Premises for fowl sperm preservation based on applied bioenergetics

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ABSTRACT: The primary goal of this work was to test whether the sperm mobility assay could be used to derive mathematical relationships from which predictions could be made about sperm cell function. A precondition was random sampling from a pool of sperm. This precondition was met by centrifuging mobile sperm through 12% (wt/vol) Accudenz containing the Ca\(^{2+}\) chelator 1,2-bis-(\(\sigma\)-aminophenoxy)ethane-\(N,N',N'',N''\)-tetraacetic acid (BAPTA) and then holding washed sperm at 20°C within buffered potassium chloride. These 2 conditions rendered washed sperm immobile at 20°C. Resumption of sperm mobility was independent of time (\(P > 0.8558\)) when sperm were reactivated at body temperature with 2 mM Ca\(^{2+}\) in isotonic sodium chloride at pH 7.4. Reactivated sperm mobility was 93% of the prewash control. Subsequent experiments served to define a dose response, predict optimal conditions for in vitro sperm mobility, and show how sperm can recover from an imposed non-physiological condition. Thus, functions were derived from which predictions were made. Whereas the utility of BAPTA treatment was confirmed in a new context, such utility did not address the question of whole-cell Ca\(^{2+}\) flux during sperm cell manipulation. This issue is pivotal for the application of bioenergetics to fowl sperm preservation. Therefore, the secondary goal of this research was to investigate sperm cell Ca\(^{2+}\) flux using a simulation of conditions encountered by sperm during centrifugation through 12% (wt/vol) Accudenz. These conditions included a temperature of 30°C, a Ca\(^{2+}\) sink, and no exogenous substrate. Sperm motion was measured with a Hobson SpermTracker. Data points conformed to parabolic functions when motile concentration and velocity were plotted as functions of time. In each case, maximums were observed, e.g., 26 min for motile concentration. The upswing was attributed to a redistribution of intracellular Ca\(^{2+}\) whereas the downswing was attributed to sperm cell Ca\(^{2+}\) depletion. A pronounced isothermal increase was observed for each variable when the Ca\(^{2+}\) sink was overcome with exogenous Ca\(^{2+}\). Experimental outcomes supported four testable premises applicable to fowl sperm preservation research: 1) the importance of sperm mobility phenotype, 2) the relationship between mitochondrial Ca\(^{2+}\) cycling and sperm mobility, 3) the utility of the sperm mobility assay for predicting experimental outcomes, and 4) understanding mitochondrial Ca\(^{2+}\) cycling in terms of whole-cell Ca\(^{2+}\) flux.

Key words: bioenergetics, calcium, chicken, semen preservation, sperm, sperm mobility

INTRODUCTION

To date, chicken semen quality has been defined in terms of volume as well as the attributes of sperm therein (e.g., concentration, viability, and motility; Wishart, 2009). Such variables have been used in attempts to discover a means by which poultry semen can be preserved. Nevertheless, poultry breeders have yet to implement a routine, reliable means of preserving chicken semen (D. P. Froman, personal communication). The author attributes the disparity between effort and application to a vague definition of semen quality. In other words, the goal for semen storage is maintenance of semen quality. However, if semen quality is ill-defined, then experimental outcomes are likely to be tangential to the goal. In contrast, if semen quality can be defined, then mechanisms affecting semen quality can be evaluated in a purposeful manner. In this regard, Froman and Rhoads (2013) used systems biology to define chicken semen quality in terms of the daily output of mobile sperm per rooster.


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3The author was awarded U.S. Patent Number 5,866,354 entitled “Method of Measuring Mobility of Sperm” on February 2, 1999.

Received November 10, 2013.
Accepted December 16, 2013.
This definition affords a new perspective for sperm preservation research. In effect, the sperm mobility assay is a bioassay for sperm cell mitochondria. Moreover, there can be no reasonable expectation that sperm preservation will be successful if freshly ejaculated viable sperm nonetheless contain moribund mitochondria at the onset of manipulation or if sperm cell manipulation induces mitochondrial damage. This new perspective presents 2 technical challenges. First, can the effects of sperm cell manipulation be predicted as opposed to merely detected? Second, even though proof of concept was established for 1,2-bis-(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid (BAPTA)-mediated manipulation of mitochondrial Ca\(^{2+}\) cycling (Froman, 2003; Froman and Feltmann, 2010; Froman, 2013), is Ca\(^{2+}\) flux through fowl sperm as simple as it appears? The present work was performed to answer these 2 questions. However, the first question required an experimental precondition: random sampling from pooled sperm without time affecting the outcome.

**MATERIALS AND METHODS**

**Experimental Birds.** Semen donors (n = 80) were selected at 40 wk of age from a line of New Hampshire chickens bred for high sperm mobility. Roosters were maintained on a 14:10 photoperiod and fed ad libitum in accordance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition. Experiments were conducted with the approval of the Oregon State University Institutional Animal Use and Care Committee.

**Reagents.** Accudenz was purchased from Accurate Chemical and Scientific Corp., Westbury, NY. All other solutes were purchased from Sigma Chemical Co., St. Louis, MO. All solutions were prepared with cell-filtered, cell culture-tested water purchased from Sigma. Buffered solutions were prepared with N-tris(hydroxymethyl) methyl-2-amino-ethanesulfonic acid (TES). The pH of buffered solutions containing NaCl was adjusted by the incremental addition of 5 M NaOH. In contrast, the pH of buffered solutions containing KCl was adjusted with 5 M KOH. Osmometer standards (100, 290, and 1,000 mmol/kg) were purchased from Wescor, Inc., Logan, UT.

**Buffer Osmolality.** The osmolality of each experimental solution was measured with a Model 5500 Vapor Pressure Osmometer (Wescor, Inc., Logan, UT). This instrument was calibrated daily with each of 3 standard solutions. If the osmolality of a solution was within ±3 mmol/kg from the desired value then no adjustment was made. In contrast, if the observed osmolality exceeded 3 mmol/kg from the expected value, then osmolality was adjusted by either the addition of solute or water. When the osmolality was increased by the addition solute, then either NaCl or KCl was added in accordance with the solution being prepared.

**Sperm Mobility Assay.** Sperm concentration was determined with a spectrophotometer as outlined by Froman and Rhoads (2012). Next, a 50-μL volume of either neat semen or washed sperm (see below) was mixed with prewarmed buffer to procure a sperm suspension containing 5 × 10\(^6\) sperm/mL at body temperature (41°C). Each suspension was prepared in a 12 × 75 mm polypolyene test tube removed from a 41°C water bath. A 60-μL volume of the sperm suspension was withdrawn and overlaid on a 600-μL volume of 6% (wt/vol) Accudenz pre-warmed to 41°C in a semi-microcuvette. The absorbance of the Accudenz solution was read at 550 nm after incubating the cuvette for 5 min at 41°C within a water bath (see Froman et al., 1999). Sperm mobility was expressed in terms of a percentage rather than absorbance units as follows. Cuvette contents were mixed after the absorbance of the Accudenz solution was measured and then absorbance was measured again. The percentage of mobile sperm within the test sample was determined by dividing the first absorbance value by the second and then multiplying the quotient by 100 (see Froman and Rhoads, 2013).

**Experimental Precondition.** A stock solution of 30% (wt/vol) Accudenz was prepared with 3 mM KCl in 5 mM TES, pH 7.4. The following solutions, each with an osmolality of 300 mmol/kg, were prewarmed in a 41°C water bath: 1) a 1-mL volume of 30% (wt/vol) Accudenz, 2) a 30-mL volume of 12% (wt/vol) Accudenz, and 3) a 2-mL volume of TES-buffered KCl, pH 7.4. These solutions were held within a 12 × 75 polypolyene culture tube, a 50-mL polypolyene centrifuge tube, and a 15-mL polypolyene centrifuge tube, respectively. Ejaculates were pooled until a 5-mL volume of semen was obtained. Sperm concentration and pre-wash sperm mobility were measured as outlined above. The prewarmed 30% (wt/vol) Accudenz solution was layered beneath the prewarmed 12% (wt/vol) Accudenz solution while the assay was being performed. Once prewash sperm mobility was recorded, a sperm suspension was prepared with TES-buffered potassium chloride as follows. A volume of pre-warmed buffer was withdrawn so that on the admixture of residual buffer with semen, a 6-mL sperm suspension would be procured with 2 × 10\(^6\) sperm per mL. However, before semen was added, 169 mg of 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-BAPTA was dissolved in the buffer to provide 5 mM BAPTA on the addition of semen.

The sperm suspension was overlaid on the 12% (wt/vol) Accudenz solution. Sperm were washed by passage through 12% (wt/vol) Accudenz and then concentrated at the interface between Accudenz solutions.
as follows. Sperm, initially at body temperature, were cooled to 30°C while they were centrifuged through 12% (wt/vol) Accudenz at 1,250 × g for 30 min (Froman, 2013). This Accudenz solution was prepared with TES-buffered potassium chloride, pH 7.4, and contained 5 mM BAPTA. Following centrifugation, the seminal plasma and 12% (w/v) Accudenz supernatants were discarded along with the 30% (wt/vol) Accudenz infranatant. Washed sperm were mixed with a 900-μL volume of TES-buffered potassium chloride at room temperature, and sperm concentration was measured. Each of seven 200-μL volumes of sperm suspension was pipetted into a 1.5-mL microcentrifuge tube, and loaded tubes were placed within a floating rack as outlined by Froman (2013). The rack was placed in a 20°C water bath. One tube was removed after 0, 10, 20, 30, 40, 50, and 60 min of incubation. In each case, a 50-μL sample was withdrawn for the sperm mobility assay. This procedure was replicated 5 times with semen pooled from different roosters each time. Data were plotted as a function of time. Parameters of the function

\[ y(x) = \alpha + \beta(x) \]

were estimated with the PROC GLM feature of SAS (SAS Inst. Inc., Cary, NC).

### Dose Response

Sperm from pooled ejaculates (n = 5 pools) were washed, six 200-μL volumes of washed sperm were pipetted into microcentrifuge tubes within a floating rack, and the rack was placed in a 20°C water bath as outlined above. Sperm mobility assays were performed at 1-min intervals after microcentrifuge tubes were held for 5 min at 20°C. Washed sperm were diluted in TES-buffered sodium chloride, pH 7.4, containing 2 mM Ca²⁺ and 0, 50, 100, 200, 400, or 800 μM cyanide. The order in which treatments were tested was assigned randomly within pools of washed sperm. Data within pools were normalized against the highest observed value. Normalized data were used to estimate the parameters of the function

\[ p(x) = \frac{1}{1 + e^{\alpha - \beta(x)}} \]

with the PROC NLIN feature of SAS (SAS Inst. Inc.).

### pH Optimum

Sperm from pooled ejaculates (n = 5 pools) were washed, seven 200-μL volumes of washed sperm were pipetted into microcentrifuge tubes within a floating rack, and the rack was placed in a 20°C water bath as outlined above. Sperm mobility assays were performed at 1-min intervals after microcentrifuge tubes were held for 5 min at 20°C. Washed sperm were diluted with 2 mM Ca²⁺ in TES-buffered sodium chloride at pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 or 8.0. In each case, the 6% (wt/vol) Accudenz used for the assay was prepared with the same buffer used in the overlay. The order in which treatments were tested was assigned randomly within pools of washed sperm. Data were plotted as a function of assay pH. Parameters of the function

\[ y(x) = \alpha + \beta(x) + \gamma x^2 \]

were estimated with the PROC GLM feature of SAS (SAS Inst. Inc.).

### Osmolality Optimum

Sperm from pooled ejaculates (n = 5 pools) were washed, seven 200-μL volumes of washed sperm were pipetted into microcentrifuge tubes within a floating rack, and the rack was placed in a 20°C water bath as outlined above. Sperm mobility assays were performed at 1-min intervals after microcentrifuge tubes were held for 5 min at 20°C. Washed sperm were diluted with 2 mM Ca²⁺ in TES-buffered sodium chloride, pH 7.4, with an osmolality of 225, 250, 275, 300, 325, 350, or 375 mmol/kg. In each case, the 6% (wt/vol) Accudenz used for the assay was prepared with the same buffer used for the overlay. The order in which treatments were tested was assigned randomly within pools of washed sperm. Data were plotted as a function of assay osmolality. Parameters of the function

\[ y(x) = \alpha + \beta(x) + \gamma x^2 \]

were estimated with the PROC GLM feature of SAS (SAS Inst. Inc.).

### Sperm Recovery

Pre-wash sperm mobility was measured for replicate semen pools (n = 5) as outlined above. Sperm from each pool were washed as above with the exception that osmolality of the 12% (wt/vol) Accudenz solution was 360 mmol/kg as was the TES-buffered potassium chloride, pH 7.4, into which washed sperm were suspended. Washed sperm were allocated into microcentrifuge tubes (n = 6) as outlined above. Sperm mobility assays were performed at 1-min intervals after microcentrifuge tubes were held for 5 min at 20°C. Sperm were diluted with 2 mM Ca²⁺ in TES-buffered sodium chloride, pH 7.4, with an osmolality of 360, 350, 340, 320, 300, or 290 mmol/kg. In each case, the 6% (wt/vol) Accudenz used for the assay was prepared with the same buffer used in the overlay. The order in which treatments were tested was assigned randomly within pools of washed sperm. Data were plotted as a function of assay osmolality. Parameters of the function

\[ y(x) = \frac{\gamma}{1 + e^{\beta(\tau - x)}} \]

were estimated with the PROC NLIN feature of SAS (SAS Inst. Inc.).

### Computer-Assisted Sperm Motion Analysis

Motile concentration and average straight line velocity (VSL)
were measured at 30°C as outlined by Froman and Feltmann (2000) using 10 replicate roosters. In brief, a 1.25-mL sperm suspension was prepared in a 12 × 75 mm polypropylene test tube. This suspension contained 1.2 × 10⁶ sperm per mL in TES-buffered sodium chloride, pH 7.4, containing 5 mM BAPTA. The tube was placed within a water bath at 30°C. Such sperm had a concentration of 5.3 ± 0.43 × 10⁹ sperm/mL (mean ± SD) before assay. Sperm were reactivated at 41°C in TES-buffered sodium chloride containing 2 mM Ca²⁺. Each open circle represents a mean ± SD. The solid line denotes the linear function $y(x) = 71 - 0.00009(x)$. The slope did not differ from zero ($P > 0.8558$). This experiment demonstrated that random samples could be withdrawn from a pool of immobile sperm and then uniformly reactivated independent of time.

for the predicted function, 93% of prewash sperm mobility was recovered on reactivation. The extent to which mitochondrial function affected sperm cell reactivation is shown in Fig. 2; for a logistic dose response was observed when cytochrome oxidase was inhibited by cyanide. The effect of extracellular pH on sperm reactivation is shown in Fig. 3. In this experiment, a parabolic relationship was observed with a maximal sperm mobility at 274 mmol/kg. Sperm were completely immobile at body temperature in the presence of Ca²⁺ when suspended in buffer with an osmolality of 375 mmol/kg (data excluded from Fig. 4). As shown in Fig. 5, osmotic inhibition of sperm mobility was reversible as data conformed to a logistic function when sperm mobility was plotted as a function of decreasing assay osmolality. Prewash sperm mobility was 74 ± 2.9% in this experiment. Whereas postwash sperm mobility was only 3% when the assay was performed at an osmolality of 360 mmol/kg, full recovery was observed when assay osmolality was 290 mmol/kg. In this case, postwash sperm mobility was 74 ± 7.6%.

Sperm mobility was 76 ± 3.3% (mean ± SD) for 5 pools of freshly ejaculated semen in the first experiment. Postwash sperm were immobile when held at 20°C before assay (data not shown). The effect of holding immobile sperm at 20°C before reactivation with Ca²⁺ at body temperature is shown in Fig. 1. The mobility of reactivated sperm was independent of time ($P > 0.8558$) over a 60-min interval. As evidenced by a $y$-intercept of 71% for the predicted function, 93% of prewash sperm mobility was recovered on reactivation. The extent to which mitochondrial function affected sperm cell reactivation is shown in Fig. 2; for a logistic dose response was observed when cytochrome oxidase was inhibited by cyanide. The effect of extracellular pH on sperm reactivation is shown in Fig. 3. In this case, a parabolic relationship was observed. Maximal sperm mobility, as estimated by the quantity $\beta/2\gamma$, was at pH 7.2. The effect of extracellular osmolality on sperm reactivation is shown in Fig. 4. In this experiment, a parabolic relationship was observed with a maximal sperm mobility at 274 mmol/kg. Sperm were completely immobile at body temperature in the presence of Ca²⁺ when suspended in buffer with an osmolality of 375 mmol/kg (data excluded from Fig. 4). As shown in Fig. 5, osmotic inhibition of sperm mobility was reversible as data conformed to a logistic function when sperm mobility was plotted as a function of decreasing assay osmolality. Prewash sperm mobility was 74 ± 2.9% in this experiment. Whereas postwash sperm mobility was only 3% when the assay was performed at an osmolality of 360 mmol/kg, full recovery was observed when assay osmolality was 290 mmol/kg. In this case, postwash sperm mobility was 74 ± 7.6%.

The motile properties of fowl sperm incubated at 30°C in TES-buffered sodium chloride, pH 7.4, containing 5 mM BAPTA are shown in Fig. 6 and 7. In each case, data approximated a parabolic function in

**RESULTS**

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which a maximal value was reached after many minutes of incubation (i.e., 26 and 49 min for motile concentration and VSL, respectively). When the effect of BAPTA was overcome by the addition of Ca\(^{2+}\), both motile concentration and average VSL increased abruptly, 3.5- and 1.6-fold, respectively.

**DISCUSSION**

The mobile sperm within an ejaculate are those that can move against resistance at body temperature. This ability is measured in vitro with the sperm mobility assay (Froman and McLean, 1996). Resistance is imposed by an interface between 2 solutions that differ in density: an overlying sperm suspension and an underlying Accudenz solution. Fowl sperm require a VSL ≥ 30 µm/s to be mobile in vitro (Froman et al., 2003). Consequently, sperm mobility is a linear function of the proportion of such sperm within an ejaculate. This relationship affords an important distinction: whereas all mobile sperm are motile, not all motile sperm are mobile.

A sperm mobility measurement has biological significance for 2 reasons. First, sperm mobility phenotype predicts male fertility when insemination dose is controlled (Froman et al., 1999). The profound effect of sperm mobility phenotype on fecundity was confirmed by competitive fertilization (Birkhead et al., 1999; Pizzari et al., 2008). Second, the sperm mobility assay can be used to estimate the proportion of immobile sperm within an ejaculate in addition to the proportion of mobile sperm. All roosters ejaculate a mixture of mobile and immobile sperm. This ratio is independent of age (Froman et al., 1999; Froman, 2006), hence the ability to assign phenotype to any given rooster. Immobile sperm contain moribund mitochondria (Bowling et al., 2003; Froman and Kirby, 2005; Froman and Feltmann, 2010), and mitochondrial failure has been explained in stochastic terms using sperm cell proteomics (Froman et al., 2011) and reproductive tract throughput (Froman and Rhoads, 2013). It appears that all sperm are at risk of oxidative stress during their passage through the excurrent ducts of the testis. However, the likelihood of premature mitochondrial failure increases if sperm have a limited glycolytic ability and undergo protracted transit through these ducts.

Mitochondria within ejaculated sperm are clearly at risk when sperm are manipulated in vitro (Froman and Feltmann, 2010). However, apart from mitochondrial Ca\(^{2+}\) overloading (Froman et al., 2006), the conditions that impose this risk are largely unknown. Therefore, the first objective of this research was to determine whether the sperm mobility assay could be used to predict relationships as opposed to assigning phenotype or testing for a treatment effect. The distinction between hypothetical and conventional applications of the sperm mobility assay can be explained as follows.
New application for sperm mobility assay

Froman and Feltmann (1998) used this assay to phenotype roosters within a population of random-bred chickens. Roosters characterized by average sperm mobility, as determined by the population mean and standard deviation, were used as semen donors in an experiment that tested the effect of protein phosphatase inhibition on sperm mobility. This experiment was performed for 2 reasons. First, the basis for phenotypic variation was unknown. Second, Ashizawa et al. (1995) reported that calyculin A, a protein phosphatase inhibitor, could increase sperm motility. Therefore, the following argument was made: if calyculin A can increase test subject sperm mobility, then axoneme malfunction is an unlikely mechanism underlying phenotypic variation. Sperm mobility doubled in response to 500 nM calyculin A, a dose deemed optimal beforehand (Ashizawa et al., 1995). Therefore, the search for a mechanism underlying phenotypic variation was directed towards the midpiece. This example illustrates how the sperm mobility assay can be used to assign phenotype and then test for a treatment effect using sperm from an individual rooster immediately after ejaculation.

Highly mobile sperm were washed by centrifugation through 12% (wt/vol) Accudenz containing 5 mM 1,2-bis-(o-aminophenoxy)ethane-N,N’,N”,N”-tetraacetic acid (BAPTA). This was achieved by centrifugation through 12% (wt/vol) Accudenz. Washed sperm (n = 5 replicate pools) had a concentration of 5.5 ± 0.20 × 10^9 sperm/mL (mean ± SD). Washed sperm were held briefly at 20°C before assay. Sperm were reactivated at 41°C with 2 mM Ca^{2+} in TES-buffered sodium chloride, pH 7.4, with varying osmolality. Each open circle is a mean ± SD. The solid line denotes the logistic function \( y(x) = \frac{75}{1 + e^{0.098 (339 - x)}} \), where 75% recovery denotes an asymptote. This experiment demonstrated that the sperm mobility assay could be used to define how sperm recover from an imposed, non-physiological condition.

Figure 5. Sperm reactivation as a function of extracellular osmolality. Before assay, sperm were immobilized by transfer from seminal plasma into 360 mmol/kg TES-buffered potassium chloride, pH 7.4, containing 5 mM 1,2-bis-(o-aminophenoxy)ethane-N,N’,N”,N”-tetraacetic acid (BAPTA). This was achieved by centrifugation through 12% (wt/vol) Accudenz. Washed sperm (n = 5 replicate pools) had a concentration of 5.5 ± 0.20 × 10^9 sperm/mL (mean ± SD). Washed sperm were held briefly at 20°C before assay. Sperm were reactivated at 41°C with 2 mM Ca^{2+} in TES-buffered sodium chloride, pH 7.4, with varying osmolality. Each open circle is a mean ± SD. The solid line denotes the logistic function \( y(x) = \frac{75}{1 + e^{0.098 (339 - x)}} \), where 75% recovery denotes an asymptote. This experiment demonstrated that the sperm mobility assay could be used to define how sperm recover from an imposed, non-physiological condition.

Figure 6. Motile concentration of sperm as a function of time. Motile concentration is a time-averaged estimate of the number of motile sperm within a volume defined by sample chamber depth and the area of the analysis field of a Hobson SpermTracker. Sperm were collected from each of 10 semen donors. Sperm were diluted to 1.2 × 10^6 sperm/mL in a 12 × 75 mm polypropylene tube as outlined by Froman and Feltmann (2010). Each sperm suspension contained 5 mM 1,2-bis-(o-aminophenoxy)ethane-N,N’,N”,N”-tetraacetic acid (BAPTA) in TES-buffered NaCl, pH 7.4. Each tube was incubated at 30°C within a water bath. A series of 7-µL samples was withdrawn over a 90-min interval. Each sample was transferred to a 50-µm microcell prewarmed to 30°C, the temperature at which data were collected. Each open circle is a mean ± SD. The solid line denotes the parabolic function \( y(x) = 0.79 + (0.009)x - (0.0002)x^2 \). As motile concentration approached zero, Ca^{2+} was added to the residual sperm suspension to overcome the effect of BAPTA. Immediately thereafter, a 3.5-fold isothermal increase in motile concentration was observed. This experiment demonstrated that intracellular Ca^{2+} enabled motility while sperm were incubated in a Ca^{2+} sink.

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To date, the author has yet to improve sperm mobility apart from genetic selection (Froman et al., 2002; Froman and Rhoads, 2013). Therefore, it was anticipated that any hypothetical relationship would most likely describe compromised sperm mobility. For this reason, roosters from the author’s high sperm mobility line were used as semen donors. Based on the author’s work to date, the difference between the semen donors used in this study and high sperm mobility males within populations of noncommercial and commercial chickens is the frequency at which such roosters are found as opposed to what determines their phenotype.
motile sperm. If so, then self-propulsion might be independent of extracellular pH within reason. In any event, the objective of this experiment was to define a function from which a prediction could be made, and this goal was realized. The importance of the experimental outcome can be explained as follows. The effect of pH on sperm motility was addressed by Ashizawa et al. (2000). The authors state that sperm motility experiments are typically performed at pH 7.4. This practice may have begun with G. Wishart, who initiated the rigorous investigation of biochemical mechanisms underlying fowl sperm motility in the early 1980s and then collaborated extensively with K. Ashizawa throughout the 1990s. Thus, it was by convention that the author adopted pH 7.4 as the operative pH for the sperm mobility assay (Froman and McLean, 1996) and washing sperm through 12% (wt/vol) Accudenz (McLean et al., 1998). What Ashizawa et al. (2000) did not mention was that the use of experimental solutions at pH 7.4 was based on reasonable assumptions and emulation rather than a predicted value that could confirmed independently. The author concedes that fowl sperm mobility may not be subject to a narrow pH optimum. However, if this is the case, this fact does not invalidate the method used to prove the point.

The pronounced effect of extracellular osmolality on sperm mobility was attributed to the relationship between the volume of the mitochondrial matrix and metabolic rate (Kaasik et al., 2007). Thus, diminution of sperm mobility below the optimum was attributed to mitochondrial damage induced by swelling whereas the diminution of sperm mobility above the optimum was attributed to mitochondrial contraction and reduced power output. The latter effect was reversible (Fig. 5). This phenomenon has 2 implications. First, it provides a hypothetical mechanism by which fowl sperm obtain the potential for progressive motility as they pass through the excurrent ducts of the testis. Second, it demonstrates that fowl sperm can recover from an imposed, nonphysiological condition.

In review, the experimental outcomes summarized in the first 5 figures confirmed that BAPTA-mediated disruption of mitochondrial Ca\(^{2+}\) cycling could be used to procure immobile sperm that could be used to derive useful functions. However, even though BAPTA proved useful in this new application (see Froman, 2003; Froman and Feltmann, 2010; Froman, 2013), such utility did not address the question of whole cell Ca\(^{2+}\) flux. This topic warranted investigation for the following reasons. First, it stands to reason that the fowl sperm’s nuclear envelope contains Ca\(^{2+}\) because this organelle is a variant of the endoplasmic reticulum, an organelle known to sequester Ca\(^{2+}\). Therefore, it may be easier to disrupt mitochondrial Ca\(^{2+}\) cycling than to eliminate intracellular Ca\(^{2+}\). Second, one’s choice of Ca\(^{2+}\)-specific fluores-
cent dye determines whether one visualizes the nucleus and midpiece (Froman et al., 2006) or just the midpiece (Froman and Feltmann, 2010). Third, thapsigargin induces Ca\(^{2+}\) efflux from the endoplasmic reticulum, and this substance induced formation of the mitochondrial permeability transition pore in fowl sperm (Froman et al., 2006). As reviewed by Nicholls and Ferguson (2002), mitochondrial Ca\(^{2+}\) uptake is electroforetic, and excess uptake leads to formation of the mitochondrial permeability transition pore. Once formed, this pore dissipates the mitochondrial membrane potential, ATP regeneration is terminated, and necrosis ensues. As stated above, this mechanism is operative within fowl sperm (Froman et al., 2006) and accounts for the ultrastructure of mitochondria within immobile sperm (Froman and Kirby, 2005).

To date, BAPTA has proven most effective when treated sperm are briefly incubated at body temperature and then cooled to 30°C while sperm are centrifuged through 12% (wt/vol) Accudenz. This temperature change could not be simulated within a water bath. Therefore, a steady state was used for simplicity. In addition, this experiment was performed with the knowledge that motile concentration decreased exponentially when sperm were incubated at 41°C in a Ca\(^{2+}\) sink (Froman, 2003). Therefore, a protracted exponential decay was anticipated at 30°C. However, as shown in Fig. 6, this did not happen. Rather, data points conformed to a parabolic relationship with a maximum after 26 min of incubation. Fowl sperm are not progressively motile without mitochondrial Ca\(^{2+}\) cycling (Froman and Feltmann, 2005, 2010). Therefore, the motility of sperm in the Ca\(^{2+}\) sink was attributed to the flux of intracellular Ca\(^{2+}\) through the mitochondria and then into the surrounding buffer. As evidenced by the instantaneous effect of supplemental Ca\(^{2+}\) on motile concentration, sperm incubated without an exogenous substrate for 90 min were not damaged. Sperm motility increased from 25 to 83% when the effect of the Ca\(^{2+}\) sink was overcome. Although average VSL did not increase in proportion (Fig. 7), the abrupt increase in average VSL is even more telling because this effect documented an isothermal increase in intracellular energy production that affected sperm cell propulsion. Parenthetically, the data summarized in Figs. 6 and 7 call into question the notion that fowl sperm are motile at 30°C in the absence of Ca\(^{2+}\). The awkward term ‘reversible temperature-dependent motility inhibition of fowl sperm’ pertains to the observation that fowl sperm, in the absence of extracellular Ca\(^{2+}\), are immotile at body temperature but motile at 30°C (see Ashizawa et al., 2000). In fact, fowl sperm become immotile at either temperature should one apply a Ca\(^{2+}\) sink and then wait long enough. In any event, computer-assisted sperm motion analysis demonstrated the challenge inherent to manipulating Ca\(^{2+}\) within fowl sperm.

Such manipulation links the experimental outcomes presented in this manuscript with a bioenergetics approach to sperm preservation. In other words, the present work is a logical step from the realization that mitochondrial Ca\(^{2+}\) cycling can be manipulated for the purpose of in vitro sperm preservation (Froman and Feltmann, 2010). In this regard, 4 premises are proposed. First, sperm mobility phenotype must be taken into account. Recall that the sperm mobility assay is, in effect, a bioassay for sperm cell mitochondria. Moreover, there can be no reasonable expectation that sperm preservation will succeed—by virtue of any method—if a minority of sperm within an ejaculate has functional mitochondria. Unfortunately, this is indeed the case for many roosters in view of published and proprietary phenotype distributions compiled by the author to date. Second, maintenance of mitochondrial Ca\(^{2+}\) cycling is perhaps the most critical variable because fowl sperm do not function as self-propelled DNA delivery vehicles when this phenomenon is disabled (Froman, 2003; Froman and Feltmann, 2005; Froman and Feltmann, 2010). Third, the sperm mobility assay can be used to define relationships that predict the status of sperm cell mitochondria in response to sperm cell manipulation. This ability adds a procedural aspect to the paradigm outlined by Froman and Feltmann (2010). Fourth, mitochondrial Ca\(^{2+}\) cycling should be understood in terms of whole-cell Ca\(^{2+}\) flux.

REFERENCES


