished results of Thirumala et al.). Clearly, further studies are needed to further delineate the effects of Me_2SO and other CPAs on membrane transport and predicted rates of optimally cooling *M. chrysops* sperm cells.

In conclusion, the water transport (cellular dehydration) data for *M. chrysops* sperm cells in the presence of extracellular ice and Me_2SO during freezing was obtained using a DSC technique at a cooling rate of 20 °C/min. The measured water transport data in the presence and absence of Me_2SO 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 $L_{pg} = 0.51$ to 1.7×10^{-15} m^3/Ns (0.003–0.01 μm/min-atm), and $E_{Lp} =$ 83.6–131.3 kJ/mol (20.0–31.4 kcal/mol). The permeability parameters obtained in this study predict an optimal rate of cooling for *M. chrysops* sperm cells of ~22 °C/min, which compares closely with empirically determined satisfactory rates of cooling WB sperm cells (~16 °C/min). These values will be invaluable for current and future experiments optimizing cryopreservation of WB sperm, and we hope will serve as an alternative model to the empirical approach of determining optimal cooling rate for cryopreservation of sperm of aquatic species.

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