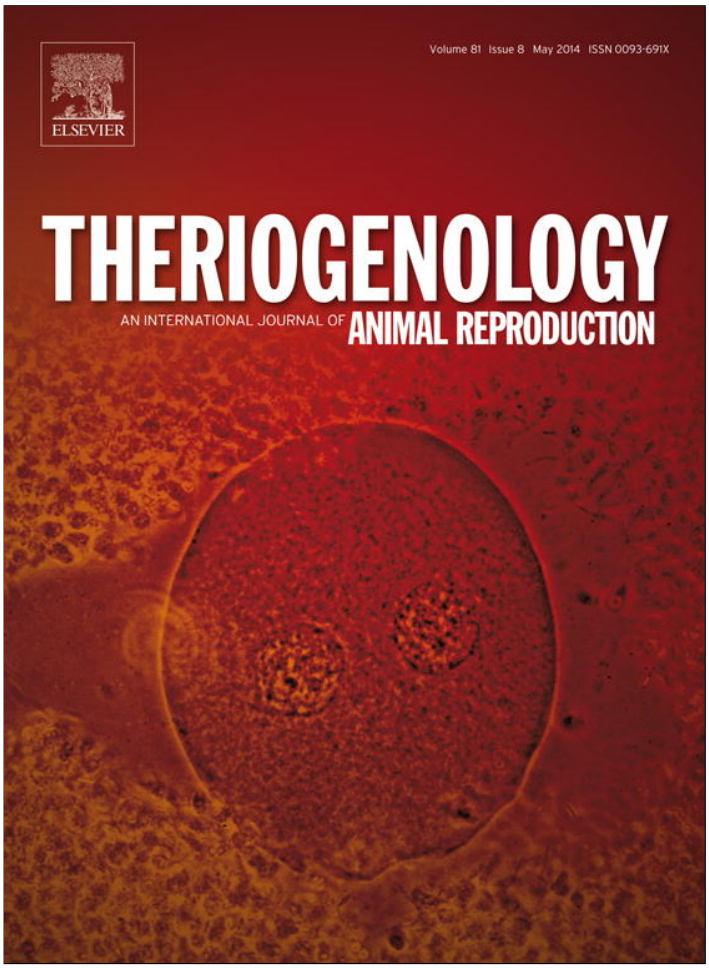

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Effects of frozen and liquid hypothermic storage and extender type on calcium homeostasis in relation to viability and ATP content in striped bass (*Morone saxatilis*) sperm

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ABSTRACT

The effect of hypothermic storage on striped bass sperm calcium homeostasis was determined by Fluo-3 flow cytometry. Calcium homeostasis was defined as the ability of cells to maintain a low concentration of intracellular free calcium as measured by Fluo-3 fluorescence. Sperm were stored frozen in striped bass extender (SBE) and Tris–NaCl medium (T350) modified with 50 mM glycine and 7.5% dimethylsulfoxide and in nonfrozen form diluted 1:3 (vol/vol) in SBE and T350 for 1, 24, and 48 hours at 4 °C in an oxygen atmosphere. Fluo-3 fluorescence was detected in less than 5% of fresh viable sperm cells indicating maintenance of calcium homeostasis. In contrast to sperm in fresh semen, frozen-thawed and nonfrozen sperm cells lost to a considerable extent the ability to maintain low intracellular free calcium even in the absence of exogenous calcium; positive Fluo-3 fluorescence was found in 26% and 39% of thawed sperm frozen in SBE- and T350-based freezing diluents, respectively, and increased ($P < 0.05$) to 67% during nonfrozen storage in SBE and T350 at 24 and 48 hours. Sperm viability measured by exclusion of propidium iodide by flow cytometry was 99% in fresh milt and maintained at 86% ($P > 0.05$) in SBE after 48 hours of nonfrozen storage but decreased ($P < 0.05$) to 55.7% after 48 hours in T350. Energy status in terms of ATP content, determined by luciferin–luciferase bioluminescence assay, was higher ($P < 0.05$) in sperm frozen in SBE than in T350 during the first 5 minutes post-thaw and decreased to essentially zero by 15 minutes post-thaw and did not differ among nonfrozen storage treatments. In conclusion, sperm cells impervious to propidium iodide after frozen or nonfrozen storage were unable to maintain low intracellular calcium content. SBE is a better medium than T350 for frozen or nonfrozen storage of striped bass sperm. The inability to regulate intracellular calcium in striped bass sperm may be associated with poor activation of motility after 4 °C storage and cryopreservation.

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1. Introduction

The hybrid striped bass aquaculture industry, an important economic component of the US finfish aquaculture industry [1], relies on the fertilization of white bass eggs with milt from the striped bass to produce the

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industry standard sunshine bass [2]. Significant efforts have previously been published to examine the effects of osmolality, cryoprotectant, and equilibration time on motility of striped bass sperm [3]; describe striped bass sperm ultrastructure, motility, and fertilization rate after cryopreservation [4]; evaluate the energetic status of striped bass sperm during storage and after activation [5]; and evaluate freezing rates to better optimize cryopreservation protocols for striped bass sperm [6].

In addition, striped bass sperm have been cryopreserved in buffered saline solutions at different cooling rates in the presence of dimethylsulfoxide [7–9] and stored in nonfrozen form for up to 48 hours [9]. However, the proportion of thawed sperm capable of motility activation was greatly reduced and the number of sperm required for *in vitro* fertilization was greatly increased after frozen storage [4].

Calcium homeostasis is important in cells to permit vital cell functions, and cells must expend energy to maintain a low level of cytosolic free calcium ($[Ca^{2+}]_i$) of 10^{-7} M or less compared with extracellular concentrations of approximately 10^{-3} M [10]. Calcium is known to play a primary role in the regulation of sperm cell function directly or as a second messenger by means of voltage- and stretch-activated Ca^{2+} channels for increasing $[Ca^{2+}]_i$ from external and intracellular storage sites in fish and other species [11,12]. Small fluctuations in $[Ca^{2+}]_i$ are involved with activation of sperm in many fish species [13,14]; however, no evidence was found for the role of Ca^{2+} in activation of freshly collected striped bass sperm [15]. Despite the absence of activation of striped bass sperm by calcium treatment [15], a subsequent study of striped bass sperm stored for 24 hours in an isotonic Tris–NaCl medium (T350) at 4 °C reported that a large influx of calcium was associated with reduced ability to activate sperm [16]. Another medium, striped bass extender (SBE), has been used for frozen [6,17] and nonfrozen storage [9] but has not been evaluated for their effects on calcium homeostasis. The effect of cryopreservation and the impact of different cooling rates on calcium homeostasis have not been reported for thawed striped bass spermatozoa using either T350 or SBE as cryodiluents.

This study was conducted to compare the ability of 2 media, T350 and SBE, to maintain sperm calcium homeostasis during nonfrozen storage and as freezing diluents for frozen storage. The capacity to maintain sperm calcium homeostasis was related to additional measures of sperm function, using markers of viability, impermeability to propidium iodide (PI), and energy status in terms of ATP content.

2. Materials and methods

2.1. Collection of spermatozoa

All reagents, unless otherwise stated, were purchased from Fisher Scientific International Inc. (Atlanta, GA). Mature, 5-year-old striped bass males were randomly selected from a population that was spawned in May 2004 from parents of Choptank River and Chesapeake Bay origin (MD, USA). They were grown to maturity over the next 4 to 5 years and were confirmed to be spermiating by the early spring of 2009 (experiment 1) and 2010 (experiment 2). The fish were maintained under controlled photothermal conditions in the 40,000-L tank in the laboratory at the University of Maryland's Crane Aquaculture Facility (located on the University of Maryland College Park campus at latitude: 38.9967 N and longitude: 76.9275 W) under approved Animal Care and Use Protocol R-07-35. Water temperature and photoperiod were computer controlled throughout the gametogenic cycles in 2009 to 2010 to mimic average conditions for the Maryland reach of the Chesapeake Bay. In the spring, experimental males were moved into a 6,400-L circular tank, part of the recirculating water system, and held at 15 ± 1 °C for the remainder of the 5-week study. Each fish was given a cholesterol cellulose implant containing 150 μg of mammalian gonadotropin-releasing hormone (Sigma Chemical Co., St Louis, MO) inserted into the dorsal lymphatic sinus to stimulate spermiogenesis [18].

Milt was recovered 3 or more days after administering the implant from live fish anesthetized in a 70 mg/L MS-222 (Finquel; Argent Chemical Laboratories, Redmond, WA, USA) bath, buffered to pH 8.0 with sodium bicarbonate, and urine was removed by applying gentle pressure around the urogenital vent [19]. Milt was expressed directly into 50-mL sterile conical tubes and placed immediately in a rack over ice. Sperm concentration was determined using a hemocytometer, and in these experiments, all the striped bass sperm samples exhibited 90% motility or more as indicted by microscopic examination. The mean and standard deviation of the sperm concentration from 13 males used in this study were 13.7×10^9 and 2.53×10^9sperm/mL, respectively.

2.2. Sperm cryopreservation procedure

Sperm ejaculates from each male were divided into two parts for extension 1:3 (v:v) using one freezing diluent composed of SBE containing 240 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, 5.5 mM glucose, and 75 mM glycine [4] and another composed of T-350 containing 20 mM Tris-free base (#1503, Sigma-Aldrich, St Louis, MO, USA), NaCl [5], and 75 mM glycine. Both media were adjusted to 550 mM with NaCl and adjusted to pH 7.6 with HCl. Then DMSO was added as a cryoprotectant to a final concentration of 7.5% (vol/vol) described previously [4,17]. Aliquots of 250 μL of milt extended in each freezing diluent were aspirated into 500-μL Cassou cryostraws (TS Scientific, Perkasie, PA) and heat sealed. The loaded cryostraws were equilibrated for 10 minutes over ice, cooled to 5 °C in a programmable N_2 vapor freezer (Planer Kryosave-Model KS30; Planer Products, Sunbury-on-Thames, England), and frozen at cooling rates of 10, 15, 20, 30, and 40 °C/min until the temperature reached −120 °C. On reaching −120 °C, the cryostraws were plunged into liquid nitrogen. For analysis, each cryostraw was thawed in a 37 °C water bath for 8 seconds and held over ice for the experiments.

2.3. Flow cytometric analysis of cytosolic free Ca^{2+} and viability

After hypothermic storage, sperm cells were loaded with the fluorescent dye Fluo-3 AM ester for 15 minutes to determine calcium homeostasis and loaded with PI as described previously [16] just before analysis as described in the appropriate experiments. Cells were analyzed on a Beckman-Coulter XL-MCL flow cytometer using an excitation of 488 nm. Data statistics including mean Fluo-3 fluorescence intensity per cell on a 4-cycle logarithmic channel number (CN) scale, the percent of viable cells with Fluo-3 fluorescence, and percent PI-negative cells were provided by the instrument's System II Software.

2.4. ATP analysis

The amount of sperm ATP was determined as described previously [16]. Briefly, a 100-μL aliquot of each sample containing 4×10^6 cells was collected after completion of the *in vitro* treatments and incubated over ice with 1 μL of a 100× phosphatase–ATPase inhibitor solution (#P5726; Sigma-Aldrich) for 30 minutes to inhibit ATP degradation and production. The samples were stored at −70 °C until boiled to release ATP, and a 25-μL aliquot representing 1×10^6 cells from each sample was quantified using a luciferin–luciferase assay adapted to measurement of bioluminescence on a SpectraFluor Plus plate reader (Tecan Group Ltd., Maennedorf, Switzerland). Data were recorded as pmoles of ATP/10^6 sperm.

2.5. Experiment 1: effects of cooling rate, freezing diluent, and incubation treatment on thawed sperm intracellular calcium, viability, and ATP

Milt samples from 13 males were frozen at 5 different cooling rates in SBE- and T350-based freezing diluents. For analysis of $[Ca^{2+}]_i$, aliquots of thawed were extended to 350×10^6 sperm cells/mL in 2 mL of T350 + 0.1% PVA and pH 8, incubated over ice (10 °C), and loaded with 1 μM Fluo-3 for 15 minutes. Without washing, Fluo-3–loaded sperm (85.7 μL) were removed to determine the level of calcium homeostasis (the ability of the cells to maintain low $[Ca^{2+}]_i$). Five aliquots of 30×10^6 sperm were assigned to five 30-minute incubation treatments at 4 °C consisting of #1: 1 mM EGTA and 0 mM $CaCl_2$, #2: 0 mM EGTA and 0 mM $CaCl_2$, #3: 0 mM EGTA and 0.01 mM $CaCl_2$, #4: 0 mM EGTA and 0.1 mM $CaCl_2$, and #5: 0 mM EGTA and 1.0 mM $CaCl_2$. After incubation, 2 μL of PI working solution was added to each sample for a final concentration of 9.6 μM to identify and exclude dead cells from the analysis.

The statistical design for Fluo-3 and viability measurements featured cooling rate, freezing diluent (SBE and T350), and EGTA/$CaCl_2$ treatment combinations as fixed effects in a $5 \times 2 \times 5$ factorial arrangement.

Aliquots of thawed milt frozen in SBE or T350 were analyzed for ATP content as described in section 2.4 as a marker for energy status at 0.5, 2.5, 5, and 15 minutes post-thaw. The statistical design for ATP measurements featured cooling rate and freezing diluent (SBE and T350) as fixed effects and post-thaw incubation time as a repeated measure in a $5 \times 2 \times 4$ factorial arrangement. The aliquots of sperm cells used for ATP measurements did not include the five 30-minute incubation treatments.

2.6. Experiment 2: sperm calcium homeostasis, viability, and ATP in nonfrozen stored milt

For analysis of $[Ca^{2+}]_i$, milt from 6 striped bass males was extended 1:3 (vol/vol) in SBE or T350 with osmolality adjusted to 350 mOsm/kg within 1 hour of collection and stored under an oxygen atmosphere at 4 °C as described previously [16]. Aliquots of diluted milt were removed at 0, 24, and 48 hours and incubated with 0 or 0.1 mM $CaCl_2$ for 30 minutes at 4 °C as a test for calcium homeostasis. Fluo-3 and PI were added for analysis of changes in Fluo-3 fluorescence and viability as described in section 2.3. The experimental design for Fluo-3 and viability measurements featured storage medium (SBE and T350) and $CaCl_2$ treatment as fixed effects and storage time as a repeated measure in a $2 \times 2 \times 3$ factorial arrangement.

Aliquots of milt were analyzed for sperm ATP content as described in section 2.6 after 0, 24, and 48 hours of storage. The statistical design for ATP measurements featured storage medium as a fixed effect and storage time as a repeated measure in a 2×3 factorial arrangement.

2.7. Statistical analysis

Data were analyzed using the mixed model analysis of variance procedure [20] in the Statistical Analysis System software, release 9.2 (SAS Institute Inc., Cary, NC) as described for each experiment. The model diagnostics included testing for a normal distribution of error residuals and homogeneity of treatment variance and selection of appropriate covariance structure for repeated measures analysis with fish as the subject in the repeated statement [20]. Compound symmetry, the simplest repeated measures structure, was used in the statistical analysis of sperm ATP content in experiment 1 and for Fluo-3 variables, viability, and ATP in experiment 2 because variance components, heterogeneous compound symmetry, and first-order autoregressive structures offered no improvement when model fitting statistics were compared. Comparison of least squares means was made by the LSMEANS statement using the DIFF option with a significance level of 0.05.

3. Results

3.1. Experiment 1: effects of freezing medium on thawed sperm characteristics

Significant sources of variation for the percent of live thawed sperm containing Fluo-3 were the main effects of freezing diluent, cooling rate, and post-thaw treatment ($P < 0.05$). The two-factor interactions of freezing diluent with post-thaw treatment and freezing diluent with cooling rate were also sources of variation ($P < 0.05$) and were the only significant sources of variation among the interactions, and their two-factor means are shown in Figures 1 and 2, respectively. The percent of control sperm

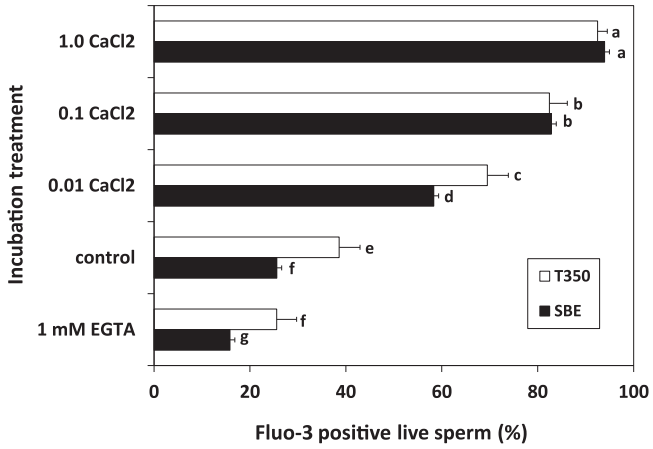

Fig. 1. Mean (±SEM) effects of five incubation treatments and two freezing diluents; SBE consisting of 240 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, and 5.5 mM glucose and T350 medium consisting of 20 mM Tris-base solution (20 mM Tris-base and $NaCl_2$) on percent Fluo-3–positive live thawed striped bass sperm (n = 13). Both media were adjusted to 350 mOsm/kg with NaCl and pH 8 with HCl and contained 40 mM glycine and 7.5% (vol/vol) dimethyl sulfoxide. Bar means without a common superscript (a–g) differed (P < 0.05). SBE, striped bass extender; T350, Tris–NaCl medium.

cells containing Fluo-3 after a 30-minute incubation after thawing was greater (P < 0.05) in T350-based freezing diluent than SBE, 23% and 38%, respectively (Fig. 1). The percentage of sperm containing Fluo-3 increased with increasing amounts of $CaCl_2$ present during the 30-minute treatment and the difference in the percent of cells containing Fluo-3 between sperm frozen in T350 and SBE disappeared in the presence 0.1 and 1.0 mM $CaCl_2$. Compared with the control treatment, the presence of 1 mM EGTA decreased (P < 0.05) the percent of sperm containing Fluo-3 fluorescence. Cooling rates had variable effects on the percentage of sperm cells containing Fluo-3 (Fig. 2). Compared with sperm cells frozen in the SBE-based freezing diluent, sperm cells frozen in the T350-

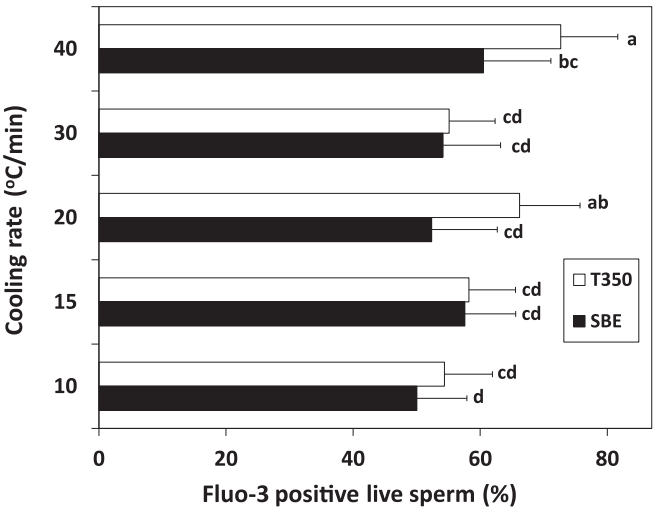

Fig. 2. Mean (±SEM) effects of five different cooling rates during freezing and two freezing diluents SBE and T350, as described in Figure 1, on percent Fluo-3–positive live thawed striped bass sperm (n = 13 fish). Bar means without a common superscript (a–d) differed (P < 0.05). SBE, striped bass extender; T350, Tris–NaCl medium.

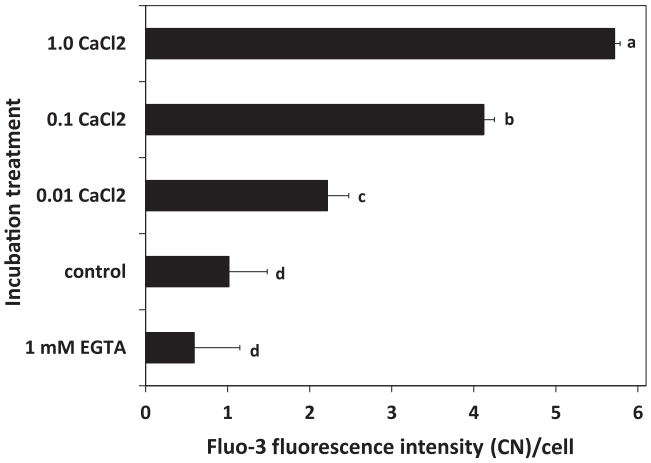

Fig. 3. Mean (±SEM) effects of five incubation treatments on Fluo-3 fluorescence intensity, CN per cell, in live thawed striped bass sperm (n = 13 fish). Bar means without a common superscript (a–d) differed (P < 0.05). CN, channel number; SBE, striped bass extender; T350, Tris–NaCl medium.

based freezing diluent contained a greater (P < 0.05) percent of sperm containing Fluo-3 at cooling rates of 40 and 20 °C/min but not at cooling rates at 30, 15, and 10 oC/min.

Significant sources of variation for Fluo-3 fluorescence intensity per cell included the main effects cooling rate and post-thaw treatment (P < 0.05) but not the freezing diluent main effect. The two-factor interaction of freezing diluent with cooling rate was the only significant source of variation among the interactions (P < 0.05). The post-thaw treatment means for Fluo-3 fluorescence intensity for the five incubation treatments are shown in Figure 3, and the two-factor means for the 10 freezing diluent and cooling rate combinations are shown in Figure 4. The mean Fluo-3 fluorescence intensity for control thawed sperm cells containing Fluo-3 after 30-minute incubation was 1 CN and increased progressively (P < 0.05) with increasing amounts of calcium (Fig. 3). Sperm cell Fluo-3 fluorescence intensity was greater (P < 0.05) in sperm frozen in T350-based freezing diluent than in SBE-based freezing diluent but only at a cooling rate of 40 °C (Fig. 4).

Sperm viability expressed as percent PI-negative sperm was increased (P < 0.05) by cooling rate from 72.1% to 84.1% between 10 and 40 °C/min (data not shown). Sperm viability was not affected (P > 0.05) by freezing diluent, by *in vitro* treatment designed to increase calcium exposure of thawed sperm or by interactions among main effects (data not shown).

Significant sources of variation for sperm ATP content included the main effects of freezing diluent and post-thaw incubation time (P < 0.05) but not the cooling rate main effect. The two-factor interaction of freezing diluent with post-thaw incubation time was the only significant source of variation among the interactions (P < 0.05). The effects of freezing diluent on mean sperm ATP content during the 15-minute interval after thawing are shown in Figure 5. Sperm ATP content decreased (P < 0.05) rapidly after thawing in both the SBE- and T350-based freezing diluents, but sperm frozen in SBE-based freezing diluent contained threefold more (P < 0.05) ATP than T350-based

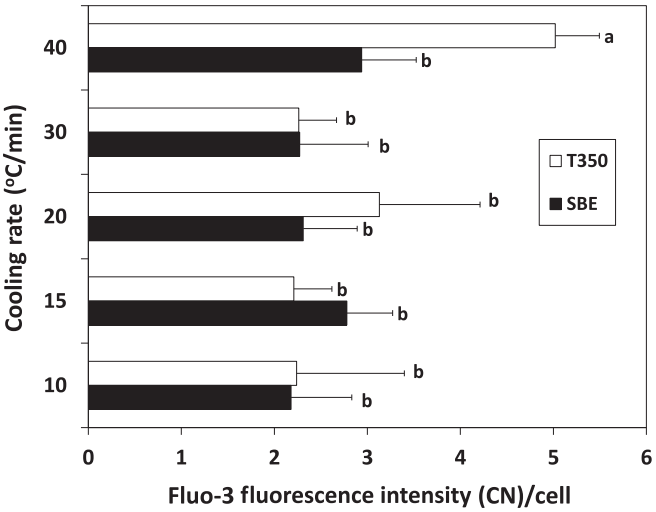

Fig. 4. Mean (±SEM) effects of five different cooling rates during freezing and two freezing diluents SBE and T350, as described in Figure 1, on Fluo-3 fluorescence intensity, CN per cell, in live thawed striped bass sperm (n = 13). Bar means without a common superscript (a–b) differed (P < 0.05). CN, channel number; SBE, striped bass extender; T350, Tris–NaCl medium.

freezing diluent when first measured at 30 seconds post-thaw. Sperm ATP was essentially depleted by 15 minutes post-thaw.

3.2. Experiment 2: sperm calcium homeostasis and viability in nonfrozen stored milt

The effects of storage time and storage medium on percent viable Fluo-3 fluorescent sperm cells, Fluo-3 fluorescence intensity, and viability are shown in Table 1. The only significant source of variation for the percent of live nonfrozen sperm containing Fluo-3 and their Fluo-3

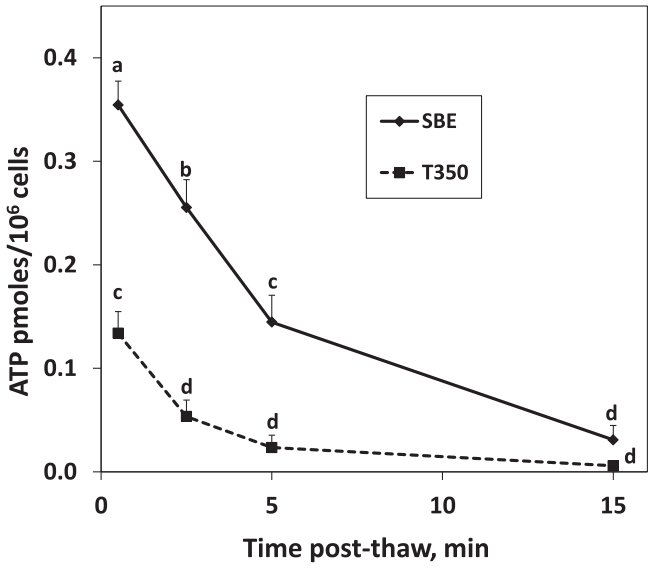

Fig. 5. Mean (±SEM) effects of two freezing diluents and time post-thaw on ATP content inthawed striped bass sperm (n = 13 fish) frozen in SBE or T350, as described in Figure 1. Line means without a common superscript (a–d) differed (P < 0.05). SBE, striped bass extender; T350, Tris–NaCl medium.

fluorescence intensity was the main effect of storage time (P < 0.05). The main effects of storage medium and 0.1 mM $CaCl_2$ treatment and the two- and three-factor interactions were not significant sources of variation. The mean percent of freshly collected sperm containing detectable Fluo-3 was less than 5% with mean fluorescence intensity of 0.22 CN. During the first 24 hours of storage, the percent of Fluo-3 fluorescent sperm increased to 67% and Fluo-3 fluorescence intensity increased to a mean of 1.83 CN and did not change significantly during the second 24-hour period.

Significant sources of variation for sperm viability were the main effects of storage medium and storage time (P < 0.05). The two-factor interaction of storage medium with storage time was the only significant source of variation among the interactions (P < 0.05). Initially, sperm cells were viable with all cells in both media essentially excluding PI (Table 1). Sperm held in SBE maintained viability well with a nonsignificant decrease to 86.6% at 48 hours. In contrast, viability of sperm held in T350 decreased progressively to 77.9% at 24 hours and 55.7% at 48 hours. Incubation with $CaCl_2$ after storage had no significant effect on percent viable sperm (P > 0.05).

Neither the main effects of storage time or storage medium or their interaction were significant sources of variation for sperm ATP content (P > 0.05). The sperm ATP content means were 1.18, 0.83, and 1.25 pmoles/10^6 cells (SEM ± 0.21) at 0, 24, and 48 hours of storage.

4. Discussion

Calcium is a universal signal transduction element in somatic cells modulating cell growth and differentiation [21] and is a major regulator of sperm function [22,23]. Normally, a 10^3- to 10^4-fold concentration gradient exists for Ca^{2+} between the intracellular and extracellular environments of sperm cells [24]. Low $[Ca^{2+}]_i$ is maintained to permit cell signaling by Ca^{2+} influx required to initiate physiological activities such as motility, hyperactivity, capacitation, acrosome reaction, and fertilization. In sperm of mammalian species and sea urchins, calcium signaling involves a specialized set of channels, transporters, and intracellular storage sites [13,22,23,25]. Although calcium channels, transporters, and intracellular storage sites have not been identified in teleost fish sperm, Ca^{2+} treatment has been shown to play an important role in the activation of motility in sperm cells of many fish species [11,14,26–28].

Our study indicated that calcium homeostasis was maintained in more than 95% of freshly collected striped bass sperm cells as indicated by Fluo-3 fluorescence found in less than 5% of live cells (experiment 2). These results were similar to those we reported previously [16] and are in agreement with data collected from boar and turkey sperm cells (H.D. Guthrie and G.R. Welch, unpublished data). In addition, 0.1 mM $CaCl_2$ was not capable of penetrating the plasma membrane in fresh striped bass sperm cells to increase Fluo-3 fluorescence compared with no $CaCl_2$ during a 30-min incubation period in this study and in one conducted previously [16]. In contrast,

Table 1
Mean (±SEM) effects of the two treatment factors storage time and two different storage extenders, SBE and T350, on intracellular free calcium content measured by Fluo-3 fluorescence and on plasma membrane integrity measured by exclusion of PI in striped bass sperm after a 30-minute incubation in experiment 2 (n = 6 fish).

Storage time, h	Storage medium	Fluo-3–positive cells, %	Fluo-3 fluorescence intensity, CN	PI-negative cells, %
0	T350	4.2 + 1.2[a]	0.22 + 0.02[a]	99.2 + 0.2[a]
	SBE	3.4 + 1.2[b]	0.22 + 0.02[a]	99.2 + 0.3[a]
24	T350	70.8 + 1.9[b]	1.99 + 0.25[b]	77.9 + 6.6[b]
	SBE	62.9 + 7.1[b]	1.70 + 0.25[b]	96.2 + 0.8[a]
48	T350	61.7 + 8.8[b]	1.67 + 0.36[b]	55.7 + 11.2[c]
	SBE	73.2 + 4.3[b]	2.30 + 0.25[b]	86.6 + 3.0[ab]

Within a column, means without a common superscript letter (a–c) differed ($P < 0.05$).
Abbreviations: PI, propidium iodide; SBE, striped bass extender; T350, Tris–NaCl medium.

we found that calcium homeostasis in viable populations of striped bass sperm were markedly disrupted in thawed sperm and in sperm stored in nonfrozen form at 4 °C for 24 and 48 hours. After 24 hours of storage, even in the absence of added $CaCl_2$, approximately 66% of the viable cells became Fluo-3 fluorescent, and Fluo-3 fluorescence intensity in these cells was increased at least eightfold compared with freshly collected sperm cells. Disrupted calcium homeostasis in thawed sperm was also demonstrated; the percent of viable sperm cells containing Fluo-3 was 23% and 38% in SBE- and T350-based freezing diluents, respectively, and Fluo-3 fluorescence intensity was fivefold greater in thawed sperm compared with freshly collected sperm cells.

In this study, two different media were used as freezing diluents, and freezing was conducted at five different cooling rates. Of the cooling rates that have been evaluated for cryopreservation of striped bass sperm, a cooling rate at 40 °C/min has been established as best to maintain sperm cell viability, motility, and ATP content after thawing [6]. With regard to calcium homeostasis, the cooling rate effect on calcium homeostasis was minimal using SBE-based freezing diluent in terms of both the percent of Fluo-3–positive cells or Fluo-3 fluorescence intensity. The SBE-based freezing diluent was marginally better than T350-based freezing diluent at maintaining a lower level of Fluo-3 fluorescence in thawed sperm cells, but neither freezing diluent could adequately protect sperm cells from calcium influx in the absence or presence of exogenous calcium.

Although SBE- and T350-based storage or freezing diluents were not greatly different or particularly beneficial for maintenance of calcium homeostasis, for other characteristics, the utilization of SBE was superior to T350. Sperm viability in nonfrozen storage for 48 hours in SBE was maintained at 86%. In contrast, viability in milt stored for 48 hours in T350 decreased to 55.7%. For cryopreservation of striped bass sperm, SBE was superior to T350 for initially maintaining ATP in thawed sperm. The primary metabolic problem in frozen-thawed striped bass sperm compared with nonfrozen storage may be the loss the ability to maintain mitochondrial inner transmembrane potential (Guthrie and Woods, unpublished) and ATP production in thawed sperm as shown in this study which could explain the more rapid loss of motility found in thawed than fresh milt [4].

Evidence of storage-induced disruption of calcium homeostasis, increased $[Ca^{2+}]_i$, has been reported during nonfrozen storage of bovine sperm [29,30] or after freeze thawing [31–33]. In general, the source of increased $[Ca^{2+}]_i$ during storage may be intracellular, from mitochondria [23] or from the excess nuclear envelope [21], or extracellular from seminal plasma.

The intact plasma membrane in stored sperm, judging by the PI exclusion criterion, could not provide a barrier to exogenous $CaCl_2$. The increased Ca^{2+} internalization has been suggested to be a result of cooling-induced plasma membrane reorganization leading to nonspecific bilayer faults [34,35] that permit influx of Ca^{2+}. Alternatively, changes in the physiochemical properties of the membrane lipid environment and cooling [36,37] may prevent extrusion of Ca^{2+} from sperm by impairing the function of the plasma membrane Ca^{2+} ATPase or the Na^{+}–Ca^{2+} exchanger enzymes present in sperm cells [22,38].

In conclusion, we have shown that the two media used for nonfrozen and modified for frozen storage did not prevent an apparent loss of calcium homeostasis in striped bass sperm. The loss of calcium homeostasis may be linked to a reduced ability to activate motility in sperm after nonfrozen storage at 4 °C or cryopreservation. Our results may indicate that calcium pumps are operating at suboptimal levels or unable to compensate for increased calcium entry into the cytoplasm. Future work to determine causation or protective measures will require analysis of the effect on nonfrozen storage and cryopreservation on the activities of Ca^{2+} channel or pore proteins *in situ* or *in vitro*.

Acknowledgments

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