University of Zagreb

Faculty of Veterinary Medicine

Pauline Jourdain

**Fatty acid composition of the dog spermatozoa and seminal plasma**

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"This paper was created in the Faculty of Veterinary Medicine University of Zagreb within the laboratories of the Department of Physiology and Radiobiology and Clinic for Obstetrics and Reproduction, under the guidance of prof. Martina Lojkić, DVM, PhD and assoc. prof. Lana Pađen, DVM, PhD and submitted to the competition for the Rector's Award in academic year 2023/2024”.

**List and explanation of abbreviations**

AA = arachidonic acid

ALH = amplitude of lateral head displacement

BCF = beat cross frequency

CASA = Computer Assisted Sperm Analysis

DHA = docosahexaenoic acid

DNA = Deoxyribonucleic Acid

ELONG = elongation

EN = eosin/nigrosin

EPA = eicosapentaenoic acid

FA = fatty acids

FAME = fatty acids methyl esters

FID = flame ionization detector

HOS = hypo-osmotic swelling test

LIN = linearity

LIN = linearity

MNS = morphologically normal spermatozoa

MUFA = monounsaturated fatty acids

n-3 PUFA = omega 3 polyunsaturated fatty acids

n-6 = omega 6 polyunsaturated fatty acids

PM = Progessive motility

PMI = plasma membrane integrity

PUFA = polyunsaturated fatty acids

SFA = saturated fatty acids

SGG = sulfogalactosylglycerolipid

STR = straightness

VAP = average path velocity

VCL = curvilinear velocity

velocity distribution: fast (RAP), medium (MED), slow (SLOW) and static (STATIC)

VSL = straight line velocity

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# Introduction

Semen is the final product from the combined input of the testes, the epididymis and the accessory glands. It is composed of a cellular portion – the spermatozoa and a fluid portion – the seminal plasma.

The spermatogenesis is a process that takes place in the testes, more particularly in the seminiferous tubules and that gives rise to highly specialized cells: sperm cell made to deliver the male nuclear content to the female gamete. Structurally, sperm cells are divided into 2 main parts: the head which includes the nucleus and the acrosomal cap and the flagellum subdivided into different pieces. The sperm cell is delimited and protected from its outer environment by a membrane: the sperm plasmatic membrane. It is made of a bilayer of phospholipids, with saturated and unsaturated fatty acids being the functional units (RAMOS ANGRIMANI et al., 2017). As the spermatozoa moves from the lumen of the seminiferous tubules through the epididymis, the spermatozoa continue to mature. They lose their cytoplasmic droplet, progressively gain motility and fertilizing ability (LOPATE, 2022) and the spermatozoa’s plasma membrane undergoes reorganization and stabilization through structural changes in its lipid profile, which allows higher membrane integrity and, eventually, acquire progressive motility (AMANN et al., 1993). This lipid remodeling of the sperm plasma membrane during epididymal maturation is mediated by protein and lipid constituents of the liquid, in which sperm is located, namely the seminal plasma.

Seminal plasma is a complex fluid composed of amino acids, fatty acids, ions, carbohydrates, organic salts and proteins (MANN and LUTWAK-MANN, 1976) mostly derived from the accessory glands with some contribution from the testes, efferent ducts, and epididymis, and is involved in the regulation of sperm function (STRZEZEK and FRASER., 2009). It is recognized that seminal plasma has a significant impact on sperm motility, viability (GRAHAM, 1994) and protection, by improving their resistance during cryopreservation (NOTHLING et al., 2005). Seminal plasma components are necessary to prevent and reverse the damage done to the plasma membrane during assisted reproduction manipulation and thus help to improve quality of semen (STRZEZEK and FRASER, 2009). Some of those important components are lipids.

Lipids are an essential part of every mammalian cell and are involved in a wide range of physiological events, serving as storage compounds, signaling molecules as well as different membrane related functions (ESMAEILI et al., 2015). In the physiology of reproduction lipids are important for spermatogenesis, sperm maturation and fertilization (MOGIELNICKA-BRZOZOWSKA and CICHOWSKA, 2024). Lipids and fatty acids are physiologically present in both the sperm cell membrane and seminal plasma. Indeed, it is thought that once matured, the plasmatic membrane final composition consists in 70% phospholipids, 25% neutral lipids (mainly cholesterol) and 5% glycolipids, which play an important role in modulating spermatozoa functions and their capacity to fuse with the oocyte (GAUTIER and AURICH, 2022). According to RAMOS ANGRIMANI et al. (2017) among the polyunsaturated fatty acids, the docosahexaenoic acid (DHA) represents 50% of the total sperm content. The sperm plasma membrane is highly specialized, where each membrane part has an important function in the capacity of the sperm to bind to the oocyte. The lipid components of the sperm plasma membrane are involved in the formation of micro-domains that contribute to the sperm motility, capacitation, acrosome reaction, and binding to the oocyte (GAUTIER and AURICH, 2022). Changes in the formation and composition of the sperm plasma membrane may contribute to compromised fertility. It has been shown that the plasma membrane lipids greatly contribute to sperm motility and fluidity, capacitation, acrosome reaction and binding to the zona pellucida in the fertilization process, therefore affecting fertility (HOSSAIN et al., 2007; TAVILANI et al., 2007). Knowledge of the sperm membrane composition and changes during spermatogenesis, sperm maturation, ejaculation and semen processing, is therefore an imperative for understanding of the sperm fertilizing potential and for improving the efficacy of assisted reproduction (GAUTIER and AURICH, 2022).

Fatty acids are important structural components of all cells, having the pivotal role in physiology of cells. Fatty acids (FAs) are classified into different groups according to the degree of hydrocarbon chain saturation, including saturated FA (SFA), monounsaturated FA (MUFA) and polyunsaturated FA (PUFA). According to National Research Council (NRC, 2006) omega 3 polyunsaturated fatty acids (n-3 PUFA), particularly eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3), and omega 6 (n-6) PUFA, mainly arachidonic acid (AA; 20:4 n-6), are essential FA for dogs and should be therefore incorporated into their diets. The presence of specific FA in sperm or seminal plasma influences fertility greatly. Docosahexaenoic FA is essential for the fluidity of the plasma membrane, and thus for the acquisition of sperm motility and acrosome reaction (ROOKE at al., 2001). Indeed, lower content of DHA and PUFA were observed in asthenozoospermic, oligozoospermic and oligoasthenozoospermic spermatozoa in man than in normozoospermic (AKSOY et al., 2006). GADELLA et al. (2008) and SHAN et al., (2021) have shown that PUFA contributes to a greater fluidity and flexibility of the sperm plasma membrane, which is necessary for physiological sperm function. Additionally, in a study conducted on boar semen, n-3 PUFA content in the sperm plasma membrane and extracellular antioxidants present in the seminal plasma were correlated to better motility, viability, and normal sperm morphology (AM-IN et al., 2011). Nonetheless, presence of double bonds, typical of unsaturated FA, especially long chain FA, increases chemical reactivity and makes sperm plasma membranes greatly susceptible to free radicals and oxidative stress through lipid peroxidation (TAPIA et al., 2012) which may result in functional disorders of sperms, including membrane damage and subsequent loss of motility (LUCIO et al., 2017).

It is reported that FA composition could be a reliable sperm quality indicator as well as an indicator for sperm maturation level and capacity to be frozen (COLLODEL et al., 2020; KOGAN et al., 2021). A study by LUCIO et al. (2017) have proposed some sperm membrane phospholipid classes being higher in motile versus asthenospermic samples. Furthermore, DI NISIO et al. (2023) proposed some lipid clusters that were associated with human semen parameters, among which sulfogalactosylglycerolipid (SGG) and PUFA represented the most important predictors of semen quality. According to DI NISIO et al. (2023), dietary PUFA and SGG have acknowledged antioxidant functions and could, therefore, represent sensitive markers of sperm quality and testicular function.  to be used as markers for sperm motility.

# Hypothesis and/or General and specific objectives of the work

Knowledge of the sperm lipids and seminal plasma composition is required for a better understanding of the sperm viability and fertility potential, with the final goal to improve the results of assisted reproductive technologies. However, there is still little knowledge regarding the FA profile of seminal plasma and spermatozoa and their effects on semen quality in canine species, therefore, the general objective of this study is to determine a FA profile of canine spermatozoa and seminal plasma. Furthermore, the specific objective of this work is to determine the relationship and effect of specific FA, especially essential FA, on ejaculate quality.

# Materials and methods

## *Animals*

Research was conducted on 46 privately owned dogs presented at Clinic for Obstetrics and Reproduction at the Faculty of Veterinary Medicine University of Zagreb. The dogs belonging to 27 breeds are shown, with accompanied body mass and age, in table 1. Median age was 6.5 years. All dogs had palpable normal genitalia and normal libido. Out of 46 semen collections that were performed on owner’s demand for semen evaluation, we analyzed 35 spermatozoa collections and 45 seminal plasmas. By signing the Consent to treatment form, the owners have given their consent to the use of data for scientific and research purposes. The research was approved by the Ethical Committee of the faculty of Veterinary Medicine University of Zagreb (Class:640-01/24-02/06; Sub. No.: 251-61-01/139-24-28).

Table 1. Age, weight and breed of dogs used in the study (n=46)

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **Breed**  | **Weight, kg** | **Age, yr** |  | **Breed**  | **Weight, kg** | **Age, yr** |
| Dog 1 | English cocker spaniel | 15 | 4 | Dog 24 | Siberian Husky | 26 | 8 |
| Dog 2 | English cocker spaniel | 15 | 4 | Dog 25 | Labrador Retriever | 30 | 7 |
| Dog 3 | English cocker spaniel | 15 | 3 | Dog 26 | Akita Inu | 43 | 2 |
| Dog 4 | English cocker spaniel | 16 | 6 | Dog 27 | Shiba Inu | 10 | 10 |
| Dog 5 | Spanish water dog | 20 | 2 | Dog 28 | Portuguese Water Dog | 21 | 8 |
| Dog 6 | Jack Russel  | 7 | 4 | Dog 29 | Portuguese Water Dog | 22 | 4 |
| Dog 7 | Schipperke | 9 | 3 | Dog 30 | Schnauzer | 23 | 3 |
| Dog 8 | Spanish water dog | 20 | 4 | Dog 31 | Baset Grifon Vendeen | 16 | 2 |
| Dog 9 | Staffordshire Bullterrier | 16 | 3 | Dog 32 | White Swiss Shepherd | 37 | 3 |
| Dog 10 | Cavalier King Charles | 9 | 2 | Dog 33 | Baset Grifon Vendeen | 17 | 3 |
| Dog 11 | Boston Terrier | 8 | 4 | Dog 34 | Weimaraner | 35 | 4 |
| Dog 12 | Miniature Schnauzer | 7 | 3 | Dog 35 | Peruvian Hairless Dog | 10 | 6 |
| Dog 13 | Cairn Terrier | 7 | 1 | Dog 36 | American Staffordshire Terrier | 30 | 7 |
| Dog 14 | Miniature Schnauzer | 8 | 4 | Dog 37 | Cane Corso | 56 | 6 |
| Dog 15 | Papillon | 5 | 8 | Dog 38 | Portuguese water dog | 22 | 7 |
| Dog 16 | Miniature Schnauzer  | 8 | 1,5 | Dog 39 | Tornjak | 50 | 7 |
| Dog 17 | Labrador Retriever | 30 | 4 | Dog 40 | Rhodesian Ridgeback | 41 | 6 |
| Dog 18 | German Shepherd | 42 | 7 | Dog 41 | Tornjak | 49 | 9 |
| Dog 19 | White Swiss Shepherd | 38 | 2 | Dog 42 | Dachshund | 11.7 | 2 |
| Dog 20 | Labrador Retriever | 31 | 4 | Dog 43 | Pomeranian  | 2 | 1 |
| Dog 21 | Boxer | 36 | 8 | Dog 44 | Portuguese water dog  | 21 | 12 |
| Dog 22 | Akita Inu | 44 | 2 | Dog 45 | Labrador Retriever | 32 | 8 |
| Dog 23 | Miniature Schnauzer | 42 | 6 | Dog 46 | English cocker spaniel  | 14 | 8 |

## *Semen collection and evaluation*

Semen was collected by manual stimulation in a presence of a teaser bitch or by olfactory stimulation with vaginal swabs of oestrus bitch. Once the pelvic thrusting began, the preputial sheet was gently massaged with gloved hand.  As partial erection occurred, the prepuce was quickly retracted, and penis was encircled proximal to bulbus glandis. Three different fractions were collected separately into pre-warmed plastic tubes attached to a plastic funnel. Only the sperm rich fraction was used for evaluation and processing. Semen parameters were evaluated as follows: volume was evaluated using graduated tubes, colour, admixtures and homogeneity were assessed macroscopically. Concentration was determined using Accuread ® photometer (IMV Technologies, France). After calibration with 4000 μL of normal saline, a volume of 40 μL of semen was mixed with 3960 μL of normal saline in a measuring cuvette and was placed in spectrophotometer and concentration was measured. Total number of spermatozoa in the ejaculate was calculated as the concentration times the volume.

Motility of spermatozoa was assessed using Computer assisted sperm analyzer (Hamilton Thorne Inc., Beverly, USA) with software version 12.3. This analyzer consists of a phase-contrast microscope, a camera, a heating plate and a computer. Immediately after the examination, the semen was diluted with normal saline to 50x106 sperm/mL. A 5 µL of diluted semen was placed in a pre-warmed counting chamber (Leja, depth 20 µm, Leja Products B.V. Niuew-Vennep, Netherlands) and analyzed for sperm motion and kinematic characteristics. A minimum of 1000 sperm cells were analyzed in at least eight microscopic fields with 30 frames acquired per field at a frame rate of 60 Hz. The following parameters were measured: total motility (%), progressive motility (%), average path velocity (VAP), straight line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), straightness (STR), amplitude of lateral head displacement (ALH) and beat cross frequency (BCF), linearity (LIN), elongation (elongation), area (area) and velocity distribution: fast (RAP), medium (MED), slow (SLOW) and static (STATIC). Regarding analysis settings, the CASA was set to standard factory settings for canine semen; sperm with straightness of >75% and VAP >50 μm/s were considered progressively motile.

Sperm viability and morphology were assessed by eosin-nigrosin staining according to BLOOM (1950). A drop of semen (5 µL) was placed on preheated slide (37°C) and mixed with one drop of Eosin and two drops of Nigrosin (Minitube ®, Germany). Smear was prepared and allowed to dry on a heat plate at 37°C. A total of 200 spermatozoa was classified using bright field microscopy (Olympus CX41, Tokyo, Japan) at x1000 magnification under immersion oil. For viability, the number of live spermatozoa (unstained) were presented as percentage. For morphology evaluation two hundred spermatozoa were evaluated and classified as normal or with morphological defects. Abnormalities were classified according to the site of defects as head, neck/midpiece, tail defects and proximal and distal cytoplasmic droplet (MENON et al., 2011). The results were expressed as percentage.

Sperm plasma membrane integrity was determined using hypo-osmotic swelling test (HOS). The HOS solution consisted of 0.73 g of sodium citrate and 1.35 g of fructose dissolved in 100 mL distilled water (osmotic pressure 100 mOsm/kg). To assess plasma membrane integrity, 10 µL of semen was diluted with 200 µL of HOS solution and incubated for 30 minutes at 37°C. A drop of incubated suspension was put onto a glass slide, covered with a coverslip and examined under phase contrast microscope at x400 magnification (Olympus BX 51, Tokyo, Japan). Two hundred spermatozoa were assessed for their swelling ability. Spermatozoa with coiled tail were considered to have intact plasma membrane (ENGLAND and PLUMMER, 1993). Results were presented as percentage of sperm with intact plasma membrane.

## *Separation of seminal plasma and spermatozoa*

After the evaluation of semen quality, the seminal plasma was separated from spermatozoa by centrifugation at 1000x g/15 min (Centrifuge 5702, Eppendorf, Germany). Separated spermatozoa were washed 3 times in normal saline by centrifugation (500xg/5 min). Both seminal plasma and spermatozoa were stored at -80 °C until analysis. For analyses, samples were thawed at room temperature.

## *Total lipid extraction and transesterification*

Total lipids were extracted using a mixture of chlorophorm/methanol (2:1 v/v) by modified method according to FOLCH et al. (1957). Lipid extracts were concentrated in a tabletop Centrivap vacuum concentrator, equipped with a Centrivap cooling unit and a diaphragm vacuum pump 230V, 50/60 HZ 1 (Labconco, Kansas City, USA). Fatty acids (FA) from the total lipid extract were converted to methyl esters (FAME) via transesterification with methanolic HCl according to international standard procedure ISO 5509 (2000).

## *Gas chromatography analysis*

Analysis of FA methyl esters was performed on a gas chromatograph (Agilent 8860; Agilent Technologies. Inc., California, USA) equipped with a flame ionization detector (FID). The temperature of the injector and detector were 200 °C and 240 °C, respectively. Chromatography was performed on a capillary column DB-23 (Agilent Technologies, California,USA), length 60 m, inner column diameter 0.25 mm, active layer thickness 0.25 μm. The temperature regime was as follows: 150 °C for 2 minutes, increased to 230 °C by 5°C/min, and held for 20 minutes. Hydrogen at a flow rate of 1 mL/min was used as the carrier gas. Processing of results was performed using the computer program OpenLAB CDS ChemStation, Workstation VL. FAME was identified by comparing retention times with methyl standards (Sigma Aldrich Chemie, GmbH and Supelco, USA). Quantification was performed using nonadecanoic acid methyl ester (C19:0). Fatty acid composition was calculated as the percentage of each individual FA relative to the total FA. FAME were categorized according to their chain length and structure, namely as: saturated (SFA), if they do not contain any unsaturated double bond and any methyl branched; monounsaturated (MUFA), if they contain one double bond; polyunsaturated (PUFA), if they contain more than one double bond.

## *Statistical analysis*

The results were processed in the statistical program STATISTICA version 12 (StatSoft, Tulsa, USA). The normality of the distribution was checked using the Kolmogorov-Smirnov and Shapiro-Wilks W tests. Results are presented as mean and standard deviation. The significance of differences in the fatty acid composition of sperm and seminal plasma was checked by Student's t-test if it was a normal distribution, and by the Mann-Whitney U test if the distribution was different from Gaussian. The correlation between the indicators was determined by linear and the Spearman correlation test. Differences are considered statistically significant if p<0.05.

# Results

## *Fatty acid composition of dog's spermatozoa and seminal plasma*

Table 2 shows distribution of FA (in %) in dog's spermatozoa and seminal plasma. The most dominated group in both sample types was SFA, with C16:0 as most represented, and both were significantly higher in spermatozoa. Second group in representation was PUFA in both sample types, while the least represented was MUFA. As shown in tabl1 1, MUFA was significantly higher in seminal plasma. The most dominant MUFA in spermatozoa and seminal plasma was C18:1c9, which was significantly higher in seminal plasma. Furthermore, essential FA, C18:2n-6 and conditionally essential FA, C20:5n-3 were significantly higher in seminal plasma.

Table 2. Fatty acid composition of spermatozoa and seminal plasma of distinctive dog’s breed in %.

|  |  |  |
| --- | --- | --- |
|  | Mean±SD |  |
|  | Spermatozoa | Seminal plasma | P value |
| **C8:0** | 1.53 ± 0.64 | 1.61 ± 0.50 | 0.54 |
| **C9:0** | 4.32 ± 1.44 | 7.88 ± 3.99 | <0.01 |
| **C11:0** | 1.83 ± 0.79 | 2.74 ± 1.22 | <0.01 |
| **C14:0** | 9.39 ± 4.39  | 11.42 ± 3.16 | <0.01 |
| **C16:0**  | 20.21 ± 6.12 | 14.13 ± 4.94 | <0.01 |
| **C16:1**  |  2.80 ± 1.42 | 2.92 ± 1.15  | 0.70  |
|  **C16:2** |  8.80 ± 2.83 | 8.75 ± 2.08 | 0.92 |
| **C18:0**  | 15.30 ± 7.21 | 10.72 ± 5.95 | <0.01 |
| **C18:1n-9**  | 13.6 1± 3.89 | 16.15 ± 4.81 | 0.01 |
| **C18:2n-6**  | 5.92 ± 2.07 | 7.05 ± 2.64 | 0.04 |
| **C18:3n-3**  | 0.97 ± 0.89 | 0.70 ± 0.57 | 0.57 |
| **C20:0** | 2.81 ± 0.97 | 2.67 ± 0.70 | 0.44 |
| **C20:1** | 2.80 ± 0.84 | 2.85 ± 0.64 | 0.77 |
| **C20:4n-6** | 3.23 ± 1.89 | 2.88 ± 1.26 | 0.74 |
| **C20:5n-3** | 0.85 ± 0.52 | 1.40 ± 0.60 | <0.01 |
| **C22:5n-3** | 1.49 ± 0.73 | 1.30 ± 0.64 | 0.65 |
| **C22:6n-3** | 4.38 ± 1.49 | 4.84 ± 1.37 | 0.13 |
| **SFA**  | 55.16 ± 9.50 | 51.16 ± 7.16 | 0.04 |
| **MUFA**  | 19.21 ± 4.55 | 21.91 ± 4.80 | 0.01 |
| **PUFA**  | 25.63 ± 5.92 | 26.9 3± 4.02 | 0.52 |
| **UFA**  | 44.84 ± 9.50 | 48.84 ± 7.16 | 0.04 |

SFA-saturated fatty acids; MUFA-monounsaturated fatty acids; PUFA-polyunsaturated fatty acids; UFA-unsaturated fatty acids

 Table 3 shows sums of FA (% of total FA), FA ratios and stearoyl-CoA desaturase (SCD) activity indices of spermatozoa and seminal plasma of dogs. Ratios of conditionally essential FA, AA/EPA and DHA/EPA were significantly higher in dog's seprmatozoa compared to seminal plasma.

Table 3. Fatty acids (FA) sums (% of total FA) and FA ratios of spermatozoa and seminal plasma of distinctive dog’s breed

|  |  |  |  |
| --- | --- | --- | --- |
|  | Spermatozoa | Seminal plasma | P value |
| Mean±SD |
| **UFA/SFA** | 0.86 ± 0.29 | 0.99 ± 0.23 | 0.04 |
| **PUFA/SFA** | 1.36 ± 0.23 | 1.27 ± 0.24 | 0.10 |
| **AA/EPA** | 4.60 ± 3.26 | 2.60 ± 2.18 | <0.01 |
| **AA/DHA** | 0.79 ± 0.49 | 0.63 ± 0.32 | 0.09 |
| **DHA/EPA** | 5.91 ± 1.67 | 4.16 ± 2.09 | <0.01 |
| **VLn-3PUFA**  | 6.72 ± 2.32 | 7.54 ± 2.04 | 0.09 |
| **n-6**  | 9.15 ± 2.94 | 9.93 ± 3.13 | 0.25 |
| **n-3**  | 7.69 ± 2.89 | 8.25 ± 2.03 | 0.11 |
| **n-6/n-3** | 1.34 ± 0.60 | 1.30 ± 0.60 | 0.61 |

AA-arachidonic fatty acid; EPA-eicosapentaenoic fatty acid, c20:5n-3; DHA-docosahexaenoic fatty acid, C22:6n-3; VLn-3PUFA-polyunsaturated fatty acids of the very long chain n-3 family

## *Parameters of dog's semen quality*

Table 4 shows parameters of dog's semen quality assessment in distinctive dog's breed. The semen quality of dogs ranged from poor to excellent, with excellent being dominant (70%). The total sperm number ranged from 25.3 x106 to 2138.5 x106 with mean total sperm number 1875.7x106. Majority of dogs (86%) had motility > 70% and 54% of dogs had progressive motility > 50%. Mean percentage of live and plasma membrane intact spermatozoa were > 80%. The proportion of morphologically normal spermatozoa ranged from 6 to 97%, and 71% of dogs had a proportion of MNS > 70%. Results are presented as mean ± SD.

Table 4. Parameters of dog's semen quality assesment

|  |  |
| --- | --- |
|  | **Mean ± SD** |
| **Basic semen parameters** |  |  |
| Volume (mL) | 3.4 **±** 2.1 |
| Concentration/mL (x106)Total sperm number (x106)  | 109.33 **±** 80.181875.7 **±** 2826.1 |
| **CASA semen parameters** |  |  |
| Total motility (%) | 81.96 **±** 13.05 |
| Progressive motility (%) | 47.83 **±** 15.68 |
| VAP µm/s | 88.10 **±** 23.29 |
| VSL µm/s | 70.58 **±** 23.39 |
| VCL µm/s | 151.57 **±** 47.46 |
| ALH µm | 8.96 **±** 8.00 |
| BCF (Hz) | 26.45 **±** 16.94 |
| STR (%) | 78.14 **±** 15.13 |
| LIN (%) | 48.29 **±** 12.88 |
| ELNG (%) | 47.21 **±** 9.92 |
| AREA µm/sq | 7.03 **±** 7.32 |
| RAPID (%) | 53.66 **±** 20.52 |
| MEDIUM (%) | 8.01 **±** 5.78 |
| SLOW (%)STATIC (%) | 21.83 **±** 11.9415.9 **±** 11.0 |
| **Vitality of spermatozoa**  |  |  |
| Live (%) | 84.7 **±**1 0.65 |
| PMI (%)  | 85.97 **±** 13.71 |
| **Sperm morphology parameters** |  |  |
| Normal morphology (%) |  71.83 ± 25.93 |
| Head (%) |  |  7.35 ± 15.69 |
| Midpiece (%) |  |  7.29 ± 15.69 |
| Tail (%) |  |  8.06 ± 13.48 |
| Proximal droplet (%) |  |  10.76 ± 20.97 |

VAP, velocity average pathway; VSL, velocity straight line; VCL, velocity curvilinear; ALH, amplitude of the lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity; ELNG, elongation; EN, eosin/nigrosin- live spermatozoa; PMI, plasma membrane integrity



Figure 1. Evaluation of sperm viability using eosin-nigrosine staining; white arrow: unstained live spermatozoa, red arrow: stained dead spermatozoa, blue arrow: tail abnormality



Figure 2. Evaluation of sperm membrane integrity with HOS test; white arrow: coiled tail- intact membrane, blue arrow: uncoiled tail - damaged membrane

## *Correlation between fatty acid composition and spermatozoa quality parameters*

Regarding spermatozoa morphological parameters and spermatozoa FA composition, we determined significant positive correlation between AA/EPA ratio and pathologically changed spermatozoa head (r=0.47).

Seminal plasma FA composition also showed significant correlations between spermatozoa morphological parameters; AA/DHA ratio correlated positively, while C22:5 correlated negatively with pathologically changed spermatozoa midpiece (r=0.49, r=-0.57; respectively). Both DHA and C22:5 in seminal plasma correlated with pathologically changed spermatozoa tail (r=0.40, r=-0.48; respectively). While C22:5 alone correlated positively with pathologically changed spermatozoa head (r=0.47). Also, SFA in seminal plasma correlated positively with VCL µm/s and elongation (r=0.48, r=0.41, respectively). Inversed correlated with BCF (Hz) was determined in C18:1c9 and FA n-3 family (r=-0.48; r=0.38, respectively).

Regarding CASA spermatozoa parameters and FA spermatozoa composition, we found that MUFA, C18:3n-3 and EPA correlated positively with VCL µm/s (r=0.41, 0.37 and 0.37; respectively). MUFA also correlated with medium velocity distribution (r=0.41,). Furthermore, C18:3n-3 showed positive correlation with motility (r=0.40). When correlating spermatozoa FA composition with progressive motility, no significant difference was found in dogs with PM lover than 50%. However, in dogs with more than 50% progressive motility C11:0 and UFA/SFA correlated significantly (r=-0.59, r=0.58; respectively). Furthermore, FA composition of seminal plasma did not correlate significantly or strong enough, with either progressive motility in dogs.

# Discussion

In this study, the most dominated group in both dog’s spermatozoa and seminal plasma was SFA, with palmitoleic FA as most represented, and both were significantly higher in spermatozoa. Second group in representation was PUFA in both sample types, while the least represented was MUFA. In seminal plasma, MUFA was significantly higher. The most dominant MUFA in spermatozoa and seminal plasma was oleic FA, which was significantly higher in seminal plasma. Furthermore, essential FA, LA and conditionally essential FA, EPA, were significantly higher in seminal plasma. Ratios of conditionally essential FA, AA/EPA and DHA/EPA were significantly higher in dog's spermatozoa compared to seminal plasma. Seminal plasma FA composition also showed significant correlations between spermatozoa morphological parameters; AA/DHA ratio correlated positively, while C22:5 correlated negatively with pathologically changed spermatozoa midpiece (r=0.49, r=-0.57; respectively). Regarding CASA spermatozoa parameters and FA spermatozoa composition, we found that MUFA, ALA and EPA correlated positively with VCL µm/s (0.41, 0.37 and 0.37; respectively). When correlating spermatozoa FA composition with progressive motility, no significant difference was found in dogs with PM lover than 50%. However, in dogs with more than 50% progressive motility C11:0 and UFA/SFA correlated significantly (r=-0.59, r=0.58; respectively).

Composition of FA in spermatozoa and seminal plasma may vary between species. Similar to this study (table 2), the predominant FA of dog seminal plasma were C16:0 (palmitic FA, 30.4%), C18:0 (stearic FA, 23.4%) and C18:1n-9 (oleic FA, 9.0%) (DIAZ et al., 2014). In boar sperm, the most abundant SFA were palmitic FA (18%) and stearic FA (16%), while the most abundant PUFA were docosapentaenoic FA (DPA, C22:5n-3) (15%) and docosahexaenoic FA (DHA, C22:6n-3) (16%) (WATERHOUSE et al., 2006). Furthermore, in stallion, the sperm contains high levels of DPA, representing on average 49.9%, followed by palmitic acid and stearic acid, representing 17.6% and 8.7%, respectively (MACIAS GARCIA et al., 2011). Similar to horses, human spermatozoa contain DHA as the most abundant n-3 PUFA, as well as large amounts of palmitic FA (LENZI et al., 2000).

In this study there was significantly higher percentage of palmitic FA in spermatozoa than in seminal plasma (table 2), which could be beneficial to spermatozoa function, since study by WATERHOUSE et al. (2006) states positive correlation of palmitic FA with sperm survival and plasma membrane integrity. MENEZES et al. (2019) indicates that the content of palmitic FA in sperm is a screening key indicator in bull for high or low fertility phenotypes. On the other hand, some research states that palmitic FA in semen of infertile men and asthenozoospermia patients was higher than that of normal sperm (ANDERSEN et al., 2016) and that increased levels lead to the sperm plasma membrane metabolism disorder (TANG et al., 2017).

In this study, we found a significantly higher percentage of oleic FA in dog’s seminal plasma compared to spermatozoa (table 2). Oleic FA is a MUFA member of the omega-9 family, with 18 carbon atoms, whose antioxidant potency has been widely recognized (THOMBARE et al., 2017). Furthermore, ZHU et al. (2020) states that oleic together with palmitic FA can positively influence boar sperm motility via enhancing mitochondrial β-oxidation for ATP generation.

Linoleic (LA, C18:2n-6) and α-linolenic FA (ALA, C18:3n-3) are essential FA that have a pivotal role in the physiological development and functioning of the body (LENOX, 2015). Since mammals lack enzymes allowing for the formation of unsaturated bonds in the omega-3 and omega-6 positions, i.e., ∆-12 desaturase and ∆-15 desaturase, they are not able to synthesize the series of omega-6 and omega-3 PUFAs, and therefore essential FA must be provided in the diet (LENOX, 2015). In this study, LA was significantly higher in dog’s seminal plasma compared to spermatozoa, while ALA was higher in spermatozoa, although not significantly (table 2). Omega-3 PUFA plays an important role in reproductive physiology, including regulation of prostaglandin synthesis, having antioxidant effects and increasing membrane stability thus allowing spermatozoa to withstand the physical stress caused by cooling and freezing (MOALLEM et al., 2015). Furthermore, PUFA improves semen quality and influence spermatogenesis (RISSO et al., 2016). If ALA content in spermatozoa membrane increases, fluidity of plasma membrane also increases (EJAZ et al., 2017). Influence of DHA on semen quality are numerous; DHA improves antioxidant capacity and reduce oxidative stress, it promotes testosterone synthesis and sperm maturation, it also improves sperm plasma membrane and DNA integrity, as well as DHA protects sperm from damage caused by cryopreservation (YUAN et al., 2023). However, LIU et al. (2015) determined that a diet rich in omega-3 PUFAs affected boar sperm, being more susceptible to lipid peroxidative damage, which can negatively influence membrane structure and function.

Regarding spermatozoa morphological parameters and spermatozoa FA composition, we determined some significant positive correlation between AA/EPA ratio and pathologically changed spermatozoa head (r=0.47). Since infertility can be related to the percentage of sperm defects (ROOT and JOHNSTON, 1994), 80% or more of the sperm in the ejaculate in dogs should be without morphological defects. Dietary supplementation with omega-3 and –6 on sperm quality of dogs did not have any influence on sperm morphology (RODRIGUES et al., 2017). Contrary to that, ATTAMAN et al. (2012) found that men who ingested a diet supplemented with omega-3 had lower sperm defects. Seminal plasma FA composition also showed significant correlations between spermatozoa morphological parameters; AA/DHA ratio correlated positively, while C22:5 correlated negatively with pathologically changed spermatozoa midpiece (r=0.49, r=-0.57; respectively). Both DHA and C22:5 in seminal plasma correlated with pathologically changed spermatozoa tail (r=0.40, r=-0.48; respectively). While C22:5 alone correlated positively with pathologically changed spermatozoa head (r=0.47). Some research observed that spermatozoa from asthenozoospermic samples have lower levels of DHA than those from normozoospermic individuals (AKSOY et al. 2006; TAVILANI et al. 2007). OLLERO et al. (2000) reported that the content of unsaturated FA, especially DHA, as well as SFA content decreased during the spermatozoa maturation. Authors concluded that beforementioned removal of DHA from sperm membrane is imperative for decrease of susceptibility of sperm to lipid peroxidation, but the critical level is retained for optimal membrane fluidity required for sperm motility and acrosome reaction.

Regarding CASA spermatozoa parameters and FA spermatozoa composition, we found that MUFA, ALA and EPA correlated positively with VCL µm/s (0.41, 0.37 and 0.37; respectively). In this study MUFA also correlated with medium velocity distribution (r=0.41). FERRAMOSCA et al. (2017) determined that MUFA influence sperm quality by increasing gamete motility, reducing oxidative stress and improving mitochondrial respiration efficiency. In our study ALA showed positive correlation with motility (r=0.40). It is known that ALA is important n-3 PUFA with anti-inflammatory (ZHU et al. 2020) and antioxidant properties (ISTIFLI et al., 2019) having positive effect on the morphology, viability and progressive motility of frozen– thawed sperm (KHOSHVAGHT et al. 2016). In mammals, ALA is needed for the conversion of other n-3 FA, such as EPA and DHA through elongation and desaturation by enzymes (STARK et al. 2016). EPA is a precursor of eicosanoids such as prostaglandins, cyclic prostaglandins, thromboxane, and leukotrienes is particularly important for sperm normal morphology, plasma membrane integrity, freezing resistance and motility (LASS and BELLUZZI, 2019) and in this study it was significantly higher in seminal plasma (table 2).

In this study, SFA in seminal plasma correlated positively with VCL µm/s and elongation (r=0.48, r=0.41, respectively). Oleic FA and n-3 family FA inversely correlated with BCF (Hz) (r=-0.48; r=0.38). When correlating spermatozoa FA composition with progressive motility, in dogs with more than 50% progressive motility, we found significant correlations of C11:0 and UFA/SFA (r=-0.59, r=0.58; respectively). In bovine semen C18:0 was incorporated into sperm *in vitro*, while SFA were associated with energy metabolism and therefore with sperm motility (ORTEGA-FERRUSOLA et al. 2009). Furthermore, HOSSAIN et al. (2007) determined that in pig sperm C18:0 has a positive effect on sperm functionality, thus affecting significantly the viability, motility and acrosome reaction. In this study, SFA was predominant group in dog’s spermatozoa (over 50%) and level of C18:0 was around 15%, as well as representation of PUFA (over 20%), which could contribute to good quality of dog’s semen.

# Conclusions

In both canine spermatozoa the SFA predominated (over 50%), with palmitic FA as most represented (around 20%). The second group in representation was PUFA (over 22%), with LA as most represented (around 6%). Canine seminal plasma also contained SFA as predominant (around 50%) and palmitic acid as most represented (around 14%). As well as in canine spermatozoa, canine seminal plasma had PUFA as second (around 25%) and LA as most represented (around 7%).

Significant correlations of FA and semen quality parameters found in this study suggest the importance of PUFA, especially n-3 family PUFA content in the physiological function of spermatozoa and thus point to the importance of quality nutrition on the fertility of dogs.

The link between the FA composition of spermatozoa and seminal plasma with the quality of the ejaculate is particularly important, as it could in further research enable the finding of markers that will indicate the quality of the ejaculate and thus serve in clinical work, considering the increasing number of dogs that have a problem with reduced fertility.

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Pauline Jourdain

**Fatty acid composition of the dog semen and seminal plasma**

Abstract**:**

The aim of this study was to determine the fatty acid composition of spermatozoa and seminal plasma of dogs and its relationship with ejaculate quality, since to our knowledge, data on the composition of fatty acids (FA) in seminal plasma, their effect on sperm, and data on the composition of fatty acids in dog sperm are limited. Out of 46 semen collections that were performed on owner’s demand for semen evaluation, we analyzed 35 spermatozoa collections and 45 seminal plasmas. Dogs were subjected to a clinical examination, B-mode ultrasonography of the testes, epididymis and prostate, semen evaluation after collection using CASA system that included 11 parameters (VAP, VSL, VCL, ALH, BCF, STR, LIN, ELONG, AREA, MOT, PMOT – divided into 4 categories of movement : RAP, MED, SLOW, STATIC) as well as standard microscopic procedure (volume, pH, concentration and semen viability – HOS test and Bloom staining). After the evaluation of semen quality, the seminal plasma was separated from spermatozoa. From both spermatozoa and seminal plasma total lipids were extracted then converted to methyl esters and analyzed on gas chromatograph. Results show that the most dominated group in both sample types was saturated FA, with C16:0 as most represented, and both were significantly higher in spermatozoa (p=0.04, p<0.01). Second group in representation was polyunsaturated FA in both sample types, while the least represented was monounsaturated FA. Furthermore, essential FA, C18:2n-6 and conditionally essential FA, C20:5n-3 were significantly higher in seminal plasma (p=0.04. p<0.01). Ratios of conditionally essential FA, AA/EPA (arachidonic/eicosapentaenoic FA) and DHA/EPA (docosahexaenoic /eicosapentaenoic FA) were significantly higher in dog's spermatozoa compared to seminal plasma (p<0.01, p<0.01). Seminal plasma FA composition also showed significant correlations between spermatozoa morphological parameters; AA/DHA ratio correlated positively, while C22:5 correlated negatively with pathologically changed spermatozoa midpiece (r=0.49, r=-0.57; respectively; p<0.05). Regarding CASA spermatozoa parameters and FA spermatozoa composition, we found that MUFA, C18:3n-3 and EPA correlated positively with VCL µm/s (r=0.41, 0.37 and 0.37; respectively; p<0.05). MUFA also correlated with medium velocity distribution (r=0.41; p<0.05). Furthermore, C18:3n-3 showed positive correlation with motility (r=0.40; p<0.05). Composition of FA in canine spermatozoa and seminal plasma as well as significant correlations of FA and semen quality parameters found in this study suggest importance of PUFA, especially n-3 family PUFA content, in the physiological function of spermatozoa and thus point to the importance of quality nutrition on the fertility of dogs. The link between the FA composition of spermatozoa and seminal plasma with the quality of the ejaculate is particularly important, could in future research enable, the finding of markers that will indicate the quality of the ejaculate and thus serve in clinical work, considering the increasing number of dogs that have a problem with reduced fertility.

Keywords: canine spermatozoa and seminal plasma, fatty acid composition, semen quality parameters

Pauline Jourdain

**Masnokiselinski sastav spermija i sjemene plazme pasa**

Sažetak**:**

Cilj ovog istraživanja bio je utvrditi sastav masnih kiselina spermija i sjemene plazme pasa i njihovu povezanost s kvalitetom ejakulata, budući da su prema našim saznanjima podaci o sastavu masnih kiselina u sjemenoj plazmi, njihovom učinku na spermije te podaci o sastav masnih kiselina spermija u pasa ograničeni. Od 46 polučivanja sjemena koje je učinjeno na zahtjev vlasnika radi procjene kvalitete sjemena, analizirano je 35 uzorkovanja spermija i 45 sjemenih plazmi. Na psima je proveden klinički pregled te je ocjenjena kvaliteta ejakulata nakon polučivanja korištenjem parametara računalno potpomognute analize spermija (VAP, VSL, VCL, ALH, BCF, STR, LIN, ELONG, AREA, MOT, PMOT - dodatno podijeljeno u četiri kategorije kretanja: RAP, MED, SLOW, STATIC) i standardnom mikroskopskom pretragom (volumen, pH, koncentracija i vitalnost spermija -HOS test i bojenje po Bloom-u). Nakon procjene kvalitete sjemena, sjemena plazma je odvojena od spermija. Iz spermija i sjemene plazme ekstrahirani su ukupni lipidi, zatim prevedeni u metilne estere i analizirani na plinskom kromatografu. Rezultati pokazuju da su zasićene masne kiseline (MK) najdominantnija skupina masnih kiselina u obje vrste uzoraka, s C16:0 kao najzastupljenijom, čiji je postotak bio značajno viši u spermijima (p=0.04, p<0.01). Druge po zastupljenosti u oba tipa uzorka bile su višestruko nezasićene MK, dok su najmanje zastupljene bile jednostruko nezasićene MK. Nadalje, esencijalna MK, C18:2n-6 i uvjetno esencijalna MK, C20:5n-3 bile su značajno više u sjemenoj plazmi (p=0.04. p<0.01). Omjeri uvjetno esencijalnih MK, AA/EPA i DHA/EPA bili su značajno viši u spermijima pasa u usporedbi sa sjemenom plazmom (p<0.01, p<0.01). Sastav MK sjemene plazme značajno korelira s morfološkim pokazateljima spermija; omjer AA/DHA korelirao je pozitivno, dok je C22:5 korelirao negativno s patološki promijenjenim srednjim dijelom spermija (r=0,49, r=-0,57). Što se tiče pokazatelja računalno potpomognute analize spermija i sastava MK spermija, utvrđeno je da MUFA, C18:3n-3 i EPA pozitivno koreliraju s VCL µm/s (r=0,41, r=0,37, r=0,37; p<0.05). MUFA također značajno korelira s distribucijom srednje brzine (r=0,41; p<0.05). Nadalje, C18:3n-3 značajno pozitivno korelira s motilitetom (r=0,40; p<0.05). Sastav MK u spermijima pasa i sjemenoj plazmi kao i značajne korelacije MK i pokazatelja kvalitete sjemena pronađenih u ovom istraživanju, upućuju na važnost višestruko nezasićenih MK, posebice sadržaja višestruko nezasićenih MK iz n-3 obitelji, u fiziološkoj funkciji spermija i time rezultati upućuju na važnost kvalitetne ishrane na plodnost pasa. Posebice je važna poveznica između masnokiselinskog sastava spermija i sjemene plazme s kvalitetom ejakulata, jer u budućim istraživanjima pruža mogućnost pronalaženja markera koji će upućivati na kvalitetu ejakulata i time poslužiti u kliničkom radu, s obzirom na sve veći broj pasa koji imaju problem sa smanjenom plodnosti.

Ključne riječi: spermiji i sjemena plazma pasa, sastav masnih kiselina, pokazatelji kvalitete sjemena

# Biography

I was born on November 20th, 1999 in Saintes, a town in south west of France. I have achieved elementary school from 2011 to 2015 and finished high school in 2017 with a “very well” score. After that I finished two years of preparatory classes in France before applying and starting at the Faculty of Veterinary Medicine University of Zagreb in September 2019. I have enrolled in integrated under-graduate and graduate course in veterinary medicine at the Faculty of Veterinary Medicine University of Zagreb in 2019 and soon I am at the beginning of a 6th year veterinary student with a prominent interest in obstetrics and physiology of dog reproduction. Stated is the primary reason why I have joined research activities on Department of Physiology and Radiobiology and on Clinics for Reproduction and Obstetrics. During the first four years of study, I was awarded as the best student of my generation. I am also volunteering at the Equine clinic of the Faculty of Veterinary Medicine from November 2020.

I have fulfilled internships in private veterinary medicine clinics during 2014 by spending 1 week at the Veterinary clinic of Plougastel-Daoulas (29) Dr. Cozien (canine oriented). I did continue with internships in several more private veterinary clinics: 2021- 1 week at the Veterinary clinic Vétéa Saintes (canine and new companion animals); 2022- 2 weeks at Veterinary clinic Vétéa Saintes (17) (canine); 2022- 1 week with an ambulatory equine veterinarian Dr. Gotz; 2023- 1 week at the Veterinary clinic Vétéa Saintes (17) (canine); 2023- 4 weeks with Dr. JM. Betizeau. Veterinary clinic du Parc. Saintes (17) (equine and bovine veterinarian). Since 2021, I have been a member of the team of equine volunteers at the Faculty of Veterinary Medicine University of Zagreb Clinic.

I am very fond of animals, and I gladly find a free time for pet sitting of different animal species as well as to work as a horse rider and manager of a breeding stable: Haras de TUS at Sainte-Même (France). During the holiday time, back in France, I am splitting my time between internships in equine and canine clinics and my job of rider and care taker of horses in a breeding stable. My hobbies are sport related activities, especially running, football and biking as well as foreign literature and cinema.