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Effects of Age and Mitochondrial Functionality on Canine Sperm Quality: Implications for Canine Fertility



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Effects of Age and Mitochondrial Functionality on Canine Sperm Quality: Implications for Fertility

By

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DISSERTATION

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## Table of Contents

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## Preface

This thesis and the research from which it is composed is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Integrative Pathobiology, with a designated emphasis in Reproductive Biology, in the office of Graduate Studies of the University of California, Davis (UCD). This work was completed in the UCD Gamete Biology Laboratory within the Department of Anatomy, Physiology, and Cell Biology of the School of Veterinary Medicine (SVM) and was made possible by funding from the Center for Companion Animal Health (CCAH; UCD SVM), and the North American Great Dane Charitable Trust.

**Dissertation Abstract**

Not all sires have sperm suitable for chilled or frozen storage, and success in artificial insemination (AI) varies highly among individual dogs and breeds. Fertilizing potential is further complicated as sperm quality declines with the aging process. The overall objective of this work was to study the effects of age and mitochondrial function on sperm quality and bioenergetics using traditional methods assessed in the clinical setting, as well as emerging methods of investigating metabolic flexibility and the heterogenous nature of sperm. This dissertation explores the physiology and pathophysiology of canine sperm through the study of a breeding colony of highly fertile Labrador retrievers, and a rapidly aging purebred population of North American Great Danes. This was accomplished in three chapters.

Chapter 1, *Comparative Oxidative Metabolism in Mammalian Sperm*, provides a review of the literature and current understanding of mitochondrial physiology and metabolic control of essential sperm functions in mammalian species of veterinary importance. We discuss the differences in fundamental oxygen and ATP substrate balance in stallions, bulls, and dogs, with an emphasis on the maintenance of sperm motility, electron transport chain (ETC) function, reactive oxygen species (ROS) production, and the balance of glycolytic and oxidative phosphorylation production of ATP in sperm.

In Chapter 2, *Sperm Parameters in the Great Dane: Influence of Age on Semen Quality*, I characterized the distribution of sperm quality parameters within a purebred population of actively showing North American Great Danes, a rapidly aging breed currently suffering declining fertility. I identified progressive declines in sperm quality that accompany aging, detailed the relationship between ROS production and sperm motility and morphology, and distinguished sources of variation in sperm quality within the breed. Age was negatively

associated with several sperm motility parameters associated with sperm fertility such as total and progressive motility, and amplitude of lateral head displacement ($p<.05$), with a predicted -9.9%, -9.0%, and +8.3% change per year of age. Sperm of younger GD dogs aged $12 \leq x < 24$ months had significantly higher total and progressive motility (TM, PM), amplitude of lateral head displacement (ALH), and nonlinear motility ($p<.05$) than older dogs ($x \geq 48$ months). Results support anecdotal reports of decline of the fertility with the advance of age in this breed, and indicate that age and ROS have significant influences on sperm parameters in the GD. The influence of selection for breed specific phenotypes could help explain the functional significance of the diversity among GD males.

In Chapter 3, *Effects of Substrate Availability and Mitochondrial Disruption on Oxidative Metabolism and Sperm Motility in Fertile Dogs,* I investigated the adaptability of canine sperm bioenergetics under differing nutrient conditions and the effects of mitochondrial dysregulation on sperm motility. With simultaneous measurement of mitochondrial oxygen consumption (MITOX) and sperm kinematics, I observed a high adaptability and metabolic flexibility of high-quality canine sperm. While all energetic substrates tested successfully maintained sperm motility, significant differences were found in MITOX and mitochondrial spare respiratory capacity (SRC), the strength with which mitochondrial energy production may rise to meet energetic challenge. In addition, the ability to maintain sperm kinematics when supplemented with various energetic substrates differed significantly with mitochondrial disruption by mitochondrial effectors. A population of Labrador retrievers from a highly fertile breeding colony managed by Guide Dogs for the Blind was chosen for this study due to their high semen quality, good health, and known fertility.

**Dissertation Introduction and Outline**

Sperm are highly specialized cells tasked with delivering the paternal haploid genome to the oocyte. Fusion with the oocyte and fertilization occurs after sperm successfully traverse the diverse and hostile conditions of the female genital tract. The maintenance of essential sperm functions such as motility patterns associated with sperm quality and fertility have a high energy requirement. Sperm are specialized for these highly energy dependent processes despite their small size and few organelles.

Unsurprisingly, significant physiological and metabolic differences have been found between mammalian species, representing the adaptation to different environmental and nutrient conditions. Though routine semen analysis includes the measurement of microscopic and biochemical endpoints, little is known regarding the pathophysiology of canine sperm. In addition to interspecies differences in bioenergetics and control of key fertilizing events, intraspecies variation reflects the heterogenous nature of sperm populations and the effects of age and mitochondrial dysfunction on sperm quality. A greater understanding of the effects of age and energy management on canine sperm quality will improve male fertility, semen cryopreservation, and breeding management.

The overall objective of this dissertation research was to study the effects of age and mitochondrial function on sperm quality and bioenergetics. This work explores the pathophysiology of canine sperm using emerging methods to investigate metabolic flexibility and the heterogenous nature of sperm, including measurement of mitochondrial oxygen consumption (MITOX) and subpopulation analysis.

This was accomplished by the works reported in the following chapters:

Chapter 1 provides a review of oxidative metabolism in mammalian sperm and explores inter- and intra-species variation in sperm bioenergetics driving essential sperm functions.

In Chapter 2, we characterized the distribution of sperm quality parameters within a purebred population of actively showing North American Great Danes. We distinguished sources of variation in sperm quality and identified changes in sperm quality that may accompany aging. Great Danes were chosen for this study due to being a large, rapidly aging breed, and the strengths of a conformationally correct, single-breed population currently experiencing a decline in fertility.

In Chapter 3, we investigated the adaptability of canine sperm bioenergetics under differing nutrient conditions and the effects of mitochondrial dysregulation on sperm motility. Through simultaneous measurement of MITOX and sperm kinematics we explored the ability of high-quality canine sperm to meet energetic challenges, and the relationship between mitochondrial function and sperm kinematics. A population of Labrador retrievers from a highly fertile breeding colony managed by Guide Dogs for the Blind was chosen for this study due to their high semen quality, good health, and known fertility.

**The dissertation includes the following publications:**

Foutouhi A, Meyers S. Comparative oxidative metabolism in mammalian sperm. Animal Reproduction Science 2022;247:107095.

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# Chapter 1: Comparative Oxidative Metabolism in Mammalian Sperm

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## Introduction

Sperm are highly specialized cells tasked with delivering genomic material to the ovum during sexual reproduction thereby forming genetically unique offspring through the fusion of the two haploid gametes. In contrast to 'primitive' spermatozoa from lower vertebrates and invertebrates, which are structurally simple and participate in external fertilization such as in aquatic conditions, the sperm of internal fertilizers are specialized to the internal environment of the female reproductive tract [1]. Sperm of internal fertilizers such as mammals have the greatest diversity in structure and function, reflecting genetic modifications and evolutionary adaptations.

Primitive sperm have a simple morphological form with a small acrosome and a short midpiece containing few or no discernable mitochondria arrayed on the basal portion of the flagellum. Broadcast sperm are deposited on or near the ova and fertilization occurs quickly. Because they are deposited into a pre-fertilization environment devoid of metabolizable nutrients they depend entirely on intracellular reserves for energy production. In contrast, the modified sperm of internal fertilizers, such as mammals, have evolved specific structural and physiologic characteristics to overcome the hazards of the internal environment and traverse great distances to the oocyte. Modifications to the sperm head and midpiece in mammals include a membrane-limited acrosomal cap over the sperm nucleus that contains the enzymes necessary for penetration of the corona radiata and zona pellucida. The head of the sperm also imparts increased structural complexity and anchoring of the flagellar motor apparatus, and an elongated middle piece (midpiece) consisting of 50-75 mitochondria that are stabilized, or fixed, and arranged helically and end-to-end within the mitochondrial sheath [1, 2].

Mitochondrial activity is essential to sperm functionality and has been proposed to be an important biomarker of sperm quality and male fertility. Mitochondria are central to sperm

bioenergetics and are the site of production of adenosine triphosphate (ATP) and reactive oxygen species (ROS), and calcium regulation which are integral to sperm motility, hyperactivation, capacitation, and acrosome reaction. Recent work has shown sperm can use different metabolic pathways to support these heavily energy-dependent processes and have identified species-specific preferences in the metabolic substrates supporting key sperm functions.

The two main pathways for ATP production in mammalian sperm are 1) glycolysis which occurs in the cytosol overlaying the sperm tail, and 2) oxidative phosphorylation (OXPHOS) in the mitochondria. OXPHOS is more energy-intense and produces 16 times more ATP than glycolysis and is a major source of ROS. OXPHOS occurs by way of the mitochondrial electron transport chain (ETC) located in the inner mitochondrial membrane (IMM). Metabolic flexibility may be advantageous wherein the oviductal environment may induce a shift from one metabolic pathway to another. The purpose of this review is to discuss current research in mitochondrial oxidative metabolism and effects of reactive oxygen species on sperm in stallions, bulls, and dogs. A brief review of mammalian sperm mitochondrial anatomy and physiology will preface the discussion.

**Anatomy and Physiology of Sperm Mitochondria**

Mitochondria are semiautonomous organelles possessing their own genome and are found in almost all eukaryotic cells due to endosymbiosis between α-proteobacteria and a eukaryotic ancestor. Sequencing studies in the last two decades have shown mitochondrial genomes are highly susceptible to DNA damage and mutation and have undergone a ‘reductive evolution’ [3]. Some mitochondrial genomes are estimated to have retained less than 1% of their

content, but ATP production coupled to electron transport, and the translation of mitochondrial proteins are common to all mitochondrial genomes [4, 5].

The mitochondrial sheath of the sperm midpiece is wrapped around the proximal portion of the sperm tail which consists of a 9+2 microtubule arrangement surrounded by nine outer dense fibers (ODF) which provide additional mechanical support and enable navigation within the highly viscous environment of the female reproductive tract [6, 7]. The midpiece is a key factor influencing motility and bioenergetics, and studies on microneedle dissected and demembranated sperm have shown the flagellar apparatus of mature sperm have ATP-dependent autonomous functionality in motility generation [8, 9]. However, mitochondrial volume has a stronger correlation with ATP content than measures of sperm velocity, suggesting additional factors influence sperm motility [10].

Mitochondria are comprised of four defined compartments: the outer mitochondrial membrane, the inner mitochondrial membrane (IMM) where the electron transport chain (ETC) is located, the intermembrane space where the mitochondrial membrane potential (MMP) is maintained, and the mitochondrial matrix where the Krebs cycle generates reduced electron carriers for the ETC. The four respiratory enzyme complexes of the ETC participate in a series of redox reactions which couple the oxidation of reduced inputs to the phosphorylation of ADP to ATP by ATP synthase. Three of these respiratory complexes use redox energy to translocate protons across the IMM into the intermembrane space, establishing the proton motive force used by ATP synthase. This strong proton gradient has both a chemical (pH) component, and electric component which can be measured as the mitochondrial membrane potential (MMP) and is correlated with sperm competency [8].

With its high reduction potential, oxygen is reduced to water at Complex IV, which is the final redox reaction in OXPHOS and a major source of oxygen consumption in cellular respiration [9]. Recent studies in mammalian sperm have used the measurement of oxygen consumption to better understand mitochondrial energetic metabolism under different conditions and have found it to be positively correlated with motility in several species and a sensitive indicator of sperm mitochondrial health [10, 11].

In addition to involvement in ATP production, redox equilibrium, calcium homeostasis, and apoptotic pathways, the ETC is a major source of ROS such as superoxide anion radical ($O_2^-$) and hydrogen peroxide ($H_2O_2$) which function in essential signaling pathways and cause oxidative damage if left unchecked [12]. Mitochondrial ROS production is a factor of oxygen availability, redox state of the ETC complexes, and the MMP, indicating a delicate balance between mitochondrial activity and the maintenance of essential sperm function that can be upset by age, disease, and cryopreservation [8, 13-15].

To maintain balance, sperm possess robust antioxidant systems in both seminal plasma and within the plasma membrane. Sperm mitochondria are significant contributors to oxidative stress, and it is generally accepted that ROS production is associated with poor sperm function and subfertility [16, 17]. However, recent work with stallions identified a positive correlation between sperm motility and oxidative stress, suggesting the most fertile ejaculates had the highest levels of oxidative stress [18, 19]. Current work in our laboratory studying age-related decline of sperm quality in North American Great Danes identified significant positive relationships between ROS and total motility and measures of velocity and distance [47]. Given a suitable ionic environment and sufficient energetic support mature sperm can achieve the fundamentally different modes of flagellar beat associated with linear swimming and

hyperactivation which are necessary for transit through the female reproductive tract and fertilization. Understanding the efficiency of mitochondrial respiration can be useful to better understand the changes in mitochondrial energetic metabolism across mammalian species, particularly with regard to domestic species and the clinical management of sperm cryopreservation. The following discussion highlights some of the similarities and differences in the metabolic substrates and pathways underlying sperm motility among the three species we have studied in our laboratory and is meant to begin a comparative discussion of species-specific adaptations of sperm bioenergetics.

**Oxygen Consumption and Physiology of Equine Sperm**

Mitochondrial oxygen consumption (MITOX) is an important parameter of mitochondrial health and is a good measure of the bioenergetics driving sperm motility. Mitochondrial activity as measured by MITOX is predictive of sperm motility and is a reliable measure of equine sperm health and function [20, 21]. There is growing evidence that OXPHOS is the essential metabolic pathway supporting sperm motility and the maintenance of membrane integrity in stallions[22]. Several studies have shown that disruption of the mitochondrial ETC with mitochondrial inhibitors results in significant reductions in sperm velocity and ATP levels, while the inhibition of glycolysis has a smaller impact on motility [18, 23].

Despite the well characterized presence of glucose transporters in equine sperm, the energy demand for motility is not typically met by glycolysis alone [24]. Nevertheless, glycolysis may have a moderate role in maintaining stallion sperm velocity after long incubation periods in depleted media [23]. However, treatment with the anti-diabetic drug rosiglitazone has been found to enhance the metabolic flexibility of equine sperm by allowing more efficient glucose

metabolism, resulting in increased motility and ATP levels accompanied by a decrease in ROS production [25]. These findings implicate novel strategies for exploiting the metabolic plasticity of equine sperm to enhance functionality and improve long-term preservation methods.

Recent work measuring MITOX under different metabolic conditions have confirmed that some level of existing versatility allows sperm to utilize a variety of substrates available in commercial medias and within the female reproductive tract [19, 20]. Results indicated a preference in equine sperm for pyruvate and lactate rather than glucose as precursors for OXPHOS and identified a dose response relationship between increasing mitochondrial function and substrate concentrations. An isoform of lactate dehydrogenase, LDH-C, has been confirmed within the mitochondrial matrix of equine sperm, allowing the conversion of exogenous lactate to pyruvate for further catabolism into highly reduced inputs for the ETC [26]. These MITOX studies have emphasized that glycolysis and OXPHOS pathways do not function exclusively in equine sperm and found that glucose alone was sufficient for sperm motility by glycolytically providing pyruvate to the mitochondria when favored substrates were unavailable. Supplementation of pyruvate containing media with L-carnitine has been shown to sustain ATP production, reduce oxidative stress, and maintain acceptable motility for use in AI, potentially enhancing the commercial viability of stallions that tend to have poor results in cooled semen [27].

Mitochondrial dysfunction in sperm is a significant result of stallion aging and cryopreservation-related damage, which result in MITOX alterations and decreased sperm motility [21]. Proteomic profiling of equine sperm suggests that cryopreservation results in alterations to key proteins involved in MITOX and redox regulation [28]. MITOX function increases with stallion age, reaching a peak of superior mitochondrial functionality and sperm

motility at 12-13 years. A subsequent reduction in MITOX is mirrored by decreases in motility and velocity parameters and highlights the need to address declining sperm mitochondrial function. Equine sperm depend on mitochondrial oxidative metabolism, so extenders and cryopreservation media should be formulated with the specific metabolic inputs to support optimal mitochondrial functionality.

**Oxygen Consumption and Physiology of Bovine Sperm**

Bovine sperm have been shown to utilize a variety of substrates such as glucose, fructose, mannose, and pyruvate as well as lipids and phospholipids to meet energy demands and sustain motility [29, 30]. Understanding that mammalian sperm bioenergetics are adapted to the substrate profile of the female reproductive tract, the low availability of oxidizable substrates in the bovine oviductal lumen raises questions about mitochondrial support of sperm motility [31]

Despite findings that ATP diffusion from the mitochondrial sheath to the distal end of the flagellum is sufficient to support contractile activity of bovine sperm, it has long been thought that OXPHOS was unnecessary to support sperm motility [32]. Nevertheless, mitochondrial activity is a crucial parameter for sperm quality and fertility in bulls [33].

Early work investigating the role of mitochondrial activity in maintaining bull sperm functionality found a dependence on OXPHOS to support capacitation but proposed that OXPHOS was not necessary to support sperm motility when provided with glycolytic substrates [34, 35]. However, the upper fraction of bovine sperm swim-up preparations has been found to possess higher motility and greater mitochondrial oxygen consumption rates, suggesting highly motile sperm utilize mitochondrial metabolism to support increased energetic demand [36].

A recent study exploring the relationship between MITOX and sperm motility in medias mimicking the female reproductive tract (low glucose) and traditional semen processing methods (high glucose) identified an inhibitory effect of glucose on bull sperm mitochondria [37]. However, there was no difference in motility between sperm incubated in either high or low glucose media. Regardless of media treatment, frozen-thawed sperm had higher mitochondrial activity, which was largely attributed to increased proton leakage due to mitochondrial damage from cryopreservation. Proton leakage was negatively associated with sire conception rate.

Recent MITOX work investigating the relationship between mitochondrial ETC function and sperm kinematics show bovine sperm flagellar motility is impacted by, but not dependent on OXPHOS [38]. Mitochondrial oxidative metabolism was negatively correlated with local motility, but positively correlated with kinematic measures of distance and velocity.

When supplemented with glycolytic substrates, reduction of mitochondrial function by inhibition of ATP synthase or with the use of uncoupling agents did not significantly reduce sperm motility. However, inhibition of the ETC with Antimycin-A significantly decreased total and progressive motility and other kinematic measures highly correlated with fertility in fresh bull semen [39]. These findings suggest bovine sperm motility can be maintained in the absence of oxidative metabolism but highlight a dependence on mitochondrial energetics to maximize motility parameters associated with fertility.

**Oxygen consumption and physiology of Canine Sperm**

Canine sperm have been observed to maintain a significant degree of motility with supplementation of a wide range of metabolic substrates, and we have found that when provided with media containing energetic substrates such as glucose and fructose, inhibition or uncoupling

of MITOX does not affect canine sperm total or progressive motility. As in equine and bovine sperm, the presence of glucose transporters (GLUTs) has been confirmed in canines, but distribution differs markedly from that of other species [24]. GLUT localization near the acrosome rather than tail structures where glycolytic enzymes are primarily located implicates energy reservoirs in the form of glycogen. The existence of a fully functional gluconeogenic pathway in mammalian sperm was first demonstrated in the dog, and gluconeogenesis-linked glycogen metabolism is important in the maintenance of In vitro capacitation of dog sperm in media devoid of glucose [40, 41].

The incredible metabolic flexibility exhibited by canine sperm highlights species-specific strategies of energy management mechanisms modulating critical sperm functions. Due to the lengthy time between ejaculation and fertilization which can reach 11 days in dogs, these long-term energy stores established by glucose and fructose availability may help maintain sperm bioenergetics during the uniquely long storage in the oviduct and transit to the oocyte in canids [42].

Previous work investigating the relationship between motility and mitochondrial function in canine sperm identified an association between progressive motility and high MMP, but suggested MMP measures assessed by JC-1 fluorescence were more strongly correlated to sperm plasma membrane integrity [43]. Recent studies in our lab show mitochondrial oxygen consumption is a reliable indicator of mitochondrial function and is correlated with canine sperm motility measures of distance and velocity. By measuring MITOX responses to ETC inhibition or uncoupling and supplementation with multiple energy substrates, we have demonstrated the following relationships between canine sperm bioenergetics and motility.

All energy substrates including lactate and pyruvate, glucose, and fructose supported total and progressive motility, as well as kinematic measures of distance and velocity. Uncoupling MITOX from ATP production revealed a large spare respiratory capacity in dog sperm, which indicates the robust ability to increase mitochondrial function during high energy demand and other stress conditions. Mitochondrial uncoupling did not affect sperm motility parameters when supplemented with either glucose or fructose, although there is evidence indicating these sugars act differently on the mechanisms controlling motility of cooled canine sperm [44].

MITOX was significantly correlated with amplitude of lateral head displacement and measures of distance and velocity in complete media. Meanwhile, MITOX was predictive of total and progressive motility, distance measures, and linear and oscillatory movement with inhibition of ETC and ATP synthase, indicating mitochondrial influence on motility patterns. Inhibition or uncoupling of MITOX reduced average pathway velocity and average pathway distance in all medias, with the largest effect seen in medias containing only lactate and pyruvate. In addition, when media containing only lactate and pyruvate were provided, inhibition of mitochondrial activity by antimycin-A and oligomycin nearly eliminated progressive motility. While eliminating ATP production by OXPHOS in the absence of alternative substrates severely affected motility, viability remained high, demonstrating that canine sperm possess alternative pathways to support membrane integrity and other essential functions. This contrasts with equine sperm, which depend on both glycolysis and OXPHOS for motility and require mitochondrial ATP production for the maintenance of membrane integrity [22].

Canine sperm enjoy greater metabolic plasticity than that of many other species including equine and bovine, with energy substrates possibly acting as function modulators beyond their role in energy production. As total and progressive motility and velocity decrease with age and

preservation in canine sperm, MITOX work emphasizes how the interplay between metabolic pathways and energy substrates can be exploited to influence sperm motility and support optimal functionality [45, 46].

## Conclusions

The aim of this review was to discuss the role of mitochondrial bioenergetics in the maintenance of mammalian sperm motility and fertility through comparison between equine, bovine, and canine comparative oxidative metabolism. Although MITOX is essential to mammalian sperm functionality and is an important biomarker of sperm quality and male fertility, the metabolic pathways underlying motility and key sperm functions are affected by substrate availability, age, and cryopreservation and can differ between species.

This metabolic flexibility is advantageous and is an evolutionary response to the differing environmental conditions through which sperm must travel and survive during the path to fertilization. Recent work has shown sperm can use different metabolic pathways to support these heavily energy-dependent processes and have identified species-specific preferences in the metabolic substrates supporting key sperm functions. Equine, bovine, and canine sperm all exhibit some degree of this metabolic flexibility.

Inhibition of the ETC does not significantly reduce motility of canine sperm when glycolytic substrates are available. ETC inhibition results in decreased ATP production and sperm velocity in equine sperm, while inhibition of glycolysis had a smaller impact on sperm motility. Highly motile bovine sperm utilize mitochondrial metabolism to support increased energy demands, but high glucose availability inhibits MITOX without affecting motility. Equine sperm depend on MITOX, while bovine sperm motility is impacted by but not dependent

on MITOX; meanwhile, canine sperm display the greatest metabolic flexibility. OXPHOS and glycolysis do not appear to function independently in either of these species, and findings implicate novel strategies for exploiting the metabolic plasticity of sperm to enhance functionality and improve long-term preservation methods.

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## Chapter 2: Sperm Parameters in the Great Dane: Influence of Age on Semen Quality

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**Abstract**

Not all sires have sperm suitable for chilled or frozen storage, and success in artificial insemination (AI) varies highly among individual dogs and breeds. Fertilizing potential is further complicated as sperm quality declines with the aging process. Due to the rapidity of aging and senescence in large breed dogs, associated health and fertility changes may be observed over a shorter period, though this period remains undefined for any breed.

Working with a population of purebred Great Danes (GD), our aims were (1) to characterize the distribution of a series of sperm parameters, (2) to distinguish sources of variation in sperm quality within this rapidly aging breed, and (3) to identify changes in sperm quality that may accompany aging. Ejaculates collected from young, middle-aged, and senior Great Dane dogs (n=50) were evaluated for semen volume, total sperm number and viability, and reactive oxygen species (ROS), in addition to sperm morphology and kinematic parameters. Total testicular volume was also determined using ultrasonography.

Testicular volume was not a predictor of sperm production in the GD, however, significant differences between coat colors were identified. Age was negatively associated with total motility, progressive motility, and amplitude of lateral head displacement (ALH) ($p<.05$). We identified significant relationships between GD male age and TM, PM, and immotility with -9.9%, -9.0%, and +8.3% change per year of age, respectively, which support the anecdotal reports of decline of the fertility with the advance of age in this breed.

Sperm of younger GD dogs aged $12\leq x<24$ months had significantly higher TM, PM, ALH, and nonlinear motility ($p<.05$) than older dogs ($x\geq 48$ months). High ROS levels were positively associated with TM and PM, average pathway distance (DAP) and straight line distance (DSL), average pathway velocity (VAP), straight line velocity (VSL), and the presence

of hairpin tails ($p<.05$). While age and ROS have significant influences on sperm parameters in the GD, the influence of selection for breed specific phenotypes could help explain the functional significance of the diversity among GD males.

## Introduction

The use of artificial insemination (AI) with cryopreserved sperm for dog breeding has been expanding in veterinary clinical practice and is useful for overcoming geographic barriers in semen transportation, the genetic improvement of elite breeding stock, and protection against the transmission of venereal diseases. However not all sires used for AI have sperm suitable for frozen storage, and despite significant advances, cryopreservation success varies highly among individual dogs.

Fertilizing success is further complicated by the aging process. Like humans, dogs display natural lifespan variation although the age a dog is considered "senior" can differ widely with breed. Mixed breed dogs live an average of 1.2 years longer than their size-matched purebred counterparts, and body size is negatively correlated with lifespan: small breeds such as the toy poodle average 16 years, while large breeds such as the Irish Wolfhound may average 6-7 years [1-3]. Due to the rapidity of aging and senescence in large breed dogs, associated health and fertility changes may be observed over a shorter period, though this period remains undefined for any breed.

In dogs, advanced age is known to result in decreased sperm motility, smaller litter sizes, and higher perinatal puppy loss [4, 5]. There is evidence of increased occurrence of testicular tumors and decreased spermatogenesis in older dogs, and a lower percentage of normal sperm in the ejaculate when compared to younger dogs [6, 7]. Sperm from senior dogs has been found to be

more susceptible to cryoinjury highlighting the current need to cryopreserve sperm of elite breeding stock during the height of reproductive maturity, often years before being proven in their discipline [8, 9].

The progressive decline in fertility with age is frequently associated at the cellular level with the production of advanced glycation end products (AGE) and oxidative stress, a state related to increased cellular damage triggered by reactive oxygen species (ROS) [10-12]. ROS are normally produced at a basal level within mitochondria during ATP production as a result of low levels of electron leakage from mitochondrial electron transport chain complexes I and III, resulting in oxidation of molecular oxygen. This results in superoxide anion ($O_2^-$) as the primary ROS formed. Sperm motility is a heavily ATP-dependent function and oxidative phosphorylation (OXPHOS) has been identified as a major source of ROS in sperm as ATP is produced in the mitochondria to support essential functions of the fertilization process [13]. Intermediate ROS levels mediate important functions of sperm required for fertilization such as the signal transduction processes facilitating capacitation. However, high ROS levels may overwhelm antioxidant capabilities resulting in reduced mitochondrial function, membrane and DNA damage, and apoptosis [14, 15].

In addition to age effects, genetic selection in dogs has resulted in well-defined breeds but may have also negatively influenced semen quality and fertility outcomes. This indicates a possible need for assisted reproductive technologies (ART) such as AI and the development of breed-specific reproductive strategies. Single nucleotide polymorphism (SNP) array data indicates large breed dogs tend to have a higher coefficient of inbreeding than smaller breeds, which can result in an increase in reduced ejaculate quality and fertility, and greater puppy loss in litters with older parents when compared to outbred dogs [1, 4, 16, 17].

Few large-scale breed-specific population studies of canine semen quality have been reported for any breed, and the understanding of sperm quality parameters that underlie optimal sperm function and fertility remain unclear, particularly in large rapidly-aging breeds such as the Great Dane (GD). Our primary objectives were to characterize the distribution of sperm parameters in a large population of purebred North American Great Danes, to determine the relationship between testicular volume and sperm production capacity as has been shown in several species including livestock [18-23]. Moreover, we sought to distinguish sources of variability within the breed and to characterize semen parameters and sperm morphologic traits associated with increased age and ROS production. This study was performed using a single representative ejaculate from a population of actively showing dogs of various ages and coat colors approved by the American Kennel Club.

## Materials and Methods

### *Animals*

Great Dane males (n=50) were evaluated at the 2019 Great Dane National Specialty (Great Dane Club of America) in Virginia Beach, VA from September 11 to September 13, 2019 with the consent of their owners. As this was a clinical field study with privately-owned dogs, IACUC approval was not required. The males represented were actively showing Great Dane dogs. Ages of the dogs sampled ranged from 11 months to 72 months. All coat colors approved for the Great Dane breed by the American Kennel Club were represented in the study. This sample population is skewed younger by the nature of the show dog population and does not have balanced representation of senior intact dogs for this study. Further, as dogs presenting to us for semen evaluation were randomly presented by owners wishing to participate in this semen

survey study, we obtained a complete health history and determined that all dogs were current on routine vaccinations and fed a wide variety of diets outside the scope of analysis of this study.

*Semen Collection*

All semen collection and sample processing was completed on-site at the 2019 Great Dane National Specialty. Semen was collected on a rubber backed mat in a quiet and isolated hotel meeting room in which a mobile laboratory was set up by us. Ejaculates were collected from each dog by a veterinarian using manual collection into sterile plastic collection sleeves attached to 15-mL conical tubes after the dog achieved erection. Total ejaculate volume was recorded and an initial evaluation confirming the presence of sperm was performed using a phase contrast microscope at X 200 magnification (Zeiss AxioLab®). No dogs had been collected within the previous seven days of their participation in the study. Due to the dogs in this study actively showing at the national specialty, semen was collected after the completion of their events and we were unable to perform a clean out prior to collection. No dogs in this study were undergoing active semen collection for breeding or shipping.

*Chemicals and Reagents*

The fluorochromes CellRox™ Deep Red Fixable and Live-Dead™ Green Fixable 488 were obtained from ThermoFisher Scientific (Greenville, NC, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated.
Culture medium used for this study was modified Tyrode's medium (TGLP hereafter) prepared without albumin, containing 1% polyvinylpyrrolidone, 75 milimolar (mM) NaCl, 2.8 mM KCl, 0.2645 mM KH2PO4, 40 mmol/L HEPES sodium salt, 2 mmol/L NaHCO3, 2 mM CaCl2 (0.1 molar solution, Ricca), and 0.4 mM MgCl2 (1 molar solution) [24]. Complete medium contained

the following metabolites: 5 mM D-glucose, 1 mM sodium pyruvate, and 0.186% v:v DL-Lactic acid syrup (21.6 mM). pH of complete medium was adjusted to 7.4 ± 0.02 and osmolality of 300 ± 10 mOsm/kg. The complete TGLP medium was prepared fresh daily for experimentation and pre-warmed to 37°C prior to semen collection.

*Determination of sperm concentration, morphology, and motility parameters*

Sperm number, concentration, and viability estimates were obtained by using the NucleoCounter® SP-100TM automated cell counter (Chemometech, Allerød, Denmark) immediately following collection, using plasma membrane status determined by propidium iodide staining as an approximation of viability [25, 26]. One hundred microliters of each dog's raw ejaculate was fixed in 500µL of 10% buffered formalin for later morphological assessment at our UC Davis laboratory. Sperm morphology was assessed by a single observer and recorded in the SpermVision®SAR computer assisted sperm analysis (CASA) system (Minitube USA, Inc. Verona, WI 53593). One hundred sperm were evaluated for each fixed sample at X 1000 magnification by differential interference contrast (DIC) microscopy with oil immersion (Olympus BX-60 with X 100 objective). All CASA motility assessments were performed by the same observer using the SpermVision®SAR CASA system. Leja chambered slides (Leja Products BV; Luzernestraat, The Netherlands) were pre-warmed on a 37°C warming plate for 5 minutes then each chamber was loaded with 3µL of semen extended in TGLP (200µL; 30-50 million/mL). Average motility parameters were evaluated using SpermVision®SAR measuring seven fields with X 200 reverse phase-contrast microscopy. Semen was evaluated for total and progressive motility (TM, PM, %), average pathway velocity (VAP, µm s-1), straight line velocity (VSL, µm s-1), curved line velocity (VCL, µm s-1), straightness (STR, ratio), amplitude

lateral head displacement (ALH, μm), average path distance (DAP, μm), straight line distance (DSL, μm), curved line distance (DCL, μm), beat cross frequency (BCF, Hz), wobble (WOB, ratio), linearity (LIN, ratio), % local motility, and % hyperactive. SpermVision®SAR CASA settings are listed in Table S1.

*Measurement of testicular volume*

Testicular volume was measured using ultrasonographic measurement of height, width, and length of each testicle using an Exapad Mini ultrasound unit with 7.5 to 4.5 mHz microconvex transducer (IMV Imaging, Rochester, MN 55901). Briefly, manual isolation of each testicle within the scrotum was performed such that length (l), width (w), and height (h) of each testicle was measured using the ultrasound digital caliper. Each dimension was scanned and evaluated for any ultrasonographic irregularities in tissue density. The l x w x h gross volume product for each testicle was calculated in cm and then combined into a total testicular gross volume (cm3) which was then fitted to the volume of an ellipsoid by the following equation: $4/3(\pi)abc$ where a= h/2; b= w/2; c=l/2 [19] in Microsoft Excel.

*Fluorescence staining and laser flow cytometry*

Stained, fixed sperm were evaluated using flow cytometry for viability and cellular ROS production using a BD Accuri C6 Flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) equipped with blue (488nm) and red (640nm) lasers. Cellular ROS production in live sperm was measured using a combination of fixable and stable stains. CellRox™ Deep Red reagent is a cell-permeable weakly fluorescent probe that exhibits a strong fluorescence signal after oxidation. Live-Dead™ Green 488 is impermeable to cells with intact membranes and allows for discrimination of live and dead cells by reacting with free amines of the cell surface and interior

to yield intense fluorescence. These stains were necessary in order to determine the viability and ROS status of sperm prior to fixation and such that the samples could be express-shipped to our laboratory at UC Davis for flow cytometric evaluation. In preliminary work, we determined optimal conditions for fixation and staining and determined that fluorescence of the stained and fixed samples were stable for at least 48 hours. Briefly, ejaculates were washed with modified Tyrode's medium (TGLP), then adjusted to a concentration of 25 x 106 sperm per mL. Aliquots (500µL) were stained with 0.5 µL of Live-Dead Green according to manufacturer's directions diluted with dimethylsulfoxide (DMSO). In viable cells the stain's reactivity is limited to the cell surface, resulting in a 50-fold difference in signal intensity between live and dead cells. Samples were immediately then counterstained with 1.25µL of CellRox™ Deep Red diluted to 1mM with DMSO. CellRox™ Deep Red localizes to the cytoplasm and specifically detects ROS in live cells. The probe is weakly- or non-fluorescent in its reduced state but exhibits strong signal upon oxidation by oxidizing agents in the cytoplasm. Samples were incubated at 37°C in the dark for 30 min and washed to remove excess probe by centrifugation at 350g for 5 min to obtain a soft pellet. After discarding the supernatant, the pellet was resuspended in 1mL of TGLP. Samples were then washed once by centrifugation at 350g for 5 min, then supernatant was discarded and pellet resuspended in 250µL of TGLP. Stained samples were then fixed by adding 250µL of 4% paraformaldehyde in Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS -/-) for 15 minutes in the dark at room temperature. Fixed stained samples were washed once by centrifugation at 350g for 5 min, then supernatant was carefully discarded, and the pellet was resuspended in 500µL of DPBS -/- and shipped overnight in a light-tight package at room temperature to our UC Davis laboratory for measurement.

Tert-butyl hydroperoxide (TBHP) was used as a positive control for ROS production, and frozen-killed sperm that were flash frozen in liquid nitrogen was used as a negative control for viability. Forward scatter and side scatter measurements were used to gate for sperm by excluding larger contaminating cells or any clusters of adherent sperm. 20,000 events were collected per sample. Red fluorescence was measured with a FL4 640/65 filter, and green fluorescence was detected with a FL1 495/520 filter.

*Data Analysis*

All statistical analysis was performed using JMP® Pro (Version 16.0. SAS Institute Inc., Cary, NC, 1989–2021). Population distributions of semen parameters represent untransformed data and are presented as mean ± SEM. Normality of data was determined using the Shapiro-Wilk test and when possible non-normal data was transformed to achieve normal distribution using either log, square-root, or arcsin square-root when appropriate. Flow cytometric data was gated and analyzed to identify live high-ROS sperm subpopulations using the BD FACSuite software (BD Biosciences) prior to statistical analysis.

Effects of age, viability, ROS, motility, and other parameters were analyzed using linear (age) and linear-log (ROS) regression with level of significance set at $p<0.05$. Interpretation of coefficients of linear and linear-log model regressions were performed to estimate the effect of increasing age and ROS on sperm parameters [27].

Means comparison testing was performed using ANOVA, or Kruskal-Wallis (KW) for non-parametric data, to determine significant differences between GD grouped by coat color, and by age in months (dogs 12 months or older and up to 24 months, dogs 24 months or older and up to 48 months, and dogs 48 months of age or older) with significance set at $p<.05$. Post hoc

analysis using the Tukey-Kramer honestly significant difference test (HSD) for parametric data, and Steel-Dwass all pairs test (Dwass) for nonparametric data was used to identify differences between coat color and age groups, with significance set at $p<.05$. Due to muliticolinearity of highly correlated semen sperm parameters, Factor Analysis was used to reduce dimensionality in the dataset and identify relationships between variables. Absolute loading values less than 0.6 were suppressed.

## Results

### *General Distribution of Sperm Parameters*

The median age of the Great Danes collected in this study was 30.4 months with a minimum of 12 and a maximum of 72 months (Table S2). Two dogs younger than 12 months of age were collected but were azoospermic and excluded from the study. Sperm viability estimates ranged from 0 to 99.3% with a mean of 80 ± 3.7%. The total sperm number was positively skewed with a mean of 2854.1 ± 404.1 million sperm.

Total testicular volume significantly differed by coat color ($p<.001$), with the testicular volume of fawn dogs (n=13) being smaller than that of mantle (n=7), blue (n=7), and harlequin (n=5)($p<.05$) dogs. Testicular volume of mantle dogs was greater than that of fawn ($p<.001$), brindle (n=6) and black dogs (n=7)($p<.05$) (Figure 1, Table S5). However, no relationships between testicular volume, total sperm number, or sperm motility were observed.

The distribution of sperm morphology parameters in the study population are shown in Table 1, and a subset are highlighted in Figure S1. Percentage of morphologically normal sperm ranged between 2% - 81% with a mean of 43.4 ± 2.8% and 7.3% of Great Danes in this study had ≥70% morphologically normal sperm.

*Effect of Age on Sperm Motility*

The distribution of motility parameters in the study population are shown in Table 2 and highlighted in Figure S2. When GD were grouped by age in months as follows: dogs 12 months or older and up to 24 months (n=16), dogs 24 months or older and up to 48 months (n=27), and dogs 48 months of age or older (n=5). Means comparisons testing indicated significant differences between age groups in TM ( $p<.05$), PM ($p<.05$), ALH ($p<.05$), and %nonlinear sperm ($p<.05$). Post-hoc analysis yielded significant differences in sperm parameters between dogs 12 to 24 months of age, and dogs older than 48 months (Figure 2, Table S5).
GD older than 48 months had significantly lower TM ($p<.05$) and PM ($p<.05$) than dogs aged between 12 and 24 months. Both TM and PM displayed bimodal characters, with two populations of sperm with <40% and >50% TM, and <30% and >40% PM. ALH and the percent of nonlinear sperm was greater in GD between 12 and 24 months than dogs older than 48 months at significance level $p<.05$. Differences between these groups approached significance for additional kinematic parameters such as local motility ($p<.05$), %linear sperm ($p<.05$), and %immotile sperm ($p<.05$).

Significant predictive relationships were identified (Table S3) in the prediction of several dependent variables based on male age and include TM ($p<.01$) and PM, local motility, immotility, ALH, and % linear sperm at significance level $p<.05$. Regression modeling predicted that TM and PM decreased 9.9% and 9.0%, respectively, with each year of age in the Great Dane. In contrast, the percent of immotile sperm is predicted to increase 8.3% per year of age. ALH is predicted to decrease 0.3 μm with each year, while linearity increases by 6.6%. A small

effect was identified between age and local motility, with a predicted 1% yearly increase in Great Danes in this study.

*Relationship Between ROS and Sperm Motility and Morphology*

Due to insufficient sperm numbers required for staining procedures (<50 million) 12 dogs were excluded from the flow cytometry portion of the study (n=38) and are not reflected in ROS data. Significant relationships were identified in the prediction of several dependent variables based on the percentage of live sperm with high cytoplasmic ROS and include motility parameters such as TM, VAP, VSL, DAP, and DSL at significance level $p<.05$ (Table S4). A 10% increase in ROS in live sperm is predicted to result in a 12.7% increase in TM, 13.7μm s−1 increase in VAP, 12.4μm s−1 increase in VSL, 5.8μm increase in DAP, and a 5.4μm increase in DSL.

Significant positive relationships were also identified between high cytoplasmic ROS and morphologic abnormalities of the tail, where a 10% increase in ROS is predicted to result in a 9.8% increase in hairpin tails ($p<.05$) and a 4.8% increase in coiled tails ($p<.05$). High cytoplasmic ROS has a significant negative relationship with immotility ($p<.05$), where a 10% increase in ROS is expected to decrease the percentage of immotile sperm by 12.7%.

*Principal Component Analysis*

Factor Analysis (FA) was performed to explore relationships and sources of variation within the data and identified several population clusters within the Great Danes in this study. Seven factors were extracted, accounting for 84.6% of the variation within the breed population we studied (Table 3).

Factor 1 captured 42.2% of variation between Great Danes and was comprised of measures of sperm motility (TM, PM), distance (DSL, DCL, DAP), velocity (VAP, VSL, VCL), viability, and normal morphology. Factor 2 captured an additional 11.2% of variation and was comprised of kinematic measures associated with final sperm maturation events (WOB, LIN, %hyperactive). Factor 3 explains 9.8% of variability and includes age, total sperm number, and an estimate of flagellar vigor (ALH). Factors 4 and 5 included morphologic abnormalities of sperm midpiece and tail, and abnormalities of sperm neck capturing 6.2% and 5.7% of variation, respectively. Factors 6 and 7 explained 4.9% and 4.6% of the variation, respectively, and included ROS and hairpin tails, and total testicular volume and proximal droplets.

The biplot of Factors 1 and 2 accounts for approximately 53.4% of total variation between dogs and indicates parameter clusters and outliers within the population (Figure S3). Total and progressive motility, kinematic measures of velocity and distance, normal morphology, total sperm number, and viability were clustered with a strong positive association with F1. This kinematic cluster shows a strong negative association with a cluster of morphologic abnormalities including proximal droplets, bent midpieces, coiled tails, and immotility. High cytoplasmic ROS grouped closely with viability and motility parameters but has a negative relationship with morphologically abnormal sperm, excepting distal droplets and hairpin tails.

Factor 1 explains 97.2% of variation in sperm immotility among Great Danes. Similarly, variation within kinematic parameters BCF (94%), DAP (98%), VAP (98%), DSL (99%), and VSL (99%) are well explained by F1 and share a positive association with ROS. Hyperactive sperm shared strong inverse associations along F2 with grouped outliers WOB and LIN and together represent 11.4% of variation between Great Danes. Variation in WOB (96%), %hyperactive (85%) and LIN (95%) are explained well by F2.

**Discussion**

A total sperm number greater than 300 million in canine ejaculates is generally considered normal, and approximately 75% of ejaculates from GD in our study met this criteria [28]. Though total scrotal width has been associated with sperm production in dogs [29], our regression analysis indicates testicular volume is not a predictor of sperm production capabilities in the Great Danes of this study as compared to that of other species. No age effect was identified in total sperm number or total testicular volume for dogs over 12 months of age, but significant differences between coat colors were identified. The testicular volume of harlequin, mantle, and blue Great Danes were all significantly larger than that of fawn dogs, yet no such relationship existed in total sperm number.

A canine ejaculate with >70% progressively motile and morphologically normal sperm is generally considered to be of high quality, but due to morphologic abnormalities few dogs (n= 4) in our study population met this criteria [30]. The Great Danes in this study had lower mean percentages (43.4%) of morphologically normal sperm than a population of young (75%), middle-aged (76%), and senior (57%) Labrador Retrievers with known high fertility that we previously studied [31]. Although not fully defined for the GD, sperm morphologic defects that were observed in this study have been otherwise associated with improper or incomplete spermatogenesis, selenium deficiency, and pathological ROS production in bulls and mice [32, 33]. Major morphologic abnormalities are negatively correlated with fertility when present in large percentages, and sperm of dogs which successfully resulted in pregnancy by AI are shown to have significantly better motility and morphology than sperm of males with a history of failed pregnancies [34, 35].

When compared to an age-matched known fertile population of Labrador Retrievers, the GD in this study had lower TM (57.8%) and PM (53.6%) than young, middle-aged, and senior dogs (TM>75%, PM>70%) [5]. These parameters significantly differed between GD aged 12 to 24 months and dogs older than 48 months and are expected to decrease further with age and cooled semen transport as we have previously observed [5, 31]. In fact, we identified significant relationships between GD male age and TM, PM, and immotility with -9.9%, -9.0%, and +8.3% change per year of age, respectively, which is roughly 5-10-fold higher than reported in humans [36].

Sperm of GD older than 48 months of age had significantly lower ALH and non-linear motility than younger dogs aged 12 to 24 months. ALH is significantly greater in highly fertile bulls and is thought to contribute to cervical mucus penetration and sperm-oocyte fusion [37, 38]. Non-linear motility has been described as sperm motility tracks with various degrees of curvature that do not approach a straight line and may include hyperactivated motility [39]. In dairy bulls bred by AI, larger percentages of highly motile non-linear sperm have greater fertilization capacity, and post-thaw sperm from low fertility bulls has lower non-linearity [40]. In humans, non-linearity is positively correlated with mitochondrial membrane potential, indicating the importance of metabolic flexibility in maintaining fertility [41].

Sperm viability estimates by membrane integrity status were high in the GD, with half of dogs having between 70% and 96% viable sperm, although dogs younger than 12 months had low viability (<20%). While sperm number and viability were observed to be high in the GD dogs in our study, the age-related decline in sperm motility is expected to lower fertilization success particularly in association with cooled and cryopreserved semen.

The ability to generate large objective data sets of physiological responses and kinematic measurements using CASA and flow cytometry has shown that mammalian ejaculates consist of a heterogenous group of sperm subpopulations [42, 43]. Using Factor Analysis we identified distinct clusters in the Great Danes associated with sperm motility and morphology, age, and ROS. In FA, communality is a useful measure for predicting a variable's value. Communality values were high for all kinematic measures in the Great Dane (>.90), indicating more than 90% of variability in sperm motility parameters is explained by the factors identified by FA. The kinematic cluster distinguished close relationships between several sperm velocity indices such as VCL, VSL, VAP, and ALH which are predictive of better post thaw velocities and associated with freezeability in canine sperm [44]. Along with distance parameters including DSL and DAP, this grouping of cryo-predictive parameters is significantly associated with age and ROS in the GD.

When accompanied with high sperm velocity indices, outliers WOB, LIN, and STR have also been associated with predicted freezability in the dog, though subpopulation distributions differed completely between males [44]. A dose-response relationship has been identified between environmental exposure to the endocrine disrupting chemical (EDC) Bisphenol A (BPA) and increased WOB, LIN, and STR and decreased ALH in human sperm, suggesting environmental exposure to EDCs could be a significant contributor to reduced fertility in males, in general, including Great Danes [45]. BPA has been detected in human and pet tissues and is commonly encountered in toys and training aids, dishes, and pet foods, and is associated with oxidative stress, reduced sperm number and quality, impaired germ cell proliferation, and morphological changes in reproductive organs [46-52]. In fact, perinatal BPA exposure in male rats has been shown to cause transgenerational reproductive impairments including smaller litter

sizes, increased resorptions, impaired spermatogenesis, and morphogenesis of testes, uterus, and mammary glands in unexposed offspring two generations later [53, 54]. Exposure to BPA and other EDCs due to environment and chewing and mouthing behavior is of particular concern in dogs, but its effects on fertilization and compromised embryo development have shown improvement with antioxidant supplementation in mice [55].

The morphologic abnormality cluster identified by FA distinguished related parameters such as immotility and major sperm defects including coiled tails, midpiece abnormalities, and proximal cytoplasmic droplets in the GD, which generally arise as defects of spermatogenesis and are not considered compensable [56]. In contrast to kinematic measures, roughly 64%-83% of variation in morphology and 72% of variation in ROS can be explained, necessitating further investigation into factors underlying variation in these parameters between GD dogs.

Though defects such as lipid peroxidation, DNA damage, and apoptosis arise when the balance between ROS generation and antioxidant activity is disturbed, ROS also positively influences sperm motility, capacitation, acrosome reaction, and sperm-oocyte fusion [57-61]. In the GD, high cytoplasmic ROS in live sperm is positively associated with measures of sperm motility rather than morphologic abnormalities of the head and neck, or cytoplasmic droplets. The positive relationship between ROS and motility parameters highlights the potentially damaging effect of the metabolic maintenance of motility in sperm. Recent single-cell imaging flow cytometry studies from our lab have demonstrated that abnormal morphotypes of the sperm head and midpiece were directly associated with elevated ROS levels in equine sperm, suggesting excessive oxidative stress can contribute to the pathophysiology of morphologic abnormalities [62].

While age and ROS have significant influences on sperm parameters within the Great Dane breed, the influence of environment and selection for breed-specific phenotypes may help explain the functional significance of the diversity among the dogs in this study. As pedigreed dogs are bred with the primary aim of conforming to breed standards, fertility and underlying reproductive traits are not generally under heavy selection. Expansion of this work through Whole Genome Sequencing or single nucleotide polymorphism (SNP) studies can identify potential biomarkers associated with fertility.

Due to conducting the study onsite at the National Great Dane Specialty, one limitation of this study is a lack of clean-out, or stabilization of extra-gonadal sperm reserves, prior to collection. Ideally, dogs should be abstinent 4-5 days prior to collection, but prolonged sexual rest may result in increased secondary abnormalities such as detached heads and distal cytoplasmic droplets [63, 64]. Additionally, our study was potentially age-biased in that the oldest dog enrolled was 72 months (6 y), with most dogs falling into the middle-aged category. The large study population may have also been inherently biased by including dogs present at the breed National Specialty since breed classes at a National Specialty tend to include younger conformationally sound dogs with unknown fertility. As most senior Great Danes are no longer showing, it is important to reach out to owners to recruit senior dogs for additional further study.

**Conclusions**

The actively showing GD dogs we studied had high total sperm number with high viability, though sperm motility and the percentage of sperm without defects was lower in comparison to a known fertile population of Labrador retrievers. While its variation within the GD is not well explained, high cytoplasmic ROS has a significant positive relationship with TM

and several kinematic measures, emphasizing the need to understand the central energetic pathways underlying sperm motility in dogs. Age related changes to sperm function and response to metabolic challenges could be used to improve timelines and identify therapeutic targets for individualized methods of reproductive management of breeding dogs.

Several significant relationships were identified between age and kinematic parameters, with decreases in TM, PM, ALH, and nonlinearity, with sperm from older GD having significantly poorer performance than young dogs. Our results suggest that the aging process could leave large breeds such as the GD susceptible to poorer reproductive outcomes, and that success by ART may be improved by targeting reproductive management between 24 and 48 months of age.

**Acknowledgements**

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**Tables and Figures**

**Table 1:** Sperm Morphology of the Great Dane

| **Parameter (%)** | **Mean ± SEM** |
|---|---|
| No Defect | 43.43 ± 2.83 |
| Proximal Droplets | 6.15 ± 1.41 |
| Distal Droplets | 4.55 ± 0.81 |
| Bent Midpieces | 5.48 ± 0.87 |
| Bent Necks | 2.9 ± 0.57 |
| Hairpin Tails | 22.95 ± 2.66 |
| Coiled Tails | 7.43 ± 1.55 |
| Multiple Tails | 0.48 ± 0.16 |
| Detached Heads | 6.45 ± 2.02 |

Table 1: Distribution of sperm morphology parameters in the Great Dane, expressed as mean ± SEM. (n=48)

**Table 2:** Sperm Motility of the Great Dane

| **Motility Parameter** | **Mean ± SEM** |
|---|---|
| Total Motility (%) | 57.83 ± 4.33 |
| Progressive Motility (%) | 53.59 ± 4.12 |
| Non-linear (%) | 13.39 ± 1.33 |
| Velocity Average Pathway (VAP, μm $s^{-1}$) | 97.84 ± 4.18 |
| Velocity Straight Line (VSL, μm $s^{-1}$) | 86.69 ± 4.0 |
| Amplitude Lateral Head Displacement (ALH, μm) | 5.01 ± 0.15 |
| Distance Average Path (DAP, μm) | 43.02 ± 1.85 |
| Distance Straight Line (DSL, μm) | 38.28 ± 1.76 |
| Distance Curved Line (DCL, μm) | 59.56 ± 2.36 |
| Velocity Curved Line (VCL, μm $s^{-1}$) | 123.73 ±5.94 |
| Local Motile (%) | 4.24 ± 0.4 |
| Beat Cross Frequency (BCF, Hz) | 25.83 ± 0.67 |
| Wobble (WOB, ratio) | 0.72 ± 0.009 |
| Hyperactive (%) | 2.14 ± 0.27 |
| Linearity (%) | 41.8 ± 3.25 |

Table 2: Distribution of sperm motility parameters in the Great Dane, expressed as mean ± SEM. (n=48)

**Table 3:** Factor Composition by Factor Analysis

| Factor | % Variation | Description | Cum. Percent |
|---|---|---|---|
| 1 | 41.7 | DSL (.99), DCL (.98), DAP (.98), BCF (.94), VSL (.99), VCL (.97), VAP (.98), %LIN (.97), PM (.97), TM (.97), Viability (.69), normal morphology (.80) | 41.7 |
| 2 | 11.4 | WOB (.96), LIN (.85), % Hyperactive (.85) | 53.2 |
| 3 | 9.8 | Age (.56), ALH (.88), Total Sperm Number (.62) | 63.0 |
| 4 | 6.2 | Distal Droplet (.75), Bent Midpiece (.64) | 69.2 |
| 5 | 5.7 | Bent Neck (.83) | 74.9 |
| 6 | 4.9 | ROS (.72), Hairpin Tails (.81) | 79.8 |
| 7 | 4.6 | Total Testicular Volume (.85), Proximal Droplets (0.79) | 84.4 |

Table 3: Description of each factor identified by Factor Analysis including total variation described by each factor, parameter composition, communality values describing proportion of variation described by the factor, and cumulative percent of variance described by the analysis. Absolute loading values less than 0.6 were suppressed.

**Figure 1:** Testicular Volume by Coat Color

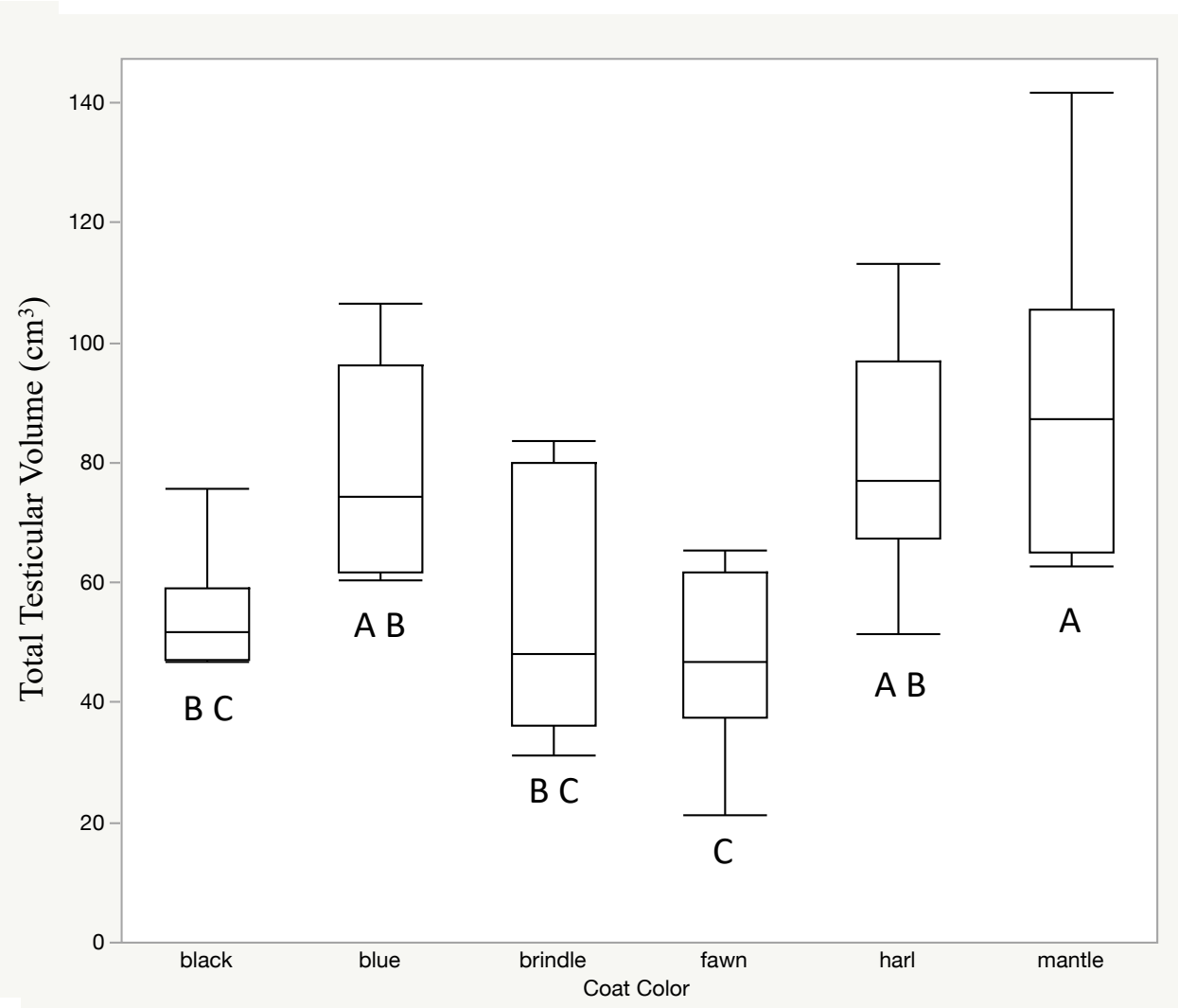


Figure 1: Means comparison of total testicular volume by coat color, where shared letters indicate lack of significance between groups. Groups which do not share letters have significantly different means ($p<.05$). Box plots indicate group means ± SEM.

**Figure 2:** Sperm Motility by Age Group

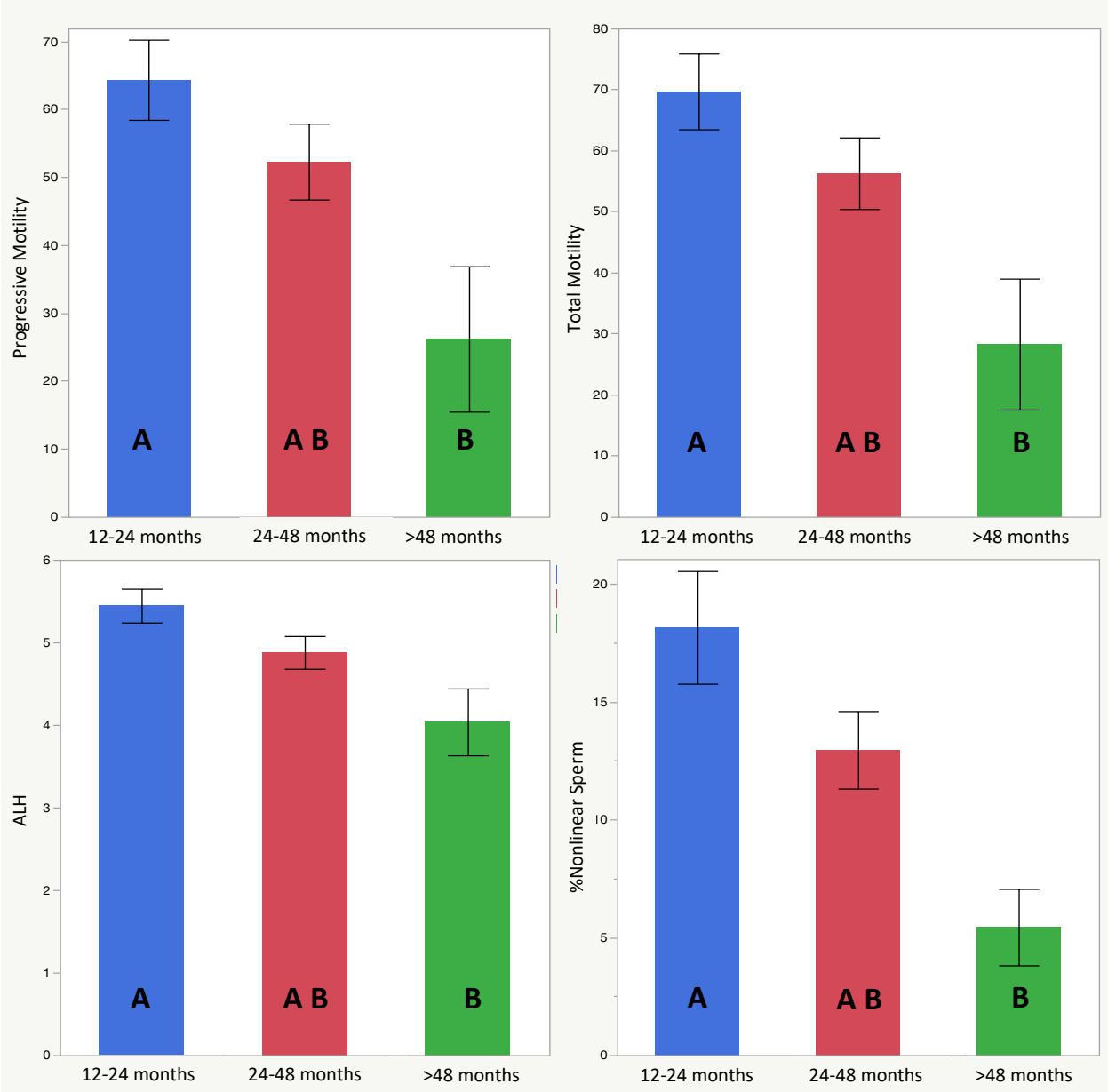


Figure 2: Means comparisons of progressive and total motility, amplitude lateral head displacement (ALH), and % nonlinear sperm by age group identified significant differences between young and older Great Dane dogs. Dogs were grouped by age as follows: dogs 12 months or older and up to 24 months, dogs 24 months or older and up to 48 months, and dogs 48 months of age or older. Groups which do not share letters have significantly different means ($p<.05$). No difference was found between group means of young and older dogs. Box plots indicate group means ± SEM.

**Table S1:** SpermVision® Technical Settings

| Settings Category | Parameter | Settings |
|---|---|---|
| General | Field of view depth | 20µm |
| | Pixel to µm ratio | 130 to 100 |
| | Cell identification area | 16 to 60µm$^2$ |
| | Assessment requirements | 4000 cells or 7 fields |
| | Additional particle filtering | none |
| | Light threshold | min 170, max 255 |
| | Points to use in cell path smoothing | 17 |
| Level 1 Cell Classifications | Immotile | VAP<20 |
| | Local | VAP<50, AOC>7, STR<0.75 |
| Level 2 Cell Classifications | Hyperactive | ALH>7.5, VAP≥0.75, LIN<0.5 |
| | Linear | STR>0.75, LIN>0.45 |
| | Nonlinear | LIN<0.35, STR<0.75 |
| | Curvilinear | DAP/Radius≥3, LIN<0.5 |

Table S1: SpermVision® CASA system technical settings used for assessment and quantification of motility parameters are displayed. Manufacturer recommended canine motility settings were used.

**Table S2:** Distribution of Semen Parameters in Study Population

| Parameter | Distribution |
|---|---|
| Age, months | 30.4 ± 2.04 |
| Coat Color | 13.1% black, 13.1% blue, 10.9% brindle, 15.2% mantle, 17.4% harlequin, 28.3% fawn |
| Viability, % | 80 ± 3.7% |
| Testicular Volume | 65.7 ± 3.5 |
| Total Sperm Number | 2854.1 ± 404.1 million |
| High Reactive Oxygen Species, % | 12.3 ± 2.2 |

Table S2: Distribution of Semen Parameters in the Great Dane, expressed as mean ± SEM. (n=48)

**Table S3:** Age and Sperm Motility

| Variable | Significance, Regression Equation |
|---|---|
| Total Motility, % | F(1,48)= 8.1314 p=0.006, $R^2$ .1502. Predicted Total Motility = 82.9331-9.9031*age |
| Local Motility, % | F(1,48)= 8.2223, p=0.006, $R^2$ .1516. Predicted Local Motility = 6.5484-0.9126*age |
| Immotile, % | F(1,48)= 4.5206, p=0.04, $R^2$ .1063. Predicted Immotile = 14.4041+8.3414*age |
| ALH, µm | F(1,48)= 5.5629, or p=0.02, $R^2$ .1276. Predicted ALH = 5.8129-0.3295*age |
| Progressive Motility, % | F(1,48)= 7.3104, p=0.009, $R^2$ .1371. Predicted Progressive Motility = 76.3786-8.9901* age |
| Linearity, % | F(1,48)= 6.1478, p=0.02, $R^2$ .1179. Predicted Linearity = 58.4397- 6.5753*age |

Table S3: Significant relationships between sperm motility and age in the Great Dane identified by regression analysis with significance level set to p<.05.

**Table S4:** ROS and Sperm Motility, Morphology

| Variable | Significance, Regression Equation |
|---|---|
| Total Motility (%) | F(1,48)= 4.4283 p=0.04, $R^2$ .1043. Predicted Total Motility = 55.21+13.39*$\log_{10}$ROS |
| Immotile (%) | F(1,48)= 4.4283 p=0.04, R2 0.1043. Predicted %Immotile =44.79 - 13.39*log10ROS. |
| DSL | F(1,48)=4.3448, p=0.04, $R^2$.1026. Predicted DSL = 45.31+5.67 *$\log_{10}$ROS |
| VAP | F(1,48)=5.0772, p=0.03, $R^2$ .1179. Predicted VAP = 115.76+14.44*$\log_{10}$ROS |
| VSL | F(1,48)=4.5917, p=0.04, $R^2$ .1078. Predicted VSL = 102.95+13.09*$\log_{10}$ROS |
| DAP | F(1,48)=4.4858, p=0.04, $R^2$ .1055. Predicted DAP = 50.53+6.06*$\log_{10}$ROS |
| Hairpin Tails (%) | F(1,48)=6.6861, p=0.01, $R^2$ .1496. Predicted Hairpin Tails = 35.81+10.36 *$\log_{10}$ROS |
| Coiled Tails (%) | F(1,48)=4.9131, p=0.03, $R^2$.1145. Predicted Coiled Tails = 0.86-5.29 *$\log_{10}$ROS |
| Progressive Motility (%) | F(1,48)=3.9895, p=0.05, $R^2$.095. Predicted Progressive Motility = 51.25+12.28*$\log_{10}$ROS |

Table S4: Significant relationships between ROS and sperm motility and morphology in the Great Dane identified by regression analysis with significance level set to p<.05.

**Table S5:** Means Comparisons Testing by Age Group and Coat Color

| Parameter | P value | df | Test Statistic | Test |
|---|---|---|---|---|
| Total Motility | p<.05 | 2 | H=7.3 | KW |
| Progressive Motility | p<.05 | 2 | H=6.2 | KW |
| ALH | p<.05 | 2 | F=3.8 | ANOVA |
| %linearity | p<.05 | 2 | H=7.9 | KW |
| Total Testicular Volume | p<.001 | 5 | F=6.9 | ANOVA |

| Parameter | Groups | | P value | Post-hoc |
|---|---|---|---|---|
| Total Motility | 12≥ mo >24 | ≥48 mo | p<.05 | Dwass |
| Progressive Motility | 12≥ mo >24 | ≥48 mo | p<.05 | Dwass |
| ALH | 12≥ mo >24 | ≥48 mo | p<.05 | HSD |
| %Linearity | 12≥ mo >24 | ≥48 mo | p<.05 | Dwass |
| | | | | |
| Total Testicular Volume | Fawn | Mantle | p<.001 | HSD |
| | Fawn | Blue | p<.05 | HSD |
| | Fawn | Harlequin | p<.01 | HSD |
| | Mantle | Fawn | p<.001 | HSD |
| | Mantle | Brindle | p<.05 | HSD |
| | Mantle | Black | p<.05 | HSD |

Table S5: Sperm parameters for which means differ significantly by age group or coat color in the Great Dane. For means comparisons testing dogs were grouped by coat color, or by age in months (mo) as follows: dogs 12 months or older and up to 24 months, dogs 24 months or older and up to 48 months, and dogs 48 months of age or older. ANOVA was performed for parametric data, or Kruskal-Wallis (KW) for non-parametric data to determine significant differences between group means at significance level p<.05. Significance by Tukey-Kramer honestly significant difference (HSD) or Steel-Dwass (Dwass) Post-hoc testing are shown. There was no significant difference between dogs older than 48 months, and dogs aged 24 to 48 months of age.

**Figure S1:** Sperm Morphology of the Great Dane

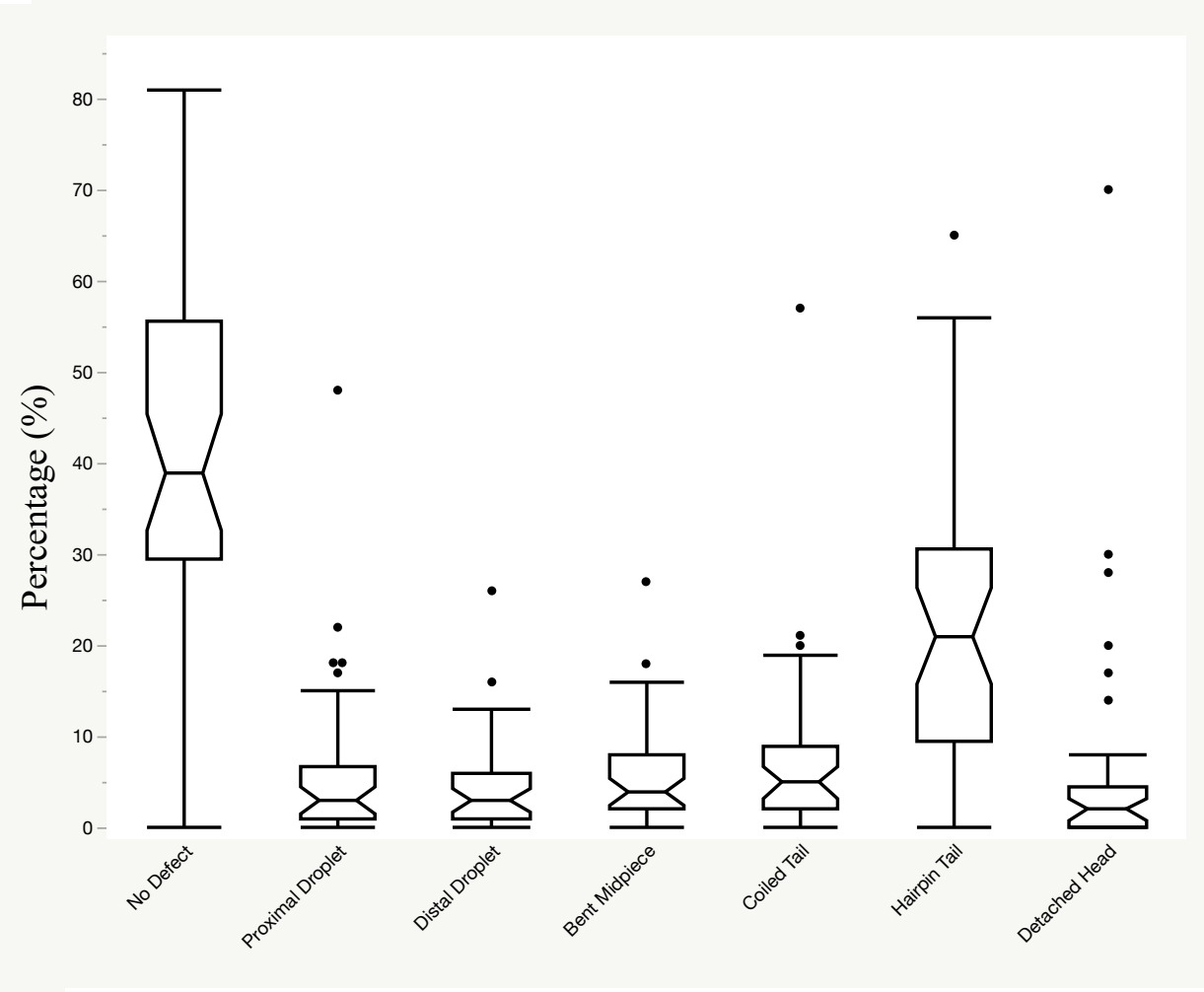


Figure S1: Notched box plots illustrating distributions of select sperm morphology parameters in the Great Dane, where notches represent the 95% confidence interval of the median. Dots indicate outliers.

**Figure S2:** Sperm Motility of the Great Dane

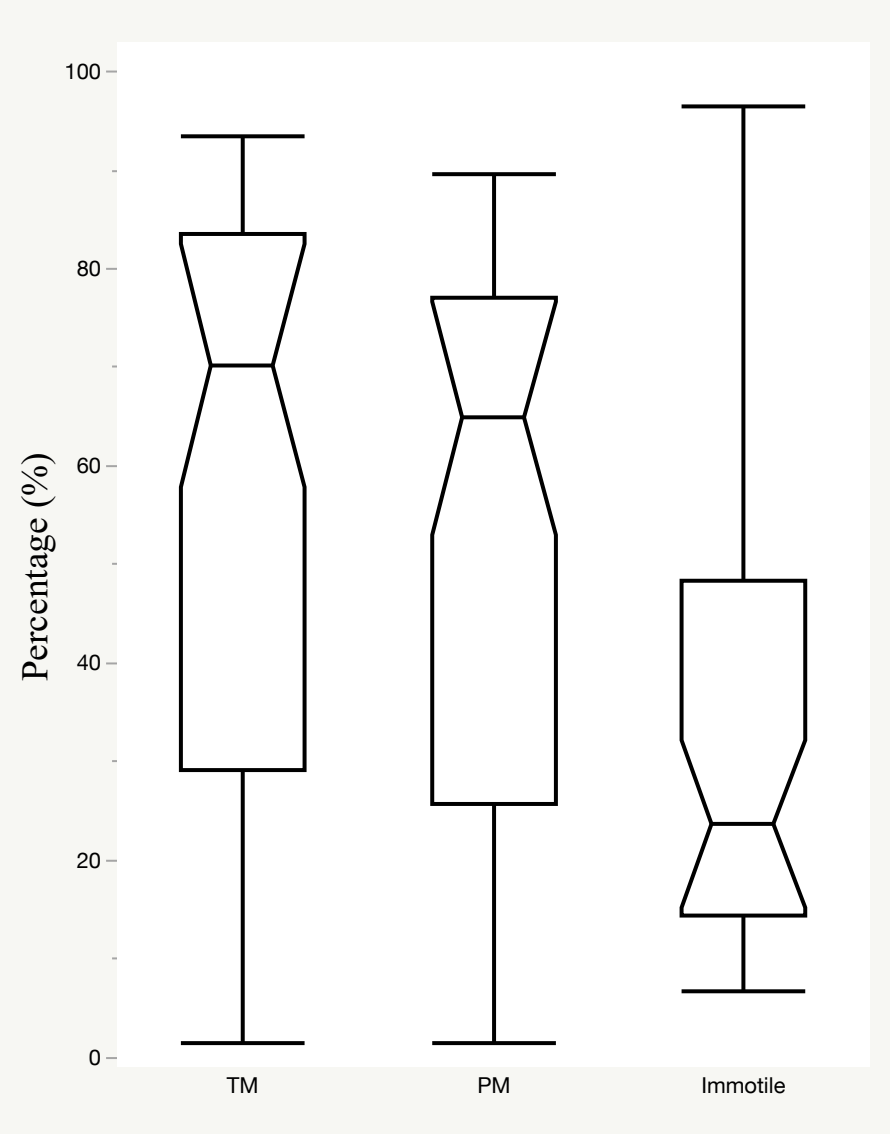


Figure S2. Notched box plots illustrating population distributions of sperm total motility (TM), progressive motility (PM), and immotility in the Great Dane, where notches represent the 95% confidence interval of the median.

**Figure S3:** Biplot of Sperm Parameters in the Great Dane

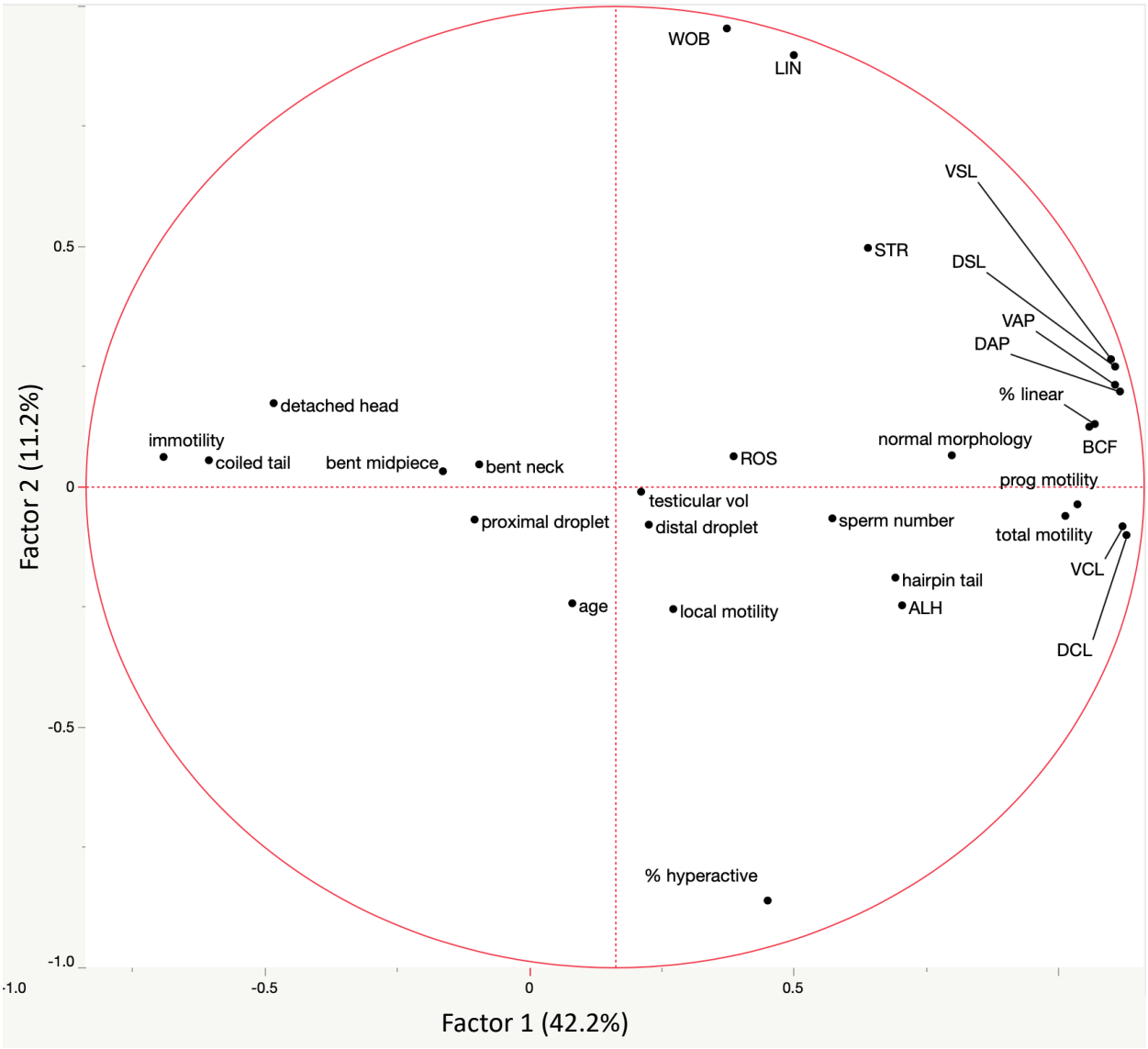


Figure S3: Biplot of factors 1 and 2 by factor analysis, representing 53.2% of total variation within Great Dane dogs studied. Biplot indicates positive and negative associations between clusters of kinematic and morphologic sperm parameters, ROS, and age.

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**Chapter 3:** Effects of Substrate Availability and Mitochondrial Disruption on Oxidative Metabolism and Sperm Motility in Fertile Dogs

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## 1. Introduction:

While the role of mitochondrial function in sperm maturation and fertilization has been studied in mammals, the relationship between mitochondrial activity and the support of specific sperm functions such as motility and oxidative metabolism vary widely between species. In general, the maintenance of sperm motility is a heavily energy-dependent process and, like many key sperm functions and fertilizing events, energy in the form of adenosine triphosphate (ATP) is required [1]. The ATP necessary for sperm motility, capacitation, and acrosomal exocytosis necessary for physiological fertilization is formed by glycolysis and oxidative phosphorylation (OXPHOS), two metabolic pathways which function in the sperm head and tail regions, and the mitochondrial sheath, respectively [2].

Although OXPHOS generates approximately 16 times more ATP per molecule of glucose than glycolysis, they are not fully independent pathways, with glycolysis supplying carbon-rich molecules to the mitochondria for further oxidation through OXPHOS. Following the oxidation of glucose to pyruvate in the sperm head and principal piece, pyruvate is further oxidized within the mitochondrial matrices located in the sperm midpiece to form reduced electron carriers (NADH and $FADH_2$). These reducing agents deliver electrons to a chain of protein complexes within the electron transport chain (ETC) and enable a series of energetically favorable oxidation-reduction reactions (redox) terminating in the reduction of oxygen to water. The energy released by this chain of redox reactions is used to translocate protons (H+) from the mitochondrial matrix to the intermembrane space, establishing a strong electrochemical gradient used by ATP synthase to convert potential energy to chemical energy in the form of ATP. In sperm of several mammalian species, the measurement of mitochondrial oxygen consumption (MITOX) under different metabolic conditions have demonstrated a positive correlation with

motility and may be a sensitive indicator of mitochondrial health, although little is known about canine sperm mitochondrial function [3-6].

Recent work investigating the importance of mitochondrial activity in the support of sperm kinematics has identified species-specific differences in the preferred metabolic pathways underlying sperm motility. For example, the glycolytic pathway is the primary energy source supporting human sperm motility, while stallion sperm rely on OXPHOS nearly exclusively [7, 8]. While mitochondrial functionality is key in bovine sperm capacitation and motility regulation, bovine sperm can maintain motility even in the event of mitochondrial inhibition when provided with glycolytic substrates [9-11]. Yet, high glucose availability produces an inhibitory effect on bull sperm mitochondria, indicating metabolic flexibility in response to substrate availability [12].

Canine sperm have been known to undergo capacitation and maintain motility with supplementation of a wide range of energy substrates and presented the first demonstration of gluconeogenesis-linked glycogen metabolism in mammalian sperm [13-15]. Dogs are unique among domestic mammals in the lengthy time between ejaculation and fertilization, which can reach 11 days, during which sperm must overcome the physical and biochemical hazards of the female reproductive environment [16]. Therefore, some degree of metabolic plasticity would prove advantageous in maintaining sperm bioenergetics during lengthy storage and transit times. Further, though canine sperm have been shown to utilize both fructose and glucose as energy sources, the two sugars have demonstrated different roles in motility, glycogen metabolism and deposition, and hexose metabolism [13, 17-19]. This suggests canine sperm employ distinct energy management systems in differing nutrient conditions, which can be exploited to improve sperm handling and storage.

However, little is known regarding the relationship between mitochondrial function and sperm motility in dogs, and whether any metabolic preferences exist. The aims of this study were to explore the metabolic flexibility of canine sperm, and to investigate the relationship between mitochondrial function and the regulation and maintenance of motility under differing nutrient conditions. By monitoring MITOX, sperm kinematics, and viability in the presence of mitochondrial effector drug treatments, we explore substrate preferences and the bioenergetics underlying canine sperm motility.

## 2. Methods:

### *2.1. Animals and semen collection*

Ejaculates were obtained from Labrador Retriever (LR, n=12) dogs over three separate days at the Guide Dogs for the Blind breeding program (GDB, San Rafael, CA). Using an estrous female for mounting in a quiet dedicated environment, semen was collected into sterile plastic collection sleeves attached to 15ml conical non-toxic polypropylene Falcon tubes (Corning Inc., Corning, NY). Immediately following collection at GDB, motility was assessed by the SpermVision®SAR computer assisted sperm analysis (CASA) system (Minitube USA, Inc. Verona, WI), and total sperm number was estimated by the Spermacue photometer (MOFA Global, Verona, WI). This preliminary assessment was used to help ensure 12 high quality ejaculates defined as having at least 70% initial total motility and total sperm number greater than 300 million sperm were used in this study. Ejaculate volume was recorded and semen was extended 1:2 (v:v) in pre-warmed (37°C) modified TALP media buffered with 40 mmol/L HEPES containing glucose, lactate, and pyruvate (TGLP hereafter) [20, 21]. One hundred microliters of each raw ejaculate were fixed in 500µL of 10% buffered formalin for

morphological assessment. Samples and extended semen were transported at ambient temperature to the UC Davis Gamete Biology Laboratory (1.5 hr transit time).

*2.2. Chemicals and media*

All chemicals were purchased from MilliporeSigma Life Sciences (Burlington, MA) unless otherwise indicated. Calcium chloride (0.1 M, RICCA- 1760-32) was purchased from Sycamore Life Sciences (Houston, TX). NucleoCounter® SP-100TM reagents were purchased from ChemoMetec (Allerød, Denmark).

Complete culture medium used for this study was modified (TGLP hereafter) prepared without albumin, containing 1% polyvinylpyrrolidone, 75 mmol/L NaCl, 2.8 mmol/L KCl, 0.2645 mmol/L KH2PO4, 40 mmol/L HEPES sodium salt, 2 mmol/L NaHCO3, 2 mmol/ L CaCl2 (0.1 M solution, Ricca), and 0.4 mmol/L MgCl2 (1 M solution) [20, 21]. Complete medium contained the following metabolites: 5 mmol/L D-glucose, 1 mmol/L sodium pyruvate, and 0.1862% (v/v; 21.6 mmol/L) DL-Lactic acid syrup. pH of complete medium was adjusted to 7.4 ± 0.02 and osmolality of 300 ± 10 mOsm/kg. The complete TGLP medium was prepared fresh daily for experimentation and pre-warmed to 37°C prior to semen collection.
In addition to TGLP, three additional modified TGLP medias containing either 67mM glucose (G media), 67mM fructose (F media), or 33mM pyruvate and 33mM lactate (LP media) as the sole energy source were studied.

*2.3 Semen Assessment*

Immediately upon arrival at the UC Davis laboratory, total sperm number and viability estimates were obtained using the NucleoCounter® SP-100TM automated cell counter

(ChemoMetech, Allerød, Denmark) as previously reported [21]. Viability was determined in the NucleoCounter using membrane status as determined by propidium iodine staining [22, 23].

Motility assessments were performed by the same observer using the SpermVision®SAR CASA system. An aliquot of each ejaculate was adjusted to 30-50 M/mL in complete TGLP and 3µl was loaded into Leja chambered slides pre-warmed on a 37°C warming plate for 5 minutes (Leja Products Luzernestraat, The Netherlands). Average motility parameters were evaluated using SpermVision®SAR measuring seven fields with 200X reverse phase-contrast microscopy. Motility parameters assessed included summary measures, and measures of distance, velocity, and linearity. Semen was evaluated for total and progressive motility (TM, PM, %), average pathway velocity (VAP, µm s-1), straight line velocity (VSL, µm s-1), curved line velocity (VCL, µm s-1), straightness (STR, ratio), amplitude of lateral head displacement (ALH, µm), average path distance (DAP, µm), straight line distance (DSL, µm), curved line distance (DCL, µm), beat cross frequency (BCF, Hz), wobble (WOB, ratio), linearity (LIN, ratio), % local motility, and % hyperactive. SpermVision®SAR CASA settings are listed in Table 1.

All sperm morphologic assessment was completed by one individual for consistency. One hundred sperm were evaluated for each fixed sample at 1000X magnification by differential interference contrast (DIC) microscopy with oil immersion (Olympus BX-60 with 100x objective).

*2.5. Oxygen consumption and simultaneous motility assessment*

Following initial sperm quality assessment, ejaculates were pooled in groups of 3 to obtain adequate sperm numbers for treatment groups. The pooled samples were then separated into four equal portions, washed once with TGLP using centrifugation at 250 x g for 8 min, then

resuspended in either TGLP, G media, F media, or LP media and adjusted to 50M/ml using NucleoCounter® SP-100TM to confirm sperm concentration and measure viability. Measurement of oxygen consumption was performed using the OP96U OxoPlates® (PreSens Precision Sensing GmbH; Regensburg, Germany), which allow monitoring of oxygen consumption without inhibiting sperm motility. An integrated optical sensor containing an oxygen dependent indicator dye (platinum porphine) and oxygen independent reference (sulforhodamine) dye were used to calculate the oxygen partial pressure [24]. Fluorescence intensity was measured using a SynergyTM H1 plate reader (BioTek; Winooski, VT), using two filter pairs for the OxoPlate® read-out (filter pair 1, indicator: 540 nm Ex/ 650 nm Em; filter pair 2, reference: 540 nm Ex/590 nm Em).

In order to minimize temperature driven fluctuations in fluorescence. OxoPlates®, media, and treatments were prewarmed to 37°C prior to plating . In preliminary work, titrations were performed to confirm sperm viability was unaffected by the assay, and to determine the optimal cell number and drug treatment concentrations. Calibration was performed according to Oxoplate® manufacturer recommendations using oxygen-free water (cal0) and air-saturated water (cal100). Oxygen-free water (cal0) was prepared with 0.2 g of sodium sulfite (Na2SO3; a molecular oxygen scavenger) added to 20 mL of pre-warmed $H_2O$. Six replicates received 300 µL of cal0 and wells were immediately sealed with adhesive foil. Air saturated water (cal100) was prepared by placing 20 mL of pre-warmed water into a 50 mL conical tube and shaking vigorously for 2 min, then slightly opened and swirled gently for 1 min to prevent oversaturation. Six replicates of 200 µL of cal100 were plated with wells left uncovered. Appropriate media (50 µL) was then pipetted into all wells designated for sperm samples or ambient controls. Next, 100 µL of each sperm sample (25 M viable cells/mL) was added into each allotted well, for a final

total of 12.5 M viable cells/well. All sperm and treatment combinations were run with 6 replicate wells.

After an initial fluorescence reading the plate was equilibrated at 37°C for 20 min. Following equilibration, another fluorescence reading was taken, and mitochondrial drug treatments were added. An inhibitor of electron transport chain Complex III, Antimycin A (ANTI, 2 mM) was used to determine non-mitochondrial oxygen consumption. ATP Synthase inhibitor (ETC complex V), Oligomycin (OLIGO, 2 mM), was used to approximate the rate of respiration linked to ATP production. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 mM), a mitochondrial uncoupler, was included for stimulation of maximal oxygen consumption.

Fluorescence readings were taken for 60 min immediately after treatment addition (t0), every 2 min for 10 min (t2, t4, t6, t8, t10), then every 5 min for 20 min (t15, t20, t25, t30), and lastly, every 10 min for an additional 30 min (t40, t50, t60) with viability assessed prior to plating, and at t30 and t60. Between measurements, plates were carefully maintained at 37°C. Parallel aliquots of each sample were prepared in microcentrifuge tubes for simultaneous motility assessment by CASA, as described previously, 6 and 30 min after treatment addition.

*2.6. Data processing and statistical analysis*

Data was processed according to manufacturer instructions to calculate oxygen concentration as a percentage of air saturation ($pO_2$) with the following equations for each well and time point [11, 24].

$IR = I_{indicator} / I_{reference}$

$pO_2$ = 100 x (k0 / IR e 1) / (k0 / k100 e 1)

All statistical analysis was performed using JMP® Pro (Version 16.0. SAS Institute Inc., Cary, NC, 1989–2021). Pearson's product moment correlation was used to explore relationships between sperm motility parameters (t6, t30), viability, and pO2 across media types and mitochondrial inhibitors tested. Normality was assessed using the Shapiro-Wilk test ($p<.05$). Means comparison testing was performed using ANOVA or Kruskal-Wallis (KW) for non-parametric data to determine significant differences between sperm motility and $pO_2$ when grouped by media type. Post-hoc analysis was performed using the Tukey-Kramer Honestly Significant Difference test (HSD) for parametric data, and Steel-Dwass All Pairs test for nonparametric data to identify differences in O2 consumption, spare respiratory capacity (SRC, FCCP-basal), and sperm motility between nutrient sources and mitochondrial effector drug treatments. Significance was set at $p<.05$ for all statistical testing.

**3. Results:**

*3.1 Oxygen Concentration ($pO_2$)*

Oxygen concentration over time for each treatment condition is displayed in Figure 1. $pO_2$ differed by both media type and mitochondrial effector drug treatments at t30. $pO_2$ of sperm treated with FCCP was greater than untreated sperm across all media types ($p<.0001$). OLIGO $pO_2$ levels were significantly lower than untreated sperm in all medias except LP media, in which OLIGO treated sperm had significantly higher $pO_2$ (all, $p<.05$). Except for media containing only lactate and pyruvate, ANTI treatment resulted in significantly lower $pO_2$ levels than that of untreated sperm ($p<.0001$).

*3.2 Spare Respiratory Capacity*

Means comparisons testing identified significant differences in spare respiratory capacity (SRC) by nutrient source (ANOVA, $F(3)=12.3$, $p<.001$) (Figure 2). Sperm in media containing fructose as the sole energy source had significantly higher SRC than sperm provided with LP media or media containing lactate, pyruvate, and glucose together ($p<0.01$) (Table 2). Sperm in media containing glucose as the sole energy source had significantly higher SRC than sperm provided with media containing lactate, pyruvate and glucose together ($p<0.05$).

*3.3 Mitochondrial Inhibition and Sperm Motility*

Sperm motility endpoints are listed in Table 3 and shown in (Figures 3a-d). In the absence of mitochondrial disruption by ANTI, OLIGO, or FCCP treatment, nutrient source had no effect on maintenance of canine sperm motility (t30) ($p>.05$).

When provided with only mitochondrial substrates (LP media), disruption of OXPHOS by mitochondrial effectors ANTI, OLIGO, and FCCP significantly reduced several measures of sperm motility including PM, TM, VSL, VCL, VAP, DSL, DAP, DCL, and %Linear (all $p<.0001$) when compared to untreated sperm. However, viability remained high (>90%) in all treatment groups. OXPHOS disruption by ANTI had no effect on ALH in LP media ($p>.05$). When provided with non-mitochondrial substrates, several motility measures were maintained regardless of OXPHOS disruption including TM, PM, and ALH, with no significant differences observed compared to untreated sperm ($p<.05$). In media containing fructose as the sole energy source, disruption of OXPHOS by mitochondrial effectors ANTI, OLIGO, and FCCP had no effect on TM, PM, or ALH ($p>.05$). Treatment with ANTI significantly reduced VSL, VAP, DAP, DCL, DSL, VCL, %HA, and %Linear ($p<.05$). Treatment with FCCP significantly

reduced VSL, VAP, and DAP ($p<.05$). Treatment with OLIGO significantly reduced VSL, VAP, DAP, DSL, VCL, %HA, and %Linear ($p<.05$).

When provided with glucose as the sole energy source, disruption of OXPHOS by mitochondrial effectors ANTI, OLIGO, and FCCP had no effect on TM, PM, or ALH ($p>.05$). ANTI, OLIGO, and FCCP treatment significantly reduced VSL, VAP, VCL, and DAP ($p<.05$). ANTI and OLIGO treatment significantly reduced DCL, and DSL, but significantly increased %HA (all $p<.01$). ANTI treatment significantly increased %linear sperm in glucose supplemented media ($p<.001$).

When provided with a complete media, disruption of OXPHOS by mitochondrial effectors ANTI, OLIGO, and FCCP had no effect on TM, PM, ALH, VSL, VAP, DAP, or DSL ($p<.05$). Treatment with FCCP significantly reduced VCL and DCL ($p<.01$), while OLIGO significantly reduced %HA and %Linear sperm ($p<.01$).

Though nutrient source had no significant effect on %HA of untreated sperm, ANTI treatment resulted in significantly higher %HA in fructose supplemented media than in complete or LP media ($p<.001$).

*3.4 MITOX and Sperm Motility*

Correlation analysis revealed that, in general, MITOX is significantly correlated with motility parameters in untreated canine sperm (Table 4). In sperm provided with LP media, significant negative relationships were identified between MITOX, TM and %linear sperm (all, $p<.05$), with PM approaching significance.

In sperm supplemented with fructose, MITOX shared a positive relationship with %HA, and a negative relationship with STR (all, $p<.05$). Relationships between MITOX and motility

approached significance for %linear and %nonlinear sperm. When glucose was provided as the sole energy source, we identified negative relationships between MITOX and motility parameters including STR, VSL, %linear sperm, WOB, and DSL, and a positive relationship with %HA (all $p<.05$). Relationships between MITOX and motility in glucose media approached significance for VAP and DAP.

There was no significant relationship observed between MITOX and motility in untreated canine sperm provided with a complete media containing glucose, lactate, and pyruvate ($p>.05$). However, %HA and linear motility approached significance ($p=.05$, $p=.07$).

**Discussion:**

As expected, maximal stimulation of oxidative metabolism in canine sperm with the ionophore FCCP significantly increased mitochondrial oxygen consumption in all media types, indicating a strong response to perturbation of the ETC regardless of nutrient source. Nevertheless, the ability to increase mitochondrial function in response to energetic challenge and stress conditions, spare respiratory capacity (SRC), significantly differed by nutrient source. Along with excessive mitochondrial ROS production, impaired SRC is an important biomarker for sperm dysfunction and is implicated in poor IVF outcomes from infertile males, and age-related declines in SRC sensitize the cell to surges in ATP demand and risks driving the affected cells towards senescence or death [25, 26].

In canine sperm, the high affinity glucose transporter GLUT3 and fructose transporter GLUT5 have been localized to the mid and principal piece, and the periacrosomal region and midpiece, respectively [17]. Fructose is a glycolytic substrate that bypasses the rate-limiting enzyme phosphofructokinase to be quickly metabolized and converted to pyruvate and lactate,

generating additional ATP. Fructose and glucose supplementation supported higher SRC than lactate and pyruvate containing medias, indicating canine sperm mitochondria achieve maximum functionality when partnered with supportive metabolic pathways.

We found that sperm from fertile dogs maintain motility in all medias regardless of nutrient composition, indicating that glycolysis, fructolysis, and OXPHOS are all capable of supporting canine sperm motility. Our results are consistent with that of Rigau et al., which found that when provided as the sole energy source, neither fructose nor glucose modify the percentage of motile canine sperm [18]. However, Rigau et al. identified subpopulations of sperm which responded differently to glucose and fructose supplementation, suggesting some degree of metabolic sensitivity and adaptability [18].

Disruption of OXPHOS by mitochondrial uncoupling, or by inhibition of ETC Complex III or ATP Synthase failed to significantly reduce TM and PM in media containing glucose or fructose, indicating sperm of fertile dogs use compensatory mechanisms to support motility in the event of decreased mitochondrial functionality.

In known fertile dogs, MITOX is correlated with several motility measures regardless of supplementation by glucose, fructose, or lactate and pyruvate. However, we found MITOX shares an inverse relationship with measures of linear movement in both fructose and glucose supplemented medias. This is in contrast to previous work showing supplementation with fructose results in more rapid and linear motility patterns than glucose when provided at lower concentrations than used in our study [18]. Disruption of the ETC by ANTI significantly reduced measures of curvilinear motility, VCL and DCL, regardless of nutrient source. Determination of %TM and %PM sperm are defined by VCL, suggesting that while canine sperm motility can be

maintained by several metabolic pathways, sperm subpopulations with low mitochondrial functionality may be responsible for low motility ejaculates.

In contrast to sperm provided with alternative substrates, mitochondrial disruption of canine sperm provided with only lactate and pyruvate resulted in several significantly reduced motility parameters associated with sperm quality, fertility, and freezability such as TM, PM, VSL, VCL, VAP, DSL, DCL, DAP, some of which are expected to decrease further with age and cryopreservation [21, 27, 28].

Though previous work has shown canine sperm maintain total motility for 30 min in sugar-free media, our results imply that gluconeogenesis and glycogen reserves previously reported in canine sperm are not capable of maintaining motility in the absence of aerobic metabolism when supplemented with only mitochondrial substrates [18, 19, 29]. Curiously, though mitochondrial disruption of sperm provided with only mitochondrial substrates significantly reduced most kinematic measures, no effect was seen in ALH. An estimate of flagellar vigor thought to contribute to cervical mucus penetration and sperm-oocyte fusion, ALH is predictive of fertility in bulls and is significantly lower in senior dogs [21, 27]. Previous canine work in has shown reduced intracellular reactive oxygen species (ROS) production due to antioxidant treatment is associated with decreased ALH in canine sperm [30]. While increased ROS production resulting from senescence and cryopreservation may overwhelm antioxidant capacity and cause cellular damage and reduced sperm viability, ROS is positively associated with several measures of sperm motility, velocity, and distance in canines [21, 31-33]. Since ALH was not significantly reduced in our study by mitochondrial disruption regardless of nutrient availability, it raises questions regarding the pathways contributing to control of sperm

motility, and the mechanism by which sperm function suffers with cryopreservation and increasing age.

In this study, we have demonstrated the adaptability and metabolic sensitivity of canine sperm in maintaining viability and sperm kinematics, highlighting both the interconnected nature of energy management pathways and the utility of diverse nutrient sources in supporting key sperm functions. Our investigation of relationships between canine sperm bioenergetics and kinematics has shown MITOX is correlated with several motility parameters in sperm supplemented with differing nutrient sources. This reveals that real time assessment of MITOX is a valuable tool for assessing mitochondrial functionality in canine sperm.

Though several substrates can maintain motility in fertile dogs in the event of mitochondrial inhibition, decreased sperm kinematics induced by mitochondrial disruption prompts questions regarding how well canine sperm function can be maintained by isolated pathways. An improved understanding of the functional effects of sperm bioenergetics across age and fertility challenges will help adapt sperm handling and storage to address challenges encountered during a dog's breeding lifetime.

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**Tables and Figures**

**Figure 1:**

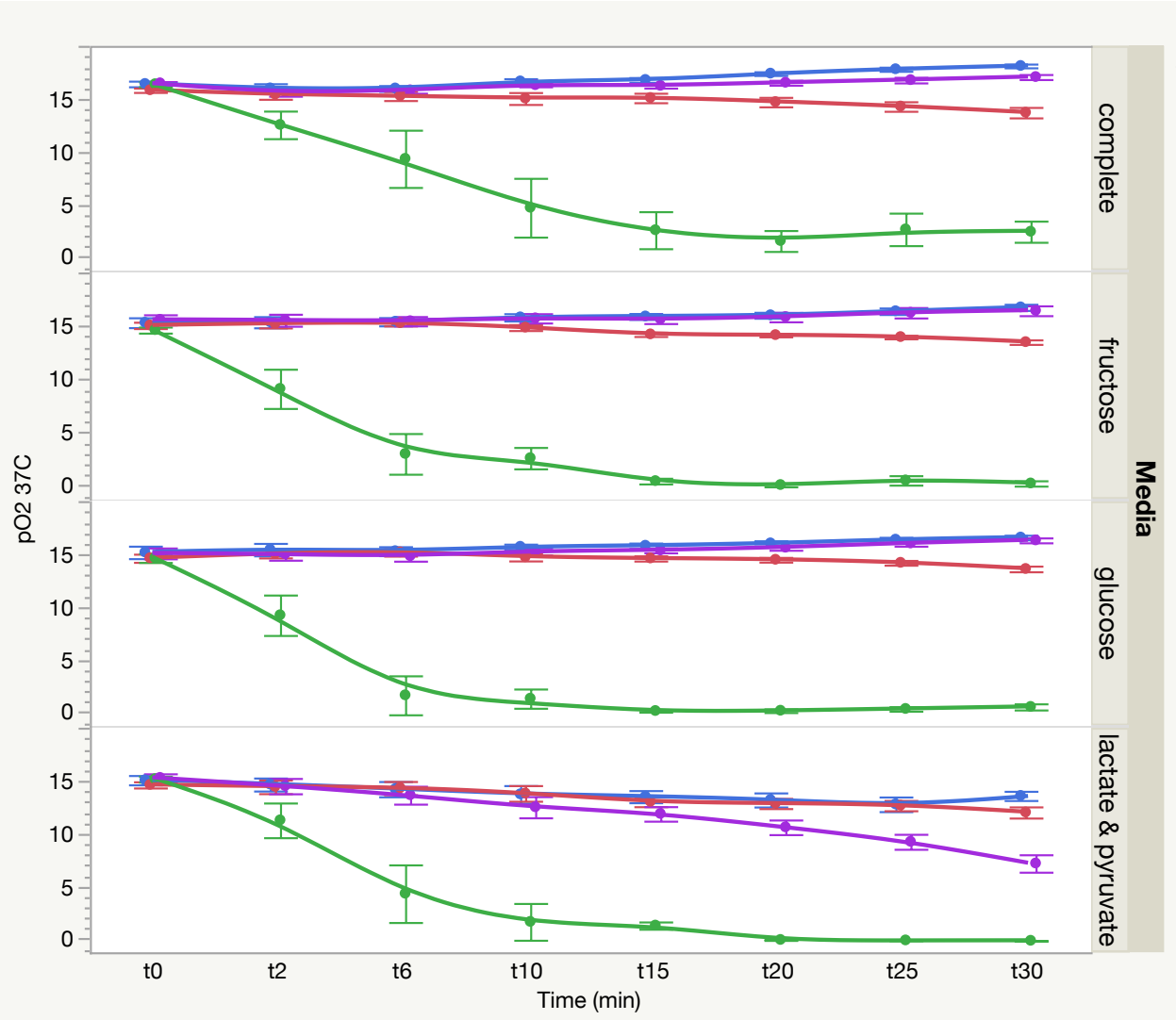


Figure 1. Oxygen Consumption by Nutrient Source. Oxygen consumption is presented in $pO_2$ values mean ± SEM. Blue lines represent basal oxygen consumption in untreated sperm. Green, red, and purple lines represent mean oxygen consumption after FCCP, Antimycin, and Oligomycin treatment, respectively.

**Figure 2:** Spare Respiratory Capacity by Nutrient Source

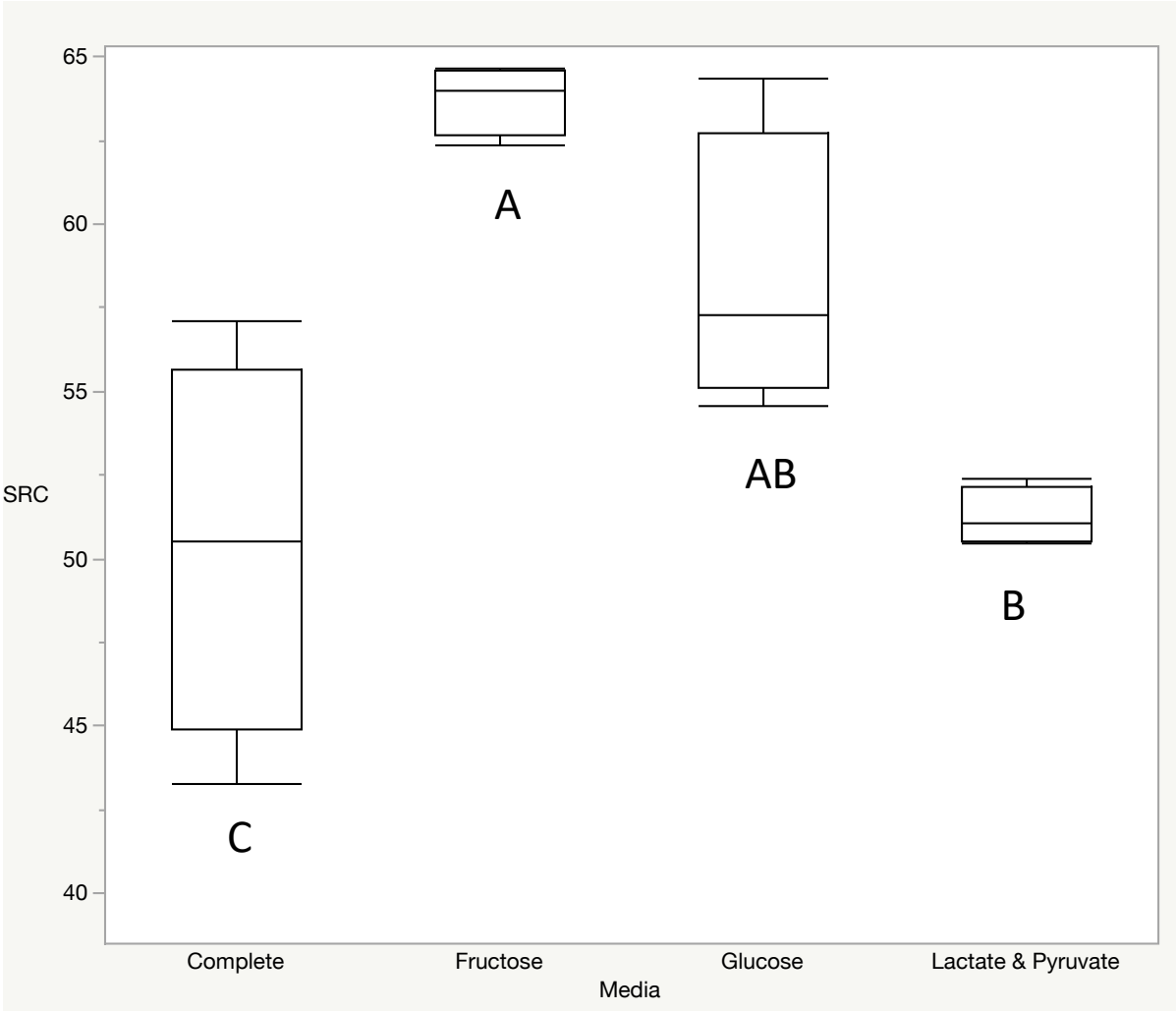


Fig.2: Mean spare respiratory capacity of untreated canine sperm by nutrient source. Media contained either Glucose or Fructose as the sole energy source, or Glucose, Lactate, and Pyruvate (Complete), or Lactate and Pyruvate. Box plots not sharing the same letter are significantly different ($p<0.05$).

**Figure 3a:** Summarized motility parameters in differing medias in the presence of mitochondrial effector treatments.

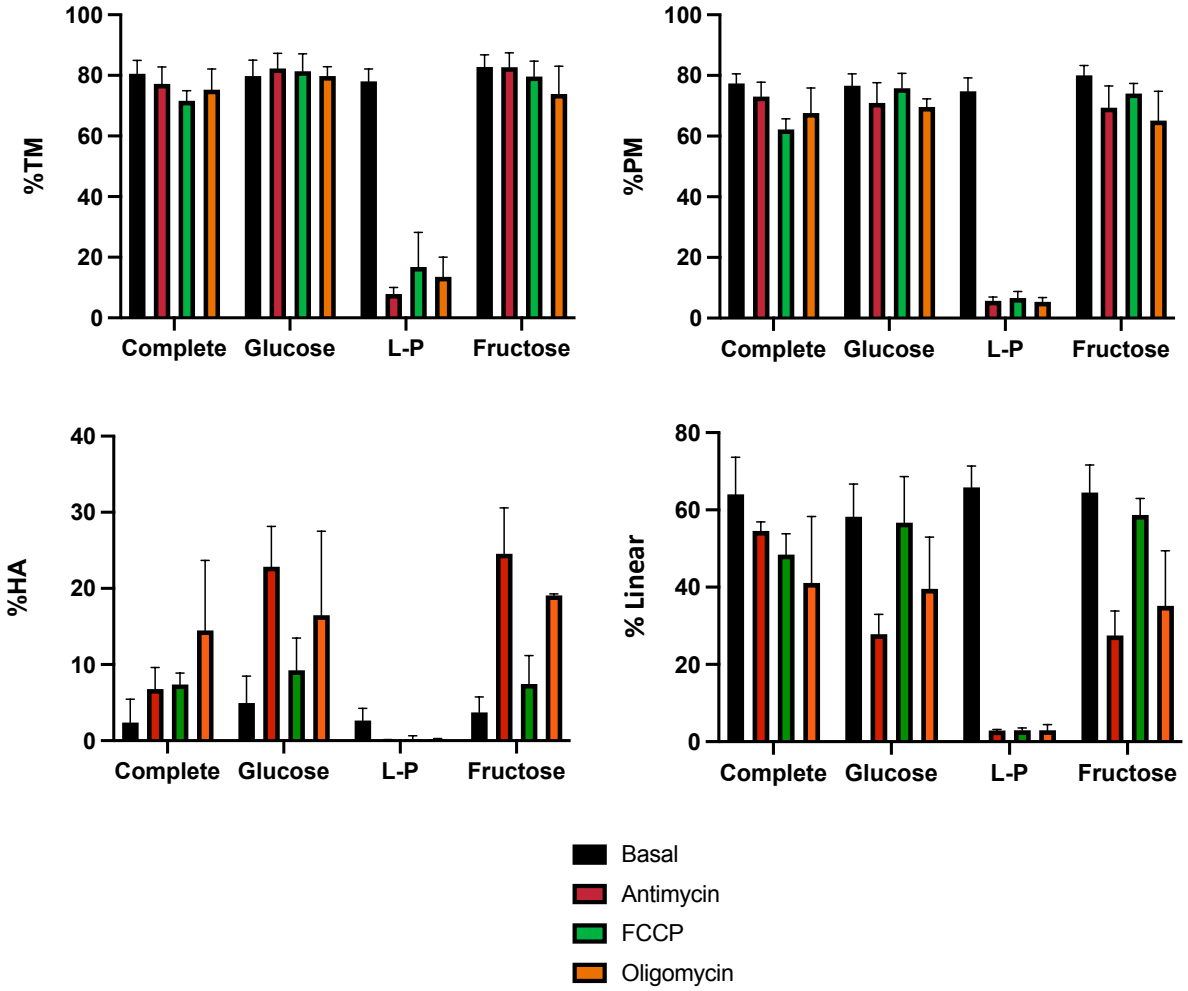


Figure 3a: Sperm motility parameters were quantified by computer-assisted sperm analysis (CASA) 30 min (t30) post-treatment addition. Plots of mean±SEM of Total Motility (TM, %), Progressive motility (PM, %), Hyperactive Motility (HA,%), and Linear Motility (Linear, %) are displayed. Sperm were tested in four medias: a complete media (containing glucose, lactate, and pyruvate), media supplemented with lactate and pyruvate, or media containing glucose or fructose as the sole energy source.

**Figure 3b:** Velocity measures in of motility in differing medias in the presence of mitochondrial effector treatments

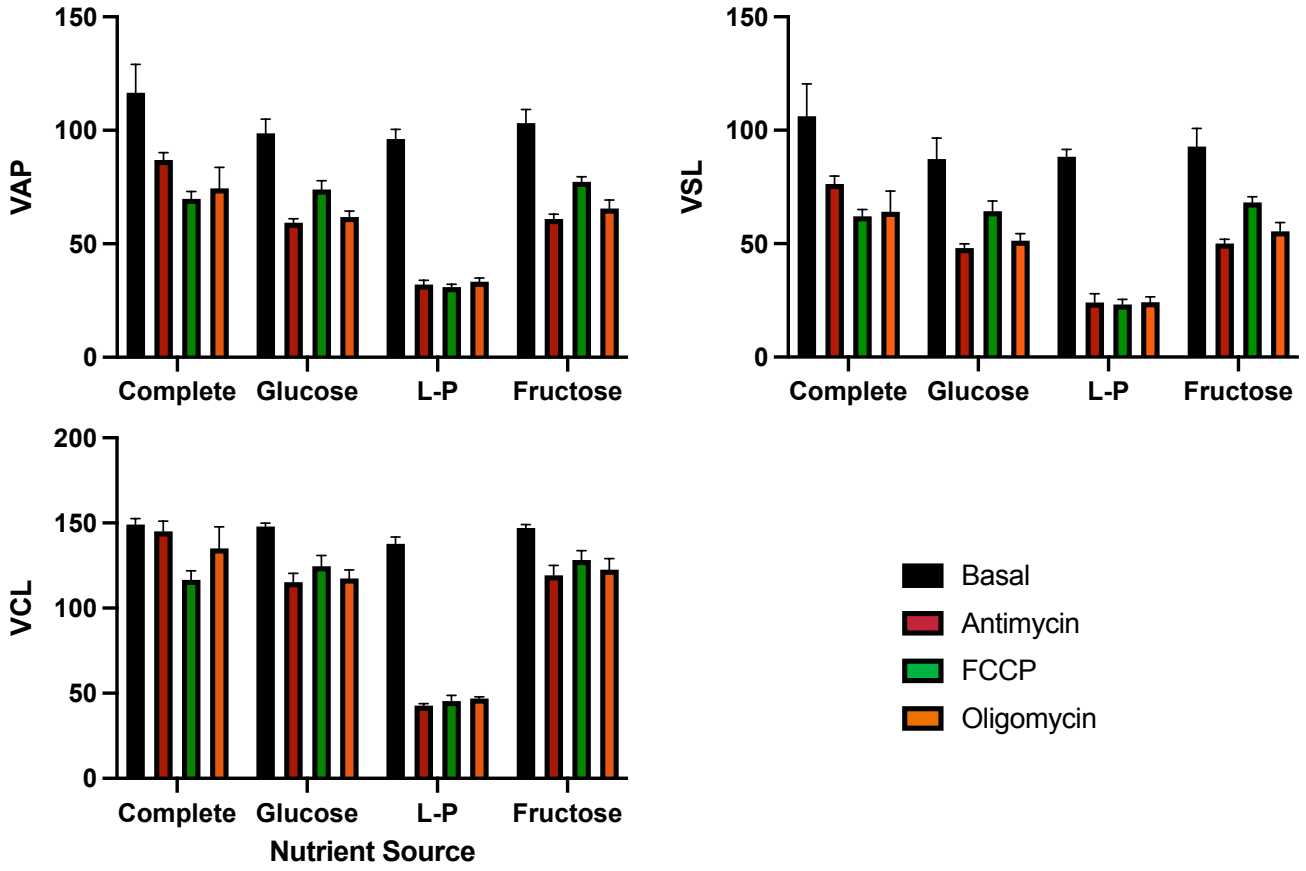


Fig. 3b: Velocity measures of motility in differing medias in the presence of mitochondrial effector treatments. Sperm motility parameters were quantified by computer-assisted sperm analysis (CASA) 30 min (t30) post-treatment addition. Plots of mean±SEM of Velocity Average Path (VAP, μm/s), Velocity Straight Line (VSL, μm/s), and Velocity Curbed Line (VCL, μm/s) are displayed. Sperm were tested in four medias: a complete media (containing glucose, lactate, and pyruvate), media supplemented with lactate and pyruvate, or media containing glucose or fructose as the sole energy source.

**Figure 3c:** Distance measures of motility in the presence of mitochondrial effector treatments

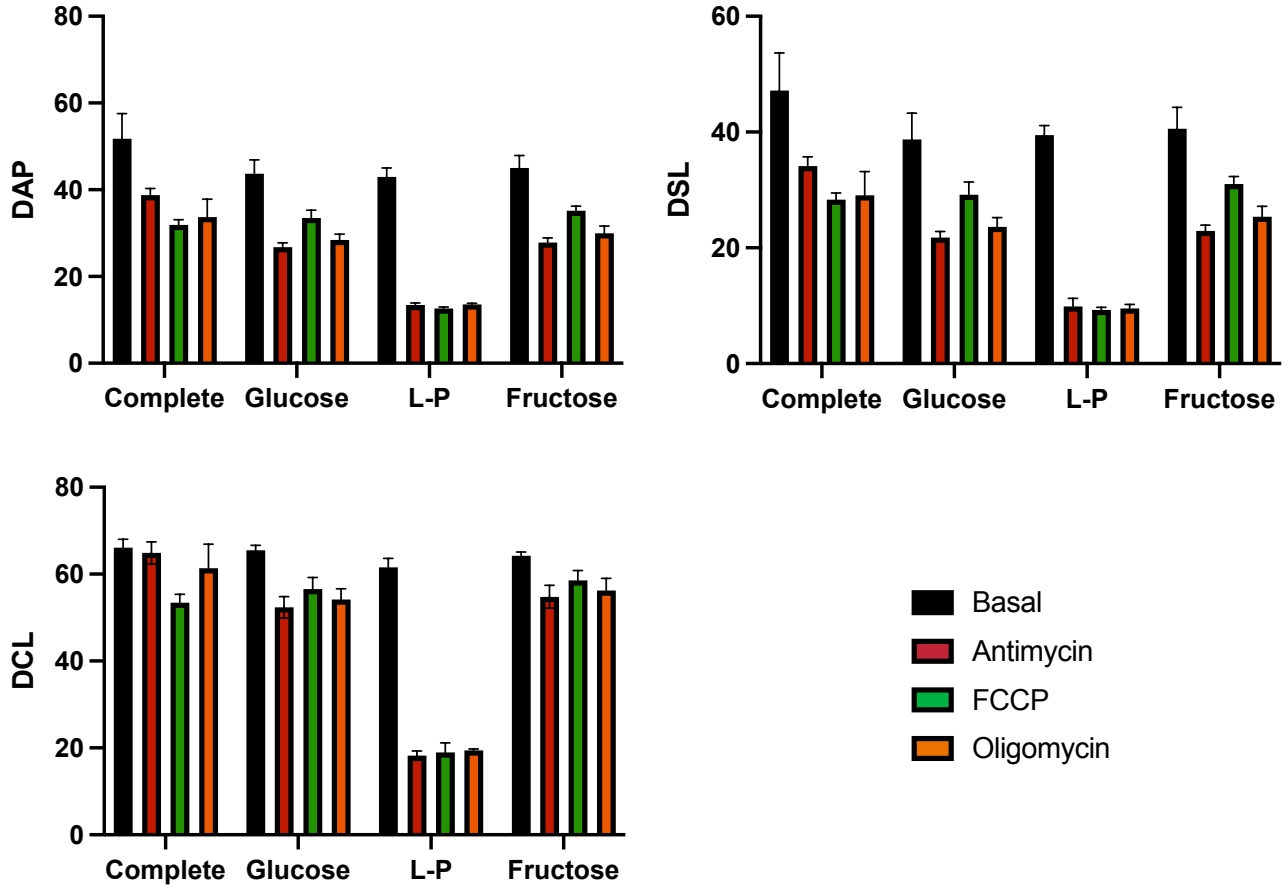


Figure 3c: Distance measures of motility in differing medias in the presence of mitochondrial effector treatments. Sperm motility parameters were quantified by computer-assisted sperm analysis (CASA) 30 min (t30) post-treatment addition. Plots of mean±SEM of Distance Average Path (DAP, μm), Distance Straight Line (DSL, μm), and Distance Curved Line (DCL, μm) are shown. Sperm were tested in four medias: a complete media (containing glucose, lactate, and pyruvate), media supplemented with lactate and pyruvate, or media containing glucose or fructose as the sole energy source.

**Figure 3d:** Linearity measures of motility in differing medias in the presence of mitochondrial effector treatments

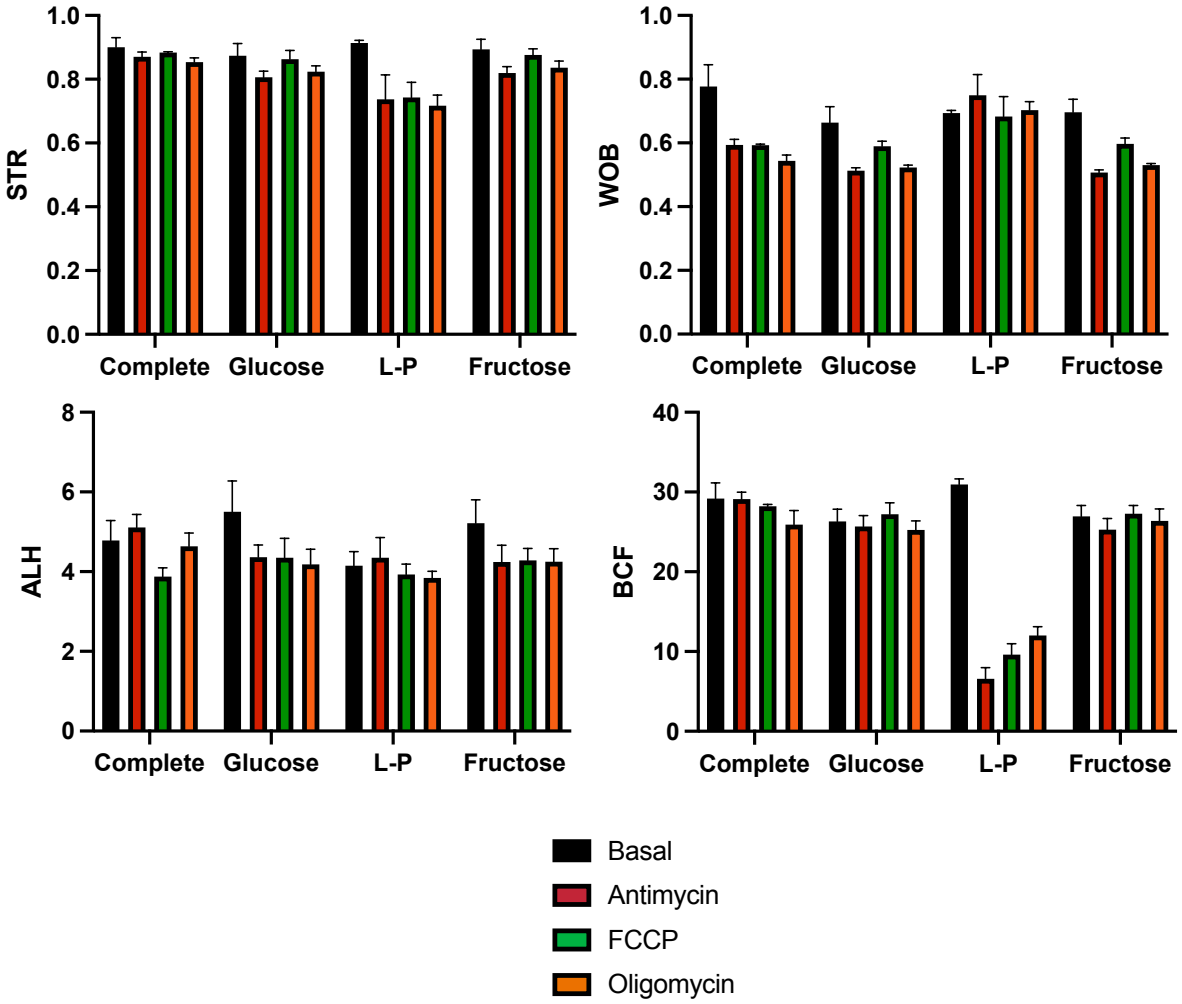


Figure 3d: Linearity measures of motility in differing medias in the presence of mitochondrial effector treatments. Sperm motility parameters were quantified by computer-assisted sperm analysis (CASA) 30 min (t30) post-treatment addition. Plots of mean±SEM of average Straightness (STR), Wobble (WOB), Amplitude of Lateral Head Displacement (ALH), and Beat Cross Frequency (BCF) are displayed. Sperm were tested in four medias: a complete media (containing glucose, lactate, and pyruvate), media supplemented with lactate and pyruvate, or media containing glucose or fructose as the sole energy source.

**Table 1:** Technical settings used for SpermVision (Minitube USA; Boulder, CO). CASA

| Settings Category | Parameter | Settings |
|---|---|---|
| General | Field of view depth | 20μm |
| | Pixel to μm ratio | 130 to 100 |
| | Cell identification area | 16 to 60μm$^2$ |
| | Assessment requirements | 4000 cells or 7 fields |
| | Additional particle filltering | none |
| | Light threshold | min 170, max 255 |
| | Points to use in cell path smoothing | 17 |
| Level 1 Cell Classifications | Immotile | VAP<20 |
| | Local | VAP<50, AOC>7, STR<0.7 |
| Level 2 Cell Classifications | Hyperactive | ALH>7.5, VAP≥0.75, LIN< |
| | Linear | STR>0.75, LIN>0.45 |
| | Nonlinear | LIN<0.35, STR<0.75 |
| | Curvilinear | DAP/Radius≥3, LIN<0.5 |

**Table 2:** Effect of Nutrient Source on Spare Respiratory Capacity

| Nutrient Source | Spare Respiratory Capacity |
|---|---|
| Glucose | 58.37(1.06)$^{a,c}$ |
| Fructose | 63.74(0.26)$^{a}$ |
| Complete | 50.35(1.42)$^{b}$ |
| Lactat and Pyruvate | 51.24(0.21)$^{b,c}$ |

Table 2. Spare respiratory capacity (SRC) of canine sperm by nutrient source. Data presented as mean (SEM). Media contained either glucose or fructose as the sole energy source, or glucose, lactate, and pyruvate (Complete) or lactate and pyruvate. Means without a common superscript significantly differed (p<.05).

**Table 3:** Sperm Motility Parameters by Nutrient Source

| Media | Treatment | TM | PM | ALH | VSL | VCL | VAP | DSL | DAP | DCL | %Linear | %HA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Glucose | Basal | 79.77(3.02) | 76.59(2.27 | 5.5(0.45) | 87.32(5.37) | 147.93(1.12) | 98.71(3.61) | 38.74(2.61) | 43.71(1.85) | 65.48(0.68) | 58.23(2.82) | 4.95(1.18) |
| | ANTI | 82.28(2.9) | 70.98(3.83) | 4.36(0.18) | 48.04(1.1) | 115.24(3.0) | 59.28(1.06) | 21.77(0.61) | 26.78(0.56) | 52.38(1.42) | 27.87(1.7) | 22.86(1.76) |
| | OLIGO | 81.35(3.33) | 75.82(2.83) | 4.35(0.28) | 64.3(2.57) | 124.61(3.66) | 73.88(2.24) | 29.19(1.25) | 33.48(1.06) | 56.59(1.53) | 56.7(3.98) | 9.24(1.42) |
| | FCCP | 79.78(1.77) | 69.59(1.57) | 4.19(0.22) | 51.27(1.85) | 117.37(2.94) | 61.79(1.56) | 23.6(0.94) | 28.4(0.79) | 54.14(1.44) | 39.59(4.47) | 16.49(3.68) |
| Lactate and Pyruvate | Basal | 78.02(2.37) | 74.78(2.54) | 4.15(0.2) | 88.32(1.85) | 137.68(2.36) | 96.17(2.45) | 39.5(0.93) | 42.99(1.19) | 61.6(1.17) | 65.8(1.85) | 2.65(0.54) |
| | ANTI | 7.86(1.28) | 5.68(0.72) | 4.35(0.29) | 34.03(2.28) | 42.71(0.66) | 32.02(1.12) | 9.86(0.83) | 13.43(0.26) | 18.22(0.62) | 2.91(0.08) | 0.08(0.02) |
| | OLIGO | 16.76(6.61) | 6.61(1.26) | 3.93(0.15) | 23.24(1.24) | 45.49(1.92) | 30.9(0.76) | 9.28(0.27) | 12.65(0.18) | 18.94(1.27) | 2.95(0.21) | 0.23(0.13) |
| | FCCP | 13.53(3.77) | 5.41(0.78) | 3.84(0.09) | 24.23(1.36) | 46.94(0.52) | 33.29(0.97) | 9.52(0.4) | 13.54(0.19) | 19.41(0.2) | 2.94(0.5) | 0.14(0.05) |
| Complete | Basal | 80.51(2.58) | 77.38(1.85) | 4.79(0.29) | 106.24(8.21) | 149.06(2.01) | 116.6(7.23) | 47.17(3.77) | 51.74(3.38) | 66.08(1.13) | 64.03(3.2) | 2.39(1.02) |
| | ANTI | 77.18(3.22) | 73.0(2.76) | 5.11(0.19) | 76.31(1.99) | 145.02(3.46) | 86.89(1.91) | 34.14(0.9) | 38.79(0.85) | 64.87(1.47) | 54.57(0.79) | 6.78(0.95) |
| | OLIGO | 71.64(1.92) | 62.2(2.0) | 3.87(0.13) | 62.02(1.78) | 116.65(3.06) | 69.83(1.88) | 28.32(0.68) | 31.89(0.7) | 53.4(1.13) | 48.44(1.79) | 7.39(0.5) |
| | FCCP | 75.27(3.98) | 67.66(4.76) | 4.64(0.19) | 64.05(5.31) | 135.03(7.31) | 74.38(5.4) | 29.07(2.36) | 33.67(2.4) | 61.39(3.18) | 41.08(5.73) | 14.49(3.07) |
| Fructose | Basal | 82.82(2.32) | 80.00(1.91) | 5.21(0.34) | 92.83(4.64) | 147.01(1.15) | 103.15(3.47) | 40.58(2.14) | 45.04(1.66) | 64.2(0.53) | 64.49(2.38) | 3.72(0.68) |
| | ANTI | 82.67(2.79) | 69.35(4.14) | 4.25(0.24) | 50.03(1.07) | 119.28(3.33) | 60.90(1.26) | 22.92(0.57) | 27.84(0.6) | 54.8(1.51) | 27.52(2.1) | 24.54(2.01) |
| | OLIGO | 79.64(2.94) | 74.02(1.92) | 4.28(0.17) | 68.14(1.46) | 128.32(3.14) | 65.54(2.17) | 31.03(0.74) | 35.17(0.62) | 58.59(1.3) | 58.72(1.41) | 7.46(1.24) |
| | FCCP | 73.83(5.3) | 65.09(5.62) | 4.25(0.19) | 55.39(2.28) | 122.56(3.77) | 77.3(1.31) | 25.38(1.05) | 29.99(0.95) | 56.25(1.62) | 35.17(4.75) | 19.08(0.07) |

Table 3. Sperm Motility endpoints by nutrient source, and mitochondrial effector treatment. Data presented as mean(SEM). Media contained either glucose or fructose as the sole energy source, or glucose, lactate, and pyruvate (Complete) or lactate and pyruvate.

**Table 4:** Regression Analysis: MITOX and Sperm Motility

| Nutrient Source | Endpoint | Significance, Regression Equation |
|---|---|---|
| Glucose | %Linear | F(1,5)= 93.83, p=0.002, R2 .97 %Linear = -0.76 + 0.02*pO2 |
| | WOB | F(1,5)= 86.83, p=0.003, R2 .97 WOB = -0.26 + 0.02*pO2 |
| | VSL | F(1,5)= 15.18, p=0.03, R2 .83 VSL = -113.95 + 2.92*pO2 |
| | VAP | F(1,5)= 7.9, p=0.07, R2 .73 VSL = -42.71 + 2.05*pO2 |
| | DSL | F(1,5)= 15.43, p=0.03, R2 .84 DSL = -58.4 + 1.41*pO2 |
| | DAP | F(1,5)= 9.88, p=0.05, R2 .77 DAP = -28.45+ 1.05*pO2 |
| Lactate and Pyruvate | %Linear | F(1,5)= 13.13, p=0.04, R2 .81. %Linear = 94.97-0.49*pO2 |
| | PM | F(1,5)= 8.72, p=0.06, R2 .74. PM = 119.21-0.7*pO2 |
| | TM | F(1,5)= 12.95, p=.04, R2 .81. TM = 122.87-0.71*pO2 |
| Complete | %Linear | F(1,5)= 7.41, p=0.07, R2 .72. %Linear = 2.51 + 0.89*pO2 |
| | %HA | F(1,5)= 9.96, p=0.05, R2 .77 %HA = 20.55 - 0.26*pO2 |
| Fructose | %HA | F(1,5)= 18.46, p=0.02, R2 .86 %HA = 36.75- 0.49*pO2 |

**Table 4:** Significant relationships between sperm motility and MITOX identified by regression analysis with significance level $p<.05$. Media contained either glucose or fructose as the sole energy source, or glucose, lactate, and pyruvate (Complete) or lactate and pyruvate.

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**Dissertation Conclusion**

The overall objective of this work was to study the effects of age and mitochondrial function on sperm quality and bioenergetics using traditional methods assessed in the clinical setting, as well as emerging methods investigating metabolic flexibility and the heterogenous nature of mammalian sperm. This dissertation explores the physiology and pathophysiology of canine sperm through the study of a breeding colony of highly fertile Labrador retrievers, and a rapidly aging purebred population of Great Danes.

In this work, I reviewed the literature and current understanding of mitochondrial physiology and the metabolic control of essential sperm functions in mammalian sperm. I presented a comparative analysis of oxidative metabolism in sperm of species of veterinary importance including Equine, Bovine, and Canine, and discussed the differences in fundamental oxygen consumption and ATP substrate balance. This review focused on the dynamic and responsive nature of sperm mitochondria, and explored how age, ROS production, and substrate availability affect the maintenance of sperm motility and other functions essential to fertilization.

Through two studies of highly contrasting but carefully managed canine populations, I explored the relationships between age, substrate availability, and sperm physiology. In a large, rapidly aging breed suffering a decline in fertility, I identified progressive declines in sperm quality that accompany aging including significant decreases in several measures of sperm motility associated with fertility and good cryopreservation outcomes. I then explored the relationship between ROS byproducts of mitochondrial function, and sperm motility and morphology, discussing the delicate balance between oxidative damage and maintenance of sperm kinematics. We showed that in a large rapidly aging breed, sperm motility parameters such as total and progressive motility decrease with each year of age at a rate 5-10 fold higher

than observed in humans. The rapid decline in semen quality indicated success by ART may be improved by targeting reproductive management between 24 and 48 months of age, though this is often earlier than a dog being proven in its discipline. Lastly, I distinguished sources of variation in sperm quality within the breed, and discussed how the selection for breed specific phenotypes, environmental exposure to toxicants such as BPA, and the heterogenous nature of sperm subpopulations can help explain the functional significance of the diversity among males.

I then investigated the adaptability of canine sperm bioenergetics and the effects of mitochondrial dysregulation on maintenance of sperm motility in a breeding population of highly fertile canines. Through simultaneous measurement of mitochondrial oxygen consumption (MITOX) and sperm kinematics in differing nutrient conditions and mitochondrial functional states, I observed the pronounced adaptability of high-quality canine sperm. Sperm of highly fertile dogs displayed great metabolic flexibility, with all energetic substrates tested successfully maintaining sperm motility. While sperm from other mammalian species may have a dependence on predominant metabolic pathways supporting sperm motility, sperm from fertile dogs maintain motility with a range of energy sources, and even in the event of mitochondrial disruption when alternative substrates are available. I found MITOX was correlated with several measures of sperm motility. However, substrate effects were identified in mitochondrial functionality, with significant differences observed in MITOX and mitochondrial spare respiratory capacity (SRC), the strength with which mitochondrial energy production may rise to meet energetic challenge. These findings suggest metabolic plasticity of mammalian sperm may be exploited to improve fertility outcomes by assisted reproductive techniques.

Unsurprisingly, significant physiological and metabolic differences have been found between mammalian species, representing the adaptation to different environmental and nutrient

conditions. Though routine semen analysis includes the measurement of microscopic and biochemical endpoints, little is known regarding the pathophysiology of canine sperm. In addition to interspecies differences in bioenergetics and control of key fertilizing events, intraspecies variation reflects the heterogenous nature of sperm populations, and effects of age and mitochondrial dysfunction on sperm quality. A greater understanding of the effects of age and energy management on canine sperm quality will improve male fertility, semen cryopreservation, and breeding management.