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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.

1. 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

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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.

2. Materials and methods

2.1. 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.

2.2. Semen collection

All semen collection and sample processing was completed onsite 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 ×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.

2.3. 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 KH_2PO_4, 40 mmol/L HEPES sodium salt, 2 mmol/L $NaHCO_3$, 2 mM $CaCl_2$ (0.1 M solution, Ricca), and 0.4 mM $MgCl_2$ (1 M 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.

2.4. Determination of sperm concentration, morphology, and motility parameters

Sperm number, concentration, and viability estimates were obtained by using the NucleoCounter® SP-100™ 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 500 μL 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 ×1000 magnification by differential interference contrast (DIC) microscopy with oil immersion (Olympus BX-60 with ×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 min then each chamber was loaded with 3 μL of semen extended in TGLP (200 μL; 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.

2.5. 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 (cm^3) 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.

2.6. 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 (488 nm) and red (640 nm) 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 h. Briefly, ejaculates were washed with modified Tyrode's medium (TGLP), then adjusted to a concentration of 25×10^6 sperm per mL. Aliquots (500 μL) 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 1.25 μL of CellRox™ Deep Red diluted to 1 mM 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 350*g* for 5 min to obtain a soft pellet. After discarding the supernatant, the pellet was resuspended in 1 mL of TGLP. Samples were then washed once by centrifugation at 350*g* for 5 min, then supernatant was discarded and pellet resuspended in 250 μL of TGLP. Stained samples were then fixed by adding 250 μL of 4% paraformaldehyde in Dulbecco's phosphate buffered saline without calcium or magnesium (DPBS −/−) for 15 min in the dark at room temperature. Fixed stained samples were washed once by centrifugation at 350*g* for 5 min, then supernatant was carefully discarded, and the pellet was resuspended in 500 μL 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.

2.7. 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 $p < .05$. 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.

3. Results

3.1. 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) (Fig. 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 Fig. S1. Percentage of morphologically normal sperm ranged between 2% and 81% with a mean of 43.4 ± 2.8% and 7.3% of Great Danes in this study had ≥70% morphologically normal sperm.

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

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

3.2. Effect of age on motility

The distribution of motility parameters in the study population are shown in Table 2 and highlighted in Fig. 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 12–24 months of age, and dogs older than 48 months (Fig. 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.

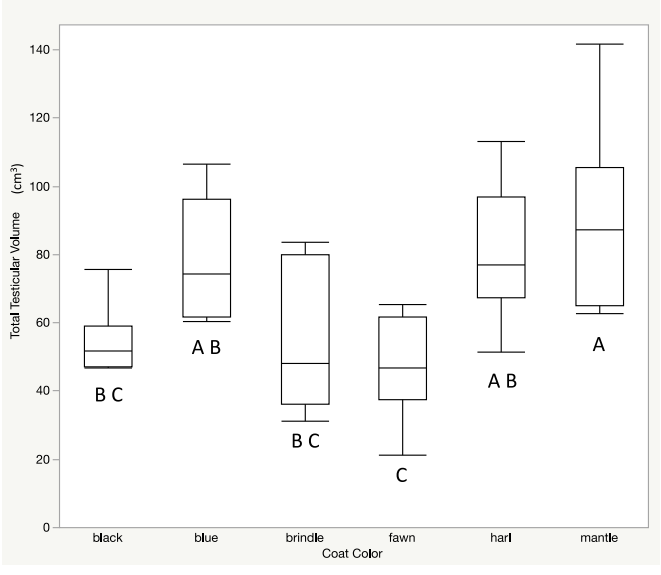

Fig. 1. Means comparison of total testicular volume by coat color, where shared letters indicate lack of significance between groups. Groups which do not share letters have significantly different means (p < .05). Box plots indicate group means ± SEM. (n = 48 total and distribution as mantle (n = 7), harlequin (n = 8), fawn (n = 13), black (n = 7), brindle (n = 6), blue (n = 7).

3.3. 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, 13.7 μm s^{-1} increase in VAP, 12.4 μm s^{-1} increase in VSL, 5.8 μm increase in DAP, and a 5.4 μm 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%.

3.4. 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

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

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

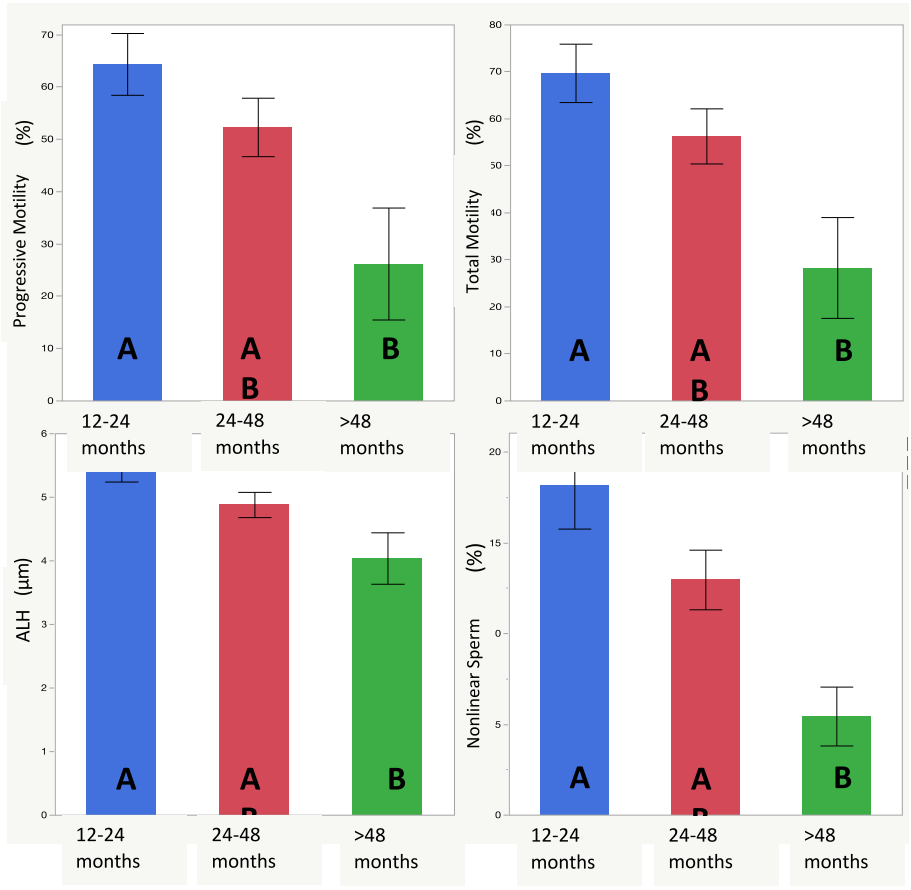

Fig. 2. Means comparisons of sperm motility parameters by age group identified significant differences between young and older Great Dane dogs. Dogs were grouped by age as follows: dogs 12 months or older and up to 24 months (n = 16), dogs 24 months or older and up to 48 months (n = 27), and dogs 48 months of age or older (n = 5). 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.

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 (Fig. 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

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.

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

(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.

4. 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 12–24 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 12–24 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.

5. 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.

CRediT authorship contribution statement

Azarene Foutouhi: Methodology, Investigation, Validation, Writing – original draft, Data curation. **Andrea Hesser:** Methodology, Investigation, Resources. **Alejandro de la Fuente:** Methodology, Investigation, Writing – review & editing. **Evelyn Bulkeley:** Methodology, Investigation, Writing – review & editing. **Pouya Dini:** Conceptualization, Writing – review & editing. **Stuart Meyers:** Conceptualization, Project administration, Supervision, Investigation, Methodology, writing; original and revision, Funding acquisition, Resources.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.theriogenology.2022.12.001.

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