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Oxidative Phosphorylation Is Essential for Felid Sperm Function, but Is Substantially Lower in Cheetah (*Acinonyx jubatus*) Compared to Domestic Cat (*Felis catus*) Ejaculate[1]

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ABSTRACT

Compared with the normospermic domestic cat, sperm metabolic function is compromised in the teratospermic cat and cheetah, but the pathway(s) involved in this deficiency are unknown. Glycolysis is essential for sperm motility, yet it appears to function normally in spermatozoa of either species regardless of structural morphology. We conducted a comparative study to further understand the mechanisms of energy production in felid spermatozoa, with the hypothesis that oxidative phosphorylation is required for normal sperm function and is impaired in teratospermic ejaculates. Electroejaculates from both species were stained with MitoTracker to quantify mitochondrial membrane potential (MMP) or were incubated to assess changes in sperm function (motility, acrosomal integrity, and lactate production) after mitochondrial inhibition with myxothiazol. Sperm midpiece dimensions also were quantified. Sperm mitochondrial fluorescence (directly proportional to MMP) was ~95% lower in the cheetah compared with the normospermic and teratospermic cat, despite the cheetah having a 10% longer midpiece. In both species, MMP was increased 5-fold in spermatozoa with retained cytoplasm compared with structurally normal cells. Inhibition of oxidative phosphorylation impaired sperm function in both species, but a 100-fold higher inhibitor concentration was required in the cat compared with the cheetah. Collectively, findings revealed that oxidative phosphorylation was required for sperm function in the domestic cat and cheetah. This pathway of energy production appeared markedly less active in the cheetah, indicating a species-specific vulnerability to mitochondrial dysfunction. The unexpected, cross-species linkage between retained cytoplasmic droplets and elevated MMP may reflect increased concentrations of metabolic enzymes or substrates in these structures.

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INTRODUCTION

Oxidative phosphorylation is an active pathway of sperm energy production in all mammalian species studied to date, including the bull, ram, boar, rabbit, mouse, and man (reviewed in Storey [1]). Although human spermatozoa were once considered to be exclusively glycolytic [1], cellular respiration now is known as a significant source of ATP [2] and is required to maintain motility in these cells [2–6]. Human infertility and/or asthenospermia are linked to various indicators of impaired oxidative phosphorylation in spermatozoa, including reduced midpiece length [7], abnormal mitochondrial organization [7–9], reduced oxygen consumption [10], and decreased mitochondrial enzyme activity [11]. However, the relationship between mitochondrial ATP production and fertilizing ability in species other than the human is less clear. For example, although oxidative phosphorylation supplies the majority of ATP in bovine spermatozoa [12], bull fertility can be predicted by sperm motility values, but not by mitochondrial membrane potential [13, 14]. Conversely, although motility and capacitation of murine spermatozoa are unaffected by chemical inhibition of oxidative phosphorylation [15, 16], *Smcp*$^{-/-}$ (sperm mitochondrion-associated cysteine-rich protein) knockout mice are infertile and experience reduced sperm motility and zona penetration ability [17].

Little is known about the mechanisms of sperm energy production in the domestic cat or its wild relatives. The cat itself is a valuable model for understanding gamete physiology, because there are two readily available phenotypes, normospermic versus teratospermic (ejaculating $\geq$60% structurally abnormal spermatozoa) [18, 19]. Because teratospermia is highly prevalent in men [20] and considered ubiquitous in certain rare felid species (most notably the cheetah, clouded leopard [*Neofelis nebulosa*], and Florida panther [*Puma concolor coryi*]) [19], understanding the physiological consequences of this condition has application to both human fertility and wildlife management/conservation. We recently determined that rates of sperm lactate production are reduced in the teratospermic domestic cat and cheetah compared with the normospermic domestic cat [21]. In both species, lactate production is positively correlated to sperm motility, acrosomal integrity, and normal morphology, yet appears to be indirectly related to cellular energy production. Glucose uptake by these

cells is minimal or absent [21], and the majority of lactate is instead produced by the reduction of exogenous pyruvate [22]. This process does not appear to serve any physiological purpose, as sperm motility and acrosomal integrity are unaffected when lactate dehydrogenase is chemically inhibited or when pyruvate is absent from the culture medium [22]. Although it has a questionable role in felid sperm fertilizing ability [22], lactate production may indirectly indicate NADH availability that, in turn, is important in maintaining REDOX potential [23] and in ATP generation via oxidative phosphorylation [24]. If this prediction is correct, reduced rates of lactate production may reflect mitochondrial dysfunction in teratospermic domestic cat and cheetah ejaculates. Alternately, the physiological mechanisms causing impaired lactate production may differ between the two species. Regardless, it is likely that a mitochondrial deficiency would significantly impair energy generation by the felid spermatozoon, particularly because our previous investigations suggest a key role of oxidative phosphorylation in these cells [21, 22]. Although cat and cheetah spermatozoa metabolize little or no exogenous glucose, we have discovered that the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is required to maintain sperm progressive motility in both species [22]. We predict that the importance of GAPDH in felid spermatozoa relates to its role in glycerol breakdown, and that these cells rely primarily on the metabolism of endogenous phospholipid to support motility and viability. Such a mechanism would explain why felid spermatozoa remain motile for at least 7 h in a substrate-free medium [22], and would suggest that mitochondrial metabolism (of fatty acids and/or glycerol-derived pyruvate) is a substantial source of cellular ATP.

To better understand the mechanisms of cellular metabolism in felid spermatozoa, we conducted a comparative assessment of sperm oxidative phosphorylation in the normospermic versus teratospermic domestic cat and the teratospermic cheetah. We tested the hypotheses that oxidative phosphorylation is required for sperm motility and viability in the cat and cheetah, and that mitochondrial function is impaired in the teratospermic phenotype. We anticipated that knowledge of these mechanisms could yield insight into the disruptions of various energy-dependent processes (e.g., sperm motility [21], protein tyrosine phosphorylation [25], and acrosome reaction [26]) usually associated with teratospermia in felids, and could provide clues for improving the success of reproductive technologies (e.g., sperm cryopreservation) useful to improving both human fertility and wildlife management/conservation.

MATERIALS AND METHODS

Animals

The domestic cat sperm donors were seven adult (ages 1.5–8 yr) males previously determined to be normospermic (n = 2 males, n = 6 total ejaculates) or teratospermic (n = 5 males, n = 8 total ejaculates) [21, 22]. Management protocols for this species at the Smithsonian Conservation Biology Institute (SCBI; Front Royal, VA) have been described in detail [21].

Electroejaculates (one per male, 16 males) were collected from adult cheetahs (ages 2–10 yr) housed at the Cheetah Conservation Fund (CCF; Otjiwarongo, Namibia; n = 1), White Oak Conservation Center (WOCC; Yulee, FL; n = 4), the SCBI (n = 5), the Philadelphia Zoo (PHL; Philadelphia, PA; n = 3), The Wilds (TW; Cumberland, OH; n = 2), and the Cleveland Metroparks Zoo (CLE; Cleveland, OH; n = 1). The male at CCF was wild born and was housed singly, as described previously [27]. Males at WOCC, SCBI, and PHL were captive born and were managed as explained previously [26], and represented one (PHL) or two (WOCC, SCBI) sibling groups at each institution. Both males at TW were captive born: one was maintained singly in a 5000-m^2 outdoor enclosure and the other was maintained with two unrelated males in a 500-m^2 outdoor enclosure. The male at CLE was captive born and managed on exhibit with a male sibling in a 900-m^2 outdoor enclosure. Males at TW and CLE were fed a commercially produced diet (Premium Beef Feline Diet; Central Nebraska Packaging Inc., North Platte, NE), and diets for this species in all other institutions have been reported previously [21, 22]. There was no statistical influence of location on any sperm quality metric ($P \geq 0.27$), and we previously have determined that ejaculate quality is similar between wild-born Namibian cheetahs and captive individuals at North American institutions [28, 29]. Reagents and semen collection equipment were transported from SCBI to each study site, and all samples were shipped back to our laboratory for analyses of metabolic rates, mitochondrial membrane potential, sperm morphology, and acrosomal integrity.

Semen Collection

A surgical plane of anesthesia was induced in domestic cats and cheetahs for semen collection according to protocols developed by institutional veterinarians and were the same as we have described previously [26, 27]. All animal procedures were approved by National Zoological Park's Animal Care and Use Committee and similar committees of collaborating institutions. Semen was collected using a rectal probe of 1 cm (domestic cat) or 1.9 cm (cheetah) in diameter and an electrostimulator (P.T. Electronics, Boring, OR) as described earlier [21, 30].

Sperm Processing

Immediately after collection, a 10-μl aliquot of raw semen containing approximately 2×10^5 spermatozoa was fixed in 0.3% glutaraldehyde in phosphate-buffered saline for assessment of sperm morphology [21, 30, 31]. Each ejaculate then was diluted with an equal volume of a chemically defined, protein-free, modified mouse tubal fluid medium (cMTF) [32] supplemented with 2% polyvinyl alcohol [33]. The cMTF medium was prepared as described previously [21] and contained 98.4 mM NaCl, 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 1 mM glucose, 1 mM Na-pyruvate, 25 mM 3-(*N*-morpholino) propanesulfonic acid buffer, and 0.02 mg/ml phenol red. All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Osmolality of the final working medium (315–345 mOsm) was determined using a vapor pressure osmometer (Wescor Inc., Logan, UT) and was within 10% of the physiological value of domestic cat semen (323 mOsm [34]). Sperm concentration was determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) [35].

Oxidative Inhibition

Each diluted ejaculate (maintained at ambient temperature, 19°C–22°C) was washed by centrifugation (8 min; 300 × *g* for domestic cat; 100 × *g* for cheetah) and resuspended in cMTF. To determine the influence of inhibition of oxidative phosphorylation, a sperm sample (3×10^6 motile sperm per milliliter) from each ejaculate (n = 6 normospermic cat, n = 6 teratospermic cat, n = 8 cheetah) was exposed to 160 nM myxothiazol (known to block transfer of electrons from complex III to cytochrome C [5]; included in the resuspension medium) and incubated in parallel with negative controls. This myxothiazol concentration has been used to study mammalian sperm metabolism previously [12] and was the minimum required to disrupt cheetah sperm motility in dose-dependent trials (0.16 nM, 16 nM, and 160 nM; data not shown). Because domestic cat spermatozoa were not influenced by the 160 nM myxothiazol treatment, a subset of these ejaculates (n = 2 normospermic, n = 2 teratospermic) also were incubated in higher inhibitor concentrations (1.6 μM and 16 μM).

Sperm samples were cultured (37°C) in microcentrifuge tubes under oil to prevent evaporation, as described previously [21]. Assessments of sperm percentage motility (% M), forward progression (FPS), and acrosomal integrity (% IA) were made at 0, 1, 3, and 7 h of incubation. Motility was assessed visually (200×), and FPS was rated on a 0 to 5 scale, with a rating of 5 equivalent to most rapid, linear progress [30]. Spermatozoa (approximately 2×10^5 cells) were fixed in 4% paraformaldehyde and stained with Coomassie Blue G-250 (Fisher Biotech, Springfield, NJ) to evaluate acrosomal integrity using previously described methods [36, 37]. Spermatozoa with an intact acrosome exhibited a uniform blue staining pattern overlying the acrosomal region, whereas nonintact cells had clear or patchy staining over this region [37]. Because we discovered in an earlier study that there was a positive correlation between lactate production and sperm motility, acrosomal integrity, and normal morphology in both the cat and cheetah [19], we monitored rates of lactate production (Δ L) in each sample during the 7-h incubation period [21]. To determine lactate concentration, medium samples were centrifuged (8 min; 1000 × *g*) through a CoStar Spin-X 0.22-μm nylon filter tube (Corning Inc., Corning, NY) and stored at −80°C until analysis using a lactate dehydrogenase/glutamine-pyruvate transaminase-linked fluorescence assay [21, 32, 38]. Assay enzymes were purchased

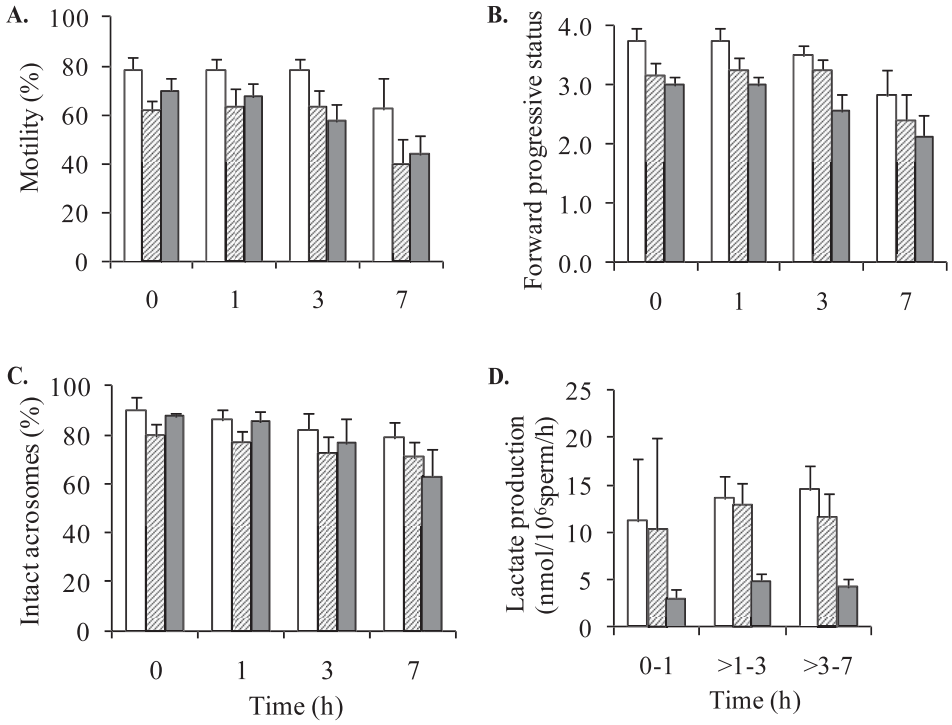

FIG. 1. Absolute values for percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in normospermic cat (white bar; n = 6), teratospermic cat (lined bar; n = 6), and cheetah (gray bar; n = 8) control sperm samples. Assessments at 0 h were made <5 min after sperm washing and resuspension in cMTF medium. Graphs illustrate least-squares means ± standard errors.

from Roche Applied Science (Indianapolis, IN) and fluorescence analyzed using a Spectra Max Gemini XPS fluorescent plate reader (340-nm excitation, 445 nm-emission) and SoftMax Pro 5 software (Molecular Devices, Sunnyvale, CA). The Δ L was calculated as the change in medium substrate concentration over time, divided by sperm concentration and reported in nanomoles per 10^6 sperm per hour. All data were normalized to control values for presentation.

Mitochondrial Membrane Potential

To assess mitochondrial membrane potential (MMP), samples of ejaculates (n = 5 domestic cat, n = 7 cheetah) were incubated (45 min; ambient temperature, 19°C–22°C) in the dark with 0.5 nM MitoTracker Red CMXRos (Molecular Probes Inc., Eugene, OR) in a volume of 100 μl at a concentration of 3×10^6 motile sperm per milliliter. To assess MMP, 100 spermatozoa per ejaculate were individually analyzed (400×) using a BX40 fluorescence microscope (555-nm excitation; Olympus America Inc., Center Valley, PA), and fluorescence was quantified using a Sensicam qe high-performance camera (Cooke Corp., Romulus, MI) and IP Lab v4.04 software (BD Biosciences, Rockville, MD). The presence of sperm structural abnormalities was recorded (as described previously [21]), except for acrosomal deformities, which were not reliably detected at this magnification. Spermatids and retained cytoplasmic droplets represented the majority (~80%) of sperm malformations in each species. For data analysis, each spermatozoon was classified into one of the following five major morphotypes: structurally normal, midpiece droplet, flagellar droplet, spermatid, and ''other'' [21]. The category of ''midpiece droplet'' primarily represented a spermatozoon with a bent midpiece encircling residual cytoplasm, but it also included spermatozoa with a proximal droplet attached to a straight midpiece. In contrast, a flagellar droplet was always associated with a structural bend in the tail region. The ''other'' category consisted of rarer type malformations that collectively comprised <10% of all spermatozoa in each species and included head deformities and midpiece anomalies or aplasia.

Sperm Morphometrics

A sperm sample (approximately 2×10^5 cells) from each ejaculate used to assess MMP also was fixed in 4% paraformaldehyde for morphometric evaluation. Dimensions of head length and width, midpiece length and width, and principal piece length were measured for 50 spermatozoa (1000×) per subsample by phase-contrast microscopy using the Sensicam qe high-performance camera and IP Lab v4.04 software. Spermatozoa with complete or partial midpiece aplasia (<2% of total) were excluded from this assessment.

Statistical Analyses

Data were analyzed with statistical analysis software (SAS) version 9.1 (SAS Institute, Cary, NC), and percentage data were arcsine transformed before evaluation. Pearson correlation was used to evaluate the relationships among sperm quality metrics (% M, FPS, % IA, and Δ L) across all samples. Data for OXPHOS-inhibited samples were normalized (i.e., expressed as percentages of control values) for figure presentation only; statistical analyses were performed on raw data (i.e., absolute values for inhibited and control samples). The interaction between inhibition of oxidative phosphorylation and domestic cat group (normospermic and teratospermic) was assessed using SAS General Linear Model (GLM) Procedures [39], with % M, FPS, % IA, and Δ L included as response variables. Treatment and domestic cat group were considered class variables, and time was included as a covariate. The interaction between inhibition of oxidative phosphorylation and species (domestic cat and cheetah) was assessed using a GLM as described above. Within species, treatment effects were analyzed separately for each time point using paired *t*-tests (treatment versus control). Pearson correlation was used to evaluate the relationships between sperm morphology and changes in % M, FPS, % IA, and Δ L (relative to controls) at the end of incubation. Differences in mean MMP, % M, FPS, and % structurally normal spermatozoa of MitoTracker-stained samples were analyzed using a GLM and Tukey test with animal group (normospermic cat, teratospermic cat, and cheetah) included as a class variable. Differences in MMP among sperm morphotypes were evaluated using a separate GLM for each animal group, and means were evaluated using Tukey test. Mean sperm dimensions were calculated for each male, and these values then were analyzed using a GLM with species included as a class variable. Results were considered significant at $P < 0.05$ and are reported as least-squares means ± SEM. Differences in sperm morphometrics among males were evaluated using Duncan new multiple-range test.

RESULTS

Inhibition of Oxidative Phosphorylation

Consistent with our previous findings [21], rates of sperm lactate production (Δ L) were correlated positively ($P < 0.0001$) to % M ($r = 0.49$), FPS ($r = 0.42$), and % IA ($r = 0.36$) across all samples. To facilitate comparisons between the two species, data for all treatment groups were normalized to controls, with absolute values for control samples presented in Figure 1. Compared with untreated controls (Fig. 1), domestic

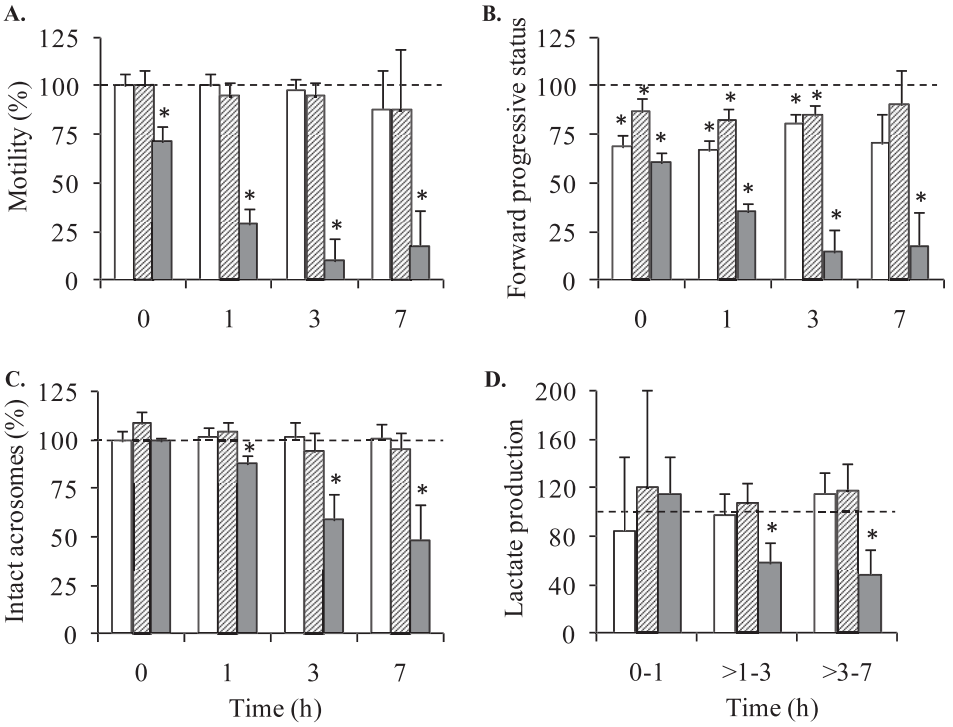

FIG. 2. Percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in normospermic cat (white bar; n = 6), teratospermic cat (lined bar; n = 6) and cheetah (gray bar; n = 8) sperm samples incubated with 0.16 μM myxothiazol. Assessments at 0 h were made <5 min after sperm washing and resuspension in cMTF with myxothiazol. Data are expressed as percentages of control values (dashed line). Within each time point and animal group, bars with an asterisk (*) differ from controls ($P < 0.05$). Graphs illustrate least-squares means ± standard errors.

cat sperm from normospermic and teratospermic donors were unaffected ($P \geq 0.36$) for sperm % M, % IA, and Δ L when exposed to 0.16 μM myxothiazol (Fig. 2, A, C, and D); there was a modest, but significant (~20%; $P < 0.05$), loss in FPS (Fig. 2B). By contrast, there was an immediate and marked decline ($P < 0.05$) in cheetah sperm % M and FPS, with a ~50% reduction ($P < 0.05$) in % IA and Δ L after 7 h of incubation (Fig. 2). This species-treatment interaction was highly significant ($P < 0.0001$) for % M, FPS, and % IA, but represented a trend ($P = 0.08$) for Δ L. There was no interaction ($P = 0.54$) between domestic cat sperm phenotype (normospermic versus teratospermic) and myxothiazol treatment.

Substantial decreases in % M, FPS, % IA, and Δ L were only observed ($P < 0.05$) in domestic cat ejaculates exposed to the highest concentration (16 μM) of myxothiazol (Fig. 3). These declines became more severe ($P < 0.05$) over time, with the exception of Δ L (due to larger standard errors). Absolute values for control samples in this treatment group (data not shown) were similar ($P = 0.83$) to those from domestic cats in the previous treatment group (Fig. 1).

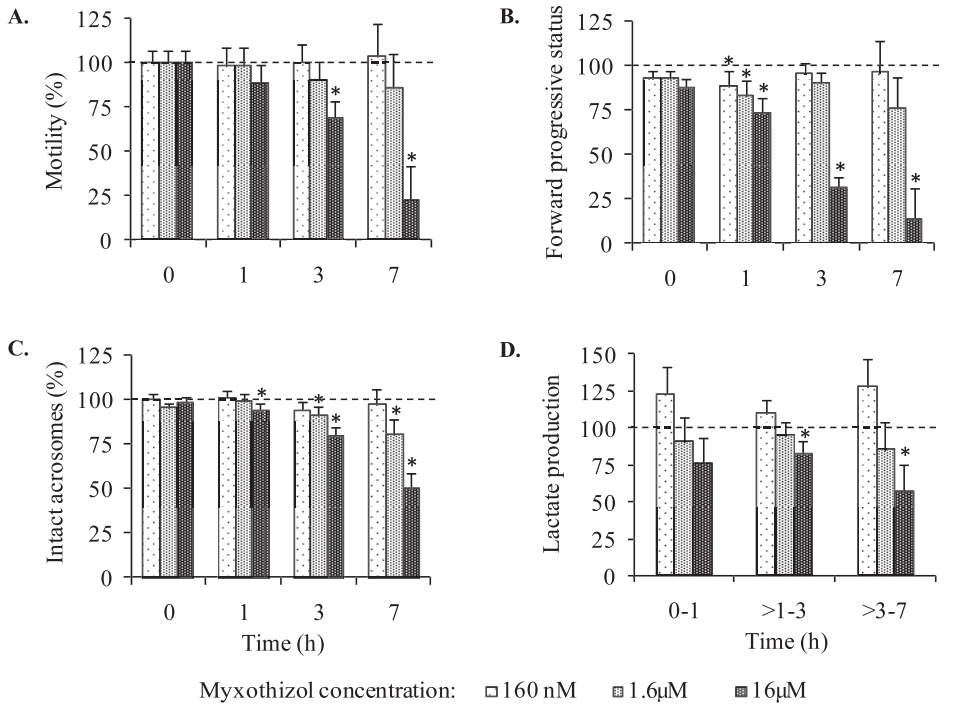

FIG. 3. Percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in domestic cat sperm samples (n = 4) incubated with increasing concentrations of myxothiazol to inhibit mitochondrial function. Assessments at 0 h were made <5 min after resuspending centrifuged sperm in cMTF medium with myxothiazol. Data are expressed as percentages of control values (dashed line). Within each time point, bars with an asterisk (*) differ from controls ($P < 0.05$). Graphs illustrate least-squares means ± standard errors.

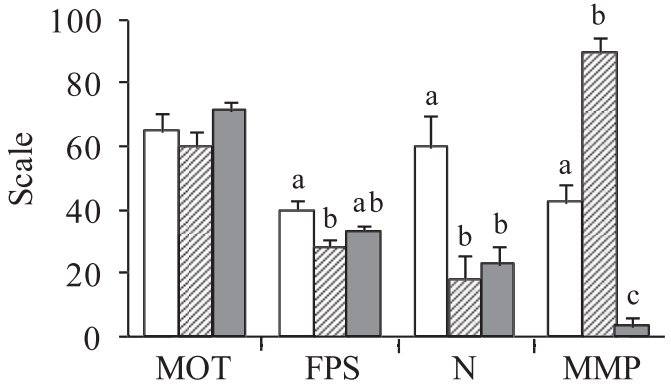

FIG. 4. Sperm MMP in normospermic cat (white bar; n = 2), teratospermic cat (lined bar; n = 3), and cheetah (gray bar; n = 7) sperm samples in relation to percent motility (MOT), FPS (scale, 0–5), and percent normal morphology (N). To allow the presentation of metrics on a single scale, values were increased 10× for FPS and decreased 1000× for MMP. Within each metric and among animal groups, bars with different superscripts differ ($P < 0.05$). Graphs illustrate least-squares means ± standard errors.

MMP and Sperm Morphometrics

Sperm fluorescence after MitoTracker staining (which is directly proportional to MMP) was substantially lower ($P < 0.0001$) in the cheetah compared with the normospermic and teratospermic domestic cat (Fig. 4). In contrast, sperm % M, FPS, and the percentage of structurally abnormal cells were similar ($P \geq 0.16$) in cheetahs compared with normospermic and/or teratospermic cats (Fig. 4). Fluorescence analysis of individual sperm morphotypes determined that the influence of cellular structure on MMP was consistent among normospermic cats (Fig. 5A), teratospermic cats (Fig. 5B), and cheetahs (Fig. 5C). Specifically, in both species, the presence of a retained cytoplasmic droplet at the midpiece was associated with a 3- to 5-fold increase ($P < 0.05$) in MMP compared with structurally normal spermatozoa (Fig. 5). Flagellar droplet and spermatid morphotypes demonstrated MMP values that were similar ($P \geq 0.20$) to midpiece droplet morphotypes and, in the teratospermic cat and cheetah, also were similar ($P \geq 0.20$) to structurally normal cells. Sperm MMP values in cells classified as ''other'' were similar ($P \geq 0.20$) to normal and/or midpiece droplet morphotypes, depending on the animal group, which was expected, given the diversity of abnormalities within this category. The use of the camera system to analyze MMP allowed determining that, although it was associated with increased fluorescence, retained sperm cytoplasm did not fluoresce in either species (Fig. 6).

Based on earlier data from teratospermic men [7], we anticipated that reduced sperm MMP activity could be associated with a shorter sperm midpiece. In contrast to this expectation, the midpiece was ~10% longer ($P < 0.05$) in the cheetah compared with the domestic cat spermatozoon (Table 1). Other sperm dimensions were similar ($P \geq 0.14$) between species (Table 1).

DISCUSSION

This was the first study of oxidative metabolism in felid spermatozoa, and we made three significant discoveries. First, oxidative metabolism was required to maintain sperm motility and viability in both the domestic cat and cheetah. Thus, certain mechanisms of sperm energy production in felids appear similar to those in the boar [40] and human [2, 3], but are different from those in the mouse [16] and dog [41, 42] (referenced in Bartlett [43]). Second, fluorescence of MitoTracker-stained spermatozoa, an indicator of MMP, was markedly lower in the cheetah compared with both the normospermic and teratospermic domestic cat. This species specificity was not explained by a reduction in sperm midpiece length, which in the human is associated with fewer mitochondrial gyres, asthenospermia, and male infertility [7]. On the contrary, the midpiece of the cheetah spermatozoon actually was longer than that of the cat counterpart. Finally, common sperm malformations were associated with elevated mitochondrial activity in both species, which may help explain why reproductive physiologists have long been perplexed by the inability of certain traditional quality metrics (e.g., MMP, motility) to accurately predict sperm fertilizing ability [44].

An advantage of our research approach was the cross-species comparative assessment that allowed us to discover a 100-fold increased sensitivity to electron transport inhibition and evidence for a remarkably low MMP in the cheetah spermatozoon compared with the domestic cat. Although fluorescence of MitoTracker-stained spermatozoa is directly proportional to MMP, it is important to note that this species difference also could be related to uncontrolled factors influencing the dye's accumulation in the mitochondrion, such

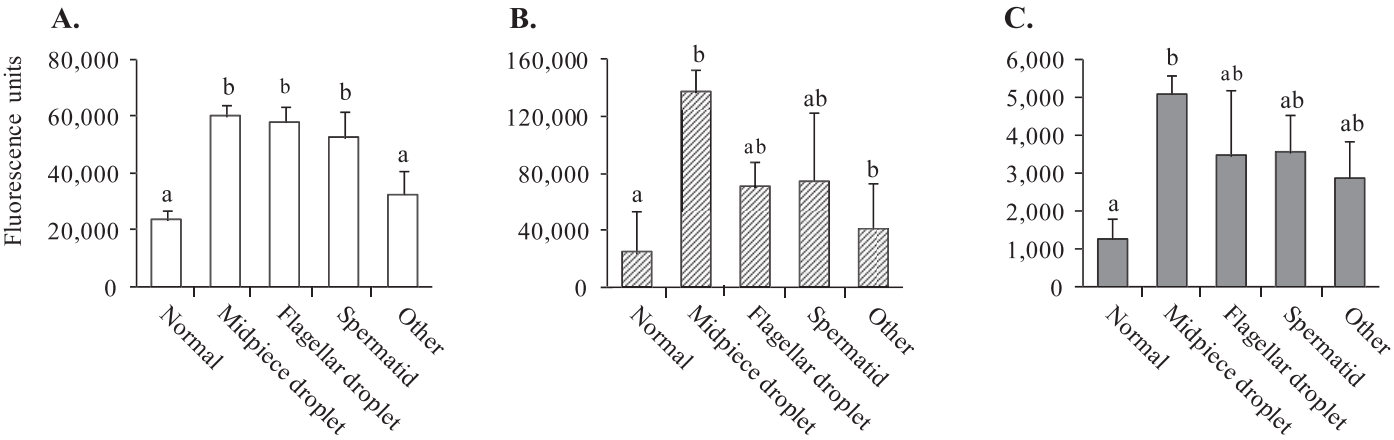

FIG. 5. Sperm MMP in normospermic cat (**A**; n = 2), teratospermic cat (**B**; n = 3), and cheetah (**C**; n = 7) ejaculates in relation to cellular morphotype. Note the differences in scale among the three graphs. Among sperm morphotypes and within each species, bars with different superscripts differ ($P < 0.05$). Graphs illustrate least-squares means ± standard errors.

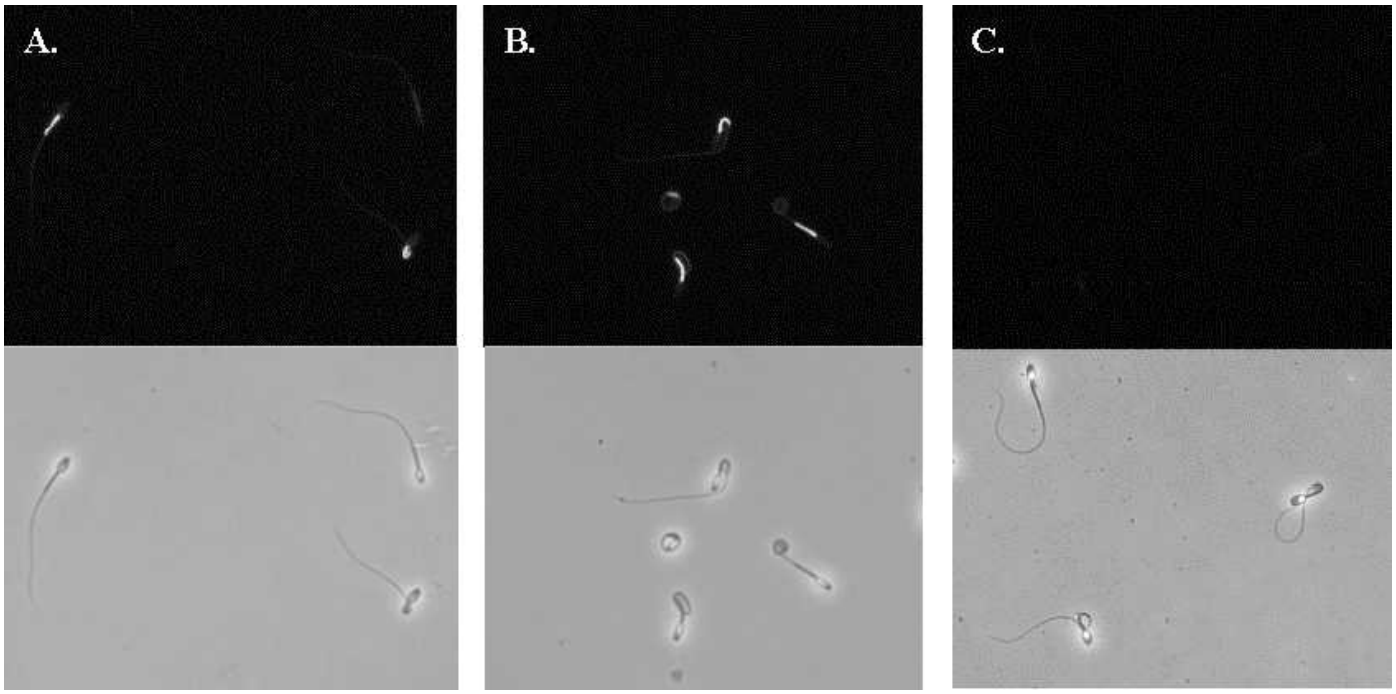

FIG. 6. Representative fluorescence (top row) and phase-contrast images (bottom row) of Mitotracker-stained normospermic cat (**A**), teratospermic cat (**B**), and cheetah spermatozoa (**C**). Original magnification ×400.

as the degree of binding to cellular structures, facilitated diffusion, or complex ion interactions [45]. However, given our standardization of MitoTracker and sperm concentrations, extended incubation time, use of the same cellular medium, and, particularly, the 20-fold magnitude of the MMP difference, we believe that this finding indicated that there was a functional difference in membrane potential between the species. Combined with the relative sensitivity to OXPHOS inhibition, these observations provided evidence for mitochondrial dysfunction in cheetah spermatozoa. This may well represent the mechanism underpinning the functional abnormalities observed in teratospermic felid ejaculates, including reduced sperm longevity in vitro [46], poor motility [47], disrupted protein tyrosine phosphorylation [25], delayed capacitation [26], compromised acrosomal function [26], and low in vitro fertilization success [48]. Interestingly, however, sperm MMP and sensitivity to oxidative inhibition were similar between the normospermic and teratospermic domestic cat, even though sperm lactate production was previously found to be compromised in a large set of ejaculates from the latter group [21]. This observation may indicate that the assemblage of genes involved in teratospermia differs between the domestic cat and cheetah [49], a reasonable hypothesis given that the trait presumably arose in the latter species after a severe population contraction that occurred ~10 000 years ago [50], long after the evolutionary divergence from other felids ~6 000 000 years ago [51].

In contrast to our previous study [21], lactate production of control sperm samples was reduced by only 20% or less in teratospermic compared with normospermic domestic cats, a trend that was insignificant. Although perhaps due to our overall smaller sample size in the present study, we suspect that this observation reflected physiological differences among the males used in the two investigations. Substantial variation in sperm motility and longevity in vitro commonly occurs among teratospermic domestic cats, and it is likely that such variation is driven by differences in metabolic function and, ultimately, the genes causing this condition. Interestingly, analogous differences in ejaculate quality are commonly detected among teratospermic cheetahs, even though we have observed little variation in metabolic function within this genetically monomorphic species [21, 22].

It was noteworthy that although sperm mitochondrial activity was ~95% lower in the cheetah compared with the domestic cat, cellular percent motility and FPS were similar between species. This may have indicated that lower sperm MMP in the cheetah was indeed ''normal'' and not representative of metabolic dysfunction. If so, glycolytic metabolism could compensate for relatively low mitochondrial activity. In this context, our previous finding that GAPDH inhibition causes less severe motility declines in cheetah versus cat spermatozoa [22] could be interpreted as evidence that the glycolytic pathway is comparatively more robust in cheetah ejaculates. It also is possible that energy demand was reduced in cheetah compared with domestic cat spermatozoa, perhaps because of differences in the efficiency of microtubule sliding or ATP transport along the flagellum [52]. However, we

TABLE 1. Dimensions of domestic cat[†] and cheetah[‡] spermatozoa (mean ± SEM).

Sperm dimension (μm)	Domestic cat	Cheetah
Head length	4.54 ± 0.15	4.38 ± 0.15
Head width	2.22 ± 0.10	2.36 ± 0.10
Midpiece length	7.68 ± 0.13[a]	8.47 ± 0.13[b]
Midpiece width	0.80 ± 0.01	0.79 ± 0.01
Principal piece length	40.06 ± 0.76	40.12 ± 0.76
Total length	52.29 ± 0.88	52.96 ± 0.88

[†] Domestic cats: n = 5 males (one ejaculate per male).
[‡] Cheetahs: n = 7 males (one ejaculate per male).
[a,b] Within rows, values with different superscripts differ ($P < 0.05$).

suspect that the most likely explanation for similar sperm motility traits between species was that the low MMP of cheetah spermatozoa was just above the threshold required to support sperm function, and that the rapid losses in motility commonly observed in these ejaculates [21, 47, 53] resulted from unsustainable ATP consumption. In this case, our previous observations of reduced sensitivity of cheetah ejaculates to glycolytic inhibition [22] could indicate that this pathway is simply less active in the cells of this species.

While determining that the low MMP in cheetah spermatozoa was unrelated to midpiece length, we also had the opportunity to compare other cellular dimensions. Such data are rare for wild felids, although we discovered that the cheetah spermatozoon had a shorter and narrower (~30%) head than a previous report for the tiger (*Panthera tigris*) [54], and a shorter (~30%) midpiece than for the leopard (*Panthera pardus*) [55], both of which are considered normospermic species. No doubt the size metrics of the cheetah spermatozoon more closely resembled the domestic cat because these species are more genetically similar compared with the tiger or leopard [51].

Also related to sperm morphology was its interesting link to MMP, an association that was remarkably consistent between the two species. A spermatozoon with retained cytoplasm is a common pleomorphism in the cat [18, 21] and cheetah [21, 28], as well as certain other species/populations that experience teratospermia [46, 56–67]. We discovered in the present study that this malformation was associated with substantially increased MMP. To a lesser but nonsignificant extent, increased MMP also was observed in spermatozoa with retained cytoplasm in the flagellar region, suggesting that the proximity of the droplet to the midpiece influenced its ultimate impact on MMP. The relationship between a retained droplet and enhanced mitochondrial function was unexpected because in the human, mouse, stallion, and boar, this residual cytoplasm is rich in lysosomal enzymes (e.g., 15-lipoxygenase [15-LOX]) that degrade organelles, including mitochondria via phospholipid peroxidation [68, 69]. We have identified three possible mechanisms by which retained cytoplasm could confer increased MMP, the first being that there simply could be functional mitochondria in the residual droplet that causes the fluorescence. Although such sperm cytoplasm in mammals generally does not contain mitochondria [43, 68], a recent study documented intact mitochondria embedded in the retained cytoplasm of sperm from *Alox15* knockout mice lacking the 15-LOX enzyme [58]. This possibility probably was unlikely in our current study because cytoplasmic droplets did not fluoresce after MMP staining, indicating an absence of functional mitochondria. Another alternative is that the retained droplets may be a source of metabolic substrates. For example, in the bull [70], ram [70], boar [71], rabbit [70], rat [72], and hamster [73], the droplet contains an abundance of membranous elements, which may be remnants of Golgi bodies or the endoplasmic reticulum and could provide substantial energy in the form of lipid [68]. A final option is that the preserved cytoplasm may contain factors that increase metabolic efficiency by directly stimulating mitochondrial activity or increasing the availability of existing substrates [74]. For example, glycolytic enzyme activity in free-lying (i.e., unattached to a spermatozoon) cytoplasmic droplets from bull, ram, and boar ejaculates can be up to 5-fold higher than in the spermatozoon itself [74]. If felid spermatozoa containing cytoplasmic droplets are enriched in enzymes involved in lipid/glycerol metabolism, a higher concentration of oxidative substrate may be available to the mitochondrion. Because the current study provides compelling evidence that oxidative metabolism plays a key role in felid sperm motility, we predict that the link between cytoplasmic droplets and increased MMP is related to the increased abundance or availability of lipid as a mitochondrial substrate.

Our observation of an association between a retained cytoplasmic droplet and increased MMP likely would confound mitochondrial-based estimates of sperm quality, because there is strong evidence that mammalian sperm function is compromised by residual cytoplasm [48, 75–80]. Although normally shed soon after ejaculation [81], retained droplets are known to reduce plasma membrane integrity [78], disrupt nuclear maturation [82], and decrease in vitro fertilization success [78] in humans. Cytoplasmic retention also reduces sperm-ovum interaction in vitro in the mouse [79], and appears to perturb capacitation in the dog [80]. Domestic cat spermatozoa with retained cytoplasm fail to penetrate the oocyte's inner zona pellucida, and therefore are incapable of fertilization [48]. Although felid spermatozoa with retained cytoplasm may demonstrate high MMP soon after ejaculation, these values could quickly decrease as lysosomal enzymes assault mitochondrial and plasma membranes, resulting in the rapid loss of viability. We suspect that this scenario may occur in many felid species because residual cytoplasm is a highly common anomaly in the Felidae taxon. Prevalence can range from ~10% to ~70% of ejaculated spermatozoa in the domestic cat [21], cheetah [21, 28], puma (*Puma concolor*) [60], leopard cat (*Prionailurus bengalensis*) [61], clouded leopard [62, 63], snow leopard (*Panthera uncia*) [64], Iberian lynx (*Lynx pardinus*) [65], fishing cat (*Prionailurus viverrinus*) [46], and sand cat (*Felis margarita*) [66]. Although several genes have been linked to cytoplasmic droplet retention using mouse knockout models [43, 57, 83], spermatozoa from these individuals commonly have other structural abnormalities (e.g., a hairpin bend in the neck or flagellum) that are rarely observed in felids [21, 28, 46, 60–66]. It is unlikely that the *ALOX15* gene is being disrupted in the cat or cheetah, because we observed no functional mitochondria in spermatozoa with retained droplets. Thus, we suspect that there remains a yet-to-be-determined genetic mechanism(s) by which sperm cytoplasmic droplet migration can become compromised in felids.

In conclusion, the results of this study demonstrate the value of a comparative approach to understanding sperm metabolic mechanisms related to distinctive species physiology versus teratospermia. Oxidative phosphorylation appears to be a critical pathway for supporting sperm motility in felids, yet the cheetah spermatozoon may operate at or below the minimum threshold of aerobic metabolism required to maintain cellular function. We predict that identifying sperm metabolic deficiencies that are driven by species-specific mechanisms or related to teratospermia will be the first step to developing effective strategies for mitigation. Given the remarkable differences in gamete physiology observed between these two closely related taxa (domestic cat and cheetah), it is clear that improving the success of assisted reproduction will require methods tailored to the unique physiology of the target species. By understanding and addressing sperm physiological disruptions at the fundamental level of energy production, we may be able to simultaneously overcome multiple functional deficiencies (e.g., poor motility, delayed capacitation) that arise from a common metabolic defect. This basic knowledge will provide a valuable foundation for future studies on the importance of sperm metabolism and teratospermia, with broad interest and, importantly, application to preserving fertility in humans and an array of domestic and wildlife species [84, 85].

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