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Glycolytic Enzyme Activity Is Essential for Domestic Cat (*Felis catus*) and Cheetah (*Acinonyx jubatus*) Sperm Motility and Viability in a Sugar-Free Medium[1]

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ABSTRACT

We have previously reported a lack of glucose uptake in domestic cat and cheetah spermatozoa, despite observing that these cells produce lactate at rates that correlate positively with sperm function. To elucidate the role of glycolysis in felid sperm energy production, we conducted a comparative study in the domestic cat and cheetah, with the hypothesis that sperm motility and viability are maintained in both species in the absence of glycolytic metabolism and are fueled by endogenous substrates. Washed ejaculates were incubated in chemically defined medium in the presence/absence of glucose and pyruvate. A second set of ejaculates was exposed to a chemical inhibitor of either lactate dehydrogenase (sodium oxamate) or glyceraldehyde-3-phosphate dehydrogenase (alpha-chlorohydrin). Sperm function (motility and acrosomal integrity) and lactate production were assessed, and a subset of spermatozoa was assayed for intracellular glycogen. In both the cat and cheetah, sperm function was maintained without exogenous substrates and following lactate dehydrogenase inhibition. Lactate production occurred in the absence of exogenous hexoses, but only if pyruvate was present. Intracellular glycogen was not detected in spermatozoa from either species. Unexpectedly, glycolytic inhibition by alpha-chlorohydrin resulted in an immediate decline in sperm motility, particularly in the domestic cat. Collectively, our findings reveal an essential role of the glycolytic pathway in felid spermatozoa that is unrelated to hexose metabolism or lactate formation. Instead, glycolytic enzyme activity could be required for the metabolism of endogenous lipid-derived glycerol, with fatty acid oxidation providing the primary energy source in felid spermatozoa.

α-chlorohydrin, assisted reproductive technology, endogenous substrates, gamete biology, glycerol, glycogen, glycolysis, lactate, male sexual function, metabolic inhibition, oxidative metabolism, sodium oxamate, sperm metabolism, sperm motility and transport, spermatozoa

INTRODUCTION

Glycolysis is widely considered to be a key pathway in mammalian sperm energy production [1–7]. In the bull, ram, dog, and mouse, glycolysis can fully support sperm motility when oxidative metabolism is blocked [1, 8–12]. In the human and rhesus macaque, glycolysis is an essential source of ATP, and motility cannot be maintained by respiration alone [5, 12–15]. Glucose is considered to be the primary metabolic sugar in mammalian spermatozoa [4], but these cells can also utilize fructose, mannose, and maltose as substrates for glycolysis [7, 12, 16]. Although producing <6% of the ATP generated by oxidative metabolism [17], the disproportionate importance of the relatively inefficient glycolytic pathway has been linked to the compartmentalization of sperm energy production [18–20]. Because active microtubule sliding occurs in the distal flagellum, far from the site of mitochondrial activity [2], glycolysis may be an obligate energy source for cellular motility. Consistent with this idea, glycolytic enzymes are tightly bound to structural elements of the sperm flagellum in several species, including the rabbit, boar, bull, rat, stallion, human, mouse, and fox [18–21]. Furthermore, even in species capable of sustaining motility by oxidative metabolism alone (e.g., mouse), glycolysis may be required for sperm capacitation [11, 22, 23], hyperactivation [24, 25], the acrosome reaction [25], zona binding [26], or fusion with the oocyte plasma membrane [26].

We recently described sperm metabolic profiles of the domestic cat and cheetah, the first knowledge about gamete energy production in any felid species [27]. One key finding was that the condition of teratospermia (where males produce ≥60% structurally abnormal spermatozoa) was linked to remarkably reduced rates of sperm lactate production. This observation suggests that sperm ATP synthesis is impaired in teratospermic felids—an intriguing idea, since metabolic disruption could explain many of the functional abnormalities observed in these ejaculates, including reduced sperm motility [27], delayed capacitation [28], compromised acrosomal function [28], disrupted protein tyrosine phosphorylation [29, 30], reduced zona penetration ability [31], and decreased fertilization success in vitro [31]. Although occurring at reduced rates in teratospermic ejaculates, lactate production is positively correlated to other components of felid sperm function (motility and acrosomal integrity [27]), suggesting a key role of anaerobic glycolysis in these cells. Yet regardless of ejaculate quality, our earlier study demonstrated that felid spermatozoa experience a surprising lack of glucose uptake, even in the absence of other glycolyzable substrates [27]. There are three possible explanations for these observed metabolic

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profiles. First, glucose may be imported and metabolized at very low rates (below the threshold of detection), but at a level still required for supporting sperm motility. Second, endogenous glucose could be present in the form of glycogen, as in the domestic dog, where this carbohydrate is localized to the sperm head and midpiece [32] and can provide an energy source for capacitation in a hexose-free medium [6]. Finally, felids may be unusual among mammals in that glycolysis is an insignificant source of sperm energy production. In this case, we would expect the oxidative metabolism of endogenous lipid to support sperm function, since our previous study detected little uptake of any extracellular substrates by cat or cheetah spermatozoa. In several species, including the mouse [33], bull [34], and boar [35], sperm motility can be maintained through endogenous phospholipid or di-/tri-glyceride metabolism, and it is conceivable that this mechanism exists in felids.

The cheetah and domestic cat are excellent models for studying sperm function in teratospermic species, including humans [36] and several endangered felids [37]. Cheetahs are well-known to consistently produce ejaculates containing at least 70% malformed spermatozoa [27, 38, 39], and domestic cats may exhibit either the normospermic or teratospermic phenotype [27, 40, 41]. Certain other physiological traits that influence fertility also are common between humans and domestic cats or cheetahs, including oligospermia [36, 38, 42] and sperm chromatin abnormalities [4, 43]. Thus, understanding the mechanisms driving sperm energy production in the teratospermic domestic cat and cheetah could provide insight into male reproductive abnormalities across a range of species. The present study extended our recent comparative findings on sperm metabolism in the cat and cheetah [27] to elucidate the contribution of endogenous substrates and glycolytic metabolism to basic sperm function. The hypothesis was that sperm motility and viability are maintained in the absence of glycolytic metabolism and are supported by endogenous energy sources, regardless of ejaculate phenotype (i.e., normospermic or teratospermic). Studying the domestic cat and cheetah, our objectives were to determine 1) the influence of exogenous glucose and pyruvate availability on sperm motility and viability; 2) the effect of blocking glycolytic ATP synthesis versus NAD^+ regeneration using chemical inhibitors of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lactate dehydrogenase (LDH), respectively; and 3) differences (or similarities) in sperm metabolic function related to species physiology or ejaculate phenotype.

MATERIALS AND METHODS

Animals

Electroejaculates were collected from adult domestic cats (ages 1.5–8 yr) that were known to consistently produce either normospermic or teratospermic ejaculates (n = 3 males per group). Ten ejaculates were collected from normospermic males (one to five per individual) and 10 ejaculates from teratospermic males (two to four per individual). Males were housed individually in 2.7-m^3 indoor cages at the Smithsonian Conservation Biology Institute (SCBI; Front Royal, VA), maintained on a 14L:10D cycle, and provided dry, commercial cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water ad libitum.

Electroejaculates (one per male, 17 males) were collected from adult cheetahs (ages 2.5–10 yr) housed at the Cheetah Conservation Fund (CCF; Otjiwarongo, Namibia; n = 7), White Oak Conservation Center (WOCC; Yulee, FL; n = 6), SCBI (n = 3), or the Philadelphia Zoo (Philadelphia, PA; n = 1). Males at CCF were wild-born and housed as described previously [44]. The six males at WOCC represented three sibling pairs, and two of these pairs were captive born. Each pair was housed separately in 2500-m^2 outdoor enclosures off exhibit and fed a mixed diet of two commercial products (Toronto Zoo Feline Diet; Milliken Meat Products Ltd., Scarborough, ON; and Carnivore Diet 10; Natural Balance Pet Foods Inc., Pacoima, CA). Males at SCBI were captive born and housed together (in a group of three) off exhibit in a 2200-m^2 outdoor enclosure and fed a commercially produced carnivore diet (Carnivore Diet 10; Natural Balance Pet Foods Inc., Pacoima, CA). The single male in Philadelphia was captive-born and housed on exhibit in a sibling group with two other males in a 1500-m^2 outdoor enclosure and fed a commercially produced carnivore diet (Carnivore Diet 10).

Semen Collection

A surgical plane of anesthesia was induced in domestic cats and cheetahs according to protocols determined by institutional veterinarians and similar to those previously used for semen collection in these two species [28, 44]. All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee and similar committees of the WOCC and Philadelphia Zoo. 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 previously [27, 45]. A sample of raw semen containing $\sim 2 \times 10^5$ spermatozoa was fixed in 0.3% glutaraldehyde in PBS for assessment of sperm morphology, as described previously [27, 45, 46].

Sperm Processing and Metabolic Assessments

Each ejaculate was diluted immediately with an equal volume of a chemically defined, protein-free, modified mouse tubal fluid medium (cMTF) [47] supplemented with 2% polyvinyl alcohol [48]. The cMTF medium was prepared as described previously [27] 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. Sperm concentration was determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) [49].

Each diluted ejaculate (maintained at ambient temperature, 19–22°C) was washed by centrifugation (8 min, $300 \times g$ for domestic cat, $100 \times g$ for cheetah) and resuspended in a modification of cMTF corresponding to each treatment. To evaluate the influence of exogenous substrate availability, four aliquots from a given, well-mixed ejaculate (n = 3 normospermic cat, n = 3 teratospermic cat, n = 8 cheetah) were incubated in cMTF containing 1) no metabolic substrates, 2) pyruvate only, 3) glucose only, or 4) both substrates (i.e., complete medium, representing the positive control). To determine the effect of blocking glycolysis, sperm aliquots from a second set of ejaculates (n = 6 normospermic cat, n = 6 teratospermic cat, n = 8 cheetah) were exposed to 50 mM α-chlorohydrin (a GAPDH inhibitor) and incubated in glucose-free cMTF in parallel with negative controls in complete cMTF. This α-chlorohydrin concentration has been used to study sperm metabolism in other species [50] and was the minimum required to impair domestic cat sperm function in a preliminary dose-dependent trial (data not shown). To a lesser extent, α-chlorohydrin also inhibits triose phosphate isomerase, the enzyme immediately preceding GAPDH in the glycolytic pathway [51]. Glucose was omitted from the α-chlorohydrin treatment medium to prevent the accumulation of cytotoxic glycolytic intermediates [52]. Finally, to determine the influence of blocking lactate production, aliquots of individual ejaculates (n = 3 normospermic cat, n = 2 teratospermic cat, n = 5 cheetah) were exposed to 50 mM sodium oxamate (a specific LDH inhibitor) and incubated in parallel with negative inhibitor-free controls in complete cMTF. This inhibitor concentration also was chosen on the basis of a preliminary dose-dependent trial (data not shown). Some of the ejaculates in the sodium oxamate group were also represented in the α-chlorohydrin treatment group (n = 2 normospermic cat, n = 1 teratospermic cat, n = 4 cheetah). Medium osmolality was maintained in GAPDH and LDH-inhibited samples by adjusting NaCl concentration. Osmolality of all final working media (300–345 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).

All sperm samples were incubated (37°C) at a concentration of 3×10^6 motile cells per milliliter in microcentrifuge tubes under oil to prevent evaporation, as described previously [27]. Assessments of sperm percentage motility (% M), forward progression (FPS), and acrosomal integrity (% IA) were made at 0, 1, 3, and 7 h of incubation by a single investigator who was blind to each treatment. 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 [45]. Spermatozoa ($\sim 2 \times 10^5$ cells) were fixed in 4% paraformaldehyde and stained with Coomassie Blue G-250 (Fisher Biotech, Springfield, NJ) to evaluate acrosomal integrity, as described previously [42, 53]. 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 [42]. Rates of pyruvate uptake and lactate production were also assessed over each time interval (except in the LDH-inhibited group, as these assays use LDH-linked fluorescence for substrate quantification). Rates of glucose uptake were assessed over each time interval in the first treatment

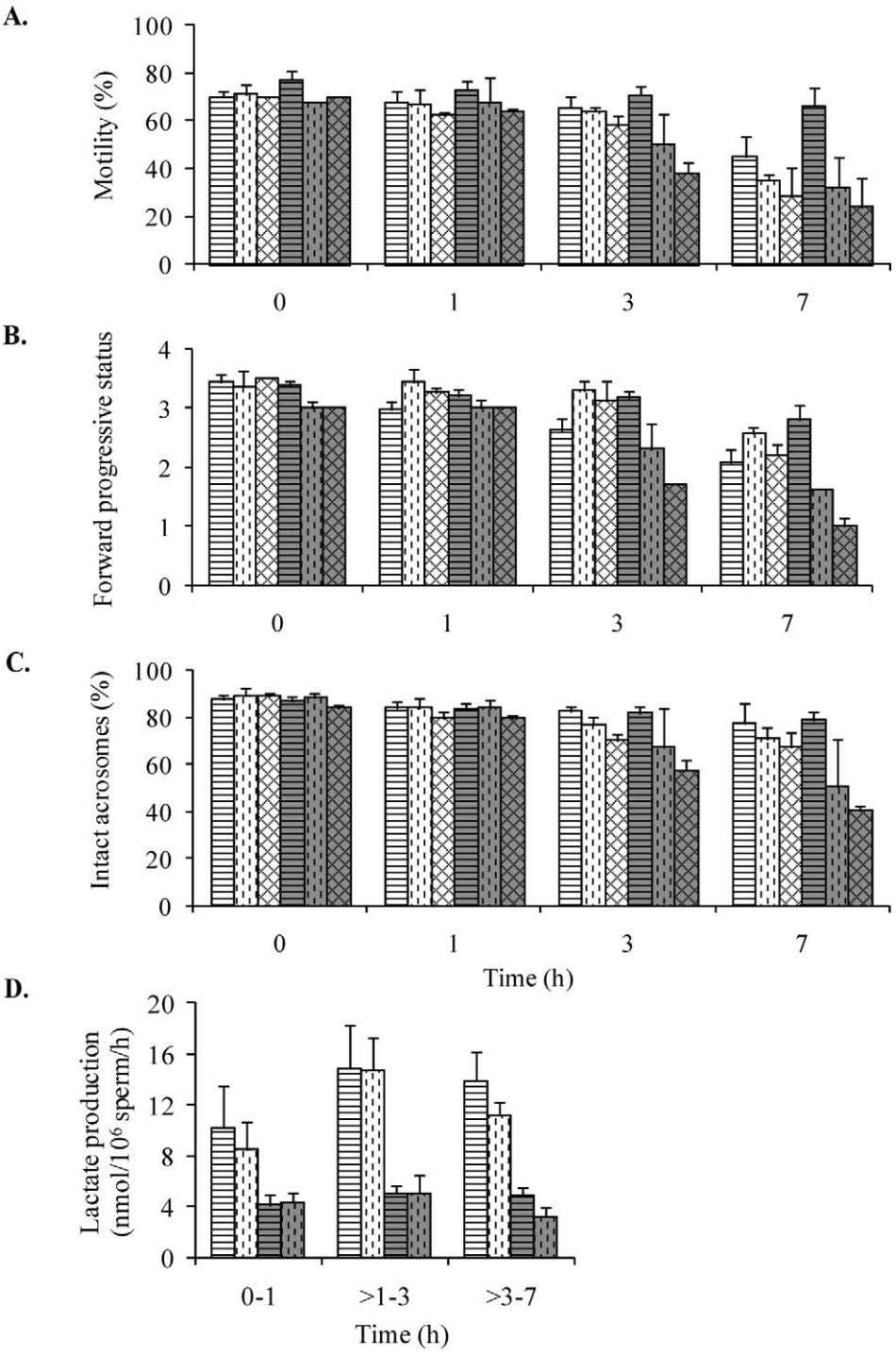

FIG. 1. Absolute values for percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in control sperm samples from domestic cats (white bars) and cheetahs (gray bars). Controls (incubated in complete cMTF medium) correspond to the following three treatment groups: (1) presence/absence of exogenous substrates (horizontal lined bars), (2) GAPDH inhibition (vertical dashed bars), and (3) LDH inhibition (diamond-patterned bars).

group (exogenous substrate availability). Although we previously had determined that sperm glucose uptake was minimal in these species [27], it was possible that the absence of oxidative substrates in the medium would stimulate glucose uptake. To determine pyruvate, lactate, and glucose concentrations, all 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 an LDH/GPT- or HK/G6PDH-linked fluorescence assay described previously [27, 47, 54]. Fluorescence was 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). In a subset of control samples, sperm cells were removed by centrifugation (8 min, 1000 × *g*) prior to medium filtration for analysis of glycogen content as described below. Rates of lactate production were calculated as the change in medium substrate concentration over time, divided by sperm concentration, and were reported in nanomoles per 10^6 sperm per hour. All data were normalized to control values for presentation.

Glycogen Assay

Glycogen content was measured in control group sperm samples (3×10^5 spermatozoa; n = 5 domestic cat, n = 5 cheetah) taken at 0 h, as described above. To address the possibility that the glycogen content of these samples might be below an unknown threshold of detection, we also prepared highly concentrated domestic cat epididymal samples for this assay. Testes (n = 6 males; ages 1–3 yr) were harvested at local veterinary clinics, transported in PBS to the laboratory within 6 h of orchiectomy, and dissected in 500 μl cMTF at room temperature. Testicular cell suspensions were combined (two males per tube) and centrifuged (8 min, 300 × *g*) to obtain pellets (n = 3) containing ~1×10^8 sperm. All samples (ejaculated and epididymal) were stored frozen (−80°C) until analysis. Sperm extracts were prepared for analysis of glycogen content using a modified protocol of Ballester et al. [32]. Briefly, sperm pellets were thawed and homogenized by sonication with 300 μl of KOH on ice.

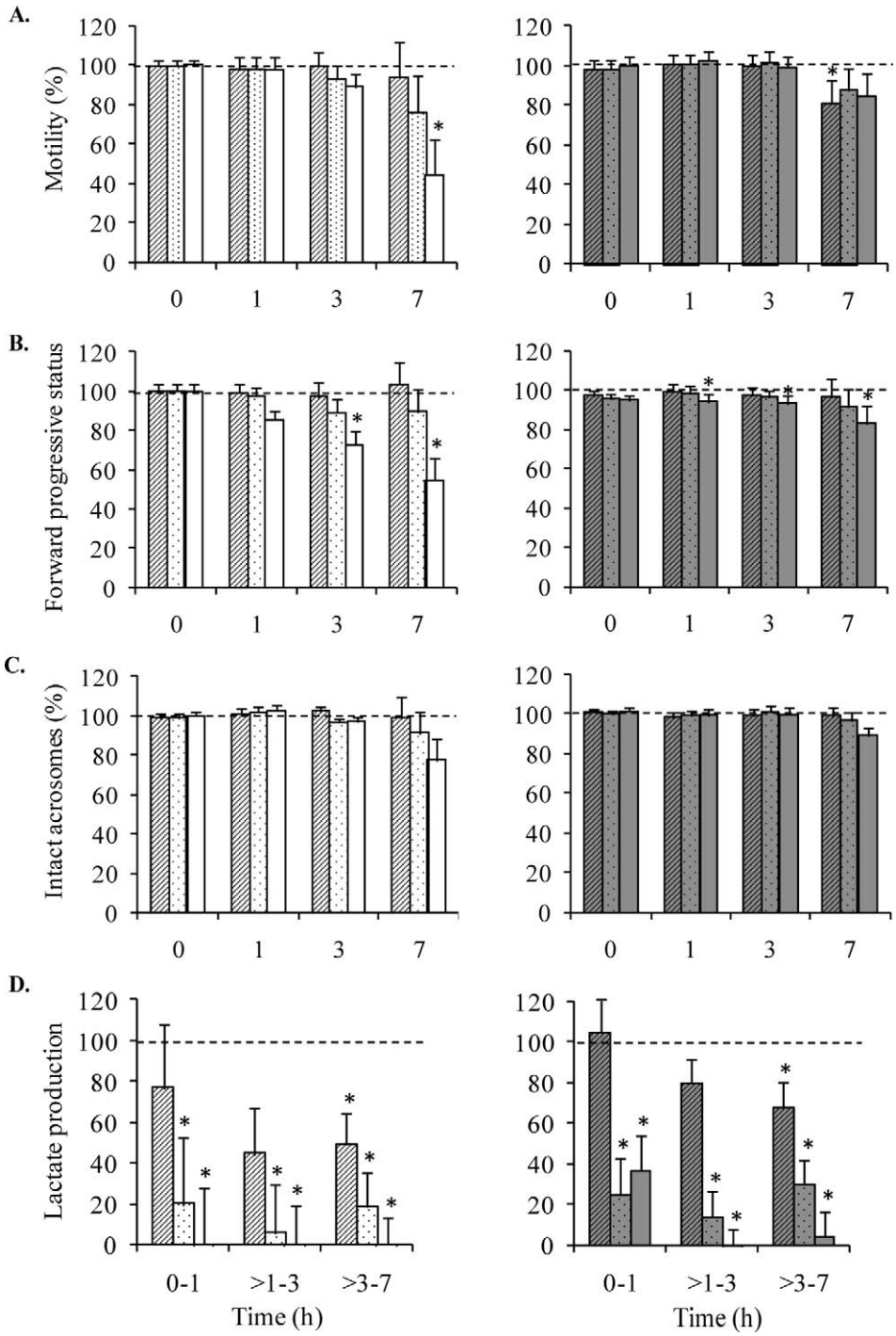

FIG. 2. Percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in domestic cat (white bars) and cheetah (gray bars) sperm samples incubated with 1 mM pyruvate (lined bars), 1 mM glucose (dotted bars), or without exogenous substrate (solid bars). Data are expressed as percentages of control values (dashed line). Within each species and time interval, bars with an asterisk (*) differed from control values ($P < 0.05$).

Homogenates were incubated at 100°C for 15 min, then incubated (37°C) 1:1 with a glycogen hydrolysis buffer (0.3 U/ml α-amyloglucosidase, 50 mM sodium acetate; pH 4.6) for 30 min. Standards of known concentration (0.00, 0.06, 0.13, 0.25, 0.50, and 1.00 mM) and controls (domestic cat sperm with 1 mM glycogen added, n = 3) were prepared using the same protocol. Standards and unknowns were assayed for glycogen content using a HK/G6PDH-linked assay described previously [27] with a 1:5 ratio of sample to reaction volume. Fluorescence was quantified as described above.

Chemicals

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise noted. Enzymes (LDH, GPT, α-amyloglucosidase, HK, and G6PDH) were obtained from Roche Applied Science (Indianapolis, IN).

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. The interaction between treatment and domestic cat group (normospermic and teratospermic) was assessed using SAS General Linear Model (GLM) Procedures [55], with % M, FPS, % IA, and lactate production included as response variables. Treatment and domestic cat group were considered class variables, and time was included as a covariate. Because there was no interaction ($P > 0.05$) between treatment and group, all domestic cat samples were combined for subsequent analysis. The interaction between treatment and species (cat and cheetah) was assessed using a GLM as described above. Within species, treatment effects were analyzed separately for each time point using paired Student *t*-tests (treatment vs. control). Pearson correlation was used to evaluate the relationships between sperm morphology and changes

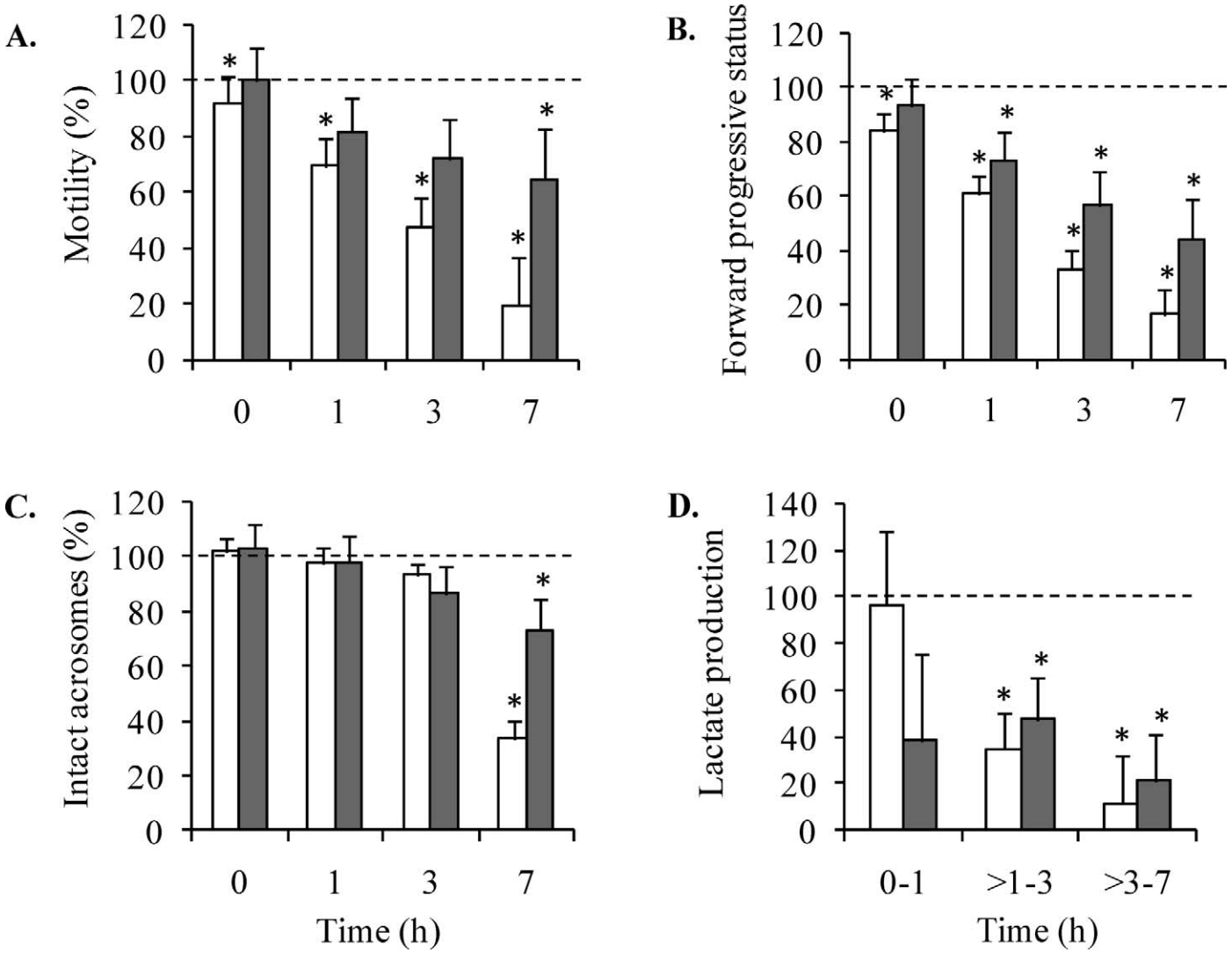

FIG. 3. Percent motility (**A**), forward progression (**B**), acrosomal integrity (**C**), and lactate production (**D**) in domestic cat (white bars) and cheetah (gray bars) sperm samples incubated with 50 mM α-chlorohydrin to inhibit GAPDH. Data are expressed as percentages of control values (dashed line). Within each species and time interval, bars with an asterisk (*) differed from control values ($P < 0.05$).

in sperm motility, FPS, % IA, and lactate production (relative to controls) at the end of incubation. Fluorescence of glycogen samples was analyzed using a GLM. Results were considered significant at $P < 0.05$ and were reported as least-squares means ± SEM.

RESULTS

Exogenous Substrate Availability

To facilitate comparisons between domestic cats and cheetahs, data for all treatment groups are presented as normalized to control values. Absolute values for control samples are provided in Figure 1. Overall, the absence of either glucose or pyruvate from the culture medium did not ($P > 0.05$) influence any sperm functional metrics (% M, FPS, or % IA) in the domestic cat or cheetah (Fig. 2, A–C). The only exception was a decrease ($P < 0.05$) in % M in cheetah ejaculates after 7 h of incubation in a pyruvate-free medium. After 7 h incubation in substrate-free medium, sperm percent motility was decreased ($P < 0.05$) only in the cat (Fig. 2A), whereas FPS was reduced ($P < 0.05$) ~50% in the cat and ~20% in the cheetah (Fig. 2B). Acrosomal integrity was not ($P > 0.05$) influenced by the lack of substrates in either species (Fig. 2C). In both cats and cheetahs, spermatozoa produced lactate in the absence of glucose, but only if pyruvate was present in the culture medium (Fig. 2D). Conversely, rates of lactate production were reduced ($P < 0.05$) by ~80% or more in the absence of pyruvate. Consistent with previous findings [27], sperm glucose uptake was minimal in both species and was not influenced ($P > 0.05$) by the absence of exogenous oxidative substrates (Supplemental Fig. S1A; all Supplemental Data are available online at www.biolreprod.org). Likewise, rates of pyruvate uptake were not affected ($P > 0.05$) by the absence of exogenous glycolytic substrates (Supplemental Fig. S1B). For all response variables, there was no interaction ($P > 0.05$) between substrate availability and species or between substrate availability and domestic cat ejaculate phenotype (i.e., normospermic vs. teratospermic).

GAPDH and LDH Inhibition

The influence of GAPDH inhibition was consistent between species and resulted in impaired ($P < 0.05$) sperm function (% M, FPS, and % IA; Fig. 3). Losses in motility (% M and FPS) became more severe ($P < 0.05$) over time (Fig. 3, A and B),

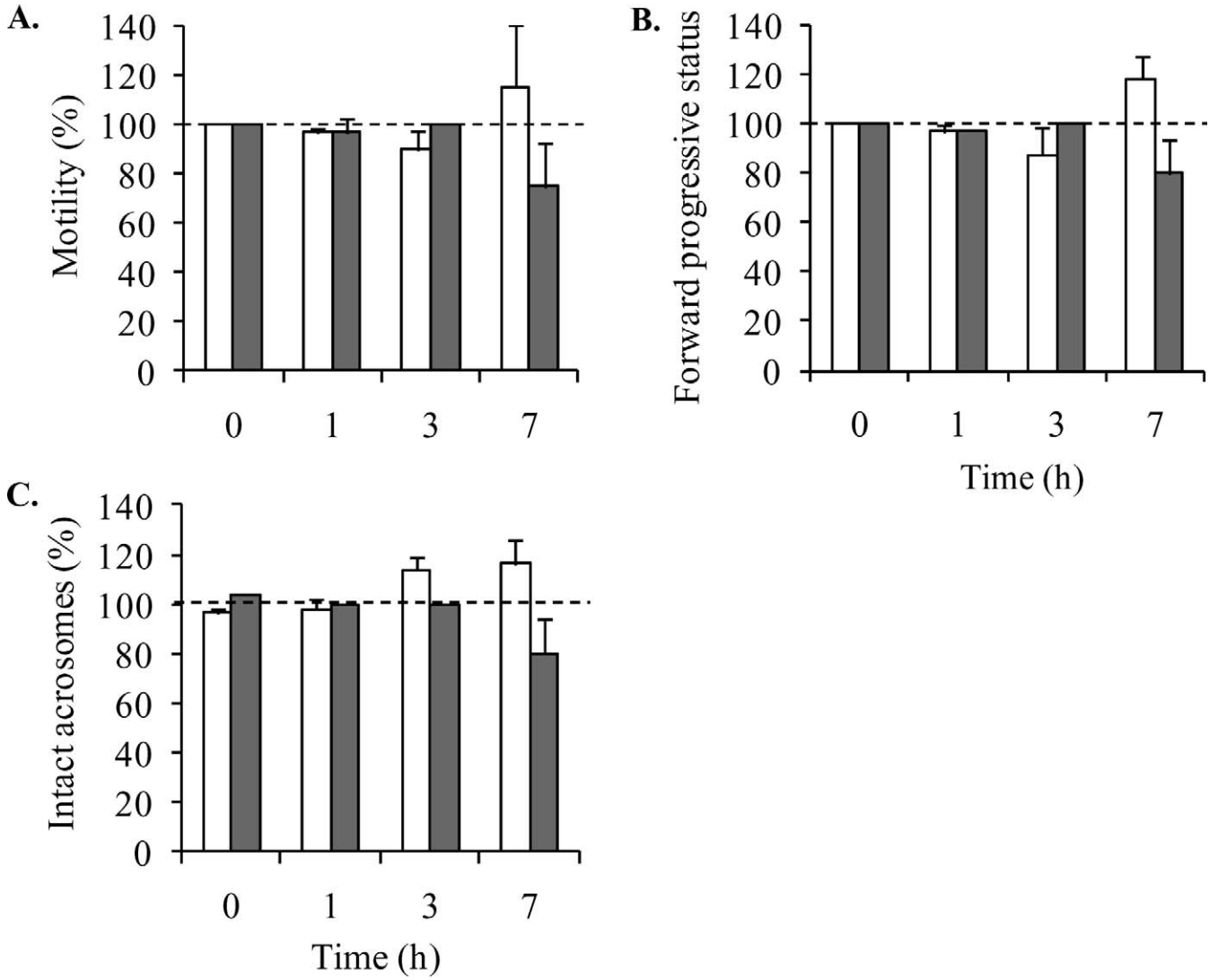

FIG. 4. Percent motility (**A**), forward progression (**B**), and acrosomal integrity (**C**) in domestic cat (white bars) and cheetah (gray bars) sperm samples incubated with 50 mM sodium oxamate to inhibit LDH. Data are expressed as percentages of control values (dashed line). All treatment values were similar to controls ($P > 0.05$).

whereas acrosomal integrity was not impaired until 7 h (Fig. 3C). An unusual motility pattern that involved rapid flagellar beating but little forward progression (i.e., vigorous twitching), was often observed within 1 h of exposure to the GAPDH inhibitor, prior to declines in % M. Within each species, rates of lactate production were positively correlated ($r = 0.30$–0.44, $P < 0.05$) to sperm function (% M, FPS, and % IA) and declined ($P < 0.05$) after 1 h of exposure to the GAPDH inhibitor (Fig. 3D). Substantial variation in rates of pyruvate uptake was observed, obscuring any potential overall treatment effect (Supplemental Fig. S2). This variation was likely related to the assay method that quantified pyruvate uptake based on a *decrease* in baseline fluorescence (in contrast to the more sensitive lactate assay that measured a fluorescence increase from zero). These data were also consistent with our previous observation that lactate production is a more reliable indicator of sperm quality than pyruvate uptake [27]. Compared to the cheetah, domestic cat spermatozoa were more sensitive ($P < 0.05$) to loss of motility and FPS following glycolytic inhibition (i.e., a treatment-species interaction was detected). However, within domestic cats, ejaculate phenotype did not ($P > 0.05$) influence sensitivity to glycolytic inhibition. None of the sperm functional metrics (% M, FPS, or % IA) was influenced ($P > 0.05$) by the inhibition of lactate production (LDH) in either species (Fig. 4).

Glycogen Content

Glycogen digest was effective across all standards ($R^2 \geq 0.945$). The concentration of glycogen in spiked controls was similar ($P > 0.05$) to the corresponding 1-mM standard. However, glycogen content of all samples (ejaculated or epididymal, domestic cat, or cheetah) did not differ ($P > 0.05$) from the 0-mM standard.

DISCUSSION

Results from our comparative investigation of sperm metabolism in the domestic cat and cheetah supported our hypothesis that these cells relied primarily on endogenous substrates to fuel motility and viability, although surprisingly GAPDH activity was essential for these functions. Because (1) sperm motility was maintained in a medium without glycolyzable substrates, and (2) there was no evidence of intracellular

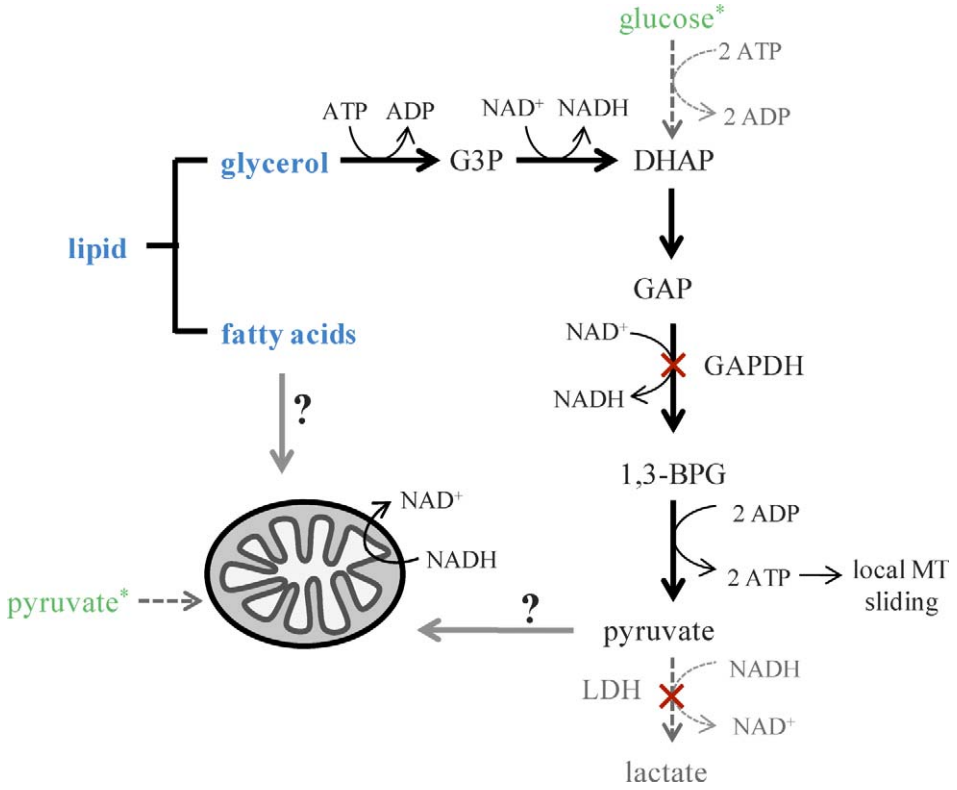

FIG. 5. Theoretical model of energy production in domestic cat and cheetah spermatozoa illustrating targets of metabolic inhibition (×). Diagram includes exogenous substrates utilized in this study (green) and hypothesized endogenous energy sources (blue). Dashed lines indicate pathways determined to be nonessential for sperm motility and viability. Certain enzymes and glycolytic intermediates are omitted from the figure for clarity. G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 1,3-BPG, 1,3-bisphosphoglycerate; LDH, lactate dehydrogenase; MT, microtubule. *Necessary to maintain full motility in the absence of other exogenous substrates.

glycogen stores, we concluded that the obligate role of the glycolytic pathway was unrelated to sugar metabolism. Furthermore, since sperm motility was impaired following GAPDH, but not LDH, inhibition, we surmised that the role of this pathway was related to ATP production rather than NAD^+ regeneration. Based on these collective results we propose a new model of energy production in felid spermatozoa whereby ATP is produced primarily by the oxidation of endogenous lipid, and the main function of the glycolytic pathway is to metabolize lipid-derived glycerol (Fig. 5). In this model, fatty acids hydrolyzed from intracellular lipid (i.e., phospholipid or di-/triglycerides) would be oxidized by the mitochondria to generate ATP, whereas glycerol would enter the glycolytic pathway via its conversion to dihydroxyacetone phosphate (DHAP) [56]. The remaining steps of glycolysis (including the GAPDH-catalyzed conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate) would ultimately produce pyruvate and two molecules of ATP, for a net gain of one ATP [17]. Cytosolic NADH produced by this process would be transported to the mitochondria via the malate-aspartate shuttle to regenerate NAD^+ for the next round of glycerol metabolism (Fig. 5) [57]. Finally, the oxidative metabolism of glycerol-derived pyruvate would provide another significant source of ATP.

Based on this model, we identified three possible mechanisms by which GAPDH inhibition could have compromised sperm function. First, although perhaps least likely, glycolytic inhibition could have caused cytotoxic pathway intermediates to accumulate within the spermatozoa [52]. We are doubtful of this possibility, as evidence in the ram and boar suggests that α-chlorohydrin does not generate cytotoxic intermediates in the absence of exogenous sugars, even when endogenous lipid is actively metabolized [58]. Secondly, the glycolytic pathway may have been an important source of mitochondrial substrate, analogous to the mechanism of sperm energy production in the boar [35]. Although boar spermatozoa actively metabolize endogenous lipid, they do not appear to oxidize fatty acids [59] and instead generate oxidative substrates from the glycolytic metabolism of glycerol [35]. However, because exogenous pyruvate was available to GAPDH-inhibited spermatozoa, we believe it is unlikely that the role of this enzyme is related to oxidative substrate production. Finally, GAPDH could have maintained active microtubule sliding along the length of the flagellum via local glycerol metabolism. This pathway is less efficient than oxidative phosphorylation, with a net yield of only one ATP per molecule of glycerol. Nonetheless, glycerol metabolism could support sperm motility if mitochondrial energy cannot reach the distal flagellum, analogous to the role of compartmentalized glucose metabolism in other species [11]. Given the abnormal pattern of sperm motility observed in the domestic cat and cheetah immediately after exposure to the inhibitor (i.e., vigorous twitching), we predict that the importance of GAPDH activity was related to the need for localized ATP production along the flagellum.

The importance of lipid metabolism in our model is supported by other studies that indicate that this substrate is endogenously present in the form of phospholipid or di/triglycerides and is a significant source of ATP in bull [34], ram [60], boar [35], mouse [33], and rhesus macaque [61] spermatozoa. Lipid can provide up to six times the energy of an equal weight of glycogen [56] and, therefore, is an ideal energy source for spermatozoa that have an inherently high ATP demand and a tightly packed, economical structure. Yet this endogenous energy supply appears to be limited in felid spermatozoa, given our observation of a gradual loss in progressive motility in substrate-free medium. In contrast, spermatozoa maintained motility with glucose or pyruvate as the sole exogenous substrate, thereby implying a capacity of these cells to alter their metabolic strategy in response to a changing microenvironment. Such an adaptive mechanism of energy production would improve chances for in vivo longevity and successful fertilization by allowing sperm to

metabolize substrates within the female tract while utilizing an endogenous energy supply when needed.

Because the domestic cat routinely produces higher-quality ejaculates compared to the cheetah [27, 38], we were surprised to discover that spermatozoa from the former were more susceptible to motility declines in the absence of exogenous substrates or when GAPDH was inhibited. This unexpected finding could indicate that ATP demand is unusually low in cheetah spermatozoa, and may be related to earlier observations that multiple energy-driven processes (e.g., motility and cellular viability [27, 39]) are consistently impaired in ejaculates from this species. One of the strengths of our study design was that sperm metabolic function could be comparatively assessed not only between species, but also in domestic cat ejaculates containing a high versus low proportion of structurally malformed spermatozoa. This remarkable phenotypic difference in the cat has had a significant influence in earlier studies on sperm motility [27, 31], chilling sensitivity [62], osmotic stress [46], protein tyrosine phosphorylation [29], capacitation [28], acrosomal function [28], zona penetration [31], and fertilizing ability [31]. Yet, the incidence of teratospermia among domestic cats in the present study had no influence on sperm sensitivity to glycolytic inhibition or substrate availability. In contrast, we previously found that rates of sperm pyruvate uptake and lactate production were greatly decreased in teratospermic cats and cheetahs compared to normospermic cats [27]. Given the proposed model in Figure 5, we predict that disruptions in sperm physiology associated with teratospermia (including substrate uptake/production) are driven primarily by reduced capacity for oxidative energy production. This is the focus of a current investigation in our laboratory.

In conclusion, our findings emphasized the value of studies that compare biological phenomena in nontraditional species (the cheetah) to more commonly used models (the domestic cat) [63]. This approach revealed conserved mechanisms of glycolytic metabolism and substrate utilization in domestic cat and cheetah spermatozoa. The consistency of our findings was noteworthy, given the significant genetic and phenotypic differences between these two felid species, and provided strong support for our proposed model of sperm energy production. Although domestic cat and cheetah spermatozoa shared a requirement for GAPDH activity, differences were observed regarding the severity of sperm functional declines following exposure to the GAPDH inhibitor. These differences suggested that sperm energy demand may be greater in the domestic cat compared to the teratospermic cheetah. If confirmed, this new finding could be mechanistically linked to previous observations that multiple energy-driven processes are disrupted in felid ejaculates with the teratospermic phenotype [28, 29, 31, 43]. Understanding the fundamental cause of these physiological disruptions could provide clues to developing methods to enhance sperm fertilizing ability, e.g., through exposure to metabolic stimulants [64]. As high (>40%) proportions of pleiomorphic spermatozoa have been documented in at least 18 of the 21 wild felid species studied [41, 65] and are nearly ubiquitous in man [36], cross-species comparisons that provide insight into teratospermia can help improve the success of assisted reproduction for biodiversity preservation and human fertility.

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