# Effects of hypothermic storage on intracellular calcium, reactive oxygen species formation, mitochondrial function, motility, and plasma membrane integrity in striped bass (*Morone saxatilis*) sperm

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## Abstract

Experiments were conducted to determine the effect of hypothermic 24 h storage on striped bass sperm cell plasma membrane integrity, free intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$), mitochondrial membrane potential ($\Delta\Psi_m$), and reactive oxygen species (ROS) formation (oxidation of hydroethidine to ethidium) as determined by flow cytometry; motion activation and ATP concentration as determined by Luciferin-Luciferase bioluminescence assay. Semen was stored for 1 or 24 h at 4 °C in an $O_2$ atmosphere undiluted or diluted (one volume semen with 3 volumes diluent) with T350 (20 mM TRIS base-NaCl, 350 mOsm/mL, pH 8) or with seminal plasma in the presence of various treatments. Viability (% cells excluding propidium iodide) approached 100% after 1 h storage in undiluted or diluted semen. After 1 h of storage the $[Ca^{2+}]_i$ marker, Fluo-3, was detected in only 3% of sperm cells in undiluted or diluted semen. In contrast to storage for 1 h, after 24 h the incidence Fluo-3 fluorescence intensity was increased (P < 0.05) in > 50% of the viable cells in undiluted and diluted semen along with increased cell death; the presence of 1 mM ethylene glycol tetraacetic acid (EGTA) blocked $CaCl_2$-induced Fluo-3 fluorescence and cell death. Activation of sperm motility was 82% after 1 h in T350 and decreased (P < 0.05) to 30% after 24 h. However, motility activation failed in the presence of EGTA at 1 or 24 h. During storage $\Delta\Psi_m$ was not affected by storage time or treatment. In contrast, sperm ATP was greater (P < 0.05) at 1 h than at 24 h and was greater in sperm stored in diluted than undiluted form. While ROS formation was induced by menadione treatment, there was no evidence of storage-induced ROS formation in the absence of menadione. The increased $[Ca^{2+}]_i$ found after 24 h indicates a storage induced defect in the maintenance of cellular calcium homeostasis which may be detrimental to sperm activation.
Published by Elsevier Inc.

*Keywords:* Flow cytometry; Fluo-3; JC-1; Sperm viability

## 1. Introduction

Aquaculture of the Moronidae family of fish uses hybrid crosses. For example hybrid Sunshine Bass are produced by *in vitro* fertilization of eggs from white bass females (*Morone chrysops*) using semen from striped bass males (*Morone saxatilis*). Semen is collected from wild striped bass males during spring spawning or from captive males hormonally induced to spermiate during the spawning season of white bass females [1]. The hybrid striped bass market has leveled off at 12 million lbs of fish/year since 2000 [2] and further progress in this industry requires a more stable source of semen and eggs. The use of striped bass semen is complicated by the lack of a good method of

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storage of the semen before use. No adequate procedures or media exist for overnight shipment from the site of semen collection to a laboratory [3] for *in vitro* fertilization or cryopreservation for later use.

Calcium is known to play a primary role in the regulation of sperm cell behavior by means of voltage- and stretch-activated $Ca^{2+}$ channels for increasing free intracellular $Ca^{2+}$ $[Ca^{2+}]_i$ from external and intracellular storage sites in fish and other species directly or as a second messenger [4,5]. However, we were not able to demonstrate a role of $Ca^{2+}$ for induction of motility in striped bass sperm [3] and to our knowledge no studies have been conducted to investigate calcium homeostasis in sperm of this species. Small fluctuations in $[Ca^{2+}]_i$ are involved with activation of sperm in other fish species [6,7,8] and play a role in mammalian sperm motility, capacitation, and the acrosome reaction [5,9]. Calcium homeostasis is important in cells to permit vital cell functions and cells must expend energy to maintain a low level of $[Ca^{2+}]_i$, $\leq 10^{-7}$ M compared to extracellular concentrations of $\sim 10^{-3}$ M [10]. Preliminary data in boar sperm indicate that the $[Ca^{2+}]_i$ must be regulated carefully because treatments resulting in high $[Ca^{2+}]_i$ not only terminated boar sperm motility, but also increased sperm cell death (Guthrie and Welch, unpublished).

Studies of turkey spermatozoa have shown that storage of oxygenated sperm at 4 °C for 24 h results in significant membrane lipid peroxidation associated with a reduction in fertility [11]. Because striped bass semen is stored under similar conditions it is possible that reduced motility in striped bass sperm might be associated with reactive oxygen species (ROS) formation.

The purpose of the current study was to determine the effects of hypothermic liquid storage on cellular regulation of $[Ca^{2+}]_i$, ROS formation, survival, motility activation, and ATP in striped bass sperm after 24 h of storage in a Tris-NaCl medium.

## 2. Materials and methods

### 2.1. Collection of spermatozoa and seminal plasma

Mature, 4-year-old striped bass males were randomly selected from a population maintained under controlled photothermal conditions in the 40,000-L tank wet lab at the University of Maryland's Crane Aquaculture Facility [12]. Water temperature and photoperiod were computer-controlled throughout the gametogenic cycles (Experiments 1–3 conducted in 2008 and Experiments 4–5 conducted in 2009) 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 $15 \pm 1$ °C for the remainder of the 5-week study. Each fish was given a cholesterol cellulose implant containing 150 $\mu$g of mammalian gonadotropin-releasing hormone (Sigma Chemical Co., St. Louis, MO) inserted into the dorsal lymphatic sinus to stimulate spermiogenesis, as previously described for striped bass [13].

Semen was recovered from the testes of fish euthanized in a 200 mg/L bath of tricaine methanesulfonate (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA) buffered with sodium bicarbonate as described previously [14]. The medium used for dilution was 20 mM Tris free base (#1503, Sigma-Aldrich, St. Louis, MO, USA) adjusted to 350 mOsm/kg with NaCl and adjusted to pH 8 with HCl (T350). Seminal plasma was isolated from semen collected from live fish to use as a diluent in one experiment by centrifugation of semen, at 10,000 g for 5 min at 4 °C, with a Heraeus Labofuge 400R refrigerated centrifuge (Thermo Scientific, Asheville, NC). The experiments were conducted under an approved Institutional Animal Care and Use Committee Experimental Protocol (R-07-35).

### 2.2. Activation and measurement of motility

Sperm density and the percentage of motile sperm were determined after diluting approximately 0.01 $\mu$L of semen with 10 $\mu$L of 20 mM Tris base, 50 mOsm/kg pH 8, in a Makler Sperm Counting Chamber (Sefi Medical Instruments, Haifa, Israel). The activity in the loaded Makler chamber was recorded at 400x on a Magnavox Model ZC320MW8 digital recorder (Wal-Mart, Laurel, MD) with a Hitachi Model KP-D20BU, high-contrast color digital camera (Hitachi Inc., Tokyo, Japan) attached to a Zeiss Model D-7082 phase-contrast microscope (Carl Zeiss Inc., Berlin, Germany) [3]. The percentage of motile sperm was determined by counting spermatozoa (approximately 200–800 cells) and then dividing the number of motile sperm by the total number of sperm cells in the field of view. Only semen samples having > 90% motility were used in these experiments. Motility after storage was determined in Experiment 4 (section 3.4).

### 2.3. Flow cytometric analysis of intracellular free $Ca^{2+}$

The relative amounts of $[Ca^{2+}]_i$ were measured in sperm cells loaded with the plasma membrane permeant ester form of the $Ca^{2+}$ probe Fluo-3 AM (Invitrogen F14218) using procedures similar to those

for boar sperm [15]. Intracellularly Fluo-3 AM is hydrolyzed by cytosolic esterases to its fluorescent, $Ca^{2+}$ sensitive form. The Fluo-3 AM stock solution was received at 1 mM in dimethyl sulfoxide (DMSO) and was stored at −20 °C. For each analysis a Fluo-3 working solution was prepared by mixing the stock solution with an equal volume of 8% Pluronic to a final concentration 0.5 mM Fluo-3 and 4% Pluronic. Pluronic F-127 was received as 20% solution in DMSO (Invitrogen P300MP) and the Pluronic working solution (8%) was prepared using 1 volume of stock and 1.5 volume of DMSO. Staining of sperm was conducted in $12 \times 75$ mm polypropylene tubes held aerobically in wire racks over ice (mean temperature 10 °C). Sperm were first loaded with 2 $\mu$L of the Fluo-3 working solution for a final concentration of 1 $\mu$M Fluo-3 for 15 min. Before analysis 2 $\mu$L of PI working solution (Invitrogen, P-4170) was added to each sample for a final concentration of 4.8 $\mu$M to identify and exclude dead cells from the analysis. Cells were analyzed on a Beckman-Coulter XL-MCL flow cytometer using 488 nm excitation from a 15 mW air cooled argon laser with the FL1 and FL3 detectors receiving green fluorescence emission from Fluo-3 and red fluorescence emission from PI. Ten thousand sperm events were selected by light scatter to exclude doublets and debris. Subsequently those events negative for PI fluorescence were analyzed for their Fluo-3 intensity. Data including the percent of viable cells positive for Fluo-3, their mean Fluo-3 fluorescence intensity/cell on a channel number (CN) on a 4 cycle logarithmic scale, and percent viable sperm (PI negative cells) were provided by the instrument's System II Software.

## 2.4. Flow cytometric analysis of high mitochondrial transmembrane potential ($\Delta\Psi_m$)

Energy status of sperm mitochondria was determined in sperm cells loaded with the mitochondrial probe 5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (T-3168, Molecular Probes Inc., Eugene, OR) as described previously [14]. In brief, $30 \times 10^6$ sperm/mL in $12 \times 75$ mm polypropylene assay tubes were suspended in 1 mL T350 containing 4 $\mu$L of PI (final concentration 9.6 $\mu$M) and 0.5 $\mu$L of JC-1 working solution (final concentration 0.5 $\mu$M) for 30 min. The cells and reagents were incubated for 30 min over ice prior to flow cytometric analysis. Sperm cells with energized mitochondria (high $\Delta\Psi_m > 80$ to 100 mV) emitted red-orange fluorescence from JC-1 aggregates ($J_{agg}$). The percent of cells with $J_{agg}$ fluorescence and their mean $J_{agg}$

fluorescence intensity/cell on a CN scale were provided by the Beckman-Coulter XL-MCL System II Software.

## 2.5. ATP analysis

The amount of sperm ATP was determined as described previously [14]. Briefly, a 100 $\mu$L aliquot of each sample containing $12 \times 10^6$ cells was collected after completion of the *in vitro* treatments and incubated over ice with 1 $\mu$L of a 100$\times$ phosphatase-ATPase inhibitor solution (#P5728, Sigma-Aldrich, St. Louis, MO, USA) for 30 min to inhibit ATP degradation and production. The samples were stored at −70 °C until boiled to release ATP and a 25 $\mu$L aliquot representing $3 \times 10^6$ 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.

## 2.6. Flow cytometric analysis of ROS formation

Reactive oxygen species derived from storage or menadione-induced superoxide (SO) formation were measured by oxidation of hydroethidine to ethidium in viable sperm cells (those excluding Yo Pro-1) modified from a flow cytometric procedure for boar sperm [16]. One mL aliquots of $30 \times 10^6$ cells were removed after incubation treatment and were stained for 30 min over ice with hydroethidine staining solution (20 mM working solution, D-1168, Molecular Probes) in DMSO (final concentration 10 $\mu$M) and with YoPro-1 working solution (0.1 mM, Y-3603, Molecular Probes Inc.) in DMSO (final concentration 0.025 $\mu$M). Data were recorded as mean ethidium fluorescence intensity/cell on a CN scale and percent viable cells from flow cytometer provided by the Beckman-Coulter XL-MCL System II Software.

## 2.7. Experiment 1. Effects of $CaCl_2$ and calcium ionophore A23187 on $[Ca^{2+}]_i$ and $\Delta\Psi_m$

Freshly recovered semen samples (n = 6) were extended to $350 \times 10^6$ sperm cells/mL in 2 mL of T350. Sperm cells were loaded with Fluo-3 and treated with PI as described in section 2.3. Aliquots of $30 \times 10^6$ Fluo-3 loaded sperm (85.7 $\mu$L) were removed without washing, and then assigned to eight different treatment combinations consisting of 0, 0.01, 0.1 and 1 mM $CaCl_2$ with or without 1 $\mu$M A23187 for 30 min and then analyzed by flow cytometry as described in section 2.3.

Aliquots of semen were assigned to the same treatment combinations of $CaCl_2$ and A23187 listed above and incubated aerobically over ice for 30 min. The

semen samples were then treated with JC-1 and analyzed by flow cytometry for $J_{agg}$ fluorescence as described in section 2.4. The statistical design for the Fluo-3 and $J_{agg}$ measurements featured the $CaCl_2$ and A23187 treatment combinations in a $4 \times 2$ factorial arrangement as fixed effects.

## 2.8. Experiment 2. Effects of $CaCl_2$, A23187, and ethylene glycol tetraacetic acid (EGTA) on $[Ca^{2+}]_i$ and viability

Freshly recovered semen samples (n = 3) were extended to $350 \times 10^6$ sperm cells/ml in 2 ml of T350 and assigned to six different treatment combinations consisting of $CaCl_2$, A32187, and EGTA, respectively: (0, 0, and 0; 0, 0, and 1; 1, 0, and 0; 1, 0, and 1; 1, 1, and 0; and 1, 1, and 1 mM). Samples were incubated aerobically over ice and aliquots were removed at 10, 30, and 60 min for addition of Fluo-3 and PI as described in section 2.3. The statistical design was a $6 \times 3$ factorial arrangement of the six treatment combinations as fixed effects and the three incubation times as a repeated measure.

## 2.9. Experiment 3. Effect of 24 h storage on $[Ca^{2+}]_i$ survival, and $\Delta\Psi_m$

Freshly recovered semen samples (n = 3) were assigned to five different storage treatments: undiluted, diluted 1:4 (one volume semen with 3 volumes diluent) with seminal plasma (with or without EGTA 1 mM), and diluted 1:4 with T350 + 0.1% PVA (with or without EGTA 1 mM). We included seminal plasma as a diluent treatment because the functionality of spermatozoa can be compromised by manipulation, such as cryopreservation, liquid hypothermic storage, or high dilution which remove seminal plasma components from spermatozoa [17,18,19] and these deleterious effects can be counteracted by the addition of variable amounts of seminal plasma. Samples were stored at 4 °C in an $O_2$ atmosphere [20] and aliquots were removed at 1 and 24 h for staining with Fluo-3 and PI for analysis of $[Ca^{2+}]_i$ as described in section 2.3 and for staining with JC-1 and PI for analysis of $\Delta\Psi_m$ in viable cells as described in section 2.4. The statistical design was a $5 \times 2$ factorial arrangement of storage treatments as a fixed effect and storage time as a repeated measure.

## 2.10. Experiment 4. Effect of 24 h storage on $[Ca^{2+}]_i$, survival, motility activation, and ATP

This experiment was conducted to confirm the effects of 24 h storage on calcium homeostasis discovered in Experiment 3 (section 2.9) of 2008, and to determine the extent of motility activation and formation of ATP. Aliquots of semen (n = 6) were assigned to receive one of four different storage treatments: undiluted, diluted 1:4 in T350 + 0.1% PVA containing no additive, 1 mM $CaCl_2$, or 1 mM EGTA. Samples were stored at 4 °C in an $O_2$ atmosphere and aliquots were removed at 1 and 24 h for staining and analysis of Fluo-3 and PI as described in section 2.3. The statistical design was a $4 \times 2$ factorial arrangement for storage treatment as a fixed effect and storage time as a repeated measure.

Aliquots of semen (n = 2) were assigned to three storage treatments. Undiluted, T350 diluted, and T350 diluted semen containing 1 mM EGTA were tested for sperm motion activation as described in section 2.2 after 1, 3, and 24 h of storage. Data from T350 diluted semen containing 1 mM EGTA were excluded from the statistical analysis because no sperm were activated. The statistical design was a $2 \times 3$ factorial arrangement of storage treatment as a fixed effect and storage time as a repeated measure.

Aliquots of semen (n = 3) were assigned to three storage treatments, undiluted and T350 diluted with and without 1 mM EGTA, and were removed at 1 and 24 h for sperm ATP analysis as described in section 2.5. The statistical design was a $3 \times 2$ factorial arrangement of storage treatment as a fixed effect and storage time as a repeated measure.

## 2.11. Experiment 5. Effect of 24 h storage and menadione on ROS formation

To evaluate the impact of ROS formation on striped bass sperm function, storage- and menadione-induced mitochondrial ROS formation was determined in the absence and presence of two different superoxide dismutase (SOD) mimetics: the SO scavengers manganese [III] tetrakis [4-benzoic acid] porphyrin (TBAP) #475870 and Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin, tetratosylate, hydroxide (TMP) #475872 (Calbiochem, San Diego, CA). Semen was collected from six different males, stored in samples containing $350 \times 10^6$ cells/mL at 4 °C in an $O_2$ atmosphere, and aliquots from each sample were assigned to four different storage treatments: diluted 1:4 in T350 + 0.1% PVA containing no additive, 1 mM EGTA, 100 $\mu$M TBAP, or 100 $\mu$M TMP. One mL aliquots were removed after 1 and 24 h of storage treatment and transferred into $12 \times 75$ mm polypropylene tubes held over ice and received 0 or 1 mM menadione (M5625, Sigma Chemical Co.) in 10 $\mu$L of DMSO, from a 20 mM stock solution dissolved in dimethyl sulfoxide. Following 60

Table 1
Mean (± SEM) effects of $CaCl_2$ and calcium ionophore A23187 on intracellular free calcium indicator fluorescence in striped bass sperm following a 30 min incubation (n= 3 fish).

| $CaCl_2$, mM | A23187, $\mu$M | Fluo-3 positive cells, % | Fluo-3 fluorescence intensity, CN |
|---|---|---|---|
| 0 | 0 | $1.4 \pm 0.3^a$ | $0.2 \pm 0.01^a$ |
|  | 1 | $5.1 \pm 2.7^b$ | $0.2 \pm 0.03^a$ |
| 0.01 | 0 | $1.3 \pm 0.4^a$ | $0.2 \pm 0.01^a$ |
|  | 1 | $5.0 \pm 2.6^b$ | $0.2 \pm 0.03^a$ |
| 0.1 | 0 | $1.4 \pm 0.3^a$ | $0.2 \pm 0.01^a$ |
|  | 1 | $4.9 \pm 2.7^b$ | $0.2 \pm 0.01^a$ |
| 1 | 0 | $75.9 \pm 1.0^c$ | $2.7 \pm 0.03^b$ |
|  | 1 | $80.9 \pm 4.1^c$ | $3.1 \pm 0.25^b$ |

Within a column, means without a common superscript (a–c) differed (P < 0.05).

min treatment the sperm were analyzed for ROS formation as described in section 2.6. The statistical design was a $4 \times 2 \times 2$ arrangement of storage treatment and menadione treatment as fixed effects and storage time as a repeated measure.

### 2.12. Statistical analysis

Data were analyzed using the mixed model analysis of variance procedure [21] 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 for homogeneity of treatment variance [21]. Comparison of least squares means was made by the LSMEANS statement using the DIFF option with a significance level of 0.05. Fluo-3 fluorescence intensity, CN, was transformed to $\log_{10}$ for statistical analysis because of positive correlation between means and variances. Data means were presented in values from the original scale.

## 3. Results

### 3.1. Experiment 1. Effects of $CaCl_2$ and A23187 on $[Ca^{2+}]_i$ and $\Delta\Psi_m$

The effects of $CaCl_2$ and A23187 treatment on changes in Fluo-3 fluorescence in viable cells after a 30 min incubation are shown in Table 1. The mean incidence of Fluo-3 positive cells in the absence of added $CaCl_2$ or with 0.01or 0.1 mM $CaCl_2$ during incubation was low, 1.4 % or less with a fluorescence intensity per cell of 0.2 CN. In contrast, treatment with 1 mM $CaCl_2$ increased (P < 0.05) the percent of Fluo-3 fluorescent cells to 76% and increased (P < 0.05) Fluo-3 fluorescence intensity to 3 CN. Treatment with A23187 in-

creased (P < 0.05) the mean percent of Fluo-3 positive cells a small amount except in the presence of 1 mM $CaCl_2$. Fluo-3 fluorescence intensity was not affected by any $CaCl_2$ concentration. Sperm viability was > 95% and not affected by $CaCl_2$ or A23187 concentrations (data not shown).

The only significant source of variation for the percent of mitochondrial energized cells ($J_{agg}$ fluorescent cells) and the estimate $\Delta\Psi_m$ ($J_{agg}$ fluorescent intensity) was $CaCl_2$ concentration. The percent of mitochondrial energized cells was 83.5% in the absence of $CaCl_2$ and was decreased (P < 0.05) only by treatment with 1 mM $CaCl_2$ to 39.1% (Table 2). Similarly mean sperm $J_{agg}$ fluorescence intensity was decreased (P < 0.05) by treatment only with 1 mM $CaCl_2$ (Table 2).

### 3.2. Experiment 2. Effects of $CaCl_2$, A23187, and EGTA on $[Ca^{2+}]_i$ and viability

The mean effects of each treatment combination (different combinations of $CaCl_2$, EGTA, and A23187) on percent of viable Fluo-3 fluorescent cells, Fluo-3 fluorescence intensity, and viability are shown in Figure 1 (panels A, B, and C, respectively). In the absence of $CaCl_2$ (treatment combinations 1 and 2) the mean percent of Fluo-3 fluorescent cells (Fig. 1A) was < 2% and mean Fluo-3 fluorescence (Fig. 1B) was 0.2 CN throughout the incubation period. In the presence of 1 mM $CaCl_2$ in absence of A23187 and EGTA (treatment combination 3) the percent of Fluo-3 fluorescent cells (Fig. 1A) and their fluorescence intensity (Fig. 1B) increased (P < 0.05) during the incubation period. The presence of A23187 in addition to $CaCl_2$ had no significant effect on the percent of Fluo-3 fluorescent cells (compare treatment combinations 3 and 5 in Fig. 1A) and had a small, but stimulatory effect on Fluo-3 fluorescence intensity at 60 min (compare treatment combinations 3 and 5 in Fig. 1B). In the presence of EGTA, the increase in percent of Fluo-3 fluorescent cells and Fluo-3 fluorescence intensity associated with $CaCl_2$

Table 2
Mean (± SEM) effects of $CaCl_2$ on mitochondrial inner transmembrane potential in striped bass sperm as measured by JC-1 fluorescence following a 30 min incubation (n= 3 fish).

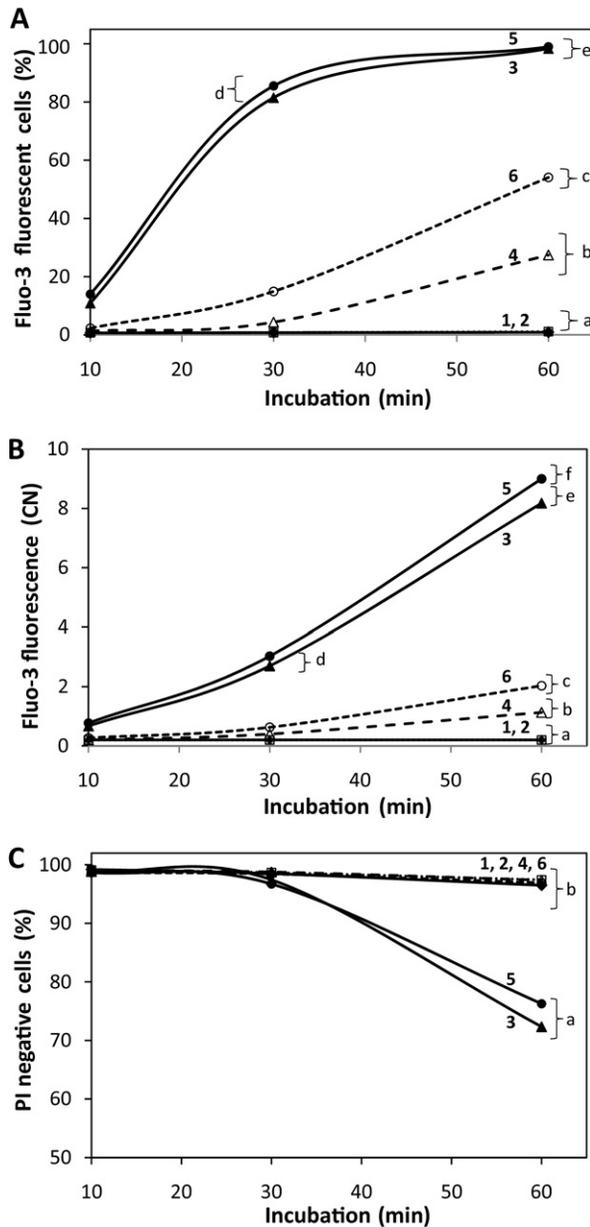| $CaCl_2$, mM | JC-1 positive cells, % | JC-1 fluorescence intensity, CN |
|---|---|---|
| 0 | $83.5 \pm 0.1^a$ | $13.6 \pm 0.6^a$ |
| 0.01 | $73.8 \pm 13.6^a$ | $12.1 \pm 7.5^a$ |
| 0.1 | $78.6 \pm 1.2^a$ | $10.0 \pm 1.9^a$ |
| 1 | $39.1 \pm 8.0^b$ | $2.3 \pm 0.5^b$ |

Within a column, means without a common a superscript (a–b) differed (P < 0.05).

Fig. 1. Mean ($\pm$ SEM) effects of six treatment combinations: 1 - ♦ - (0 mM $CaCl_2$ , 0 $\mu$M A23187, and 0 mM ethylene glycol tetraacetic acid [EGTA]), 2 ··□·· (0 mM $CaCl_2$ , 0 $\mu$M A23187, and 1 mM EGTA), 3 -▲- (1 mM $CaCl_2$ , 0 $\mu$M A23187, and 0 mM EGTA), 4 --Δ-- (1 mM $CaCl_2$ , 0 $\mu$M A23187, and 1 mM EGTA), 5 -●- (1 mM $CaCl_2$ , 1 $\mu$M A23187, and 0 mM EGTA), and 6 --○-- (1 mM $CaCl_2$ , 1 $\mu$M A23187, and 1 mM EGTA) in striped bass sperm (n = 3 fish) following 10 to 60 min of incubation in TRIS free base-NaCl extender (osmolality 350 mOsm/kg, pH8) on the percentage of viable cells containing Fluo-3 fluorescent cells (A), Fluo-3 fluorescence intensity channel number (Fluo-3, CN) in these cells (B), and cell viability, % PI negative cells (C) using flow cytometry. The treatment combination plots in each panel are identified by the number identifier above the lines adjacent to data point at 60 min. Data means on the vertical axis outside a different bracket differed (P < 0.05).

treatment was attenuated (compare treatment combinations 4 and 6 to 3 and 5). Viability was > 95% (Fig. 1C) in the absence of $CaCl_2$ (treatment combinations 1 and 2) or in the presence of $CaCl_2$ and EGTA (treatment combinations 4 and 6). However, with $CaCl_2$ treatment in the absence of EGTA viability decreased (P < 0.05) to a mean of 75% between 30 and 60 min (compare treatment combinations 3 and 5 to the rest).

### 3.3. Experiment 3. Effect of 24 h storage on $[Ca^{2+}]_i$, survival, and $\Delta\Psi_m$

The effects of storage treatment and storage time on the percent viable Fluo-3 fluorescent cells, and Fluo-3 fluorescence intensity, and cell viability are shown in Fig. 2 (panels A, B, and C respectively). At 1 h of storage < 5% of sperm cells were Fluo-3 fluorescent (Fig. 2A) with a fluorescence intensity mean of 0.2 CN (Fig. 2B). Compared to 1 h, 24 h of storage sperm increased (P < 0.05) the mean incidence of Fluo-3 fluorescent cells to over 50% of the cells for all treatments and increased (P < 0.05) fluorescence intensity to 2.2–2.3 CN in undiluted semen and in semen extended in seminal plasma and to 1.5 CN in sperm diluted in T350. The presence of EGTA during 24 h of storage maintained the percent of Fluo-3 fluorescent sperm cells and their fluorescence intensity at low levels not significantly different than those found at 1 h of storage.

Sperm cells stored for 1 h were 99% viable; after 24 h storage the viability of sperm in undiluted semen and those extended in T350 in the absence of EGTA decreased (P < 0.05) to 67.5 and 42.8%, respectively. Sperm diluted in seminal plasma (with or without EGTA) and T350 with EGTA for 24 h maintained mean viability that was not different from the means at 1 h of storage.

The storage treatment and storage time had no significant effect on mitochondrial energy status measured by the percent of viable $J_{agg}$ fluorescent cells (P > 0.38) or by their fluorescence intensity (P > 0.26). Of viable sperm cells analyzed, 94.4% possessed high $\Delta\Psi_m$ with a mean fluorescence intensity of 18.9 CN (data not shown).

### 3.4. Experiment 4. Effect of 24 h storage on $[Ca^{2+}]_i$, survival, motility activation, and ATP

The effects of storage treatment and storage time on the percent viable Fluo-3 fluorescent cells, and Fluo-3 fluorescence intensity per cell, and cell viability are shown in Fig. 3 (panels A, B, and C respectively). After 1 h of storage the percent of cells containing Fluo-3
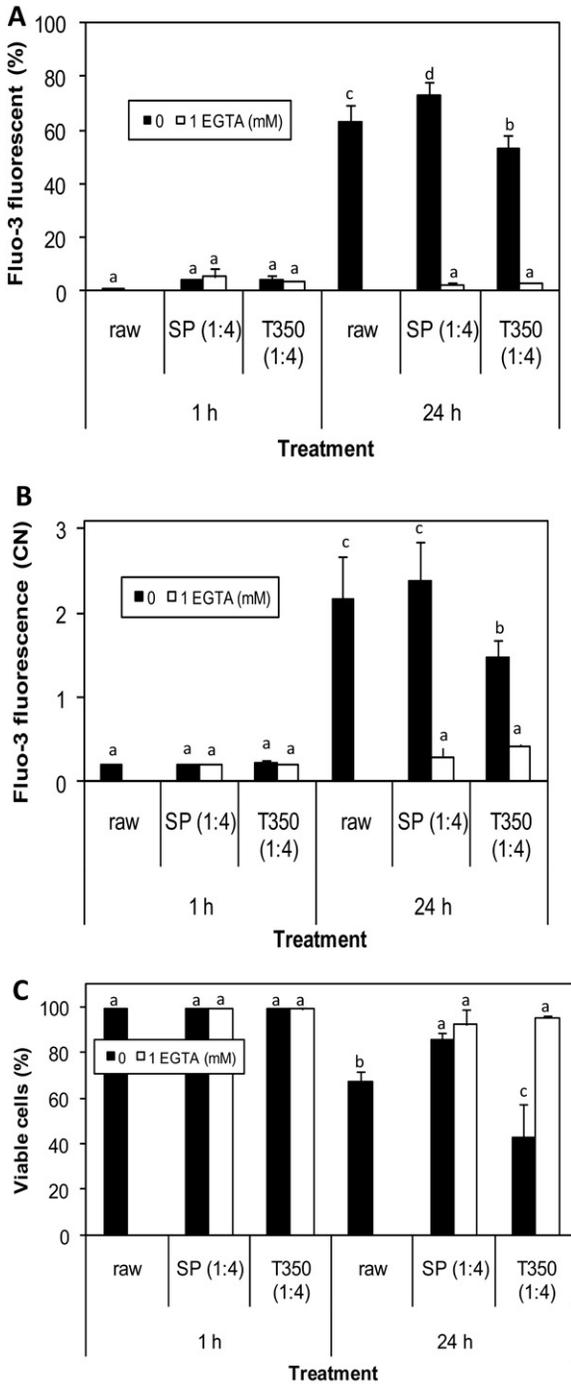
Fig. 2. Mean ($\pm$SEM) effects of storage time (1 and 24 h) and storage treatment (undiluted and diluted 1:4 in seminal plasma [SP] or TRIS free base-NaCl extender [T350] containing ethylene glycol tetraacetic acid [EGTA] [0 or 1 mM]) at 4 °C in an $O_2$ atmosphere on striped bass sperm (n = 6 fish) on the percentage of viable cells containing Fluo-3 fluorescent cells (A), Fluo-3 fluorescence intensity channel number (CN) in these cells (B), and cell viability, % PI negative cells (C) using flow cytometry (Experiment 3). [a-d]Bar means without a common superscript differed (P < 0.05).
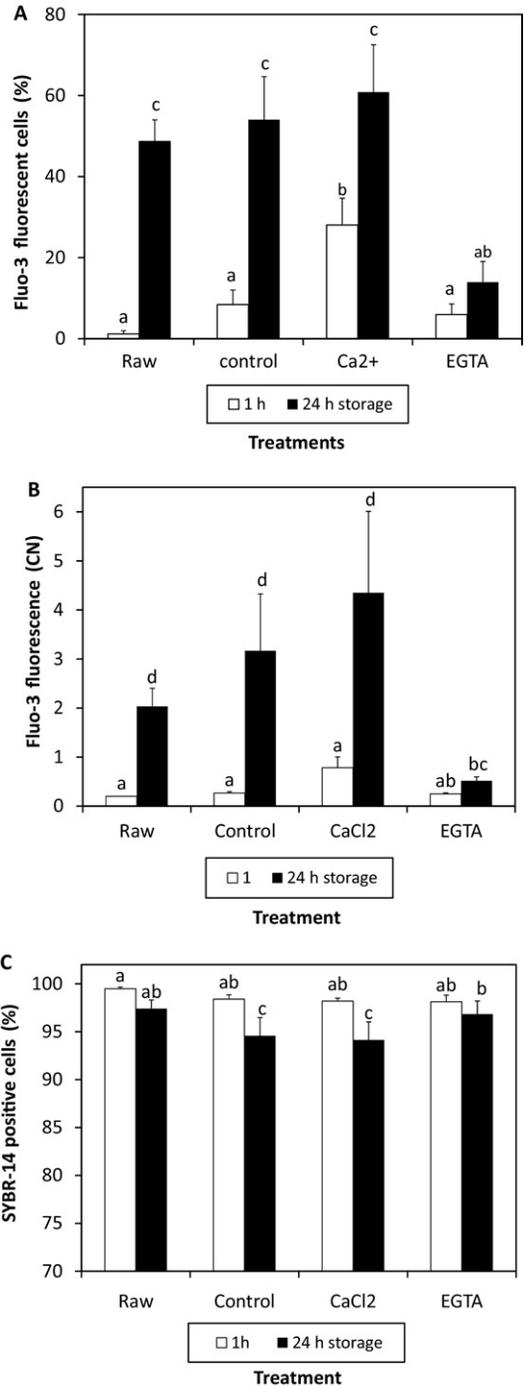
Fig. 3. Mean ($\pm$ S.E.M.) effects of storage time (1 and 24 h) and storage treatment (undiluted and diluted 1:4 in seminal plasma [SP] or TRIS free base-NaCl extender [T350] containing CalCl$_2$ [0 or 2 mM] or ethylene glycol tetraacetic acid [EGTA] [0 or 1 mM]) at 4 °C in an $O_2$ atmosphere on striped bass sperm (n = 6 fish) on the percentage of viable cells containing Fluo-3 fluorescent cells (A), Fluo-3 fluorescence intensity channel number (CN) in these cells (B), and cell viability, % PI negative cells (C) using flow cytometry (Experiment 4). [a-d] Bar means without a common superscript differed (P < 0.05).

Table 3
Mean (± SEM) effects of storage time and storage treatment (undiluted and diluted 1:4 in T350) on the percentage sperm cells activated (n = 2 fish).

| Storage time, h | Dilution treatment | Motility, % |
|---|---|---|
| 1 | Undiluted | $100.0 \pm 0.0^a$ |
|  | T350 | $85.5 \pm 2.5^a$ |
| 3 | Undiluted | $45.0 \pm 12.5^{bc}$ |
|  | T350 | $62.5 \pm 5.0^b$ |
| 24 | Undiluted | $5.0 \pm 0.0^d$ |
|  | T350 | $30.0 \pm 0.0^c$ |

Within a column, means without a common superscript (a–d) differed (P < 0.05).

fluorescence did not differ among treatments except in the absence of $CaCl_2$ (Fig. 3A). Increasing storage time from 1 to 24 h increased (P < 0.05) the percentage of Fluo-3 fluorescent sperm (Fig. 3A) and their fluorescence intensity (Fig. 3B) in undiluted, diluted control and diluted $CaCl_2$-treated sperm except in the presence of EGTA. Viability at 1 h was > 96% and did not vary significantly among treatments. However, compared to 1 h, viability at 24 h was decreased slightly and significantly to 94% in diluted control and $CaCl_2$-treated semen, but not in undiluted or EGTA-treated diluted semen (Fig. 3C).

Storage time and the interaction of storage time and storage treatment were significant sources of variation (P < 0.05) for percent motility following activation (Table 3). The ability to activate motility decreased during storage in both undiluted and T350 extended semen; however the loss of motility was more severe in undiluted semen after 24 h storage.

Storage time and storage treatment were significant sources of variation for sperm ATP content (Table 4). Over all storage times sperm diluted in T350 contained more ATP than undiluted semen (P < 0.05) and sperm

Table 4
Mean (± SEM) effects of storage time and storage treatment (undiluted and diluted 1:4 in T350 in the absence or presence of ethylene glycol tetraacetic acid (EGTA)) on the ATP content (n = 3 fish).

| Storage time, h | Storage treatment | ATP, pmoles/$10^6$ cells |
|---|---|---|
| 1 | Undiluted | $4.6 \pm 1.8^{bc}$ |
|  | T350 | $8.9 \pm 0.9^a$ |
|  | T350-EGTA (1 mM) | $10.5 \pm 1.2^a$ |
| 24 | Undiluted | $2.3 \pm 0.4^c$ |
|  | T350 | $7.4 \pm 1.5^{ab}$ |
|  | T350-EGTA (1 mM) | $4.9 \pm 1.0^{bc}$ |

Within a column, means without a common superscript (a–d) differed (P < 0.05).

Table 5
Mean (± SEM) effects of storage time and menadione treatment on reactive oxygen species formation (n = 6 fish).

| Storage time, h | Menadione, mM | Ethidium fluorescence, CN |
|---|---|---|
| 1 | 0 | $1.8 \pm 0.1^c$ |
|  | 1 | $3.2 \pm 0.1^a$ |
| 24 | 0 | $1.5 \pm 0.1^d$ |
|  | 1 | $2.7 \pm 0.1^b$ |

Within a column, means without a common superscript (a–d) differed (P < 0.05).

ATP was greater (P < 0.05) at 1 h than at 24 h of storage. Mean ATP content in diluted semen stored in the absence or presence of EGTA did not differ significantly.

### 3.5. Experiment 5. Effect of 24 h storage and menadione on ROS formation

Significant sources of variation for sperm ROS formation in diluted semen were storage time and menadione treatment after storage; storage treatment (including EGTA and two different SO scavengers) was not a significant source of variation (Table 5). Cells were sensitive to menadione treatment with an increase (P < 0.05) in mean ethidium fluorescence in the presence of menadione compared to its absence and ethidium fluorescence in the absence and presence of menadione was greater (P < 0.05) at 1 than 24 h of storage time.

## 4. Discussion

Calcium is a major regulator of sperm function. Normally a $10^3 – 10^4$-fold concentration gradient exists for $Ca^{2+}$ between the intracellular and extracellular environments of sperm cells [22]. 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 several mammalian species as well as sea urchins, calcium signaling involves a specialized set of channels, transporters, and intracellular storage sites [5,9,23,24]. While many calcium channels, transporters, and intracellular storage sites have been specifically identified by genomic or proteomic analysis in many species [5,9,23,24], comparable identification has not been accomplished in teleost fish sperm. However $Ca^{2+}$ treatment has been shown to play an important role in the activation of motility in sperm cells of many fish species [4,8,25,26,27]. Channel blockers used to restrict $Ca^{2+}$ influx and inhibitors of calcium pumps used to restrict $Ca^{2+}$ extrusion have demonstrated the importance of $Ca^{2+}$ as a secondary messenger and

transmembrane $Ca^{2+}$ transport in activation of motility [4,26,28].

In our current study striped bass sperm $[Ca^{2+}]_i$ did not increase in response to $CaCl_2$ treatment in the range of 0.01 to 0.1 mM. However when cells were treated with 1 mM $CaCl_2$ the increase in Fluo-3 fluorescence indicated an influx of $Ca^{2+}$ indicative of leakage through plasma membranes still impermeant to PI, or alternatively by voltage-gated opening of $Ca^{2+}$ channels [4,5]. The calcium ionophore A23187 under our experimental conditions appeared to have little effect on $Ca^{2+}$ uptake. While A23187 promoted a small, significant increase in the number of cells containing Fluo-3 fluorescence in the absence of or in the presence of low concentrations of $CaCl_2$ (0.01 and 0.1 mM), fluorescence intensity was not increased. This result differs from the effect of A23187 in mammalian sperm [15,29] which may be related to the differences in incubation temperature or the composition of the plasma membranes. Once calcium leakage was established or $Ca^{2+}$ channels were opened in response to 1 mM $CaCl_2$-induced hyperpolarization, no additional effect of A23187 on Fluo-3 fluorescence was detected. The design of this study did not permit us to determine the source of $Ca^{2+}$ which could have been from intracellular organelles or the seminal plasma [9]. The influx of $Ca^{2+}$ initiated by 1 mM $CaCl_2$ in Experiment 1 was detrimental to cell viability and partially disruptive of mitochondrial function.

With regard to short-term (24–48 h), non-frozen storage of striped bass semen a number of NaCl based diluents have been evaluated [30,31]. Based on recent studies of striped bass sperm energetics [14] and the normal osmolality of striped bass sperm seminal plasma [30,31] we adopted a calcium-free Tris-NaCl solution adjusted to 350 mOsm/kg and pH8. During the first hour of storage $[Ca^{2+}]_i$ remained low in undiluted and diluted semen with only few of the cells being positive for Fluo-3. However, storage of sperm in undiluted or diluted form for 24 h increased the number of sperm cells containing Fluo-3 fluorescence to > 50% and increased Fluo-3 fluorescence almost 10-fold indicating liberation of calcium from internal stores and/or external sources. This pattern of calcium accumulation was similar to that found after 30 min incubation in the presence of 1 mM $CaCl_2$ in Experiments 1 and 2. However, in contrast to the decrease in $\Delta\Psi_m$ associated $CaCl_2$-induced $Ca^{2+}$ influx, the percentage of sperm with energized mitochondria did not change even as Fluo-3 fluorescence increased during the 24 h storage period. Perhaps the increase in $[Ca^{2+}]_i$ associated with

a 30 min exposure to 1 mM $CaCl_2$ had a greater negative impact on mitochondrial $\Delta\Psi_m$ than a more gradual increase in $[Ca^{2+}]_i$ that might be expected during 24 h storage in the absence of $CaCl_2$ treatment.

The ability of EGTA to reduce sperm exposure to $CaCl_2$ and block its lethal effect of $CaCl_2$ in Experiment 2 led us to determine if EGTA might be beneficial to sperm survival during a 24 h storage period. We found that the presence of EGTA prevented the intracellular accumulation of $Ca^{2+}$ in Experiments 3 and 4. In the course of evaluating the presence of EGTA on sperm during the 24 h storage period during 2009, sperm cells were tested for activation. While T350 semen maintained motility better than undiluted semen at 24 h, the presence of EGTA in T350 semen, which would be expected to largely remove calcium from the cell suspension, blocked activation completely. This result indicates that some calcium is important for activation of motility in striped bass sperm.

While the two experiments run in the 2008 and 2009 seasons showed a similar pattern in $[Ca^{2+}]_i$ accumulation during the 24 h storage period, the effect on viability differed greatly between the two years. In both years viability at 1 h in undiluted or diluted form was > 95%, however in 2008 (Experiment 3) cell viability after 24 h of storage of semen in undiluted and extended form decreased to 64 and 42%, respectively. In contrast in 2009 (Experiment 4) viability was decreased by only a few percent and may not have been biologically significant. We have not been able to explain this difference except that different fish were sampled in different years.

An accumulation of $[Ca^{2+}]_i$ has been reported during liquid hypothermic storage of bovine and porcine sperm [32,33,34] or following freeze-thawing [29]. One explanation of this influx of calcium may be a disruption of sperm cell $[Ca^{2+}]_i$ homeostasis. The source of increased $[Ca^{2+}]_i$ during storage may have been intracellular from mitochondria [24] or the excess nuclear envelope [9], or external from the seminal plasma, or reagent contamination. The increased $Ca^{2+}$ internalization may to be a result of cooling-induced plasma membrane reorganization leading to non-specific bilayer faults [35,36] that permit influx of $Ca^{2+}$. Some of the bilayer faults may result from irreversible phase changes and changes in the lipid composition of the membranes themselves [37]. Alternatively, changes in the physiochemical properties of the membrane lipid environment and cooling [38,39] may provide a possible mechanism of $Ca^{2+}$ ATPase dysfunction to prevent

the $Na^+/Ca^{2+}$ antiporter from extruding in $Ca^{2+}$ from sperm cells [40].

Storage in an $O_2$ atmosphere is required during low temperature liquid storage to maintain motility and fertility for both striped bass [20] and turkey sperm [11]. We considered that $O_2$ exposure might be detrimental to striped bass sperm function and survival because storage time for turkey sperm is limited by lipid peroxide formation during storage [11]. While striped bass sperm did respond to menadione treatment by formation of ROS, there was no evidence of increased ROS formation between 1 and 24 h of storage. The striped bass sperm response to menadione treatment indicated a disruption of mitochondrial electron transport. However, the presence of EGTA and the SO scavengers TBAP and TMP did not alter the baseline level of ROS or menadione-induced ROS formation at 1 or 24 h of storage indicating a possible non-involvement of $Ca^{2+}$ in menadione induced ROS formation and the failure of EGTA and the SO scavengers to penetrate the plasma membrane. Sperm ATP decreased between 1 and 24 h of storage. While menadione was likely disruptive of electron transport in the sperm mitochondria, intracellular calcium flux or sperm ATP were not measured in the presence of menadione so we do not know whether menadione would have altered calcium flux and ATP production or whether EGTA would have prevented these potential effects.

In our current experiments sperm $[Ca^{2+}]_i$ increased during storage for 24 h and was accompanied by a loss of viability in one experiment. During storage EGTA blocked the influx of free calcium into the cytosol and the increase in cell death, and did not depress cell energetics in terms of sperm and $\Delta\Psi_m$ or ATP content. However, the presence of EGTA in the extender blocked activation of motility so that the overall objective of this study to improve striped bass sperm storage was not realized. We suggest that the increased intracellular calcium found after 24 h indicates a storage induced defect in the maintenance of cellular calcium homeostasis which may be detrimental to sperm activation. Future studies will be required to determine whether inhibitors of $Ca^{2+}$ channel function will inhibit $Ca^{2+}$ influx during storage and maintain sperm motility.

## Acknowledgments

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