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ORIGINAL ARTICLE

Effects of substrate availability and mitochondrial disruption on oxidative metabolism and sperm motility in fertile dogs

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Abstract

In several mammalian species, the measurement of mitochondrial oxygen consumption (MITOX) under different metabolic conditions has demonstrated a positive correlation with sperm motility and may be a sensitive indicator of mitochondrial health. In general, the maintenance of sperm motility and many key sperm functions and fertilizing events are heavily energy-dependent processes, and some species-specific substrate preferences exist. Although canine sperm have been known to undergo capacitation and maintain motility with supplementation of a wide range of energy substrates, the relationship between mitochondrial function, and the maintenance of oxidative metabolism and sperm motility remain unclear. The objective of this study was to explore the metabolic flexibility of canine sperm, and to investigate the relationship between mitochondrial function, and maintenance of motility under differing nutrient conditions. We explored substrate preferences and the bioenergetics underlying maintenance of canine sperm motility by monitoring mitochondrial oxidative function and sperm kinematics in the presence of mitochondrial effector drug treatments: FCCP, antimycin (ANTI), and oligomycin (OLIGO). We hypothesized that canine sperm possess the ability to use compensatory pathways and utilize diverse nutrient sources in the maintenance of motility. Oxygen consumption (change in pO_2, oxygen partial pressure) and sperm kinematics (CASA) were

measured concurrently (t0–t30) to assess the relationship between oxidative metabolism and maintenance of sperm motility in dogs. Four media were tested: containing glucose, lactate, and pyruvate (GLP), containing glucose (G), fructose (F), or lactate and pyruvate (LP). In the absence of pharmacological inhibition of the electron transport chain, energetic substrate had no effect on sperm kinematics in fertile dogs. Following mitochondrial disruption by ANTI and OLIGO, mitochondrial oxygen consumption was negatively correlated with several sperm motility parameters in GLP, G, F, and LP media. In every media, FCCP treatment quickly induced significantly higher oxygen consumption than in untreated sperm, and spare respiratory capacity, the maximal inducible oxidative metabolism, was high. With respiratory control ratios RCR >1 there was no indication of bioenergetic dysfunction in any media type, indicating that sperm mitochondria of fertile dogs have a high capacity for substrate oxidation and ATP turnover regardless of substrate. Our results suggest MITOX assessment is a valuable tool for assessing mitochondrial functionality, and that canine sperm employ flexible energy management systems which may be exploited to improve sperm handling and storage.

Keywords

canine | mitochondrial respiration | oxidative metabolism | sperm

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1 | INTRODUCTION

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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 remains unclear. 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 (Cao et al., 2006). The ATP necessary for sperm motility, capacitation, acrosomal exocytosis, and 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 (du Plessis et al., 2015).

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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 of 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 favourable 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 which is 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 oxygen consumption and mitochondrial oxygen consumption (MITOX) under different metabolic conditions has demonstrated a positive correlation with motility and may be a sensitive indicator of mitochondrial health, although little is known specifically about canine sperm mitochondrial function (Ferramosca et al., 2008; Giaretta et al., 2022; Moraes & Meyers, 2018; Nesci et al., 2020).

Recent work investigating the importance of mitochondrial activity in the support of sperm kinematics has identified species-specific differences in the preferred metabolic pathways supporting sperm motility. For example, the glycolytic pathway is the primary energy source supporting human sperm motility, while stallion sperm rely on OXPHOS nearly exclusively (Darr et al., 2016; Nascimento et al., 2008). While mitochondrial functionality is key in bovine sperm capacitation and motility regulation, bovine sperm can maintain motility in the event of mitochondrial inhibition when provided with glycolytic substrates (Bulkeley et al., 2021; Hutson et al., 1977; Krzyzosiak et al., 1999). Yet, high glucose availability produces an inhibitory effect on bull sperm mitochondria, indicating metabolic flexibility in response to substrate availability (Moraes et al., 2021).

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 (Ballester et al., 2000; Ponglowhapan et al., 2004; Rota et al., 1999). 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 (Birkhead & Møller, 1993). Therefore, some degree of metabolic plasticity would prove evolutionarily 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 (Ballester et al., 2000; Palomo et al., 2003; Rigau et al., 2001, 2002). This suggests canine sperm employ flexible 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 aim of this study was 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 oxygen consumption and sperm kinematics 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

Fresh semen was collected from actively breeding, fertile Labrador Retriever (LR, $n = 12$) dogs under University of California IACUC-approved guidelines at Guide Dogs for the Blind (GDB). Each dog was owned by GDB and resided with a guardian owner in the San Francisco metropolitan area. They were under the medical care of veterinarians and staff at GDB during visits to the GDB campus. All animals were current in vaccinations with Body Condition Score (BCS) ranging 4–5/9. The dogs were fed similar diets, and in good overall health per annual veterinary evaluation at GDB.

Semen was collected into sterile plastic collection sleeves attached to 15 mL conical non-toxic polypropylene tubes (Corning Inc.) using a female in estrus for mounting in a quiet dedicated environment. Immediately following collection at GDB, motility was assessed by the SpermVision®SAR computer-assisted sperm analysis (CASA) system (Minitube USA, Inc.), and total sperm number was estimated by the SpermaCue photometer (MOFA Global, Verona, WI). Ejaculate volume was recorded and semen was extended 1:2 (v:v) in pre-warmed (37°C) modified TALP medium buffered with 40 mM HEPES containing glucose, lactate, and pyruvate (GLP hereafter) (Foutouhi et al., 2023; Parrish, 2014). One hundred microliters of each raw ejaculate were fixed in 500 µL of 10% buffered formalin for morphological assessment. Samples and extended semen were transported by car at ambient temperature to the UC Davis Veterinary Assisted Reproduction Laboratory (1.5 h 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). The culture medium used for this study was modified Tyrode's medium prepared without albumin, containing 1% polyvinylpyrrolidone, 75 mM NaCl, 2.8 mM KCl, 0.26 mM KH2PO4, 40 mM HEPES sodium salt, 2 mM $NaHCO_3$, 2 mM $CaCl_2$ (0.1 M solution, Ricca), and 0.4 mM $MgCl_2$ (1.0 M solution) (Foutouhi et al., 2023; Parrish, 2014). GLP medium contained the following metabolites: 5 mM D-glucose, 1 mM sodium pyruvate, and 0.19% (21.6 mM) DL-Lactic acid syrup. pH of GLP medium was adjusted to 7.4 ± 0.02 and osmolality of 300 ± 10 mOsm/kg. GLP medium was prepared fresh daily for experimentation and pre-

warmed to 37°C prior to semen collection. In addition to GLP, three additional modified Tyrode's medium containing either 67 mM glucose (G media), 67 mM fructose (F media), or 33 mM pyruvate and 33 mM lactate (LP media) as the sole energy source were studied.

2.3 | Sperm assessment

Immediately upon arrival at the UC Davis Veterinary Assisted Reproduction Laboratory, total sperm number and viability estimates were obtained using the NucleoCounter® SP-100TM automated cell counter (ChemoMetec) as previously reported (Foutouhi et al., 2023). Viability was estimated by using membrane status as determined by propidium iodine staining in the NucleoCounter (Daub et al., 2016; McCue, 2021). Motility assessments were performed by the same observer using the SpermVision®SAR CASA system. An aliquot of each ejaculate was adjusted to a sperm concentration of 30–50 x 10^6/mL in GLP media and 3 μL was loaded into Leja chambered slides pre-warmed on a 37°C warming plate for 5 min (Leja Products Luzernestraat) for determination of sperm motility. 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), straight line velocity (VSL, μm/s), curved line velocity (VCL, μm/s), 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 (%HA). SpermVision®SAR CASA settings are listed in Table 1.

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

Settings category	Parameter	Settings
General	Field of view depth	20 μm
	Pixel to μm ratio	130–100
	Cell identification area	16–60μm^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	Immotile	VAP <20

Classifications	Local	VAP <50, AOC >7, STR <0.75
Level 2 Cell Classifications	Hyperactive	ALH >7.5, VAP≥0.75, LIN <0.5

2.4 | Oxygen consumption and simultaneous motility assessment

Following initial sperm quality assessment, ejaculates were pooled into groups of 3 to obtain adequate sperm numbers for MITOX and kinematic evaluation of treatment groups. The pooled samples were then separated into four equal portions, washed once with GLP media using centrifugation at 250 × *g* for 8 min, then resuspended in either GLP, G media, F media, or LP media and adjusted to 50 × 10^6/mL using NucleoCounter® SP-100TM to confirm sperm concentration and measure viability.

Oxygen consumption measurements were performed using the OP96U OxoPlates® (PreSens Precision Sensing GmbH; Regensburg), 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 dye (sulforhodamine) were used to calculate the oxygen partial pressure (Regensberg, personal communication, January 1, 2016). Measurements were taken using a SynergyTM H1 plate reader (Agilent Technologies, Inc), set in dual kinetic mode and 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).

OxoPlates®, media, samples and treatments were prewarmed to 37°C prior to plating to minimize temperature-derived fluctuations in fluorescence. Titrations were performed in preliminary work to determine optimal cell number and drug treatment concentrations, and to identify testing endpoints after oxygen consumption curves have reached saturation levels. All controls and treatment groups were plated into 6 replicate wells. Two-point calibration was performed according to Oxoplate® manufacturer recommendations using oxygen-free water (cal0) and air-saturated water (cal100). Specifically, oxygen-free water (cal0) was prepared with 0.2 g of sodium sulfite (Na_2SO_3; a molecular oxygen scavenger) to 20 mL of pre-warmed H_2O, and 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, followed by slightly opening the tube and moving the liquid with a gentle swirling motion for 1 min to prevent oversaturation. Each well then received 300 µL of cal0, and the wells were immediately covered with adhesive foil. 200 µL of the air-saturated cal100 was plated into each well, leaving the wells uncovered. Appropriate media (100 µL) was then pipetted into all wells designated for sperm samples or ambient controls. 100 µL of each sperm sample (70 × 10^6 viable cells/mL) was added into each allotted well, for a final total of 7 × 10^6 viable cells/well.

An initial fluorescence reading was taken both before and after the plate was equilibrated in an incubator (37°C) for 20 min. Following equilibration, mitochondrial drug treatments (ANTI, OLIGO, FCCP) were quickly added and fluorescence measured (t0). Antimycin A (ANTI, 1 mM), an inhibitor of electron transport chain Complex III, was included to determine the baseline for non-mitochondrial oxygen consumption. Oligomycin (OLIGO, 2 mM), an ATP Synthase inhibitor (ETC complex V), was included to approximate the rate of ATP- linked respiration and observe the effects of proton leak. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP, 1 mM), a protonophore and mitochondrial uncoupler, was included for stimulation of maximal oxygen consumption.

Fluorescence readings were taken over the course of 60 min immediately following 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). Plates were incubated at 37°C between measurements. While preparing the OxoPlates®, aliquots of all samples were prepared in microcentrifuge tubes for parallel motility assessment. Motility was assessed by CASA as described previously, at t6 when drug treatments have taken effect, and t30 after FCCP oxygen consumption curves have reached saturation.

2.5 | Data processing and statistical analysis

Data was processed according to manufacturer instructions to calculate oxygen partial pressure (pO2) with the following equations for each well and time point (Bulkeley et al., 2021).

$$\mathrm{IR} = \mathrm{I}_{\mathrm{indicator}} / \mathrm{I}_{\mathrm{reference}}$$

$$\mathrm{pO}_2 = 100 \ \mathrm{x} \ ((\mathrm{k0} / \mathrm{IR} \ \mathrm{e} \ 1) / (\mathrm{k0} / \mathrm{k100} \ \mathrm{e} \ 1))$$

Oxygen partial pressure (pO_2) was used to calculate Spare Respiratory Capacity (SRC, 'reserve capacity') and Respiratory Control Ratio (RCR) at t30 with the following equations.

$$\mathrm{SRC} = \left| \left(\mathrm{pO}_{2\,(\mathrm{FCCP})} - \mathrm{pO}_{2\,(\mathrm{basal})} - \mathrm{pO}_{2\,(\mathrm{ANTI})}\right)\right.$$

$$\mathrm{RCR}_{\mathrm{max}} = \left(\mathrm{pO}_{2\,(\mathrm{FCCP})} - \mathrm{pO}_{2\,(\mathrm{ANTI})}\right) / \left(\mathrm{pO}_{2\,(\mathrm{OLIGO})} - \mathrm{pO}_{2\,(\mathrm{ANTI})}\right)$$

SRC is the difference between maximal and basal mitochondrial respiration and represents the maximal inducible oxidative metabolism which can be mobilized in case of increased energy demand (Desler et al., 2012). RCR is a measure of substrate oxidation and ATP turnover; RCR >1 implies there is no overall bioenergetic dysfunction in the mitochondria (Hill et al., 2012).

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), and pO_2 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 O_2 consumption, spare respiratory capacity, and sperm kinematics between nutrient sources and mitochondrial effector drug treatments. Significance was set at $p < .05$ for all statistical testing. Visualizations summarizing the effect of media type and mitochondrial treatments on canine sperm motility parameters were produced using GraphPad Prism version 9.5.1 for MacOSX GraphPad Software, www.graphpad.com.

3 | RESULTS

3.1 | Oxygen partial pressure (pO_2), and oxygen consumption

Oxygen partial pressure (pO_2) over time for each treatment condition is displayed in Figure 1. Subsequent decreases in oxygen over time represent oxygen consumption, measured as a decrease in pO_2. We observed significant differences in oxygen consumption over time by media type and mitochondrial effector treatments ($p < .0001$).

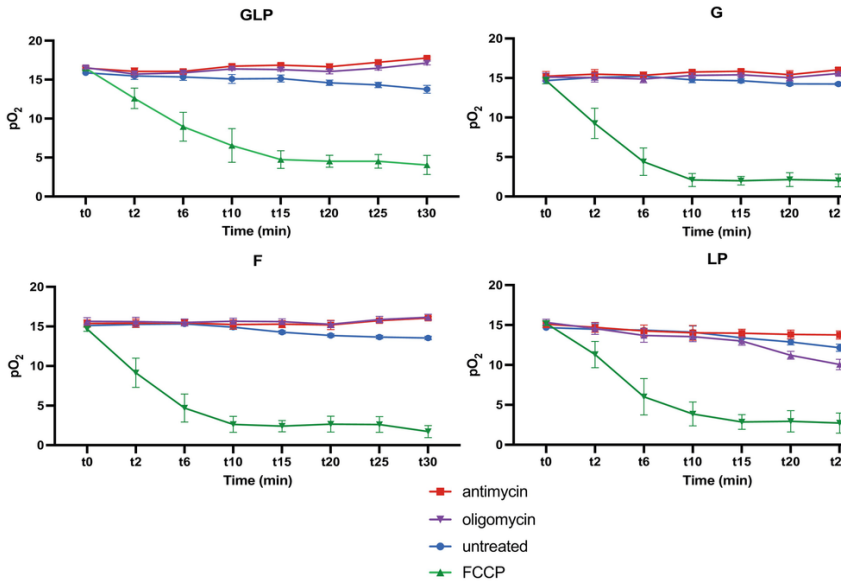

FIGURE 1.
Average oxygen partial pressure over time by Nutrient Source presented in pO_2 mean ± SEM. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

FCCP pO_2 levels were lower than ANTI, OLIGO, and untreated sperm in all media types by t6 and reached maximal oxygen consumption rate by t15 ($p < .0001$). ANTI pO_2 levels remained high in all media

types, and except for LP media ($p < .0001$), ANTI pO_2 levels were indistinguishable from OLIGO treatment.

OLIGO pO_2 levels significantly differed from untreated sperm by t30 in GLP and LP media ($p < .05$, $p < .0001$). OLIGO pO_2 levels remained high in all media except LP, where oxygen consumption increased and pO_2 significantly decreased compared to OLIGO treatment in G, F, and GLP media ($p < .0001$).

3.2 | Spare respiratory capacity (SRC), MITOX, and respiratory control ration (RCR)

Means comparisons testing identified significant differences in spare respiratory capacity (SRC) by nutrient source (ANOVA, F[3] = 12.3, $p < .001$), and represents the maximal increase in mitochondrial oxygen consumption (MITOX) that can be leveraged during a state of increased energy demand (Figure 2a). Sperm in GLP media had significantly higher MITOX (all, $p < .05$), and significantly higher SRC (all, $p < .0001$) than sperm provided with G, F, or LP media.

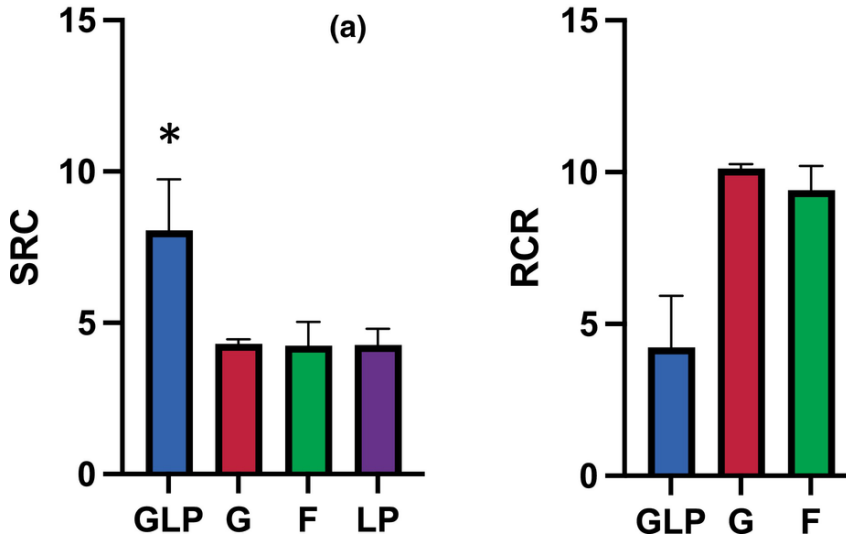

FIGURE 2.

Spare Respiratory Capacity and Reserve Capacity Ratio by Nutrient Source. (a) Mean spare respiratory capacity (SRC), defined as the maximal amount of mitochondrial respiration that can be leveraged by oxidative phosphorylation in case of a sudden increase in energy demand. (b) reserve capacity ratio (RCR) of untreated canine sperm by nutrient source (t30). RCR >1 asserts no overall bioenergetic dysfunction. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate. Box plots not sharing the same letter are significantly different ($p < .05$).

Respiratory control ratios (RCR) were calculated to assess substrate and treatment dependent mitochondrial dysfunction (Figure 2b). RCR >1 in all media types asserts there is no overall bioenergetic dysfunction in the mitochondria of sperm tested regardless of substrate.

3.3 | Mitochondrial inhibition and sperm motility

Sperm motility endpoints are listed in Table 2 and shown in (Figures 3–6). Nutrient source had no effect on maintenance of motility in untreated sperm (t30) ($p < .05$). When provided only mitochondrial substrates (LP media), disruption of OXPHOS by ANTI, OLIGO, and FCCP significantly reduced PM, TM, VSL, VCL, VAP, DSL, DAP, DCL, BCF, HA and LIN (all $p < .0001$) compared to untreated sperm. ANTI, OLIGO, and FCCP had no effect on ALH or WOB in LP media.

TABLE 2. Sperm Motility endpoints by nutrient source, and mitochondrial effector treatment.

Media	Treatment	TM	PM	ALH	VSL	VCL	VAP
GLP	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)
	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)
	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)
	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)
G	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)
	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)
	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)
	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)

Note: Data presented as mean(SEM).
Abbreviations: F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

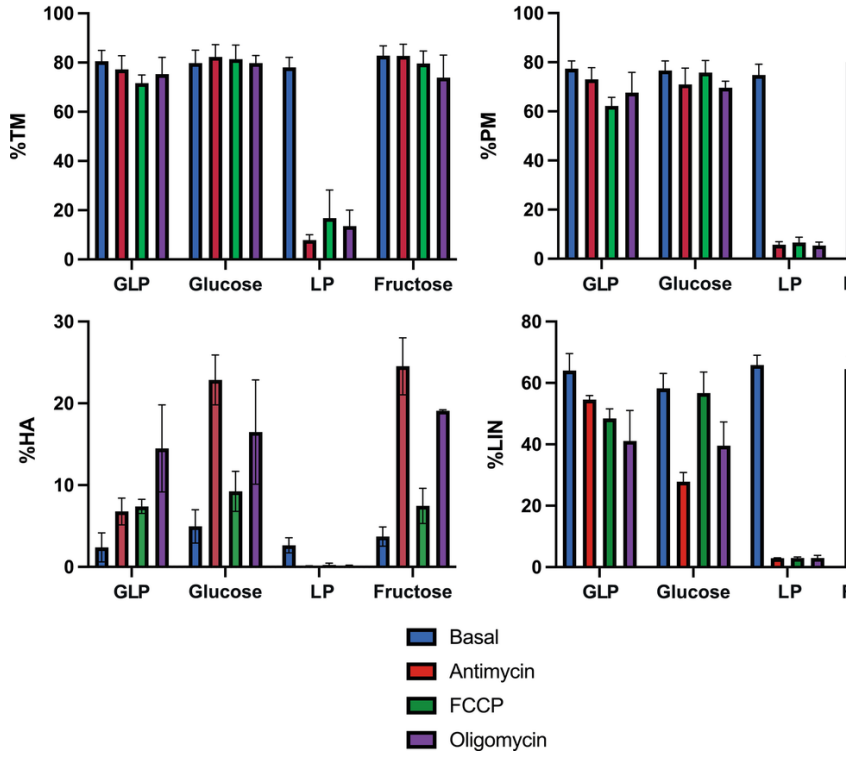

FIGURE 3.
Summarized canine sperm motility parameters in differing medias in the presence of mitochondrial effector treatments. Sperm motility parameters were quantified by computer-assisted sperm analysis (CASA) 30 min (t30) post-treatment addition. Plots of mean ± SEM of Total Motility (TM, %), Progressive motility (PM, %), Hyperactive Motility (HA, %), and Linear Motility (Linear, %) are displayed. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

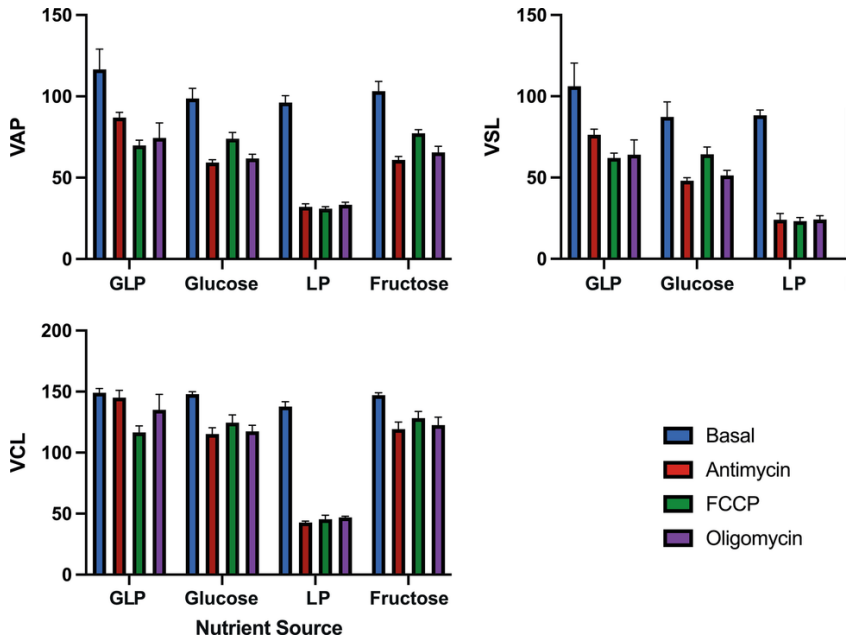

FIGURE 4.
Velocity measures of canine sperm motility in differing medias in the presence of mitochondrial effector treatments. Velocity measures of motility in differing medias in the presence of mitochondrial effector

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. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

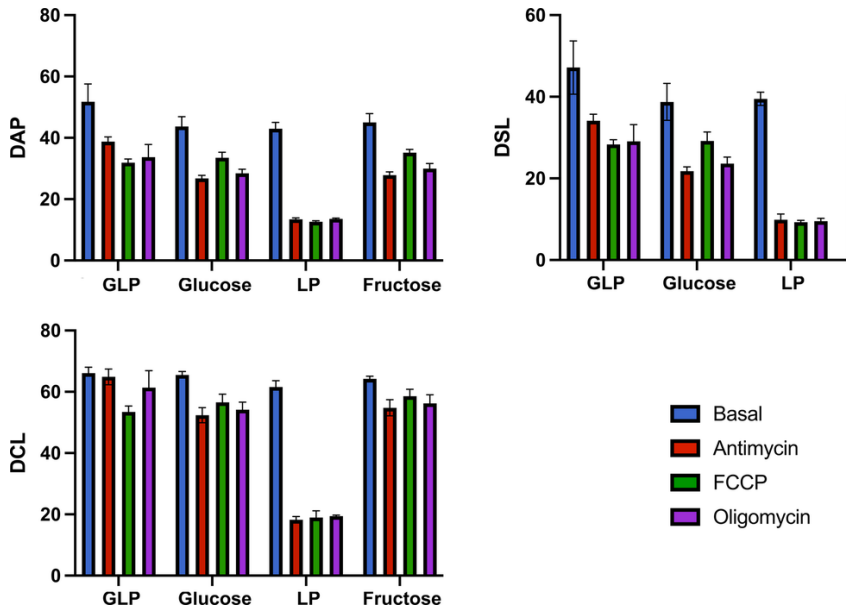

FIGURE 5.

Distance measures of canine sperm motility in the presence of mitochondrial effector treatments. 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. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

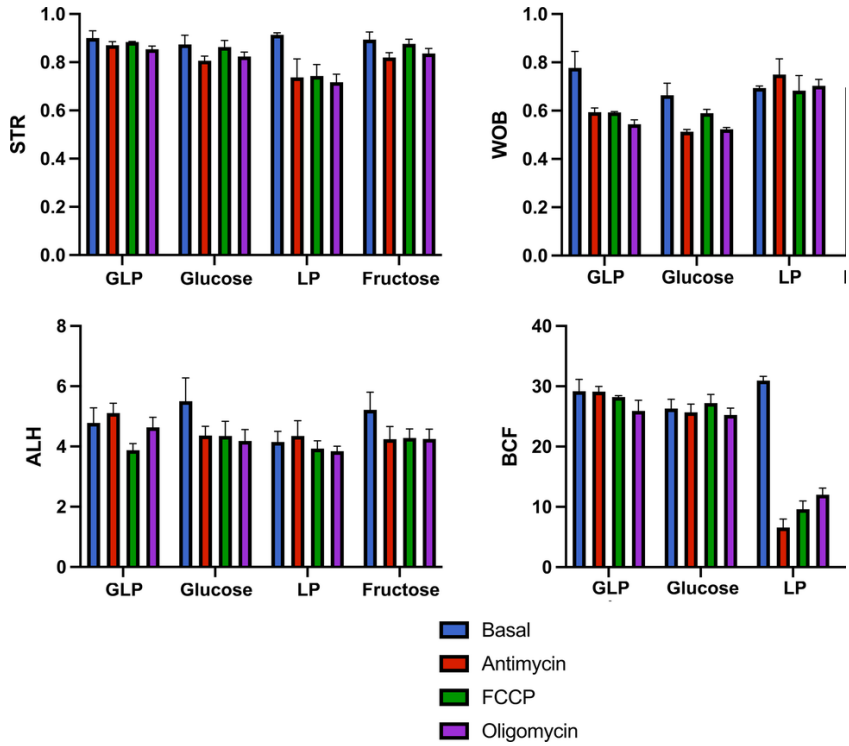

FIGURE 6.
Linearity measures of canine sperm motility in differing medias in the presence of mitochondrial effector treatments. Linearity measures of motility in differing medias in the presence of mitochondrial effector treatments. Sperm motility parameters were quantified by computer-assisted sperm 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. F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

Compared to untreated sperm, ANTI, OLIGO and FCCP had no effect on TM, PM, and BCF in GLP, G, and F media. FCCP significantly reduced VSL, VAP, and DAP (all $p < .05$) in F media, while OLIGO significantly reduced VSL, VAP, DAP, DSL, VCL, HA, and Linear (all $p < .05$). ANTI significantly increased HA, and significantly reduced VAP, VSL, VCL, DAP, DSL, WOB, and LIN in F media (all $p < .05$). FCCP significantly reduced VAP, VSL, VCL, DAP, DCL in GLP media (all $p < .05$).

HA did not significantly differ by nutrient source in untreated sperm, but was abolished by ANTI, OLIGO, and FCCP treatment in LP (all $p < .001$). In G and F media ANTI treatment resulted in significantly higher HA than untreated sperm (all $p < .05$).

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

3.4 | Oxygen consumption and sperm motility

Correlation data is displayed in Table 3. No significant correlations were identified between pO_2 and motility parameters in untreated sperm regardless of media type. Several significant correlations were identified between pO_2 and motility parameters in sperm treated with ANTI, OLIGO, and FCCP.

TABLE 3. Pearson's correlation of pO_2 with sperm motility (t30).

Substrate	Treatment	Motility parameter	Mean ± SD	Correlation with pO_2	*p*-value
GLP	FCCP	VCL	116.65 ± 9.17	−0.9994	.0211
		DCL	53.4 ± 3.38	−0.984	.0296
	OLIGO	WOB	0.54 ± 0.03	0.9995	.0191
G	ANTI	ALH	4.36 ± 0.54	−0.9985	.0348
	OLIGO	WOB	0.52 ± 0.01	0.9988	.0307
F	OLIGO	BCF	26.39 ± 2.60	−0.9973	.0464
		WOB	0.53 ± 0.01	−0.9991	.0273
LP	ANTI	VSL	24.03 + 6.83	−0.9978	.0422

Note: Significant relationships between sperm motility and oxygen consumption identified by Pearson product–moment correlation with significance level $p < .05$ (t30).
Abbreviations: F, fructose; G, Media contained either glucose; GLP, glucose lactate and pyruvate; LP, lactate and pyruvate.

In sperm treated with OLIGO, mean pO_2 was positively correlated with WOB in GLP, G, and F media (all $p < .05$). In LP media, mean pO_2 was negatively correlated with TM ($p < .05$). In sperm treated with ANTI in LP media, mean pO_2 was negatively correlated with VSL, DSL, and STR (all $p < .05$). ALH was negatively correlated with mean pO_2 in ANTI treated sperm in G media ($p < .05$). In sperm treated with FCCP, mean pO_2 was negatively correlated with VCL and DCL in GLP media (all $p < .05$).

4 | 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 collapse of the proton gradient regardless of nutrient source.

We found that untreated 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 the report 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 (Rigau et al., 2001). We identified little variation between glucose and fructose supplementation in measures of velocity, distance, and linearity. This contrasts 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 (Rigau et al., 2001); (Rigau et al., 2002).However, Rigau et al. identified subpopulations of sperm which responded differently to glucose and fructose supplementation, suggesting some degree of variation in metabolic sensitivity and adaptability (Rigau et al., 2001).

Disruption of OXPHOS by mitochondrial uncoupling, or by inhibition of either ETC Complex III or ATP Synthase failed to significantly reduce total and progressive motility in media containing non-mitochondrial substrates. The metabolic requirements and aetiology of decreased sperm function in sub fertile dogs is unknown, but results indicate sperm of fertile dogs use compensatory mechanisms to support motility in the event of decreased mitochondrial functionality, and substrate availability.

AQ7 Measurement of oxygen consumption has been used as a sensitive indicator of mitochondrial health in stallions, humans, and bulls (Bulkeley et al., 2021; Darr et al., 2016; Ferramosca et al., 2013). Although no relationships were identified between MITOX and kinematics in untreated sperm from fertile dogs, oxygen consumption is negatively correlated with several motility measures following mitochondrial disruption.

Oxygen consumption and sperm kinematics were more highly correlated in sperm treated with ANTI and OLIGO in LP media, suggesting oxygen consumption and MITOX may be better descriptors of mitochondrial function under stress in fertile dogs.

Spare respiratory capacity (SRC) is emerging as a qualitative indicator of mitochondrial energetic status and is likely regulated by energy demand and substrate availability (Hill et al., 2012). SRC being significantly greater in GLP media suggests the use of multiple energetic substrates could best support mitochondrial respiration during increased energy demand.

The respiratory control ratio (RCR) has been used as an index of mitochondrial respiration efficiency and sperm metabolic status in stallions and men (Irigoyen et al., 2022). RCR >1 in all media, implying mitochondria of sperm from fertile dogs have a high capacity for substrate oxidation and ATP turnover, with no indication of bioenergetic dysfunction (Ferramosca et al., 2008). 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 (Foutouhi & Meyers, 2022; Fuente-Lara et al., 2019; Martínez et al., 2006). 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 (Ballester et al., 2000; Palomo et al., 2003; Rigau et al., 2001).

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 vigour 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 (Foutouhi & Meyers, 2022; Foutouhi et al., 2023; Fuente-Lara et al., 2019); (Hesser et al., 2017). 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 (Setyawan et al., 2016). Similarly, we found that inhibition of a major source of mitochondrial ROS, complex III, was negatively correlated with ALH in glucose media. 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 dogs (de Lamirande & Gagnon, 1995; Foutouhi & Meyers, 2022; Gibb et al., 2014, 2020).

In addition to substrate availability and ROS production, inducible mitochondrial proton leak may influence sperm kinematics. Treatment with ATP synthase inhibitor OLIGO results in an inability to harness the free energy of the electrochemical proton gradient to synthesize ATP, thereby reducing dissipation of the H^+ gradient through the enzyme. As the rate of diffusion increases proportionately to concentration of the gradient, proton leak increases. In our study, OLIGO treated sperm in LP media showed significantly higher oxygen consumption than untreated and OLIGO treated sperm in any media. Proton leak includes both basal and inducible components, the latter catalysed by adenine nucleotide translocase (ANT) and uncoupling proteins (UCP) which are highly conserved in mammals and regulate mitochondrial bioenergetics and ROS production in human sperm (Jastroch et al., 2010; Moraes et al., 2021; Skinner et al., 2022). As a result, modulation of proton leak is a therapeutic target for many aspects of age-related disease.

In this study, we have demonstrated the adaptability and metabolic sensitivity of canine sperm in maintaining oxidative metabolism 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 examination of

canine sperm bioenergetics and kinematics has shown oxygen consumption and MITOX are correlated with several motility parameters in sperm suffering mitochondrial disruption. 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.

5 | CONCLUSION

In this study we have demonstrated that in the absence of mitochondrial disruption, energetic substrate (GLP, G, F, LP) had no effect on sperm kinematics of fertile dogs. RCR calculations assert that there is no overall bioenergetic dysfunction of sperm mitochondria in any of the medias tested, and that sperm mitochondria of fertile dogs have a high capacity for substrate oxidation and ATP turnover with several substrates. The maximal inducible oxidative metabolism which can be mobilized in case of increased energy demand, SRC, was highest in GLP media, suggesting canine sperm may benefit from supplementation with multiple energetic substrates. When provided with non-mitochondrial substrates, we observed no effect of mitochondrial disruption of total or progressive motility. Oxygen consumption was correlated with several sperm motility measures in all media types following mitochondrial disruption. Our results suggest canine sperm employ flexible energy management systems which can be exploited to improve sperm handling and storage, but questions remain regarding the pathways contributing to control of sperm motility, and the mechanism by which sperm function suffers with cryopreservation and increasing age.

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CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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