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Metabolism and cryo-sensitivity of domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) spermatozoa

A Dissertation

Submitted to the graduate faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy
in
Conservation Biology

by

Kimberly Ann Terrell

B.S. Tulane University, 2005
B.A. Tulane University, 2005

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Abstract (350 words max)

Teratospermia (ejaculation of ≥ 60% structurally abnormal spermatozoa) is prevalent among felids facing extinction risk, including the cheetah. This trait also occurs in certain domestic cat populations, providing a valuable research model. Multiple components of sperm function are disrupted in teratospermic cats, and even structurally normal spermatozoa from these ejaculates may be functionally compromised. Teratospermic ejaculates are highly sensitive to damage during cryopreservation, limiting the success of genome resource banking programs for species conservation. Although both teratospermia and cryopreservation are linked to disruptions in multiple energy-dependent sperm processes, the metabolism of these cells has not been investigated. This project explored how cellular metabolism of domestic cat and cheetah spermatozoa is influenced by species physiology, teratospermia, and sperm cryopreservation.

The project scope was divided into four studies that collectively examined the two main energy-producing pathways in spermatozoa, i.e., glycolysis and oxidative phosphorylation. Each study compared three animal populations: normospermic cat, teratospermic cat, and cheetah. First, rates of glycolytic and oxidative substrate utilization were correlated to standard metrics of sperm function. Second, the influence of exogenous substrate availability and glycolytic enzyme activity was investigated. Third, mitochondrial activity and the role of oxidative metabolism were assessed. Lastly, sperm metabolic function was examined after cryopreservation and post-thaw processing.

Patterns of substrate utilization were similar in spermatozoa of the cat and cheetah, including an unexpected lack of glucose uptake. However, rates of sperm pyruvate uptake and lactate production were reduced in the teratospermic cat and cheetah compared to the normospermic cat. Lactate production predicted ejaculate quality in each study. Glycolytic

enzyme activity was essential for sperm function, but, unexpectedly, the importance of this pathway appeared to be linked to glycerol rather than glucose metabolism. Sperm oxidative metabolism was severely compromised in the cheetah, and comparison with the teratospermic cat proved this defect to be species-specific. Spermatozoa from both species experienced metabolic damage during cryopreservation. Post-thaw processing recovered a metabolically-normal sperm subpopulation in the cat, but cheetah spermatozoa remained functionally compromised. Collectively, these studies provided key insight into metabolism and cryo-sensitivity of felid spermatozoa and highlighted the importance of domestic animal models for wildlife research.

Keywords: Cryopreservation, Teratospermia, Teratozoospermia, Felid, Sperm, Glucose, Pyruvate, Lactate, Mitochondria, Glycolysis

CHAPTER 1: GENERAL INTRODUCTION

1.1 Teratospermia and Sperm Cryo-Sensitivity

Felidae comprises nearly 40 species and is one of the most phylogenetically diverse carnivore families in existence [1]. Teratospermia (ejaculation of ≥ 60% structurally abnormal spermatozoa) is remarkably prevalent in this taxon, and nearly all (~90%) felid species studied to date consistently ejaculate high proportions (> 40%) of malformed spermatozoa [2]. In addition to sperm pleiomorphisms, teratospermic ejaculates generally contain low numbers of spermatozoa, and these cells are highly susceptible to damage during cryopreservation [2, 3]. Severe teratospermia (> 85% structurally abnormal cells) is linked to reduced fecundity and infertility in certain wild felids [4-6]. Because most (70%) wild felids are at risk of extinction and nearly half of the non-threatened species are in decline [7], understanding the physiology of this intriguing reproductive phenomenon is important both for conservation efforts and to manage genetically valuable populations *ex situ*. Such knowledge also would benefit human health, as teratospermia is nearly ubiquitous in man (representing a significant source of male infertility [3]) and is common among domestic cat (*Felis catus*) lineages studied as models of human disease [8, 9].

Teratospermia has been linked to a loss of genetic diversity through opportunistic studies of rare felids, including the cheetah (*Acinonyx jubatus*) [10, 11], lion (*Panthera leo*) [4, 12], Florida panther (*Puma concolor coryi*) [5, 13], and leopard cat (*Felis bengalensis*) [4]. In each case, ejaculates containing high proportions of structurally abnormal spermatozoa, low sperm numbers, and/or poor motility were associated with a lack of heterozygosity from an ancient population bottleneck (cheetah [14, 15]), recent population decline (lion [12] and Florida panther [13]), or captive inbreeding (leopard cat [4]). Prospective inbreeding in the domestic cat

confirmed the link between ejaculate quality and genetic diversity [2]. After a single generation of incestuous mating, proportions of malformed spermatozoa ejaculated increased to > 85% (compared to < 45% in outbred males). In contrast to the low numbers of spermatozoa previously reported in teratospermic ejaculates, inbred toms experienced an ~80% *increase* in total sperm production compared to normospermic cats [2]. High numbers of ejaculated spermatozoa were linked to an increase in testicular volume and a greater spermatogenic/Sertoli cell ratio [16]. This physiological response presents the intriguing possibility that certain individuals or populations may possess a compensatory mechanism to maintain fertility despite abnormal spermatogenesis [2].

The ability to empirically test the link between teratospermia and genetic diversity in the domestic cat highlights the value of this species as a model for wildlife conservation and human health. Comparative studies of domestic cat populations that produce different proportions of pleiomorphic spermatozoa have yielded insight into the extreme cryo-sensitivity and potential for decreased fertility of teratospermic ejaculates. Specifically, spermatozoa from these ejaculates exhibit increased susceptibility to osmotic stress [17] and cold-induced acrosome damage [18], as well as delayed capacitation [19], impaired acrosomal function [19], reduced levels of protein tyrosine phosphorylation [20], chromatin instability [21], decreased zona pellucida penetration [22], and compromised fertilization ability in vitro [22]. Importantly, many of these cellular functions also are impaired in structurally normal spermatozoa from teratospermic ejaculates [19, 20, 22]. In contrast to these detailed studies, there is a lack of knowledge about cellular metabolism in felid spermatozoa, including the possible influence of teratospermia or cryopreservation. Given the spermatozoon's extraordinarily high ATP demands and the disruption of multiple energy-dependent processes (e.g., motility, viability, protein

tyrosine phosphorylation) in teratospermic ejaculates, cellular metabolism is a priority research focus. Identifying the primary biochemical pathway responsible for sperm energy production and understanding its role in teratospermia/cryo-sensitivity could help improve the success of assisted reproductive technologies (e.g., sperm cryopreservation, in vitro fertilization) for wildlife management and human fertility [23].

1.2 Mammalian Sperm Metabolism

Mammalian spermatozoa produce energy in the form of ATP almost exclusively by two pathways: glycolysis and oxidative phosphorylation (OXPHOS) [24]. Each pathway's relative importance varies substantially among the small number of species studied, which includes the human, mouse, boar, bull, ram, rabbit, and dog (reviewed in [25]). Sperm energy sources are equally diverse, likely reflecting species variation in metabolic substrate availability within the female tract [24]. Potential energy substrates include exogenous hexoses (primarily glucose) and monocarboxylates (pyruvate and lactate) [24], as well as endogenous lipid [26, 27] and even glycogen [28, 29]. The localization of mitochondria to the midpiece and glycolytic enzymes to the flagellum results in a compartmentalized mode of sperm energy production [24]. Therefore, the relative contribution of each pathway is dictated by its capacity to fulfill local energy demands and/or by the operation of intracellular ATP transporters [30]. This compartmentalization likely explains the essential role of glycolysis in human [31] and mouse [32] spermatozoa, despite extremely inefficient rates of ATP production relative to OXPHOS. Both glycolytic [33] and oxidative [34-36] pathways may be disrupted by sperm cryopreservation, although substantial variation in susceptibility to metabolic damage can exist, even within a species [37]. Given the remarkable differences in mechanisms of energy

production among mammalian spermatozoa, comparative investigations of multiple species and/or populations are essential for generating reliable conclusions about these unstudied processes in felids.

1.3 Study Focus and Scope

The overall aim of this project was to understand how metabolism of felid spermatozoa is influenced by teratospermia, sperm cryopreservation, and species physiology. To achieve this goal, each study comparatively assessed three felid populations: 1) normospermic domestic cat, 2) teratospermic domestic cat, and 3) cheetah (an entirely teratospermic species). The cheetah population included wild- and captive-born individuals of the southern African subspecies *Acinonyx jubatus jubatus* housed at the Cheetah Conservation Fund (Otjiwarongo, Namibia) or in North American institutions within the Association of Zoos and Aquarium's Species Survival Plan (SSP).

To assess the importance of glycolytic and oxidative metabolism in felid spermatozoa, and to elucidate the mechanisms of sperm cryo-sensitivity, the specific foci of this project included:

1) Glycolytic versus oxidative substrate utilization and metabolic indicators of cellular function (Chapter 2).

   Published manuscript:

   Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LM, Marker LL, Crosier AE. Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*). Biol Reprod 2010; 83: 833-841.

2) The role of exogenous substrate availability and glycolytic enzyme activity in sperm function (Chapter 3).

   Published manuscript:

   Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LM, Marker LL, Crosier AE. Glycolytic enzyme activity is essential for domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) sperm motility and viability in a sugar-free medium. Biol Reprod 2011; 84: 1198-1206.

3) The importance of oxidative phosphorylation for sperm function (Chapter 4).

   Published manuscript:

   Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LP, Crosier AE. Oxidative metabolism is essential for felid sperm function, but is substantially lower in cheetah (*Acinonyx jubatus*) compared to domestic cat (*Felis catus*) Ejaculate. Biol Reprod 2011; 83: 833-841.

4) Changes in metabolic function resulting from cryopreservation and/or post-thaw processing (Chapter 5).

   Manuscript in review:

   Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LP, Crosier AE. Different patterns of metabolic cryo-damage in domestic cat (*Felis catus*) and cheetah (*Acinonyx jubatus*) spermatozoa. Cryobiology.

1.4 Status of Wild and Captive Cheetah Populations

The cheetah (Fig. 1.1) is a species well known to consistently ejaculate high (~80%) proportions of abnormal spermatozoa, regardless of geographic range, season, age, or

Figure 1.1. The cheetah.

environment (captive versus wild) [11, 38]. This trait has been linked to an extreme lack of genetic diversity from a severe population contraction ~10,000 years ago [14, 15, 39], coincident with the widespread extinctions of Pleistocene megafauna [40]. Subsequent analysis revealed a second population bottleneck in the twentieth century due to a ~90% decline in wild populations from poaching and habitat loss [41]. Today fewer than 10,000 cheetahs remain in nature, although the effective population size may be less than half of that number [42]. The species has disappeared from ~80% of its historic African range [42]. Surviving cheetahs exist in geographically isolated populations throughout the continent, with a very small number of individuals remaining in Iran (Fig. 1.2) [42]. The largest extant cheetah population (~4,500 adults) occurs in southern Africa, primarily in Namibia and Botswana [43]. Since the vast majority of these individuals inhabit private farmland, shooting or trapping by farmers (due to the perceived threat of livestock predation) continues to pose a major threat to the species' survival [44]. For example, ~7,000 Namibian cheetah were killed or brought into captivity during the 1980s [45], approximately three times the number of free-ranging individuals in Namibia today [42].

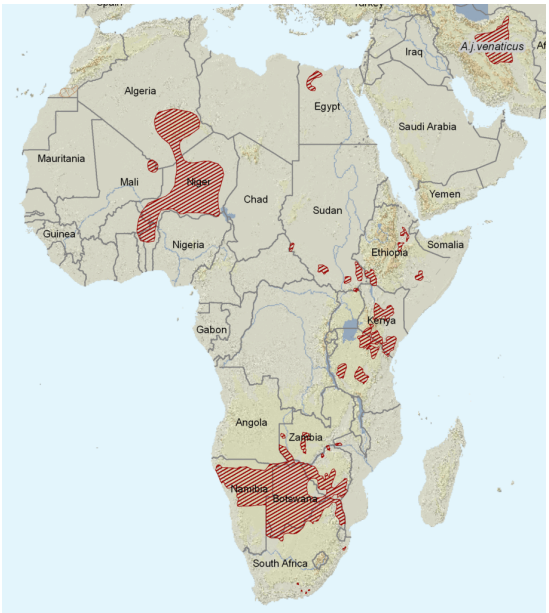

Figure 1.2. Current range (red shading) of the cheetah [7].

Due to the ongoing decline in wild cheetah populations (suspected to be ≥ 30% over the past three generations), the species is considered by the IUCN (International Union for the Conservation of

Nature and Natural Resources) to be 'vulnerable' to extinction [42]. Captive cheetahs have the potential to contribute to the survival of wild populations by providing 1) a long-term reservoir of genetic diversity, 2) an opportunity for increasing basic knowledge about a species that is challenging to study in the wild, and 3) 'ambassador' animals that educate the public, engage non-scientists in biodiversity conservation, and generate funds to support *in situ* conservation. However, cheetahs reproduce poorly in captivity (likely due to suboptimal management), and these populations are not self-sustaining. As a result, ~30% of individuals in the global captive population are wild-caught [46]. Within the North American Species Survival Plan (SSP) population, only ~20% of the 281 cheetahs have ever reproduced, and many founding lineages are severely under-represented (Fig. 1.3) [47]. Based on recent population growth rates, genetic diversity is predicted to decline to 90% of current levels in only 32 years, in contrast to the 100 year goal established by the SSP [47].

Technologies associated with assisted reproduction (e.g., sperm cryopreservation, artificial insemination, in vitro fertilization) can facilitate the management of rare felid populations through increased reproductive success, reduced translocations of stress-sensitive animals for breeding, and the long-term (in theory, infinite) preservation of genetic diversity. An

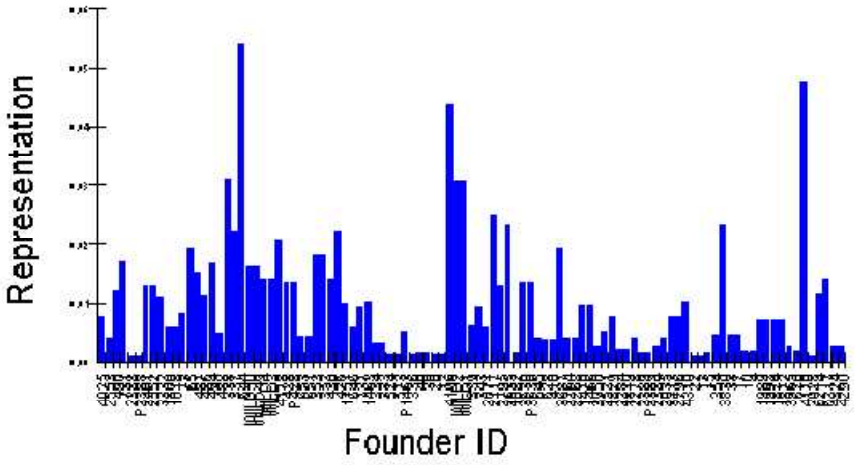

Figure 1.3. Unequal representation of founding lineages in the North American SSP cheetah population [47].

excellent example of the potential of these tools to contribute to species survival is the black-footed ferret. A successful captive breeding and genome resource banking program (including routine artificial insemination using cryopreserved spermatozoa) was instrumental in rescuing this carnivore from the brink of extinction [48]. Artificial insemination has been successful in the cheetah [48], and one surviving cub was produced using cryopreserved spermatozoa imported from Africa [49]. Yet despite a demonstrated potential, the success of these technologies in the cheetah remains limited [48]. Poor ejaculate quality and extreme cryo-sensitivity of cheetah spermatozoa represent significant challenges to effective genetic management in this species. Therefore, the cheetah was a priority target species for our investigation of felid sperm metabolism. Because sperm energy production is vital for successful fertilization, understanding this process likely will provide clues to optimizing assisted reproduction and, ultimately, preserving genetic diversity in this remarkable species.

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CHAPTER 2: EVIDENCE FOR COMPROMISED METABOLIC FUNCTION AND LIMITED GLUCOSE UPTAKE IN SPERMATOZOA FROM THE TERATOSPERMIC DOMESTIC CAT (*FELIS CATUS*) AND CHEETAH (*ACINONYX JUBATUS*)

2.1 Abstract

Cheetahs and certain other felids consistently ejaculate high proportions (≥ 60%) of malformed spermatozoa, a condition known as teratospermia that is prevalent in humans. Even normal-appearing spermatozoa from domestic cat teratospermic ejaculates have reduced fertilizing capacity. To understand the role of sperm metabolism in this phenomenon, we conducted a comparative study in the normospermic domestic cat versus the teratospermic cat and cheetah with the general hypothesis that sperm metabolic function is impaired in males producing predominantly pleiomorphic spermatozoa. Washed ejaculates were incubated in chemically-defined medium containing glucose and pyruvate. Uptake of glucose and pyruvate, and production of lactate were assessed using enzyme-linked fluorescence assays. Spermatozoa from domestic cats and cheetahs exhibited similar metabolic profiles, with minimal glucose metabolism and approximately equimolar rates of pyruvate uptake and lactate production. Compared to normospermic counterparts, pyruvate and lactate metabolism were reduced in teratospermic cat and cheetah ejaculates, even when controlling for sperm motility. Rates of pyruvate and lactate (but not glucose) metabolism were correlated positively with sperm motility, acrosomal integrity, and normal morphology. Collectively, our findings revealed that pyruvate uptake and lactate production were reliable, quantitative indicators of sperm quality in these two felid species, and that metabolic function was impaired in teratospermic ejaculates. Furthermore, patterns of substrate utilization were conserved between these species, including the unexpected lack of exogenous glucose metabolism. Because glycolysis is required to support

sperm motility and capacitation in certain other mammals (including the dog), the activity of this pathway in felid spermatozoa is a target for future investigation.

2.2 Introduction

An interesting trait of certain felid species and genotypes is the production of unusually high proportions of sperm malformations. Species, populations, or individuals that express this condition are considered teratospermic [1]. This phenomenon is especially common in species or subspecies that have low levels of gene diversity (cheetah [2-4]; Florida panther [5, 6]; Asia lion [7]) and in domestic cats that have been purposefully inbred [8]. Teratospermia (defined here as the production of ≥ 60% structurally-abnormal spermatozoa) also is common among men. A recent meta-analysis of semen characteristics by the World Health Organization revealed that > 95% of men can be classified as teratospermic under this definition [9]. Researchers at the Smithsonian Conservation Biology Institute have used certain felid species and genetic lineages to better understand the impact and etiology of teratospermia. Various studies have revealed that spermatozoa from teratospermic ejaculates demonstrate delayed capacitation [10], compromised acrosomal function [10], disrupted protein tyrosine phosphorylation [11, 12], increased osmotic sensitivity [13, 14], reduced zona penetration ability [15], and increased sensitivity to cooling [16] and cryopreservation [17]. These mechanisms no doubt contribute to the reduced fertilizing ability of teratospermic ejaculates in vitro, even after processing to isolate structurally-normal spermatozoa for insemination [15].

Some of these physiological impairments (e.g., tyrosine phosphorylation) could be related to a diminished capacity for energy production in malformed spermatozoa, but there is currently no knowledge of gamete metabolism in felids. Studies of mammalian sperm energy

production, although conducted since the 1940s, have generally been confined to humans and fewer than 10 domesticated species [18]. Yet as Storey detailed in a recent review [18], there are considerable differences in metabolic function of male gametes, even within this small group of species. It is known that spermatozoa are capable of generating energy in the form of adenosine triphosphate (ATP) through glycolysis and/or oxidative phosphorylation. However, the relative importance of each pathway to sperm functions, such as motility and capacitation, varies among species [18-25]. Oxidative phosphorylation is 18 times more efficient than anaerobic glycolysis and provides a significant proportion of the ATP supply in spermatozoa of most species [21]. The notable exception is the human, whose sperm appear to rely entirely on glycolysis for motility and hyperactivation [26]. Despite the efficiency of oxidative metabolism, its ability to fulfill energy demands in the distal flagellum is questionable [23, 24, 27] as mitochondria are confined to the sperm midpiece. Therefore, glycolysis may be an important supplemental source of ATP to fuel sperm motility, and glycolytic enzymes have been localized along the fibrous sheath of the flagellum in the boar, bull, rat, stallion, human and mouse [28, 29]. Sperm production of lactate (presumably by glycolysis) is correlated positively with motility, normal morphology, acrosomal integrity, and osmotic resistance in the boar and donkey [30, 31]. One of the latter studies has suggested that these relationships are more than casual in that litter size in the pig is enhanced after artificial insemination using sperm producing high lactate concentrations [30].

In contrast to livestock species, there is a lack of information on sperm metabolism in carnivores. Gamete metabolism has been fairly well-studied in the domestic dog [19, 32-39], but, to our knowledge, these pathways have not been investigated in any other carnivore species. The dog apparently is uniquely capable of sperm gluconeogenesis [19], which is surprising given

that glucose synthesis requires three times more ATP than is produced by glycolysis [40]. Utilization of this pathway may explain how dog spermatozoa are able to maintain motility and achieve capacitation in a medium without glucose [19, 38]. This is especially interesting as glucose is required for capacitation in the mouse [41] and human [26], but inhibits this process in the bull [42] and guinea pig [43].

Felids are attractive models for studying gamete metabolism. The availability of multiple species within the family Felidae provides opportunities for comparative studies to understand the conservation (or diversification) of physiological processes. Furthermore, the existence of teratospermia in certain species or genetic lineages provides the opportunity to explore linkage between a complex biological phenomenon and potential causative factors. Our aim in this study was to determine the relationship between rates of glycolytic and oxidative sperm metabolism and conventional indices of cellular function (i.e., structural morphology, motility, and acrosomal integrity). The approach was unique because we took advantage of two domestic cat populations that consistently produce differing proportions of pleiomorphic spermatozoa. To increase the robustness of our findings, we conducted a cross-species comparison using the cheetah, a species that is well known to be teratospermic regardless of season or living in free-ranging versus captive conditions [2-4]. Our hypotheses were that: 1) metabolic rates are useful indicators of sperm quality in felids; and 2) metabolic function is compromised in spermatozoa from teratospermic ejaculates compared to normospermic counterparts. We expected that elucidating the pathways of felid sperm energy production not only would provide insight into the physiological basis of teratospermia, but also might yield a reliable, quantitative indicator of ejaculate quality. The latter information has potential applied benefits. For example, identifying metabolic substrate requirements would be highly informative for enhancing the use of certain

types of assisted reproductive technologies for genetically managing wild felid species [44] or domestic cat lineages studied as models of human disorders [45].

2.3 Materials and Methods

2.3.1 Animals

Electroejaculates were collected from adult domestic cats (ages, 1.5 – 8 years) that were known to consistently produce either normospermic (≥ 60% normal sperm/ejaculate, n = 3 males) or teratospermic (<40% normal sperm/ejaculate, n = 3 males) ejaculates. A total of 15 ejaculates was collected from normospermic males (3 – 8 per individual) and 19 ejaculates from teratospermic males (3 – 9 per individual). Males were housed individually in 2.7 m^3 indoor cages at the Smithsonian Conservation Biology Institute (Front Royal, VA), maintained on a 14:10 h light:dark cycle and provided dry, commercial cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water *ad libitum*.

Electroejaculates (1 per male, 22 males) were collected from adult cheetahs (ages, 2.5 – 10 years) housed at the Cheetah Conservation Fund (CCF, Otjiwarongo, Namibia; n = 18), White Oak Conservation Center (WOCC, Yulee, FL; n = 3), or the Smithsonian's National Zoological Park (NZP, Washington, D.C.; n = 1). Males at CCF were wild-born and housed as described previously [46]. Males at WOCC were wild-born and housed together (in a group of 3) in a 2,000 m^2 outdoor enclosure and fed a commercially-produced Nebraska Carnivore diet (Central Nebraska Packaging Inc, North Platte, NE). The single male at NZP was captive-born and housed on exhibit with two other males in a 1,400 m^2 outdoor enclosure and fed a commercially-produced carnivore diet (Carnivore - 10; Natural Balance Pet Foods Inc., Pacoima, CA).

2.3.2 Semen Collection and Evaluation

A surgical plane of anesthesia was induced in domestic cats and cheetahs according to protocols determined by institutional veterinarians and similar to those previously utilized for semen collection in these two species [10, 15, 46]. All animal procedures were approved by NZP's Animal Care and Use Committee (ACUC) and the WOCC-ACUC. Methods for semen collection/evaluation were similar to those described in previous studies [10, 15, 46]. A rectal probe of 1 cm (domestic cat) or 1.9 cm (cheetah) in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, OR) were used to deliver 80 stimuli (at a low voltage of 2 – 5 V) over a 30 min interval [47]. Ejaculates (n = 56 total) were collected in sterile, pre-warmed vials as previously described [4, 47].

The volume of each ejaculate was measured using a pipette, and 3 µl of ejaculate were immediately assessed visually for sperm percentage motility and forward progressive status (FPS or speed of forward progression; scale = 0 – 5, with a rating of 5 equivalent to most rapid, straightforward progress [47]). A sperm motility index was calculated using the formula (percent motility + (FPS × 20) ÷ 2 [48]. A 5 µl sample of raw semen was fixed in 0.3% glutaraldehyde in phosphate-buffered saline (pH 7.4, 340 mOsm) for subsequent assessment of sperm morphology [13]. For each sample, 100 spermatozoa were assessed (1,000× magnification) and classified as normal or having (in order of precedence) a head, acrosomal, midpiece, flagellar, or other abnormality, as previously described [47]. For all ejaculates, a second 5 µl aliquot of raw semen was fixed in 4% paraformaldehyde to evaluate acrosomal integrity. Fixed samples were processed using a modified Coomassie Blue G-250 (Fisher Biotech, Springfield, NJ) staining technique, as described earlier [49, 50], and 200 spermatozoa from each sample were evaluated (1,000×) and classified as having an intact or non-intact

acrosome. Spermatozoa with an intact acrosome exhibited a uniform blue staining pattern overlying the acrosomal region, whereas non-intact cells had clear or patchy staining over this region [50].

2.3.3 Sperm Processing and Metabolic Assessments

Each ejaculate was diluted immediately with an equal volume of a chemically-defined and protein-free, modified mouse tubal fluid medium (cMTF) [51] supplemented with 2% polyvinyl alcohol (PVA) [52]. The final cMTF medium (pH 7.45) 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 (MOPS) buffer, and 0.02 mg/mL phenol red. All reagents were purchased from Sigma Aldrich (St. Louis, MO). The cMTF medium was prepared fresh daily from five concentrated stock solutions containing: 1) $NaHCO_3$ and phenol red; 2) $CaCl_2$; 3) glucose and pyruvate; 4) MOPS and phenol red; and 5) all remaining reagents. All stock solutions were kept at 4^{o}C and discarded after 2 weeks (stocks 1 – 3) or 3 months (stocks 4 and 5). PVA was added, and the medium was sterilized through a 0.22 μm syringe filter immediately prior to use. Osmolality of the final working medium (295 – 341 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 [53]).

Diluted ejaculates (maintained at ambient temperature, 19 – 24^{o}C) were washed by centrifugation (8 min; × 300 *g* for domestic cat; × 200 *g* for cheetah) and resuspended in fresh cMTF at a concentration of 3×10^6 motile sperm/ml. Sperm concentration was determined using a Nucleocounter SP-100 (Chemometec, Denmark) [54]. Sperm samples (0.5 – 1.0 ml) were incubated (37^{o}C) in 1.5 ml centrifuge tubes under oil (200 μl) to prevent evaporation. Based on

rates of sperm oxygen consumption in the dog and fox [55], we estimated that dissolved oxygen in cat/cheetah sperm samples would decrease by < 1% after 24 h. Because sperm respiration is not limited until 90% of oxygen is depleted from the medium (starting at the atmospheric value) [56], hypoxia due to culture under oil was not of concern. A sample (130 µl) of sperm suspension was taken at 0, 1, 3, 7, and 24 h of incubation, and cells were removed by centrifugation (3 min; × 1,000 *g*) through a CoStar Spin-X 0.22 µm nylon filter tube (Corning Incorporated, Corning, NY). The filter was removed from the tube, and the sperm-free medium was stored at -80^{o}C until analysis. Acrosomal membrane integrity and sperm motility were assessed at 0, 1, 3, 7, and 24 h as described above, and are reported as average values over each time interval to facilitate comparison with metabolic rates.

Samples of sperm-free medium were analyzed for glucose, pyruvate, and lactate concentrations using enzyme-linked fluorescence assays [51, 57]. Each assay is linked to the oxidative status of the coenzyme NADP (glucose) or NAD (pyruvate and lactate). The reduced forms of these coenzymes (NADPH and NADH) fluoresce at 445 nm when excited at 340 nm, while the oxidized forms do not. For the glucose assay, sperm-free medium (10 µl) was incubated (5 min, 37^{o}C) with an enzyme cocktail (200 µl) containing 0.42 mM dithiothreitol, 3.1 mM $MgSO_4$, 0.42 mM ATP, 1.25 mM NADP, and 0.1 U/ml hexokinase/glucose-6-phosphate dehydrogenase (HK/G6PDH) in 50 mM EPPS buffer, pH 8.0. The cocktail was stored in the dark at -80^{o}C for up to 3 months prior to use. The conversion of glucose to 6-phosphogluconate was carried out as shown in equation 1. Glucose concentration determined by this assay was directly proportional to NADPH fluorescence.

Eqn. 1:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-phosphate} + \text{ADP}$$

$$\text{Glucose-6-phosphate} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{6-phosphogluconate} + \text{NADP}^+ + \text{H}^+$$

For the pyruvate assay, sperm-free medium was incubated with an enzyme cocktail (as above) containing 0.14 mM NADH and 0.12 U/ml lactate dehydrogenase (LDH) in 50 mM EPPS buffer (4-(2-hydroxyethyl)-1-piperazine propane-sulfonicacid), pH 8.0. The cocktail was stored in the dark at -80^{o}C for up to 3 months prior to use. The conversion of pyruvate to lactate was carried out as shown in equation 2. Pyruvate concentration measured by this assay was inversely proportional to NADH fluorescence.

Eqn. 2:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{lactate} + \text{NAD}^+$$

For the lactate assay, sperm-free medium (25 µl) was incubated (5 min, 37^{o}C) with an enzyme cocktail (250 µl) containing 1.92 U/ml LDH, 0.2 U/ml glutamate-pyruvate transaminase (GPT), 0.42 mM NAD^+, and 100 mM glutamate in 1 M glycine buffer containing 5.6 mM ethylenediaminetetraacetic acid. The buffer was stored at 4^{o}C for up to 1 month prior to use, and the cocktail was prepared fresh daily using NAD^+ and glutamate stock solutions stored at -80^{o}C for up to 3 months prior to use. This assay is a non-toxic alternative to the LDH/hydrazine assay. The conversion of lactate to alanine was carried out as shown in equation 3. Lactate concentration in this reaction was directly proportional to NADH fluorescence.

Eqn. 3:

$$\text{Lactate} + \text{NAD}^+ \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+$$

$$\text{Pyruvate} + \text{glutamate} \xrightarrow{\text{GPT}} \text{alanine} + \alpha\text{-ketoglutarate}$$

Enzymes (LDH, product # HK/G6PDH and GPT) were purchased from Roche Applied Science (Indianapolis, IN), and all other assay reagents were obtained from Sigma Aldrich (St. Louis, MO). Fluorescence was analyzed using a Spectra Max Gemini XPS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) and SoftMax Pro 5 software (Molecular Devices, Sunnyvale, CA). Metabolic rates were calculated as the change in substrate concentration over time, divided by sperm concentration and are reported in nmol/10^6 sperm/h.

2.3.4 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. Differences in ejaculate characteristics and sperm morphology among animal groups (normospermic domestic cat, teratospermic domestic cat, cheetah) were assessed using SAS General Linear Model Procedures (GLM) [58]. To evaluate changes in sperm motility index (SMI), acrosomal integrity (% IA) and metabolic rate over time, data were analyzed using a separate GLM for each animal group [58]. Within each domestic cat group (normospermic and teratospermic), there was no interaction ($P > 0.05$) between individual and time as well as no main effect of individual ($P > 0.05$) on SMI, % IA, or metabolism; thus, these variables were omitted from the final model. Differences in SMI, % IA, and metabolic rate among animal groups were assessed using a separate GLM for each time interval [58]. To determine if variation in sperm motility was responsible for differences in metabolic rates among animal groups, data from all individuals and time points were combined and analyzed using a GLM, with percent motile spermatozoa included as a covariate. When a significant ($P < 0.05$) *F*-statistic was measured in any GLM, differences among means were assessed using Duncan's multiple-range test. Pearson's

correlation was used to evaluate the relationships between metabolic rate and sperm morphology, SMI and % IA within and across animal groups. Results were considered significant at $P < 0.05$.

2.4 Results

2.4.1 Ejaculate and Sperm Characteristics

Semen volume and sperm concentration were similar ($P > 0.05$) in normospermic and teratospermic domestic cats (Table 2.1). Cheetah ejaculates were less ($P < 0.05$) concentrated than those from domestic cats, but due to larger ($P < 0.05$) seminal volumes, the total number of spermatozoa per ejaculate did not ($P > 0.05$) differ among the three animal groups (Table 2.1). The average sperm motility index and percentage of structurally-normal spermatozoa were similar ($P > 0.05$) between teratospermic domestic cats and cheetahs, both of which were less ($P < 0.05$) than in normospermic cats (Table 2.1). A bent midpiece encompassing a cytoplasmic droplet was the most prevalent deformity observed in each animal group, and constituted ~45% of all abnormalities (Table 2.1). This was followed by acrosomal abnormalities and proximal droplets, which were more ($P < 0.05$) common in the cheetah (19% and 14% of all deformities, respectively) compared to the domestic cat (~8% and 5%). A bent flagellum encircling a cytoplasmic droplet was a less ($P < 0.05$) frequent deformity in the cheetah (3%) than domestic cat (~12%). In both species, spermatids and midpiece bends (without a droplet) constituted < 10% of all abnormalities. More than a dozen other deformities were observed rarely (≤ 5%) in each group, but collectively comprised a significant proportion (~15%) of total anomalies. These malformations were classified as 'other' and included macro/micro-cephaly, bi/tri-cephaly, a misshapen head, residual cytoplasm attached to the head, a bent neck, partial or complete midpiece aplasia, a distal midpiece droplet, a misshapen midpiece, a coiled flagellum

Table 2.1. Ejaculate characteristics of domestic cats and cheetahs. Values represent least-squares means ± standard errors.

Parameter	Normospermic cat	Teratospermic cat	Cheetah
No. males	3	3	22
No. ejaculates	15	19	22
Semen volume (mL)	0.15 ± 0.12^{a}	0.17 ± 0.11^{a}	1.98 ± 0.10^{b}
Sperm concentration ($x10^6$ cells/ml)	345 ± 41^{a}	267 ± 37^{a}	50 ± 34^{b}
Spermatozoa per ejaculate ($x10^6$)	57 ± 21	48 ± 19	93 ± 17
Sperm motility index*	80 ± 1^{a}	66 ± 1^{b}	69 ± 1^{b}
Structurally-abnormal spermatozoa (%)	37 ± 4^{a}	73 ± 4^{b}	76 ± 3^{b}
Deformity type (% of total abnormalities)			
Abnormal acrosome	8.8 ± 2.6^{a}	7.0 ± 2.2^{a}	19.3 ± 2.1^{b}
Bent midpiece with cytoplasmic droplet	49.2 ± 5.0	43.4 ± 4.7	39.8 ± 4.7
Bent midpiece without droplet	6.3 ± 1.2^{a}	$3.4 \pm 1.1^{a,b}$	2.1 ± 1.0^{b}
Proximal midpiece droplet	5.0 ± 2.2^{a}	5.4 ± 2.1^{a}	13.7 ± 1.9^{b}
Bent flagellum with droplet	12.9 ± 2.1^{a}	10.3 ± 1.9^{a}	3.0 ± 1.7^{b}
Spermatid	2.2 ± 2.1^{a}	9.0 ± 2.0^{b}	$6.8 \pm 1.8^{a,b}$
Other*	15.7 ± 3.0	21.8 ± 2.8	15.4 ± 2.5
Intact acrosomes (%)	92 ± 2^{a}	84 ± 2^{b}	92 ± 2^{a}

* Calculated as percent (motility + forward progressive status × 20) ÷ 2.
† Macro/micro-cephaly, bi/tri-cephaly, misshapen head, residual cytoplasm attached to head, midpiece aplasia, abnormal midpiece, distal midpiece droplet, coiled flagellum with or without a droplet, biflagellate, and bent neck.
[a,b] Within rows, values with different superscript letters differed ($P < 0.05$).

with or without a droplet, and a bi/tri-flagellate spermatozoon. Images of the more prevalent deformities (i.e., constituting ≥ 10% of abnormalities in any group) are provided in Supplemental Figure 2.1. These depictions along with most of the uncommon malformations listed above also are available in earlier publications [2, 4].

All three animal groups ejaculated high percentages of acrosome-intact spermatozoa (> 80% overall; Table 2.1), although teratospermic cats produced a smaller ($P < 0.05$) proportion of cells with intact membranes compared to normospermic counterparts or cheetahs.

2.4.2 Sperm Motility, Acrosomal Integrity, and Metabolism

Within each time interval, sperm motility index (SMI) and acrosomal integrity were similar ($P > 0.05$) in teratospermic cats and cheetahs, but reduced ($P < 0.05$) compared to normospermic cats (Fig. 2.1). Sperm pyruvate uptake from 0 – 1 h was higher ($P < 0.05$) in teratospermic cats compared to cheetahs, and rates in normospermic cats were similar ($P > 0.05$) to both of these groups (Fig. 2.2A). However, after 1 h, rates were ~70% lower ($P < 0.05$) in teratospermic cats and cheetahs compared to normospermic cats. Due to this inconsistency and large standard error values for the 0 – 1 h interval, rates of pyruvate uptake for this time interval were omitted from Pearson's correlations.

Exogenous glucose was minimally utilized by domestic cat spermatozoa, and rates of uptake were similar ($P > 0.05$) overall between normospermic and teratospermic males (Fig 2.2B). Extracellular glucose concentration of cheetah sperm medium samples did not change ($P > 0.05$) from 0 – 24 h, indicating no metabolism of this substrate by this species. Sperm lactate production occurred at comparatively high rates in all three groups, given the expected ratio (2:1) of lactate production to glucose uptake (Fig. 2.2C). Compared to normospermic cats, rates of

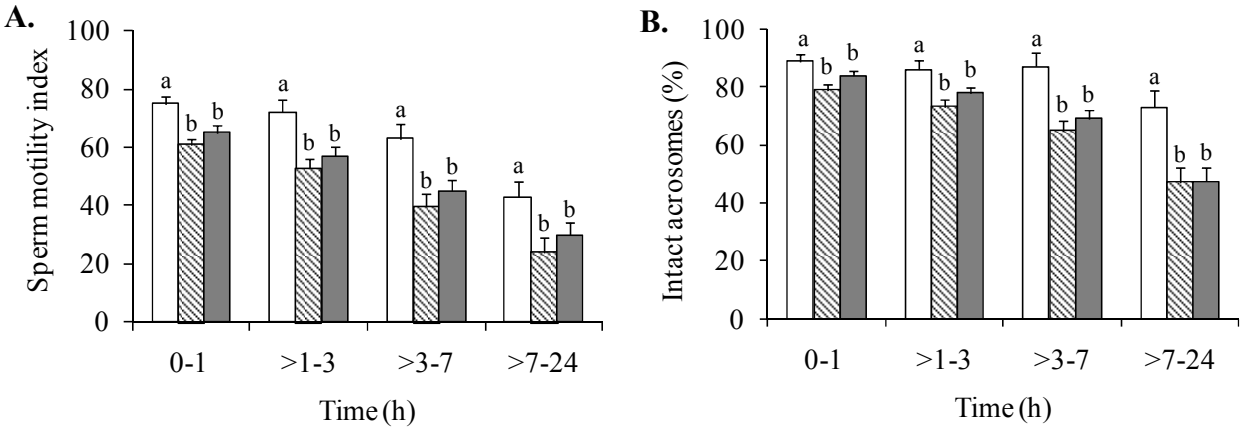

Figure 2.1. Sperm motility index (A) and acrosomal integrity (B) in normospermic domestic cats (open bars), teratospermic domestic cats (lined bars) and cheetahs (solid bars). Among animal groups and within each time point, bars with different superscripts differed ($P < 0.05$). Error bars represent means ± SEM.

sperm lactate production were ~60% less ($P < 0.01$) in teratospermic cats and ~80% less ($P < 0.001$) in cheetahs. Extreme variation in stoichiometric ratios (glucose or pyruvate uptake as a proportion of lactate production) was observed within each time interval for all three groups, with most CV values being ≥50% (data not shown). This variation likely was related to minimal (or sometimes zero) changes in metabolic substrate concentration between consecutive time points (even in samples with high percentages of motile spermatozoa). In support of this supposition, normospermic cats demonstrated higher sperm metabolic rates and overall less variation in stoichiometric ratios (CV = 12 – 392%) compared to the other two groups. To eliminate as much assay 'noise' as possible, stoichiometric ratios were recalculated based on the total change in substrate concentration from 0 – 24 h of incubation. Using this method, most (60

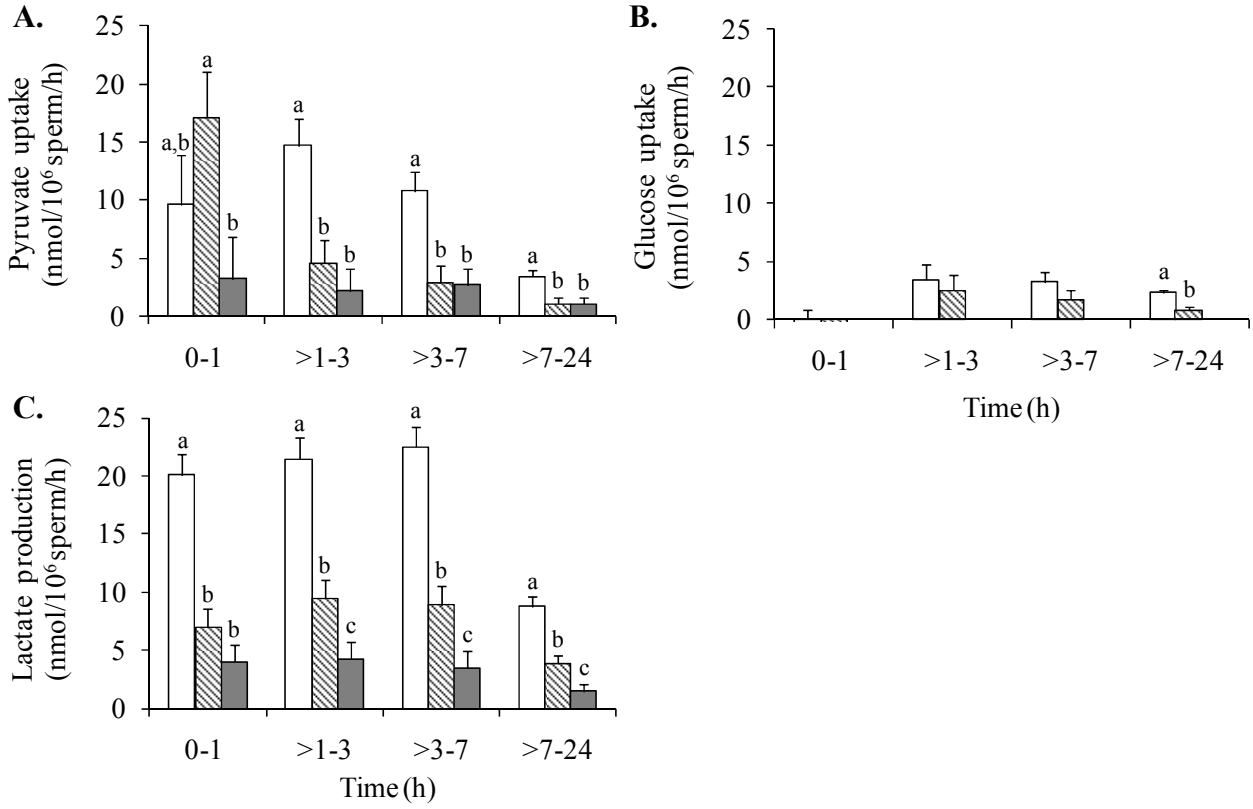

Figure 2.2 Sperm pyruvate uptake (A), glucose uptake (B) and lactate production (C) in normospermic domestic cats (open bars), teratospermic domestic cats (lined bars) and cheetahs (solid bars). Among animal group and within each time point, bars with different superscripts differed ($P < 0.01$). Error bars represent least-squares means ± standard errors.

– 70%) lactate production was attributed to the uptake and reduction of pyruvate, whereas a lesser portion (0 – 40%) was credited to the metabolism of exogenous glucose metabolism (Table 2.2).

Table 2.2. Substrate uptake/lactate production ratios in domestic cat and cheetah spermatozoa.

Parameter	Normospermic Cat	Teratospermic Cat	Cheetah*
No. males	3	3	22
No. ejaculates	15	19	22
Glucose	0.2 ± 0.4	0.1 ± 0.4	ND
Pyruvate	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1

* ND, not detected.

2.4.3 Relationship Between Metabolic Rates and Sperm Quality

When data from all ejaculates (n = 56) were combined, rates of pyruvate uptake were correlated positively ($P < 0.001$) to sperm motility index (SMI; $r = 0.44$) and the percentage of intact acrosomes (% IA; $r = 0.43$; Table 2.3). Because rates of lactate production were correlated positively ($P < 0.001$) to normal sperm morphology across all ejaculates, as well as with SMI and % IA ($r = 0.42 – 0.50$; Table 2.3), this metric was found to be a more accurate indicator of overall cellular quality than pyruvate uptake. Furthermore, rates of lactate production were correlated positively ($P < 0.05$) to SMI and % IA within each group ($r = 0.34 – 0.67$). Rates of glucose uptake were not ($P > 0.05$) correlated with SMI or % IA in any group.

To determine if decreased metabolic rates in spermatozoa of teratospermic cats and cheetahs were an artifact of reduced motility in these cells, data were reanalyzed with sperm motility (% motile and SMI) included as covariates in the GLM. Results were consistent with the previous analysis: sperm pyruvate uptake and subsequent lactate production were decreased

Table 2.3. Correlation coefficient (r) values for metabolic rate versus sperm quality in domestic cats and cheetahs[*].

Parameter	Overall	Normospermic cat	Teratospermic cat	Cheetah
No. males	28	3	3	22
No. ejaculates	56	15	19	22
Pyruvate uptake (nmol/10^6sperm/h)				
Sperm motility index	0.44[b]	0.44[a]	0.50[b]	NS
Intact acrosomes (%)	0.43[b]	0.41[a]	0.52[b]	NS
Structurally-normal spermatozoa (%)	NS	NS	NS	NS
Lactate production (nmol/10^6sperm/h)				
Sperm motility index	0.45[b]	0.37[a]	0.50[b]	0.67[b]
Intact acrosomes (%)	0.42[b]	0.34[a]	0.51[b]	0.62[b]
Structurally-normal spermatozoa (%)	0.50[b]	NS	NS	NS

[*] NS = not significant ($P > 0.05$).
[a] $P < 0.05$; [b] $P < 0.001$.

($P < 0.05$) in teratospermic cats and cheetahs compared to normospermic cats, thereby indicating a direct relationship to the teratospermic condition.

Given that rates of lactate production were correlated positively to normal sperm morphology across all felid ejaculates, we were curious about the metabolic function of cheetah ejaculates containing relatively high proportions (~40%) of structurally-normal spermatozoa. Specifically, do these high-quality cheetah ejaculates demonstrate 'normal' metabolic function when compared to domestic cat counterparts? Conversely, is metabolic function more severely compromised in cheetah ejaculates with very low (~5%) proportions of structurally-normal spermatozoa? To address these questions, cheetah ejaculates from the existing dataset were ranked in order of increasing percentages of structurally-normal sperm (% N), and those with the lowest and highest percentage values were selected for comparison of lactate production (n = 5 per group; Fig. 2.3). The mean % N in each ejaculate group was 7 ± 3% (range, 5 – 10%) and 42 ± 3% (range, 35 – 58%), respectively. Domestic cat ejaculates (n = 9 total, 4 males) having % N values within the 35 – 58% range (mean, 44 ± 2%) were selected from the existing dataset as a

control for 'normal' rates of lactate production. Despite the large difference in % N between the two cheetah groups, rates of sperm lactate production were similar ($P > 0.05$) and were reduced ($P < 0.05$) compared to the domestic cat control group (Fig. 2.3). Pyruvate and glucose metabolism was not reassessed because rates of uptake were not correlated with any sperm quality index in the cheetah (Table 2.3).

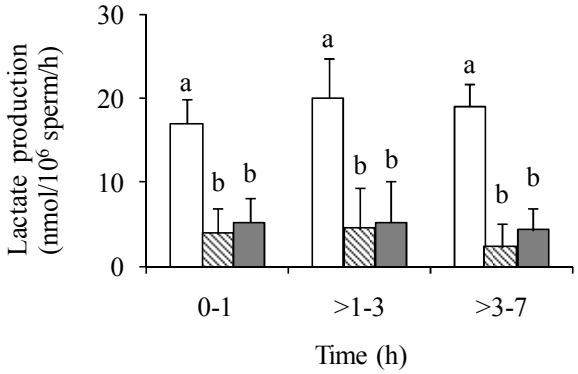

Figure 2.3 Sperm lactate production in cheetah ejaculates selected for either normal (lined bards) or abnormal (solid bars) morphology, compared to a domestic cat control group (open bars). Among animal group and within each time point, bars with different superscripts differed ($P < 0.01$). Error bars represent least-squares means ± standard errors.

2.5 Discussion

This was the first study of sperm metabolism in any felid species, and we made four significant discoveries. First, we determined that felid spermatozoa (at least from the two species studied here) did not rely on exogenous glucose as a source of energy. Rather, based on the observed ratios of substrate uptake/production, it appeared likely that these cells generated ATP from the catabolism of one or more unidentified endogenous sources. Secondly, certain cellular mechanisms related to sperm energy production were conserved between the domestic cat and cheetah, indicated by similar patterns of substrate metabolism between the species. Third, metabolic function was impaired in spermatozoa from teratospermic ejaculates as revealed by relatively low rates of pyruvate uptake and lactate production in males producing high proportions of pleiomorphisms. This observation was consistent with previous reports that linked teratospermia to disruptions in multiple components of sperm function, including several energy-dependent processes [10, 11, 15, 59]. Finally, rates of lactate production were correlated

positively to multiple measures of sperm function in both the domestic cat and cheetah. Therefore, this substrate may prove to be a valuable indicator of ejaculate quality.

The lack of glucose uptake by domestic cat and cheetah spermatozoa is unexpected, given that glycolysis is required to support sperm motility [21, 26, 34] and capacitation [19, 26, 60, 61] in the human and domestic dog. This finding perhaps could result from low hexokinase activity in felids compared to other species or, in the case of the cheetah, the complete absence of this enzyme. Reduced hexokinase activity would limit NADPH production, a key component of the glutathione-mediated defense system that protects cellular membranes against lipid peroxidation damage [62]. This is an intriguing possibility, as spermatozoa from teratospermic felids (including the domestic cat and cheetah) are unusually susceptible to membrane damage [13, 16, 46]. However, it also remains possible that felid spermatozoa possess fully functional hexokinase, but metabolize glucose at modest rates relative to oxidative substrates. We currently are using the glycolytic inhibitor α-chlorohydrin to more thoroughly understand the role of glycolysis in felid sperm cellular function.

Given the observed lack of glucose uptake by cat and cheetah spermatozoa, we were surprised to discover that these cells consistently produced lactate, which is an end-product of glycolysis [63]. Under our experimental conditions, we consider there to be three possible sources of lactate: 1) endogenous glycogen; 2) endogenous phospholipid; and/or 3) imported pyruvate. These potential mechanisms of lactate production are not mutually exclusive and may also contribute to varying degrees in the generation of NADH and ATP (Fig. 2.4). In each case, lactate formation could occur in cytosol or mitochondria as sperm-specific lactate dehydrogenase (LDHC) has been found in these compartments [64-67]. Lactate and NADH production also could occur in separate cellular locations, since reducing equivalents can be transferred between

the cytosol and mitochondria by the malate-aspartate shuttle (present in spermatozoa of several species [68-70]).

Glycogen is known to be metabolized by spermatozoa of the domestic dog, another carnivore [36]. Intracellular glycogen breakdown would yield ATP, and cytosolic NAD^+ would be regenerated by lactate production (Fig. 2.4). However, phospholipid is considered the primary endogenous substrate for most mammalian spermatozoa [21] and could provide greater

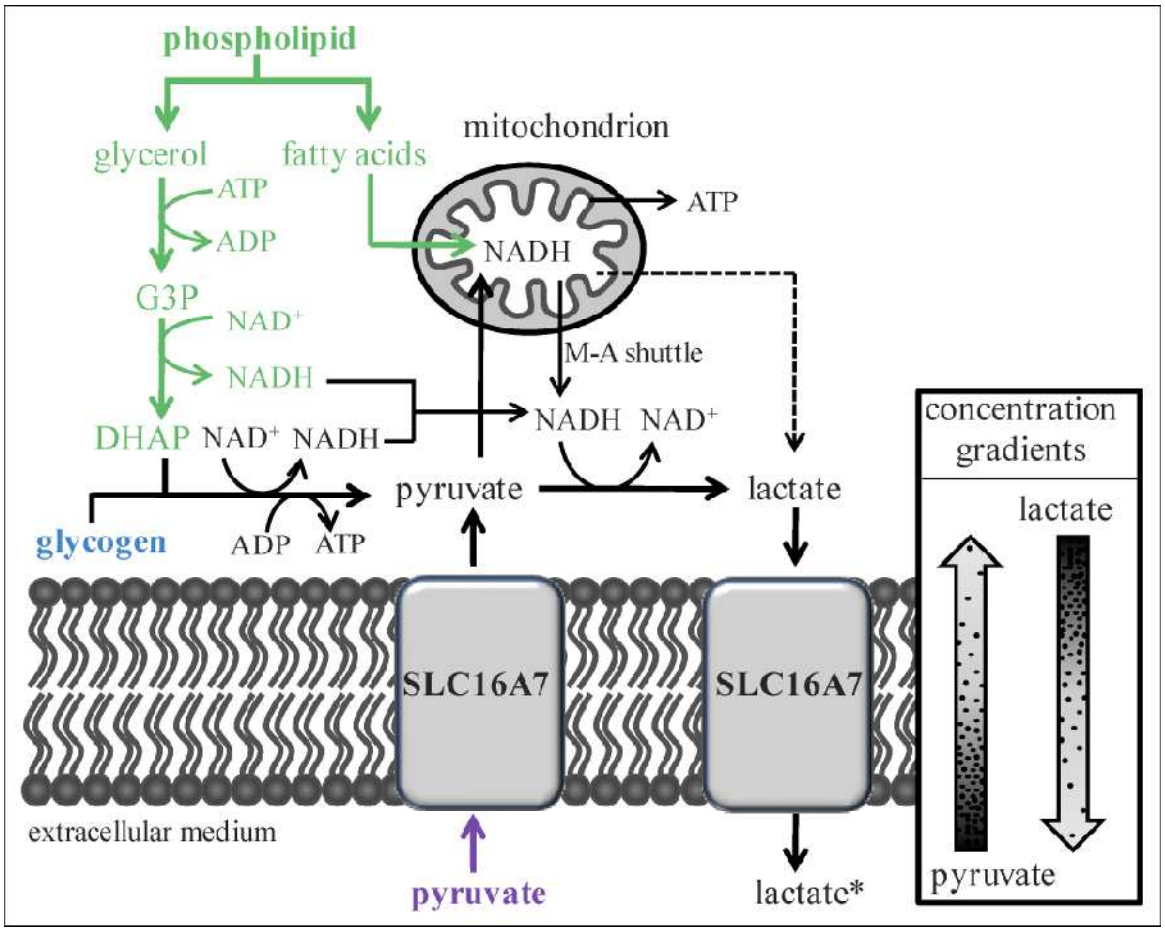

Figure 2.4. Theoretical model showing three possible mechanisms of lactate production by cat and cheetah spermatozoa, with NADH, lactate, and ATP generated from the metabolism of endogenous phospholipid (green), glycogen (blue), and/or extracellular pyruvate (purple). Common metabolic products or intermediates are in black. Dashed line indicates possible intramitochondrial lactate formation. Enzymes and certain metabolic products or intermediates are omitted from the figure for clarity. The absence of lactate in the starting medium is noted by an asterisk (*). SLC16A7 = monocarboxylic acid transporter 2; G3P = glycerol-3-phosphate; DHAP = dihydroxyacetone phosphate; M-A shuttle = malate-aspartate shuttle.

amounts of cellular ATP. Phospholipid hydrolysis would yield glycerol and fatty acids (Fig. 2.4). Glycerol would enter the glycolytic pathway via conversion to dihydroxyacetone phosphate (DHAP) and would be metabolized to produce lactate, NADH, and ATP [71]. Mitochondrial oxidation of fatty acids would provide substantial amounts of NADH, which could contribute to ATP and/or lactate production (Fig. 2.4).

Because we used a protein-free, chemically defined medium, the only possible exogenous source of lactate in our study was by the uptake and reduction of extracellular pyruvate. Although pyruvate reduction would require a NADH source and would not generate ATP [63], these molecules could be provided by endogenous substrate metabolism (as described above). Pyruvate uptake likely would occur via the facultative transporter SLC16A7 (monocarboxylic acid transporter 2, previously known as MCT2), the primary monocarboxylate transporter in mature spermatozoa of species studied to date [72-74]. Given the starting composition of our cMTF medium (1 mM pyruvate, 0 mM lactate) and the kinetic properties of SLC16A7 and LDH [75-77], rapid pyruvate uptake and reduction theoretically should occur independently of sperm energy production. Examination of stoichiometric ratios revealed that 60 to 70% of produced lactate could have been explained on the basis of pyruvate uptake and reduction (in contrast to only 0 – 40% generated from exogenous glucose catabolism). These are important observations given that lactate production (measured by enzyme-linked fluorescence) has been used as an indicator of glycolytic metabolism for other species [25, 30, 31]. Ongoing research in our laboratory supports this mechanism of lactate production in felid spermatozoa. Initial findings determined that sperm lactate production was approximately four times greater in the presence of exogenous pyruvate compared to equimolar amounts of exogenous glucose. Furthermore, in the absence of both substrates in the culture medium no lactate was produced. Collectively, these

observations suggested that mammalian spermatozoa may produce lactate independently of glycolysis, implying that the functional importance of this pathway could have been misinterpreted in earlier studies. More detailed studies are in progress in our laboratory using chemical inhibitors of oxidative and glycolytic metabolism to identify the primary energy substrates for felid spermatozoa.

Although the source of lactate was unclear, rates of production provided a consistent indicator of sperm motility and acrosomal function in both felid species. Intriguingly, lactate production also was correlated to proportions of structurally-normal spermatozoa among domestic cat, but not cheetah, ejaculates. This finding may be related to previous reports that even structurally-intact cells from teratospermic ejaculates can be functionally-compromised. Specifically, these spermatozoa may demonstrate increased osmotic sensitivity, delayed acrosome reaction, or reduced zona penetration ability [10, 11, 15]. Therefore, rates of lactate production may provide an accurate indicator of ejaculate quality in both species, and may reveal disrupted cellular physiology in apparently-normal cheetah spermatozoa. However, lactate production should be validated against a more direct measure of sperm fertilizing ability such as the zona pellucida assay, particularly given its unclear relationship to ATP generation. Because spermatozoa must be capable of multiple energy-dependent processes to achieve successful fertilization, a zona penetration assay could provide a more robust test of the functional relevance of lactate production than any single measure of sperm function (e.g., motility). Understanding the source and biological significance of lactate produced by felid spermatozoa could yield a quantitative, field-friendly indicator of ejaculate quality, which would significantly facilitate developing and refining reproductive technologies for improving felid management and conservation.

Because so little is known about gamete metabolism in felids, this taxon is an excellent target for more detailed investigation in this area of study. The existence of >30 species in the family Felidae (including several with the teratospermic phenotype [78]) also provides important opportunities to determine the etiology and evolution of certain physiological attributes influencing reproductive success. Indeed, direct comparisons across species are fundamental to identifying differences in the mechanisms involving energy production in mammalian gametes, particularly given the ambiguities of published findings relating to sperm metabolism. For example, Storey et al. have reported that mouse spermatozoa metabolize endogenous oxidative substrates to remain motile for >4 h in vitro in the absence of supplemental energy sources [79]. Yet Mukai and Okuno have found that mouse spermatozoa become non-motile within 30 min in a substrate-free medium and have presented evidence for an obligate role of glycolysis in supporting cellular motility [80]. Contradictory findings also have been reported regarding the need for exogenous glucose to achieve capacitation in dog spermatozoa [61, 81]. Such equivocations often can be avoided and more confidence generated by comparatively evaluating two taxonomically-related species using a standardized experimental protocol. In the present study, we found similar patterns of substrate uptake/production in the domestic cat and cheetah, suggesting that spermatozoa of both species relied on the same energy source(s). Relatively low rates of substrate uptake/production in cheetah ejaculates revealed that sperm metabolic efficiency was compromised in this species. More importantly, comparison with the teratospermic cat model then allowed us to link this finding to ejaculate phenotype. Still, we uncovered a subtle, but key physiological difference between these species. Specifically, in contrast to the domestic cat, even apparently 'high-quality' cheetah ejaculates (i.e., containing higher proportions of structurally-normal spermatozoa) demonstrated compromised metabolic

function. Thus, while the collective results confirmed that the teratospermic cat was an excellent model for expanding knowledge about gamete physiology in wild felids, findings revealed important functional differences between these two related species.

In conclusion, study results underscored the importance of species diversity in fundamental reproductive phenomena, as has been emphasized recently in the contexts of biological conservation and human health [82]. We predict that understanding the unique mechanisms of energy production in felid spermatozoa will facilitate increased efficiency of assisted reproductive technologies that have been used for producing offspring in both wild felids [83, 84] and domestic cats, including at least eight models of human genetic disease (Swanson, W.F., personal communication) [85]. For example, such information would be important for optimizing medium composition to maximize sperm survival for artificial insemination, in vitro culture (including for IVF), and cryopreservation. Furthermore, we observed that metabolic efficiency reflected other sperm quality metrics across both individuals and species, thus providing the first objective, field-friendly indicator of gamete function in felids. Such findings also offer new insights for improving reproduction in small populations of endangered species or rare biomedical genotypes. Indeed, a basic understanding of the reproductive uniqueness of a previously unstudied species has been critical to recovery or development of self-sustaining populations [82]. The opportunity to conduct these fundamental studies is one of the invaluable contributions of ex situ (hedge) felid populations to the conservation of Earth's biodiversity [86].

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CHAPTER 3: GLYCOLYTIC ENZYME ACTIVITY IS ESSENTIAL FOR DOMESTIC CAT (*FELIS CATUS*) AND CHEETAH (*ACINONYX JUBATUS*) SPERM MOTILITY AND VIABILITY IN A SUGAR-FREE MEDIUM

3.1 Abstract

We previously have 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 (α-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 α-chlorohydrin resulted in an immediate decline in sperm motility, particularly in the domestic cat. Collectively, our findings revealed an essential role of the glycolytic pathway in felid spermatozoa that was unrelated to hexose metabolism or lactate formation. Instead, glycolytic enzyme activity could have been required for the metabolism of

endogenous lipid-derived glycerol, with fatty acid oxidation providing the primary energy source in felid spermatozoa.

3.2 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 also can 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 [27] 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 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], while 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 impact 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.

3.3 Materials and Methods

3.3.1 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). A total of 10 ejaculates was collected from normospermic males (1 – 5 per individual) and 10 ejaculates from teratospermic males (2 – 4 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 14:10 h light:dark cycle, and provided dry, commercial cat food (Purina Cat Chow; Ralston Purina Co., St. Louis, MO) and water ad libitum.

Electroejaculates (1 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 2,500-m^2 outdoor enclosures off exhibit and fed a mixed diet of two commercial products (Toronto Zoo Feline Diet; Milliken Meat Products Ltd., Scarborough, Ontario, Canada; 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 2,200-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 1,500-m^2 outdoor enclosure and fed a commercially produced carnivore diet (Carnivore Diet 10; Natural Balance Pet Foods Inc., Pacoima, CA).

3.3.2 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 utilized 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) diameter and an electrostimulator (P.T. Electronics, Boring, OR), as described previously [27, 45]. A sample of raw semen containing ~2×10^5 spermatozoa was fixed in 0.3% glutaraldehyde in phosphate-buffered saline for assessment of sperm morphology, as described previously [27, 45, 46].

3.3.3 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 (PVA) [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 (MOPS) 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 *g* for domestic cat; × 100 *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 (TIM), 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 (~2×10^5 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 utilize LDH-linked fluorescence for substrate quantification). Rates of glucose uptake were assessed over each time interval in the first treatment 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; × 1,000 *g*) through a CoStar Spin-X 0.22-µm nylon filter tube (Corning Incorporated, Corning, NY) and stored at –80°C until analysis using a 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; × 1,000 *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 nmol/10^6 sperm/h. All data were normalized to control values for presentation.

3.3.4 Glycogen Assay

Glycogen content was measured in control group sperm samples (3 x 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 (2 males per tube) and centrifuged (8 min; 300 × *g*) to obtain pellets (n = 3) containing ~1 x 10^8 sperm. All samples (ejaculated and epididymal) were stored frozen (-80^{o}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. Homogenates were incubated at 100^{o}C for 15 min, then incubated (37^{o}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.

3.3.5 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).

3.3.6 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's t-tests (treatment vs. control). Pearson's correlation was used to evaluate the relationships between sperm morphology and changes 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.

3.4 Results

3.4.1 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 3.1. 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. 3.2A-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. 3.2A), whereas FPS was reduced ($P < 0.05$) ~50% in the cat and ~20% in the cheetah (Fig 3.2B). Acrosomal integrity was not ($P > 0.05$) influenced by the lack of substrates in either species (Fig. 3.2C). In both cats and cheetahs, spermatozoa produced lactate in the absence of glucose, but only if pyruvate was present in the culture medium (Fig. 3.2D). 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 (Sup. Fig. 3.1A). Likewise, rates of pyruvate uptake were not affected ($P > 0.05$) by the absence of exogenous glycolytic substrates (Sup. Fig. 3.1B). 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).

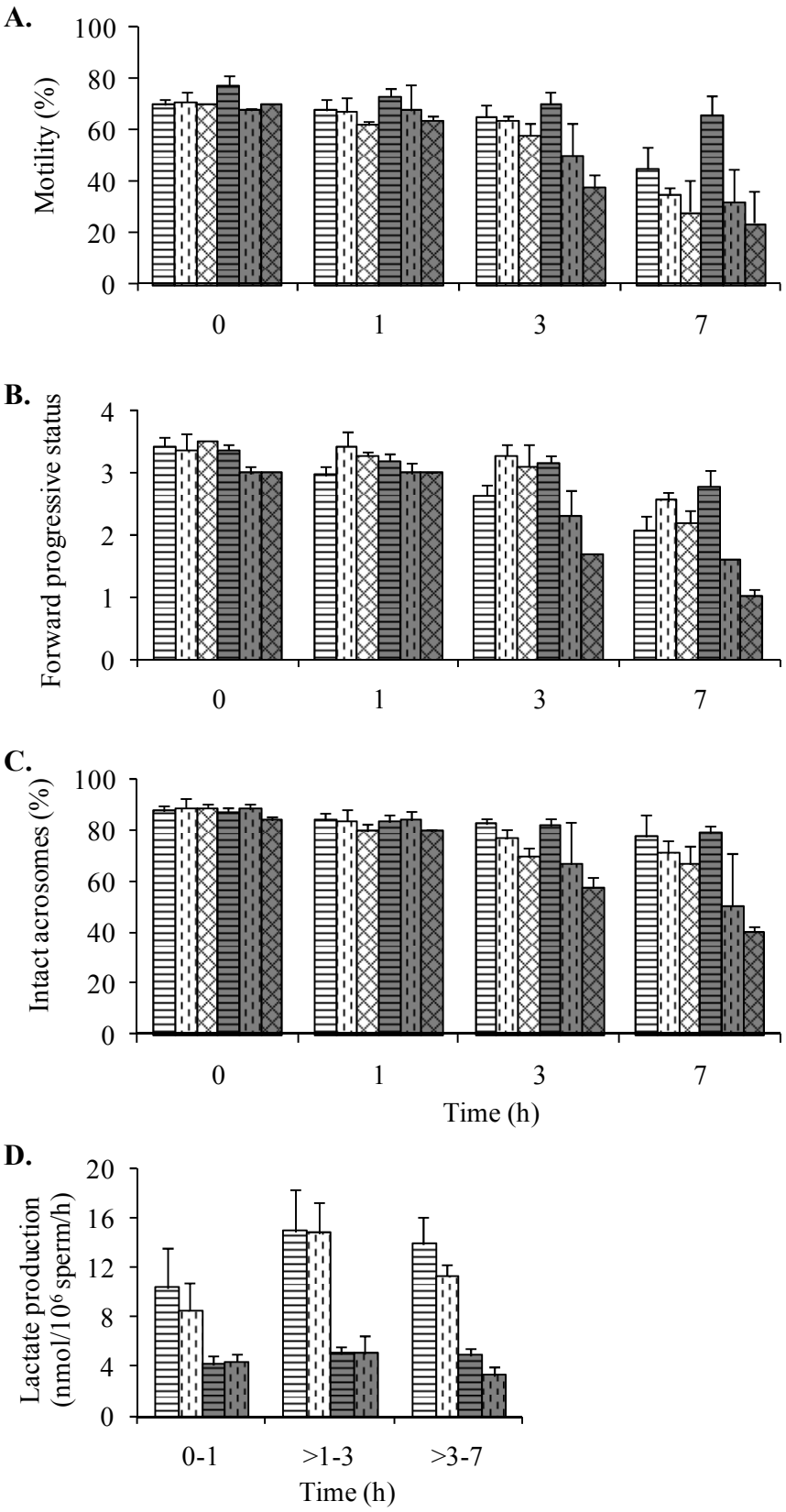

Figure 3.1 Absolute values for percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in control sperm samples from domestic cats (white bars) and cheetahs (grey bars). Controls (incubated in complete cMTF medium) correspond to the following three treatment groups: 1) presence/absence of exogenous substrates (horizontal lined bars); 2) GAPDH inhibition (vertical dashed bars); and 3) LDH inhibition (diamond-patterned bars). Error bars illustrate least-squares means ± standard errors.

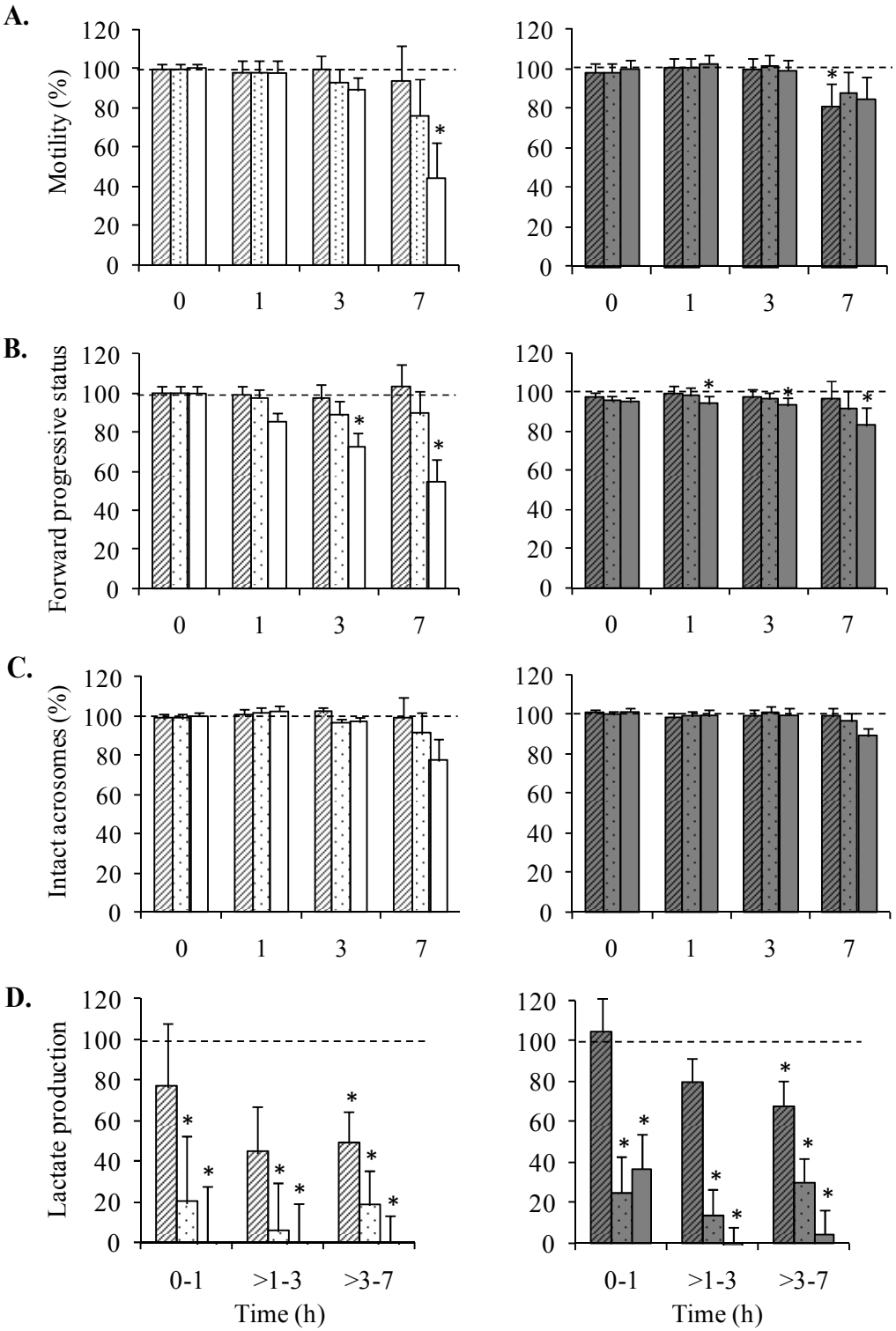

Figure 3.2 Percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in domestic cat (white bars) and cheetah (grey bars) sperm samples incubated with 1 mM pyruvate (lined bars), 1 mM glucose (dotted bars), or without exogenous substrate (solid bars). Data are expressed as percentages of control values (dashed line). Within each species and time interval, bars with an asterisk (*) differed from control values ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

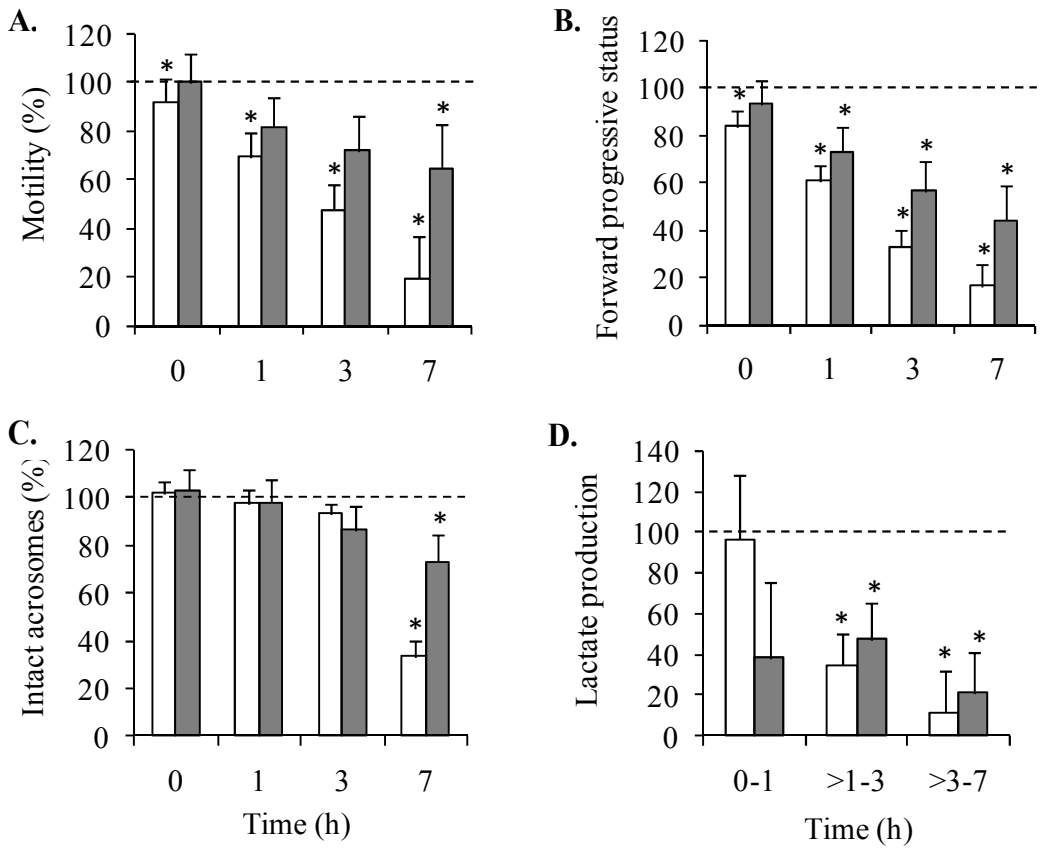

Figure 3.3 Percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in domestic cat (white bars) and cheetah (grey bars) sperm samples incubated with 50 mM α-chlorohydrin to inhibit GAPDH. Data are expressed as percentages of control values (dashed line). Within each species and time interval, bars with an asterisk (*) differed from control values ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

3.4.2 GAPDH and LDH Inhibition

The influence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) inhibition was consistent between species and resulted in impaired ($P < 0.05$) sperm function (% M, FPS, and % IA; Fig. 3.3). Losses in motility (% M and FPS) became more severe ($P < 0.05$) over time (Fig. 3.3A&B), whereas acrosomal integrity was not impaired until 7 h (Fig. 3.3C). An unusual motility pattern that involved rapid flagellar beating, but little forward progression (i.e., vigorous twitching) often was 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

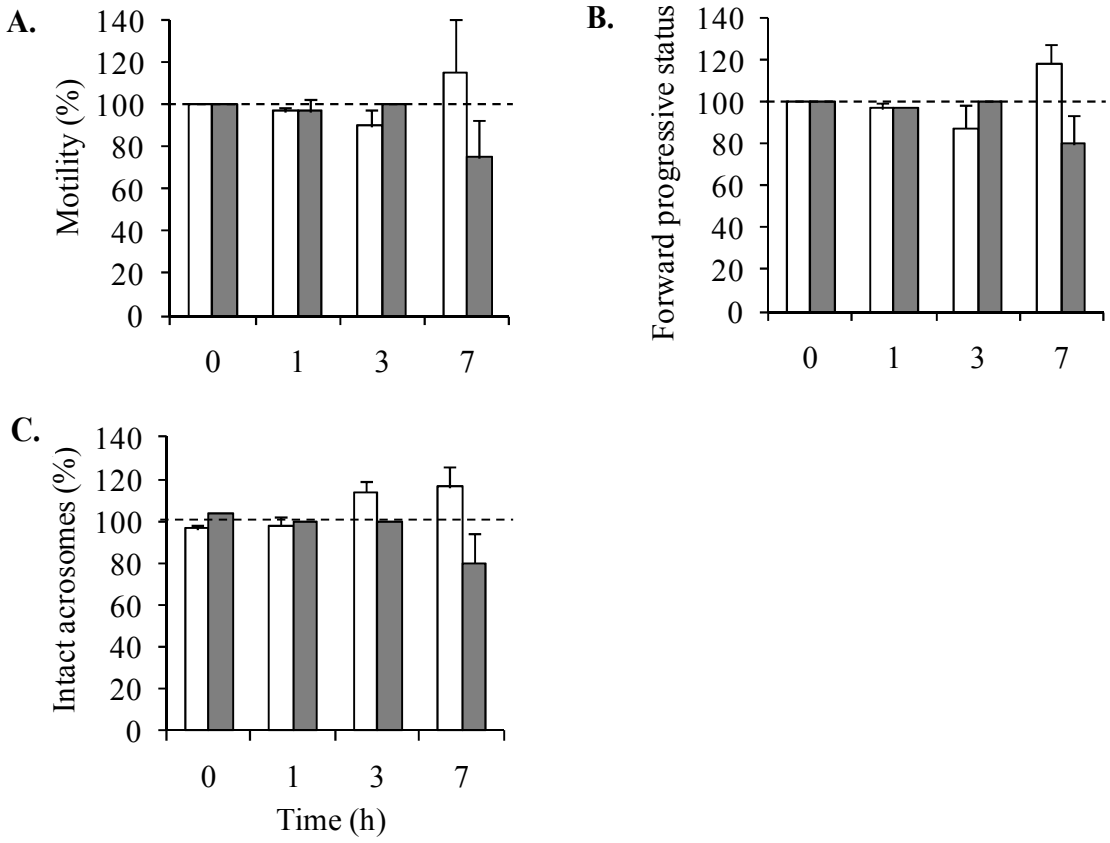

Figure 3.4 Percent motility (A), forward progression (B), and acrosomal integrity (C) in domestic cat (white bars) and cheetah (grey bars) sperm samples incubated with 50 mM sodium oxamate to inhibit LDH. Data are expressed as percentages of control values (dashed line). All treatment values were similar to controls ($P > 0.05$). Error bars illustrate least-squares means ± standard errors.

exposure to the GAPDH inhibitor (Fig. 3.3D). Substantial variation in rates of pyruvate uptake was observed, obscuring any potential overall treatment effect (Sup. Fig. 3.2). This variation likely was 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 also were consistent with our previous observations 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. 3.4).

3.4.3 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.

3.5 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 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. 3.5). In this model, fatty acids hydrolyzed from intracellular lipid (i.e., phospholipid or di-/tri-glycerides) 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. 3.5) [57]. Finally, the oxidative metabolism of glycerol-derived pyruvate would provide another significant source of ATP.

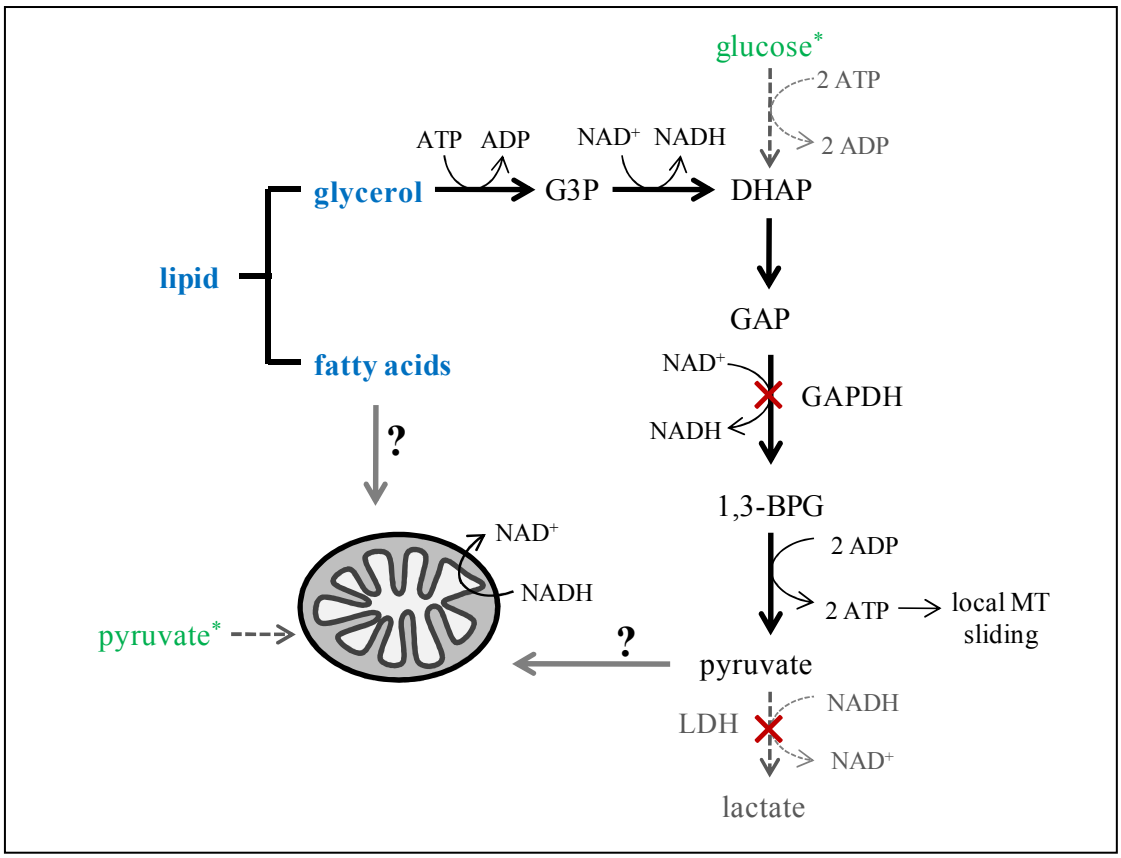

Figure 3.5 Theoretical model of energy production in domestic cat and cheetah spermatozoa illustrating targets of metabolic inhibition (×). Diagram includes exogenous substrates utilized in this study (green) and hypothesized endogenous energy sources (blue). Dashed lines indicate pathways determined to be non-essential 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.

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 actively metabolizing endogenous lipid, boar spermatozoa 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/tri-glycerides 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 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 spermatozoa 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]) consistently are 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 3.5, 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 non-traditional 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. Yet while 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, for example, 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.

3.6 References

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CHAPTER 4: OXIDATIVE METABOLISM IS ESSENTIAL FOR FELID SPERM FUNCTION, BUT IS SUBSTANTIALLY LOWER IN CHEETAH (*ACINONYX JUBATUS*) COMPARED TO DOMESTIC CAT (*FELIS CATUS*) EJACULATE

4.1 Abstract

Compared to 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 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 metabolism is required for normal sperm function and is impaired in teratospermic ejaculates. Ejaculates 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 to the normospermic and teratospermic cat, despite the former having a 10% longer midpiece. In both species, MMP was increased 5-fold in spermatozoa with retained cytoplasm compared to structurally normal cells. Inhibition of oxidative phosphorylation impaired sperm function, but a 100-fold higher inhibitor concentration was required in the cat compared to 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 link between retained cytoplasmic droplets and elevated MMP may have reflected increased concentrations of metabolic enzymes or substrates in these structures.

4.2 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 [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) knock-out 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 to 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 having 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 since 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 usually associated with teratospermia in felids, including sperm motility [21], protein tyrosine phosphorylation [25], and the acrosome reaction [26]. Elucidation of these functional defects could provide clues for improving the success of reproductive technologies (e.g., sperm cryopreservation) useful to improving both human fertility and wildlife management/conservation.

4.3 Materials and Methods

Animals

Electroejaculates were collected from adult (1.5 – 8 yr old) domestic cats 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 (1 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 housed singly, as described previously [27]. Males at WOCC, SCBI, and PHL were captive-born and managed as explained earlier [26], and represented one (PHL) or two (WOCC, SCBI) sibling groups at each institution. Both males at TW were captive-born, one of which was maintained singly in a 5,000-m^2 outdoor enclosure and the other 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 (ACUC) 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 ~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 (PVA) [33]. The cMTF medium was prepared as described earlier [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 (MOPS) 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].

Inhibition of Oxidative Phosphorylation

Each diluted ejaculate (maintained at ambient temperature, 19–22^{o}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/ml) 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 previously to study mammalian sperm metabolism [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 (~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; × 1,000 *g*) through a CoStar Spin-X 0.22-μm nylon filter tube (Corning Incorporated, Corning, NY) and stored at –80°C until analysis using a LDH/GPT-linked fluorescence assay

[21, 32, 38]. Assay enzymes were purchased 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 nmol/10^6 sperm/h. 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, 19–22^{o}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/ml. To assess MMP, 100 spermatozoa/ejaculate were individually analyzed (400 ×) using a BX40 fluorescence microscope (Olympus America Inc., Center Valley, PA; 555 nm excitation), 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 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 (~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 (1,000 ×) 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's 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 vs. control). Pearson's 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's 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's 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. Differences in sperm morphometrics among males were evaluated using Duncan's new multiple range test. Results were considered significant at $P < 0.05$.

4.4 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 percentage sperm motility (% M; $r = 0.49$), forward progression (FPS; $r = 0.42$), and acrosomal integrity (% 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 4.1. Compared to untreated controls (Fig. 4.1), domestic cat sperm from normospermic and teratospermic donors

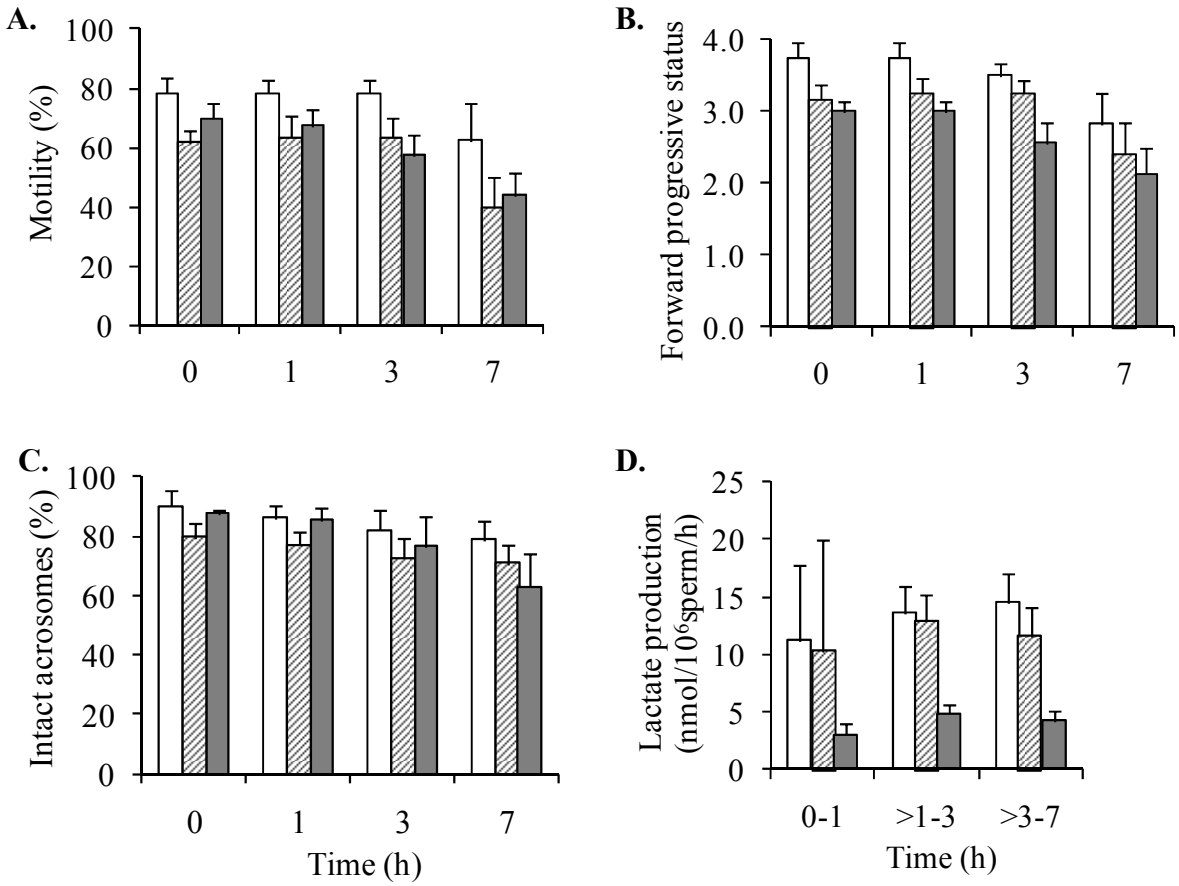

Figure 4.1 Absolute values for percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in normospermic cat (white bar; n = 6), teratospermic cat (lined bar; n = 6), and cheetah (gray bar; n = 8) control sperm samples. Assessments at 0 h were made < 5 min after sperm washing and resuspension in cMTF medium. Error bars illustrate least-squares means ± standard errors..

were unaffected ($P \geq 0.05$) for sperm %M, % IA, and Δ L when exposed to 0.16 μM myxothiazol (Fig. 4.2A, C-D); there was a modest, but significant (~20%; $P < 0.05$) loss in FPS (Fig. 4.2B). 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. 4.2A-D). 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 ($P \geq 0.05$) between domestic cat sperm phenotype (normospermic versus teratospermic) and myxothiazol treatment.

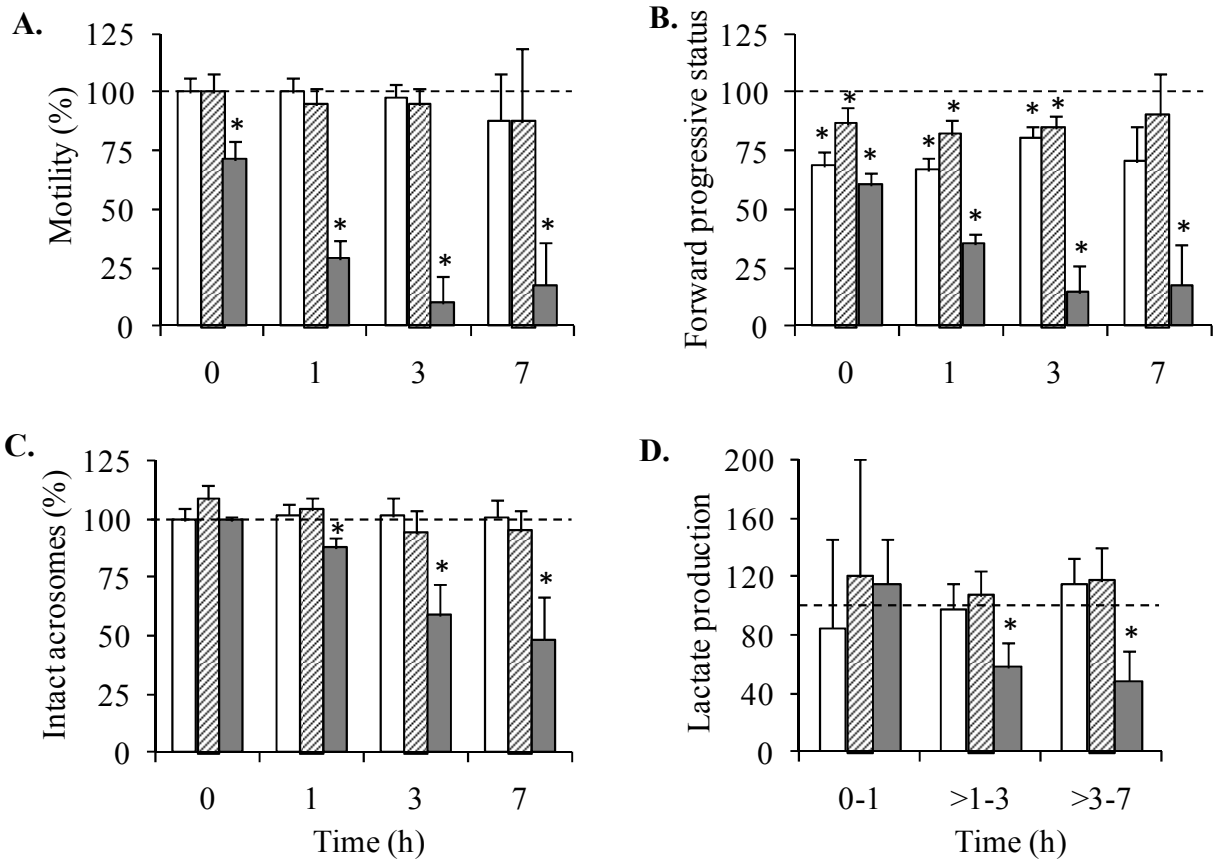

Figure 4.2 Percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in normospermic cat (white bar; n = 6), teratospermic cat (lined bar; n = 6) and cheetah (gray bar; n = 8) sperm samples incubated with 0.16 μM myxothiazol. Assessments at 0 h were made < 5 min 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 ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

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. 4.3A-D). 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 ($P \geq 0.05$) to those from domestic cats in the previous treatment group (Fig. 1).

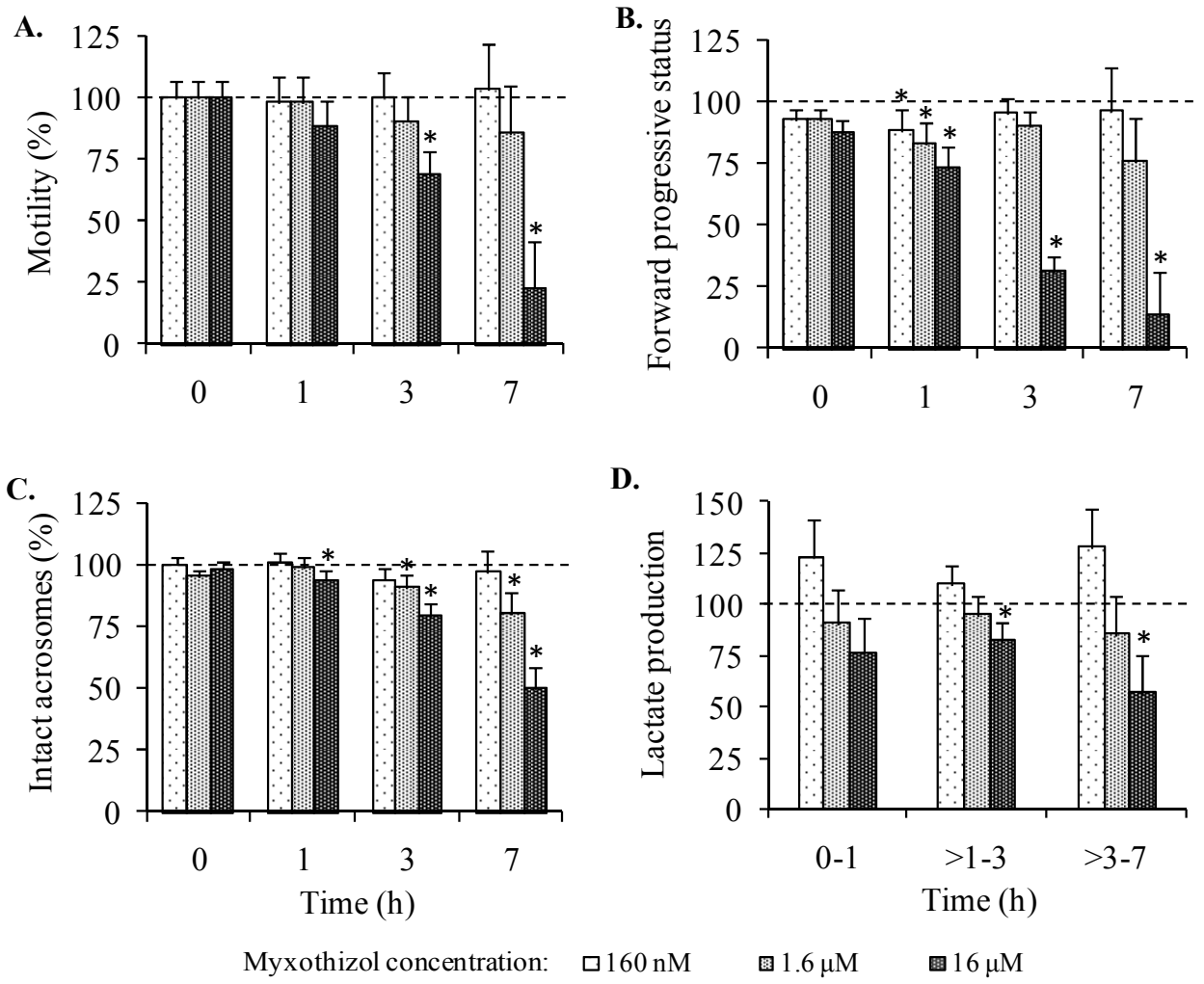

Figure 4.3 Percent motility (A), forward progression (B), acrosomal integrity (C), and lactate production (D) in domestic cat sperm samples (n = 4) incubated with increasing concentrations of myxothiazol to inhibit mitochondrial function. Assessments at 0 h were made < 5 min after resuspending centrifuged 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 ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

Mitochondrial membrane potential and sperm morphometrics

Sperm fluorescence after MitoTracker-staining (which is directly proportional to mitochondrial membrane potential, MMP) was substantially lower ($P < 0.0001$) in the cheetah compared to the normospermic and teratospermic domestic cat. In contrast, sperm % M, FPS, and the percentage of structurally abnormal cells were similar ($P \geq 0.16$) in cheetahs compared to normospermic and/or teratospermic cats (Fig. 4.4). Fluorescence analysis of individual sperm

morphotypes determined that the influence of cellular structure on MMP was consistent among normospermic cats (Fig. 4.5A), teratospermic cats (Fig. 4.5B), and cheetahs (Fig. 4.5C). 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 to structurally normal spermatozoa (Fig. 4.5 A-C). 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 associated with increased fluorescence, retained sperm cytoplasm did not fluoresce in either species (Fig. 4.6 A-C). 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 to the domestic cat spermatozoon (Table 4.1). Other sperm dimensions were similar ($P \geq 0.14$) between species (Table 4.1).

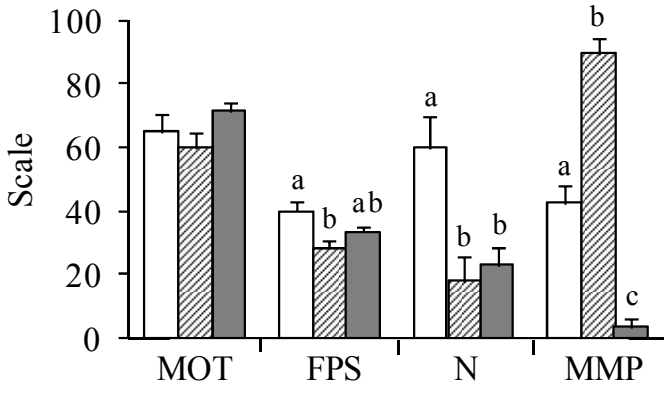

Figure 4.4 Sperm mitochondrial membrane potential (MMP) in normospermic cat (open bar; n = 2), teratospermic cat (linec bar; n = 3), and cheetah (shaded bar; n = 7) sperm samples in relation to percent motility (MOT), forward progressive status (FPS; scale, 0 – 5), and percent normal morphology (N). To allow presenting metrics on a single scale, values were increased 10× for FPS and decreased 1,000× for MMP. Within each metric and among animal groups, bars with different superscripts differ ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

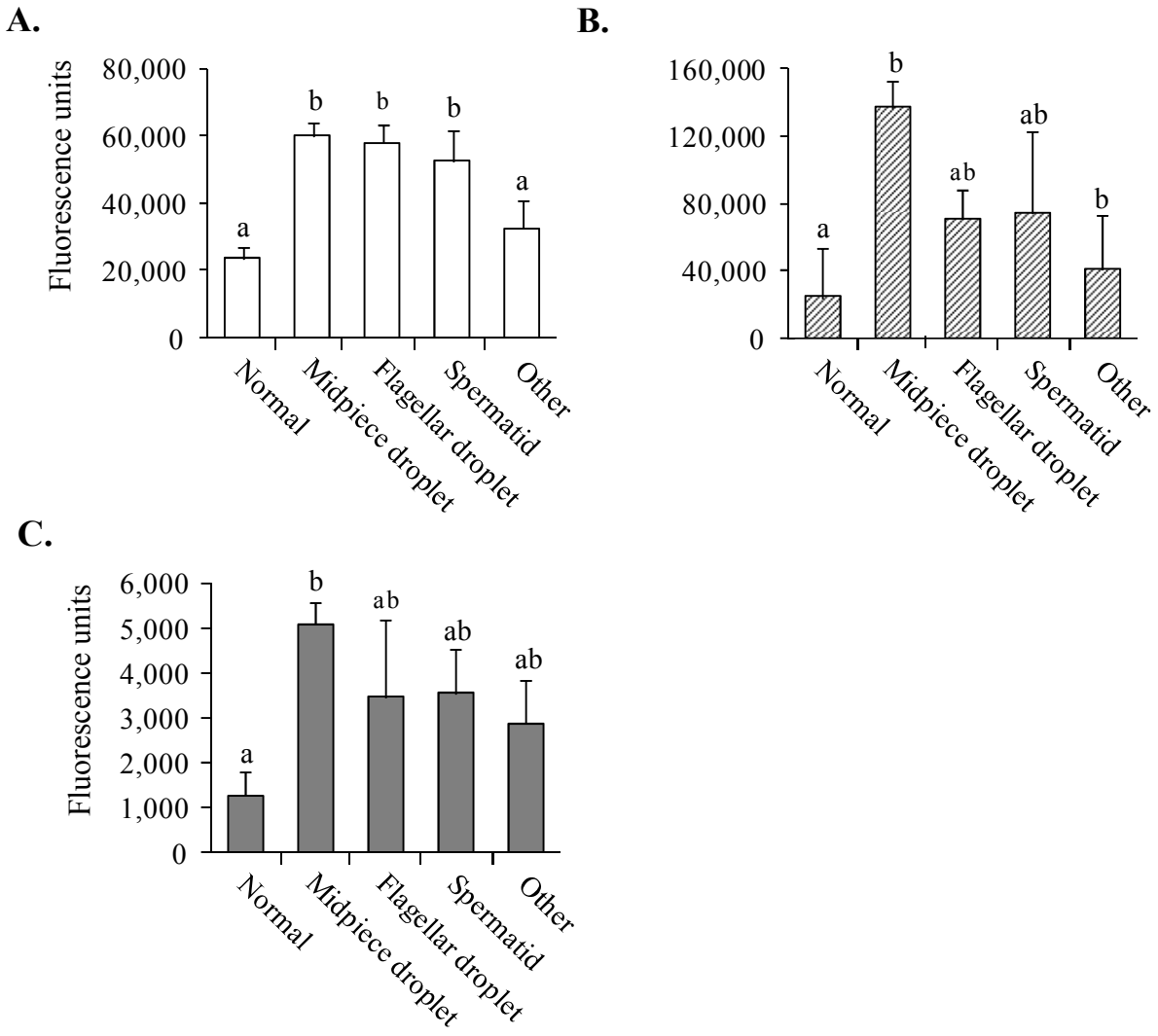

Figure 4.5 Sperm mitochondrial membrane potential (MMP) in normospermic cat (A; n = 2), teratospermic cat (B; n = 3), and cheetah (C; n = 7) ejaculates in relation to cellular morphotype. Note the difference in scale between the two graphs. Among sperm morphotypes and within each species, bars with different superscripts differ ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

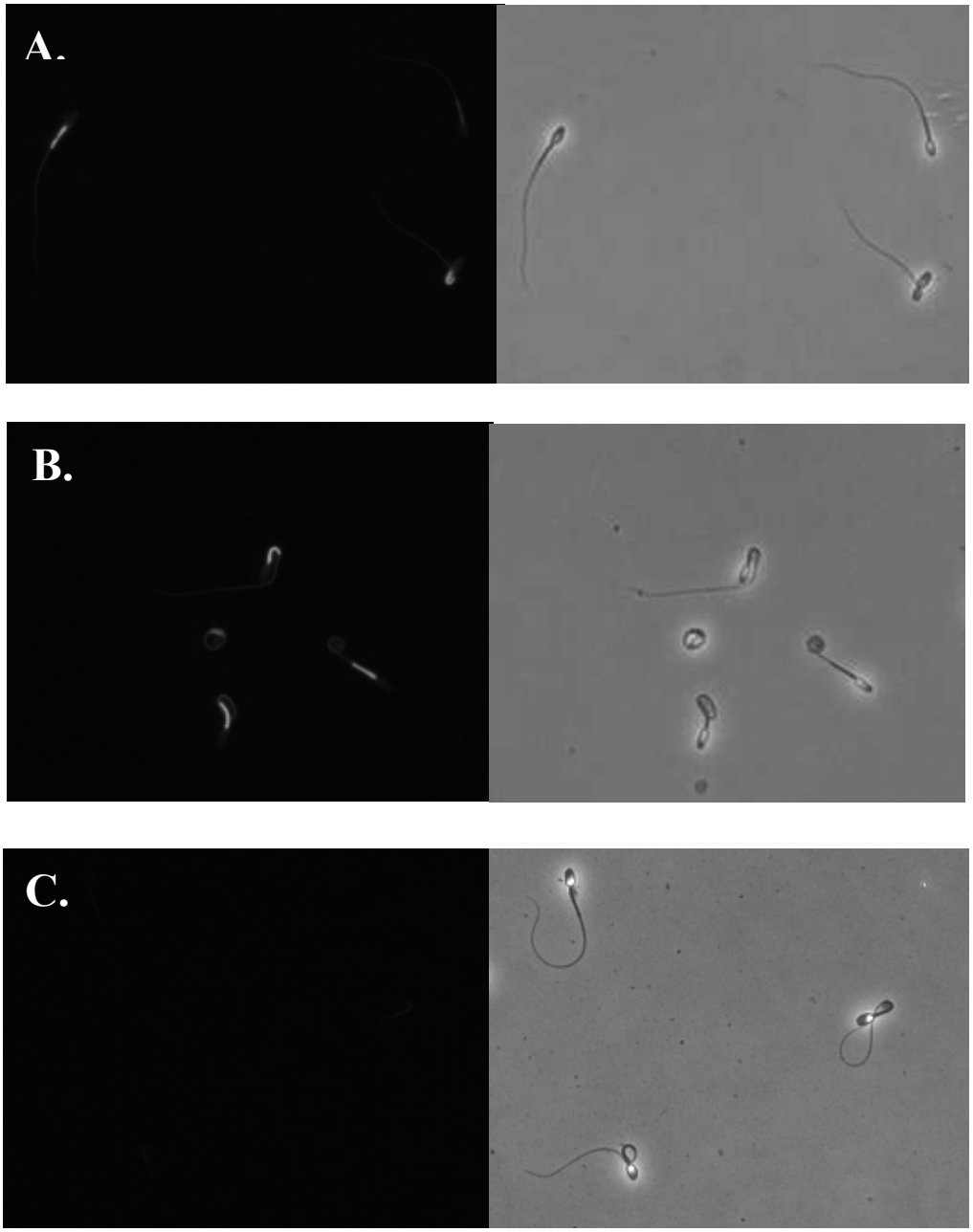

Figure 4.6 Representative fluorescence (left column) and phase-contrast images (right column) (400×) of Mitotracker-stained normospermic cat (A), teratospermic cat (B), and cheetah spermatozoa (C).

Table 4.1. Dimensions of domestic cat[†] and cheetah[‡] spermatozoa. Values represent least-squares means ± standard errors.

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

[†]n = 5 males (1 ejaculate per male).
[‡]n = 7 males (1 ejaculate per male).
[a,b]Within rows, values with different superscripts differ ($P < 0.05$).

4.5 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 [43]). Second, fluorescence of MitoTracker-stained spermatozoa, an indicator of mitochondrial membrane potential (MMP), was markedly lower in the cheetah compared to 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 to the domestic cat. While 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 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 IVF 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 former 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 to 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 to the domestic cat, cellular percent motility and forward progression were similar between species. This may have indicated that lower sperm MMP in the cheetah was indeed 'normal' and not representing 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 to domestic cat spermatozoa, perhaps due to differences in the efficiency of microtubule sliding or ATP transport along the flagellum [52]. However, we 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 those of the domestic cat because these species are more genetically similar compared to 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 pleiomorphism 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 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* knock-out mice lacking the 15-LOX enzyme [58]. This possibility 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, as 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 IVF 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]. While 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 *15-LOX* 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 demonstrated 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].

4.6 References

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CHAPTER 5: DIFFERENT PATTERNS OF METABOLIC CRYO-DAMAGE IN DOMESTIC CAT (*FELIS* CATUS) AND CHEETAH (*ACINONYX JUBATUS*) SPERMATOZOA

5.1 Abstract

Felid spermatozoa are sensitive to cryopreservation-induced damage, but functional losses can be mitigated by post-thaw swim-up or density gradient processing methods that selectively recover motile or structurally normal spermatozoa, respectively. Despite the importance of sperm energy production to achieving fertilization, there is little knowledge about the influence of cryopreservation or post-thaw processing on felid sperm metabolism. We conducted a comparative study of domestic cat and cheetah sperm metabolism after cryopreservation and post-thaw processing. We hypothesized that freezing/thawing impairs sperm metabolism and that swim-up, but not density gradient centrifugation, recovers metabolically-normal spermatozoa. Ejaculates were cryopreserved, thawed, and processed by swim-up, Accudenz gradient centrifugation, or conventional washing (representing the 'control'). Sperm glucose and pyruvate uptake, lactate production, motility, and acrosomal integrity were assessed. Mitochondrial membrane potential (MMP) was measured in cat spermatozoa. In both species, lactate production, motility, and acrosomal integrity were reduced in post-thaw, washed samples compared to freshly-collected ejaculates. Glucose uptake was minimal pre- and post-cryopreservation, whereas pyruvate uptake was similar between treatments, due to variation in the data. In the cat, swim-up, but not Accudenz processing, recovered spermatozoa with increased lactate production, pyruvate uptake, and motility compared to controls. Although confounded by differences in non-specific fluorescence among processing methods, MMP within treatments was positively correlated to sperm motility and acrosomal integrity. Cheetah spermatozoa isolated by either selection method exhibited

improved motility and/or acrosomal integrity, but remained metabolically compromised. Collectively, findings revealed a metabolically-robust subpopulation of cryopreserved cat, but not cheetah, spermatozoa, recovered by selecting for motility rather than morphology.

5.2 Introduction

Despite extensive research to optimize sperm cryopreservation protocols for the domestic cat [1] and various wild felids [2], freezing/thawing causes significant damage to these cells [3-6]. The degree of cryo-injury is particularly severe in the cheetah and other species that produce teratospermic ejaculates (i.e., those containing high proportions of sperm pleiomorphisms) [7-12]. In such cases, thawed spermatozoa consistently exhibit reduced motility and decreased proportions of cells with intact acrosomes [3-12]. This cellular damage is linked to disruption of sperm membranes from osmotic stress during feezing, thawing, and/or cryoprotectant removal [9, 13, 14]. It also is possible that the physiological stress experienced by spermatozoa during cryopreservation disrupts cellular metabolism. For example, one recent investigation of epididymal spermatozoa from the domestic cat determined that mitochondrial membrane potential (MMP) declined rapidly after cryopreservation and post-thaw washing [15]. Sperm MMP also is impaired in frozen-thawed ejaculates from the human [16], boar [17], ram [18], stallion [19], bull [20], and elephant [21]. Such published observations reinforce the need to explore the impact of cryopreservation on metabolic function of felid spermatozoa. Our recent studies of the domestic cat and cheetah have provided an understanding of baseline sperm metabolism in felids and have revealed intriguing species differences in metabolic function [22-24]. In both species, sperm glucose metabolism was minimal, while rates of pyruvate uptake and lactate production were correlated positively to cellular function [22]. Sperm MMP was

essential for lactate production [24], and these metabolic indicators were substantially reduced in cheetah compared to domestic cat ejaculates [22, 24].

Although being a potential source of osmotic stress, post-thaw removal of sperm cryoprotectant [25] provides an opportunity to isolate functionally-normal spermatozoa. For example, sperm 'swim-up' into fresh medium increases the proportions of motile, structurally intact cells in cat ejaculates [26, 27] and improves sperm morphology in cryopreserved cheetah samples [28]. Evidence in other mammals indicates that, in addition to improved motility and/or morphology, spermatozoa isolated by selective processing also exhibit high metabolic rates. In the ram, density gradient processing of thawed ejaculates improves sperm MMP [18], whereas swim-up processing has the same influence on bull sperm [20] and increases oxygen consumption by ~20-fold in freshly-ejaculated human semen [29]. While either approach could enhance metabolic function in thawed felid ejaculates, swim-up processing (targeting vigorous, motile cells with high rates of ATP production) [30-32] might be expected to recover the most metabolically-robust spermatozoa. By contrast, density gradient processing (targeting cells with normal morphology) may be less effective, because it is known that metabolism can be impaired in structurally normal cheetah spermatozoa [22].

Our general aim was to determine the impact of cryopreservation and post-thaw processing on the metabolic function of felid spermatozoa. This novel approach compared two species (domestic cat and cheetah) for which baseline patterns of sperm metabolism are well-characterized [22, 23]. Because felid sperm metabolism is influenced both by teratospermia [22] and species physiology [22-24], domestic cats producing low proportions of structurally normal spermatozoa were included for comparison with the teratospermic cheetah. To assess the influence of cellular function versus morphology, metabolism was assessed in thawed

spermatozoa selected on the basis of motility (swim-up) or normal morphology (density gradient). We hypothesized that the metabolism of cat and cheetah spermatozoa, specifically rates of pyruvate and lactate utilization, would be impaired by cryopreservation. Considering the known differences in cryo-sensitivity and baseline metabolism in spermatozoa from these species, we expected sperm metabolic cryo-damage to be more severe in the cheetah compared to the domestic cat. We also predicted that a subpopulation of thawed spermatozoa in each species would exhibit robust metabolic function (similar to baseline values) and would be recovered more effectively by isolating motile rather than normal-appearing cells.

5.3 Materials and Methods

5.3.1 Animals

All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee (ACUC) and similar committees of the WOCC and the SDZSP. Electroejaculates were collected from domestic cats and cheetahs using methods described below. For the former, a total of 16 semen samples was recovered from five adult males (ages, 1.5 – 8 yr) that were known to consistently produce either normospermic or teratospermic ejaculates [22-24]. Teratospermia has been described previously in felids and is defined as the ejaculation of < 40% structurally normal spermatozoa [33]. Three normospermic cats were used to produce five ejaculates (1 – 2 per individual) and two teratospermic males produced five samples (2 – 3 per individual). Three additional ejaculates were collected from one male in each group for the optimization of Accudenz density gradients (described below). The management protocol for domestic cats maintained at the Smithsonian Conservation Biology Institute (SCBI; Front Royal, VA) has been described in detail [22].

Electroejaculates (1 per individual) were collected from eleven adult cheetahs (ages, 2.5 – 10 yr). These animals were managed at the Cheetah Conservation Fund (CCF; Otjiwarongo, Namibia; n = 9), White Oak Conservation Center (WOCC; Yulee, FL; n = 1), or the San Diego Zoo Safari Park (SDZSP; Escondido, CA; n = 1). Males at CCF and the WOCC were wild caught and captive born, respectively, and maintained under previously described protocols [22, 28]. The male at SDZSP was captive born and managed with two male siblings off exhibit in a 1,300-m^2 outdoor enclosure and fed a commercial carnivore diet (Natural Balance Pet Foods Inc., Pacoima, CA).

5.3.2 Semen Collection

A surgical plane of anesthesia was induced in domestic cats and cheetahs according to protocols determined by institutional veterinarians for these species [28, 34]. 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 [22, 35]. A sample of raw semen containing ~2×10^5 spermatozoa was fixed in 0.3% glutaraldehyde in phosphate-buffered saline for assessment of sperm morphology according to previous descriptions for our laboratory [9, 22, 35].

5.3.3 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) [36] supplemented with 2% polyvinyl alcohol (PVA) [37]. The cMTF medium was prepared as described previously [22] 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 (MOPS) buffer and 0.02 mg/mL phenol red. Sperm concentration was determined using a Nucleocounter SP-100 (Chemometec, Allerød, Denmark) [38]. Osmolality of the final cMTF medium (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).

A sample containing ~3×10^6 motile spermatozoa was removed from each diluted ejaculate, washed by centrifugation for 8 min (× 300 *g* for domestic cat; × 100 *g* for cheetah), resuspended in fresh cMTF, and then incubated (37°C) in the dark in a microcentrifuge tube (3×10^6 motile sperm/ml) under oil to prevent evaporation, as described previously [22]. Assessments of sperm percentage motility (% M), forward progression (FPS), and acrosomal integrity were made at 0, 1, 3, 5, and 7 h of incubation. However, the low percentages of spermatozoa recovered in samples processed by swim-up or gradient centrifugation (see below) precluded sampling at more than three time points. Therefore, data obtained at 1 and 5 h were omitted from analysis. 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 [35]. A sperm motility index was calculated using the formula (% M + [FPS × 20] ÷ 2) [27]. Spermatozoa (~2×10^5 cells) were fixed in 4% paraformaldehyde and stained with Coomassie Blue G-250 (Fisher Biotech, Springfield, NJ) to evaluate acrosomal integrity, as described previously [7, 39].

Glucose and pyruvate uptake and lactate production also were assessed at each time point, and metabolic rates were calculated from substrate concentrations at 0, 3, and 7 h of incubation. To determine metabolic substrate concentrations, medium samples (110 µl) were centrifuged for 8 min (× 1,000 *g*) through a CoStar Spin-X 0.22-µm nylon filter tube (Corning Incorporated,

Corning, NY) and stored at –80°C until analysis using a HK/G6PDH (glucose), or LDH (pyruvate and lactate) fluorescence assay, as described previously [22, 40]. Rates of substrate utilization were calculated as the change in medium substrate concentration over time, divided by sperm concentration.

5.3.4 Sperm Cryopreservation

After removing a sperm sample for metabolic assessment, the remainder of each diluted ejaculate was cryopreserved in 4% glycerol as described previously [7, 28]. Briefly, each diluted ejaculate was centrifuged for 8 min (× 300 *g* domestic cat; × 100 *g* cheetah), resuspended in TEST-yolk buffer (TYB) refrigeration medium without glycerol (Irvine Scientific, Santa Ana, CA), and cooled slowly (~3.5 h) in a water bath from ambient temperature (19 – 22°C) to 4°C. After cooling, an equal volume of TYB containing 8% glycerol (prepared as a 1:2 mixture of refrigeration medium and TYB with 12% glycerol; Irvine Scientific) was added in a step-wise manner over 30 min (¼ volume at 0 min, ¼ volume at 15 min, and ½ volume at 30 min). Samples were loaded into 0.25-ml plastic straws (Veterinary Concepts, Spring Valley, WI) with a final sperm concentration of ~60 × 10^6 motile spermatozoa/ml, frozen in liquid nitrogen vapor (1 min at ~7.5 cm and 1 min at ~2.5 cm above liquid nitrogen), and then plunged directly into liquid nitrogen. Samples were stored in liquid nitrogen until thawing (2 days to 30 mo).

5.3.5 Comparison of Post-Thaw Processing Methods

Cryopreserved straws were thawed individually for 10 sec in air (19 – 22°C) followed by immersion in a 37°C water bath for 30 sec [7, 28]. Each straw was dried, and its contents emptied into a sterile microcentrifuge tube. After thorough mixing, sperm motility, acrosomal

integrity, and morphology were assessed as described above. Each sample was divided among three processing treatments: 1) wash; 2) swim-up; and 3) Accudenz density gradient. For wash and swim-up treatments, 100 µl of the thawed sample was diluted to 1 ml by the slow, drop-wise addition of cMTF medium [28]. Each washed sample was centrifuged for 8 min (× 100 *g*), the supernatant removed, and the pellet resuspended in 500 µl of cMTF medium. Each swim-up sample was centrifuged as above, the supernatant removed, and the sperm pellet gently overlaid with 100 µl of cMTF medium. These samples were maintained at ambient temperature for 45 min in the dark to allow motile spermatozoa to enter the supernatant. The top 90 µl of supernatant was removed and diluted to 500 µl in fresh cMTF.

For the Accudenz treatment, 100 µl of thawed sperm solution was gently layered on top of a 4% to 10% (wt/vol) discontinuous density gradient in cMTF (except for domestic cat samples, described below) [28]. The Accudenz gradient was created by layering 100 µl of 10% solution underneath 500 µl of 4% solution in a microcentrifuge tube. After adding the sperm sample, each gradient was centrifuged for 8 min (× 100 *g*). The entire suspension formed three distinct layers after centrifugation: 1) the top layer (predominantly composed of TYB); 2) the interphase layer containing motile spermatozoa (except for domestic cat samples, described below); and 3) the bottom layer containing non-motile cells. The interphase (~80 µl) was removed and diluted to 500 µl. To recover sufficient Accudenz and swim-up processed spermatozoa for metabolic assessments, treatments were replicated in separate microcentrifuge tubes (3 – 5 replicates/treatment for each thawed ejaculate, depending on the ejaculate volume available) and combined before determining sperm concentration. For each processing treatment, the combined sample was diluted to a standard concentration (3×10^6 motile spermatozoa/ml), and final volumes ranged from 0.5 ml to 1.4 ml. Processed samples were

incubated (37°C) and motility, forward progression, acrosomal integrity, and metabolic rates were evaluated at 0, 3, and 7 h, as described above. A volume of sample (containing ~2 × 10^5 cells) was removed at the start of incubation for assessment of sperm morphology, as described above.

Sperm mitochondrial membrane potential (MMP) of domestic cat samples was evaluated immediately post-thaw and after processing at 0, 3, and 7 h of incubation. Cheetah sperm MMP was not assessed because too few spermatozoa were recovered from swim-up and Accudenz treatments. Additionally, a recent study from our laboratory demonstrated that MMP values of fresh cheetah ejaculate are extremely low [24], suggesting that it would be impossible to detect differences among post-thaw treatments for this species. At each time point, 48 µl of domestic cat sperm suspension were removed, combined with 2 µl of 12.5 nM MitoTracker® Red CMXRos (Molecular Probes, Inc., Eugene, OR) in cMTF (0.5 nM final concentration), and incubated (1 h at ambient temperature) in the dark. To quantify MMP, 100 spermatozoa per ejaculate were individually analyzed (400 ×) using a BX40 fluorescence microscope (Olympus America Inc., Center Valley, PA; 555 nm excitation) and a Sensicam qe high performance camera (Cooke Corp., Romulus, MI) and IP Lab v4.04 software (BD Biosciences, Rockville, MD). Sperm with MMP values below the background fluorescence threshold were considered to have non-functional mitochondria. The presence of sperm structural abnormalities in MMP samples was recorded as described previously [24], with each spermatozoon classified as being one of the following five morphotypes: structurally normal, midpiece droplet, flagellar droplet, spermatid, and 'other.' The 'other' category included less common abnormalities (head deformities, malformation of the midpiece or flagellum) that collectively represented ≤ 10% of sperm in each species.

5.3.6 Accudenz Gradient Optimization for Domestic Cat Spermatozoa

Initial attempts to process domestic cat samples by Accudenz density gradient centrifugation resulted in very low sperm recovery (< 5%), compared to percentages of cheetah spermatozoa recovered by the same method in this study and previously (~25%) [28]. This double layer gradient (4% – 10%) centrifugation for 8 min (× 300 *g*) method resulted in a high concentration of motile spermatozoa in the pellet, suggesting that the density of the medium was below that needed to localize these cells in the target interphase layer. Therefore, we tested the efficacy of a triple layer gradient (4% – 10% – 30%) and reduced centrifugation speed (100 *g*). This higher concentration was chosen because a 12% to 30% discontinuous Accudenz gradient has been successfully used to recover motile spermatozoa in the chicken, with the cells isolated from the interface between the two layers [41, 42]. Cryopreserved sperm samples from two domestic cats (one normospermic, the other teratospermic, n = 3 ejaculates per male) were thawed as above. Each thawed sample was mixed thoroughly, and the total volume (90 – 150 µl) was divided equally among three centrifugation treatments: 1) double gradient at 300 *g*; 2) triple gradient at 300 *g*; and 3) triple gradient at 100 *g*. Double gradients were prepared as described above. Triple gradients were prepared using 200 µl each of 4%, 10%, and 30% (wt/vol) Accudenz in cMTF. The solutions were added to the microcentrifuge tube in order of increasing density, with each new layer added underneath the previous. After centrifugation, each layer was transferred to a new tube (in the order of interphase, top, and bottom layer) and assessed for sperm motility index and percent recovery. Sperm morphology and acrosomal integrity also were evaluated in the interphase layer. The interphase was considered to be the volume at the interface of the 4% and 10% solutions for the double gradient or the 10% and 30% solutions for the triple gradient.

5.3.7 Chemicals

All reagents were purchased from Sigma Aldrich (St. Louis, MO) except enzymes (LDH, GPT, HK, and G6PDH) that were obtained from Roche Applied Science (Indianapolis, IN) and Accudenz, which was from Accurate Chemical and Scientific Corporation (Westbury, NY).

5.3.8 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. Within species, the influence of post-thaw processing method was assessed at each time point using a General Linear Model (GLM) [43], with sperm motility index, acrosomal integrity, metabolic rates, percent normal morphology (0 h only), mean MMP (domestic cat only), and mean percent non-functional mitochondria (domestic cat only) included as response variables. Differences in MMP (0 h only) among sperm morphotypes were assessed using a GLM with data for all processing treatments combined. Accudenz gradient optimization data were analyzed using a separate GLM for each gradient layer (top, interphase, and bottom). Gradient type and male were considered class variables, and sperm percent recovery, motility index, acrosomal integrity (interphase only), and morphology (interphase only) were included as response variables. When treatment effects were significant, differences among means were evaluated using Duncan's Multiple Range Test. Pearson's correlation was used to evaluate the relationships among sperm motility index, acrosomal integrity, and metabolic metrics. Results were considered significant at $P < 0.05$.

5.4 Results

5.4.1 Accudenz Density Gradient Optimization for Domestic Cat Spermatozoa

Motile spermatozoa were recovered from all layers of each gradient. However, most (≥ 73%) of these cells in the gradients centrifuged at 300 × *g* were isolated from the pellet-containing bottom layer (Table 5.1). Compared to these treatments, the percentage of spermatozoa recovered from the target interphase layer of the 100 × *g* triple gradient was increased ($P < 0.05$) ~3 to 20-fold (Table 5.1). Reduced centrifugation speed also was associated with a ~50% improvement ($P < 0.05$) in sperm motility index (SMI) compared to the double gradient (Table 5.1). Acrosomal integrity (% IA) and percentages of structurally normal sperm were not different ($P > 0.05$) among gradient treatments (Table 5.1). Primary head abnormalities

Table 5.1. Characteristics of domestic cat* post-thaw sperm samples recovered from Accudenz density gradients. Values represent least-squares means ± standard errors.

	Density gradient		
Parameter	Double†	Triple†	Triple, low speed‡
Interphase (target layer)			
Sperm recovered (%)	4 ± 7[a]	24 ± 7[a]	70 ± 7[b]
Sperm motility index	32 ± 5[a]	40 ± 5[a,b]	48 ± 4[b]
Acrosomal integrity (%)	34 ± 6	43 ± 6	43 ± 6
Normal sperm morphology (%)	54 ± 4	65 ± 4	52 ± 4
Macro/microcephaly (%)	12 ± 1[a]	4 ± 1[b]	3 ± 1[b]
Top layer			
Sperm recovered (%)	2 ± 4[a]	3 ± 4[a]	18 ± 4[b]
Sperm motility index	35 ± 7	25 ± 7	39 ± 7
Bottom layer and sperm pellet			
Sperm recovered (%)	94 ± 7[a]	73 ± 7[a]	12 ± 7[b]
Sperm motility index	53 ± 5	42 ± 5	44 ± 5

*n = 2 males, 3 ejaculates per male.
†8 min at 300 *g*; ‡8 min at 100 *g*.
[a, b]Within rows, values with different superscripts differed ($P < 0.05$).

(i.e., macro/microcephaly) were more prevalent ($P < 0.05$) in the double versus either triple gradient (Table 5.1). Other malformations did not differ ($P < 0.05$) among gradient treatments (data not shown). There was no interaction ($P > 0.05$) between male and gradient treatment.

5.4.2 Comparison of Post-Thaw Processing Methods

Domestic Cat

Cryopreservation and post-thaw washing of domestic cat spermatozoa resulted in decreased (~30 – 70%, $P < 0.05$) SMI, % IA, and lactate production (Δ L) compared to freshly-

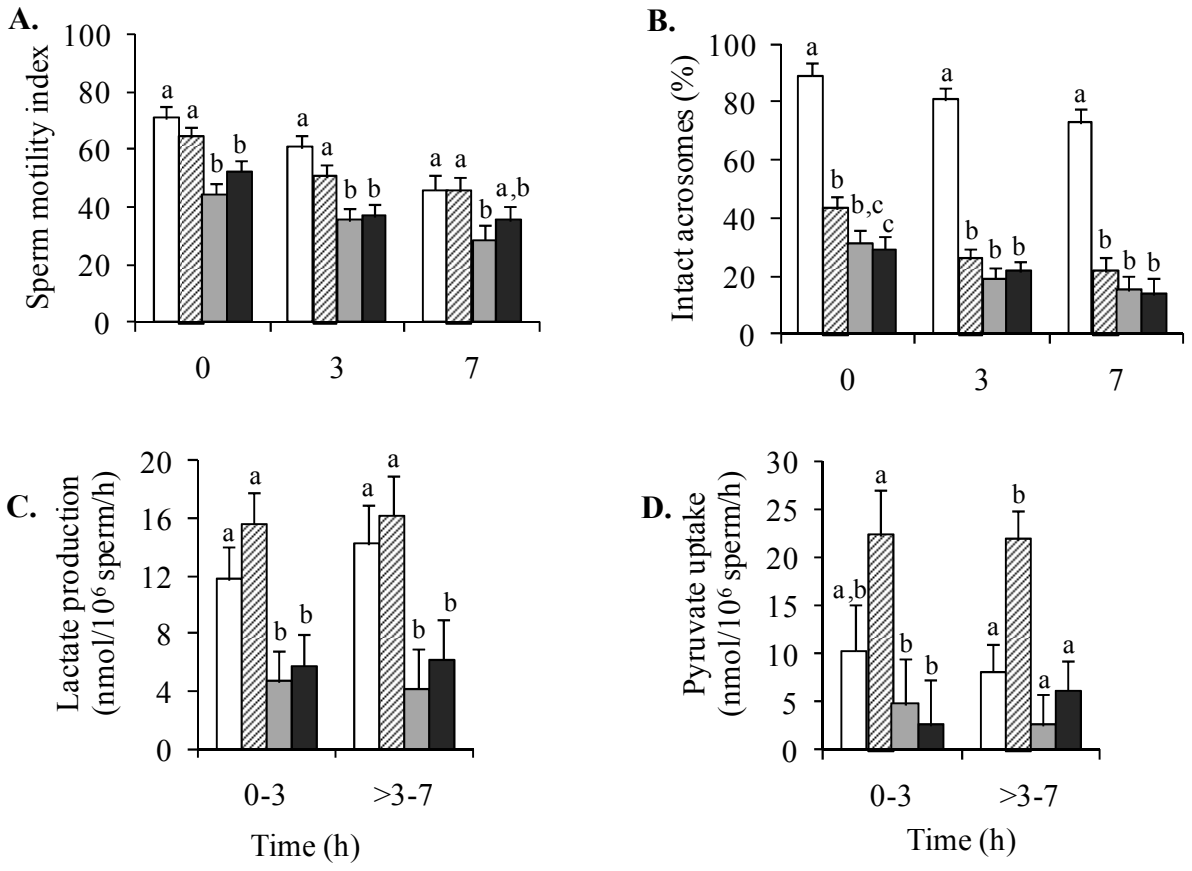

Figure 5.1 Sperm motility index (A), acrosomal integrity (B), lactate production (c), and pyruvate uptake (D) in domestic cat ejaculates before cryopreservation (open bar) and after post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) method. Within each time interval, bars with different superscripts differed ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

collected ejaculates (Fig. 5.1A-C). Although values of pyruvate uptake (Δ P) were lower in washed versus freshly-collected samples, this difference was non-significant ($P > 0.05$) due to large variation in the data (Fig. 5.1D). Consistent with previous findings [22], sperm lactate production was correlated positively ($P < 0.05$) to SMI and % IA (Table 2), whereas glucose uptake (Δ G) was minimal and was not influenced ($P > 0.05$) by cryopreservation or post-thaw processing (Sup. Fig. 5.1A). Swim-up processing recovered metabolically-robust cells with SMI, Δ L, and Δ P that were increased ($P < 0.05$) relative to washed samples and similar to ($P > 0.05$) or greater than ($P < 0.05$) corresponding values for freshly collected ejaculates (Fig. 5.1A, C, & D). There was no change ($P > 0.05$) in Δ L and Δ P of swim-up samples after 3 h, indicating that normal metabolic function was maintained for an extended time period (Fig. 5.1C & D). Although swim-up processing increased ($P < 0.05$) % IA relative to washed samples, this improvement was not ($P > 0.05$) sustained after 0 h, and values remained substantially reduced ($P < 0.05$) compared to fresh ejaculates (Fig. 5.1B). In contrast to spermatozoa isolated by swim-up, the subpopulation recovered by density gradient centrifugation experienced impaired

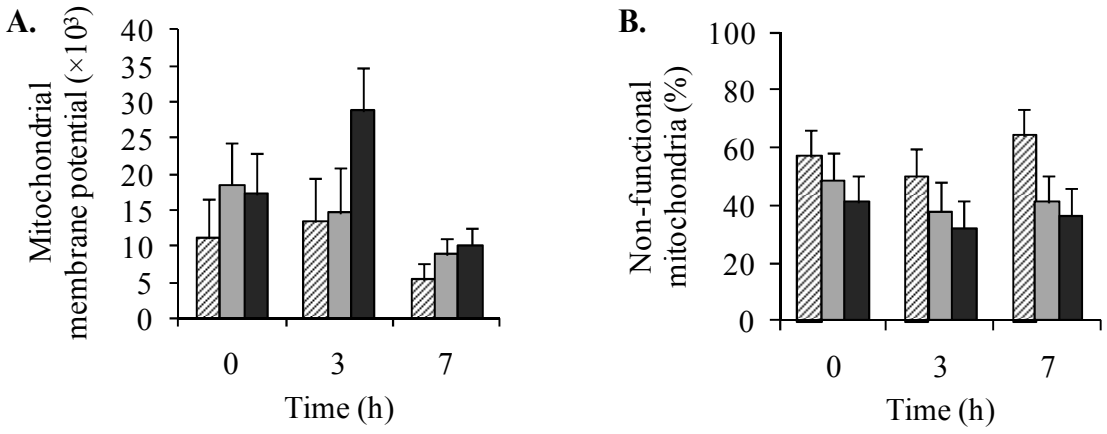

Figure 5.2 Sperm mitochondrial membrane potential (A) and percentages of cells with non-functional mitochondria (B) in domestic cat ejaculates after cryopreservation and post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) method. Error bars illustrate least-squares means ± standard errors.

cellular function (SMI, % IA, Δ L, and Δ P) similar ($P > 0.05$) to washed samples (Fig. 5.1A-D).

Post-thaw processing did not influence ($P > 0.05$) sperm MMP or the percentage of cells with non-functional mitochondria (% NFM) (Fig. 5.2A & B). This likely was because of substantial variation within the data set due, at least in part, to an increase in non-specific fluorescence of spermatozoa in washed and Accudenz treatments relative to swim-up samples (Sup. Fig. 5.2A-C). We suspected that non-specific fluorescence was related to the amount of cryopreservation buffer (TYB) carryover, which likely was minimal in swim-up samples. We also observed high percentages (85 – 100%) of spermatozoa with non-fluorescent mitochondria immediately post-thaw (data not shown), suggesting that high concentrations of TYB inhibited MitoTracker® staining.

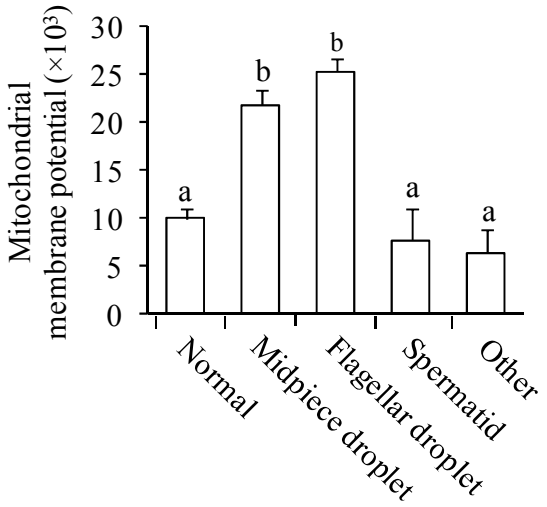

Figure 5.3 Sperm mitochondrial membrane potential in relation to cellular morphotype for all domestic cat post-thaw processing treatments combined. Among sperm morphotypes, bars with different superscripts differed ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

When data from all sperm treatments were combined, both MMP and % NFM were correlated (positively and negatively, respectively; $P < 0.05$) to sperm motility and acrosomal integrity, although the relationship between MMP and motility represented a trend ($P = 0.07$; Table 5.2). Compared to sperm lactate production, MMP and % NFM were less accurate predictors of motility and acrosomal integrity (Table 5.2), likely due to the confounding influence of non-specific fluorescence after MitoTracker® staining. Sperm MMP and % NFM

were better correlated to motility and acrosomal integrity when treatment groups were analyzed individually (Table 5.3), although certain relationships became non-significant due to the smaller sample sizes. Finally, consistent with previous findings in freshly collected cat and cheetah ejaculates [24], MMP was greater ($P < 0.05$) in spermatozoa with a retained cytoplasmic droplet at the midpiece or flagellum compared to other cellular morphotypes (Fig. 5.3).

Table 5.2. Correlation coefficient (*r*) values for metabolic indicators versus domestic cat[†] or cheetah[‡] sperm quality metrics after cryopreservation.

Metabolic indicator	Sperm motility index	Intact acrosomes (%)
Domestic cat		
Lactate production (nmol/10^6sperm/h)	0.54[c]	0.55[c]
Mitochondrial membrane potential	0.25[a]	-0.28[b]
Non-functional mitochondria (%)	0.33[b]	-0.27[b]
Cheetah		
Lactate production (nmol/10^6sperm/h)	0.41[c]	0.25[b]

[†]n = 5 males, 10 total ejaculates; [‡]n = 11 males, 11 total ejaculates.
[a] $P = 0.07$; [b] $P < 0.05$; [c] $P < 0.0001$.

Table 5.3. Correlation coefficient (*r*) values for mitochondrial function versus domestic cat[†] sperm quality.

	Mitochondrial membrane potential		Non-functional mitochondria (%)	
Treatment	SMI	% IA	SMI	% IA
Wash	NS	0.56[a]	NS	-0.60[a]
Swim-up	0.63[a]	0.48[a]	-0.63[a]	-0.47[a]
Accudenz	0.59[a]	0.44[b]	-0.51[a]	NS

[†]n = 5 males, 10 total ejaculates.
NS = not significant ($P > 0.05$).
[a] $P < 0.05$; [b] $P = 0.07$.

Cheetah

The influence of cryopreservation and post-thaw washing on cheetah spermatozoa was consistent with results for the domestic cat. Sperm SMI, % IA, and Δ L in washed samples was

markedly reduced (~30 – 70%, $P < 0.05$) compared to freshly-collected ejaculates (Fig. 5.4A-C), and, although Δ P also was decreased, this difference was non-significant due to data variation ($P > 0.05$; Fig. 5.4D). Similar to results for cat spermatozoa, cheetah sperm Δ G was minimal before and after cryopreservation (Sup. Fig. 5.1B). Selective processing by swim-up and Accudenz methods increased ($P < 0.05$) SMI compared to washed samples, but values remained decreased ($P < 0.05$) relative to fresh ejaculates, and this improvement was not maintained after 3 h (Fig. 5.4A). Similarly, Accudenz centrifugation improved ($P < 0.05$) % IA compared to washed samples, but after 3 h there were no differences ($P > 0.05$) among processing treatments

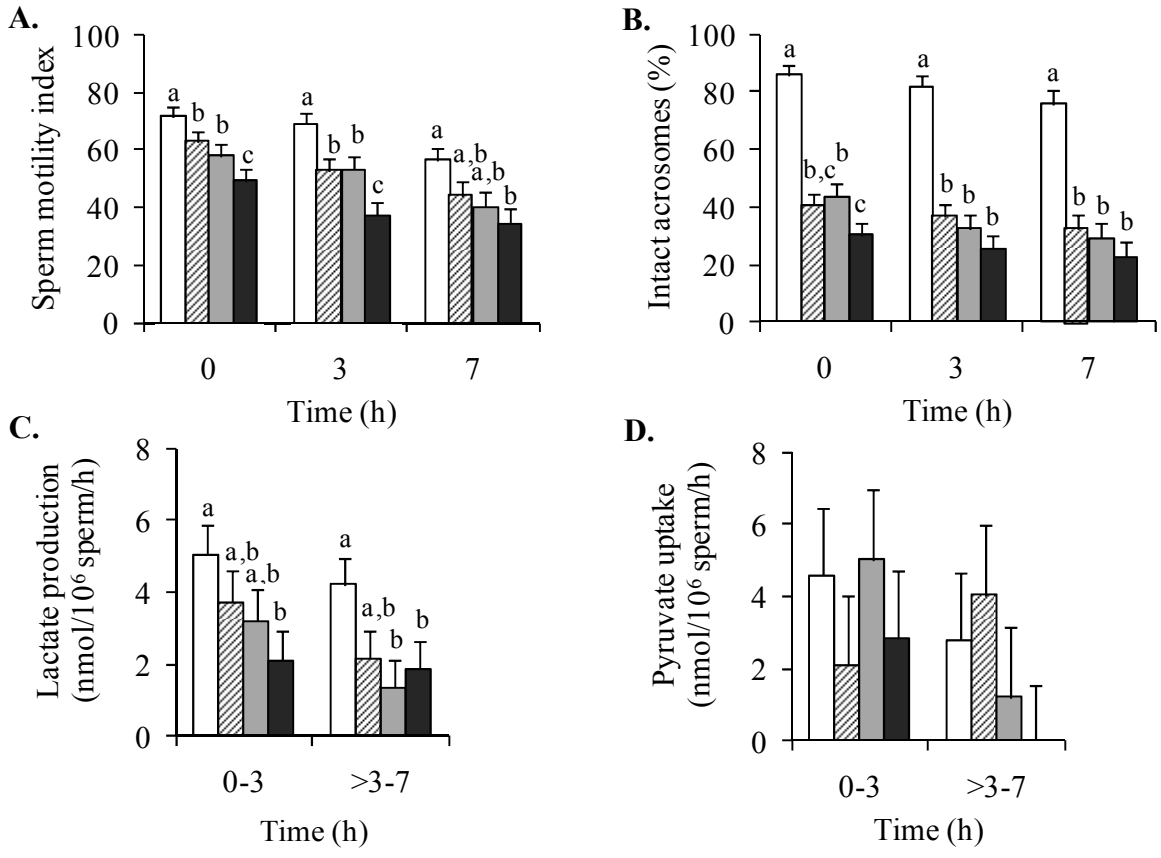

Figure 5.4 Sperm motility index (A), acrosomal integrity (B), lactate production (c), and pyruvate uptake (D) in cheetah ejaculates before cryopreservation (open bar) and after post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) method. Within each time interval, bars with different superscripts differed ($P < 0.05$). Error bars illustrate least-squares means ± standard errors.

(Fig. 5.4B). Sperm Δ L and Δ P were similar ($P > 0.05$) among all three processing treatments (Fig. 5.4C & D). Finally, Δ L was correlated positively with SMI and % IA (Table 5.2).

5.5 Discussion

Our investigation of cellular metabolism in cryopreserved felid spermatozoa yielded four significant discoveries. First, conventional cryopreservation protocols for felid spermatozoa not only impaired sperm motility and membrane integrity (as determined previously [7, 8]), but also disrupted pathways of cellular metabolism, as demonstrated by decreased rates of lactate production. Second, despite an overall reduction in metabolic function of post-thaw ejaculates, a subpopulation of domestic cat spermatozoa appeared to be unaffected by cryopreservation, as indicated by high rates of pyruvate uptake and lactate production. Third, in contrast to the domestic cat, cheetah spermatozoa were unable to sustain baseline metabolic profiles after thawing (despite using an identical cryopreservation protocol), indicating species-specificity in metabolic cryo-sensitivity. Perhaps the mechanistic basis of this finding is related to the more overt deficits of spermatozoa from this genetically impoverished species [44-46]. Finally, we determined that it was possible to isolate the most metabolically-robust domestic cat spermatozoa post-thaw by targeting motile rather than structurally normal cells. Given the link between sperm metabolism and cellular function [22-24], we predict that this subpopulation of thawed cells has the greatest chance of achieving fertilization.

Although the extent of injury was different between domestic cat and cheetah spermatozoa, our observations revealed that both species routinely experience sperm metabolic cryo-damage. Therefore, felids appear to be similar to other mammalian species, including in the human [16], boar [17], ram [18], stallion [19], bull [20], and elephant [21], that produce

ejaculates exhibiting impaired post-thaw metabolism. The exact origin of this disruption remains to be elucidated. However, it was important to consider that cryopreservation-induced stress to plasma membranes could cause leakage of sperm glycolytic enzymes [47]. Therefore, we postulated that sperm metabolic rates (measured by changes in medium glucose, pyruvate, and lactate concentrations) might falsely appear to be *increased* post-thawing due to greater activity of enzymes released into the medium. Cytoplasmic droplets (a common abnormality in cat and cheetah spermatozoa [22, 46]) are particularly susceptible to post-thaw LDH leakage [47], and this could confound the use of lactate production as an indicator of cellular quality in thawed ejaculate. Nonetheless, there was no evidence of glycolytic enzyme leakage from cat or cheetah spermatozoa. On the contrary, rates of pyruvate and lactate metabolism were reduced (non-significantly in the case of pyruvate), and glucose uptake remained minimal post-thaw. Furthermore, rates of lactate production were correlated positively to sperm motility, acrosomal integrity, and MMP. This finding provides strong support of our previous studies [22-24] and indicates that lactate production is a reliable metric of sperm function in both the domestic cat and cheetah.

Although rates of lactate production revealed metabolic cryo-damage in both the domestic cat and cheetah, we also identified an important species difference in the extent of cellular injury. Unlike the domestic cat, metabolic function in cheetah spermatozoa was uniformly impaired by cryopreservation and not improved by selective processing via swim-up or density gradient centrifugation. Therefore, future efforts to improve the post-thaw function of cheetah spermatozoa should focus on preventing damage to the freshly-collected cells, perhaps by testing alternative cryoprotectants (e.g., non-permeating sugars [48]) or preservation approaches (e.g., vitrification [49]). Regardless, the revealed differences re-emphasize the value

of conducting cross-species comparisons, and, for felids, the importance of the domestic cat as a research model [8, 22-24, 27, 34, 50, 51]. Besides determining what reproductive mechanisms are conserved, this comparative approach can identify unique traits for a given species that eventually may permit large-scale, systematic cryo-banking of germplasm. Because the present study demonstrated that a proportion of domestic cat spermatozoa survived cryopreservation with their metabolic machinery intact, this cellular subpopulation could be especially useful for future research studies designed to understand the key biological factors that contribute to sperm cryo-survival.

The relatively high rates of substrate utilization in cat ejaculates processed by swim-up versus Accudenz centrifugation confirmed our suspicion that post-thaw sperm metabolic function was more closely linked to cellular motility than morphology. Although impaired in teratospermic felids, sperm lactate production also is compromised in freshly-collected cheetah ejaculates that contain high proportions of structurally normal spermatozoa [22]. We have speculated [22] that disrupted metabolism could explain why even structurally normal spermatozoa from teratospermic ejaculates often are incapable of fertilization [26]. Furthermore, we previously discovered important differences in sperm glycolytic [23] and oxidative metabolism [24] between the cat and cheetah that were related to species physiology rather than sperm morphology. For example, sperm MMP was ~95% lower in the cheetah compared to both normospermic and teratospermic domestic cats [24]. Based on these collective observations, it makes sense that metabolically-robust spermatozoa are recovered post-thaw by isolating cells on the basis of motility rather than structural integrity. Furthermore, results of the present study suggested that structurally normal cells were as susceptible to metabolic cryo-damage as their abnormal counterparts. However, because only structurally intact spermatozoa are capable of

achieving fertilization [26], cellular morphology remains an important consideration for optimizing post-thaw sperm fertilizing potential. Indeed, it now appears that the most effective cryopreservation and processing protocols should yield post-thaw samples containing high proportions of structurally normal, motile, and metabolically-robust cells.

In conclusion, our findings confirmed that important differences existed in gamete physiology among felids and in the inherent ability of spermatozoa to retain metabolic function after cryopreservation. In this case, cheetah spermatozoa, well known for a high incidence of structural pleiomorphisms [44, 46, 52, 53], was particularly susceptible to metabolic cryo-damage. In contrast, a proportion of domestic cat spermatozoa retained normal metabolism after thawing, and perhaps this cellular subpopulation can provide clues for improving sperm cryo-survival in other species or populations with less vigorous ejaculate quality. Finally, our results confirmed that sperm metabolism, specifically the rate of lactate production, was a reliable metric of cellular quality that can be applied to evaluate and enhance sperm cryo-survival.

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CHAPTER 6: GENERAL DISCUSSION

6.1 Metabolic Profiles of Cat and Cheetah Spermatozoa

Teratospermia is an intriguing reproductive phenomenon common among felid species facing extinction risk [1], including the cheetah [2-4]. Although multiple energy-dependent processes are compromised in spermatozoa from teratospermic ejaculates [1], sperm cellular metabolism has not been previously investigated in wild felids. Through comparative studies of the cheetah and domestic cat, this dissertation aimed to elucidate the mechanisms of felid sperm energy production, including the influences of species physiology, teratospermia, and sperm cryopreservation. Access to biological samples from a rare species (i.e., the cheetah) required extensive collaboration with conservation institutions in the United States and in southern Africa. This collaborative approach was facilitated by access to the Smithsonian Conservation Biology Institute's Mobile Research Laboratory and a novel, field-friendly strategy to quantify sperm metabolic rates (described in Chapter 2).

Because there was almost no previous knowledge about sperm metabolism in felids, understanding the impacts of teratospermia and sperm cryopreservation required first identifying 'normal' metabolic profiles for each species (Chaper 2). Patterns of metabolic substrate utilization were remarkably similar between cat and cheetah spermatozoa: glucose uptake was minimal, but spermatozoa imported pyruvate and produced lactate at comparatively high rates. These results were enigmatic because the opposite pattern of substrate utilization was expected, i.e., high rates of glucose uptake, corresponding with total glycolytic activity, and lower rates of lactate production, proportional to anaerobic glycolysis. The investigation of sperm function in the presence/absence of exogenous substrates (Chapter 3) elucidated this finding through the discovery that cat and cheetah spermatozoa produced lactate independently of glycolysis and

ATP production via the reduction of exogenous pyruvate. Furthermore, cellular function (motility and viability) was maintained in a substrate-free culture medium, indicating the active metabolism of endogenous substrates. Intriguingly, the activity of the glycolytic enzyme GAPDH was required for sperm motility, even in the complete absence of exogenous and endogenous (i.e., glycogen) sources of glucose. These findings suggested that glycerol (most likely derived from endogenous lipid) was an important metabolic substrate in felid spermatozoa and hinted at a key role of lipid oxidation in cellular energy production. The results of Chapter 4 supported this model by confirming the essential role of oxidative metabolism in felid sperm function. Collectively, the studies described in Chapters 2 through 4 generated informative sperm metabolic profiles for the cat and cheetah. Felid spermatozoa appeared to be fueled primarily by the metabolism of endogenous lipid, with the glycolytic pathway serving an essential role in the breakdown of glycerol rather than glucose. The consistency of findings in the cat and cheetah increases confidence in the proposed model of sperm energy production and will provide an excellent starting point for future studies to elucidate these mechanisms in other felid species.

6.2 Metabolic Indicators of Sperm Function

Initial assessments of metabolic profiles in cat and cheetah spermatozoa (Chapter 2) suggested that rates of pyruvate uptake and lactate production were accurate predictors of sperm function. Because higher variation was observed among pyruvate data, subsequent studies focused primarily on lactate as a sperm quality indicator. Lactate production was impaired (together with cellular motility and viability) by chemical inhibition of metabolism (Chapters 3 and 4) and following cryopreservation (Chapter 5). Although the link between lactate production

and sperm function had been identified previously in the boar [5] and donkey [6], the current studies were the first to reveal an indirect relationship between lactate and sperm energy production. Given the important role of oxidative metabolism established in Chapter 4, it is likely that the link between lactate and sperm function is the mitochondrial production of NADH, a coenzyme required for the formation of both ATP (via OXPHOS) and lactate.

The results of Chapter 3 determined that rates of lactate production were largely dependent on sperm medium composition (specifically the presence of pyruvate), and chemical inhibition of this process did not impair sperm function. The influence of medium composition highlights the critical importance of a standardized, chemically-defined cellular environment for generating reliable conclusions about sperm metabolism. Given the findings of Chapter 3, the link to sperm function must be validated before lactate is applied as a cellular indicator in different culture media. But despite this caveat, lactate production can provide an objective, quantitative indicator of cellular function at relatively low-cost (e.g., compared to Computer Assisted Sperm Analysis) and offers exciting possibilities for future research.

6.3 Influence of Species Physiology

Although sperm metabolic profiles were generally similar in the cat and cheetah, results of Chapters 2 through 4 revealed two intriguing differences between the species. First, higher rates of glucose uptake (Chapter 2) combined with increased sensitivity to GAPDH inhibition (Chapter 3) indicated that glycolytic metabolism was more active in domestic cat compared to cheetah spermatozoa. Secondly, low mitochondrial membrane potential combined with extreme sensitivity to OXPHOS inhibition (Chapter 4) suggested that sperm oxidative metabolism was impaired in the cheetah. Although minimal rates of oxidative metabolism could be considered

normal for cheetah spermatozoa, this trait is likely to limit sperm fertilizing ability since OXPHOS appears to be the primary pathway of energy production.

6.4 Influence of Teratospermia

An important strength of this project's comparative design was the ability to distinguish between sperm metabolic differences related to species physiology versus those resulting from teratospermia. These comparisons centered on the teratospermic domestic cat (Fig. 6.1) – a valuable model for studying reproductive physiology in humans and wild felids. Initial assessment of sperm metabolic profiles (Chapter 2) revealed that rates of pyruvate uptake and lactate production (Chapter 2) were decreased in teratospermic cats and cheetahs compared to normospermic cats. This discovery supported the prediction that sperm metabolism was impaired in teratospermic ejaculates. Given the apparent link between lactate production and mitochondrial activity (described above), it was expected that sperm mitochondrial membrane potential (MMP) also would be reduced in teratospermic ejaculates. But in contrast to the cheetah, sperm MMP was robust in the teratospermic cat (Chapter 4). This unexpected finding suggested that other factors (e.g., LDH activity) also contribute to depressed lactate production in

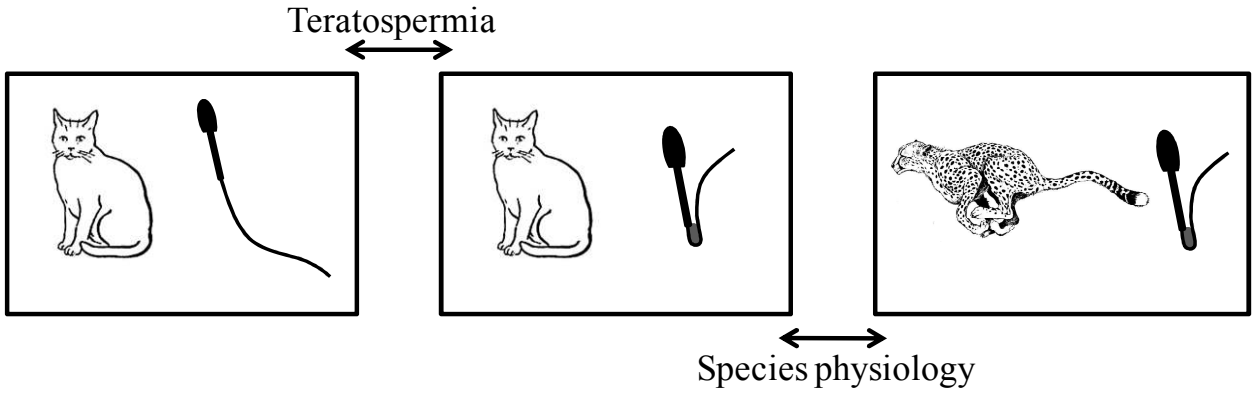

Figure 6.1 Animal groups included in each study (from left to right: normospermic domestic cat, teratospermic domestic cat, and cheetah). The two levels of comparison among these groups are indicated by double-ended arrows.

felid spermatozoa. Such an interpretation is supported by the observation that sperm lactate production was more severely depressed in the cheetah than in the teratospermic cat. Thus, teratospermic felids may share a common mechanism of impaired lactate production, while the low MMP of cheetah spermatozoa exacerbates this defect. Although requiring further study, these findings illustrate the importance of the domestic cat model for identifying physiological consequences of teratospermia in felids, particularly in species such as the cheetah that lack a normospermic 'control' population.

6.5 Influence of Sperm Cryopreservation

Consistent with previous studies [7-9], cryopreservation of cat and cheetah spermatozoa resulted in reduced post-thaw motility and severe losses in acrosomal integrity (Chapter 5). These cells also exhibited decreased rates of lactate production, providing the first evidence of metabolic cryo-damage in felid spermatozoa. The same pattern was observed with respect to pyruvate uptake, but differences were obscured by the large variation in those data. Although lactate production was markedly reduced following cryopreservation, it was unclear whether metabolic machinery was impaired directly (e.g., mitochondrial damage) or indirectly (e.g., increased plasma membrane permeability) – an intriguing question for further study.

Evaluation of post-thaw processing methods revealed that a metabolically-robust sperm subpopulation could be recovered in the domestic cat by selectively isolating highly motile spermatozoa. In contrast, metabolic function remained compromised in spermatozoa isolated on the basis of morphology, suggesting that structurally normal cells are as susceptible to metabolic cryo-damage as their abnormal counterparts. Results of post-thaw processing were similar between normospermic and teratospermic domestic cats (data were presented as a single group to

increase sample size). In the cheetah, sperm lactate production remained compromised after thawing, regardless of processing method. This key finding indicated that, in contrast to the domestic cat, a metabolically 'normal' subpopulation of cheetah spermatozoa did not exist after cryopreservation. Therefore, efforts to improve the fertilizing potential of cryopreserved cheetah ejaculates should focus on preventing, rather than mitigating metabolic cryo-damage. Finally, because metabolically-robust cells were recovered from the teratospermic cat, but not the cheetah, the mechanism allowing certain felid spermatozoa to retain metabolic function post-thaw appears to be species-specific.

6.5 Conclusions and Recommendations

Preserving and/or enhancing ejaculate quality in felids requires understanding the sperm metabolic pathways that support, directly or indirectly, every individual process required for fertilization. Both glycolysis and oxidative phosphorylation were essential for sperm motility and viability in the cat and cheetah, but the role of each pathway in other cellular processes (e.g., capacitation) should be investigated. Felid spermatozoa actively metabolized endogenous substrates, but this energy source may be limited, especially in poor quality ejaculates. Various forms of lipid (e.g., phospholipid) should be evaluated as supplemental energy sources to optimize sperm culture conditions. Furthermore, differences in the amount or composition of substrates stored intracellularly may contribute to the significant variation in ejaculate quality observed within felid species. Therefore, the characterization of sperm lipid profiles in felid ejaculates is a high research priority. Mitochondrial activity was particularly important for sperm energy production, but unidentified compounds present in the cryopreservation medium interfered with mitochondrial probes, presenting a challenge to post-thaw evaluation of MMP. A

chemically-defined sperm cryopreservation medium is needed to overcome this challenge and elucidate the mechanisms of metabolic cryo-damage. Cheetah spermatozoa were unable to survive cryopreservation with metabolic function intact, indicating a need to further optimize freezing/thawing protocols. However, certain domestic cat spermatozoa (even those from teratospermic ejaculates) somehow were able to escape metabolic cryo-damage. Identifying the factors that cause these cells to respond to freezing/thawing differently from other sperm populations could generate innovative strategies for improving cryopreservation success.

Overall, this project emphasizes the importance of domestic models and comparative research for understanding complex biological phenomena in rare animal species. Similarities in sperm metabolic profiles between the cat and cheetah generated reliable conclusions about energy production in felid spermatozoa. Yet even more exciting were the differences revealed by these comparative studies. Teratospermia and species physiology influenced sperm metabolism and/or cryosensitivity in unexpected, but important ways. These intriguing differences provide an excellent starting point for future studies of felid sperm physiology that can ultimately benefit both wildlife conservation and human health.

6.7 References

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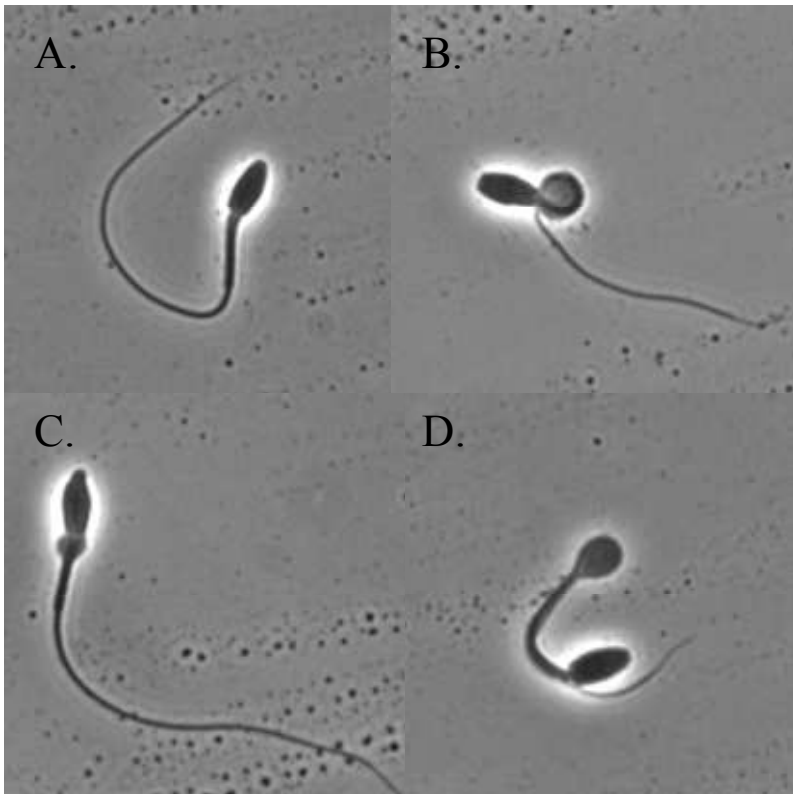
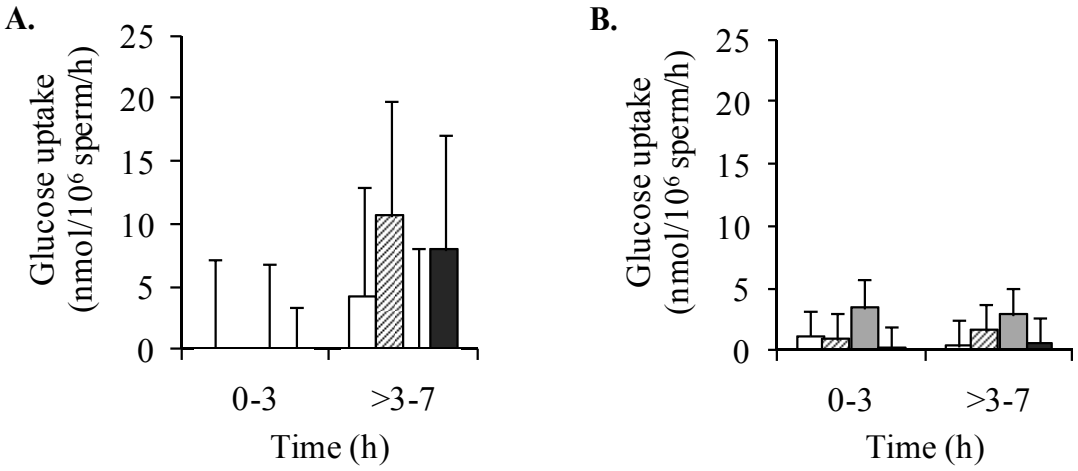
APPENDIX A

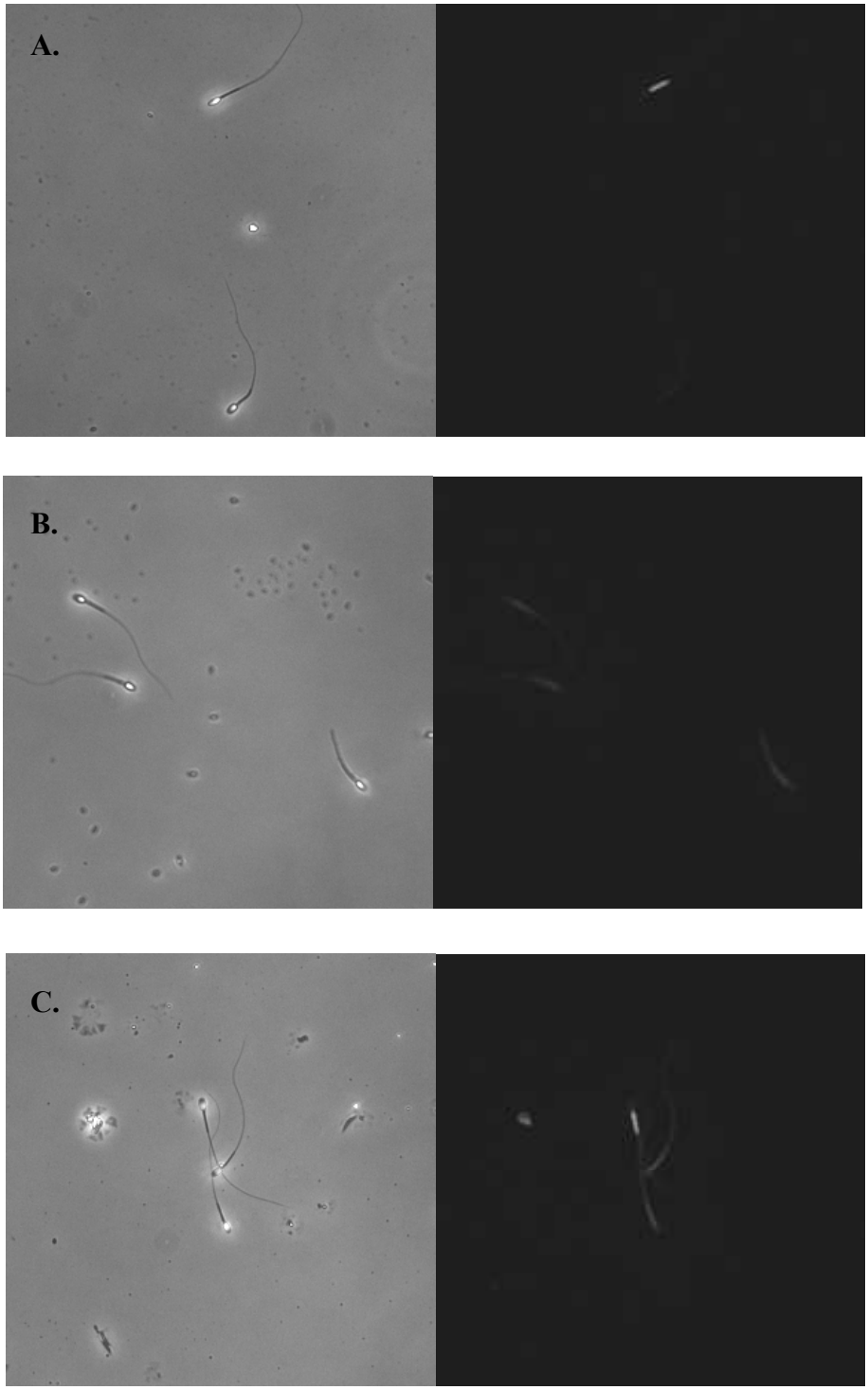


Supplemental Figure 2.1. Common sperm abnormalities in felids. Domestic cat spermatozoa exhibiting normal morphology (A); a bent midpiece encompassing a cytoplasmic droplet (B); an abnormal acrosome and a proximal cytoplasmic droplet (C); and a bent flagellum encircling a cytoplasmic droplet (D).

APPENDIX B



Supplemental Figure 5.1. Sperm glucose uptake in domestic cat (A) and cheetah (B) ejaculates before cryopreservation (open bar) and after post-thaw processing by swim-up (lined bar), Accudenz (gray bar), or wash (black bar) method. Error bars represent means ± SEM.



Supplemental Figure 5.2. Representative phase-contrast (left panel) and fluorescence (right panel) images of domestic cat sperm in swim up (A), Accudenz (B), and washed (C) samples after incubation with MitoTracker®. Images illustrate differences in the degree of non-specific fluorescent staining among sperm processing treatments.

VITA

The author was born in Park Ridge, Illinois. She earned a B.S. in Cell and Molecular Biology and a B.A. in Political Science from Tulane University in 2005. The following year, she joined the University of New Orleans Department of Biological Sciences to pursue a Ph.D. in Conservation Biology. She was the first student of the ZUNO program, a research partnership between UNO and the Smithsonian's National Zoological Park (NZP) established by her mentor, Dr. Barry Bavister. In 2007 she joined Dr. Nicola Anthony's laboratory, and the following year relocated to Virginia to complete her dissertation research at the NZP's Front Royal campus (now the Smithsonian Conservation Biology Institute, SCBI). Dr. David Wildt (head of SCBI's Department of Reproductive Sciences) and Dr. Nicola Anthony (UNO) served as her dissertation co-advisors. Drs. Barry Bavister, Adrienne Crosier, Stanley Leibo, and Bernard Rees were members of her dissertation committee.