ABSTRACT

Title of Document: INVESTIGATION OF SPERMATOZOAL METABOLISM AND SUBSTRATE UTILIZATION IN DOMESTIC AND NON-DOMESTIC FELIDS

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Cryopreservation of spermatozoa is a critical aspect of assisted reproduction. However in most cases, this process causes diminished spermatozoal function and low cellular survival. The effects of cryopreservation are further compounded in spermatozoa from individuals with disorders known to affect fertility. Teratospermia, characterized by the ejaculation of >60% morphologically abnormal spermatozoa, is one such disorder. Sperm from individuals with this condition exhibit reduced motility, impaired mitochondrial function, and a reduced ability to bind, penetrate, and fertilize an oocyte compared to sperm from normospermic males. Interestingly, even spermatozoa from teratospermic males that appear normal exhibit reduced functional capacity and following cryopreservation all cells are further impaired. More than 90% of felid species are affected by this condition, and it is prevalent among humans. Previous research suggested that impaired sperm metabolism in cheetahs – a ubiquitously teratospermic species – contributes to their poor

cryosurvival and function, but the specific mechanisms are unknown. In this research, we hypothesized that inherent differences exist between the substrates and metabolic pathways utilized by sperm from normospermic and teratospermic individuals and on a species-specific basis. Gas chromatography - mass spectrometry (GC-MS) was used to investigate felid sperm metabolism using semen collected from domestic cats (*Felis catus*), cheetahs (*Acinonyx jubatus*), and clouded leopards (*Neofelis nebulosa*). The main objectives of this research were to: **1)** characterize the metabolome of spermatozoa and seminal fluid; **2)** investigate the role of β-oxidation in sperm motility and metabolism; and **3)** interrogate the significance of specific metabolic pathways using metabolic activity profiling and heavy isotope tracers. Using GC-MS enabled the identification of metabolites unique to each species and sperm status (normospermic vs. teratospermic). Additionally, treatment of sperm with a β-oxidation inhibitor caused impaired motility in cheetah - but not domestic cat or clouded leopard - spermatozoa. Finally, fluxomic analysis demonstrated that glucose, fructose, and pyruvate are metabolized by felid sperm, but that pathway utilization is species-specific. This is the first study to utilize a metabolomic and fluxomic approach to studying felid sperm, and illustrates the complexity of sperm metabolism on a species-specific basis.

INVESTIGATION OF SPERMATOZOAL METABOLISM AND SUBSTRATE UTILIZATION IN DOMESTIC AND NON-DOMESTIC FELIDS

By

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Chapter 1: Introduction and Literature Review

Conservation of Felids

Across phyla, improving reproduction is a focal point of conservation biology. One way conservationists approach this mission is by developing assisted reproductive techniques (ARTs); however, development of these techniques requires a basic understanding of the reproductive biology of the pertinent species. The goal of this research was to investigate felid spermatozoal energy metabolism using the novel approach of combining ^{13}C-fluxomic technology and metabolomic analysis. Information gained through these studies will contribute to the basic understanding of sperm function in felids.

The *Felidae* family consists of 37 species, and is one of the most phylogenetically diverse carnivore families. *Felidae* can be divided into eight groups of related species which have been confirmed using mitochondrial DNA sequencing, allozymes, albumin immunological distance, and Y-chromosome sequences, among other monophyletic clustering techniques [6–8]. Briefly, these groups are described as consisting of cat species belonging to the lineages of either the ocelot, domestic cat, puma, bay cat, caracal, Asian leopard cat, *Lynx* genus, or *Panthera* genus [6]. The felid species in this study (domestic cat, cheetah, and clouded leopard) are parts of the domestic cat lineage [6], puma lineage [6], and *Panthera* lineage [8], respectively.

More than half of the species within the *Felidae* family have experienced a significant population decline within the past decade [9]. Habitat fragmentation and

loss is a major problem for these carnivores due to diminished resources and disrupted territory patterns. In many circumstances, natural habitats for these animals have been replaced with farmlands and subsequently used to graze livestock. As a result, wild felids utilize this artificial prey base and by doing so have initiated conflicts with land and livestock owners in countries all over the world. In the shadow of persistent population decline, basic research to improve *ex situ* management and reproductive strategies is necessary to maintain a sustainable population in captivity as a hedge against extinction.

Cheetah (*Acinonyx jubatus*)

Cheetahs are believed to have gone through two distinct population bottleneck events. The first is thought to have occurred during the Pleistocene era along with the widespread extinctions of many of the other extant megafauna at the time [10–13]. This was determined by examining the genetic diversity at the major histocompatibility complex (MHC) locus, which is one of the most genetically variable regions in mammals, and mitochondrial DNA (mtDNA), which has been shown to evolve 5-10 times faster than nuclear genes in primates and other mammals [11]. It was shown that cheetahs demonstrated 90-99% less genetic variation than other outbred felid species studied, and a back calculation of mtDNA divergence places the bottleneck approximately 10,000 years ago toward the end of the Pleistocene era [11]. The second population bottleneck has occurred throughout the 20th century and is a result of ecological perturbations [9,12]. This bottleneck event has been evidenced by the loss of allelic heterozygocity in two genetic

polymporphisms that are exhibited between the two subspecies of cheetah (*A. j. raineyi*, the east African subspecies, vs. *A. j. jubatus*, the south African subspecies) [12]. According to the International Union for the Conservation of Nature's (IUCN) Red list of threatened species, cheetahs are listed as vulnerable for these reasons, and it is estimated that fewer than 10,000 individuals exist [9]. Cheetahs have disappeared from 76% of their historic range, which once spread entirely across sub-Saharan Africa, throughout the Arabian Peninsula, and westward into central India. One reason for the disappearance of Asian populations of cheetah is due to the use of captive cheetahs in sport hunting, similar to the practice of falconry. However, the depletion of native prey base and the development of land by humans has been a major cause for population decline in both Africa and Asia. The impact of human development on wildlife is evidenced by the steady fragmentation of natural habitat areas driven by the development of rural areas in sub-Saharan Africa [9]. The cheetah still maintains a stronghold in southern and eastern Africa, where they are most densely populated in Namibia, Botswana, and South Africa (see Figure 1.1). In sub-Saharan Africa, cheetahs inhabit open grassy habitats, dry forests, and semi-desert, but are known to show preference for open savannah woodland [14]. Habitats in Iran consist mostly of desert.

The cheetah social structure is unique among felids. Most felids live solitary lifestyles and socialize for the purposes of breeding and caring for dependent offspring. Male cheetahs, however, live either solitary lifestyles or in small bachelor groups of 3-5 individuals[15]. These groups, called "coalitions," are often comprised of brothers but can consist of unrelated individuals. A coalition of cheetahs will

remain together throughout their lives to mate, hunt, and protect a common territory together [9]. In captivity, cheetahs suffer from disease, stress, and improper management. Reproduction is challenging due mainly to a lack of knowledge about female estrous cycles and sexual receptivity, as well as the persistence of teratospermia, which is characterized by the production of a high proportion (>60%) of malformed spermatozoa and is the cause of diminished survival and function following cryo-storage and *in vitro* handling [16].

Clouded Leopard (*Neofelis nebulosa*)

The clouded leopard is also listed as a vulnerable species on the IUCN's Red List due primarily to illegal hunting and habitat loss and fragmentation [17,18]. Historically, this species has been found from the Himalayan foothills in Nepal to just south of the Yangtze River in China, but due to the rapid rates of deforestation, their habitat has been fragmented across southeast Asia, and their presence in China is currently unknown (see Figure 1.2). These cats prefer to live in an arboreal forested habitat, occupying primary evergreen tropical rainforest. Little is known about the behavior of wild individuals, as clouded leopards are secretive and well-camouflaged. Like most felids, it is believed that they are solitary and only come into contact with one another to breed and when caring for dependent offspring. Territories can range from 20 to 50 km^2 for both males and females [19]. Efforts to conserve this species through captive breeding and management have been challenged by remarkably high levels of aggression [20], and high levels of pleiomorphic spermatozoa which makes cryopreservation and assisted reproduction challenging [21–23].

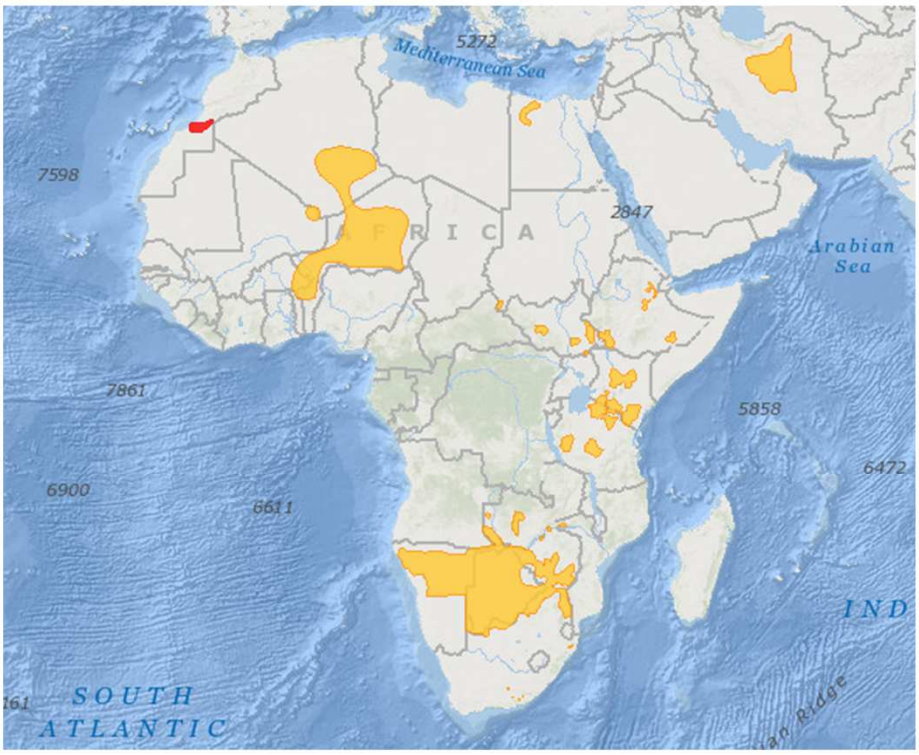

Figure 1.1: Geographic range of the cheetah (Acinonyx jubatus) througout Africa and the Middle East. Ranges are estimated based on measurements taken from 2002 - 2005. Cheetahs are believed to only inhabit 24% of their historic range (International Union for Conservation of Nature (IUCN) 2014. Acinonyx jubatus. The IUCN Red List of Threatened Species. Version 2014.3)

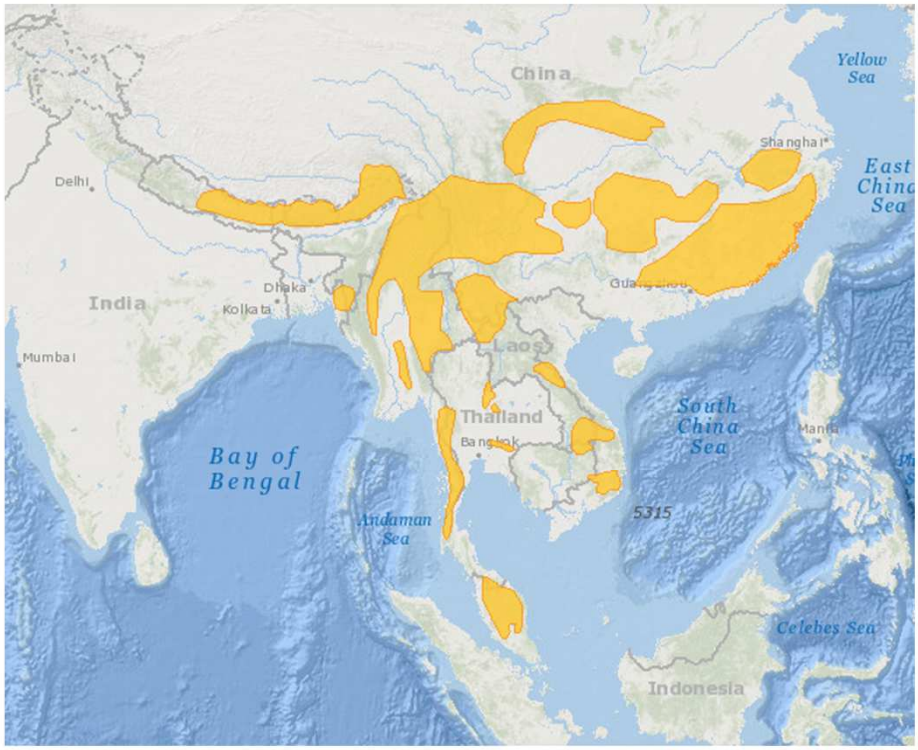

Figure 1.2: Geographic range of the clouded leopard (Neofelis nebulosa) throughout southeast Asia. Ranges are based on measurements taken from 1996 - 2007 (International Union for Conservation of Nature (IUCN) 2014. Neofelis nebulosa. The IUCN Red List of Threatened Species. Version 2014.3)

Domestic Cat (*Felis catus*)

The domestic cat inhabits all six developed continents. Much is known about these animals from a veterinary perspective, and it has been widely accepted that these animals can be used as models for other wild felid species, as well as for humans [10,24,25]. The domestic cat shares more than 200 heritable genetic defects with humans, and their genome has been shown to be much more similar to humans than other commonly used laboratory mammals, which makes them a valuable model for studying human genetic disorders. Additionally, by studying the feline immunodeficiency virus (FIV), domestic cats can serve as a valuable model for studying acquired immunodeficiency syndrome (AIDS) and the human immunodeficiency virus (HIV). Additionally, the cat has also been used as a model for studying human infertility, in that females of both species experience ovarian hyperstimulation and luteal dysfunction after going through exogenous gonadotropin therapy, and critical aspects of embryonic implantation are similar as well [26,27]. These similarities make cats a suitable model for studying human disease [10]. With relation to being a model for other felid species, much of the behaviors and physical characteristics of the domestic cat have been retained from their wild ancestors. Their similar biology, as well as the ability to breed and maintain them in captivity, makes them an ideal model species for studying felid species that are rare or difficult to care for. Finally, domestic cats have been strategically inbred to generate a teratospermic phenotype [10]. This enables us to study how this condition might be affecting spermatozoa in other species which are known to be ubiquitously teratospermic, such as the cheetah. By being able to compare normospermic domestic cats to

teratospermic domestic cats, using the domestic cat as a model becomes even more critical in defining the effects and possible solutions to this condition.

Assisted Reproduction

Various forms of assisted reproductive technologies (ARTs) have been used to collect information about endocrine and reproductive processes, preserve genetic material, improve genetic diversity, and increase the overall populations of a variety of threatened or endangered species. These techniques have been conducted and perfected in humans and many domestic species, and are becoming increasingly employed in wildlife conservation efforts. For exotic species in captivity, hormone monitoring aids in understanding and monitoring reproductive cycling, as well as measuring the success of ARTs by identifying hormones associated with pregnancy [28]. Traditional ARTs such as artificial insemination (AI), *in vitro* fertilization (IVF), *in vitro* oocyte maturation (IVM), intra-cytoplasmic sperm injection (ICSI) and sub-zonal insemination (SUZI), embryo transfer (ET), and cryopreservation genome resource banking (GRB) can supplement or be used in place of natural breeding [24]. Somatic cell nuclear transfer (SCNT), or cloning, is an ART that is most often utilized in domestic species and mainly for research purposes, but is used infrequently and often unsuccessfully in exotic species. This is because even in domestic species, in which cloning has been successfully implemented, results are variable and embryo mortality is high. In species where even less is known about the fundamentals of fertilization and embryo development, cloning is rarely successful.

The most common and relevant of these techniques to the scope of this study is cryopreservation, or the deep freezing of gametic or embryonic material. This technique provides three main advantages for the conservation field: 1) it allows gametic material to be transported across vast distances without disrupting the daily lives of animals and inducing unnecessary stress, 2) it reduces the risk of disease transfer between animals and across facilities, 3) it allows for increased genetic diversity through the transmission of genetic material between isolated populations in the wild as well as in captivity [25]. Access to gametic material from wild individuals reduces the need for continual removal of individuals from their natural habitats in the wild [29]. Cryopreservation has become a staple in the industry of assisted reproduction - especially for the study of spermatozoa - and many conservation would not be possible if it weren't for this method of gametic storage. By preserving sperm through cryopreservation, genetic material is able to be transferred when the logistics of moving the entire animal are too complex for reasons of cost, distance, rules and regulations, disease, or a lack of available space. An additional benefit to cryopreservation is that there is no limit to the amount of time biological materials can be stored. If individuals die before they are able to produce offspring, gametic materials can be collected shortly post-mortem and stored for later use. Therefore, individuals who may not have been able to contribute to the genetic diversity of a population while alive may be able to do so after death. The successes of ART in contributing to the conservation of wildlife species have been reviewed by Pukazhenthi et al. [30], Andrabi et al. [24], as well as many others. ARTs have been used to help the propagation of many species, including the black footed ferret, the

Florida panther, both Asian and African elephants, Eld's deer, the Przewalski's horse, and others at zoological institutions around the world.

ARTs have long been thought to be the answer to helping captive populations of endangered felid species reproduce in captivity, but are not always successful and can result in serious cellular trauma for sperm, oocytes, and embryos. While this trauma is often unavoidable, finding ways to mitigate the effects of such cellular trauma, such as impaired cellular metabolism, could ultimately lead to a higher rate of successful ART implementation. Therefore, continued research of feline reproductive biology is important for the improvement and eventual success of these techniques in these species.

Spermatozoa Structure and Function

Spermatozoa are the male haploid gamete responsible for fertilizing an oocyte to form an embryo in species that undergo sexual reproduction. They are generated by spermatogonial stem cells adjacent to the basement membrane of the seminiferous tubules in the testis, and undergo extreme morphological changes and differentiation to become a highly specialized means of packaging and delivering the paternal chromosomal contribution to future offspring. This process is called spermatogenesis, and it has been extensively studied throughout the last century. An extensive review details the molecular signaling, hormonal regulation, and physiological changes associated with this process [31–35].

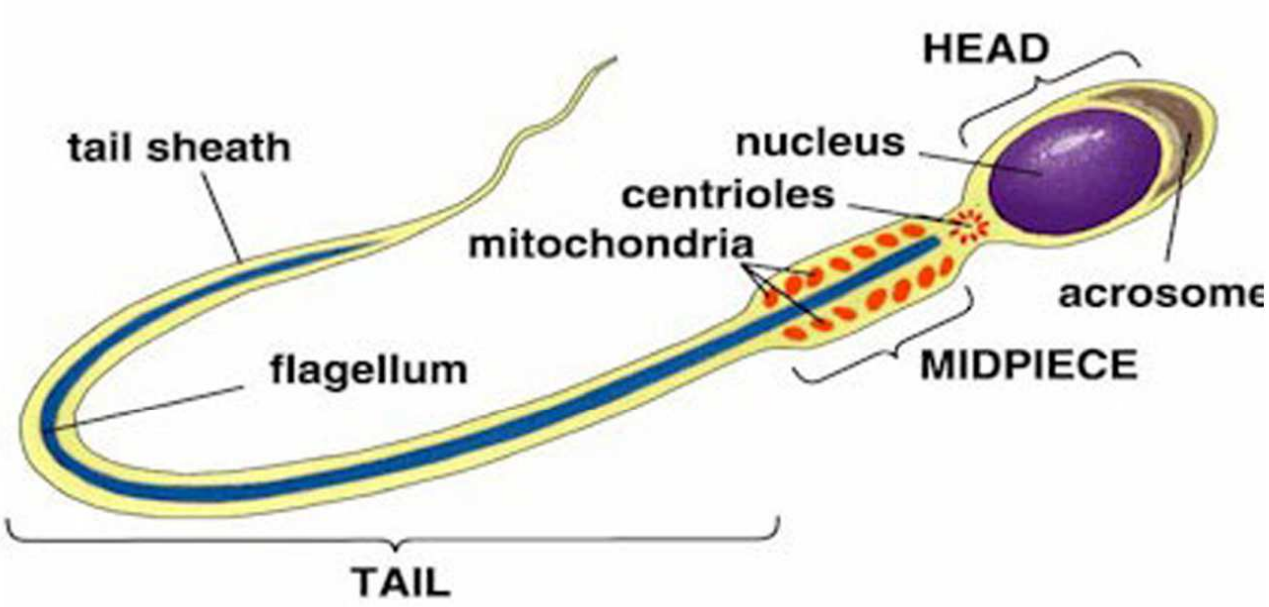

Figure 1.3: Simple illustration of a typical spermatozoon. Genetic material is stored in the head, while the centrioles and mitochondria are located in the midpiece. The flagellum is made up of fibrous sheaths and dynein which function together to sustain sperm motility (Image used with permission from Borg et. al., 2009).

Sperm are compartmentalized into a head, midpiece, and a tail or principle piece (see Figure 1.3). The head contains the nucleus and the acrosome, and is the part of the sperm that interacts directly with the oocyte and surrounding somatic cells through the acrosome reaction, zona binding, and penetration during the process of fertilization. The midpiece contains the mitochondria, and is typically thought of as the power house of the sperm cell due to the production of energy in the form of ATP following oxidative phosphorylation which occurs only along the inner mitochondrial membrane. Finally the flagellum, or tail, is responsible for propulsion of the spermatozoon through the female tract and toward the oocyte.

Following spermatogenesis and spermiation into the lumen of the seminiferous tubules, sperm pool from the seminiferous tubules into the rete testis where the lumens of the seminiferous tubules combine, migrate through the epididymis, and move through a distinct progression of micro-environments which aid in the spermatozoa's final stages of maturation [36]. The epididymal proteome and secretome have been characterized in a variety of species, and such information is indicative of the transformations that sperm are undergoing during passage through each section [37–43]. The final portion of the epididymis – the cauda, or tail – is where sperm achieve final maturation and motile status [44]. Once they reach the tail of the epididymis, they remain in a quiescent state of reduced motility until ejaculation occurs [45]. Upon ejaculation, they are propelled out of the epididymis, through the vas deferens, and past a species-specific assortment of accessory glands that deposit the various components of seminal plasma. In felines, the accessory glands responsible for the composition of seminal fluid include the epididymis,

prostate gland, and bulbourethral gland. Seminal fluid is known to contain substrates beneficial to sperm function (reviewed in [46]).

Once in the female tract, sperm must sustain forward progressive motility, undergo capacitation, reach a stage of hypermotility, and undergo the acrosome reaction in order to reach the oocyte and penetrate the zona pellucida. Thus, sperm require an immense amount of energy to carry out these functions [47]. A significant amount of research has been done to characterize the physical and molecular processes required for fertilization in domesticated animals and humans, but little has been done in non-domestic species.

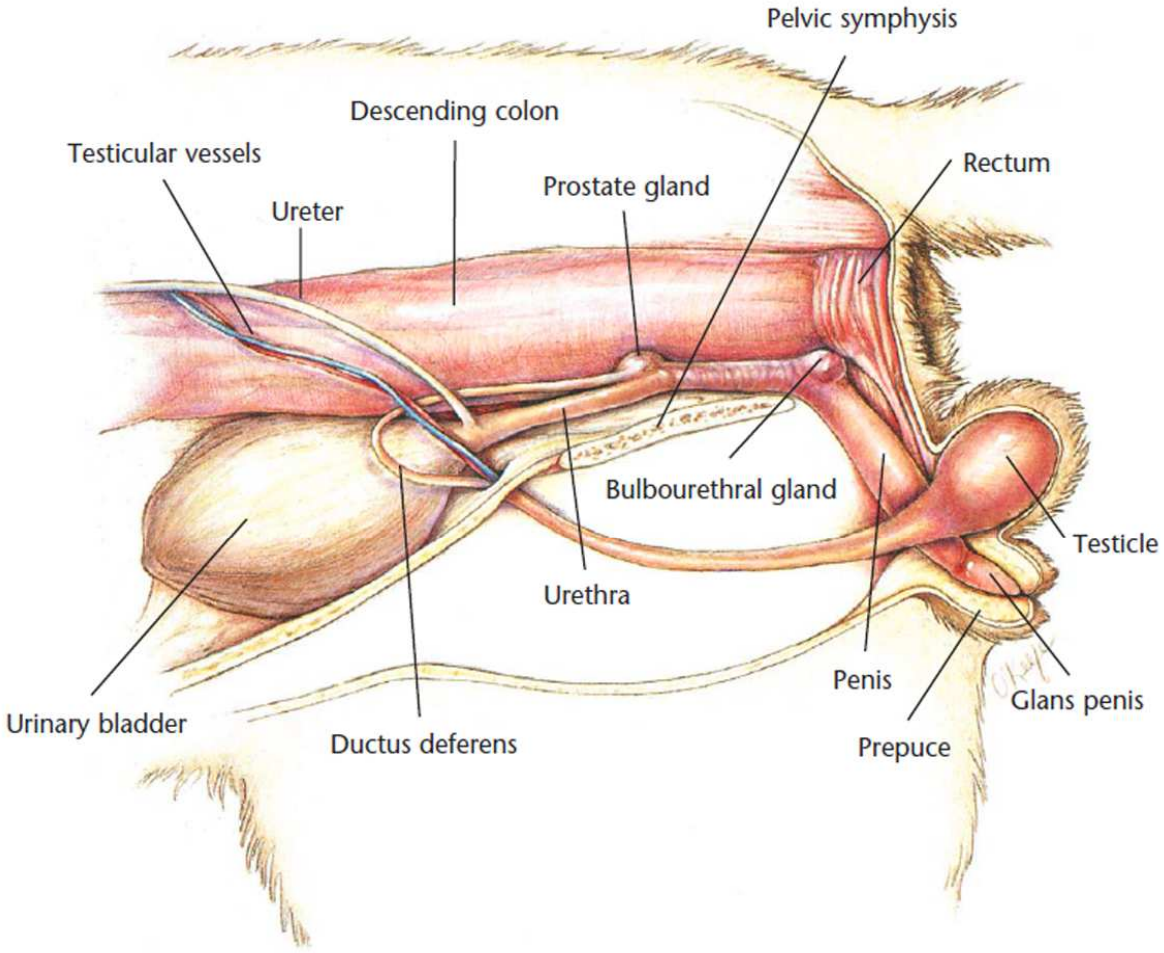

Figure 1.4: Illustration of the reproductive tract in the tom (male) cat.
(Reprinted with permission by the copyright owner, Hill's Pet Nutrition, Inc.)

Teratospermia

A remarkably high proportion of wild felid species exhibit a condition known as teratospermia. This condition is characterized by the ejaculation of spermatozoa in which more than 60% exhibit an abnormal morphology [10]. Pukazhenthi et al. (2006) described the various abnormalities that occur in the head, acrosome, midpiece, and tail of felid sperm, as well as the presence of a cytoplasmic droplet or the incidence of immature spermatozoa in the ejaculate [10]. Some sperm can exhibit multiple types of abnormalities, some of which are depicted in Figure 1.5 [10].

Individuals who are characterized as being severely teratospermic have also been shown to have low fecundity: Some males have been deemed infertile due to a decrease in the total number of sperm produced in addition to the high proportion of malformed cells present [10]. Studies in a variety of exotic felids, such as cheetah, lion, leopard cat, and Florida panther, have shown that teratospermia could be linked to a loss of genetic diversity [48]. In the cheetah, the loss of genetic diversity is thought to be derived from an ancient population bottleneck event which occurred toward the end of the Pleistocene era [10–13]. Inbreeding experiments conducted in the domestic cat demonstrated that after just a single generation of incestuous breeding, the proportion of malformed spermatozoa reached 85% [10]. Interestingly, inbred toms that exhibited high proportions of pleiomorphic sperm were also shown to produce a higher concentration of sperm cells per ejaculate in conjunction with an exhibited increase testicular volume. This suggests that compensatory mechanisms may aid in maintaining fertility despite impaired spermatogenesis [10,49]. For example, some species will alter sperm concentration, seminal fluid volume, or other

measures to improve the likelihood that their sperm will fertilize when mate competition is high [50–54].

Teratospermia has been most well-defined and researched in the cheetah. Historically, this species does not reproduce well in captivity, which is thought to be due to stress, disease, genetic homogeneity, poor management, diet, or a variety of other factors [55,56]. Additionally, spermatozoa from teratospermic individuals exhibit an increased susceptibility to osmotic stress [57], cold-induced acrosome damage [57], impaired acrosomal function [2], and compromised fertilization ability in vitro [49]. After cryopreservation, cheetah spermatozoa exhibit a significant loss in motility, and in doing so become even less capable of completing the fertilization process. In order to achieve fertilization, spermatozoa require an immense amount of energy to maintain motility, transition to hypermotility, complete capacitation and the acrosome reaction, penetrate and fuse with the oocyte. A recent comparative study between domestic cats and African cheetahs showed that felid reproduction is not driven by exogenous glucose [58]. Additionally, oxidative phosphorylation was shown to be significantly impaired in teratospermic individuals [59]. These two findings raised additional questions: What is the identity of the substrate used for continued ATP production and are other metabolic processes impaired?

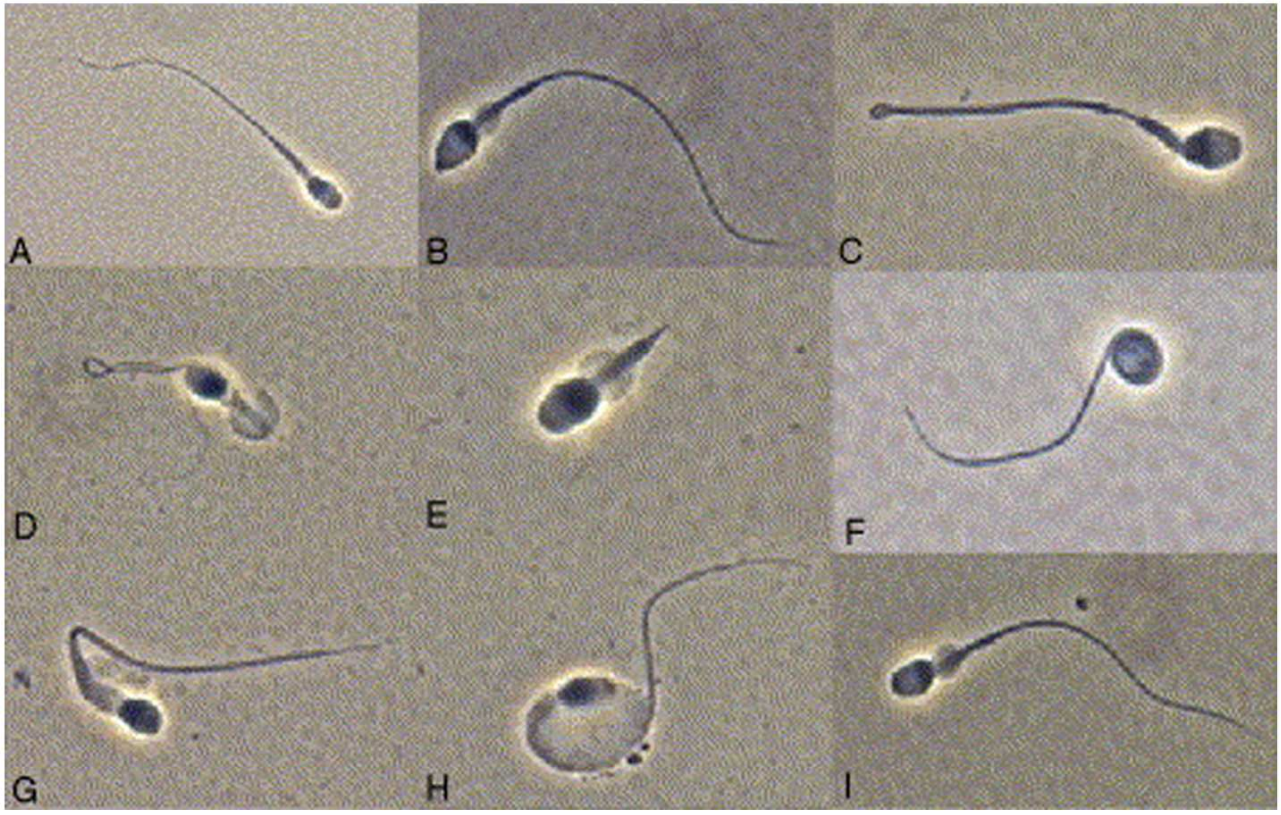

Figure 1.5: Phase-contrast photomicrographs of normal and abnormal spermatozoa commonly observed in felid electroejaculates. Depictions of (A) normal sperm; and abnormalities including (B) abnormal acrosome with proximal cytoplasmic droplet; (C) partial midpiece aplasia with bent flagellum; (D) bent midpiece with cytoplasmic droplet; (E) aplasia of the flagellum with cytoplasmic droplet; (F) spermatid; (G) abnormal acrosome with abnormal midpiece; (H) spermatid; (I) proximal cytoplasmic droplet (Image used with permission from Pukazhenthi et al., 2006).

Cellular Metabolism

Energy Production Overview

In eukaryotes, energy is produced in the form of adenosine tri-phosphate (ATP) in the cytosol by glycolysis and within mitochondria as the end-product of cellular respiration. It is used to fuel many cellular processes, including biosynthesis pathways, motility, and cell division. Metabolism is defined as the overall process through which living systems acquire and use energy to carry out and maintain cellular functions, and a metabolic pathway is a series of interconnected enzymatic reactions that use specific substrates to produce specific products [60]. Despite the fact that significant differences in form and function exist on the cell, organ, tissue, and species level, the fundamental principles of metabolism are the same in all organisms. Metabolic pathways are conserved across all eukaryotic cells, so rather than the pathways themselves being different, the source of variation is due to the presence or absence of metabolic enzymes, differences in substrate availability, and the amount of energy required by the cell or organism to perform a specific function. In eukaryotic cells, each organelle hosts a variety of signature functions and pathways that contribute to metabolism. Table 1.1 (modified from Voet et al., 2008) summarizes this information:

Of the hundreds of reactions involved in cellular metabolism, energy metabolism can be summarized in most living organisms through four major metabolic pathways: glycolysis, β-oxidation, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. Figure 1.6 illustrates how these pathways work together within a cell.

Table 1.1: Cellular organelles and their major metabolic functions

Organelle	Major functions
Mitochondrion	TCA cycle, electron transport & oxidative phosphorylation, fatty acid oxidation, amino acid breakdown
Cytosol	Glycolysis, pentose phosphate pathway, fatty acid biosynthesis, gluconeogenesis
Lysosome	Enzymatic digestion of cell components and ingested matter
Nucleus	DNA replication and transcription, RNA processing
Golgi Apparatus	Posttranslational processing of membrane and secretory proteins, formation of plasma membrane and secretory vesicles
Rough ER	Synthesis of membrane-bound and secretory proteins
Smooth ER	Lipid and steroid biosynthesis
Peroxisome	Oxidative reactions catalyzed by amino acid oxidases and catalase

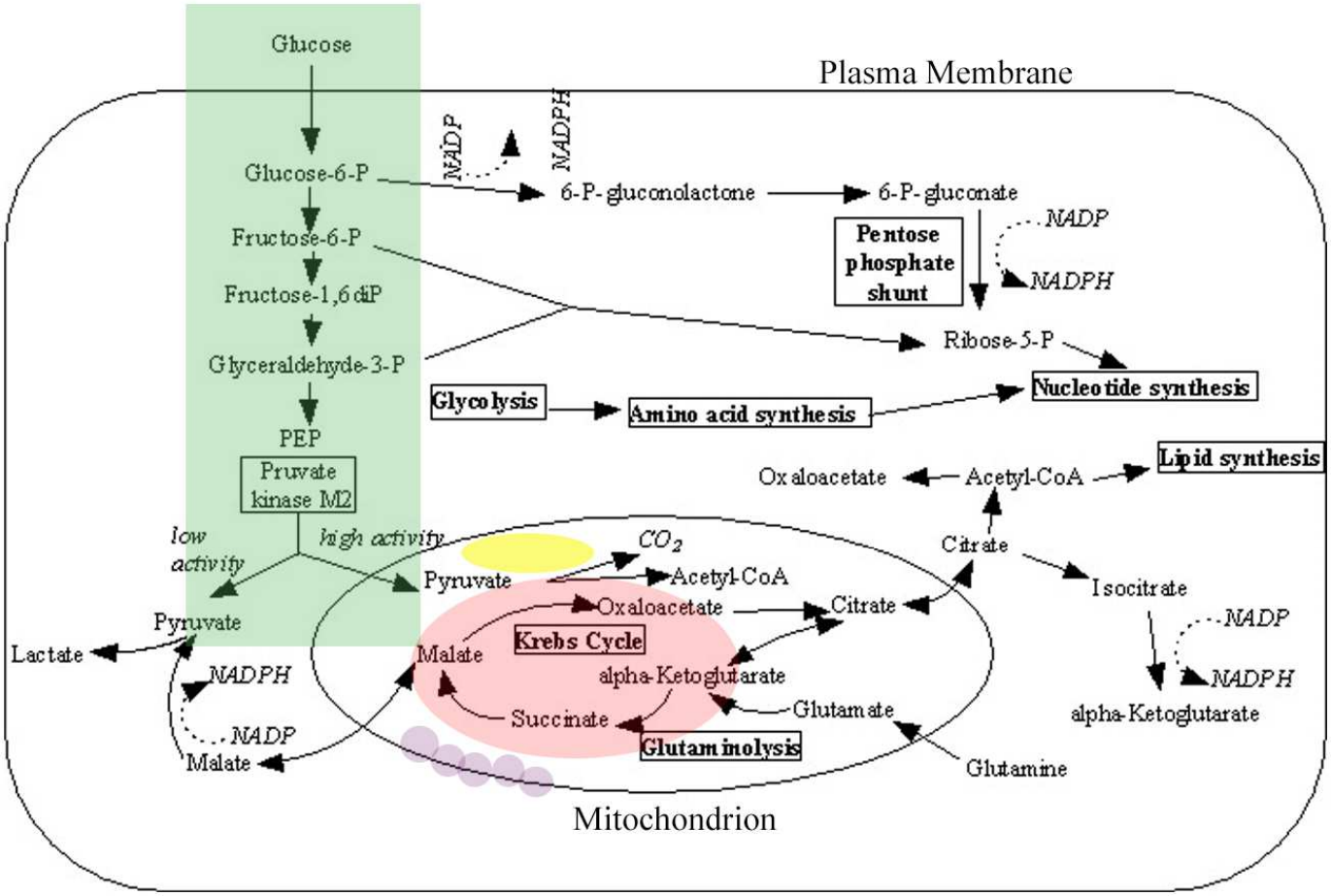

Figure 1.6: Eukaryotic energy metabolism pathways. Pathways of focus are highlighted: Green = glycolysis; red = TCA cycle; yellow = β-oxidation; purple = oxidative phosphorylation (Modified from Bequette, 2014).

Glycolysis

The most commonly considered group of energy substrates are hexoses, or sugars, and their derivatives. These molecules are broken down into pyruvate via the glycolytic pathway which is comprised of ten enzymatic steps, three of which are irreversible and therefore rate-limiting. Glucose is the conventional substrate that begins glycolysis, and when the process is completed it yields two pyruvate molecules, two NADH molecules and a net of four ATP. In addition to glucose, other hexose substrates are able to enter into glycolysis as well.

Fructose enters glycolysis toward the beginning of the glycolytic pathway, and ultimately yields two ATP and two NADH in the same way that the catabolism of glucose does. However, fructose catabolism bypasses the first four enzymatic reactions of glycolysis – hexokinase, G-6-P-isomerase, phosphofructokinase, and aldolase – and therefore does not require the consumption of two ATP. This also means that fructose catabolism bypasses the regulation associated with PFK1, which is a rate-limiting enzyme. Thus, the production of pyruvate from fructose is not regulated the same way, and an excess of this sugar can lead to an overproduction of fats and cholesterol in cells and tissues that synthesize these metabolites.

Galactose can also enter glycolysis, but it does so much earlier than fructose. It is converted to glucose 1-phosphate by galactokinase, which is phosphorylated to form glucose 1-phosphate. Phosphoglucomutase transfers a phosphoryl group to form G6P, which can enter glycolysis. In mammals, the conversion of galactose to glucose 1-phosphate takes place in the liver [61].

Finally, mannose can enter glycolysis after it is converted to mannose 6-phosphate by hexokinase, and then to fructose 6-phosphate by phosphomannose isomerase. Unlike fructose, which is more energy efficient to metabolize, galactose and mannose require the same amount of energy input as glucose to be converted to pyruvate. However, it is important to note that other substrates contribute to the cellular energy pool via glycolysis. Seminal plasma is known to contain varying amounts of hexose substrates, including glucose and fructose, which have been quantified in the seminal plasma of alpaca, camel, bull, ram, goat, buffalo, boar, human, and mouse (reviewed in [46,62]).

Lipid Metabolism and β-oxidation

The oxidation of fatty acids is an important contributor to energy production within a variety of cell types, including the heart and skeletal muscle [63]. These long heterocarbon chains are bound together with glycerol to form phospholipids and triglycerides, which can be hydrolyzed to produce free fatty acids. Fatty acids can be imported into the mitochondria and degraded methodically for entry into the TCA cycle, fueling the electron transport chain and oxidative phosphorylation to produce ATP. To do this, a fatty acid must first be activated by acyl-CoA synthetase. In this reaction, fatty acids are converted to thioesters of coenzyme A by one of four isoforms of acyl-CoA synthetase specific for the length of the hydrocarbon chain. Fatty acids exist in four types: short-chain ($\leq$6 carbons), medium-chain (6-12 carbons), long-chain (13-21 carbons), and very-long-chain ($\geq$22 carbons) varieties. Short- and medium-chain fatty acids are able to passively diffuse across the mitochondrial membranes into the matrix for β-oxidation, but long-chain fatty acids

(LCFAs) must be actively shuttled across. Very-long-chain fatty acids, due to their large size, are not metabolized in the mitochondria and are broken down within peroxisomes. In order to transport LCFAs into the mitochondria, a shuttle system is required.

The carnitine shuttle system is utilized to regulate β-oxidation. In the research presented in this dissertation, this shuttle system is blocked to study the role of β-oxidation by incubating sperm with a chemical inhibitor called etomoxir. This non-specific small molecule is used to inhibit β-oxidation by irreversibly binding to carnitine:palmitoyltransferase I, the enzyme responsible for LCFA transport into mitochondria. It is has been used extensively to measure β-oxidation in heart muscle, cumulus-oocyte complexes, sperm, and many other cell-types and tissues [64–66].

β-oxidation is named as such because it is the beta- (or second) carbon that is oxidized to degrade the fatty acid into consecutive 2-carbon fragments of acetyl-CoA. As acyl-CoA is broken down, what is left of the molecule continues along this spiraling pathway until its resources are exhausted [61]. The entire process of β-oxidation takes place in four major steps. These steps can be summarized as (1) an oxidation step, (2) a hydration step, (3) a second oxidation step, and (4) a thiolysis step [61]. Acetyl-CoA, NADH, and ubiquinol (QH_2), the three major co-products of this pathway, can be further oxidized by the electron transport chain to produce ATP. When acetyl-CoA is released into the matrix of the mitochondria, it moves into the TCA cycle to be completely oxidized and form ATP. This process is depicted in Figure 1.8.

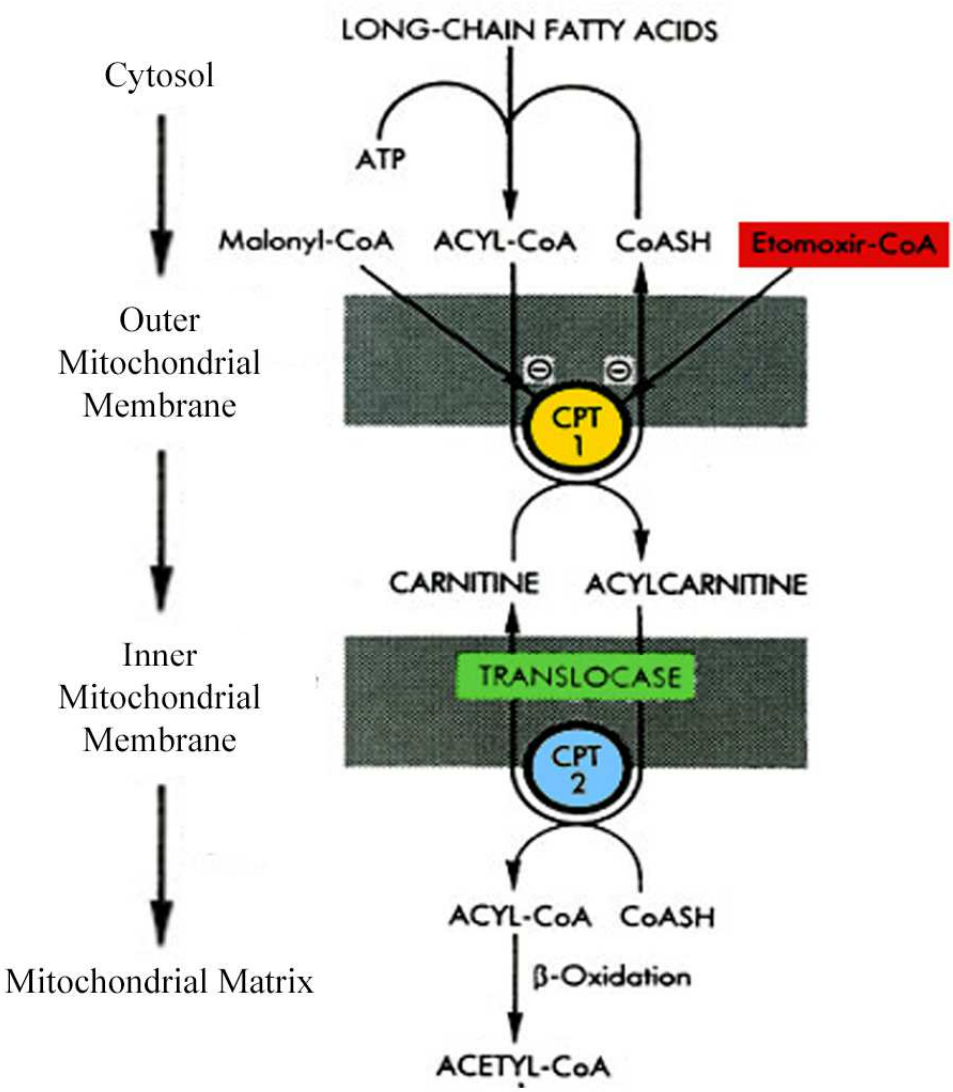

Figure 1.7: Long chain fatty acid transport into the mitochondria, and the regulatory effect of etomoxir. CPT = carnitine palmitoyl-transferase (Modified from Anstalt et. al., 2013)

The complete oxidation of fatty acids provides more energy than the complete oxidation of an equivalent amount of glucose. In even-chain fatty acids (those containing an even number of carbons), β-oxidation can occur enough times to oxidize the entire molecule [61]. For example, a single stearate molecule (C18) can complete this process 8 times to yield 9 molecules of acetyl CoA, 8 molecules of ubiquinol (QH_2), and 8 molecules of NADH. Odd chain or unsaturated fatty acids can also be oxidized to produce acetyl-CoA, but the final thiolysis of the molecule produces propionyl CoA rather than acetyl CoA. This can later be converted into succinyl CoA, and then to oxaloacetate which is the first substrate in the TCA cycle. Each of these co-products contributes to the net ATP produced by the original LCFA molecule. When these numbers are extrapolated, one molecule of stearate will produce 120 molecules of ATP [61].

In contrast, the complete metabolism of glucose will only produce 32 molecules of ATP through the processes of glycolysis, the TCA cycle, and oxidative phosphorylation [61]. Even by comparing an equal number of carbons (three glucose molecules = 18 carbons vs. one stearate molecule = 18 carbons), the fatty acid will produce more carbons by going through β-oxidation than the hexose will by going through glycolysis. In an energy-dependent cell type like spermatozoa, the ability to oxidize lipids could be advantageous by more efficiently providing the ATP necessary to fuel the cellular processes involved in motility, capacitation, and fertilization.

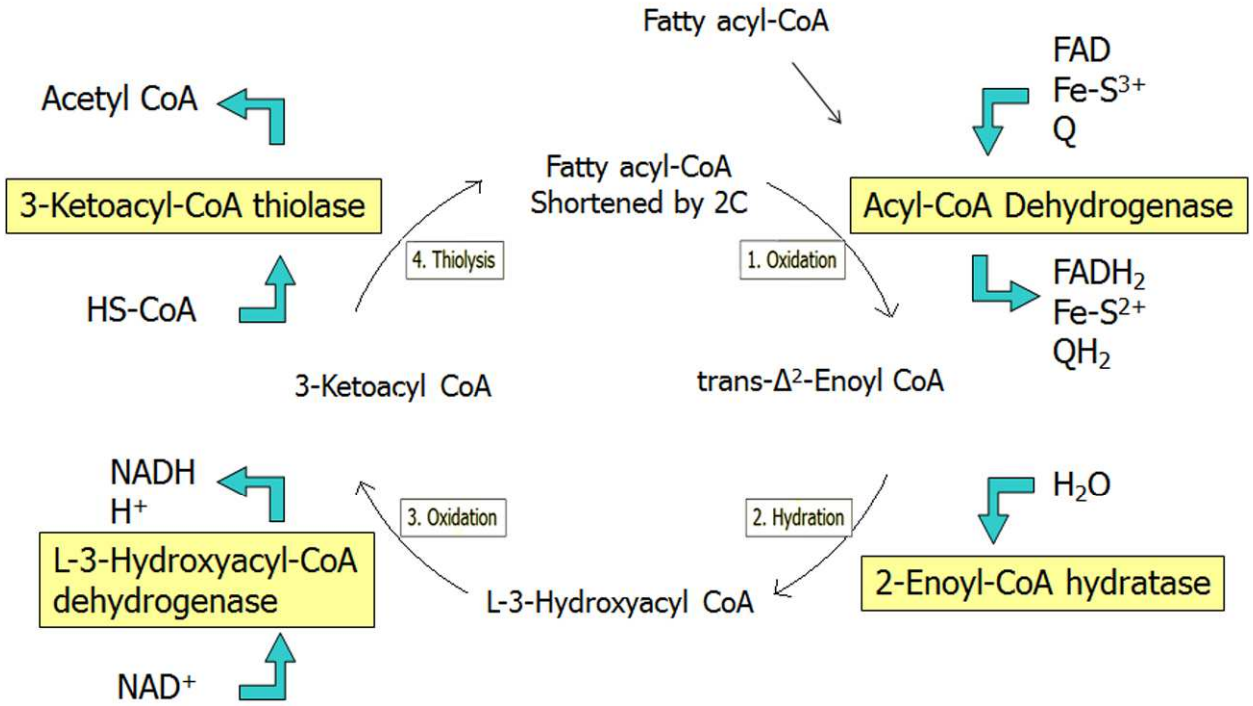

Figure 1.8: The four steps of β-oxidation. Fatty acids, after being converted to fatty acyl-CoA and transported through the mitochondrial membranes to the matrix, are broken down through a series of four steps. The result is a fatty acyl-CoA molecule that is shortened by two carbons, which can continue through the cycle until only one carbon is left on the molecule. Enzymes are listed in yellow boxes; Co-factors and co-products are indicated before and after blue arrows, respectively; white boxes indicate the type of reaction taking place at each step.

The Tricarboxylic Acid (TCA) Cycle

The citric acid cycle, also known as the tricarboxylic acid or TCA cycle, is the axis around which the precursors for ATP and energy production revolve. Glycolysis, β-oxidation, and amino-acid metabolism all contribute substrates to the cycle, producing a series of products and co-products which serve to generate ATP after further oxidation by the electron transport chain and oxidative phosphorylation. The flow of carbon and the production of energy-rich molecules are this pathway's trademark. As discussed previously, acetyl-CoA formed from either pyruvate (glycolysis) or fatty acids (β-Oxidation) is the molecule which contributes two new carbons to the TCA cycle. For each turn of the cycle, two carbons enter as acetyl-CoA and two carbons are released as CO_2; however the two contributed carbons are not the same two carbons to leave the cycle [61]. Rather, the carbons donated by acetyl-CoA make up half of succinate (a symmetric, four-carbon dicarboxylic acid) – the product and subsequent substrate of the fifth and sixth reactions in the cycle, respectively. The two halves of this molecule are chemically equivalent, so carbons originating from acetyl-CoA become evenly distributed in molecules subsequently formed from succinate [61]. For each acetyl group entering the TCA cycle, one NAD+ and one ubiquinone (Q) are reduced to form NADH and ubiquinol (QH_2), one ADP is phosphorylated to produce ATP, and oxaloacetate is reformed enabling the cycle to continue its course. In total, each turn of the TCA cycle produces two HS-CoA, three NADH, two CO_2, one ATP, and one QH_2, and there are eight enzymatic reactions responsible for these products.

Briefly, acetyl CoA enters the TCA cycle and is hydrolyzed alongside oxaloacetate by citrate synthase to form HS-CoA and citrate. Citrate is then rearranged by aconitase to form isocitrate, which is oxidized by isocitrate dehydrogenase to form NADH, CO_2, and α-ketoglutarate. This is then followed by a second oxidative decarboxylation by α-ketoglutarate dehydrogenase complex to form the second NADH and CO_2, and succinyl CoA. From succinyl-CoA, succinyl-CoA synthetase conducts a substrate-level phosphorylation to produce ATP from inorganic phosphate (P_i) and ADP, as well as succinate. Succinate is a symmetrical compound which is then oxidized by succinate dehydrogenase complex to form QH2 and fumarate, which is then hydrated by fumarase to form L-malate. This compound interacts with malate dehydrogenase to form the final NADH molecule produced by this pathway, as well as oxaloacetate. The reformation of one of the initial substrates enables the continuation of the TCA cycle, which exists in virtually every cell type to fuel cellular activities.

Oxidative Phosphorylation

Oxidative phosphorylation is a well-known energy producing pathway that takes place in the matrix of the mitochondria along the inner mitochondrial membrane. This pathway utilizes the combination of reduction and oxidation reactions to produce energy, and is comprised of two parts: the electron transport chain and ATP synthase. These two mechanisms are coupled, which means that one cannot happen without the other. A typical dysfunction of this system is called "uncoupling," where the link between the two processes is broken [67]. As its name suggests, the electron transport chain utilizes the transport of electrons to harness

energy. It does this by creating a negative proton gradient between the mitochondrial matrix and the intermembrane space (between the inner and outer mitochondrial membranes). ATP synthase relies on this gradient to drive the synthesis of ATP from ADP and P_i.

Sperm Metabolism

Mammalian spermatozoa are dependent on the efficient generation and utilization of ATP to maintain progressive motility, to transition into hyperactive motility, and finally to undergo capacitation and fertilization [68]. Parts of this process have been characterized in domestic animals and by doing so scientists have demonstrated the varied mechanisms of sperm metabolism that can be seen across species. Different pathways are more or less active in different species, and the sperm of some species use substrates that are completely different from the substrates used by sperm of another species. Much of this work has been done in the bull, boar, mouse, and human [69–74]. Mammalian sperm has been thought to rely heavily on glycolysis and oxidative phosphorylation as the major energy producing pathways. To determine sperm metabolic activity, two approaches have been utilized. The first approach involves incubating sperm with a known concentration of substrate and measuring the final concentration of substrate. This type of study conveys whether the sperm are utilizing the substrate and at what rate, but is limited by the sensitivity of the detection assay. The second common method of measuring sperm metabolism is by blocking enzymes which play a critical role in hypothesized pathways present. For example, if glycolysis is thought to be an important contributor to sperm function or energy production, then blocking the rate-limiting enzymes can be used to

determine the relative importance of glycolysis to sperm function and energy production. This approach also has limitations in that certain enzymes can perform functions in multiple metabolic pathways or chemical reactions. Care must be taken to ensure that blocking a specific enzyme is not depleting the cells in question of a necessary enzyme or impairing more than one critical pathway.

Many studies have been conducted throughout the last century that attempt to characterize sperm metabolism, most of which utilize one of the two aforementioned techniques. Human sperm metabolism has been shown to be almost entirely dependent on glucose-derived ATP (through glycolysis and oxidative phosphorylation) to undergo capacitation [69,71,75]. Energy metabolism in boar spermatozoa, like human sperm, depends heavily on glycolysis for ATP production [76]. However, proteins associated with β-oxidation have also been identified in human, bull, boar, and stallion sperm, and is known to play important roles in each [66,77,78].

Lipids and phospholipids, as potential sources for energy metabolism, have been a recent subject of focus. To date, these processes have been studied in the human [79], boar [80], and bull [43,81,82]. It has yet to be determined whether endogenous or exogenous lipids are utilized by the sperm. The sperm plasma membrane is made up of various phospholipids and proteins which are located in distinct regions, including the acrosomal, post-acrosomal, midpiece, and flagellar areas [79,83]. Membrane phospholipids exist as a fluid mosaic and are able to migrate across most of the plasma membrane and could be an endogenous source of lipids and phospholipids.

In addition to mobility within the plasma membrane, phospholipids have been shown to be drawn in from the surrounding environment [80]. Human spermatozoa that are of higher quality before undergoing cryopreservation have been shown to uptake a greater number of exogenous lipids after thawing [80]. However, not all membrane phospholipids move throughout the plasma membrane equally. Fluorescence photo-bleaching showed that phospholipids located in the midpiece region of spermatozoa were more stationary than phospholipids in other regions of the plasma membrane [79]. One possible reason for this phenomenon could be because they are being utilized for energy production via β-oxidation. As previously stated, spermatozoal mitochondria are located exclusively in the midpiece, and β-oxidation occurs in the matrix of the mitochondria. The rigidity of the plasma membrane in the midpiece region could affect the use of these molecules for energy production. In normozoospermic men, phosphatidylethanolamine (PE) was the most prevalent phospholipid in the plasma membrane, whereas phosphatidylcholine (PC) was the most populous phospholipid in men that were asthenozoospermic (i.e., had low motility) [84]. Compared to those from normozoospermic individuals, sperm from asthenozoospermic men also had a lower quantity of polyunsaturated fatty acids (PUFAs) and a higher prevalence of saturated fatty acids [84]. PUFAs are classified as unsaturated due to the presence of double bonds which cause the lipid tails to become kinked, and thus are less able to move throughout the plasma membrane. This means that sperm from normospermic individuals have a higher degree of membrane fluidity than asthenozoospermic individuals, as the double bonds in PUFAs causes reduced fluidity in phospholipid membranes. Additionally, the motility

of boar spermatozoa is directly related to the amount of *n-3* PUFAs in the plasma membrane, as well as extracellular antioxidants. Antioxidants protect spermatozoa from lipid peroxidation during oxidative stress, and a deficiency may be a source of the dysfunction in teratospermic individuals [85]. The marked difference between the phospholipid content in normozoospermic and asthenozoospermic men suggests the possibility of a similar difference between normospermic and teratospermic felids.

Previous work in bovine sperm found evidence of lipid metabolism. Ether lipids were stable and static components of the plasma membrane, whereas diacyl analogs were shown to undergo degradation and re-synthesis [86]. The breakdown of phospholipids is known to be conducted through phospholipases enzymes, which cleave the bonds connecting fatty acids to the glycerol backbone of a phospholipid molecule. Bovine sperm contain proteins which bind to PC on the plasma membrane and inhibit the catabolic actions of phospholipase A2. This protein also plays a significant role in capacitation and the acrosome reaction [87]. Identifying such proteins in the felid model could eventually be used to validate the presence of lipid metabolism within sperm from these species.

There have been a limited number of studies surrounding feline sperm metabolism. Recent discoveries indicate that glycolysis is not critical to the production of energy in the domestic cat and cheetah. Spermatozoa from both species were supplemented with exogenous glucose as a potential energy source, but its uptake was negligible [58]. However, when glycolytic enzymes were inhibited, a marked decline in spermatozoal function was observed [58]. Oxidative phosphorylation was then shown to be significantly impaired in teratospermic

individuals [59]. This could indicate the glycolytic pathway as an important component of sperm metabolism in teratospermic felines, but that the initial substrate may be glycerol derived from phospholipid metabolism rather than from traditional hexoses such as glucose or fructose. Hexoses (typically glucose or fructose) [1], monocarboxylates (pyruvate and lactate) [1], and even glycogen [88] have been identified as energy sources in mammalian sperm metabolism. The substrate diversity seen across species is thought to be attributed to the availability of such resources in the female tract, and reflects its availability to sperm after copulation *in vivo* [89].

Metabolomics

Metabolomics is the systematic analysis of the metabolic composition of cells, tissues, and organs that make up their functional phenotype. In the last several decades, many other "-omics" technologies have become the hot topics of scientific innovation (e.g., proteomics, genomics, transcriptomics, etc.), but metabolomics is arguably more valuable because it measures compounds which exist downstream of genetic, enzymatic, and environmental controls.

Metabolomics provides a window through which a snapshot can be taken of the metabolism within a biological system, and it can depict the changes that occur due to both intrinsic and extrinsic control factors [90]. There are two types of metabolomic techniques: targeted and untargeted. As the names suggest, targeted metabolomics focuses on quantifying selected metabolites of interest for a given study. Analytical procedures for targeted analysis must include identification and absolute quantification of the selected metabolites of interest in the sample. The

advantage of targeted metabolomics is that the output is directly related to the research question being asked in a given experiment. Unfortunately, this approach lends itself to a significant disadvantage when working with or looking for novel compounds. Because targeted metabolomics only measures the specific compounds of interest to the researcher, metabolites that are not well characterized and difficult to quantify can often be overlooked. This limitation can often lead to incomplete characterization and misinterpretation of metabolic events [91].

Untargeted metabolomics, or metabolic profiling, involves the rapid analysis of a large number of different metabolites with the objective to characterize a given sample. This approach is often not quantitative, and tends to be used to generate further hypotheses to test in subsequent studies. This approach to metabolomics can be sub-divided into either metabolic fingerprinting or metabolic footprinting [91]. In metabolic fingerprinting, a large number of intracellular metabolites are scanned and detected by a selected analytical method or technique. Not all metabolites within the sample must be identified and quantified, as the purpose is to simply profile the milieu of metabolites that are present. For this approach, raw data may be presented without any further analysis as long as the method delivers reproducible results [91]. Metabolite footprinting is similar to fingerprinting, but it is focused on measuring all of the extracellular metabolites present in a spent culture medium. In this approach, the compounds that are measured are either secreted by, or are being supplied to and/or transformed by the biological sample in question. Untargeted metabolomics can be incredibly beneficial when investigating a cell or tissue type about which very little is known. The amount of information that can be gained by profiling the

metabolomic fingerprint or footprint is huge, which can also serve as a disadvantage of the technique. Datasets generated from an untargeted approach can be complicated, and metabolite identification and quantification can be limited [92].

Metabolomics platforms include nuclear magnetic resonance (NMR) spectroscopy, mass spectroscopy (MS), near infrared spectroscopy, Fourier transformation infrared spectroscopy, and raman spectroscopy. Each of these techniques is used in a variety of disciplines [93]. Currently, metabolomic profiling is most often performed using nuclear magnetic resonance spectroscopy (NMR) and mass spectral (MS) technologies. While there are advantages and disadvantages to both of these techniques, one of the key differences is that NMR is less sensitive than MS, and requires that metabolite concentrations be in micro-molar amounts [90]. For this research, there are several characteristics that make MS an ideal candidate for metabolomic analysis. First, we are interested in identifying a broad range of metabolites. Additionally, semen volumes in felids are low and spermatozoa represent a very minimal size cell-type; therefore it is not guaranteed that there will be enough cellular material to accurately detect metabolites at a level high enough for NMR spectroscopy.

While MS technologies are often used for metabolite identification, the detection and identification of specific metabolites can be improved by pairing MS with an on-line separation technique. Gas chromatography MS (GC-MS) is a mature and well-researched technique for analysis of metabolites that has been extensively used due to its high separation efficiency [91]. Newer platforms have demonstrated advantages over GC-MS, including ease of sample preparations and the ability to

detect high and low molecular-weight compounds. However, GC-MS has the advantage of having available numerous mass spectral libraries for compound identification. Interfacing the GC and MS enables reliable identification of compounds when paired with such specialized chemical libraries. Finally, much in the way of analysis has been developed for this technology, making data-processing relatively easy and complete [91,94,95].

Fluxomics

Fluxomics is one of the newest of the "-omics" fields and, unlike metabolomics which takes a metaphorical snapshot of metabolism, is able to yield quantitative measures of metabolic turnover through operational pathways within a biological sample [96]. In this way, fluxomics builds upon metabolomics through the use of isotope-enriched compounds, or "tracers," which are able to be tracked as they are metabolized and used for the generation of new compounds. The mass isotopomer distribution (MID) is then calculated by quantifying the ion fragments containing stable isotopes as a ratio of the ion containing the unlabeled atoms and labeled atoms of interest, thereby demonstrating the proportion of metabolic products that are generated using the labeled substrate [97]. Fluxomic technologies have several advantages over metabolomics: first, it provides a dynamic "moving picture" of pathway activities, whereas metabolomics can only provide a static snapshot at a given point in time. Second, fluxomics allows the undeniable determination of substrate use in a biological sample. While heavy ions do exist in small amounts naturally, the ratios of *naturally* heavy isotopes are well established. By adjusting for

these well-known ratios, the use of heavy isotopes can be directly quantified in the production of new metabolites by the biological sample of interest. Third, fluxomics can provide the necessary information for calculating the rate of metabolism, or flux rate, of a metabolic pathway of interest. This is done by calculating the heavy-isotope substrate ratios before, during, and after a treatment, and comparing the results. Finally, fluxomics allows for a more directed evaluation of metabolites. When using fluxomics, it is not necessary to measure every compound that is present within a sample. Rather, focus can be applied to downstream metabolites that indicate certain pathways. Thus, while metabolomics enables the identification of the metabolites that are present, fluxomics provides a dynamic picture of metabolic pathway activities [98–100].

Tracers and Central Carbon Metabolism

The most well-known means of labeling compounds in the past has been the use of radioisotopes. However, concerns of health and safety, decay, and complications surrounding the measurement of specific activity has made this source of tracer isotopes less than ideal. Stable isotopes have been increasingly used throughout the last few decades, most commonly involving ^{13}C, ^{15}N, and ^{2}H labeled organic compounds and metabolites. Briefly, this means that each stable isotope ion exists as a mass unit heavier than the most abundant natural isotope (^{12}C, ^{14}N, and ^{1}H, respectively). In addition to being more reliable than radioisotopes, this means of labeling lends itself well to analysis via MS technology. As in any sample introduced into an MS system, the ionizing source causes labeled compounds to predictably break apart such that fragment ions containing the incorporated stable isotope can be

selectively monitored. Tracer selection is largely dependent on the research question at hand. In mammals, the pentose phosphate pathway is often interrogated using [1,2-$^{13}C_2$], and [3-$^{13}C_1$] glucose is used to evaluate pyruvate oxidation [101]. The objective of our study is to evaluate *which* pathways are active in felid sperm, not *how* active a specific pathway is, and the use of uniformly labeled substrates is ideal for this purpose. Rather than a single atom within a molecule being a heavy ion, all carbon atoms within a uniformly labeled substrate are labeled with a heavy ion [102]. This technique has additional advantages: first, the incorporation of an intact tracer molecule can be determined. In other words, incubating a sample with both labeled and unlabeled [U-$^{13}C_6$] glucose will lead to the synthesis of unlabeled and labeled pyruvate molecules after the substrate is passed through the glycolytic pathway. Second, the incorporation of ^{13}C-labeled atoms into metabolites can only occur when the tracers are utilized by the biological sample in question. Thus, the appearance of ^{13}C labeled molecules that were not initially introduced into a sample can be undeniably linked to the metabolism of the given substrate.

Cellular metabolism consists of complex networks of interconnecting pathways. Intermediates from one pathway serve as substrates or intermediates in other pathways, and measuring the effectiveness of a single enzyme can only provide a glimpse into the dynamic processes that take place within a biological sample. By understanding how pathways are interconnected, and which intermediates serve as substrates for other pathways, fluxomics can provide information regarding the movement of metabolites. The major pathways that will be discussed in this dissertation include the TCA cycle, glycolysis, and β-oxidation. Amino acids can also

be measured to determine the flux of metabolites through the TCA cycle. Table 1.2 showcases the amino acids that are linked to TCA cycle intermediates.

The easiest way to measure glycolysis using fluxomics is by introducing glycolytic substrates to a biological system and measuring the ratio of labeled products. For example, the introduction of uniformly ^{13}C-labeled glucose will yield labeled pyruvate if glucose is being metabolized by the sample. Additionally, the incidence of labeled lactate is indicative of the cytosolic pool of pyruvate. The only way to get labeled lactate is through the utilization of labeled pyruvate by lactate dehydrogenase. Figure 1.7 summarizes the connected pathways of carbon metabolism.

Table 1.2: TCA cycle intermediates and the amino acids that result from their further metabolism

TCA Intermediate	Amino Acids
Pyruvate	Alanine, Cysteine, Glycine, Threonine, Tryptophan, Serine
Acetyl-CoA	Isoleucine, Leucine, Lysine, Phenylalanine, Theonine, Tryptophan, Tyrosine
α-Ketoglutarate	Arginine, Glutamate, Glutamine, Histidine, Proline
Succinyl-CoA	Isoleucine, Methionine, Valine
Fumarate	Phenylalanine, Tyrosine
Oxaloacetate	Aspartate, Asparagine

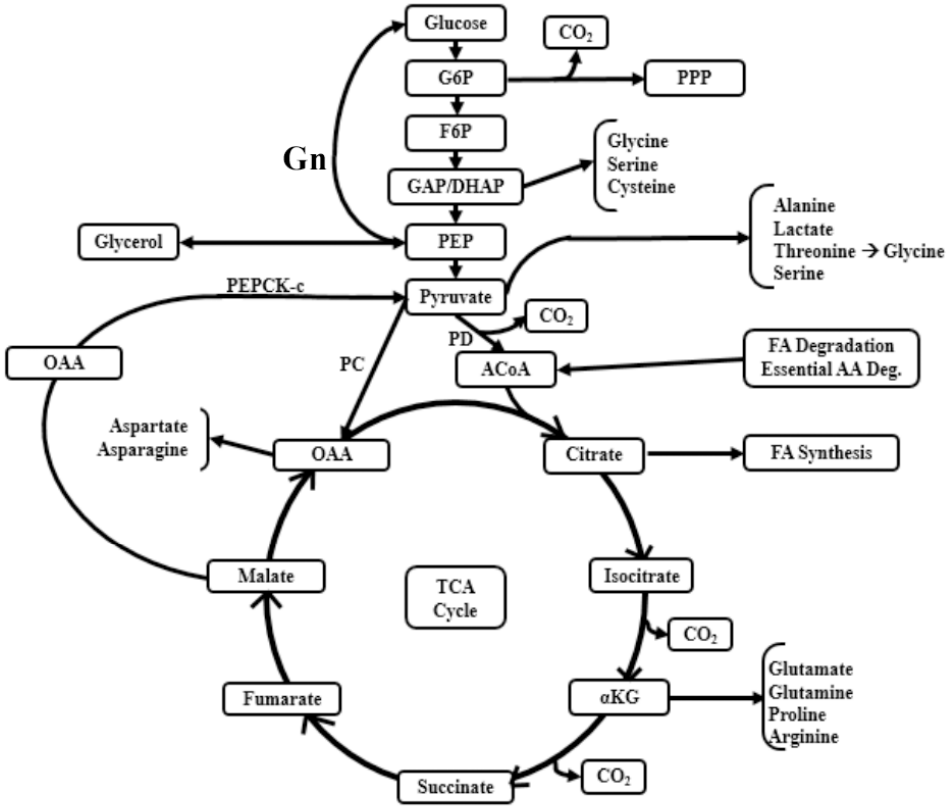

Figure 1.9: Central carbon metabolism through glycolysis and the TCA cycle. Amino acids produced from metabolic intermediates are listed in an open bracket. Other pathways included in this diagram are the pentose phosphate pathway (PPP) and gluconeogenesis (Gn) (Bequette, 2014).

In the research discussed in this dissertation, U-$^{13}C_6$ glucose, U-$^{13}C_6$ fructose, and U-$^{13}C_3$ pyruvate were introduced to sperm from three felid species (domestic cat, cheetah, and clouded leopard) to better understand if and how these substrates are utilized. Previous research claims that glucose is not utilized by felid sperm, but the methods by which this was determined may not have been sensitive enough to detect the small quantities of substrate being consumed. One of the greatest benefits to utilizing a fluxomic approach is its sensitivity for measuring substrate utilization and metabolic activity, which is much higher than that of traditional enzyme-blocking approaches. While blocking enzymes could illustrate the importance of a single pathway, this approach enables the visualization of which pathways a substrate moves through. This allows us to monitor multiple pathways, thereby putting together a more complete picture of metabolism in the given cell type.

Gas Chromatography – Mass Spectrometry: Overview

Both metabolomics and fluxomics rely on analytical platforms that can reliably measure metabolites. GC-MS is a combination of two powerful analytic techniques: the GC separates the components of a mixture in time, and the MS provides information that helps to structurally identify each compound.

Gas chromatography was first introduced in 1952, and has been used ever since to separate components in a mixture over time. There are several parts to the GC, including the injector, the column, and the detector. One of the most important components of this system is the use of a carrier gas. Typically, either hydrogen or helium is used for this purpose. The carrier gas is responsible for transferring the

sample from the injector, through the column, and into the detector. Additionally, the carrier gas must be inert, and unable to bind to the stationary phase of the column.

During the injection phase of GC processing, samples must be introduced into the GC system. Gas sampling valves, split and splitless injectors, on-column injection systems, programmed temperature injectors, and concentrating devices are all involved in this process [103]. Our system uses a split injection system. In order for the sample to be injected into the system, it is first vaporized into the stream of carrier gas. A portion of the sample and solvent is then directed onto the head of the GC column, and the rest of the sample is vented. Whether the method is "split" or "splitless" depends on what proportion of the sample is being injected onto the column. Typical split ratios range from 10:1 to 100:1, but in samples with low concentrations, a split mode as high as 2:1 can be used. Typically 1-2 μL of sample is injected onto the column at a time.

After the sample is injected into the system, the separation of compounds takes place in the column. The column is a heated hollow tube which contains a thin layer of a non-volatile chemical, or the "stationary phase." Our equipment includes a capillary column, which has the stationary phase coated onto the walls of the column. The other type of column is a "packed", where an inert solid is coated with the stationary phase and inserted into the column. Once the sample is injected, each compound is selectively retained by the stationary phase. Stationary phases can be thinner or thicker, depending on what types of compounds need to be separated. Thin (0.3 μm) phases are better for compounds that have higher boiling points, while thicker phases (1.0 μm) are better for separating compounds with lower boiling

points. This is because thicker phases are better able to retain compounds. As the temperature of the oven containing the GC rises, components are successively released based upon their affinity for the carrier, or gas phase. What this means, however, is that GC is limited by the compounds that it can successfully process: only compounds which are volatile – or that can be made volatile – can be separated using this method. Therefore, means of derivatization have been developed to increase the volatility and stability of certain types of compounds. A variety of detectors are available for GC, including flame ionization detectors, thermal conductivity detectors, thermionic specific detectors, flame photometric detectors, and electron capture detectors [103]. Finally in a GC-MS system, there must be a device responsible for transporting the material from the chromatograph to the mass spectrometer. This is done in a variety of ways, but is also dependent on the type of column and MS system that are in place. For capillary columns, the end of the GC column is often inserted directly into the ion source [103].

Mass spectrometry has been utilized for decades to discover and describe molecular compounds within a given (often biological) sample [104]. These entities can be detected according to mass, size, shape, hydrophobicity or hydrophilicity, depending on the MS system in place. Briefly, MS relies on the ionization and detection of compounds based on their mass to charge (m/z) ratios. Typically, compounds are ionized with a charge of either (+1) or (-1), which causes the m/z to be directly proportional to the mass of the molecule. There are a variety of ways in which the mass of a molecule is analyzed in an MS system, including magnetic-sector, quadrupole, time-of-flight (TOF), quadrupole ion trap (QIT), linear ion trap

(LIT), Fourier transform ion cyclotron resonance (FTICR), orbitrap, and ion mobility analyzers. While each of these analyzers works in a slightly different way, they all: 1) separate molecules on the basis of m/z, and 2) focus the mass-resolved molecules (ions) at a single focal point [104]. Once these ions are focused, they can then be passed to an ion detector for interpretation. Ion detectors convert the beam of ions from the sample into an electrical signal that can be amplified, stored, and displayed for analysis.

Study Aims

The goal of this research was to generate essential information on global metabolite composition and the flux of metabolites in felid sperm. It is well established that sperm metabolism is species-specific, and while there are a few studies investigating the role of glycolysis and oxidative phosphorylation in felid sperm, those studies did not characterize the metabolome or interrogate metabolic pathways to the same level of detail as the approaches in this research were able to. This research had three objectives pertaining to sperm collected from domestic cats, cheetahs, and clouded leopards: (1) To characterize the metabolomic profile of sperm and seminal plasma; (2) To investigate the effects of blocking long-chain fatty acid metabolism on sperm motility and metabolomic profile over time; and (3) To interrogate the relative contribution of glucose, fructose, and pyruvate to the pathways of sperm metabolism by using fluxomic and tracer technologies. This study used GC-MS as the major analytical platform, and represents the first time that this technology has been used to measure the sperm metabolism in these three species.

Chapter 2: Characterization of Felid Sperm Metabolome

Introduction

Following spermatogenesis and spermiation into the rete testis, sperm migrate through the epididymis and a distinct progression of micro-environments which aid in the spermatozoa's final stages of maturation [36]. The proteome and secretome have been characterized in the epididymides of a variety of species, and such information is indicative of the transformations that sperm are undergoing during passage through each section [37–43]. The final portion of the epididymis – the cauda, or tail – is where sperm achieve final maturation and motile status [44]. Once they reach the tail of the epididymis, they remain in a quiescent state of reduced motility until ejaculation occurs [45]. Upon ejaculation, they are propelled out of the epididymis, through the vas deferens, and past a species-specific assortment of accessory glands that deposit the various components of seminal plasma.

Once in the female tract, sperm must sustain forward progressive motility, undergo capacitation, reach a stage of hypermotility, and proceed through the acrosome reaction in order to reach the oocyte and penetrate the zona pellucida. Thus, sperm require an immense amount of energy in the form of ATP to carry out these functions [47]. A significant amount of research has been done to characterize the physical and molecular processes required for fertilization in a variety of species, but how sperm fulfill such energy requirements has only just begun to be investigated in

non-domestic species. Previous studies have identified glycolysis, oxidative phosphorylation, the Krebs cycle, and β-oxidation as essential ATP synthesizing processes in spermatozoa of mammalian species; however, the specific importance of each pathway can vary by species and is not fully understood in the felid model.

Of the 37 species which make up the felid family, 70% are threatened or endangered and over half of the remaining 30% have experienced consistent population decline throughout the last decade [9]. Assisted reproduction and cryopreservation is commonly used to mitigate the effects of a declining population in captive felids, but offspring production in captivity is still limited [10,25,105]. Felid sperm often exhibit severely reduced motility and low fertilization following cryopreservation, a technique commonly used in conjunction with other assisted reproduction technologies [10,105,106]. Additionally, 90% of felid species are teratospermic, a condition in which more than 60% of ejaculated sperm are malformed or misshapen and that has been well documented in the cheetah (*Acinonyx jubatus*) [107] and clouded leopard (*Neofelis nebulosa*) [23]. Males with this condition have been shown to have reduced fertility compared to their normospermic counterparts [5]. This condition also complicates efforts to cryopreserve sperm for use in assisted reproductive techniques [10,22]. In the limited metabolic studies that have been conducted in felid sperm, sperm from teratospermic individuals have been shown to exhibit reduced metabolism, as evidenced by low rates of pyruvate uptake and lactate production compared to normospermic individuals [58]. However metabolic pathways are complex and interconnected; therefore monitoring the

activity of a single enzyme or pathway may not provide a complete understanding of cellular activity.

Historically, sperm metabolism has been studied by investigating the role of specific enzymes and pathways by using chemical inhibitors [64,108–112], but newer methods allow more complex analyses. Mass spectrometry provides a systematic way of determining the unique chemical fingerprints of the molecular metabolites within a cell-type, fluid, or tissue. The relatively new field of metabolomics has well-established platforms for applications across disciplines. The preferred platform for identification and quantification of metabolites has historically been gas chromatography – mass spectrometry (GC-MS), despite the advantages that other platforms such as nuclear magnetic resonance (NMR) imaging, or liquid chromatography may present (ease of sample preparation, or expanded range of detection for molecular weights) [90,91]. Numerous mass-spectral libraries exist for compound identification using GC-MS, which provides a distinct advantage over other methods. GC-MS has been used to evaluate the metabolome within a variety of biological samples, including blood serum and plasma [94], urine [113], plant material, and microbial populations [114]. While the seminal fluid metabolome and proteome has been defined to a limited extent in several domestic species [72,115–117], this study is the first of its kind to investigate the metabolome of non-domestic felid sperm and seminal fluid. In this study, GC-MS was used to characterize the metabolic profiles of ejaculated fluid and spermatozoa from the domestic cat (*Felis* catus), cheetah (*Acinonyx jubatus*), and clouded leopard (*Neofelis nebulosa*), as well as epididymal fluid and spermatozoa from the domestic cat.

Materials and Methods

Media Preparation

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified. A chemically defined, protein-free, cat-modified mouse tubal fluid (cMTF) medium was used for all procedures [118]. The final cMTF medium (pH 7.45) contained 98.4 Mm NaCl (Acros, New Jersey, USA), 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 25 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Fisher Scientific, Rockford, IL), and 2% polyvinyl alcohol (PVA), and was sterilized through a 0.22 μm syringe filter prior to use [58]. Osmolality of the final working medium (300 – 340 mOsm) was within 10% of the physiological value of domestic cat semen (323 mOsm) [59], and determined using a vapor pressure osmometer (Wescor, Inc. Logan, UT). The cMTF medium was prepared from concentrated 1 M stock solutions in deionized water. All stock solutions were kept at 4°C and discarded after 3 months, or if precipitates were present.

Epididymal Fluid and Sperm Collection and Evaluation

Male reproductive tissues were obtained after routine castration procedures of normal (FIV negative, non-chryptorchidic) sexually mature ($\geq$ 1 yr) feral cats at a local veterinary clinic. Tissue was maintained and transported at 4°C until processing. Sperm and epididymal fluid from the cauda epididymis from 2-4 males were physically extruded through the ductus deferens, collected, diluted, and pooled in

cMTF at a ratio of approximately 1:10 (v/v) (n = 6). A 2.8 µL sample was taken to assess percent motility (%MOT) and forward progressive status (FPS) to calculate the sperm motility index (SMI = %MOT + (FPS x 20) ÷ 2) [119], and concentration was determined using a standard hemocytometer (Hausser Scientific, Horsham, PA). Epididymal fluid was separated via centrifugation at 27°C (8 minutes at 800 × *g*), and sperm were resuspended in 30µL cMTF. Both fractions were stored at -80°C until GC-MS processing.

Semen Collection and Evaluation

All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee (ACUC) and the University of Maryland's Institutional ACUC. A surgical plane of anesthesia was induced in domestic cats (n =7), cheetahs (n = 7), and clouded leopards (n = 4) according to protocols established by institutional veterinarians and in accordance with those previously utilized for semen collection in these species [2,120]. Methods for semen collection and evaluation were similar to those previously described [2,120]. Briefly, a rectal probe of 1 cm (domestic cat), 1.9 cm (cheetah), or 1.6 cm (clouded leopard) in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, OR) were used to deliver 90 stimuli (at a low voltage of 2 – 5 V) over a 30 min interval [121]. Ejaculates were collected in sterile, pre-warmed vials [29,107,121,122]. A 2.8µL sample was taken to assess motility using CASA (Hamilton Thorne, v.1.3.1), and percent motility (%MOT) and forward progressive status (FPS) were also estimated for the purposes of calculating the sperm motility

index (SMI = %MOT + (FPS x 20) ÷ 2) [119]. Sperm concentration was established using a standard hemocytometer (Hausser Scientific, Horsham, PA). Following motility assessment, sperm and seminal fluid were separated via centrifugation at 27°C (8 minutes at 800 × *g*). Individuals were classified as teratospermic if more than 60% of sperm were abnormal according to gross morphological assessment by the CASA system. However, our assessment of morphology did not account for acrosomal abnormalities, and therefore the teratospermic individuals described in this study were part of a more selective screening process than individuals in previous reports, resulting in fewer individuals being classified as teratospermic.The supernatant was collected and sperm were resuspended in 100µL cMTF. Both fractions were stored at -80°C until GC-MS processing.

GC-MS Processing

Prior to being analyzed, samples were first extracted and derivatized based on previous protocols [94]. Each sample (30µL) was spiked with 30µL of 5mM L-norleucine (TCI-GR, Tokyo, Japan) to serve as an internal standard, then incubated for 24 hours in extraction solution: methanol was used for epididymal and seminal fluid samples, and a 5:2:2 v/v solution of methanol:chloroform:water (MeOH:CHCl2:H2O) was used for sperm samples at -80˚C. Following extraction, samples were centrifuged and the supernatant was transferred to a new 2mL Eppendorf microcentrifuge tube and dried completely using a RotoVap SpeedVac (Savant, Farmingdale, NY). Derivatization occurred after adding 30µL of Methoxylamine (Acros) in pyridine (30 mg/mL) (ThermoScientific, Rockford, IL)

and 30μL of N,O-bis(trimethylsilyl trifluoroacetamide containing 1% trimethyl-chlorosilane (BSTFA +1% TCMS) (Thermo Scientific) was added to form the methoxime-trimethylsilyl derivative of the various metabolites. Samples were incubated on a rotor for 20 minutes at room temperature, microwaved for 2 minutes (200W), and incubated on a rocker overnight at room temperature. Following derivatization, samples were run through the GC-MS system (Agilent 6890 gas chromatography system, Palo Alto, CA). Samples were injected in one microliter aliquots (5:1 split mode) and separated on a mid-polarity capillary column (ZB50; Phenomenex, Torrance, CA). The initial oven temperature was set at 60˚C and held for 5 minutes, then heated at a rate of 5˚C per minute to 310˚C, then held for 5 minutes. Cooling took place at a rate of 50˚C per minute and held for 5 minutes for a total run time of 70 minutes. The mass spectrometer (Agilent 5973 inert EI/CI MSD system) was operated in electron impact mode, and ions were monitored in full scan mode (mass range 50 – 650 *m/z*). Agilent's GC-MSD Chem-Station software was used to capture raw GC-MS data files, which were viewed and quantitated using Agilent's Data Analysis software. The Agilent-Fiehn Mass Spectral Library was used to identify entities based on their mass, retention time (RT), and mass spectra. The compounds identified were cross-checked with the 2008 NIST Mass Spectral Library, and by comparing ion fragmentation patterns to spectral libraries and known standards [95,123].

Statistical Analysis

Sperm motility and morphology were assessed using a computer aided sperm analysis (CASA v 1.3.1) system (Hamilton Thorne, Beverly, MA), and were averaged within groups based on species (domestic cat, cheetah, or clouded leopard) and sperm status (normospermic or teratospermic by gross morphology but excluding measures of acrosomal integrity). The relative abundance of metabolites was quantified using the Mass Hunter analysis software (Agilent Technologies), and then used for significance testing. GC-MS output files were analyzed using Mass Profiler Professional (Agilent Technologies), and principal component analysis was used to characterize the variation between species and sperm status. Differences between compounds detected in epididymal vs. ejaculated domestic cat sperm and fluid were compared with an unpaired Student's T-Test with no multiple testing corrections. Normospermic and teratospermic samples were compared using a one-way ANOVA with a post-hoc Tukey HSD test to perform all pairwise comparisons. Compounds that were differentially detected between species were analyzed using a one-way ANOVA and the Bonferroni-Holm multiple comparisons correction. Entities were considered to be significantly different across samples when $P < 0.05$.

Results

Over 100 metabolites were annotated using the Agilent-Fiehn Mass Spectral Library. Samples were analyzed and compared between species (domestic cat, cheetah, and clouded leopard), fraction (sperm or fluid), collection method (epididymal or electroejaculated), and sperm status (normospermic or teratospermic).

Of the metabolites identified, 10 were present across all species and sample types (Table 2.1). These compounds are involved with a variety of metabolic pathways including oxidative phosphorylation, glycolysis and carbohydrate metabolism, amino acid metabolism, and lipid metabolism.

Of the 72 compounds identified in domestic cat sperm and fluids, 34 were unique to the domestic cat (Table 2.2). One of 29 metabolites identified in the cheetah was unique to this species (Table 2.3), as were 8 of the 37 metabolites identified in the clouded leopard (Table 2.4).

Table 2.1: Compounds detected across al species and sample types. Species: domestic cat, cheetah, and clouded leopard; sample types: epididymal sperm, epididymal fluid, ejaculated sperm, ejaculated fluid; Compound = [Fiehn Chemical Library Reference Number] Compound Name; RT = Retention Time

Compound	Mass	RT	Description
[1004] phosphoric acid [9.966]	299	20.629002	Product of Oxidative Phosphorylation
[107689] L-(+) lactic acid [6.851]	147	13.237	Glycolysis Product
[1176] urea [9.599]	147	21.091	Amino Acid Metabolism
[21236] L-norleucine 2 [10.607]	158	20.297667	Internal Standard
[5281] stearic acid [20.675]	341	40.637333	Fatty Acid; Lipid Metabolism
[69217] 1-methylhydantoin 2 [11.161]	73	13.730832	Amino Acid Metabolism
[753] glycerol [9.941]	73	17.958834	Lipid Metabolism; Energy Metabolism
[892] allo-inositol [17.245]	217	35.069836	Carbohydrate Metabolism
[971] oxalic acid [7.883]	147	17.0145	Energy Metabolism; Carbohydrate Metabolism
[985] palmitic acid [18.846]	313	37.18	Fatty Acid; Lipid Metabolism

Table 2.2: **Metabolites unique to domestic cat sperm and seminal fluid**. X = detected, and minus (-) = not detected; Compound = [Fiehn Chemical Library Reference Number] Compound Name; EPI = Epididymal; EEJ = Electroejaculated; RT = Retention Time.

Compound	EPI Sperm	EPI Fluid	EEJ Sperm	EEJ Fluid	Mass	RT	Description
[107812] hypotaurine	X	X	-	-	188	28.69	Amino acid metabolism
[16219560] lactobionic acid	X	X	-	-	204	45.42	Antioxidant; disaccharide (gluconic acid + galactose)
[5280450] linoleic acid	X	X	-	-	73	41.01	Lipid metabolism
[5951] L-serine	X	X	-	-	204	21.32	Amino acid metabolism
[750] glycine	X	X	-	-	174	19.69	Amino acid metabolism; Energy metabolism
[757] glycolic acid	X	X	-	-	147	14.25	Energy metabolism; carbohydrate metabolism
[92824] D-malic acid	X	X	-	-	73	25.53	Energy metabolism
[1174] uracil	-	X	-	-	241	23.70	Nucleotide metabolism
[138] 5-aminovaleric acid	-	X	-	-	174	33.59	Amino acid metabolism
[159793] 2-keto-L-gulonic acid	-	X	-	-	204	29.94	Carbohydrate metabolism; energy metabolism
[192826] maltotriose	-	X	-	-	204	54.77	Carbohydrate metabolism
[5785] L-ascorbic acid	-	X	-	-	73	35.61	Energy metabolism
[5960] aspartic acid	-	X	-	-	232	26.27	Energy metabolism; Amino acid metabolism
[6262] L-ornithine	-	X	-	-	142	31.17	Amino acid metabolism
[6989] thymol	-	X	-	-	207	9.88	Antimicrobial; Allosteric $GABA_A$ modulator; Related to anesthetic propofol
[790] hypoxanthine	-	X	-	-	265	36.95	Nucleotide metabolism
[8299] acetol	-	X	-	-	217	46.95	Amino acid metabolism; pain drug
[9316] methyl linolenate	-	X	-	-	67	47.29	Lipid metabolism
[439240] D-lyxose	X	-	-	-	73	28.12	Carbohydrate metabolism
[33032] L-glutamic acid	-	X	-	X	174	28.44	Amino acid metabolism
[1000] 2-amino-1-phenylethanol	X	X	X	X	174	17.44	Vasoconstriction
[12035] N-acetyl-L-cysteine	-	-	-	X	103	41.10	Amino acid metabolism
[12647] L-homoserine	-	-	-	X	103	35.66	Amino acid metabolism
[439227] pipecolic acid	-	-	-	X	142	9.16	Amino acid metabolism
[5280795] cholecalciferol	-	-	-	X	129	57.05	Carbohydrate metabolism; Vitamin D
[697] erythrose 4-phosphate	-	-	X	-	207	31.98	Carbohydrate metabolism; Energy metabolism

Table 2.3: **Metabolites present in cheetah sperm and seminal fluid**. X = detected, and minus (-) = not detected; (*) = compounds unique to cheetah samples; Compound = [Fiehn Chemical Library Reference Number] Compound Name; RT = Retention Time.

Compound	Sperm	Fluid	Mass	RT	Description
[1004] phosphoric acid	X	X	299	20.64	Product of Oxidative Phosphorylation
[1060] pyruvic acid	X	X	73	15.70	Product of Glycolysis
[107689] L-(+) lactic acid	X	X	147	13.23	Product of Glycolysis
*[10820] 4-isopropylbenzoic acid	-	X	221	11.41	Hippuric acid (urea cycle)
[1176] urea	X	X	147	21.80	Amino Acid Metabolism
[21236] L-norleucine	X	X	158	20.31	Standard
[239] Beta- alanine	-	X	191	31.15	Amino Acid Metabolism, Nucleotide Metabolism
[243] benzoic acid	X	-	179	21.72	Urea cycle (hippuric acid)
[304] cholesterol	X	X	329	55.77	Membrane stability
[33032] L-glutamic acid	-	X	246	28.64	Amino acid metabolism
[5281] stearic acid	X	X	341	40.64	Fatty Acid; Lipid Metabolism
[568] dibenzofuran	X	X	56	15.92	Xenobiotics biodegradation and metabolism
[58] 2-ketobutyric acid	-	X	73	16.87	Amino acid metabolism
[65065] N-acetyl-L-aspartic acid	-	X	274	29.41	Amino acid metabolism
[69217] 1-methylhydantoin	X	X	73	13.72	Amino acid metabolism
[753] glycerol	X	X	73	17.96	Lipid metabolism; Energy metabolism
[754] glycerol 1-phosphate	-	X	299	31.94	Lipid metabolism; Energy metabolism
[867] malonic acid	X	-	147	15.21	Energy metabolism
[892] allo-inositol	X	X	217	35.08	Carbohydrate metabolism
[971] oxalic acid	X	X	147	17.02	Energy metabolism; Carbohydrate metabolism
[985] palmitic acid	X	X	313	37.18	Fatty Acid; Lipid metabolism

Table 2.4: **Metabolites present in clouded leopard sperm and seminal fluid**; X = detected, and minus (-) = not detected; (*) = compounds unique to cheetah samples; Compound = [Fiehn Chemical Library Reference Number] Compound Name; RT = Retention Time.

Compound	Sperm	Fluid	Mass	RT	Description
[1004] phosphoric acid	X	X	299	20.62	Product of Oxidative Phosphorylation
[1060] pyruvic acid	-	X	73	15.69	Product of Glycolysis
[107689] L-(+) lactic acid	X	X	147	13.22	Product of Glycolysis
[1176] urea	X	X	147	21.21	Amino acid metabolism
[206] D (+) galactose	X	-	73	32.26	Carbohydrate metabolism
[21236] L-norleucine	X	X	158	20.29	Standard
[239] Beta- alanine	X	X	191	15.92	Amino acid metabolism
[243] benzoic acid	X	X	179	21.70	Urea cycle (hippuric acid)
[304] cholesterol	X	X	129	55.75	Cell membrane stability
[311] citric acid	X	X	273	32.73	Energy & Carbohydrate metabolism
*[3893] lauric acid	X	-	257	29.31	Fatty Acid / Lipid Metabolism
[441035] talose	-	X	73	32.26	Carbohydrate / Energy metabolism
*[444266] maleic acid	X	-	147	22.05	TCA cycle intermediate
*[445929] galacturonic acid	-	X	73	33.78	Energy metabolism
*[45] tartronic acid	-	X	73	25.34	Dicarboxylic acid
[5280335] D-sphingosine	X	-	204	34.15	Cell Membrane; Lipid Metabolism
[5281] stearic acid	X	X	341	40.63	Fatty Acid / Lipid Metabolism
*[601] D-Ala-D-Ala	X	X	75	11.75	Amino Acid Metabolism
[604] gluconic acid	-	X	73	32.84	Energy & Carbohydrate Metabolism
[65065] N-acetyl-L-aspartic acid	-	X	274	29.39	Amino Acid Metabolism
*[6912] xylitol	-	X	73	31.67	Energy Metabolism
[69217] 1-methylhydantoin	X	X	73	13.71	Amino acid metabolism
[753] glycerol	X	X	73	17.95	Lipid & Energy metabolism
[754] glycerol 1-phosphate	-	X	299	31.95	Lipid & Energy metabolism
*[785] hydroquinone	-	X	239	46.77	Xenobiotic Metabolism
[791] DL-isoleucine	-	X	86	19.35	Amino acid metabolism
*[811] itaconic acid	X	-	147	26.98	TCA intermediate
[8343] dioctyl phthalate	X	-	149	49.27	Plasticizer
[867] malonic acid	X	X	147	15.19	Energy metabolism
[8871] 2-hydroxypyridine	-	X	101	14.52	Nucleotide metabolism
[892] allo-inositol	X	X	217	35.06	Carbohydrate metabolism
[94214] methyl-β-D-galactopyranoside	-	X	204	34.15	Carbohydrate metabolism
[971] oxalic acid	X	X	147	17.01	Energy & Carbohydrate metabolism
[985] palmitic acid	X	X	313	37.18	Fatty acid / Lipid metabolism

Metabolic Comparison: Species

Following metabolite identification, the metabolomic profile of sperm and fluid samples from all three species was compared using principal component analysis (PCA). Figures 2.1 and 2.2 depict representative total ion chromatograms (TICs) of the spermatozoal metabolome and seminal fluid metabolome, respectively, of all four sample types used in this study. By visual comparison, many more metabolites are detected (and in higher relative abundance) in epididymal (A) vs. ejaculated (B-D) sperm. However, all four profiles display similar peak patterns and many of the same components are present in a majority of the samples (see Table 2.1).

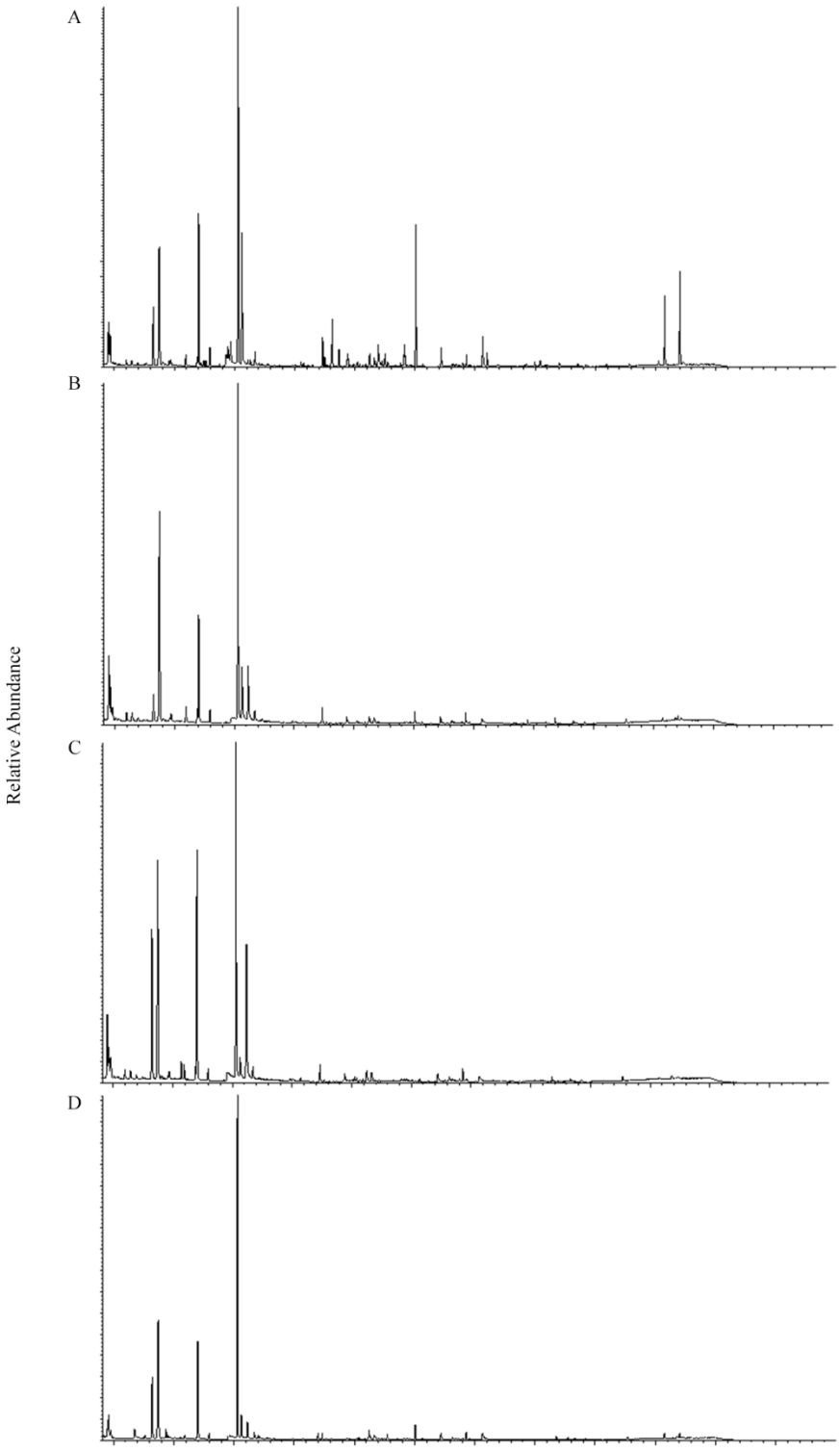

Figure 2.1: Total Ion Chromatograms of feline spermatozoa. (A) = domestic cat epididymal; (B) = ejaculated domestic cat; (C) = ejaculated cheetah; (D) = ejaculated clouded leopard.

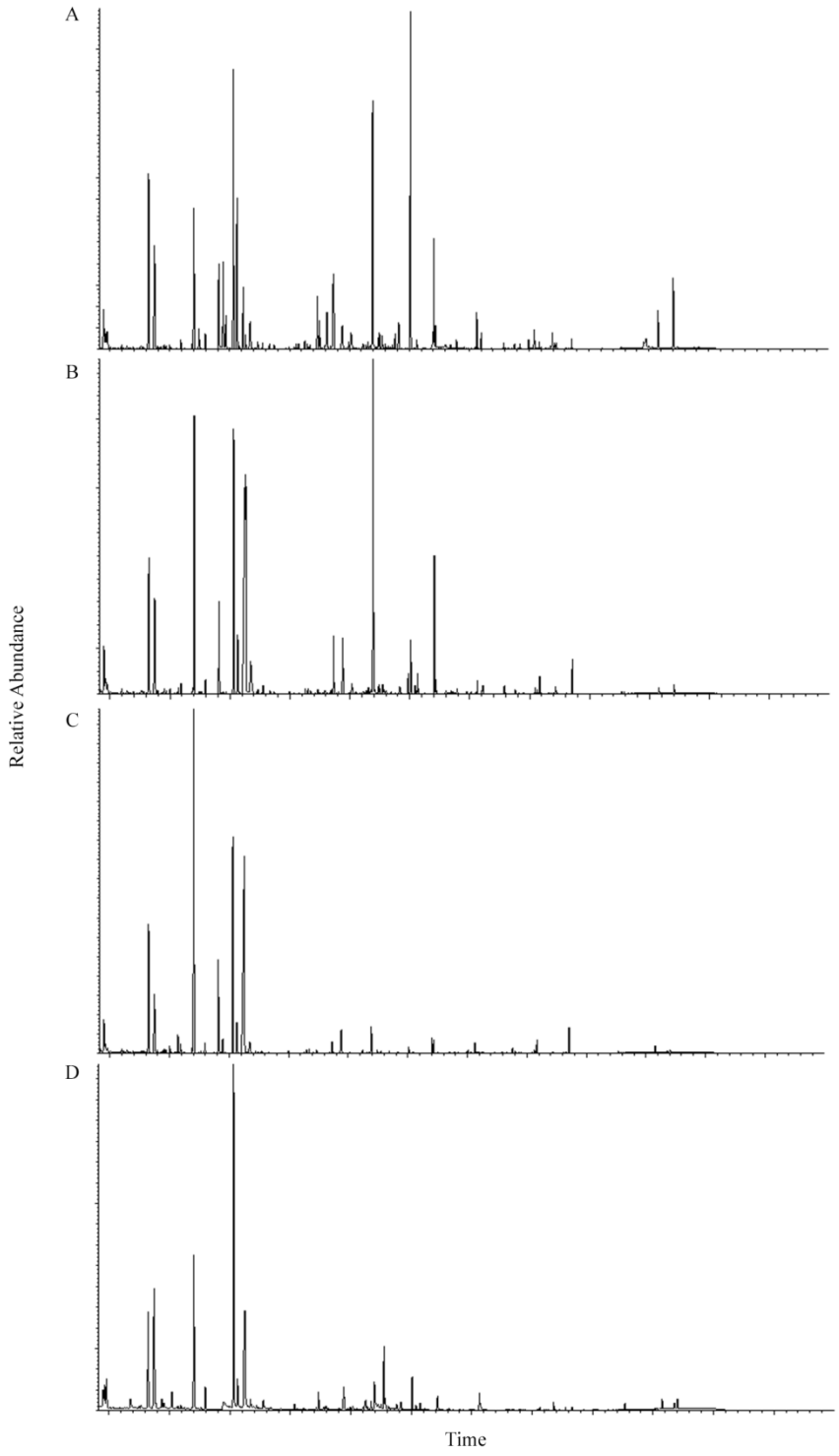

Figure 2.2: Total Ion Chromatograms of feline seminal fluid. (A) = domestic cat epididymal; (B) = ejaculated domestic cat; (C) = ejaculated cheetah; (D) = ejaculated clouded leopard.

A PCA of ejaculated sperm samples illustrates the variation seen between samples and species (Figure 2.3). Briefly, PC analysis is used to define the variation of a group of samples into two or three linearly uncorrelated variables. Each of these variables is called a "principal component (PC)," and sequentially accounts for as much variation within the data set as possible (i.e. PC1 accounts for more variation than PC2, and, subsequently, PC3). In comparing domestic cat, cheetah, and clouded leopard (A and B), sperm from cheetah and domestic cat appeared to group together, but clouded leopard sperm formed a distinctly separate cluster (with the exception of a single individual). The large degree of sample variation that occurs along the x-axis reveals that PC1 explains a majority of the variation seen from sample to sample. Samples were then reassigned in order to compare normospermic and teratospermic individuals, which revealed that the effect of teratospermia on sperm metabolome could be a source of variation due to the shift in the 3D PCA visualization when cell status became a factor (Figure 2.3 C and D). However, normospermic and teratospermic samples did not tend to cluster together, which demonstrates that species-specific differences account for more overall variation than the incidence of teratospermia. To describe the PCs that define this variation further, the results of the PCA can be correlated with specific compounds or phenotypes through a factor analysis. In this study, compounds that were differentially detected between species were analyzed using a one-way analysis of variance (ANOVA) and the Bonferroni-Holm multiple comparisons correction. Entities were significant when $P < 0.05$. Significant metabolites are represented in Table 2.5.

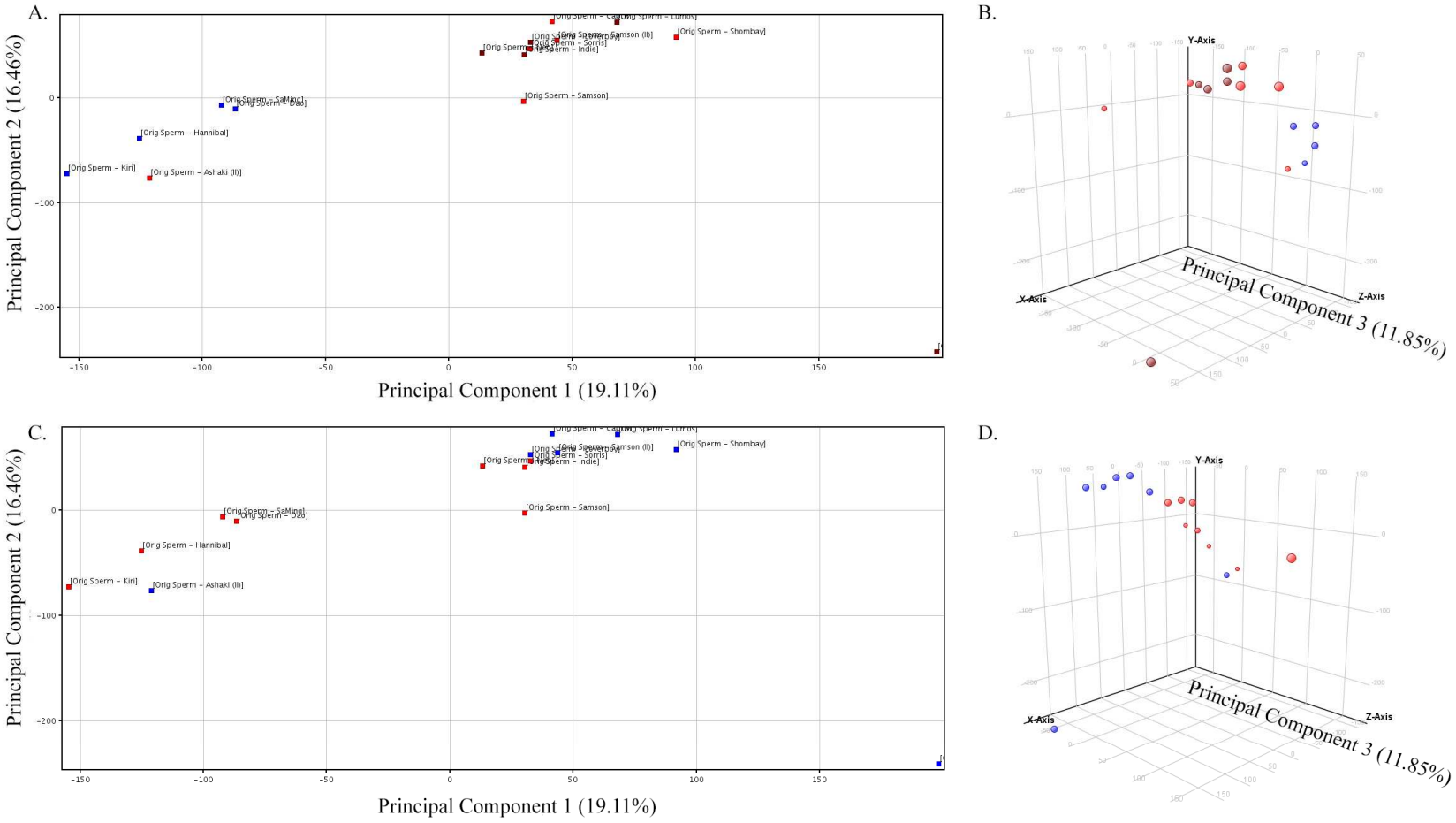

Figure 2.3: Principal component (PC) analysis of the felid spermatozoal metabolome. (A) 2-Dimensional and (B) 3-Dimensional PC analysis of sperm from domestic cats (brown), cheetahs (red), and clouded leopards (blue). Samples are re-grouped according to sperm status (normospermic = red; teratospermic = blue) in (C) and (D). PCs 1 (x-axis, 19.11%), 2 (y-axis, 16.46%), and 3 (z-axis, 11.85%) account for 47.42% of the total variation betwen samples.

Table 2.5: Differentially detected metabolites found in sperm between three different felid species. Grey indicates lower relative abundance compared to the second species listed, white indicates higher relative abundance; Compound = [Fiehn Chemical Library Reference Number] Compound Name; DC = Domestic cat, Ch = Cheetah, CL = Clouded leopard; compounds were significant when $P < 0.05$.

Compound	Corrected p-value	Differential Detection			Order of Species	Description
		Ch vs. CL	Ch vs. DC	CL vs. DC		
[10820] 4-isopropylbenzoic acid	0.0021	up	down	down	DC > Ch > CL	Hippuric Acid
[568] dibenzofuran	<0.0001	up	down	down	DC > Ch > CL	Xenobiotic metabolism
[58] 2-ketobutyric acid	0.0260	up	down	down	DC > Ch > CL	Amino Acid Metabolism
[601] D-Ala-D-Ala2	<0.0001	down	up	up	CL > Ch > DC	Amino Acid Metabolism
[811] itaconic acid	<0.0001	down	up	up	CL > Ch > DC	Carbohydrate Metabolism

Seminal fluid was also compared across species and sperm status using a PCA. Similar to the sperm results, samples of cheetah and domestic cat seminal fluid tended to group together, but clouded leopard fluid formed a distinctly separate and tighter cluster (Figure 2.4; A and B). Phenotypic assignment of sperm status (normospermic and teratospermic) showed a clustering of teratospermic samples, while normospermic samples had a higher degree of variation (Figure 2.4; C and D). Further analysis is necessary to determine whether the metabolite or group of metabolites responsible for this separation can be defined. Differentially detected compounds were identified using a one-way ANOVA and the Bonferroni-Holm multiple comparisons correction, and are represented in Table 2.6.

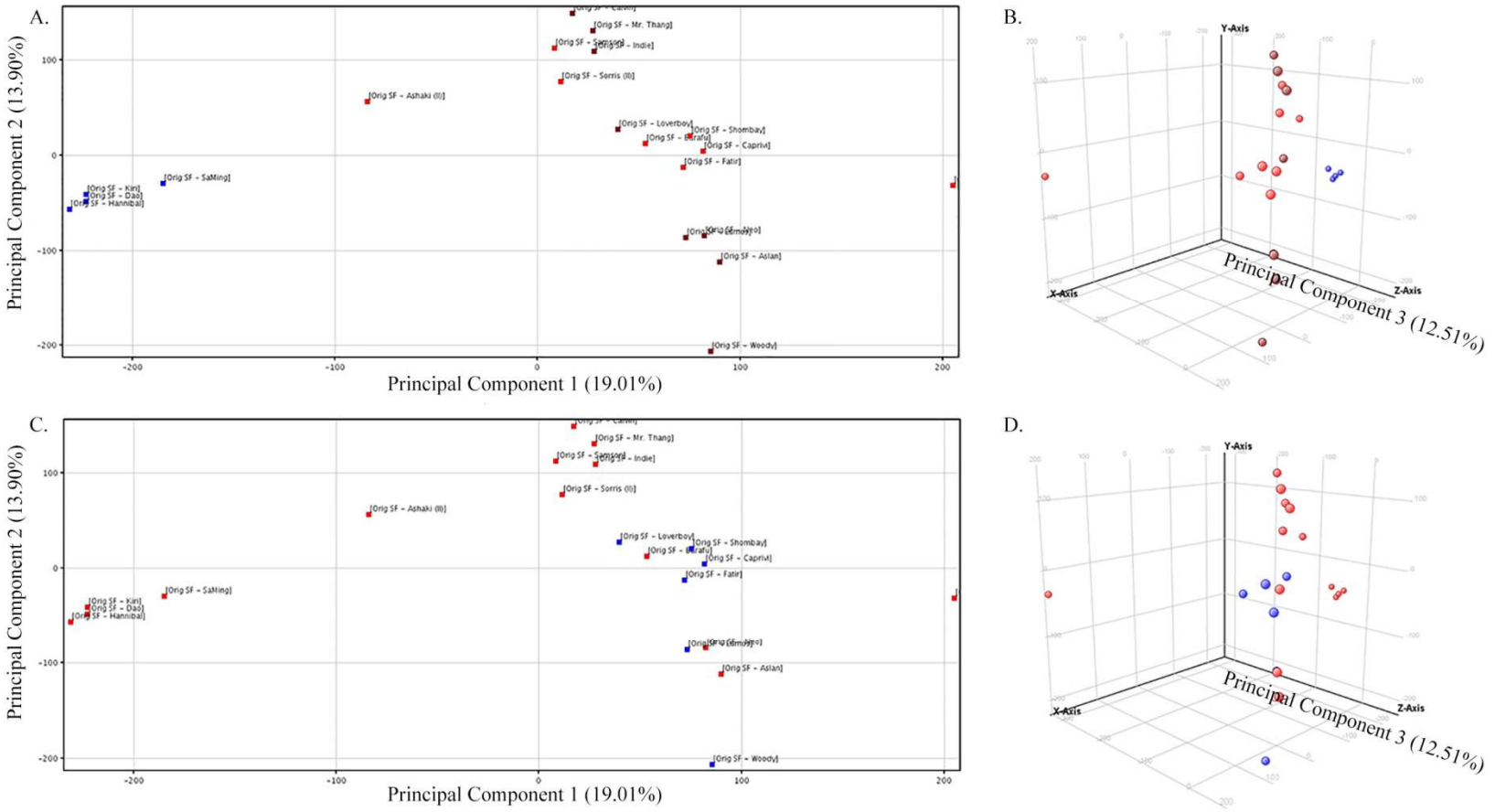

Figure 2.4: Principal component (PC) analysis of the felid seminal fluid metabolome. (A) 2-Dimensional and (B) 3-Dimensional PC analysis of seminal fluid from domestic cats (brown), cheetahs (red), and clouded leopards (blue). Samples were re-grouped according to sperm status (normospermic = red; teratospermic = blue) in (C) and (D). PCs 1 (x-axis, 19.01%), 2 (y-axis, 13.90%), and 3 (z-axis, 12.51%) acount for 45.42% of the variation seen between samples.

Table 2.6: **Differentially detected metabolites found in seminal fluid between three different felid species**. Grey indicates lower relative abundance compared to the second species listed, white indicates higher relative abundance; Compound = [Fiehn Chemical Library Reference Number] Compound Name; DC = Domestic Cat, Ch = Cheetah, CL = Clouded Leopard; compounds were significant when $P < 0.05$.

Compound	Corrected p-value	Differential Detection			Order of Species	Significance
		Ch vs. CL	Ch vs. DC	CL vs. DC		
[1060] pyruvic acid	0.0121	down	up	up	CL > Ch > DC	Energy metabolism
[239] Beta- alanine	<0.0001	down	up	up	CL > Ch > DC	Amino acid metabolism
[243] benzoic acid	<0.0001	down	down	up	CL > DC > Ch	Urea cycle
[311] citric acid	0.0314	down	down	up	CL > DC > Ch	Energy metabolism
[441035] talose	0.0242	down	down	up	CL > DC > Ch	Carbohydrate metabolism
[601] D-Ala-D-Ala2	<0.0001	down	up	up	CL > Ch > DC	Amino acid metabolism
[785] hydroquinone	<0.0001	down	down	up	CL > DC > Ch	Xenobiotic metabolism
[867] malonic acid	0.0253	down	down	up	CL > DC > Ch	Energy metabolism
[8871] 2-hydroxypyridine	<0.0001	down	up	up	CL > Ch > DC	Nucleotide metabolism
[892] allo-inositol	<0.0001	down	down	up	CL > DC > Ch	Energy metabolism

Metabolic Comparison: Sperm Status

In addition to assessing the metabolite differences between species, the compounds found in normospermic and teratospermic individuals was compared. Individuals were characterized as "teratospermic" if abnormal sperm morphology exceeded 60% (Table 2.7), based on the gross morphological measurements taken by CASA, but not on acrosomal integrity [5]. Epididymal samples were not categorized as teratospermic or normospermic due to the potential for immature sperm to be present[44]. Rather, they remained a separate category and were compared alongside normospermic and teratospermic individuals. Differentially detected compounds were identified using a one-way ANOVA and the Tukey HSD pairwise comparison correction under a 95% confidence interval. These metabolites are represented in Tables 2.8 and 2.9.

Table 2.7: Sperm motility and morphology assessed with CASA. Values expressed as mean ± S.D.; EEJ = electroejaculated; FPS = forward progressive status.

Species	Sperm (n)	Fluid (n)	Motility		Morphology				
			% Motile	FPS	% Bent Tail	% Coiled Tail	% Proximal Droplet	% Distal Droplet	Total % Abnormal
Domestic Cat (Epididymal)	6	5	58.33 ± 14.91	3.0 ± 0.9	--	--	--	--	--
Domestic Cat (EEJ) – Normospermic	2	4	78.60 ± 1.20	2.75 ± 0.8	4.35 ± 0.85	0.10 ± 0.00	1.20 ± 0.00	0.10 ± 0.00	5.70 ± 1.40
Domestic Cat (EEJ) – Teratospermic	3	3	53.40 ± 14.60	4.1 ± 0.3	49.27 ± 14.90	4.83 ± 4.57	9.33 ± 9.82	1.25 ± 0.45	64.27 ± 28.46
Cheetah (EEJ)	6	8	59.86 ± 10.41	3.4 ± 0.9	26.30 ± 24.60	0.37 ± 0.52	17.60 ± 12.40	1.90 ± 2.62	41.20 ± 36.72
Clouded Leopard (EEJ)	4	4	65.83 ± 15.18	3.6 ± 0.4	34.63 ± 10.34	2.73 ± 2.62	5.70 ± 1.42	1.00 ± 0.37	44.05 ± 11.92

Table 2.8: Sperm metabolites differentially detected between sperm status. Grey = compound has a lower relative abundance in the first phenotype compared to the second phenotype listed; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds were significant when $P < 0.05$.

Compound	p-value	Differential Detection			Order of Phenotype	Significance
		Epididymal vs Normospermic	Epididymal vs Teratospermic	Normospermic vs Teratospermic		
[1004] phosphoric acid	0.0039	down	up	up	N > E > T	Cellular Signaling
[107689] L-(+) lactic acid	<0.0001	down	down	up	N > T > E	Energy Metabolism
[107812] hypotaurine	<0.0001	up	up	down	E > T > N	Amino Acid Metabolism
[304] cholesterol	0.0065	up	up	up	E > N > T	Membrane Stability
[439240] D-lyxose	<0.0001	up	up	down	E > T > N	Carbohydrate Metabolism
[5280450] linoleic acid	<0.0001	up	up	down	E > T > N	Lipid Metabolism
[5281] stearic acid	<0.0001	up	up	up	E > N > T	Lipid Metabolism
[65065] N-acetyl-L-aspartic acid	0.0037	up	up	up	E > N > T	Amino Acid Metabolism
[750] glycine	<0.0001	up	up	up	E > N > T	Amino Acid Metabolism
[892] allo-inositol	0.0097	up	up	up	E > N > T	Carbohydrate Metabolism
[971] oxalic acid	0.0075	up	up	down	E > T > N	Energy Metabolism
[985] palmitic acid	0.0023	down	up	up	N > E > T	Lipid Metabolism

Table 2.9: Fluid metabolites differentially detected between sperm status. Grey = compound has a lower relative abundance in the first phenotype compared to the second phenotype listed; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds were significant when $P < 0.05$.

Compound	Corrected p-value	Regulation		
		Epididymal vs. Normospermic	Epididymal vs. Teratospermic	Normospermic vs. Teratospermic
[1000] 2-amino-1-phenylethanol	<0.0001	up	up	up
[107812] hypotaurine	<0.0001	up	up	down
[156807] D-lyxosylamine	<0.0001	up	up	down
[16219560] lactobionic acid	0.0284	up	up	down
[5280450] linoleic acid	<0.0001	up	up	up
[750] glycine	<0.0001	up	up	up
[8897] iminodiacetic acid	0.0283	up	up	down
[892] allo-inositol	<0.0001	up	up	up
[94214] methyl-beta-D-galactopyranoside	<0.0001	up	up	up

Discussion

This was the first metabolomic study of sperm conducted in felid species using GC-MS: Two important discoveries were made. First, significant differences exist between both the sperm metabolomic profiles and the seminal fluid metabolomic profiles of domestic cats, cheetahs, and clouded leopards. This supports previous reports as well as our hypothesis that metabolism in sperm is species-specific, and indicates that this is true even between different species within the same taxonomic family [124,125]. Second, this study demonstrates that significant differences exist between the metabolomic profiles of sperm and seminal fluid collected from normospermic vs. teratospermic individuals. While it has been reported that teratospermia causes spermatozoal metabolic dysfunction, the specific differences in metabolite composition between teratospermic and normospermic individuals has not been characterized in felids until now. Metabolites found in sperm across all species indicate potential roles for metabolic pathways including the TCA cycle, glycolysis, oxidative phosphorylation, and lipid metabolism. Such pathways have also been identified as important pathways in other species (Table 2.10).

Table 2.10: Important metabolic pathways reported to be critical for the function of mammalian spermatozoa

Pathway	Species (common name)	Reference(s)
TCA Cycle	All	--
Glycolysis	Boar, Mouse, Human	[76], [126], [73]
Oxidative Phosphorylation	Cat, Bull, Mouse, Human	[127], [47]
Lipid Metabolism (β-oxidation)	Human, Boar	[66], [128]
Gluconeogenesis	Dog	[129], [75]

When the sperm metabolome from all three species were compared, five compounds were differentially detected (Table 2.5). Three of these (2-ketobutyric acid, alanine, and itaconic acid) are known to be involved in energy metabolism. It was not surprising to find hippuric acid in these samples, as a common problem with samples obtained via electroejaculation is urine contamination [130]. While this compound was differentially detected between species, it is unlikely that this is a result of inherent metabolic differences, but is more likely present as a response to electroejaculation.

The presence of 2-ketobutyric acid, or α-ketoburyrate, is a result of threonine catabolism. This reaction is catalyzed by serine dehydratase, which is the same enzyme that catalyzes serine to pyruvate. When it is degraded, α-ketoburyrate can be used to synthesize isoleucine, or it can be converted to propionyl CoA, a precursor of succinyl CoA, which is a TCA cycle intermediate [61]. The levels of α-ketoburyrate could be representative of TCA cycle activity, and in this case may be indicative of differences in metabolic rate between species [131]. Our evidence indicates that levels of 2-ketobutyric acid are highest in the domestic cat ($P < 0.05$) and lowest in clouded leopards ($P < 0.05$).

Alanine is an amino acid derived from pyruvate, which exists in two distinct pools within a cell: the cytosolic pool and the mitochondrial pool. Glycolysis takes place in the cytosol and yields pyruvate which can be reduced to lactate by lactate dehydrogenase. However in most cells, much of the pyruvate that is produced is transported into the mitochondria, is converted to Acetyl-CoA, and enters the TCA cycle. Glutamate dehydrogenase converts 2-oxoglutarate – a TCA cycle intermediate

– into glutamate, which is exported out of the mitochondria and converted by alanine aminotransferase into alanine [132]. Thus, in order to distinguish between the two pools of pyruvate when determining relative abundance, cytosolic pyruvate can be estimated by measuring lactate while the relative abundance of alanine reflects only the mitochondrial pool. Terrell et al. established that pyruvate uptake is an indicator of sperm quality in felids [58]. The relative concentration of alanine, reflecting the mitochondrial pyruvate pool, was higher in clouded leopard sperm than in cheetah sperm, and lowest in sperm from the domestic cat. Interestingly, clouded leopards also had a higher percentage of motile sperm (65.83% $\pm$ 15.18) than cheetah and teratospermic domestic cats. Alanine is also a key element utilized by the liver to conduct gluconeogenesis – a process which has been shown to be active in dog spermatozoa [129] although further studies are needed to define it utilization in felids.

Itaconic acid (itaconitate) is derived from the TCA cycle intermediate citric acid, and like alanine, could be used to indicate mitochondrial function. The work of two enzymes is responsible for the production of itaconic acid from citric acid: Aconitase converts citric acid to *cis*-aconitic acid, which reacts with *cis*-aconitic acid decarboxylase to release CO_2 and form itaconitate [133]. Similar to alanine, the relative abundance of itaconic acid was higher in clouded leopard sperm than in cheetah sperm, and lowest in sperm from the domestic cat. If itaconic acid does represent mitochondrial function, this finding could indicate that the mitochondria of clouded leopard sperm are more active than the mitochondria in cheetah sperm and domestic cat sperm. Given that domestic cat sperm are generally assumed to be of better quality than both cheetah and clouded leopard sperm, the accumulation of TCA

intermediates may indicate inherent dysfunction or rate limiting conditions in clouded leopard and cheetah sperm. Further investigation is needed to support this claim.

In felines, the accessory glands responsible for the composition of seminal fluid include the epididymis, prostate gland, and bulbourethral gland. Seminal fluid is known to contain substrates beneficial to sperm function (reviewed in [46]). Therefore, it is not surprising that the metabolites identified in felid seminal fluid play critical roles in a variety of metabolic pathways. Similar to our findings in the sperm, some metabolites in the seminal fluid were differentially detected between species. These include pyruvate, citrate, allo-inositol, and malonic acid (energy metabolism); alanine and β-alanine (amino acid metabolism); talose (carbohydrate metabolism); 2-hydroxypyridine (nucleotide metabolism); and hydroquinone (xenbiotic metabolism).

As previously discussed, pyruvate uptake has been identified as an indicator of sperm quality in felids [58]; therefore it might not be surprising that pyruvate was differentially detected across species in seminal fluid. However, despite pyruvate stimulating motility only in the domestic cat in our studies, pyruvate was present in the highest relative abundance in the seminal fluid of clouded leopard, followed by cheetah and then by domestic cat ($P < 0.05$). This could indicate that differences exist with regards to how efficiently sperm are able to obtain the resources they need. Perhaps, the resources provided in the female reproductive tract differ or different pyruvate requirements exist for each of the three species studied. The present study does not indicate which of these options is the more likely explanation, but prompts further questions about the source and availability of substrates to sperm throughout their journey toward fertilization.

Malonic acid (or, malonate) is an interesting compound because of the role that it plays in energy metabolism. Malonic acid is a competitive inhibitor of succinate dehydrogenase – a key enzyme in the citric acid cycle, and the second enzymatic complex in the electron transport chain. When malonate binds to this complex, it causes an accumulation of succinate, α-ketoglutarate, and citrate to accumulate [61]. Our results demonstrate – with similarity to the relative abundances of citrate – that malonate is at its highest concentration in the sperm of clouded leopards, followed by domestic cats, and is lowest in cheetah spermatozoa ($P < 0.05$). Further investigations may help to explain the intriguing differences in relative abundance of these key metabolites.

Allo-inositol, which was also differentially detected in seminal fluid, is a stereoisomer of myo-inositol, which is the structural basis of important secondary messengers in a wide variety of eukaryotic cellular pathways, some of which are known to occur in spermatozoa [134–136]. It does not appear to play a role in cellular respiration, nor does it appear to be correlated with concentrations of fructose in human semen [137]. However, it is known to be a crucial regulator of cell growth, lipid synthesis, cell membrane formation, and signal transduction [138]. Ironically, many of these functions do not seem to be relevant to sperm cells. Once differentiated from spermatogonial stem cells, spermatozoa do not increase in size. In fact, as sperm continue to differentiate toward their final state as a spermatid, cellular organelles and excess material are sloughed off during the meiosis and differentiation phases of spermatogenesis [139]. This results in a significant loss of cytoplasm and a

subsequent reduction in size [32]. It therefore seems unlikely that the role of allo-inositol in spermatozoa is related to the regulation of cell growth.

The role of inositol in cell membrane formation is an interesting one, especially in the context of spermatozoa. Sperm cellular membranes go through many changes as they transition from development to fertilization. However, none of these changes appear to involve the regeneration of the cellular membrane. Rather, in processes like the acrosome reaction, the outer cellular membrane is broken down to release enzymes which aid in the sperm's progress toward reaching the oocyte. Therefore, while it is possible that inositol is playing a role in membrane formation in immature spermatozoa, inositol likely does not contribute to membrane formation in mature spermatozoa. Another possibility for the role of inositol in feline seminal plasma could be to influence the epithelium within the female reproductive tract, and possibly affect the processes necessary for fertilization.

It was observed by Terrell et al. (2010) that while felid sperm do not uptake glucose directly, glycolysis is a necessary pathway for proper function [112] and suggested that other substrates are required to fuel the process. Talose, an epimer of galactose, was detected in felid seminal fluids. Galactose can be converted to glucose via the Leloir Pathway [140]. To date, this pathway has not been investigated relating to reproduction and fertilization, but perhaps this could serve as an alternate glycolytic source of energy. Talose was detected at its highest levels in clouded leopard seminal fluid, to a lesser degree in the domestic cat, and at its lowest in the cheetah ($P < 0.05$). It did not appear to be differentially detected between normo- and

teratospermic individuals. Further studies will be necessary to determine whether this substrate is able to be taken up and metabolized by felid sperm.

Teratospermic individuals exhibit reduced fertility following assisted reproduction, and ejaculated sperm are known to exhibit impaired metabolism [5,22,59,119]. Therefore, in addition to investigating species-specific differences in metabolite concentration, we also investigated the differential detection of compounds between normo- and teratospermic individuals which could potentially lead to insight into the metabolic pathways that may be involved in teratospermic dysfunction. Comparison of seminal fluids could indicate whether inherent glandular dysfunctions contribute to the poor fertility of teratospermic individuals.

Lactic acid was higher in sperm from normospermic individuals than teratospermic individuals ($P < 0.05$). This finding is in agreement with previously published data which implies lactic acid production as an indicator of sperm quality [58]. Allo-inositol was also detected at significantly higher relative abundance in sperm from normospermic individuals compared to sperm from teratospermic individuals ($P < 0.05$).

There were three types of LCFAs that were differentially detected between sperm status: linoleic (18:2 cis, cis-9,12), stearic (C18:0), and palmitic (C16:0) acids. Palmitic and stearic acids are two of the most common fatty acids that exist in nature, and all three of these fatty acids are able to contribute to β-oxidation by way of the carnitine:acylcarnitine shuttle.

Linoleic acid is an essential fatty acid, which means that it cannot be synthesized by vertebrates and therefore must be consumed through the diet. This

fatty acid has been identified as a component of phospholipids in the plasma membranes of human [84], bull [86], ram [141], and mouse sperm [142]. In rams, it has been shown that a diet supplemented with n-3 fatty acids was able to mitigate the natural effects of seasonal declines in semen quality [141]. Additionally, in a comparative study between normo- and asthenozoospermic men, there was a significant positive correlation between content of linoleic acid and sperm motility grade [84]. It is possible that relative abundance of these fatty acids could also correlate with quality of felid sperm. The relative concentrations of stearic and palmitic acids were significantly higher in sperm from normospermic individuals than in teratospermic individuals ($P < 0.05$), but the trend for linoleic acid was reversed: sperm from normospermic individuals had significantly lower relative concentration of linoleic acid than sperm from teratospermic individuals ($P < 0.05$). This is puzzling, because sperm from normospermic domestic cats tended to have higher motility than sperm from teratospermic domestic cats (Table 2.7), which means that these findings in felids contradict what was seen in rams and humans. One possible interpretation of these results is that β-oxidation is active, yet impaired, in sperm from teratospermic felids as compared to normospermic felids. β-oxidation is the process by which fatty acids are broken down to produce energy. If this pathway is active in a given cell type, the relative amount of LCFAs would be less than in a cell type with an impaired or nonexistant β-oxidation metabolic pathway. The heightened presence of LCFAs in sperm from teratospermic individuals (as compared to sperm from normospermic individuals) could therefore indicate that β-oxidative pathways are

impaired in felids afflicted with teratospermia. Further investigation is needed to validate this claim.

Cholesterol is a critical component in the maintenance of cell membrane integrity, and plays a key role in capacitation. It was detected in epididymal sperm at a significantly higher relative abundance than ejaculated sperm from normospermic individuals ($P < 0.05$), which in turn was also at a significantly higher abundance than ejaculated sperm from teratospermic individuals ($P < 0.05$). This could indicate the relative stability of the cell membrane of each sperm type: in the epididymis, sperm are still not ready to fertilize an oocyte, and may not have even finished maturing yet. Therefore, the cell membranes are still intact and potentially stronger than older, more mature sperm populations. Ejaculated sperm have come out of their quiescent state in the epididymis, and are preparing to undergo the processes necessary for fertilization. As more metabolically active cells, sperm could be utilizing internal or membrane-bound stores of energy substrates, resulting in a "leakier" cell membrane. Sperm from teratospermic individuals, however, are known to be especially susceptible to membrane damage following cryopreservation. It is possible that a debilitating loss or lack of cholesterol could be responsible for weaker cell membranes, and thus a more highly cryo-sensitive population of sperm. Alternatively, it could be possible that sperm from teratospermic individuals capacitate more quickly and/or prematurely.

Interestingly, phosphoric acid showed differential detection amongst the three groups. While relevant to many cellular processes that occur in most cell types, phosphoric acid also makes up the energy-rich component of the cellular energy currency, ATP. While the assumption that phosphoric acid detected in this study is

correlated to the relative amount of energy being used by the sperm sample is appealing, further studies are required to determine if this is the case.

Conclusions

Recent advances in GC-MS methods and technology have allowed for the analysis and detection of compounds in "ultra-small" samples [143]. Our study demonstrates that metabolites can be detected on a species-specific and phenotypic basis, all while utilizing very small sample volumes (30 μL). We were able to identify compounds which were differentially detected between three different felid species (domestic cat, cheetah, and clouded leopard), and across three distinct sperm cell phenotypes (epididymal, normospermic, and teratospermic). We identified compounds which confirm previous reports of spermatozoal metabolism, as well as compounds which point toward promising new areas for future research. Such research could include investigating the potential role of inositol and other differentially detected compounds within spermatozoa prior to fertilization, and the potential role for lipid metabolism in fueling sperm function.

The metabolome characterization reported in this study provides a deeper understanding of the processes that may be important for spermatozoal function and survival. This information could be used to aid in improving assisted reproduction of captive felids through the species-specific tailoring of media to accommodate the specific needs of the spermatozoa in question [10,105,106]. The information revealed in this study provides a critical step in defining the metabolites that are present in spermatozoa, and the metabolic pathways that could be active in these cells.

Chapter 3: Long-Chain Fatty-Acid Metabolism and Sperm Function

Introduction

Mammalian spermatozoa are dependent on the efficient generation and utilization of ATP to maintain progressive motility, transition into hyperactive motility, and undergo capacitation and fertilization [1,47,68,73]. Glycolysis and oxidative phosphorylation are known to contribute to sperm motility and viability in many domestic species, but the relative importance of each pathway is largely species-specific (reviewed in [47]). In somatic cells, energy is produced via the breakdown of substrates from three main sources: carbohydrates, amino acids, and lipids. Carbohydrates, or hexoses, enter glycolysis in the cytosol to produce pyruvate, reducing agents, and a small amount of ATP. Pyruvate is able to enter the mitochondria, undergo the conversion to Acetyl-CoA via pyruvate dehydrogenase, and fuel the citric acid cycle (TCA cycle). Amino acids are able to enter into the TCA cycle at various points and through a number of tangential pathways. Lipids and fatty acids can be broken down to produce Acetyl-CoA which enters the TCA cycle. All three of these pathways are present and well characterized in somatic cells and tissues, but not as much is known about these pathways in germ cells.

Sperm metabolism has been studied for decades, but mostly in domesticated species and humans. The most common assumption made about metabolism is that energy must come from glucose. However, there is considerable evidence that other substrates can contribute to energy production as well. For example, Jones and

Connor were able to demonstrate that boar sperm could metabolize fructose [144]. Boar sperm were also shown to have active lipid metabolism and synthesis [145]. Human sperm, however, are almost entirely dependent on glucose-derived ATP in order to undergo capacitation [69,71,75], while in the bull phospholipid catabolism and oxidative phosphorylation are crucial for proper sperm function [86,108]. Energy metabolism in mouse spermatozoa has also been investigated, and like human sperm, it depends heavily on glycolysis for ATP production [76,126]. These examples only confirm the notion that sperm metabolism is highly species-specific, and that more than one energy-producing pathway could be responsible for sustaining sperm function.

The purpose of this study was to determine whether lipid metabolism is active in felid spermatozoa, and if so, to what extent. Recent studies by Terrell et al. demonstrated that while glycolysis is necessary for sperm function, sperm uptake of glucose was limited [58]. One possible explanation for this phenomenon is the utilization of phospholipids as energy sources, as shown and implicated in human sperm [66]. When a phospholipid is cleaved apart to form glycerol and free fatty acids, the glycerol molecule that is released could serve as the substrate that contributes to glycolysis while the fatty acids are oxidized independently. Additionally, lipid metabolism is able to produce more than six times the number of ATP molecules than what is typically produced by an equal volume of glycogen [60]. For example, the oxidation of a single glucose molecule via glycolysis yields 32 ATP molecules. However, when a fatty acid such as stearic acid is completely oxidized, a total of 112 ATP molecules are produced. Due to the massive difference between the

energy produced in these two pathways, lipid metabolism should be preferable to spermatozoa – a cell type that has a high ATP requirement.

Of the 37 species that make up the *Felidae* family, 70% are listed as threatened or endangered, and 30% of the remaining species have experienced a trend of population decline throughout the last decade [9]. Many of these species are part of intense captive management programs in which the basic biology of these animals can be studied with the hope of one day being able to improve their endangered status. Of the various techniques that are used to help wildlife, assisted reproduction is one of the most effective ways to boost captive populations as a hedge against extinction. Assisted reproductive technologies such artificial insemination, embryo transfer, and in vitro embryo production have been developed over the course of the last century. Having been perfected in many domestic species, they are becoming increasingly prominent in wildlife conservation efforts [24].

The most common ART used in wild animals is cryopreservation, or the deep freezing of gametic or embryonic material. Not only is cryopreservation an ART on its own, but it often is required in conjunction with other techniques. Cryopreservation provides four main advantages for conservation efforts: 1) it facilitates transport of gametic material across large distances without the hassle and stress of having to move the entire animal, 2) it reduces the risk of disease transfer among facilities, 3) it promotes increased genetic diversity by allowing genetically valuable individuals to contribute to the captive gene pool, and most importantly 4) it serves as an accessible means of gamete storage for rare or underrepresented

individuals [25,29]. Thus, cryopreservation has become a staple for assisted reproduction, the study of spermatozoa, and global conservation efforts.

Unfortunately, a remarkably high proportion (90%) of feline species exhibit teratospermia, which is characterized by 60% or more of the sperm exhibiting abnormal morphologies [10]. Individuals characterized as being teratospermic typically demonstrate low fecundity, and some are classified as infertile due to low sperm concentration and diminished overall morphology [10]. In a variety of wild felids including the cheetah, lion, leopard cat, and Florida panther, teratospermia has been linked to a loss of genetic diversity resulting from population bottleneck events [10–13,48]. Inbreeding experiments in the domestic cat confirmed these assumptions when more than 85% of sperm in the ejaculates were malformed from progeny after even a single generation of incestuous breeding [10]. Inbred tomcats exhibiting high proportions of pleiomorphic sperm were shown to produce a higher concentration of sperm cells, as well as an increase in testicular volume. This may imply that compensatory mechanisms could be present in historically teratospermic species to maintain fertility under normal breeding conditions despite impaired spermatogenesis [10,49]; however, conditions during ART may expose impaired functions. Critically, sperm from teratospermic individuals has been shown to be especially sensitive to cryopreservation [22,23,29]. For all of these reasons, it is important that the basic knowledge of physiological processes are further understood. By further understanding sperm metabolism, efforts can be made to improve the compositions of media used for the collection and handling of sperm.

To test whether felid sperm utilize long chain fatty acids (LCFAs) as a means of energy production, we assessed the effect of etomoxir, a β-oxidation inhibitor, on epididymal and ejaculated spermatozoa in the presence and absence of pyruvate. Etomoxir irreversibly binds to carnitine:palmitoyltransferase I, the enzyme responsible for LCFA transport into mitochondria. It has been utilized in a variety of studies to test the importance of LCFA β-oxidation, including bovine embryos [65] and human sperm cells [66]. By incubating sperm with etomoxir, LCFAs are blocked from entering the mitochondria to undergo β-oxidation. If β-oxidation of LCFAs is critical for sperm motility, the use of etomoxir will result in a significant decrease in sperm motility compared to the control. This was done in three different felid species: the domestic cat (*Felis catus*), cheetah (*Acinonyx jubatus*), and clouded leopard (*Neofelis nebulosa*). We hypothesized that treatment with etomoxir would result in a dose-dependent decrease in sperm motility; moreover, we expect that pyruvate should stimulate sperm motility as it is a known energy source for domestic cat and cheetah sperm[58]. If LCFAs contribute significantly to energy production, then blocking LCFA in the absence of pyruvate will result in a greater decrease of motility. Additionally, as sperm metabolism and motility were assessed over 4.5 hours of incubation, this study will allow a deeper insight into the metabolism of felid sperm over time. Sperm and spent media were analyzed using gas chromatography mass spectrometry (GC-MS), a unique technology to study sperm metabolism in that it allowed us to measure the relative abundance of metabolites present within the sample. By doing this, we were able to not only monitor for indicators of β-oxidation, but also to also monitor other metabolites present in or secreted from sperm samples,

which could provide clues to alternate metabolic processes that may be present. Understanding whether this pathway is active in felid sperm could be an important step in developing a better understanding of the energy requirements, which could lead to improved media used in assisted reproductive technologies.

Materials and Methods

Media Preparation

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified. A chemically defined, protein-free, cat-modified mouse tubal fluid (cMTF) medium was used for all procedures [118]. The final cMTF medium (pH 7.45) contained 98.4 Mm NaCl (Acros, New Jersey, USA), 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 25 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Fisher Scientific, Rockford, IL), and 2% polyvinyl alcohol (PVA), and was sterilized through a 0.22 µm syringe filter prior to use [58]. Osmolality of the final working medium (300 – 340 mOsm) was within 10% of the physiological value of domestic cat semen (323 mOsm) [59], and determined using a vapor pressure osmometer (Wescor, Inc. Logan, UT). The cMTF medium was prepared from concentrated 1 M stock solutions in deionized H_2O. All stock solutions were kept at 4°C and discarded after 3 months, or if precipitates were present.

Epididymal Fluid and Sperm: Collection and Evaluation

Male reproductive tissue was obtained following routine castration procedures of normal (FIV negative, non-cryptorchidic), sexually mature (≥ 1 yr) feral cats at a local veterinary clinic. Tissue was maintained and transported at 4°C until processing. Sperm and epididymal fluid from the cauda epididymis of ≥2 males was physically extruded through the ductus deferens, collected, diluted, and pooled in cMTF at a ratio of approximately 1:10 (v/v) (n = 6). A 2.8µL sample was taken to assess percent motility (%MOT) and forward progressive status (FPS) for the purposes of calculating the sperm motility index (SMI = %MOT + (FPS x 20) ÷ 2)[119], and concentration was determined using a standard hemocytometer (Hausser Scientific, Horsham, PA). Epididymal fluid was separated via centrifugation at 27°C (8 minutes at 800 × *g*) and stored at -80°C until GC-MS processing. Sperm were resuspended in 100 µL, then aliquoted into treatments at ~3.0 x 10^6 sperm/mL. Due to the higher concentration of sperm and the pooled nature of epididymal samples, a total of 8 treatments were employed: (1) 0 µM Etomoxir, (2) 10 µM Etomoxir, (3) 100 µM Etomoxir, and (4) 1 mM Etomoxir, each repeated in combination with (100 µM) or without (0 µM) pyruvate. All treatments were incubated at 37˚C under mineral oil to prevent evaporation, and motility (%) and forward progressive status (FPS; scored 0 – 5; 0 = non-motile, 5 = strong forward progression) were assessed every 30 min for 4.5 hrs following extrusion. A sample of sperm suspension (100 µL) was taken at each time point for metabolomic assessment, centrifuged to separate the cellular material from the spent media (8 minutes; 800 × *g*), and then stored at -80˚C prior to GC-MS processing.

Semen Collection and Evaluation

A surgical plane of anesthesia was induced in domestic cats (n =7), cheetahs (n = 7), and clouded leopards (n = 4) according to protocols previously established [2,120]. All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee (ACUC) and the University of Maryland's Institutional ACUC. Methods for semen collection and evaluation were similar to those previously described [2,120,121]. Briefly, a rectal probe of 1 cm (domestic cat), 1.9 cm (cheetah), or 1.6 cm (clouded leopard) in diameter with three longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, OR) were used to deliver 90 stimuli (at a low voltage of 2 – 5 V) over a 30 min interval [121]. Ejaculates were collected in sterile, pre-warmed vials [29,107,121,122]. A 2.8 μL sample was taken to assess motility using CASA (v.1.3.1: Hamilton Thorne, Beverly, MA), and percent motility (%MOT) along with forward progressive status (FPS; scored 0 – 5; 0 = non-motile, 5 = strong forward progression) were also estimated for the purposes of calculating the sperm motility index (SMI = %MOT + (FPS x 20) ÷ 2) [119]. Individuals were classified as teratospermic if more than 60% of sperm were abnormal according to gross morphological assessment by the CASA system. However, our assessment of morphology did not account for acrosomal abnormalities, and therefore the teratospermic individuals described in this study were part of a more selective screening process than individuals in previous reports, resulting in fewer individuals being classified as teratospermic. Sperm concentration was established using a standard hemocytometer (Hausser Scientific). Following motility assessment, sperm and seminal fluid were separated via centrifugation at

27°C (8 minutes at 800 × *g*). Sperm were washed with cMTF and resuspended at ~3.0 x 10^6 sperm/mL into four treatment groups: 1) 0 μM etomoxir, 0 μM pyruvate; 2) 100 μM etomoxir, 0 μM pyruvate; 3) 0 μM etomoxir, 100 μM pyruvate; and 4) 100 μM etomoxir, 100 μM pyruvate. All treatments were incubated at 37˚C under mineral oil to prevent evaporation, and motility (%) and forward progressive status (FPS; scored 0 – 5; 0 = non-motile, 5 = strong forward progression) were assessed every 30 min for 4.5 hrs following extrusion. A sample of sperm suspension (100μL) was taken at each time point for metabolomic assessment and centrifuged to separate the cellular material from the spent media (8 minutes; 800 × *g*), then stored at -80˚C prior to GC-MS processing.

GC-MS Processing

Prior to being analyzed, samples were first extracted and derivatized based on previous protocols [94]. Each sample (30 μL) was spiked with 30 μL of 5 mM L-norleucine (TCI-GR, Tokyo, Japan) to serve as an internal standard, then incubated for 24 hours in extraction solution (methanol for epididymal and seminal fluid samples; a mixture of methanol, chloroform, and water (MeOH:$CHCl_2$:H_2O, 5:2:2 v/v) for sperm samples) at -80˚C. Following extraction samples were centrifuged and the supernatant was transferred to a new 2 mL Eppendorf microcentrifuge tube, and then dried completely using a RotoVap SpeedVac (Savant, Farmingdale, NY). Derivatization occurred after adding 30 μL of Methoxylamine (Acros, New Jersey, USA) in pyridine (30 mg/mL) (ThermoScientific, Rockford, IL) and 30 μL of BSTFA +1% TCMS (ThermoScientific) was added to form the methoxime-trimethylsilyl

derivative of the various metabolites. Samples were incubated on a rotor for 20 minutes at room temperature, microwaved for 2 minutes (200 W), and incubated on a rocker overnight. Following derivatization, samples were run through the GC-MS system (Agilent 6890 gas chromatography system, Palo Alto, CA; ZB50 Capillary Column). Samples were injected in one microliter aliquots (5:1 split mode) and separated on a mid-polarity capillary column. The initial oven temperature was set at 60˚C and held for 5 minutes, then heated at a rate of 5˚C per minute to 310˚C, then held for 5 minutes. Cooling took place at a rate of 50˚C per minute and held for 5 minutes for a total run time of 70 minutes. The mass spectrometer (Agilent 5973 inert EI/CI MSD system) was operated in electron impact mode, and ions were monitored in full scan mode (mass range 50 – 650 *m/z*). Agilent's GC-MSD ChemStation software was used to capture raw GC-MS data files, which were viewed and quantitated using Agilent's Data Analysis software. The Agilent-Fiehn Mass Spectral Library was used to identify entities based on their mass, retention time, and mass spectra. The compounds identified were cross-checked with the 2008 NIST Mass Spectral Library.

Statistical Analysis

Motility data were analyzed with statistical analysis software (SAS) version 9.3 (SAS Institute, Cary, NC). The interaction between the effects of time, treatment, exogenous pyruvate, and sperm status (normospermic vs. teratospermic) was assessed using an ANOVA analysis in the SAS MIXED model procedures, with motility as the response variable. Treatment and exogenous pyruvate were included as class variables, and time was assessed using repeated measures analysis. To analyze mass

spectral data, metabolite relative abundance was calculated using the Mass Hunter analysis software (Agilent Technologies). GC-MS output files were analyzed using Mass Profiler Professional software package (Agilent Technologies) for significance testing (specific tests are discussed with the resulting data) and principal component analysis (PCA). Entity abundances were considered significantly different across samples when $P < 0.05$.

Results

The Effects of LCFA Inhibition on Sperm Motility

The interaction between the effects of time and pyruvate was significant ($P < 0.0001$), and therefore these two explanatory variables cannot be discussed independently. Rather, the effect of pyruvate can only be discussed with relation to specific time points. Epididymal sperm were incubated in cMTF media at four concentrations of etomoxir for 4.5h in the presence (+) or absence (-) of exogenous pyruvate (100μM). Because epididymal sperm are not activated immediately upon collection, the SMI tended to increase in both groups during the first 30 minutes of incubation (Figure 3.1, A and B). Percent motility was significantly decreased after 2 hours in both the presence and absence of pyruvate ($P = 0.0079$ and $P = 0.0331$, respectively). Sperm motility in cMTF(-) continued to decline for the remainder of the experiment, but sperm incubated with exogenous pyruvate had a motility index which followed a biphasic pattern. After 3 hours, sperm incubated in cMTF(+) (72.12 $\pm$ 6.98 SMI units) had a significantly higher motility index than sperm incubated in cMTF(-) (46.33 $\pm$ 7.06 SMI units), and the same was true after 3.5 hours (70.63 $\pm$

6.98 vs. 36.73 $\pm$ 7.15 SMI units) and 4 hours (58.00 $\pm$ 6.98 vs. 27.29 $\pm$ 758 SMI units) of incubation (motility expressed as mean $\pm$ SEM) (Figure 3.1D). When the effect of etomoxir on SMI was assessed, sperm were significantly impaired in cMTF supplemented with 1mM etomoxir compared to the control ($P < 0.0477$) (Figure 3.1C).

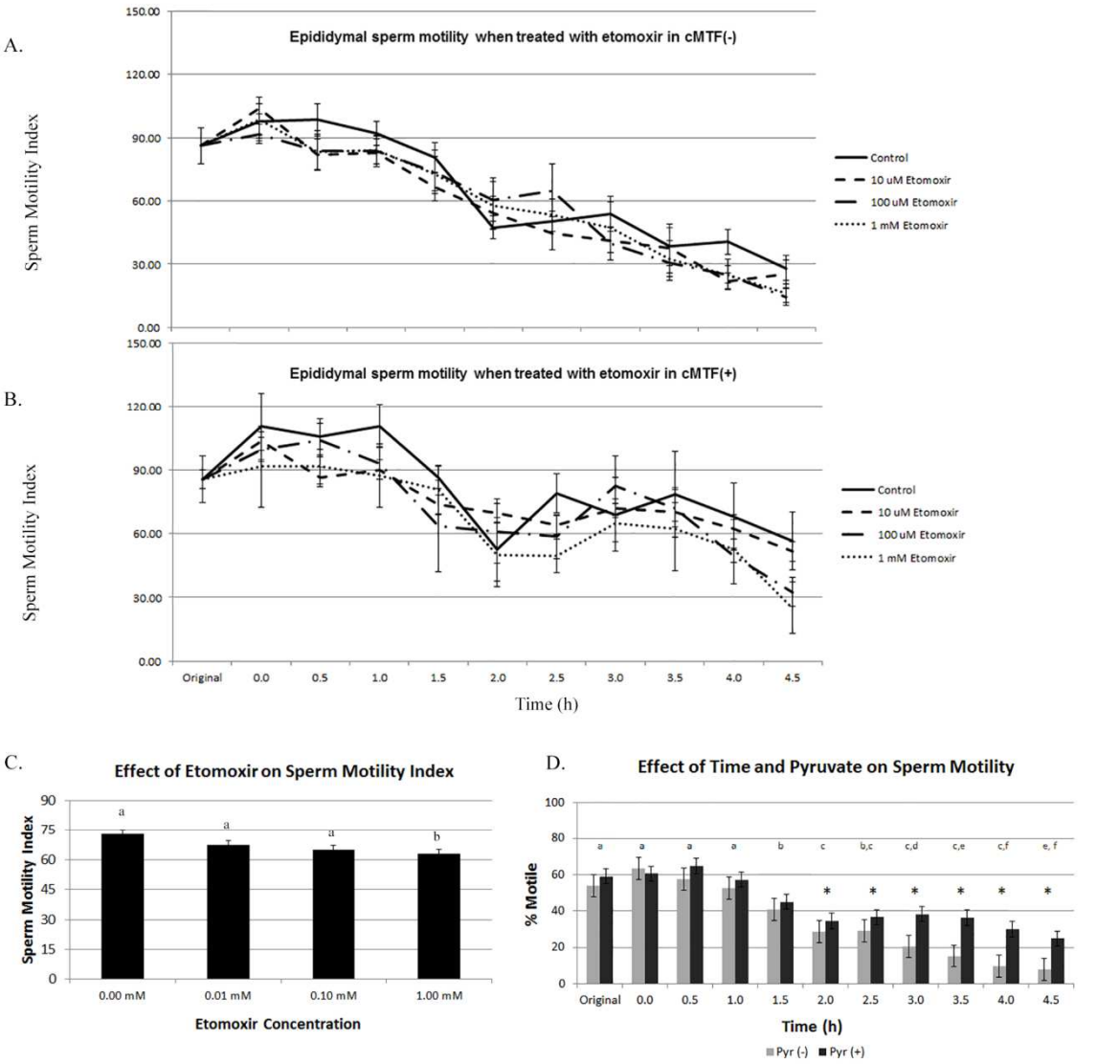

Figure 3.1: Epididymal domestic cat sperm motility after treatment with pyruvate and etomoxir over time. (A) Motility (sperm motility index) (mean ± SEM) of pooled epididymal sperm was assessed every 30 minutes for 4.5 hours while incubated in substrate free media or (B) with pyruvate; (C) the combined effect of etomoxir on sperm motility; (D) both time and pyruvate had a significant effect on % motile sperm. Significant differences in % motility over time are indicated by letters ($P < 0.05$); (*) denotes time points where a significant effect of pyruvate was detected ($P < 0.05$)..

In sperm collected from domestic cats via electroejaculation, there was a significant effect of both time ($P < 0.0001$) and exogenous pyruvate ($P = 0.0400$) on the percentage of motile sperm in a sample (Figure 3.2). As in epididymal sperm, ejaculated domestic cat sperm showed significantly reduced motility at 2hrs of incubation ($P < 0.0001$) compared to previous time points, but all subsequent time points did not significantly differ. Etomoxir did not have a significant effect on ejaculated domestic cat sperm motility ($P > 0.05$), which indicates that the β-oxidation of LCFAs does not contribute significantly to the production of energy required to fuel motility in felid sperm.

Contrary to the results obtained by treating ejaculated domestic cat sperm with etomoxir, ejaculated cheetah sperm was significantly impaired in the presence of 0.1 mM etomoxir ($P = 0.0014$), but was not significantly affected by the presence of exogenous pyruvate ($P = 0.4186$). This is an interesting finding, and demonstrates that the β-oxidation of LCFAs is contributing significantly to spermatozoal energy production in the cheetah to be used to fuel motility. Unsurprisingly, time had a significant effect on sperm % motility ($P < 0.0001$). These results are displayed in Figure 3.3.

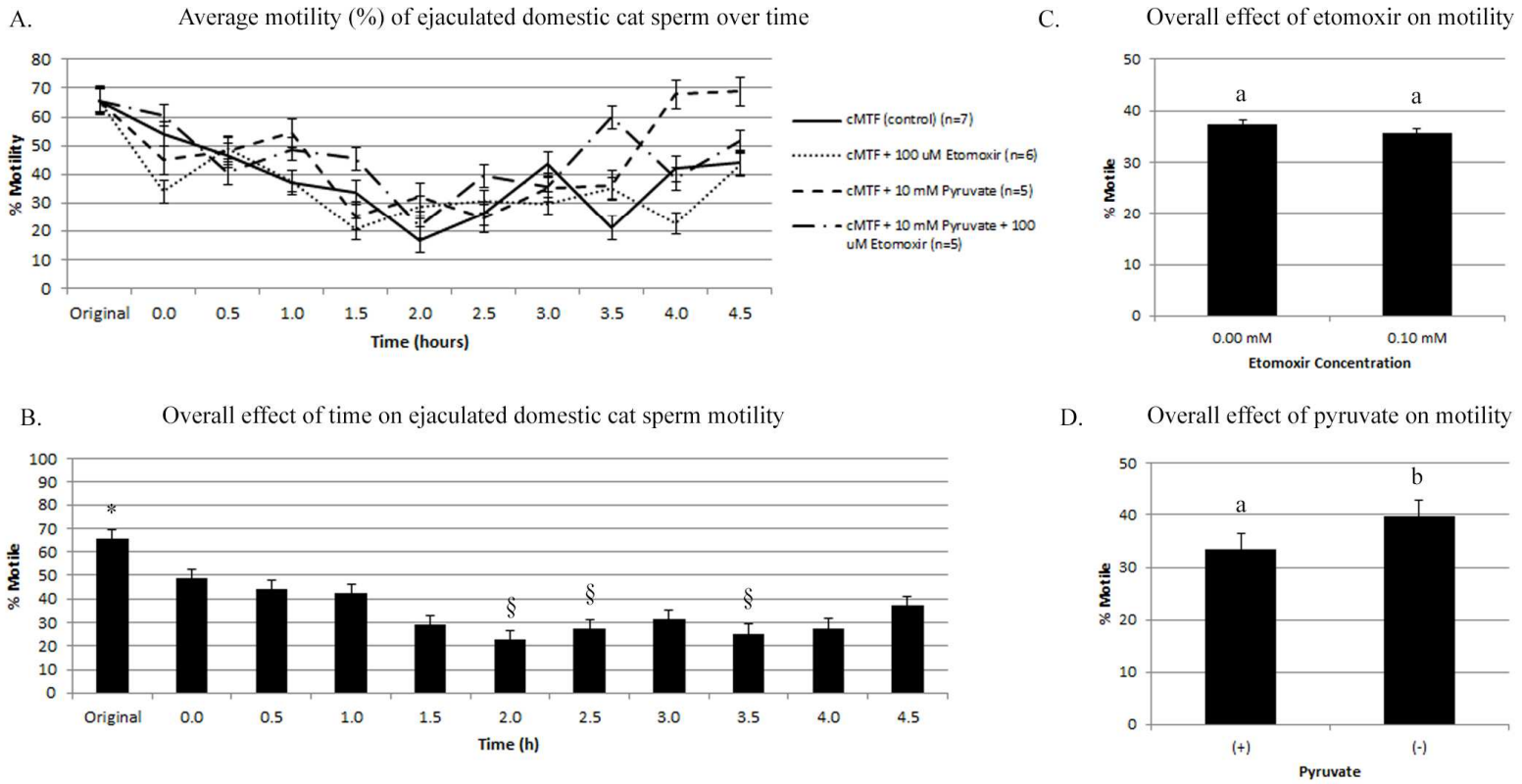

Figure 3.2: Ejaculated domestic cat sperm motility after treatment with pyruvate and etomoxir over time. (A) Motility (%) (average ± SEM) of treatment groups was measured every 30 minutes for 4.5 hours using CASA; (B) the overal effect of time had a significant effect on motility; (*) denotes a time at which motility is significantly higher than all other time points; (§) denotes a time at which motility significantly differs from most others; (C) etomoxir did not have a significant effect on sperm motility; (D) the overall effect of pyruvate significantly improved sperm motility; significance was determined when ($P < 0.05$).

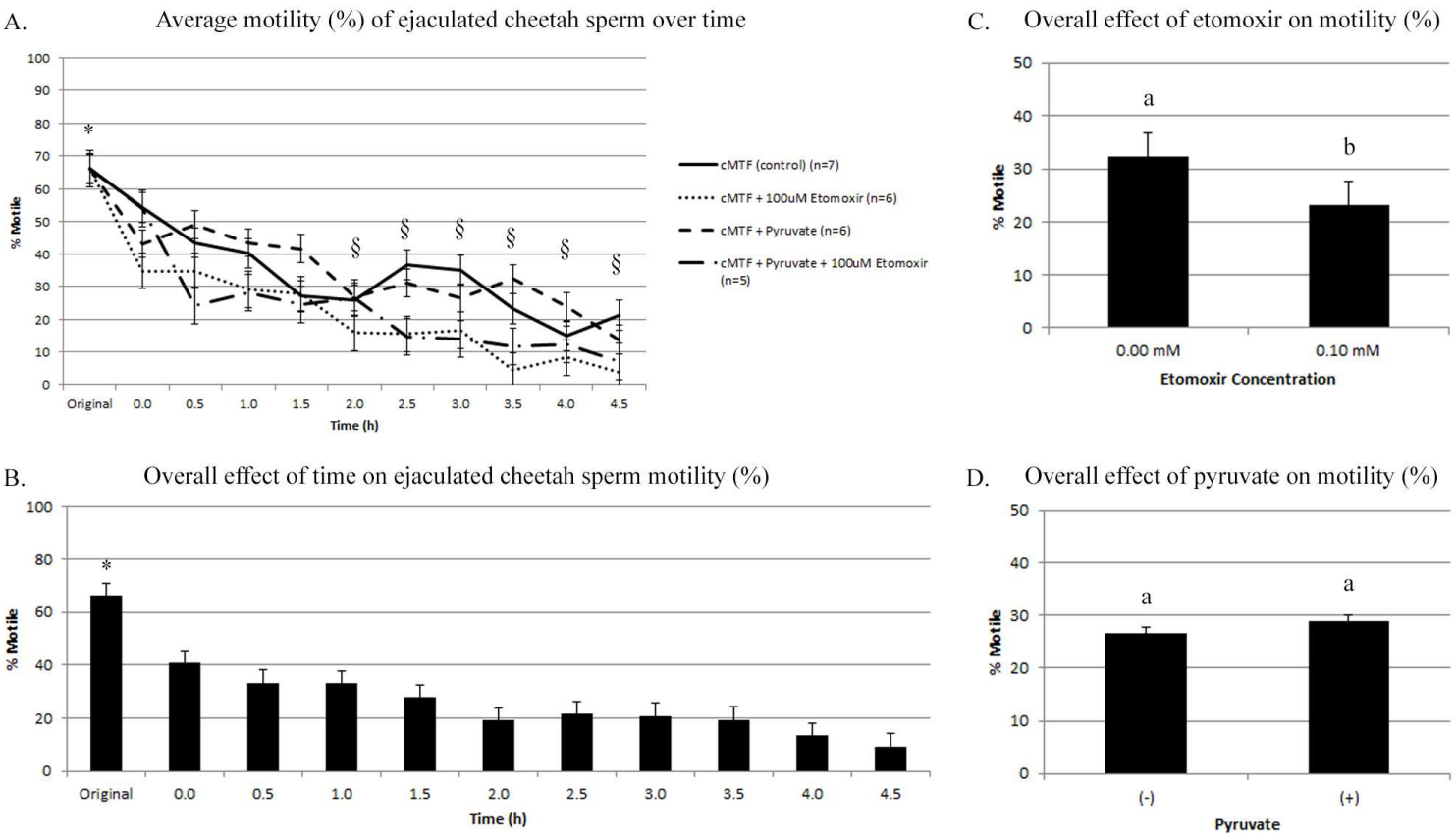

Figure 3.3: Ejaculated cheetah sperm motility after treatment with pyruvate and etomoxir over time. (A) Motility (%) (average ± SEM) was measured every 30 minutesfor 4.5 hours using CASA; (B) etomoxir caused a significant decrease in sperm motility; (C) no significant effect of pyruvate was detected; (*) denotes a time point at which motility significantly differs from all other time points; and (§) denotes time points at which there is a significant difference between control and etomoxir treated sperm; significance was determined at ($P < 0.05$).

In clouded leopards, semen was not concentrated enough to repeat all four treatment combinations that were completed in ejaculated sperm from cheetahs and domestic cats. Rather, sperm were incubated in cMTF(-) in the absence (control) or presence of etomoxir (0.1 mM), and motility was measured using CASA every 30 minutes for 4.5 hours (Figure 3.4). As with the domestic cat and cheetah, time had a significant effect on % motility ($P = 0.0071$), but there was no effect of etomoxir treatment on the percentage of motile sperm ($P > 0.05$).

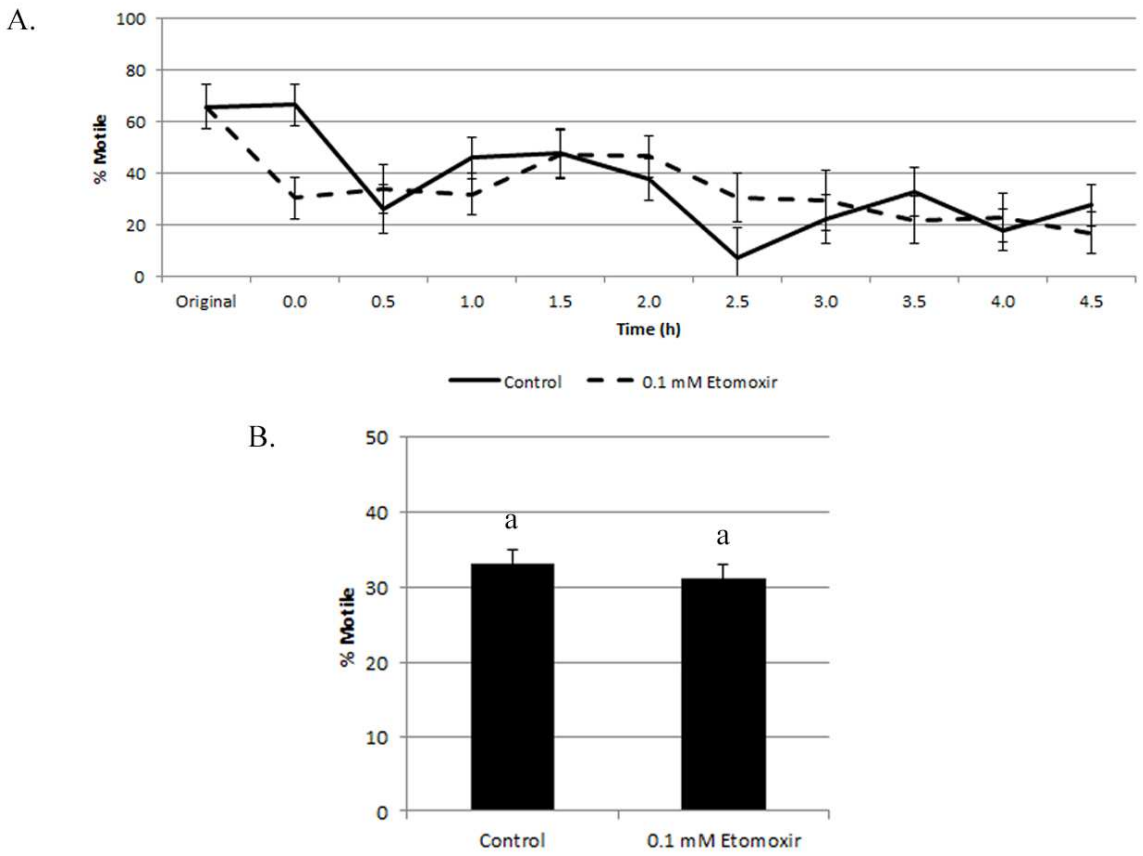

Figure 3.4: Ejaculated clouded leopard sperm motility after treatment with etomoxir over time. (A) Motility (%) (mean + SEM) was measured every 30 minutes for 4.5 hours using CASA; (*) denotes a time point at which motility significantly differs between treatments; (B) treatment with etomoxir had no significant effect on % motility; significance was determined when ($P < 0.05$).

The Effects of LCFA Inhibition on Entity Detection via GC-MS

Metabolism is a dynamic network of systems and pathways that is constantly changing. Therefore, it is expected that the relative abundance of certain metabolites in sperm will change over time and will be indicative of active pathways. Table 3.1 lists the metabolites which were differentially detected between treatment groups in domestic cat epididymal sperm and spent media after 2h and 4h of incubation in the presence (+) or absence (-) of exogenous pyruvate. Relative abundance refers to whether the compound in question was found to be in higher (up) or lower (down) abundance in the first time point listed compared to the control (0 hours). In the absence of pyruvate, there were six compounds which were differentially detected over time in sperm, but none in the spent media portions. In contrast, when sperm were incubated in the presence of exogenous pyruvate, two compounds were differentially detected in those sperm populations, and two compounds were detected in the respective spent media samples. This increase in the number of differentially detected metabolites in sperm incubated in the absence of pyruvate could be due to use of alternate internal stores of energy or energy substrate.

Only one compound – methyl-palmitoleate – was differentially detected due to etomoxir treatment in epididymal sperm (Table 3.2). Methyl-palmitoleate is a derivative of palmitoleate, which is a fatty acid biosynthesized from palmitic acid. Here we see that methyl-palmitoleate was only differentially detected in sperm and not in spent media, and was notably diminished as concentrations of etomoxir increased ($P = 0.0212$).

Table 3.1: **The effect of time on differentially detected entities in domestic cat epididymal sperm and spent media**. Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm					Media					Pathway
		p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	
(-)	[107689] L-(+) lactic acid	0.0377	1.79	up	1.22	up	--	--	--	--	--	Energy Metabolism
(-)	[107812] hypotaurine	0.0399	1.79	up	1.39	up	--	--	--	--	--	Amino Acid Metabolism
(-)	[243] benzoic acid	0.0023	1.61	up	1.00	up	--	--	--	--	--	Urea Cycle
(-)	[304] cholesterol	0.0493	1.33	up	-1.15	down	--	--	--	--	--	Membrane Stability
(-)	[750] glycine	0.0360	1.10	up	1.50	up	--	--	--	--	--	Amino Acid Metabolism
(-)	[971] oxalic acid	0.0018	2.11	up	1.41	up	--	--	--	--	--	Energy Metabolism
(+)	[11005] myristic acid	0.0035	-1.80	down	-1.81	down	--	--	--	--	--	Lipid Metabolism
(+)	[69217] 1-methylhydantoin 1	0.0348	1.50	up	1.50	up	--	--	--	--	--	Amino Acid Metabolism
(+)	[107689] L-(+) lactic acid	--	--	--	--	--	0.0446	-1.00	down	1.70	up	Energy Metabolism
(+)	[65065] N-acetyl-L-aspartic acid	--	--	--	--	--	0.0147	1.77	up	1.61	up	Amino Acid Metabolism

Table 3.2: **The effect of etomoxir on differentially detected entities in domestic cat epididymal sperm and spent media**. Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$)

Exogenous Pyruvate	Compound	Sperm (100 µM vs 0 µM Etomoxir)							Media (100 µM vs 0 µM Etomoxir)					
		p-value	Fold Change (10µM vs. 0µM)	Differential Detection (10µM vs. 0µM)	Fold Change (100µM vs. 0µM)	Differential Detection (100µM vs. 0µM)	Fold Change (1000µM vs. 0µM)	Differential Detection (1000µM vs. 0µM)	p-value	Fold Change (10uM vs. 0uM)	Differential Detection (10uM vs. 0uM)	Fold Change (100uM vs. 0uM)	Differential Detection (100uM vs. 0uM)	Fold Change (1000uM vs. 0uM)
(-)	[643801] methyl palmitoleate	0.0212	-1.88	down	-1.88	down	-1.88	down	--	--	--	--	--	--

When the combined effects of time and etomoxir were investigated using a 2-way ANOVA (Table 3.3), only compounds that were affected by time were shown to be differentially detected. After 4 hours, control sperm (no β-oxidation inhibition) and sperm incubated in 100 μM etomoxir had lower levels of lactate than samples incubated at 10 μM and 1 mM etomoxir (P = 0.221). However in the spent media, lactic acid was diminished at all concentrations of etomoxir at every time point with the exception of 10 μM and 1 mM etomoxir at 4 hours (P = 0.0293). These results imply that internal stores of lactate are increased after 2 hours, but are not increasing in the surrounding environment due to treatment with etomoxir, and therefore etomoxir is having an effect on lactate over time. Additionally, myristic acid was significantly diminished in sperm treated with etomoxir over time (P = 0.0189). Finally, oxalic acid was differentially detected in sperm treated with etomoxir over time. Oxalic acid was found in significantly higher relative abundances in sperm treated with etomoxir over time (P = 0.0022).

Table 3.3: **The combined effects of time and etomoxir on differentially detected entities in domestic cat epididymal sperm and spent media**. Control = 0 h, no etomoxir treatment; Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$)

Exogenous Pyruvate	Compound	p-value	Differential Detection based on Treatment											Pathway
			Time 0h	Time 0h	Time 0h	Time 2h	Time 2h	Time 2h	Time 2h	Time 4h	Time 4h	Time 4h	Time 4h	
			Etomoxir 10μM	Etomoxir 100μM	Etomoxir 1000μM	Etomoxir 0μM	Etomoxir 10μM	Etomoxir 100μM	Etomoxir 1000μM	Etomoxir 0μM	Etomoxir 10μM	Etomoxir 100μM	Etomoxir 1000μM	
Sperm														
(-)	[107689] L-(+) lactic acid	0.0221	down	down	down	up	up	up	up	down	up	down	up	Energy Metabolism
(-)	[107812] hypotaurine	0.0229	Up	up	up	up	up	up	up	up	up	up	up	Amino Acid Metabolism
(-)	[69217] 1-methylhydantoin	0.0379	Up	up	up	up	up	up	up	up	up	down	up	Amino Acid Metabolism
(-)	[971] oxalic acid	0.0022	down	up	up	up	up	up	up	up	up	up	up	Energy Metabolism
(+)	[11005] myristic acid	0.0189	up	up	down	down	down	down	down	down	down	down	down	Lipid Metabolism
Media														
(+)	[107689] L-(+) lactic acid	0.0293	down	down	down	down	down	down	down	down	up	down	up	Energy metabolism
(+)	[65065] N-acetyl-L-aspartic acid	0.0122	down	up	down	up	down	up	up	up	up	up	up	Amino Acid Metabolism

When the effects of pyruvate on compounds in epididymal sperm and media were assessed using an unpaired T-Test (Table 3.4), 11 compounds were differentially detected. As a prominent energy substrate known to be utilized by felid sperm [58], it is not surprising that lactate levels were increased in pyruvate-supplemented samples ($P = 0.025$). The higher relative concentrations of glycolic acid in pyruvate-supplemented sperm ($P < 0.0001$) aligns with the hypothesis that treating sperm with pyruvate as an energy substrate will increase energy production. Interestingly, malonic acid was detected to be at a significantly higher relative abundance in pyruvate-treated supernatant compared to supernatant from the substrate-free treatment group ($P < 0.0001$). In concert with this finding, oxalic acid was up-regulated in both sperm and spent-media ($P = 0.0067$).

Table 3.4: **The effect of pyruvate on entities in domestic cat epididymal sperm and media**. Control = 0.0 µM pyruvate; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$)

Compound	Sperm (100 µM vs 0 µM Pyruvate)			Media (100 µM vs 0 µM Pyruvate)			Pathway
	p-value	Fold Change	Differential Detection	p-value	Fold Change	Differential Detection	
[1000] 2-amino-1-phenylethanol	0.0241	-1.35	down	0.0037	-1.32	down	Vasoconstriction
[1004] phosphoric acid	0.0410	-1.31	down	--	--	--	Cellular Signaling; Energy Metabolism
[107689] L-(+) lactic acid	0.0254	1.35	up	0.0023	1.36	up	Energy Metabolism
[10820] 4-isopropylbenzoic acid	0.0288	1.34	up	--	--	--	Urea Cycle
[304] cholesterol	0.0094	-1.41	down	--	--	--	Membrane Stability
[65065] N-acetyl-L-aspartic acid	0.0102	-1.41	down	--	--	--	Amino Acid Metabolism
[757] glycolic acid	< 0.0001	1.73	up	--	--	--	Carbohydrate / Energy Metabolism
[8343] dioctyl phthalate	0.0147	1.38	up	--	--	--	Plasticizer
[867] malonic acid	--	--	--	<0.0001	1.69	up	Energy Metabolism
[892] allo-inositol	< 0.0001	1.57	up	--	--	--	Carbohydrate Metabolism
[971] oxalic acid	0.0067	1.43	up	<0.0001	1.95	up	Energy Metabolism

In ejaculated samples from domestic cats, eight compounds were positively identified and differentially detected over time (Table 3.5). Two compounds important for energy metabolism appeared to be affected in the opposite way to what was seen in domestic cat epididymal sperm: Lactic acid was significantly reduced at 2h and 4h of incubation in the absence of exogenous pyruvate ($P = 0.04$), whereas in the epididymal sperm lactic acid was shown to increase (Table 3.1). Additionally, oxalic acid showed an opposing trend to what was seen in epididymal sperm, as it was significantly decreased at 2h and 4h when sperm were incubated in the presence of exogenous pyruvate ($P = 0.0296$). Other compounds detected were involved in amino acid metabolism and the urea cycle.

When the effects of etomoxir on differentially detected compounds were assessed using an unpaired T-Test analysis (Table 3.6), two metabolites were identified: phosphoric acid was significantly decreased in etomoxir-treated sperm in the absence of exogenous pyruvate ($P = 0.0486$), and beta-alanine was significantly decreased in the media from etomoxir-treated sperm in the presence of exogenous pyruvate ($P = 0.0420$).

Table 3.5: **Differentially detected entities over time in ejaculated domestic cat sperm and media**. Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm					Media					Pathway
		p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	
(-)	[107689] L-(+) lactic acid	0.0429	-1.81	down	-0.85	down	--	--	--	--	--	Energy Metabolism
(-)	[243] benzoic acid	--	--	--	--	--	0.0429	-1.81	down	-0.85	down	Urea Cycle
(+)	[239] Beta- alanine	0.0392	-1.76	down	-1.33	down	--	--	--	--	--	Amino Acid Metabolism
(+)	[243] benzoic acid	0.0075	-1.98	down	-2.34	down	--	--	--	--	--	Urea Cycle
(+)	[487] methylmalonic acid	--	--	--	--	--	0.0036	1.19	up	-1.26	down	Amino Acid Metabolism
(+)	[601] D-Ala-D-Ala2	0.0396	-1.75	down	-1.32	down	--	--	--	--	--	Amino Acid Metabolism
(+)	[6106] L-leucine	0.0393	-1.75	down	-1.32	down	--	--	--	--	--	Amino Acid Metabolism
(+)	[971] oxalic acid	0.0296	-2.08	down	-1.73	down	--	--	--	--	--	Energy Metabolism

Table 3.6: The effect of etomoxir on differentially detected entities in ejaculated domestic cat sperm and media. Control = 0.0 µM etomoxir; Exogenous Pyruvate: (-) = 0µM, (+) = 100µM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm (100 µM vs 0 µM Etomoxir)			Media (100 µM vs 0 µM Etomoxir)			Pathway
		p-value	Fold Change (100µM vs. 0µM)	Differential Detection (100µM vs. 0µM)	p-value	Fold Change (100µM vs. 0µM)	Differential Detection (100µM vs. 0µM)	
(-)	[1004] phosphoric acid	0.0486	1.55	down	--	--	--	Cellular Signaling
(+)	[239] Beta- alanine	--	--	--	0.0420	-1.66	down	Amino Acid Metabolism

When the effects of time and etomoxir were assessed using a 2-way ANOVA (Table 3.7), three compounds were differentially detected in domestic cat ejaculated sperm. All three compounds (lactic acid, benzoic acid, and oxalic acid) were significantly diminished over time and in the presence of etomoxir (P = 0.0361, 0.0129, and 0.0499, respectively). Lactic acid showed a significant increase at 0h in the presence of etomoxir, but this could be the result of there being an internal store of this metabolite present. The differential detection of these compounds occurred in the absence (lactic and oxalic acids) and the presence (benzoic acid) of exogenous pyruvate.

Table 3.7: **The combined effects of time and etomoxir on differentially detected entities in ejaculated domestic cat sperm and spent media**. Control = 0.0 h, no etomoxir treatment; Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Differential Detection based on Treatment in Sperm						Differential Detection based on Treatment in Media						Pathway
		p-value	Time 0h	Time 2h	Time 2h	Time 4h	Time 4h	p-value	Time 0h	Time 2h	Time 2h	Time 4h	Time 4h	
			Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM		Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	
(-)	[107689] L-(+) lactic acid	0.0361	up	down	down	down	down	--	--	--	--	--	--	Energy Metabolism
(+)	[243] benzoic acid	0.0129	down	down	down	down	down	--	--	--	--	--	--	Urea Cycle
(-)	[971] oxalic acid	0.0499	down	down	down	down	down	--	--	--	--	--	--	Energy Metabolism

Pyruvate uptake has been shown to be an indicator of sperm quality [58], therefore, it was expected that pyruvate would have a substantial effect on sperm metabolism as determined by detection of relative abundance of metabolites. An unpaired T-test was conducted to analyze the effect of pyruvate on the differential detection of metabolites (Table 3.8), and variation was identified in both sperm and media. In sperm, compounds which were decreased in the presence of exogenous pyruvate included alanine ($P = 0.0237$), beta-alanine ($P < 0.0001$), glycine ($P = 0.0017$), and allo-inositol ($P = 0.0066$). Metabolites that showed a significant increase included leucine ($P = 0.0273$), glycolic acid ($P < 0.0001$), malonic acid ($P < 0.0001$), and oxalic acid ($P = 0.0226$).

Table 3.8: **The effect of pyruvate on differentially detected entities in ejaculated domestic cat sperm and media**. (+) = 100 μM Pyruvate, (-) = Control = 0.0 μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; Compounds are significant when $P < 0.05$.

Compound	Sperm (100 μM vs 0 μM Pyruvate)			Media (100 μM vs 0 μM Pyruvate)			Pathway
	p-value	Differential Detection	Fold Change	p-value	Differential Detection	Fold Change	
[107689] L-(+) lactic acid	--	--	--	0.0447	up	1.40	Energy Metabolism
[239] Beta- alanine	<0.0001	down	-2.46	<0.0001	down	-2.23	Amino Acid Metabolism
[243] benzoic acid	--	--	--	0.0423	down	-1.40	Urea Cycle
[601] D-Ala-D-Ala2	0.0237	down	-1.43	0.0245	down	-1.46	Amino Acid Metabolism
[6106] L-leucine	0.0273	up	1.47	--	--	--	Amino Acid Metabolism
[65065] N-acetyl-L-aspartic acid	--	--	--	<0.0001	up	2.60	Amino Acid Metabolism
[750] glycine	0.0017	down	-1.60	--	--	--	Amino Acid Metabolism
[753] glycerol	--	--	--	0.0171	down	-1.49	Carbohydrate Metabolism
[757] glycolic acid	<0.0001	up	2.27	<0.0001	up	3.77	Energy Metabolism
[867] malonic acid	<0.0001	up	3.55	<0.0001	up	2.07	Energy Metabolism
[892] allo-inositol	0.0066	down	-1.59	<0.0001	down	-1.96	Carbohydrate Metabolism
[971] oxalic acid	0.0226	up	1.56	--	--	--	Energy Metabolism

In cheetah sperm, three compounds were differentially detected as an effect of time in the absence of pyruvate when analyzed using an unpaired T-test (Table 3.9). Alanine had a significantly higher relative abundance at 2h and 4h of incubation (P = 0.0460), which could indicate high levels of mitochondrial activity during the later stages of incubation. Palmitic acid had a significantly lower relative abundance in sperm samples at 2h, but then a significantly higher relative abundance at 4h of incubation (P = 0.0159). In spent media, malonic acid was differentially detected in the presence of exogenous pyruvate at both 2h and 4h of incubation (P = 0.0434).

When the effects of etomoxir on cheetah sperm were assessed using an unpaired T-Test (Table 3.10), only two compounds were identified to be differentially detected. Phosphoric acid was shown to be significantly reduced in etomoxir-treated sperm (P = 0.0403). Phosphoric acid is involved in many cellular-signaling pathways. It is also the critical energy-producing component in ATP. Allothreonine is a compound involved in amino acid metabolism, and was shown to be significantly increased in etomoxir-treated sperm (P = 0.0069).

The combined effects of time and etomoxir, as analyzed using a 2-way ANOVA, yielded a single compound, stearic acid, which was differentially detected in the absence of exogenous pyruvate (Table 3.11). Stearic acid is a long-chain fatty acid, and its relative abundance in the spent media of cheetah sperm was significantly diminished over time and with increasing concentration of etomoxir (except at 4h) compared to the control (P = 0.0236). After 4h of incubation with 0.1 mM etomoxir, stearic acid was significantly higher in etomoxir-treated sperm than in sperm that were not treated with etomoxir.

Pyruvate had a significant effect on the differential expression of many different metabolites in cheetah sperm when analyzed using an unpaired T-test (Table 3.12). Unlike the domestic cat, lactic acid was significantly diminished in cheetah sperm incubated with exogenous pyruvate ($P = 0.0082$). Additionally, glycolic acid in the domestic cat was significantly increased by the presence of exogenous pyruvate, but this effect was reversed in the cheetah ($P = 0.0320$). Malonic acid and allo-inositol were found to be differentially detected in the spent media of sperm incubated with and without exogenous pyruvate. Malonic acid was significantly higher in spent media from pyruvate-treated sperm ($P < 0.0001$), and allo-inositol was significantly lower in the same group ($P = 0.0261$).

Table 3.9: **The effect of time on differentially detected entities in ejaculated cheetah sperm and spent media**. Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm					Media					Pathway
		p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	
(-)	[601] D-Ala-D-Ala2	0.0460	1.00	up	1.50	up	--	--	--	--	--	Amino Acid Metabolism
(-)	[69217] 1-methylhydantoin	0.0435	-1.15	down	1.39	up	--	--	--	--	--	Amino Acid Metabolism
(+)	[867] malonic acid	--	--	--	--	--	0.0434	1.79	up	1.34	up	Energy Metabolism
(-)	[985] palmitic acid	0.0159	-1.19	down	1.29	up	--	--	--	--	--	Lipid Metabolism

Table 3.10: **The effect of etomoxir on differentially detected entities in ejaculated cheetah sperm and spent media**. Control = 0.0 μM; Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm (100 μM vs 0 μM Etomoxir)			Media (100 μM vs 0 μM Etomoxir)			Pathway
		p-value	Fold Change	Differential Detection	p-value	Fold Change	Differential Detection	
(-)	[1004] phosphoric acid	0.0403	-0.61	down	--	--	--	Cellular Signaling
(+)	[99289] L-allothreonine	0.0069	0.84	up	--	--	--	Amino Acid Metabolism

Table 3.11: **The combined effects of time and etomoxir on differentially detected entities in ejaculated cheetah sperm and spent media compared to the control**. Control = 0 h, 0.0 μM etomoxir; Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Differential Detection based on Treatment in Sperm						Differential Detection based on Treatment in Media						Pathway
		p-value	Time 0h	Time 2h	Time 2h	Time 4h	Time 4h	p-value	Time 0h	Time 2h	Time 2h	Time 4h	Time 4h	
			Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM		Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	Etomoxir 0μM	Etomoxir 100μM	
(-)	[5281] stearic acid	--	--	--	--	--	--	0.0236	down	down	down	down	Up	Lipid Metabolism

Table 3.12: **The effect of pyruvate on differentially detected entities in ejaculated cheetah sperm and spent media**. Control = 0.0 µM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Compound	Sperm (100 µM vs 0 µM Pyruvate)			Media (100 µM vs 0 µM Pyruvate)			Pathway
	p-value	Differential Detection	Fold Change	p-value	Differential Detection	Fold Change	
[107689] L-(+) lactic acid	0.0082	down	-1.49	--	--	--	Energy Metabolism
[239] Beta- alanine	--	--	--	0.0388	down	-0.44	Amino Acid Metabolism
[487] methylmalonic acid	<0.0001	up	2.17	<0.0001	up	1.95	Amino Acid Metabolism
[5281] stearic acid	0.0398	up	1.36	--	--	--	Lipid Metabolism
[601] D-Ala-D-Ala2	0.0142	up	1.45	0.0427	up	0.45	Amino Acid Metabolism
[65065] N-acetyl-L-aspartic acid	--	--	--	<0.0001	up	0.82	Amino Acid Metabolism
[69217] 1-methylhydantoin	--	--	--	0.0225	down	-0.49	Amino Acid Metabolism
[757] glycolic acid	0.0320	down	-1.38	--	--	--	Carbohydrate/Energy Metabolism
[867] malonic acid	--	--	--	<0.0001	up	1.24	Energy Metabolism
[892] allo-inositol	0.0261	down	-1.40	0.0019	down	-0.67	Carbohydrate Metabolism
[99289] L-allothreonine	<0.0001	up	2.42	--	--	--	Amino Acid Metabolism

Due to small sample volumes and research limitations, clouded leopard sperm was incubated only in the presence or absence of etomoxir (100 μM) with no addition of pyruvate. Thus, any changes observed in the relative abundance of compounds occurred in the absence of exogenous energy substrates. After a repeated measures analysis of variance in clouded leopard sperm and spent media, the only metabolite that was significantly affected by time was lactic acid which was significantly reduced over time (P = 0.0464). This effect was observed only in the spent media (Table 3.13). In response to etomoxir, clouded leopard sperm showed an increased abundance of malonic acid (P = 0.0286) compared to the control (Table 3.14). Additionally, beta-alanine was higher in the spent media from etomoxir-treated sperm than in the control (P = 0.0298).

Table 3.13: **The effect of time on differentially detected entities in ejaculated clouded leopard sperm and spent media**. Exogenous Pyruvate: (-) = 0μM, (+) = 100μM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm					Media					Pathway
		p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	p-value	Fold Change (2h vs. 0h)	Differential Detection (2h vs. 0h)	Fold Change (4h vs. 0h)	Differential Detection (4h vs. 0h)	
(-)	[107689] L-(+) lactic acid	--	--	--	--	--	0.0464	-1.73	down	-1.69	down	Energy Metabolism

Table 3.14: **The effect of etomoxir on differentially detected entities in ejaculated clouded leopard sperm and media**. Control = 0.0 µM; Exogenous Pyruvate: (-) = 0µM, (+) = 100µM; Compound = [Fiehn Chemical Library Reference Number] Compound Name; compounds are significant when $P < 0.05$.

Exogenous Pyruvate	Compound	Sperm (100 µM vs 0 µM Etomoxir)			Media (100 µM vs 0 µM Etomoxir)			Pathway
		p-value	Fold Change	Differential Detection	p-value	Fold Change	Differential Detection	
(-)	[239] Beta- alanine	--	--	--	0.0298	1.64	up	Amino Acid Metabolism
(-)	[867] malonic acid	0.0286	1.83	up	--	--	--	Energy Metabolism

Discussion

GC-MS allows the visualization of a broad spectrum of metabolites and, therefore provides a more thorough assessment of energy metabolism than monitoring just a single compound of interest. Using this technique, we were able to assess the role that β-oxidation plays in fueling motility for sperm of three different felid species revealing for the first time that β-oxidation is an important energy producing pathway in the cheetah. Moreover, we observed a biphasic motility response that could be indicative of a shift from endogenous substrate to exogenesis pyruvate utilization after 2 hours of incubation both the presence and absence of etomoxir.

As pyruvate has been shown to be an energy substrate in felid sperm [58], we evaluated lipid metabolism in the presence and absence of pyruvate – a product of glycolysis and a precursor for the TCA cycle – in addition to inhibiting LCFAs from being metabolized. The rationale for provision of pyruvate was to determine whether the presence of a known exogenous energy substrate utilized by a different pathway (glycolysis) could mitigate any of the effects seen by the application of a β-oxidation inhibitor, and thereby indicate a preference by felid sperm for one metabolic pathway over another. In domestic cat epididymal sperm, a visible shift in motility was observed after 2 hours of incubation, but only in the pyruvate-treated groups. This was true also of ejaculated domestic cat sperm, as well as in ejaculated cheetah sperm. One explanation for this observation could be the availability of exogenous energy substrates. Endogenous energy substrates are often cited as being a potential source of energy for sperm. Endogenous substrates have been established to fuel motility in the boar, as evidenced by sustained motility for more than five hours in the

absence of exogenous energy substrates [76]. Given that some felid sperm are also able to maintain motility for several hours, we hypothesize that the biphasic motility pattern could be due to the expenditure of an energy sources present and a switch from endogenous to exogenous energy substrates. A biphasic motility response was only seen in sperm incubated with exogenous pyruvate, but not in sperm incubated without exogenous pyruvate, which supports this hypothesis. This also confirms previous reports that the presence of exogenous pyruvate is able to mitigate the deleterious effects of incubation time on felid sperm motility [58,142]. Further investigation is necessary to confirm the identity of endogenous energy substrates within felid sperm, but we hypothesize that the breakdown of phospholipids in the plasma membrane could be a significant contributor to this endogenous pool of energy substrate.

As previously mentioned, etomoxir is an irreversible β-oxidation inhibitor. By incubating sperm of various species with such an inhibitor and measuring motility, we were able to evaluate the relative importance of this pathway to providing energy to fuel such motility processes. Cheetah sperm motility was significantly impaired when treated with etomoxir ($P < 0.05$), but domestic cat and clouded leopard sperm motilities were unaffected by β-oxidation inhibition. Despite there being no visible effects of etomoxir on motility, the relative abundance of several compounds was significantly affected by LCFA β-oxidation inhibition in all three species. This data suggests that while β-oxidation is a critical energy-producing pathway necessary for only cheetah sperm motility, this pathway is still active in all three species. The metabolites that are most affected by the treatment of etomoxir are relevant to energy,

lipid, and amino acid metabolism pathways, which could serve as target pathways for future investigation.

In addition to etomoxir, cheetah sperm also responded differently than domestic cat sperm to supplementation with pyruvate. In cheetah sperm, the relative abundance of lactate was decreased in the presence of pyruvate, contrary to what was observed for domestic cat sperm. If cheetah sperm use both lipids and carbohydrates as energy sources, then these complex interactions and contributions require further study to parse out the relative activity each. These findings could indicate different pathway utilization with a preference for lipid metabolism by cheetah sperm - as suggested by the decreased motility in presence of etomoxir. The response of cheetah sperm to etomoxir raises intriguing questions regarding their energy metabolism: are these responses related to teratospermia, and therefore related to having an impaired metabolism? Could an impaired glycolytic pathway be forcing a dependence on alternate energy substrates, like lipids? Or are the responses seen in this study simply due to the complex interactions of multiple energy pathways? Answering these questions will require further examination of these pathways in sperm.

Conclusions

In summary, the results of this study demonstrate three key findings: (1) etomoxir had a significant effect on the sperm metabolism in all three species, as indicated by the differential detection of metabolites; (2) cheetah sperm motility was affected by treatment with etomoxir; and (3) motility in the domestic cat and the cheetah over time demonstrated a biphasic pattern, which could implicate the

presence of an endogenous substrate used for energy production. If the source of endogenous substrates for energy production is related to fatty-acid or lipid metabolism, a variety of lipid stains could be used to visualize any potential stores of these molecules within the spermatozoa. However, because there is little cytoplasmic space to hold vacuoles or storage vesicles within the sperm, studies investigating the break-down and utilization of the phospholipids in the plasma membrane could elucidate whether this structure plays a metabolic role in felid spermatozoa.

Further studies are also needed to determine the pathways affected by altered substrate conditions, which resulted in the various differentially detected compounds. This could be done by incubating sperm in the presence or absence of an excess of these metabolites, or by further interrogating relevant pathways by blocking various associated enzymes. Shifts in metabolic processes are known to occur during key steps in sperm function such as capacitation and hyperactivity, therefore further exploration using metabolomics and fluxomic approaches could provide novel insights into the metabolic requirements for these critical processes.

Chapter 4: Fluxomic Analysis of Felid Sperm Metabolism

Introduction

Spermatozoa require energy for motility and the many events leading up to fertilization such as capacitation and the acrosome reaction [4,73,146]. Sperm metabolism has been studied in a variety of species to varying degrees, but relatively little is known about sperm metabolism in felids. The *Felidae* family is comprised of 37 different species, almost all of which are experiencing population decline on account of loss of habitat, loss of prey base, and illegal poaching. Despite efforts to maintain captive populations of these animals as a hedge against extinction, reproduction in captivity has its own set of problems. Nearly 90% of all felid species are known to consistently produce high populations of malformed spermatozoa, a condition known as teratospermia [5]. Teratospermia is characterized when more than 60% of ejaculated sperm are abnormal, and even cells which appear normal have been shown to exhibit an impaired metabolism [59]. This further compounds the inherent difficulties associated with assisted reproductive techniques (ARTs) and cryopreservation [10,22]. In order to better understand sperm physiology and improve assisted reproductive technologies in endangered felids, basic information regarding how their sperm metabolize various energy substrates is needed. In this study, we aimed to further understand the activity of various energy-producing pathways in sperm from three different felid species: the domestic cat (*Felis catus*), the cheetah (*Acinonyx jubatus*), and the clouded leopard (*Neofelis nebulosa*). This information

will assist conservation efforts by providing a more in-depth insight into the energy-producing pathways within spermatozoa, and could indicate which substrates are important to sperm function and survival.

To do this, we assessed metabolism using a fluxomics approach. As the newest of the "-omics" fields, little has been done to utilize this technique to its fullest in reproduction science, and even less has been done in wildlife species. Broadly speaking, fluxomics is a means by which cellular metabolic activity can be interrogated using labeled tracers and yield quantitative measures of metabolic turnover through operational pathways within a biological sample [96]. Typical tracers have one or more heavy ions incorporated into their chemical composition creating isotopmers of the unlabeled molecule. The breakdown of these isoptomers can be tracked by the presence of heavy ion(s) in the resulting products. The distribution of these ions can be calculated by quantifying the ion fragments containing stable isotopes. To do this, the relative abundance of the unlabeled (M+0) and labeled (M+*n*) ions are detected, where M is the typical mass of a molecular fragment and *n* is the added mass when one or more heavy ion is incorporated. These abundances are then corrected for background signal, and the contribution of the tracer to the labeled product is calculated as a ratio of the ion containing the unlabeled atoms to the unlabeled atoms of interest ([M+*n*] / [M+0]) [97]. Fluxomics and ^{13}C-labeling are best paired with mass spectrometry which identifies compounds based on their fragmentation patterns. When paired with gas-chromatography, such a system is ideal for measuring multiple labeled or unlabeled compounds within a sample. This is ideal for studying metabolism, in which several interconnected pathways can be

involved in a single cellular activity, such as breaking down a substrate to produce energy. For example, glucose can be measured in fluxomic experiments to indicate the activity of glycolysis and the TCA cycle, as well as many different off-shoot pathways like the pentose phosphate pathway. Figure 4.1 shows the typical compounds that are measured in a fluxomic study and denotes the indicative [M+*n*] ions used to do so.

In order to measure the contribution of a labeled substrate to a pathway or metabolic product, it is important to ensure that the tracer does not comprise 100% of the available substrate, but is a 50:50 mixture of labeled and unlabeled substrate. Doing so ensures that downstream ion ratios can be determined accurately. If a tracer exists at 100% in a medium, there will be no basis for comparison regarding whether or not any endogenous stores of such substrate in the cell, tissue, or system will dilute the labeled substrate and, therefore, calculations will be inaccurate. However, when labeling exists initially as a 50:50 mixture, endogenous substrate stores will have less of an effect on the media, and labeled ions can be quantified based on the natural occurrence of the heavy ion in the same sample. We chose to investigate the role of three different substrates that could contribute to energy production in the sperm: glucose, fructose, and pyruvate. Despite the previous report of glucose's absent role from felid sperm metabolism, it was important for us to include it in our study for two main reasons: first, measurements of glucose uptake may not have been sensitive enough to detect whether or not the sperm were actually taking up the substrate slowly or in low amounts. Additionally, the means by which glycolysis was blocked in a follow-up study could have actually impaired metabolism as the enzyme that was

blocked came after an ATP-dependent step in glycolysis [147]. Therefore, energy was being used to fuel a pathway that was artificially non-functional, and thus potentially depriving other pathways of ATP. Fluxomic assessment of [U-^{13}C]-labeled glucose, fructose, and pyruvate can indicate definitively whether or not these substrates are being utilized by felid sperm.

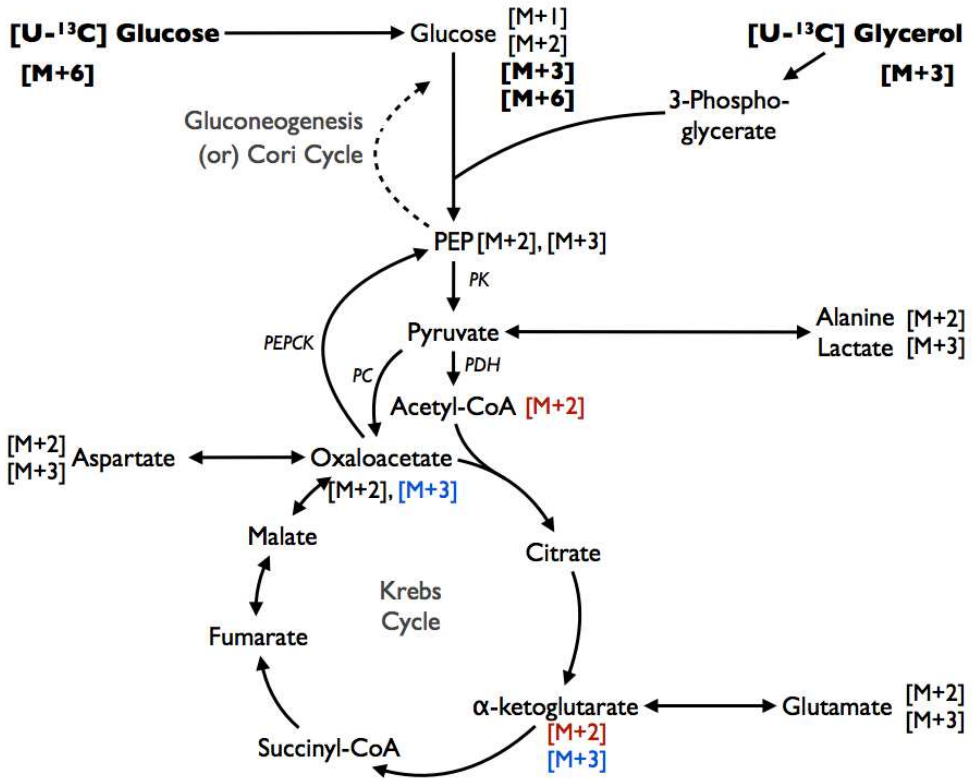

Figure 4.1: **Glucose and glycerol carbon flux through metabolic pathways.** (M+*n*) indicates the monitored ion, and glucose carbon contribution to pathway intermediates.

Materials and Methods

Media Preparation

All reagents were purchased from Sigma Aldrich (St. Louis, MO) unless otherwise specified. A chemically defined, protein-free, cat-modified mouse tubal fluid (cMTF) medium was used for all procedures [118]. The final cMTF medium (pH 7.45) contained 98.4 Mm NaCl (Acros, New Jersey, USA), 4.78 mM KCl, 1.19 mM $MgSO_4$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.71 mM $CaCl_2$, 25 mM 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (Fisher Scientific), and 2% polyvinyl alcohol (PVA), and was sterilized through a 0.22 μm syringe filter prior to use [58]. Osmolality of the final working medium (300 – 340 mOsm) was within 10% of the physiological value of domestic cat semen (323 mOsm) [59], and determined using a vapor pressure osmometer (Wescor, Inc. Logan, UT). The cMTF medium was prepared from concentrated 1 M stock solutions in deionized H_2O. All stock solutions were kept at 4°C and discarded after 3 months, or if precipitates were present. Fluxomics media stocks (20mM) were prepared using a 50:50 mixture of unlabeled to labeled substrate ([U-^{13}C]-Glucose, [U-^{13}C]-Fructose, or [U-^{13}C]-NaPyruvate; Cambridge Isotopes, Tewksbury, MA).

Epididymal Fluid and Sperm: Collection and Evaluation

Male reproductive tissues were obtained following the routine castration of normal (FIV negative, non-cryptorchidic) sexually mature ($\geq$ 1 yr) feral cats at a local veterinary clinic as available. Tissue was maintained and transported at 4°C until

processing. Sperm from the cauda epididymis of ≥2 males was physically extruded through the ductus deferens, and epididymal fluid was separated via centrifugation at 27°C (8 minutes at 800 × *g*). Sperm were diluted in cMTF at a ratio of approximately 1:10 (v/v) (n = 4). A 2.8 µL sample was taken to assess percent motility (%MOT) and forward progressive status (FPS; scored 0 – 5; 0 = non-motile, 5 = strong forward progression) for the purposes of calculating the sperm motility index (SMI = %MOT + (FPS x 20) ÷ 2) [119], and concentration was established using a standard hemocytometer (Hausser Scientific, Horsham, PA). Sperm were aliquoted into four treatment groups at a concentration of ~3.0 x 10^6 sperm/mL: 1) control (no energy substrates), 2) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled glucose, 3) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled fructose, and 4) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled Na-Pyruvate. All treatments were incubated at 37˚C under mineral oil to prevent evaporation, and motility (%) and FPS were assessed after 2 and 4.5 hrs of incubation. A sample of sperm suspension (400 µL) was removed at each time point and stored at -80˚C until being prepared for GC-MS analysis.

Semen Collection and Evaluation

A surgical plane of anesthesia was induced in domestic cats (n=4), cheetahs (n=4), and clouded leopards (n=4) according to previously described protocols [2,120]. All animal procedures were approved by the National Zoological Park's Animal Care and Use Committee (ACUC) and the University of Maryland's Institutional ACUC. Methods for semen collection and evaluation were similar to those previously described [2,120,121]. Briefly, a rectal probe of 1 cm (domestic cat), 1.9 cm (cheetah), or 1.6 cm (clouded leopard) in diameter with three longitudinal

electrodes and an electrostimulator (P.T. Electronics, Boring, OR) were used to deliver 90 stimuli (at a low voltage of 2 – 5 V) over a 30 min interval [121]. Ejaculates were collected in sterile, pre-warmed vials [29,107,121,122]. A 2.8 µL sample was taken to assess motility using CASA (Hamilton Thorne, v.1.3.1), and percent motility (%MOT) along with forward progressive status (FPS; scored 0 – 5; 0 = non-motile, 5 = strong forward progression) were also estimated for the purposes of calculating the sperm motility index (SMI = %MOT + (FPS x 20) ÷ 2) [119]. Sperm concentration was established using a standard hemocytometer (Hausser Scientific, Horsham, PA). Individuals were classified as teratospermic if more than 60% of sperm were abnormal according to gross morphological assessment by the CASA system. However, our assessment of morphology did not account for acrosomal abnormalities, and therefore the teratospermic individuals described in this study were part of a more selective screening process than individuals in previous reports, resulting in fewer individuals being classified as teratospermic. Seminal fluid was removed following motility assessment and centrifugation at 27°C (8 minutes at 800 × *g*). Sperm were washed with cMTF and resuspended at ~3.0 x 10^6 sperm/mL into four treatment groups: 1) control (no energy substrates present), 2) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled glucose, 3) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled fructose, or 4) 1 mM 50:50 unlabeled to [U-^{13}C]-labeled pyruvate. All treatments were incubated at 37˚C under mineral oil to prevent evaporation. Motility (%) and FPS were assessed after collection, centrifugation, 2 hours and 4.5 hours of incubation. A sample of sperm suspension (400 µL) was removed at each time point and stored at -80˚C until being prepared for GC-MS analysis.

GC-MS Processing

Samples were slowly thawed on ice, dried completely using a RotoVap SpeedVac (Savant, SC100), and then chemically extracted by adding 0.8 mL of a 1:1 mixture of methanol and water (MeOH:H_2O) and 0.8 mL of chloroform ($CHCl_3$) and left to incubate at room temperature on a rotary spinner for 30 minutes. To remove proteins and cellular debris, samples were centrifuged at 13,000 x *g* for 5 minutes, and the aqueous layer was transferred into a glass v-vial. Samples were then dried completely under N_2 gas at 37˚C, and derivatized by adding 30µL hydroxylamine (Sigma) in pyridine (30 mg/mL) (ThermoScientific, Rockford, IL) and incubating for 30 minutes at 60˚C. Metabolites were converted to their tert-butyldimethylsilyl derivatives by adding an equal volume of N-(tert-butyldimethylsilyl)-N-methyltrifluroacetamide with 1% t-butyldimethylchlorosilane (MTBSTFA + 1%TBDMCS; Pierce, Rockford, IL). To catalyze this process, samples were microwaved for 2 minutes (200W), and then run through the GC-MS system (Agilent 6890 gas chromatography system, Palo Alto, CA; ZB50 Capillary Column). Samples were injected in two microliter aliquots (1:1 split mode) and separated on a mid-polarity capillary column. The initial oven temperature was set at 100˚C and held for 3 minutes, then heated at a rate of 3.5˚C per minute to 280˚C. An additional heating phase took place at a rate of 50˚C per minute to 300 ˚C and held for 2 minutes for a total run time of 70 minutes. The mass spectrometer (Agilent 5973 inert EI/CI MSD system) was operated in electron impact mode, and ions were selectively monitored (SIM mode). Agilent's GC-MSD ChemStation software was used to capture raw GC-MS data files, which were viewed and quantitated using Agilent's Data Analysis

software. Compounds were identified by their mass spectra and retention time, which was confirmed through the use of purified algal standards. To determine substrate metabolism, the ration of the [M+3] ion to the [M+0] ion in each compound of interest (lactate, pyruvate, and alanine) was calculated. Table 4.1 describes the ions monitored for all samples.

Following fluxomic analysis, calculations were performed to determine the contribution of [U-^{13}C]-glucose to sperm-produced lactate and pyruvate (both mitochondrial and cytosolic) carbon pools. The crude ion abundances were corrected based on the natural abundance of isotopes present in the molecule and in the derivative using a matrix approach derived from pure standards [148]. These values were then corrected to and used to determine the isotopic enrichment of precursors, intermediates, and products (reported as moles of tracer [M+*n*] per 100 moles of tracee [M+0]). Glycolytic flux, which requires additional sample processing and analysis, could not be calculated due to sample volume limitations.

Statistical Analysis

Motility data were analyzed as a randomized design with covariate analysis using the PROC MIXED function in SAS, version 9.3 (SAS Institute, Cary, NC). Fluxomics data were analyzed by the Student's *t*-test procedure using SAS, version 9.3. Results are presented as mean $\pm$ SEM, and labeling was found to be statistically significant when $P < 0.05$.

Table 4.1: Ions selectively monitored for metabolites in felid sperm samples; (*) indicates metabolites which were able to be accurately and consistently quantified in this study

Compound	Ions Monitored
*Lactate	[M+0]:261.2 – [M+3]:264.2
*Alanine	[M+0]:260.2 – [M+3]:263.2
*Pyruvate	[M+0]:274.2 – [M+3]:277.2
Aspartate	[M+0]:418.3 – [M+4]:422.4
Glutamate	[M+0]:432.4 – [M+5]:437.4
Asparagine	[M+0]:417.3 – [M+5]:422.3
Glutamine	[M+0]:431.3 – [M+5]:436.3
Citrate	[M+0]:459.3 – [M+6]:465.3

Results

Sperm Motility

Glucose did not have a significant effect on sperm motility ($P > 0.05$) for any of the species in this study. Epididymal domestic cat sperm motility was higher when incubated with pyruvate compared to control media ($P = 0.0529$). Incubation with fructose significantly improved epididymal sperm by 32.5 $\pm$ 8.4 SMI units (mean $\pm$ SEM; $P = 0.0184$). No significant differences in motility were found between treatments in ejaculated domestic cat sperm ($P > 0.05$). Cheetah sperm motility was also not significantly affected by the presence of glucose, fructose, or pyruvate ($P > 0.05$), but clouded leopard sperm motility was significantly improved in the presence of pyruvate (35.0 $\pm$ 9.4, mean $\pm$ SEM; $P = 0.0266$). These results are depicted in Figure 4.2.

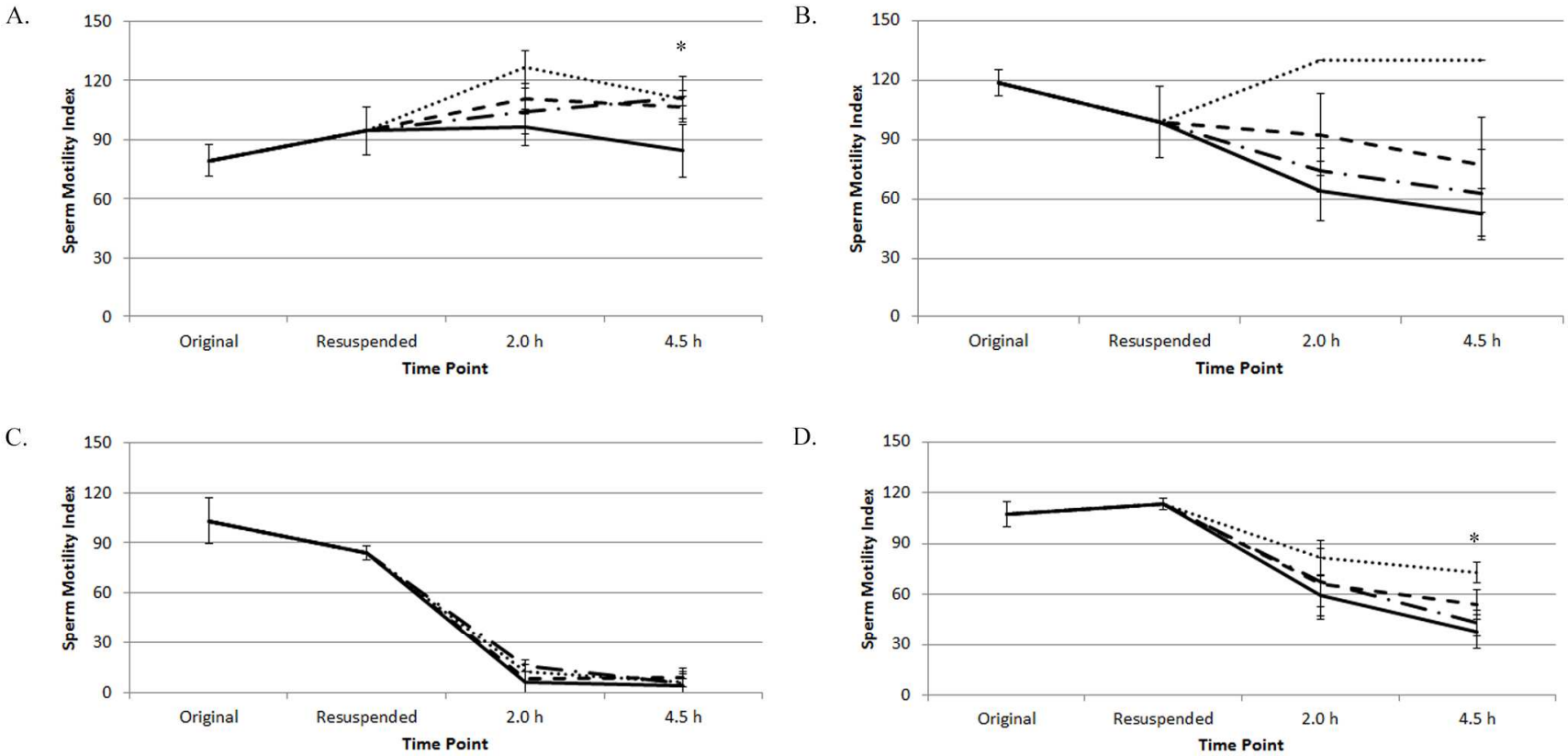

Figure 4.2: **Felid sperm motility index after treatment with U13C-labeled tracers.** (A) domestic cat epididymal sperm, (B) ejaculated domestic cat sperm, (C) ejaculated cheetah sperm, and (D) ejaculated clouded leopard sperm incubated with no energy substrate (control, solid), U13C-labeled glucose (dash), U13C-labeled fructose (dash-dot), or U13C-labeled pyruvate (dot); (*) denotes a significant difference between treatments; significance was determined at ($P < 0.05$).

^{13}C-Tracer Utilization

Uptake of labeled substrates is indicated by the presence of a "heavy ion" at the [M+3] position in the mass spectrum of the sperm-produced compound. In this study, the sperm-produced compounds measured were pyruvate, lactate, or alanine. Figure 4.3 shows representative mass spectra for these three compounds derived from U^{13}C-glucose, U^{13}C-fructose, and U^{13}C-pyruvate.

The quantification of tracer-derived, sperm-produced substrates are shown in Tables 4.2 – 4.4. The average moles of tracer per 100 moles of tracee ([M+3]/[M+0]) is reported for each monitored compound, along with the p-value stating whether this percentage is significantly different ($P < 0.05$) from the natural abundance of environmental [M+3] ions. The percentage of [M+3] ions that would exist naturally in the environment is also listed. P-values indicated with an asterisk (*) denote potential false negatives due to low sample size and high individual variability.

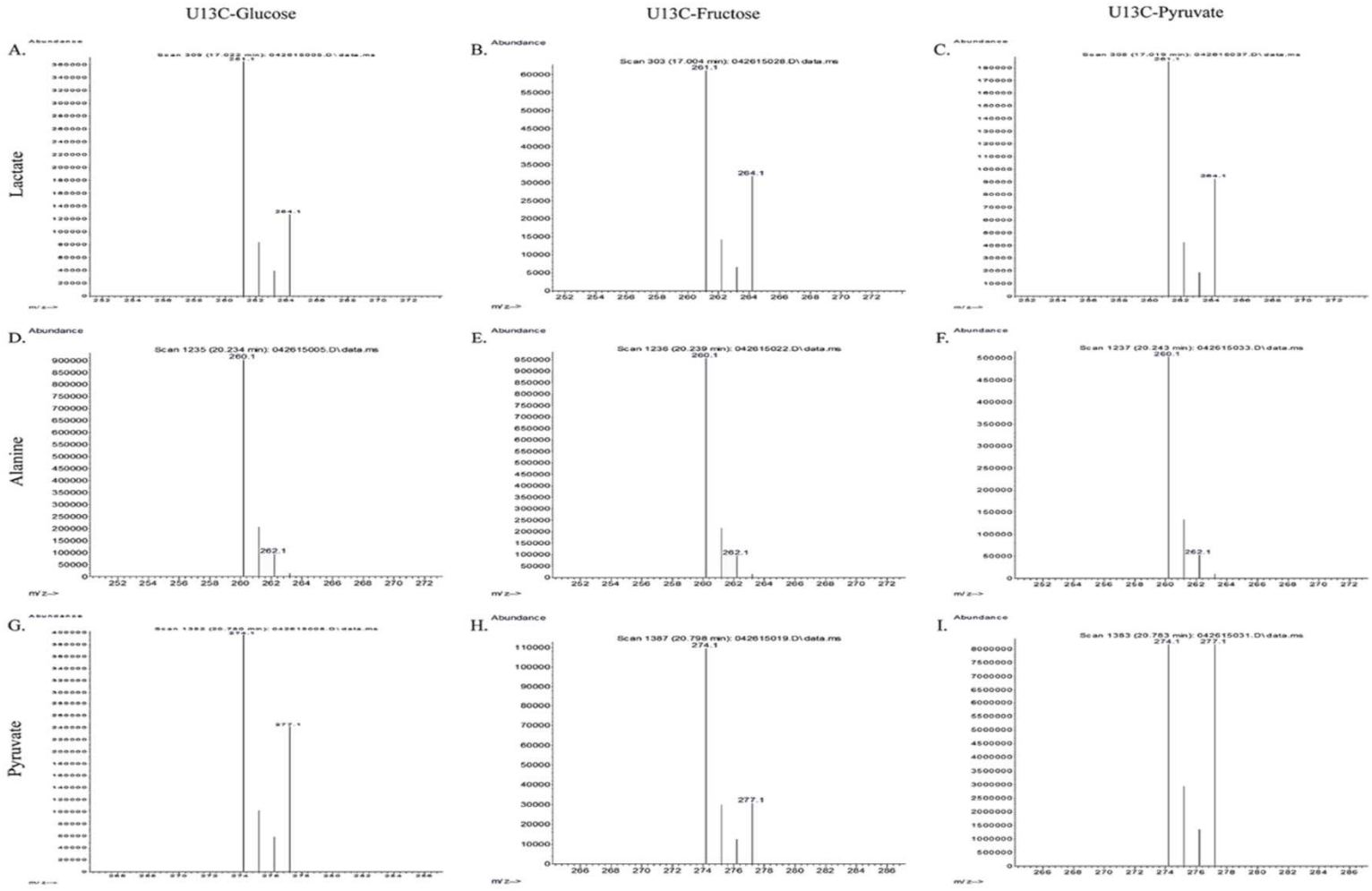

Figure 4.3: Representative mass spectra of sperm-produced lactate, pyruvate, and alanine. Spectra depict the [M+0], [M+1], [M+2], and [M+3] ions of lactate (A, B, C), pyruvate (D, E, F), and alanine (G, H, I) from U13C-glucose (A, D, G), U13C-fructose (B, E, H), and U13C-pyruvate (C, F, I); [M+0] and [M+3] ions are labeled with their respective *m/z* peak.

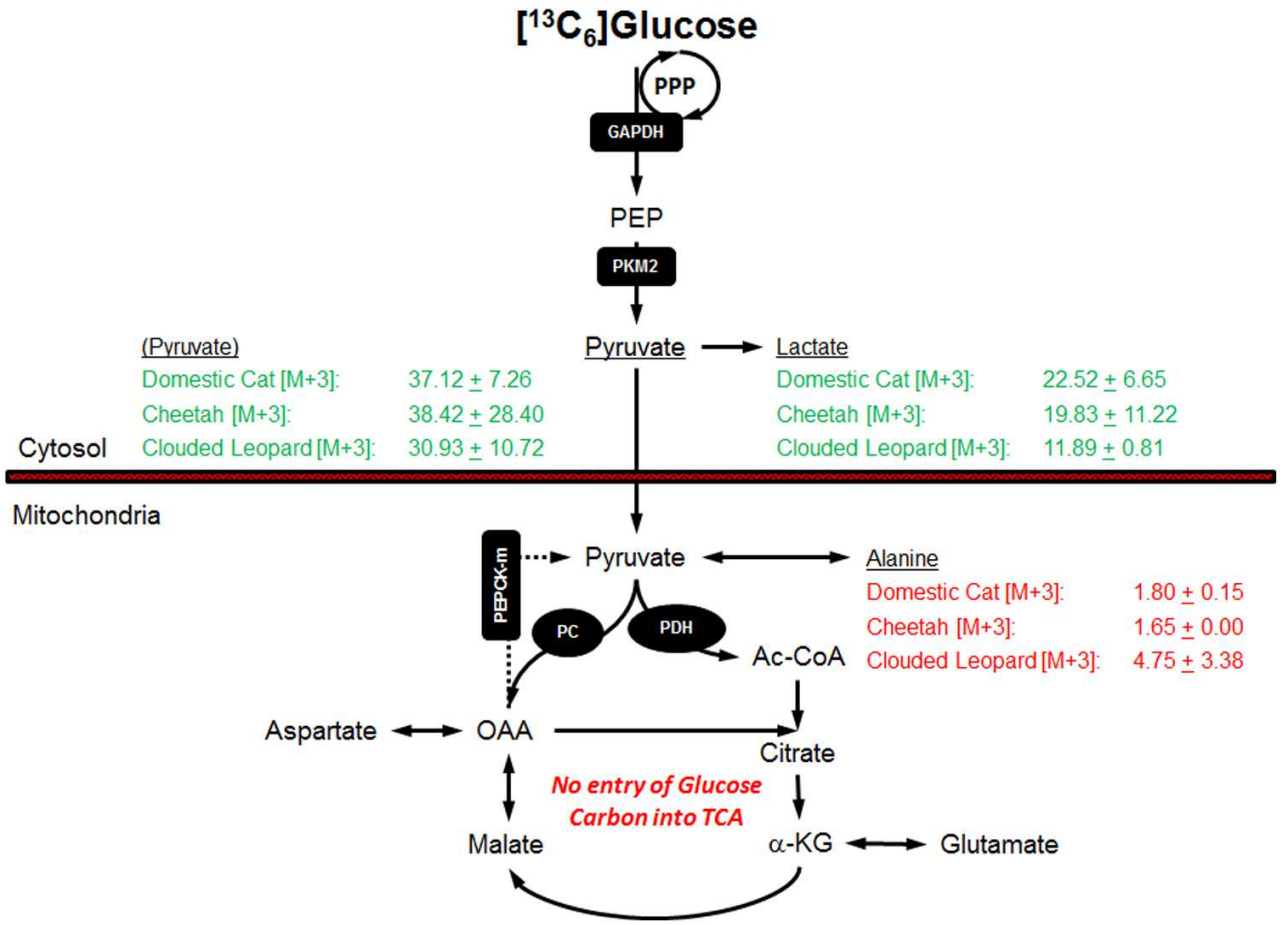

Figure 4.4: Contribution of U13C-glucose tracer to sperm-produced pyruvate, lactate, and alanine. [M+3] expressed as (mean + SEM); green = [M+3] signal is significantly different from environmental levels (i.e. significant tracer contribution); red = [M+3] signal is not significantly different from environmental levels (i.e. no significant tracer contribution).

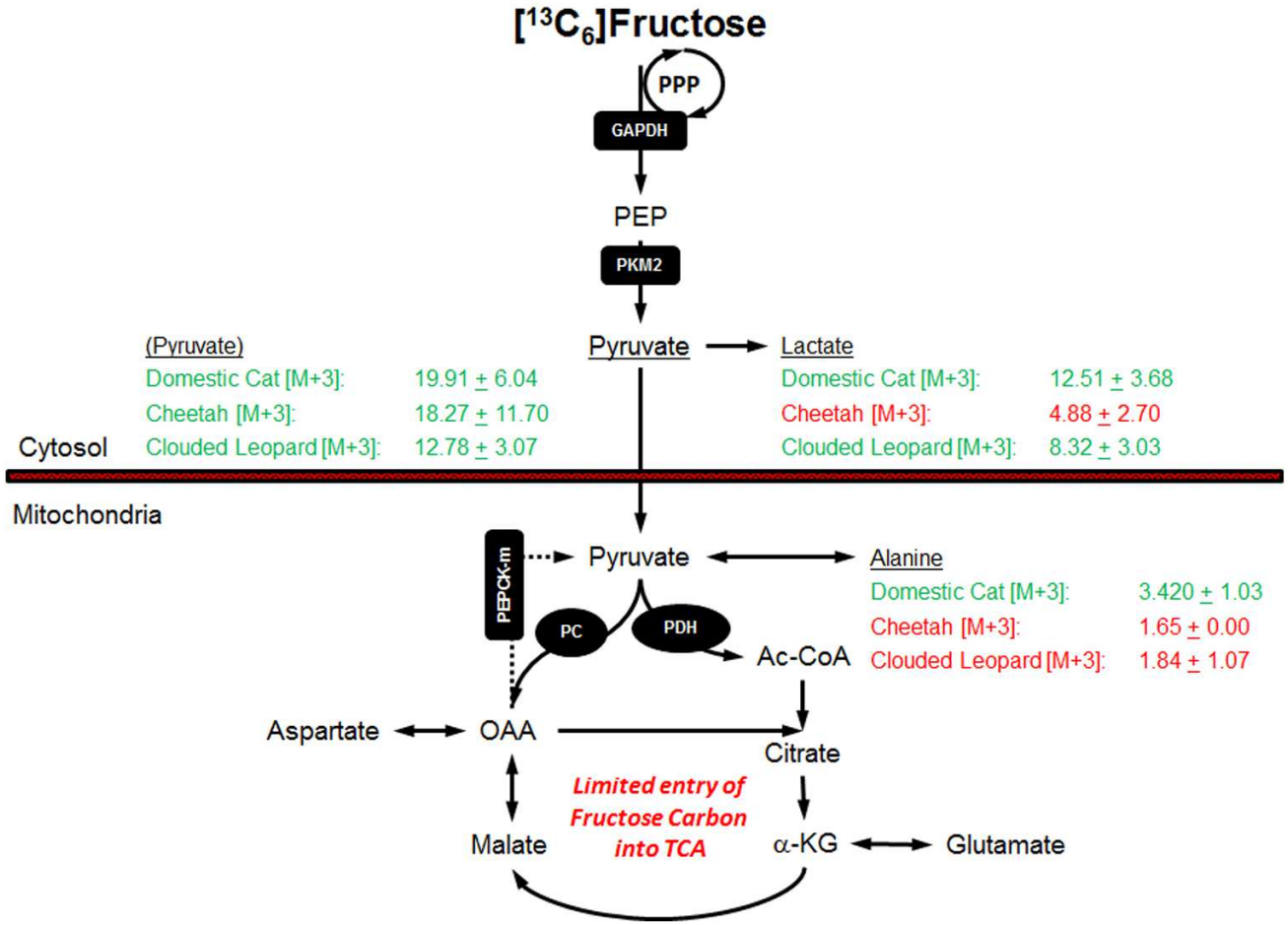

Figure 4.5: Contribution of U13C-fructose tracer to sperm-produced pyruvate, lactate, and alanine. [M+3] expressed as (mean + SEM); green = [M+3] signal is significantly different from environmental levels (i.e. significant tracer contribution); red = [M+3] signal is not significantly different from environmental levels (i.e. no significant tracer contribution).

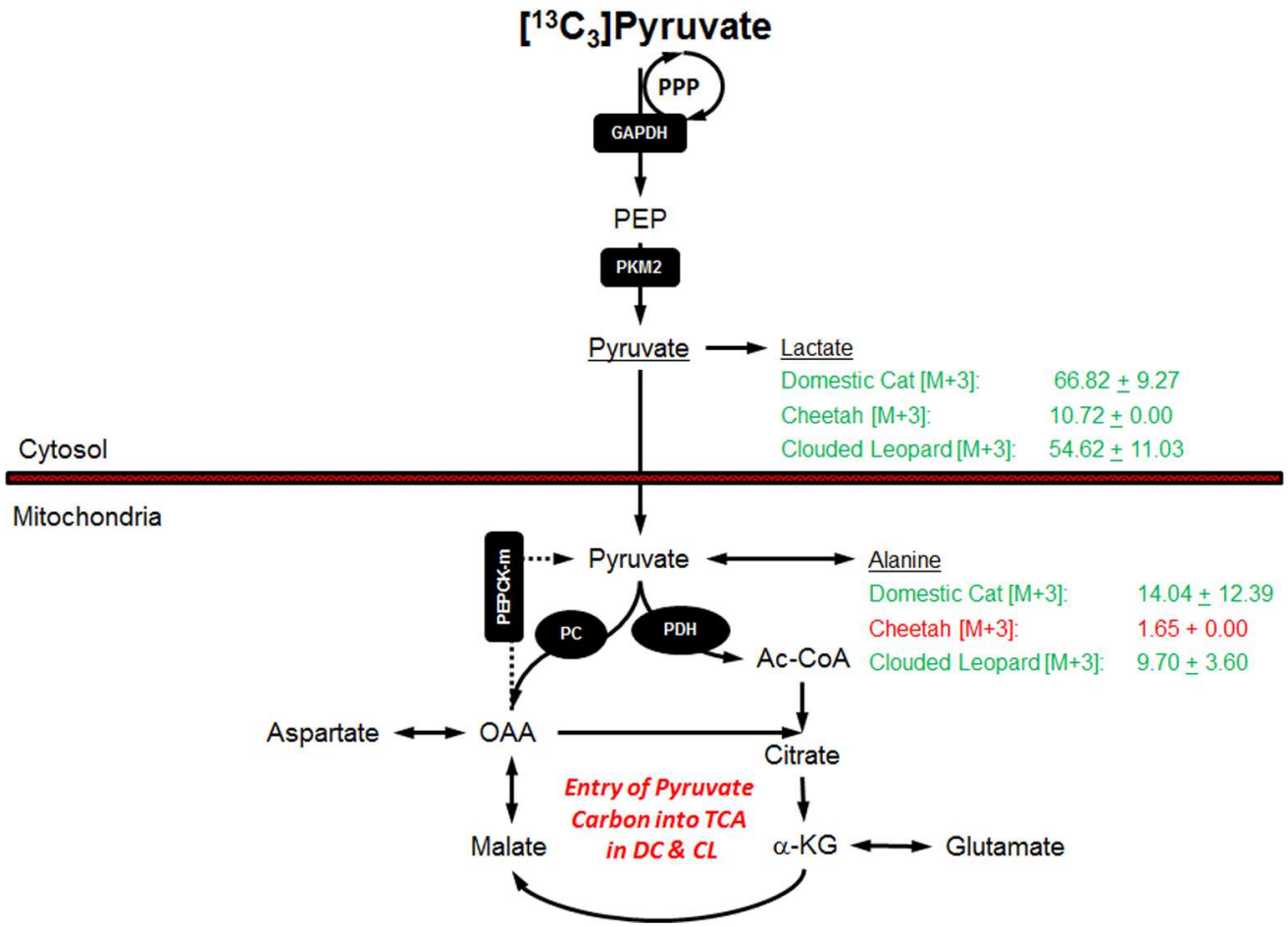

Figure 4.6: Contribution of U13C-pyruvate tracer to sperm-produced pyruvate, lactate, and alanine. [M+3] expressed as (mean + SEM); green = [M+3] signal is significantly different from environmental levels (i.e. significant tracer contribution); red = [M+3] signal is not significantly different from environmental levels (i.e. no significant tracer contribution).

Table 4.2: **Detection of sperm-produced lactate [M+3] ion after incubation with $U^{13}C$-tracers**. Media were supplemented with either $U^{13}C$-Glucose, $U^{13}C$-Fructose, or $U^{13}C$-Pyruvate; E[M+3] = environmental incidence of the [M+3] ion; Epi = Epididymal sperm; EEJ = Electro-ejaculated sperm; (*) = potential falsely negative results due to low sample size; ion ratios were significant when $P < 0.05$.

Species	Tracer	E[M+3]	[M+3]	p-value
Domestic Cat (Epi)	$U^{13}C$-Glucose	1.442	38.94 ± 11.77	0.025
	$U^{13}C$-Fructose	1.442	23.20 ± 8.72	0.044
	$U^{13}C$-Pyruvate	1.442	66.82 ± 9.27	0.003
Domestic Cat (EEJ)	$U^{13}C$-Glucose	1.442	22.52 ± 6.65	0.025
	$U^{13}C$-Fructose	1.442	12.51 ± 3.68	0.029
	$U^{13}C$- Pyruvate	1.442	--	--
Cheetah (EEJ)	$U^{13}C$-Glucose	1.442	19.83 ± 11.22	*0.174
	$U^{13}C$-Fructose	1.442	4.88 ± 2.70	*0.166
	$U^{13}C$- Pyruvate	1.442	--	--
Clouded Leopard (EEJ)	$U^{13}C$-Glucose	1.442	11.89 ± 0.81	0.003
	$U^{13}C$-Fructose	1.442	8.32 ± 3.03	0.054
	$U^{13}C$- Pyruvate	1.442	54.62 ± 11.03	0.008

Table 4.3: **Detection of sperm-produced pyruvate [M+3] ion after incubation with U^{13}C-tracers**. Media were supplemented with either U^{13}C-Glucose or U^{13}C-Fructose; E[M+3] = environmental incidence of the [M+3] ion; Epi = Epididymal sperm; EEJ = Electro-ejaculated sperm; (*) = potential falsely negative results due to low sample size; ion ratios were significant when $P < 0.05$.

Species	Tracer	E[M+3]	[M+3]	p-value
Domestic Cat (Epi)	U^{13}C-Glucose	1.501	30.93 ± 10.72	0.036
	U^{13}C-Fructose	1.501	12.78 ± 3.07	0.018
Domestic Cat (EEJ)	U^{13}C-Glucose	1.501	37.12 ± 7.26	0.008
	U^{13}C-Fructose	1.501	19.91 ± 6.04	0.028
Cheetah (EEJ)	U^{13}C-Glucose	1.501	38.42 ± 28.40	*0.209
	U^{13}C-Fructose	1.501	18.27 ± 11.70	*0.144
Clouded Leopard (EEJ)	U^{13}C-Glucose	1.501	30.93 ± 10.72	0.036
	U^{13}C-Fructose	1.501	12.78 ± 3.07	0.018

Table 4.4: **Detection of sperm-produced alanine [M+3] ion after incubation with $U^{13}C$-tracers**. Media were supplemented with either $U^{13}C$-Glucose, $U^{13}C$-Fructose, or $U^{13}C$-Pyruvate; E[M+3] = environmental incidence of the [M+3] ion; Epi = Epididymal sperm; EEJ = Electro-ejaculated sperm; (*) = potential falsely negative results due to low sample size; ion ratios were significant when $P < 0.05$.

Species	Tracer	E[M+3]	[M+3]	p-value
Domestic Cat (Epi)	$U^{13}C$-Glucose	1.464	1.65 ± 0.00	> 0.05
	$U^{13}C$-Fructose	1.464	1.65 ± 0.00	> 0.05
	$U^{13}C$- Pyruvate	1.464	14.04 ± 12.39	*0.192
Domestic Cat (EEJ)	$U^{13}C$-Glucose	1.464	1.80 ± 0.15	0.058
	$U^{13}C$-Fructose	1.464	3.420 ± 1.03	*0.076
	$U^{13}C$- Pyruvate	1.464	--	--
Cheetah (EEJ)	$U^{13}C$-Glucose	1.464	1.65 ± 0.00	> 0.05
	$U^{13}C$-Fructose	1.464	1.65 ± 0.00	> 0.05
	$U^{13}C$- Pyruvate	1.464	--	--
Clouded Leopard (EEJ)	$U^{13}C$-Glucose	1.464	4.75 ± 3.38	0.217
	$U^{13}C$-Fructose	1.464	1.84 ± 1.07	0.376
	$U^{13}C$- Pyruvate	1.464	9.70 ± 3.60	*0.053

The contribution of $U^{13}C$-glucose, $U^{13}C$-fructose, and $U^{13}C$-pyruvate carbons to sperm-produced metabolites are also represented in Figures 4.4 – 4.6, respectively. Here, the ratio of tracer to tracee ([M+3]/[M+0]) are listed by metabolite to compare substrate utilization by species. $U^{13}C$-Glucose and $U^{13}C$-fructose both entered glycolysis and yielded significant [M+3] pyruvate ions in the sperm of all three species studied. $U^{13}C$-glucose and $U^{13}C$-pyruvate contributed to the production of lactate in the sperm of all three species, while $U^{13}C$-fructose contributed to lactate production only in domestic cat and clouded leopard sperm. Regarding the production of labeled alanine, $U^{13}C$-fructose only contributed to a significant [M+3] alanine ion in the sperm of domestic cats, and $U^{13}C$-pyruvate contributed to a significant [M+3] alanine ion in the sperm of domestic cats and clouded leopards, but not cheetahs.

Discussion

This is the first fluxomics study to be conducted in felid sperm, and provides valuable insights into the roles that glycolysis and the TCA cycle play in this cell type. The information gained from this study reveals several new facts related to felid sperm metabolism: First, we provide undeniable evidence that glucose and fructose are utilized by domestic cat, cheetah, and clouded leopard spermatozoa. Second, the relative abundance of the [M+3] lactate ion is significantly higher than the [M+3] alanine ion, which indicates that carbons donated by the labeled substrates provided in this study were not significantly imported into the mitochondria to contribute to energy production via the TCA cycle and oxidative phosphorylation. Finally, these

findings imply that another source of energy substrate must be present within felid spermatozoa, given that sperm mitochondria have been demonstrated as being functional in felid sperm, and essential for sperm function [59].

The presence of a significant heavy ion in sperm-produced lactate, pyruvate, and alanine demonstrates that felid sperm do utilize exogenous glucose, fructose, and pyruvate as a carbon source, which is contrary to previous reports that felid sperm did not take up exogenous glucose [58]. The innovative use of fluxomics to visualize the uptake of isotopically-labeled hexose substrates proves without a doubt that these substrates are taken up by felid sperm cells and metabolized using glycolysis. This is evident by the presence of a high [M+3] signal in sperm-produced lactate and pyruvate which could only be derived from the labeled hexose substrate. Because it was also demonstrated that the addition of these substrates did not influence motility, the function that hexose substrates (glucose and fructose) play in sperm metabolism is not clear and will require additional research.

The second significant finding in this study relates to the comparison of the ratios of sperm-produced lactate, pyruvate, and alanine. Pyruvate exists in two pools within a cell: the cytoplasmic pool, and the mitochondrial pool. Pyruvate in the cytoplasm exists as an end-product of glycolysis or after uptake from the extracellular environment. However, pyruvate can also traverse the mitochondrial membranes to contribute carbons to the TCA cycle, and thereby contribute to energy production. Measuring pyruvate directly is problematic, as doing so would yield the sum of both the mitochondrial and the cytosolic pools. However, mitochondrial pyruvate can be estimated by measuring alanine due to the conversion of TCA intermediates to

alanine within the mitochondria. Once pyruvate crosses into the mitochondrial matrix to enter the TCA cycle, one of the TCA intermediates – 2-oxoglutarate – can be converted into glutamate by glutamate dehydrogenase, which can then be converted into alanine by alanine aminotransferase. Thus, labeled alanine is representative of the amount of substrate entering the TCA cycle and contributing to oxidative phosphorylation. Alternatively, lactate exists in equilibrium with the cytosolic pool of pyruvate due to the reversible action of lactate dehydrogenase residing in the cytosol. In tracer studies, the only way that lactate and alanine can be labeled is if they are derived from the isotopically labeled substrate. Therefore, by measuring the amount of labeling that occurs in lactate and alanine, we can estimate the amount of pyruvate that is retained in the cytoplasm vs. used in the mitochondria for energy metabolism.

When the lactate pool is more highly labeled than the alanine pool (which is what we see in all four sperm sample types in our study), we can assume two things: first, that the heavy-isotope substrate is not being metabolized in such a way that it is entering the mitochondria for further degradation and energy production through the TCA cycle and oxidative phosphorylation. This is interesting because until recently, it has been assumed that mitochondria were the sole source of energy production in felid spermatozoa. In the mouse, it has been demonstrated that dynein-localized glycolysis could fuel motility along the length of the sperm tail [66,149]. Such findings have led to the hypotheses that as ATP must be readily available within the sperm tail, which may not be possible with mitochondria being located only in the midpiece, other mechanisms for its provision are necessary. Additionally, rather than transporting ATP from the mitochondria to the sperm tail, it would be more efficient

for the sperm to simply produce ATP via glycolysis within the flagellum itself. While this process may not produce as many ATP molecules as mitochondrial energy production would, it may provide just enough energy to fuel motility as necessary. Despite the fact that our findings demonstrate no significant improvement in motility after incubation with glucose or fructose, the low sample size of this study and high individual variability could be masking significant effects of incubating sperm with exogenous hexose substrates. For example while most [M+3] alanine signals were not significantly above what are considered to be normal environmental levels, there are cases where the signal appears to be higher than normal. These signals, while not significant in this study, could become significant with a larger sample size. Therefore, while these results indicate that carbons from glucose and fructose are not entering the TCA cycle or oxidative phosphorylation to any significant extent, further investigation with more biological replicates would help to validate these findings.

In the presence of $U^{13}C$-glucose, $U^{13}C$-fructose, and $U^{13}C$-pyruvate, sperm from all three felid species produced an average [M+3] lactate peak that was significantly higher than the average [M+3] alanine peak. This uneven labeling of lactate and alanine indicates that another source of energy may be contributing to the TCA cycle and fueling oxidative phosphorylation, as the glycolytic substrates/products did not appear to be doing so. Oxidative phosphorylation has been shown to be present, active, and critical for felid sperm function [59], therefore there must be another carbon source present to contribute to the TCA cycle within the mitochondria. As evidenced in figure 4.3, glucose and pyruvate were demonstrated to be contributing almost entirely to the cytosolic pool of pyruvate as evidenced by the

high and significant [M+3] lactate signal compared to the low and not significant [M+3] alanine signal. The conversion of pyruvate to lactate generates NAD+, a cofactor which is required for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an important intermediate enzyme involved in glycolysis as well as the entrance of glycerol into the glycolytic pathway. Thus, since hexoses are typically available in the environment, it is possible that felid sperm use these substrates to keep the glycolytic pathway active. The fact that sperm are able to remain motile in a substrate-free medium for several hours before the percentage of motile sperm declines to 0% indicates that an additional endogenous energy source must exist. Further investigation is necessary to determine the identity of this source, but based on the findings in this chapter, as well as the findings from chapter 3, we hypothesize that these carbons are coming from the breakdown of fatty acids.

Conclusions

From this study, we are able to conclude three things: first, felid sperm do metabolize glucose, fructose, and pyruvate. This is evident by the significant [M+3] ions that are seen in sperm-produced lactate and sperm-produced pyruvate in all three treatment groups ($P < 0.05$). Second, glucose and fructose do not serve as a carbon source that contributes to energy production through the TCA cycle and oxidative phosphorylation. These hexose substrates could be contributing to glycolysis which occurs to fuel minimal motility throughout the sperm tail, but more studies are needed to test this hypothesis. Finally, the incidence of uneven labeling between lactate and alanine implies an alternative carbon-source for energy production within the

mitochondria. Such carbons could come from the metabolism of fatty acids through β-oxidation (as demonstrated in chapter 3). Future research utilizing uniformly labeled fatty acid tracers would enable the visualization of this process, and allow us to determine whether fatty acid oxidation truly plays a role in sperm metabolism.

Chapter 5: Discussion and Future Directions

The research presented in this dissertation represents three distinct steps in understanding felid sperm metabolism. In the first study, the metabolomic profiles of sperm and seminal fluid were characterized in the domestic cat (*Felis catus*), cheetah (*Acinonyx jubatus*), and clouded leopard (*Neofelis nebulosa*). Compounds which differed by sperm status (epididymal, normospermic, teratospermic) and on a species-specific basis were identified, and the relative abundance of each compound was compared. The culmination of the findings in this study set the ground work for future investigations of the differentially detected compounds described. In doing so, we identified target pathways for further investigation of sperm metabolism in felids.

The second study assessed the role of long-chain fatty acid (LCFA) metabolism in felid spermatozoa. In terms of energy production efficiency, the most cost-effective way to generate large amounts of energy is by oxidizing fatty acids through β-oxidation. We therefore hypothesized that spermatozoa – a small, motile cell that performs only for the purpose of fertilization – would benefit by generating as much energy as possible with the least amount of substrate cost. We investigated the potential role of β-oxidation in felid sperm by blocking LCFAs from entering the mitochondria using the chemical inhibitor etomoxir. As previously mentioned, etomoxir has been proven effective in a variety of different cell types (including human spermatozoa), and works by blocking the regulatory carnitine:palmitoyl-transferase I enzyme which modifies fatty acids for transport through the

mitochondrial membrane. From this study, we are able to make three conclusions about the effect of etomoxir on felid sperm: (1) treatments with etomoxir and pyruvate enabled us to see a biphasic shift in motility after two hours of incubation in the domestic cat and the cheetah, (2) etomoxir had a measurable effect on the overall motility of cheetah sperm, but not on the motility of domestic cats or clouded leopards, and (3) etomoxir had a measurable effect on the relative abundance of a variety of different metabolites. During key steps in sperm function such as capacitation and hyperactivity, sperm go through dramatic physiological changes [139,142]. The observed shift in motility that we see after two hours of incubation with etomoxir and pyruvate could indicate a shift in the utilization of different metabolic pathways due to the presence of endogenous and exogenous energy substrates. Not only was it important to identify compounds that were differentially detected among treatment groups, but the detection of such compounds provides targets for future research. Further investigation of these metabolites could establish how they were affected by altered substrate conditions and what their role is in relation to sperm function. In summary, this study revealed that β-oxidation is an active metabolic pathway in felid sperm based on the change in motility of cheetah sperm, and the differentially detected compounds resulting from etomoxir treatment.

The third study utilized an innovative fluxomic approach to measure the contribution to cellular metabolism of substrates labeled with heavy isotopes. We made several critical findings. First, sperm from all three species demonstrated the active metabolism of glucose and fructose through the glycolytic pathway. Additionally, measurements of ion ratios demonstrated that a significant proportion of

the hexose substrate entering into spermatozoal metabolism is converted to lactate rather than entering the mitochondria to further energy production via the TCA cycle and oxidative phosphorylation. This confirms that glycolysis is active in felid sperm; however, the data presented suggest that glycolysis may be important for functions other than motility. Additionally, the fact that glucose and fructose contributed to the production of lactate but did not have an effect on motility, and that sperm motility continued in the absence of any exogenous substrates, indicates the presence and role of alternate energy substrates.

There are many future directions that are prompted by this research. Topics of interest include investigating the potential roles of differentially detected compounds between sperm from normo- and teratospermic individuals, the existence of endogenous energy sources, and the role of β-oxidation in felid sperm using ^{13}C-labeled fatty acids. In addition to the metabolomic and fluxomic approach that we have utilized in the studies presented in this dissertation, investigation of proteomic expression could be conducted to illustrate the enzymes and regulatory mechanisms, as well as the location of such processes that govern sperm metabolism in different species. Additionally, how teratospermia asserts its influence on sperm function is a topic that continues to puzzle reproductive biologists. Whether teratospermia is the cause of metabolic dysfunction, or if metabolic dysfunction causes teratospermia is still up for debate. If we can understand how and why this condition persists, we may be able to approach assisted reproduction with afflicted individuals in a species or condition-specific manner. Finally, while the presence of endogenous energy substrates is not a new idea in the study of sperm biology and metabolism, little is

known with regards to what these substrates are and how they are stored and/or carried by spermatozoa. Further investigation is necessary to determine what other endogenous metabolic substrates are present in felid sperm, where these substrates are stored, and how they are mobilized for use in energy production.

With relation to endogenous energy substrates, the differential detection of lactate in our research presents a puzzling concept: in addition to a product of glycolysis, could lactate also be an energy substrate? And if so, why do we see a shift in relative lactate abundance when β-oxidation is inhibited? Previous research in rat spermatids (immature spermatozoa) showed lactate as an energy substrate after being converted to pyruvate within the cell [150]. Perhaps this could also be the case with felid spermatozoa, in the sense that these cells maintain an immature-like metabolic state. If teratospermia is highly prevalent in felid species, and lactate is an indicator for sperm quality in these animals [58], perhaps teratospermia manifests from incomplete or dysfunctional spermatogenesis, and the ejaculation of spermatozoa which are not fully mature. This hypothesis requires extensive further investigation, but such research could generate a better understanding of what teratospermia is and how to mitigate its effects.

Unfortunately, investigating these processes in felid sperm lends itself to many challenges. The animals we work with are not specifically used for research in the way that mice or other domestic species can be which makes the use of advanced manipulation techniques more difficult and expensive. Furthermore, studying the spermatozoa of these animals is limited to what we are able to observe on a post-translational and phenotypic level. Our research involving metabolomic and fluxomic

analysis of metabolism has demonstrated substantial advantages to studying metabolism in wildlife species. Unlike research which requires the use of species-specific antibodies or primers, it is of no consequence that such antibodies or primers are not readily available: the techniques behind metabolomics and fluxomics are not species-specific, as metabolic pathways across taxa are known to be highly conserved. Additionally, limited manipulation is required to visualize the enzymatic activity of metabolic pathways. Simply by conducting brief incubations with tracer molecules, we are able to measure enzymatic activity through the transformation of substrate to product.

In addition to being an innovative approach, the combination of metabolomics and fluxomics holds tremendous potential for increasing our understanding of the basic biology of wildlife species. These approaches do not require invasive procedures, but rather only more discrete manipulation of the substrates that are entering cellular and biological processes. Isotopic tracers hold much promise in the way of studying nutrient utilization in that they are safe, easy to use and store, and have a wide variety of applications. Stable, non-radioactive isotopes exist naturally in the environment, and do not cause deleterious effects in the same way that radioisotopes could. Therefore, the implementation of this technique in rare and endangered species could be an efficient and worthwhile way to gain information quickly and reliably. Furthermore, the storing of samples for metabolomic or fluxomic analysis is simple. Isotopically labeled substrates typically do not require refrigeration (dependent on the substrate of interest) and sample storage only requires that metabolic processes be halted, which typically only requires a freezer. Such

methods could easily be conducted in remote field locations, as is done with other more complicated research methods for studying wildlife. Finally, using a fluxomic approach is an informative way to study cellular metabolism, enzymatic function, and countless other applications for previously un-characterized processes in wildlife species. In a captive situation, it would even be possible to conduct whole-animal studies, simply by feeding isotopically labeled substrate to the study animal in question through the diet. With regards to identifying an endogenous energy source for felid sperm, feeding an animal labeled hexoses, fatty acids, or amino acids through the diet for several weeks or months would allow such substrates to be incorporated throughout the animal's physiology. A study could take place for the length of the spermatogenesis, and then sperm could be analyzed using mass spectrometry to determine whether such substrates were evident. This, and many other similar studies, could raise the bar on biochemical research in wildlife species. The fields of metabolomics and fluxomics are just beginning to find their way into the world of conservation biology, but there is great potential for the use of these techniques to gain critical and basic information about the inner workings of rare and endangered species.

In summary, the culmination of the three studies presented in this dissertation demonstrated many new facts about felid sperm metabolism. A summary of the information presented in this dissertation can be seen in Figure 5.1.

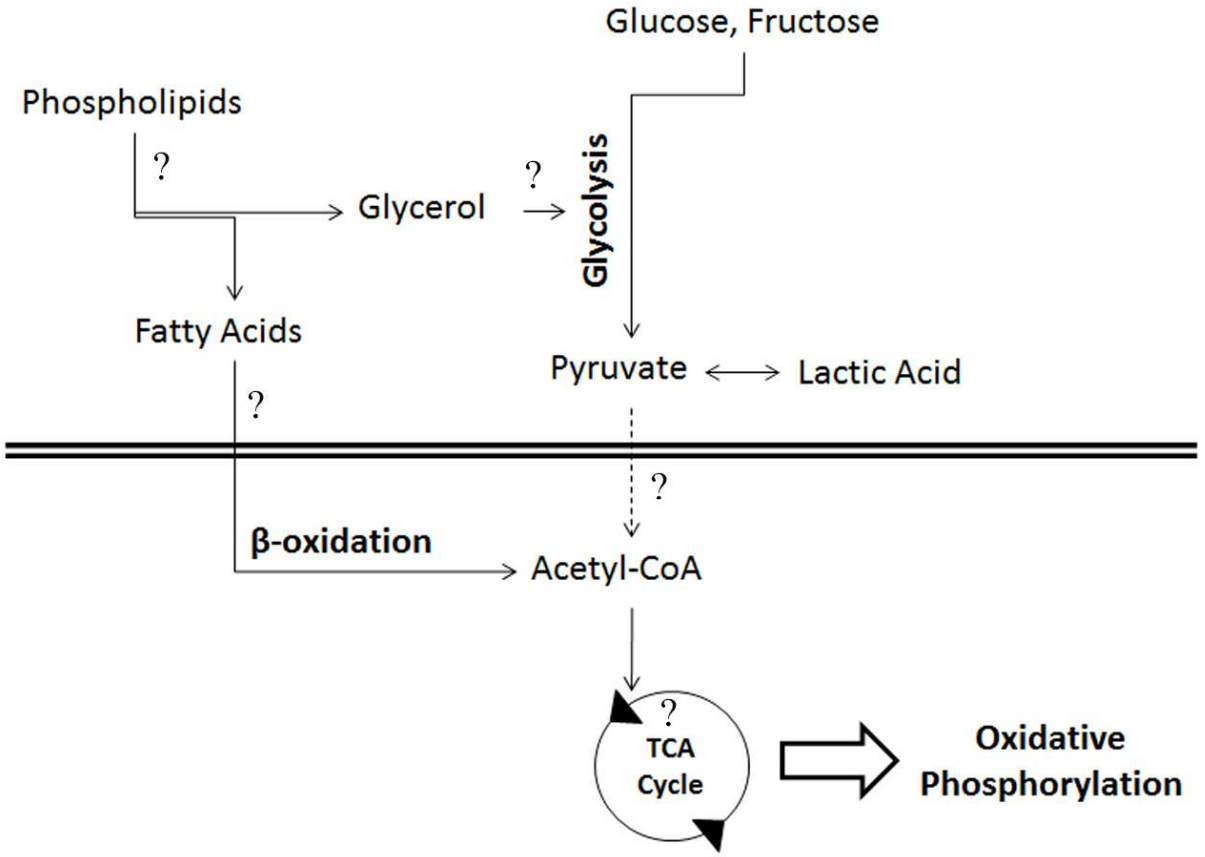

Figure 5.1: Summary and proposed mechanism of felid sperm metabolism. Bold text = metabolic pathways; double line = mitochondrial membranes; dotted arrow = limited movement of substrate; ? = targets of future investigation.

These studies demonstrate that glucose and fructose are metabolized via glycolysis in the domestic cat, cheetah, and clouded leopard, and these substrates are further utilized to synthesize lactate. Additionally, there was evidence to suggest that pyruvate derived from glycolysis is not contributing to mitochondrial energy production. We hypothesize that the breakdown of fatty acids through β-oxidation could be the carbon source that provides substrates to fuel the TCA cycle and oxidative phosphorylation, possible derived from the breakdown of phospholipids. Future research will focus on further elucidating the role of β-oxidation to felid sperm function, and the role that teratospermia plays in the function of these critical metabolic pathways. It is my hope that the information presented in this dissertation will inspire further investigation of metabolic processes related to reproduction, as well as lead to the development of improved assisted reproductive techniques for wildlife species.

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