UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE REPRODUÇÃO ANIMAL E RADIOLOGIA VETERINÁRIA

AVALIAÇÃO DO SÊMEN REFRIGERADO COM OU SEM PLASMA SEMINAL E SUA CORRELAÇÃO COM AS PROTEÍNAS DO EJACULADO DE DIFERENTES RAÇAS CANINAS

MICHELLE SILVA ARAUJO

Botucatu - SP
Dezembro/2018

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Tese apresentada à Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista “Júlio de Mesquita Filho”, Campus de Botucatu, para obtenção do título de Doutor do Programa de Pós-graduação em Biotecnologia Animal

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“(..) tenho para mim que o amor é o que há de mais importante no mundo. Analisar o mundo, explicá-lo, menosprezá-lo talvez caiba aos grandes pensadores. Mas a mim me interessa exclusivamente que eu seja capaz de amar o mundo, de não sentir desprezo por ele, de não odiar nem a ele e nem a mim mesmo, de contemplar a ele, a mim, a todas as criaturas com amor, admiração e reverência.”

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SUMÁRIO

CAPÍTULO 1 xii
1. INTRODUÇÃO E JUSTIFICATIVA 13
2. REVISÃO DE LITERATURA 15
2.1. Plasma seminal 15
2.2. Proteínas do plasma seminal canino 17
2.3. Proteínas da célula espermática canina 23
2.4. Espécies reativas de oxigênio 24
2.5. Antioxidantes 26
2.6. Estresse oxidativo durante a criopreservação do sêmen 29
3. CONSIDERAÇÕES FINAIS 30
4. REFERÊNCIAS 31
HIPÓTESES 53
OBJETIVOS 53
Objetivo Geral 53
Objetivos Específicos 53
CAPÍTULO 2 13
Insights into the influence of canine breed on proteomic profile of the spermatozoa and seminal plasma 55
Abstract 56
Introduction 57
Materials and Methods 58
Ethical aspects 58
Reagents 58
Semen collection and sample preparation 58
Flow cytometry assay 59
Protein analysis 61
Data analysis 64

Results.....65
Discussion 75
Acknowledgment 82
References 82
Supplement 1. 97
Supplement 2. 133
Supplement 3. 166
Seminal plasma positively influences the quality of canine semen stored at 5ºC 171
Abstract 172
1. Introduction.....173
2. Material and Methods 174
2.1. Ethical aspects 174
2.2. Reagents..... 174
2.3. Semen collection and immediate analysis..... 175
2.4. Laboratorial analysis..... 176
2.4.1. Sperm kinematic evaluation 176
2.4.2. Assessment of sperm membranes integrity, mitochondrial potential, superoxide production, hydrogen peroxide production and lipid peroxidation 177
2.4.3. Oxidative stress..... 180
2.4.4. Antioxidant enzymes activity 181
2.5. Data analysis..... 181
3. Results.....182
Table 1. 184
4. Discussion 186
5. Conflicts of interest 188
6. Acknowledgments.....188
7. Funding Sources.....189
8. References 189
CAPÍTULO 3 182
CONSIDERAÇÕES FINAIS.....199

RESUMO

ARAUJO, M. S. (2018) **Avaliação do sêmen refrigerado com ou sem plasma seminal e sua correlação com as proteínas do ejaculado de diferentes raças caninas.** Botucatu – SP. 2018, 199p. Qualificação (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho", Departamento de Reprodução Animal e Radiologia Veterinária.

O plasma seminal e suas proteínas influenciam a qualidade espermática do sêmen refrigerado e congelado em várias espécies, porém sua influência no sêmen refrigerado de cães ainda não está esclarecida. Ainda, diferenças interraciais na expressão de proteínas do plasma seminal que podem interferir na qualidade do sêmen canino não foram relatadas. O objetivo deste estudo foi verificar se a raça canina interfere na composição proteica do plasma seminal e das proteínas espermáticas e correlacioná-las com a refrigeração do sêmen canino na presença e ausência do plasma seminal. Desta maneira, dois experimentos foram realizados. No primeiro experimento as proteínas do plasma seminal e das células espermáticas de 4 raças caninas foram caracterizadas. O sêmen foi colhido, avaliado macro e microscopicamente. O plasma seminal e as células espermáticas foram separadas por centrifugação, refrigerados a 5º C e transportados ao laboratório para análise proteômica. Proteínas do plasma seminal e das células espermáticas foram digeridas, submetidas à espectrometria de massas, identificadas e caracterizadas quanto à ontologia gênica. A relação entre as proteínas encontradas e as raças caninas foi avaliada por análise multivariada. Foram encontradas 10 proteínas no plasma seminal e cinco proteínas espermáticas consideradas relevantes. Notou-se diferença na expressão de proteínas espermáticas e do plasma seminal de diferentes raças caninas. Para o segundo experimento, 3 pools do ejaculado de 8 cães, provenientes do experimento 1 foram utilizados. A morfologia, motilidade espermática computadorizada (CASA), espermatozoides íntegros e lesados (eosina / nigrosina), integridade de membranas plasmática e do acrossomo, produção de superóxido, produção de peróxido de hidrogênio, potencial mitocondrial, peroxidação lipídica, produção de

espécies reativas ao oxigênio (técnica do ácido tiobarbitúrico induzido e espontâneo) e produção de enzimas antioxidantes (ELISA) foram avaliadas a cada 48 e 72 horas (M0, M48, M96 e M168) durante 168 horas (7 dias), em amostras de sêmen canino refrigeradas a 5º C com e sem plasma seminal. As amostras seminais foram divididas em dois grupos: sêmen diluído em TRIS-gema-glicose com plasma seminal e sêmen diluído em TRIS-gema glicose sem plasma seminal. Os dados obtidos foram analisados pelo two-way ANOVA e a análise multivariada pelo teste de Bonferroni. As amostras refrigeradas com plasma seminal apresentaram maior motilidade total, motilidade progressiva, velocidade média do trajeto, velocidade em linha reta, espermatozoides rápidos, índice de movimento espermático, espermatozoides com membranas plasmática e do acrossomo íntegras, maior potencial mitocondrial, menor movimento lateral de cabeça e menos espermatozoides médios e lentos. Com relação ao tempo de refrigeração, foi observada diferença estatística para a motilidade total, velocidade em linha reta, movimento lateral de cabeça, movimento retilíneo, linearidade, coeficiente de oscilação e índice de movimento. O perfil proteômico das proteínas espermáticas e do plasma seminal de quatro raças caninas foi descrito. Foi observada similaridade no padrão de expressão de proteínas espermáticas entre as raças Golden Retriever e Pastor de Maremmano Abruzzese, e entre as raças Dogue Alemão e Bernese. As raças Pastor de Maremmano Abruzzese, Dogue Alemão e Bernese apresentaram similaridade no padrão de expressão das proteínas do plasma seminal. O plasma seminal exerceu influência positiva sobre os parâmetros de motilidade e viabilidade espermática e função mitocondrial durante a refrigeração do sêmen a 5 ºC.

Palavras-chave: peroxidação lipídica, refrigeração, proteômica, sêmen, cães

ABSTRACT

ARAUJO, M. S. (2018). **Evaluation of chilled semen with or without seminal plasma and its correlation with the ejaculate proteins of different canine breeds.** Botucatu – SP. 2018, 199p. Thesis (Doctoral) – Faculty of Veterinary Medicine and Animal Science, Botucatu Campus, São Paulo State Univeristy, Departament of Animal Reproduction and Veterinary Radiology.

Seminal plasma and its proteins influence the sperm quality of chilled and frozen semen in several species, but its influence on the cooled semen of dogs has not yet been clarified. Furthermore, interracial differences in the expression of seminal plasma proteins that may interfere with the quality of canine semen have not been reported. The objective of this study was to verify if the racial pattern interferes in the protein composition of seminal plasma and sperm proteins and to correlate them with the cooling of canine semen in the presence and absence of seminal plasma. In this way, two experiments were performed. In the first experiment the seminal plasma proteins of 5 canine breeds and sperm proteins of 4 breeds were characterized. The semen was collected, evaluated macro and microscopically. Seminal plasma and sperm cells were separated by centrifugation, refrigerated at 5° C and transported to the laboratory for proteomic analysis. Seminal plasma and spermatozoa proteins were digested, submitted to mass spectrometry, identified and characterized for gene ontology. The relationship between the proteins found and the canine breeds was evaluated by multivariate analysis. In the seminal plasma, 10 relevant proteins were found and in the spermatozoa five proteins were considered relevant. Differences were observed in the expression of sperm proteins from different canine breeds. For the second experiment, 3 ejaculate pools of 8 dogs from experiment 1 were used. The morphology, computer-assisted sperm motility (CASA), intact and damaged spermatozoa (eosin / nigrosin), plasma and acrosome membrane integrity, superoxide production, hydrogen peroxide production, mitochondrial potential, lipid peroxidation, oxygen reactive species production (induced and spontaneous thiobarbituric acid technique) and antioxidant enzyme production (ELISA) were evaluated every 48 and 72 hours (M0, M48, M96 and M168) until 168 hours (7 days) in samples of canine semen cooled at 5º C with and

without seminal plasma. Seminal samples were divided into two groups, semen extended in TRIS-egg yolk-glucose with seminal plasma and semen extended in TRIS-egg yolk-glucose without seminal plasma. Data were analyzed by two-way ANOVA and multivariate analysis using the Bonferroni test. Seminal plasma refrigerated samples showed high total motility, progressive motility, mean velocity (VAP), straight velocity, rapid spermatozoa, movement index, plasma and acrosomal sperm membranes, greater mitochondrial potential, low lateral head movement and low medium and slow spermatozoa. According to refrigeration time, statistical was significant for total motility, straight velocity, lateral head movement, straightness coefficient, linearity, oscillation coefficient and movement index. The profile of seminal plasma and spermatozoa proteins from the ejaculate of four canine breeds were described. Similarities between Golden Retriever and Maremmano-Abruzzese Sheepdog; and between Great Dane and Bernese Mountain Dog in proteomic profile of spermatozoa exist; while Maremmano-Abruzzese Sheepdog, Gread Dane and Bernese were correlated in proteomic profile of seminal plasma. Seminal plasma exerted a positive influence on the parameters of motility and sperm viability and mitochondrial function during semen refrigeration at 5 ºC.

Keywords: lipidic peroxidation, cooling, proteomic, semen, dogs

CAPÍTULO 1

1. INTRODUÇÃO E JUSTIFICATIVA

A possibilidade de armazenamento e transporte do sêmen de cães de alto destaque na cinofilia, com a finalidade de reduzir custos e riscos com o transporte dos animais, é um dos fatores que tem despertado o interesse dos criadores pela refrigeração e congelação espermática (UCHOA, 2011). Neste contexto, a inseminação artificial com sêmen refrigerado e congelado tem contribuído de forma significativa para a preservação de raças de alto valor zootécnico (EVANGELISTA et al., 2016), sendo o refrigerado mais utilizado no Brasil em relação ao congelado (CHIRINEA et al., 2013).

Apesar das vantagens da criopreservação, as células espermáticas, quando submetidas a baixas temperaturas, podem sofrer danos em suas estruturas celulares, devido à alta produção de espécies reativas ao oxigênio (ROS) (LUCIO et al., 2016; VIEIRA et al., 2018). As ROS são importantes durante alguns processos fisiológicos espermáticos, tais como a capacitação espermática, fertilização do oócito, reação do acrossomo e hiperativação. Porém, quando em grandes quantidades, estão relacionadas a danos ao DNA, lipídios, carboidratos e proteínas que compõem a estrutura celular do espermatozoide (DE LAMIRANDE et al., 1997; VIEIRA et al., 2018).

Para evitar estes danos, durante os eventos fisiológicos, o plasma seminal canino possui enzimas antioxidantes, como a glutationa peroxidase, a superóxido dismutase e a catalase, que minimizam a ação deletéria promovida pelas ROS aos espermatozoides (STRZEZEK et al, 2009; ANGRIMANI et al., 2014). No entanto, ainda há controvérsias quanto a real função do plasma seminal durante a criopreservação do sêmen, uma vez que já foi demonstrada influência negativa (ROTA et al., 1995; SIRIVAIDYAPONG et al., 2001; YAMASHIRO et al., 2009) e positiva (HORI et al., 2004) deste fluido sobre a qualidade espermática em cães.

Além de enzimas antioxidantes, o plasma seminal também apresenta uma variedade de proteínas que tem sido foco de vários estudos, com o objetivo de elucidar as suas funções biológicas em diferentes espécies animais (DUBÉ et al., 1994; AQUINO-CORTEZ et al., 2016; AQUINO-CORTEZ et al., 2017). Há evidências experimentais da participação destas proteínas durante os processos de peroxidação lipídica, capacitação espermática, reação do acrossomo, reações

no sistema imune feminino, interações com o epitélio da tuba uterina e fertilização (RODRÍGUEZ-MARTÍNEZ et al., 2011; AQUINO-CORTEZ et al., 2016). Várias proteínas foram identificadas no plasma seminal canino, exercendo funções relacionadas à qualidade espermática e à fertilidade (KIKUCHI et al., 2003; SOUZA et al, 2007b; SAENGSOI et al., 2011; MOGIELNICKA-BRZOZOWSKA; KORDAN, 2011). Tais moléculas são potenciais candidatas a marcadores de fertilidade e de processos patofisiológicos, além de auxiliarem o desenvolvimento de novos protocolos de criopreservação de sêmen, podendo melhorar a qualidade do sêmen após a descongelação e, consequentemente, as taxas de fertilidade (SOUZA et al., 2007a). No entanto, estudos mais detalhados sobre a composição do plasma seminal canino e sua específica função biológica ainda são necessários (AQUINO-CORTEZ et al., 2016).

Apesar dos estudos quanto às proteínas do plasma seminal, as proteínas das células espermáticas do cão ainda não foram descritas. Os espermatozoides são células diferenciadas, com compartimentos celulares específicos como a membrana celular, estrutura de maior importância durante a fertilização e que merece destaque, uma vez que permite a ligação do espermatozoide ao oócito durante a fecundação (RODRÍGUEZ-MARTÍNEZ et al., 2011).

A proteômica auxilia na identificação de marcadores de fertilidade ou de ejaculados aptos à congelação (BARROS et al., 2013). Desta forma, estudos que visam compreender as proteínas que compõem o plasma seminal e as células espermáticas caninas podem auxiliar no melhoramento das biotecnologias aplicadas à reprodução já existentes para esta espécie.

Em vista à grande variedade de raças e às diferenças entre as raças relacionadas à qualidade espermática, o objetivo deste estudo foi verificar se o padrão racial interfere na composição proteica do plasma seminal e das proteínas espermáticas e correlacioná-las com a refrigeração do sêmen canino na presença e ausência do plasma seminal.

2. REVISÃO DE LITERATURA

2.1. Plasma seminal

O plasma seminal é constituído pelas secreções produzidas pelos testículos, epidídimos, e pelas glândulas sexuais acessórias (MANN; LUTWAK-MANN, 1981; REGO et al., 2014). No cão, a próstata é a única glândula sexual acessória, e o fluido prostático contribui com 95% do total dos constituintes do ejaculado nesta espécie (IGUER-OUADA; VERSTEGEN, 2001). O ejaculado canino é composto por três frações, e em sua maioria, formado por líquido prostático (KUTZLER, 2005; BRITO-NERY et al., 2014). A primeira fração (pré-espermática) é transparente, de menor volume, contém pouco ou nenhum espermatozoide, e é originária de glândulas uretrais e próstata. A segunda fração (fração espermática), se origina principalmente do epidídimo, e contém pouco ou nenhum fluido prostático, apresentando grande concentração de espermatozoides. A terceira fração compõe o maior volume do plasma seminal e é de origem prostática, podendo conter pouca ou nenhuma célula espermática (YAMASHIRO et al., 2009; BRITO-NERY, 2014).

Durante o acasalamento, todas as frações do ejaculado do cão são lançadas no interior do trato reprodutivo da fêmea, podendo ocorrer a fertilização dos oócitos e a consequente gestação quando há condições favoráveis. Durante a inseminação artificial com sêmen fresco ou refrigerado, preconiza-se apenas a colheita da segunda fração do ejaculado, associada ou não a uma porção da terceira fração (KUTZLER, 2005). Porém, a separação de cada uma das frações durante a colheita do sêmen muitas vezes não é realizada adequadamente devido à rapidez durante a ejaculação de alguns cães, à movimentação do animal e à inexperiência ou menor agilidade do manipulador (PEÑA et al., 2006). Nestes casos, parte do plasma seminal é inseminada e refrigerada com os espermatozoides.

Assim como em equinos (MOORE et al., 2005), o plasma seminal é retirado durante a criopreservação do sêmen de cães, pois a adição do fluido prostático à fração contendo os espermatozoides altera de forma negativa a motilidade e a viabilidade espermática após a descongelação (SIRIVAIYAPONG et al., 2001; YAMASHIRO et al., 2009). Yamashiro et al. (2009) observaram vários efeitos deletérios às características de motilidade espermática após a descongelação do sêmen de cães da raça poodle, quando adicionaram fluido prostático proveniente

da primeira ou terceira fração do ejaculado à segunda fração na criopreservação.

Em contrapartida, ao descongelar espermatozoides colhidos do epidídimo de cães para estudar o efeito promovido pela presença ou ausência do fluido prostático, durante o processo de congelação sobre a taxa de concepção por inseminação artificial, Hori et al. (2004) reportaram que a sensibilização dos espermatozoides epididimários com o fluido prostático antes da congelação, apresentou efeito benéfico na viabilidade espermática e na taxa de fertilidade. Em touros, o plasma seminal de animais de alta fertilidade adicionado às células espermáticas de animais de baixa fertilidade promoveu aumento das taxas de fertilidade de touros considerados subférteis, demonstrando a importância deste fluido para a fertilidade *in vivo* (RODRIGUES et al, 1999; SOUZA, 2007a).

As investigações a respeito do principal papel desempenhado pelo plasma seminal na espécie canina, principalmente durante a refrigeração, foram pouco mencionadas e sua atuação permanece controversa (GILBERT; SUAREZ, 2013, BRITO-NERY et al., 2014; AQUINO-CORTEZ et al., 2017). Quando o sêmen canino foi mantido refrigerado no plasma seminal autólogo a 4º C por 4 dias, houve significativa redução da motilidade, velocidade e porcentagem de espermatozoides com membrana íntegra nas primeiras 24 horas (ROTA et al., 1995). Quando prostassomos, um componente do plasma seminal, foram adicionados ao sêmen canino refrigerado a 5º C por duas horas, ocorreu aumento da motilidade (MONGIELNICKA-BRZOZOWSKA et al., 2015).

O plasma seminal do cão é composto por enzimas antioxidantes (catalase, GPx e SOD) (STRZEZEK et al., 2009; ANGRIMANI et al., 2014), íons e minerais (zinco, cobre, ferro, magnésio, sódio, cloreto, potássio, cálcio) (BARTLETT, 1962; KRIEGER; REIN, 1982; SOUZA et al., 2006b), carboidratos (BARTLETT, 1962), vesículas membranosas lipídicas (prostassomos) (ZELLI et al., 2013; MOGIELNICKA-BRZOZOWSKA et al., 2015) e várias proteínas (SOUZA et al., 2006a; SOUZA et al., 2007; SOUZA et al., 2009; SAENGSOI et al., 2011; BRITO-NERY et al., 2014; WARINRAK et al., 2015; AQUINO-CORTEZ et al., 2016; AQUINO-CORTEZ et al., 2017). Estes componentes desempenham várias funções que participam ativamente dos processos reprodutivos.

2.2. Proteínas do plasma seminal canino

Proteínas do plasma seminal são produzidas e secretadas pelos diferentes órgãos que compõem o sistema reprodutor masculino, principalmente pelas glândulas sexuais acessórias, testículos, epidídimos e ductos deferentes. O plasma sanguíneo também confere proteínas ao plasma seminal por exsudação do seu conteúdo. A prealbumina, a albumina, globulina, transferrina, α-antitripsina, β-lipoproteina, β-glicoproteína, orosomucoide, cininogênio, hormônios peptídeos, IgF, IgA e IgM são algumas das proteínas de origem sanguínea que já foram identificadas no plasma seminal humano (DASKALOVA et al., 2017).

As proteínas do plasma seminal contribuem para o desempenho das funções e a sobrevivência espermática; participam, ainda da proteção do espermatozoide, ao se ligarem a superfície da célula durante a ejaculação, conferindo proteção e auxiliando nos processos de maturação espermática, capacitação, reação acrossomal, hiperativação e fecundação. Além disso, participam da modulação do sistema imune feminino e masculino e refletem o *status* fisiológico das glândulas sexuais acessórias e dos epidídimos, sendo, portanto, importantes biomarcadores de fertilidade (VARILOVÁ et al., 2006; MOGIELNICKA-BRZOZOWSKA; KORDAN, 2011; DAVALIEVA et al., 2012; MILARDI et al., 2012).

Escassos estudos sobre proteínas que compõem o plasma seminal canino foram realizados. Algumas proteínas foram isoladas e identificadas como a fosfatase ácida e alcalina (MANN; LUTWAK-MANN, 1981), esterase específica da próstata canina (CPSE) ou arginina esterase (CHAPDELAINE et al., 1984; BELL et al., 1995; ALONGE et al., 2018), lactoferrina, transferrina (KIKUCHI et al., 2003), metaloproteinases (SAENGSOI et al., 2011), enzimas antioxidantes [superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GP)] (CASSANI et al., 2005; STRZEZEK et al., 2009; ANGRIMANI et al., 2014), proteínas ligadoras de zinco (MOGIELNICKA-BRZOZOWSKA et al., 2015), e proteínas ligadoras de heparina (SOUZA et al., 2006a; SOUZA et al., 2009).

A fosfatase alcalina presente no plasma seminal canino, é produzida pela cauda do epidídimo e liberada em maior concentração na primeira fração do ejaculado. É considerada importante indicador da função epididimária (FRENETTE et al., 1986; STRZEZEK et al., 2009). Já a fosfatase ácida é produzida pela próstata, e sua secreção no plasma seminal é andrógeno dependente (STRZEZEK;

JANOWSKI, 2003; STRZEZEK et al., 2015). Embora controversa (BELL et al., 1995; SOUZA; TONIOLLO, 2001; GADELHA et al., 2013), alguns autores relataram a atuação desta proteína como biomarcador da integridade estrutural do epitélio secretor da próstata em cães, sendo um importante indicador de alterações prostáticas, tais como a hiperplasia prostática benigna e o adenocarcinoma prostático (MCENTEE et al., 1987; CORAZZA et al., 1994).

A CPSE canina, também conhecida como arginina esterase, é uma proteína similar ao antígeno específico prostático humano (PSA). Essa proteína compõe 90% das proteínas secretadas pela próstata e 30% das proteínas que compõem o plasma seminal (ISAACS; CONFFEY, 1984; BELL et al., 1995). Sua concentração nesse fluido é regulada pela testosterona e sua atividade é regulada pela quantidade de zinco disponível no ejaculado (JUNIEWICZ et al., 1990; MOGIELNICKA-BRZOZOWSKA et al., 2015). A CPSE se liga às regiões da cauda e pós-acrossomal dos espermatozoides localizados na cauda do epidídimo (ISAACS; COFFEY, 1984). Alta concentração sérica desta proteína já foi observada em cães com hiperplasia prostática benigna, prostatite bacteriana e carcinoma prostático, sugerindo sua atuação como biomarcador para o diagnóstico precoce de alterações prostáticas. No entanto, a real função da CPSE durante estes processos ainda não está esclarecida (LEVY; MIMOUNI, 2009; WOLF et al., 2012; ALONGE et al., 2018).

A lactoferrina é uma proteína ligadora de ferro de massa molecular de 75,2 kDa, quelante de íons do plasma seminal, que protege os espermatozoides dos danos provocados pela peroxidação lipídica, além de possuir função antimicrobiana (OCHSENDORF, 1999; KIKUCHI et al., 2003). A origem da lactoferrina em cães é desconhecida, porém sua secreção está relacionada com a espermatogênese, uma vez que a quantidade de lactoferrina presente no plasma seminal canino apresentou correlação positiva com a concentração de espermatozoides no ejaculado.

A transferrina, homóloga à lactoferrina, é secretada pelas células de Sertoli (HOLMES et al., 1984) e desempenha papel fundamental na espermatogênese ao fornecer ferro às células germinativas em desenvolvimento via receptores específicos presentes nestas células (HOLMES et al., 1983; SYLVESTER; GRISWOLD, 1994). A concentração de transferrina no plasma seminal de cães é

menor do que a observada em outras espécies animais (BARTHELEMY et al., 1988; GILMONT et al., 1990). Diferente da lactoferrina, a transferrina não apresenta correlação com a concentração de espermatozoides no ejaculado canino, portanto, não é um bom indicador de função gonadal nesta espécie (KIKUCHI et al., 2003).

As metaloproteinases fazem parte de uma ampla família de enzimas proteolíticas que degradam componentes da matriz extracelular e da membrana basal, regulando a interação célula-célula ou célula-matriz extracelular (SALAMONSEN; WOOLLEY, 1996; HULBOY et al., 1997; STERNLICHT; WERB, 2001). Sua atividade parece estar relacionada à remodelação da membrana basal durante o desenvolvimento dos túbulos seminíferos, remodelação da diferenciação espermática, modificação morfológica, liberação das diferentes células germinativas e apoptose da célula espermática (METAYER et al., 2002; SAENGSOI et al., 2011). São expressas em diversos órgãos reprodutivos e durante processos celulares, sendo destacadas as metaloproteinases da matriz 2 (MMP-2) e as metaloproteinases da matriz 9 (MMP-9) (HULBOY et al., 1997). Estas proteínas foram encontradas em plasma seminal de cães normais, oligospérmicos e azoospérmicos. A MMP-2 apresenta 62 kDa e a MMP-9 80 kDa (SAENGSOI et al., 2011). A MMP-2 foi positivamente correlacionada com a motilidade espermática, portanto, sua presença pode melhorar a motilidade e viabilidade do sêmen em cães (SAENGSOI et al., 2011).

A MMP-9 foi detectada em maior quantidade no plasma seminal de cães azoospérmicos, porém ainda não se conhece se esta proteína é a causa ou a consequência da reduzida concentração espermática no sêmen (SAENGSOI et al., 2011). Já foi demonstrado que esta proteína parece ser a principal responsável por acelerar a quebra da matrix extracelular em casos de reação inflamatória, podendo estar aumentada nos casos de inflamações ou infecções do trato reprodutor masculino (GARG et al., 2009). Sua expressão parece ocorrer na próstata e nas células de sertoli (YIN et al., 1990; BUCHMAN-SHAKED et al., 2002). Portanto, uma alta expressão de MMP-9 pode estar correlacionada à espermatogênese anormal (SAENGSOI et al., 2011). Acredita-se que a inibição da atividade da MMP-9 no ejaculado pode melhorar a qualidade seminal, uma vez que o inibidor de MMP-9 melhorou a angiogênese no caso de lesão provocada pelo infarto do miocárdio (SAENGSOI et al., 2011; LINDSEY et al., 2006).

Os prostassomos são nanovesículas membranosas de formato esférico (117,66 ± 86,9 nm em cães), secretadas no fluido prostático e misturadas ao sêmen durante a ejaculação (RONQUIST; BROALIYN, 1985; ARDVISON et al., 1989; ZELLI et al., 2013). Produzidas pelas células epiteliais da próstata, estão presentes no plasma seminal, contribuindo com a maturação espermática e aquisição da capacidade fertilizante. Também possuem função antibacteriana e antioxidante (SAEZ et al., 2003; ARDVISON et al., 1989; CABALLERO et al., 2011; ZELLI et al., 2013). O conteúdo dessas vesículas é formado por íons, proteínas e lipídios com alta proporção de colesterol e fosfolipídios (ARDVISON et al., 1989; ZELLI et al., 2013). Nos cães, apresentam coloração clara ou escura e contém adenosina deaminase, 5'-nucleotidase, ATPase, ADPase, dipeptidilpeptidase IV, fosfatase alcalina, fosfatase ácida total e fosfatase ácida prostática em seu interior (ZELLI et al., 2013). Após a ejaculação, se fundem à membrana celular dos espermatozoides, conferindo novas atividades enzimáticas e proporcionando novas propriedades espermáticas que podem ser exercidas no trato reprodutor feminino (SAEZ et al., 2003; ZELLI et al., 2013). Quando adicionadas ao sêmen canino a 5º C, na concentração de 2% da proteína total do plasma seminal, os prostassomos promoveram efeito benéfico nos parâmetros de motilidade, na integridade da membrana plasmática e do acrossomo (MOGIELNICKA-BRZOZOWSKA et al., 2015).

As proteínas ligadoras de zinco (ZnBPs) estão predominantemente presentes nos prostassomos caninos, apresentando significativo efeito no metabolismo do zinco no sêmen (MOGIELNICKA-BRZOZOWSKA et al., 2015). São responsáveis por manter o equilíbrio entre o conteúdo de zinco no espermatozoide e no plasma seminal (necessário para alcançar e manter a motilidade dos espermatozoides), a reação do acrossomo, e a estabilidade da membrana plasmática e da cromatina (AONUMA et al., 1981; BJORNDHAL; KVIST, 1990; MOGIELNICKA-BRZOZOWSKA et al., 2015). Sete ZnBPs foram encontradas no plasma seminal canino sendo denominadas ZnBP-1 (15,1 – 15,5 kDa), ZnBP-2 (8,8 – 12,9 kDa), ZnBP-3 (13,9 – 15,3 kDa), ZnBP-4 (14,1 – 15,8 kDa), ZnBP-5 (8,5 – 12,9 kDa), ZnBP-6 (14,2 – 15,7 kDa) e ZnBP-7 (10,8 – 13,1 kDa). Estas proteínas foram identificadas como sendo isoformas da arginina esterase pelo método de espectrometria de massas (MOGIELNICKA-BRZOZOWSKA et al., 2015).

As proteínas ligadoras de heparina atuam favorecendo a capacitação espermática, a reação do acrossomo e alteram a resposta do sistema imune aos espermatozoides. São produzidas em resposta aos andrógenos, pelas glândulas sexuais acessórias, e são secretadas com o fluído seminal (MILLER et al., 1990; DAR et al., 2018). As proteínas ligadoras de heparina se ligam à superfície dos espermatozoides no epidídimo e desestabilizam a membrana espermática ao interagirem com a heparina e outras proteínas da zona pelúcida, como os glicosaminoglicanos, induzindo à reação do acrossomo (AX et al., 2002). Ainda possuem a função de proteger o espermatozoide dos danos promovidos pela congelação/descongelação, da peroxidação lipídica durante a criopreservação, e mantém a homeostase de proteínas intracelulares (KUMAR et al., 2008; DAR et al., 2018).

Souza et al. (2007b) encontraram no plasma seminal canino um total de 37 bandas de proteínas no método de eletroforese unidimensional, as quais foram comuns a todos os cães avaliados. Foi observada correlação positiva entre os parâmetros seminais (motilidade, vigor, morfologia espermática normal e integridade de membranas celular e do acrossomo) e duas bandas (67,0 kDa e 58,6 kDa). A proteína de 58,6 kDa foi identificada como sendo uma proteína ligadora de heparina. No entanto, a fertilidade dos animais utilizados neste mesmo estudo não foi comprovada, o que impossibilitou uma correlação entre as proteínas encontradas e a fertilidade. Outras 19 bandas de proteínas ligadoras de heparina também foram identificadas com massas moleculares variando entre 5,2 kDa a 61,5 kDa (SOUZA et al., 2006a).

A osteopontina, proteína secretada no plasma seminal pela ampola dos ductos deferentes e glândula vesicular de touros (CANCEL et al., 1999), foi correlacionada com a fertilidade. Esta proteína foi originalmente identificada como um constituinte da matriz extracelular óssea e é conhecida por mediar a adesão celular e a sinalização intracelular (DENHARDT; GOU, 1993). Maior concentração da osteopontina no plasma seminal de touros e garanhões foi associada ao aumento da fertilidade (KILLIAN et al., 1993; BRANDON et al., 1999; MOURA et al., 2005). Em cães, diferentes isoformas desta proteína foram encontradas no plasma seminal e na membrana espermática, porém se desconhece seu local de

produção e sua função sobre a fertilidade nesta espécie (SOUZA et al., 2009; AQUINO-CORTEZ et al., 2016).

Os trabalhos realizados até o momento focaram na caracterização das proteínas em todas as frações (SOUZA et al., 2006a), frações isoladas do ejaculado (AQUINO-CORTEZ et al., 2017) e na membrana espermática de cães (SOUZA et al., 2009); utilizando sêmen de animais sem raça definida ou pools de ejaculados de cães de diferentes raças. Trabalhos que objetivaram avaliar a diferença do perfil proteico do ejaculado de diferentes raças caninas ainda não foram realizados. No entanto, esta diferença já foi observada em outras espécies.

Diferenças no perfil proteico do plasma seminal de touros *B. taurus* e *B. indicus* foram descritas por Assumpção et al. (2005). Embora o perfil proteico de touros *B. taurus* e *B. indicus* seja semelhante, foram encontradas proteínas no plasma seminal de touros *B. indicus* (*seminal plasma protein BSP – 30K precursor* e *bovine spermidine synthase - SPS*) que não foram descritas anteriormente nesta espécie, e que são importantes para a reprodução (ASSUMPÇÃO et al. 2005).

Em cães, Aquino-Cortez et al. (2017) identificaram por espectrometria de massas as proteínas do plasma seminal obtidas da segunda e terceira frações do ejaculado da raça Rotweiller e Pastor Alemão. Ao todo foram identificadas 268 proteínas, sendo que 251 foram comuns à segunda e terceira fração, e 17 proteínas foram exclusivas da segunda fração, demonstrando que as duas frações do ejaculado possuem composição proteica semelhante. A arginina esterase e a precursora da lactoferrina foram as proteínas mais expressas nas duas frações.

Além de encontrarem proteínas que já foram descritas no plasma seminal de cães e de outras espécies animais, estes autores encontraram proteínas que ainda não haviam sido descritas em cães (*epididymal-specific lipocalin-9*, *ras related protein Rab-8a*, *cofilin 1 isoform x2*) e que desempenham funções associadas à maturação espermática, motilidade, capacitação, reação do acrossomo e fertilidade. Outras proteínas que já haviam sido descritas no plasma seminal canino, tais como a catalase e a osteopontina, não foram encontradas por estes autores, sendo este resultado atribuído às diferenças entre as técnicas de obtenção das proteínas. Embora o sêmen utilizado neste experimento tenha sido obtido de duas raças caninas distintas (Rotweiller e Pastor Alemão), os autores não relataram

diferenças na expressão e na composição proteica do plasma seminal das duas raças.

2.3. Proteínas da célula espermática canina

A célula espermática foi uma das primeiras células a serem estudadas com relação a sua composição proteica, e teve início com o estudo pioneiro realizado por Friedrich Miescher em 1874 (OLIVA et al., 2009). A primeira proteína espermática a ser identificada foi a protamina, a qual é a mais abundante em várias espécies, e foi identificada no núcleo do espermatozoide, atuando na condensação do genoma (MIESCHER 1874; OLIVA; DIXON, 1991; OLIVA et al., 2009).

Após a identificação das protaminas, várias outras proteínas que compõem a célula espermática foram detectadas como a tubulina, proteínas do acrossomo, das mitocôndrias, da matriz nuclear, do citoesqueleto e da membrana celular (MOHRI et al., 1968; GILBOA et al., 1973; HADEN et al., 2000; SHAMAN et al., 2007). A análise destas proteínas que compõem os espermatozoides não é apenas importante para esclarecer aspectos relacionados à estrutura, função e maturação celular; mas também para elucidar os mecanismos patofisiológicos que estão envolvidos com a infertilidade (AMARAL et al., 2014).

A célula espermática é subdivida em cabeça contendo o acrossomo e o núcleo, o qual é envolto em membrana nuclear; peça intermediária que contém os centríolos proximais, um colum segmentado, várias mitocôndrias, fibras densas e anulus; e a região da cauda com o flagelo e uma bainha fibrosa, dividida em peça principal e peça terminal (FAWCETT, 1975). De acordo com a função desempenhada pelos diferentes compartimentos celulares que compõem o espermatozoide, há várias subpopulações de proteínas que as caracterizam, sendo algumas relacionadas à fertilidade em humanos (BAKER et al., 2013; AMARAL et al., 2013).

Como descrito no plasma seminal, há diferenças no perfil proteico de espermatozoides de touros mestiços (*B. taurus* x *B. indicus*) comparados a touros *B. taurus* e *B. indicus* (ASLAM et al., 2015; ASHRAFZADEH et al., 2013). Em espermatozoides de touros mestiços, proteínas importantes para o processo de fertilização e fisiologia espermática foram pouco expressas, enquanto proteínas

envolvidas com a capacitação e reação do acrossomo foram mais expressas, demonstrando a influência negativa de raças mestiças sobre a fertilidade (THIPPESWAMY et al., 2014; ASLAM et al., 2015). Diferenças no perfil proteico de células de Sertoli e espermatogônias, entre touros mestiços e touros de raça pura, também foram observadas e podem estar relacionadas à fertilidade (TRIPATHI et al., 2014).

Outro estudo realizado com touros *B. taurus*, *B. indicus* e mestiços (*B. taurus* x *B. indicus*) (ASLAM et al., 2015) mostrou que espermatozoides de touros mestiços apresentaram menor expressão de 9 proteínas e maior expressão de 8 em relação a touros *B. taurus*; e 4 proteínas mais expressas e outras 4 menos expressas em relação a touros *B. indicus*. Quando as três raças foram comparadas, foram detectadas 14 proteínas diferentes. Dentre as proteínas que foram menos expressas em touros mestiços em relação aos *B. taurus* estão a *heat shock protein HSP 90-β*, *60S ribosomal protein L5*, *40S ribosomal protein S29*, *NADH dehydrogenase*, *prostaglandina E2 receptor EP3*, as quais participam de importantes funções no espermatozoide, que são necessárias à fertilização. Desta forma, os autores concluíram que a expressão diferenciada de proteínas espermáticas pode ser um dos fatores que contribui para a alta incidência de subfertilidade em touros mestiços.

Com relação à espécie canina, há apenas um trabalho publicado até o momento com relação às proteínas de membrana espermática (SOUZA et al., 2009). Este estudo detectou a osteopontina no plasma seminal e na membrana espermática, no entanto também não foi realizado um comparativo entre as raças avaliadas.

2.4. Espécies reativas de oxigênio

As espécies reativas ao oxigênio (ROS) são moléculas derivadas do oxigênio quimicamente reativas, por apresentarem elétrons não pareados, que mantém o equilíbrio antioxidante dentro do sistema de oxidação e redução (também conhecido como sistema redox) (AGARWAL et al., 2012; WAGNER et al., 2018). O adequado equilíbrio do sistema redox é necessário para as funções fisiológicas essenciais desempenhadas pelo espermatozoide como a compactação da

cromatina durante sua maturação, a capacitação, hiperativação, reação do acrossomo e para a fertilização, por meio da estimulação da produção intracelular de AMPc e da fosforilação da tirosina (DE LAMIRANDE; GAGNON, 1993; AITKEN et al., 2004; WRIGHT et al., 2014).

Algumas ROS foram identificadas como sendo clinicamente importantes durante a infertilidade, como os radicais peroxila (ROO^-) e hidroxila (OH), o ânion superóxido (O_2^-), o peróxido de hidrogênio (H_2O_2), o óxido nítrico (NO) e o ânion peroxinitrito ($ONOO^-$) (HENKEL, 2011). Nem todas as ROS são radicais livres verdadeiros, como por exemplo o H_2O_2, que não apresenta elétrons não pareados, apesar de ser altamente reativo (WAGNER et al., 2018). As ROS são produzidas fisiologicamente durante alguns processos celulares como durante o metabolismo aeróbico celular realizado pelas mitocôndrias durante a fosforilação oxidativa para a produção de ATP (BOVERIS; CHANCE, 1973). Outra fonte intracelular de ROS é a produção citoplasmática de creatina quinase, ácido lático desidrogenase e glicose-6-fosfato desidrogenase (CASANO et al., 1991; HUSZAR; VIGUE, 1993; AITKEN et al., 1994), sendo observada relação positiva entre a gota citoplasmática espermática e o estresse oxidativo (RAO et al., 1989).

Durante os processos inflamatórios e infecciosos o recrutamento leucocitário para o local da lesão pode levar à produção de até mil vezes mais ROS do que o produzido normalmente pelo processo aeróbico celular (PLANTE et al., 1994). Diversos fatores externos e internos como infecções das glândulas sexuais acessórias, varicocele, hiperglicemia, exposição a metais pesados, pesticidas, poluição, cigarro, álcool, tratamento quimioterápico (cisplatina, doxorrubicina, ciclofosfamida), alta ou baixa exposição à radiação, podem aumentar a produção de ROS, promovendo danos aos espermatozoides e ao testículo em humanos (DEPUYDT et al., 1996; SALEH et al., 2002; TILIGADA, 2006; MANDA et al., 2007; TREMELLEN, 2008; DESAI et al., 2009; AGARWAL et al., 2014; WRITH et al.,2014; OLIVEIRA et al., 2015).

Quando uma grande quantidade de oxidantes ou de moléculas que aceitam elétrons de outro reagente é produzida, devido a um desequilíbrio entre as reações de redução e oxidação, ocorre o estresse oxidativo (HENKEL, 2011). As membranas celulares lipídicas e os carboidratos dos ácidos nucleicos que compõem o DNA são moléculas que comumente recebem elétrons não pareados

e, portanto, estão susceptíveis aos danos provocados pelas ROS (TREMELLEN, 2008). O estresse oxidativo nuclear e ao DNA mitocondrial alteram as proteínas espermáticas, as quais também sofrem oxidação pelas ROS e seus subprodutos, sendo os aminoácidos cisteína e metionina os mais susceptíveis por apresentarem compostos sulfurados na sua estrutura (LOBASCIO et al., 2015; AYAZ et al., 2018). Alterações da estrutura, solubilidade e atividade enzimática são observadas nos espermatozoides devido às modificações proteicas provocadas pelo estresse oxidativo (ZHANG et al., 2013).

O espermatozoide apresenta uma grande quantidade de ácidos graxos poli-insaturados que o torna susceptível ao estresse oxidativo, e grandes danos a sua membrana plasmática podem acontecer (WAGNER et al., 2018). Os ácidos graxos poli-insaturados possuem várias duplas ligações, que ao reagirem com as ROS resultam em peroxidação lipídica e formação de subprodutos como o malonaldeído e o 4-hidroxinonenal (MAKKER et al., 2009; WEBER et al., 2013).

Durante o estresse oxidativo as ROS atuam sobre as bases purina e pirimidina que compõem o DNA espermático, promovendo a quebra das fitas de DNA, rearranjos dos cromossomos, e ativação da apoptose celular mediada pelas caspases (BISHT et al., 2017). Desta forma, quando a quantidade de ROS produzida é maior que a capacidade antioxidante há dano celular e, consequente infertilidade, perdas gestacionais ou mutações hereditárias genéticas (DINESH et al., 2012; WAGNER et al., 2018).

Ao danificarem o DNA mitocondrial pela redução da produção de ATP, e ao promoverem a peroxidação lipídica da membrana celular espermática ao diminuir sua flexibilidade, as ROS causam queda da motilidade do espermatozoide (DE LAMIRANDE; GAGNON, 1993; MORIELLI; O'FLAHERTY, 2015). Espermatozoides com altos níveis de estresse oxidativo, principalmente após ciclos de congelamento/descongelamento, apresentam altos níveis de ativação de caspases que podem levar à apoptose (PAASCH et al., 2004).

2.5. Antioxidantes

Os antioxidantes são agentes enzimáticos e não enzimáticos que protegem as células dos danos oxidativos ao reagirem e se ligarem às ROS. Os antioxidantes

não enzimáticos podem ser produzidos por via endógena ou são adquiridos via exógena por meio da suplementação e/ou alimentação (SIES, 1993). Os antioxidantes de maior importância presentes no ejaculado canino são a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) (ANGRIMANI et al., 2014).

Nos casos de infertilidade, os antioxidantes enzimáticos que conferem maior proteção à célula espermática são a superóxido dismutase, a glutationa peroxidase e a catalase (WAGNER et al., 2018). A superóxido dismutase (SOD) é encontrada em altas concentrações nas células de Sertoli. Esta enzima promove a redução dos marcadores de estresse oxidativo, protege o espermatozoide contra a peroxidação lipídica, reduz o dano ao DNA, e está associada à motilidade em espermatozoides humanos (KOBAIASHI et al., 1991; BAUCHÉ et al., 1994; CHEN et al., 2003; TAVILANI et al., 2008). Em cães a SOD já foi detectada em todas as frações do ejaculado e na cabeça, corpo e cauda do epidídimo (NEAGU et al., 2011; ANGRIMANI et al., 2014).

Assim como a superóxido dismutase, a glutationa peroxidase também está presente nas células de Sertoli, sendo secretada pela cabeça do epidídimo e encontrada no sêmen protegendo a membrana espermática da peroxidação lipídica (BAUCHÉ et al., 1994; TAVILANI et al., 2008). Em cães a glutationa peroxidase é observada em todas as frações do ejaculado (principalmente na segunda fração), e na cabeça, corpo e em maior quantidade na cauda do epidídimo (NEAGU et al., 2011; ANGRIMANI et al., 2014). O epidídimo é a maior fonte de glutationa peroxidase, o que reforça a sua importância durante o processo de maturação espermática na espécie canina (ANGRIMANI et al., 2014).

A catalase é uma enzima encontrada no interior dos peroxissomos, está presente em baixa concentração nos espermatozoides, e realiza a conversão do H_2O_2 em O_2 e água (H_2O) (BAUCHÉ et al., 1994). Assim como a glutationa peroxidase e a superóxido dismutase, está presente em todas as frações do sêmen canino, porém em menor quantidade na primeira fração. No entanto, esta enzima não foi encontrada nas diferentes regiões do epidídimo (cabeça, corpo e cauda), o que indica que a próstata é a principal fonte de catalase no ejaculado desta espécie, conferindo proteção antioxidante aos espermatozoides após a ejaculação (ANGRIMANI et al., 2014b).

A glutationa, o ácido ascórbico, a vitamina E, o selênio, o zinco e o ubiquinol compõem os antioxidantes não enzimáticos presentes no sêmen humano (BAUCHÉ et al., 1994; LEWIS et al., 1997; BISHT et al., 2017; ZAGO; OTEISA, 2001; ALLEVA et al., 1997). A glutationa atua como uma coenzima da glutationa peroxidase, e está presente nas células espermáticas em desenvolvimento (BAUCHÉ et al., 1994). O ácido ascórbico neutraliza as ROS, protegendo os espermatozoides dos danos ao seu DNA (LEWIS et al., 1997). A vitamina E e o zinco reduzem a peroxidação lipídica espermática (LEWIS et al., 1997; ZAGO; OTEISA, 2001). O selênio está presente nas selenoproteínas como a glutationa peroxidase, atuando como um componente chave nos sistemas que mantém a homeostase antioxidante (BISHT et al., 2017). O ubiquinol é uma redução da coenzima Q10 que está presente no plasma seminal, sendo associado a baixos níveis de ROS e um correspondente aumento da motilidade e concentração espermáticas (ALLEVA et al., 1997).

Em cães a suplementação oral de Vitamina E tem promovido efeitos benéficos ao sêmen canino fresco (CASSANI et al., 2005; HATAMOTO et al., 2006). A adição de 0,5 mM de N-acetil-cisteína, um precursor intracelular da biossíntese de glutationa, ao diluente TRIS-gema-glicose durante a refrigeração do sêmen canino por 72 horas a 4ºC foi benéfica à motilidade espermática (MICHAEL et al., 2010). A adição de chá verde (polifenol) ao diluente TRIS-gema-frutose na dose de (0,5, 0,75 ou 1 mg/mL) melhorou a motilidade e a viabilidade do sêmen canino refrigerado a 5ºC por quatro semanas (WITTAYARAT et al., 2013). A suplementação de 20mM do antioxidante glutationa peroxidase reduzida ao diluente de criopreservação de sêmen canino aumentou os danos espermáticos, enquanto a adição na concentração de 10mM resultou em proteção do acrossomo e preservou a fertilidade do sêmen (LUCIO et al., 2016a).

Durante a espermatogênese a célula espermática perde citoplasma, o que limita o seu estoque intracelular de antioxidantes, tornando-a dependente de antioxidantes presentes no plasma seminal para sua proteção contra o estresse oxidativo (YEUNG et al., 1998). No entanto, durante o trânsito no lúmen epididimário os espermatozoides de cães apresentam gota citoplasmática, que em associação com as enzimas antioxidantes do interior do epidídimo, são importantes

para manter a homeostase oxidativa do espermatozoide canino durante o processo de maturação espermática (ANGRIMANI et al., 2014a).

Embora os antioxidantes atuem protegendo as células contra o estresse oxidativo, uma suplementação exagerada destes componentes pode levar a um desbalanço entre a redução e a oxidação, promovendo um paradoxal aumento da produção de ROS (HALLIWELL, 2000). O excesso de catalase previne a capacitação pela inibição da oxidação local (AITKEN et al., 1995); da mesma forma, o excesso de glutationa peroxidase e catalase podem prevenir a reação do acrossomo (DE LAMIRANDE et al., 1998). Tanto a capacitação espermática e a reação do acrossomo são eventos indispensáveis à fertilização.

2.6. Estresse oxidativo durante a criopreservação do sêmen

A criopreservação envolve a realização de várias etapas nas quais o sêmen é submetido a diferentes temperaturas e osmolaridades devido à adição de crioprotetores. Durante a criopreservação do sêmen há formação de radicais livres e peroxidação lipídica devido à remoção do plasma seminal, ao estresse térmico e à exposição do sêmen ao oxigênio atmosférico (WATSON, 2000; BUCAK et al., 2008; BECCAGLIA et al., 2009). A produção de ROS é uma das principais causas dos danos celulares observados após a criopreservação (BILODEAU et al., 2000), a qual promove aumento da translocação da fosfatidilserina, alto nível de peróxido de hidrogênio intracelular e a fragmentação do DNA da célula espermática (KIM et al., 2010).

Dentre as diferentes etapas realizadas durante a criopreservação, foi detectada maior produção de radicais livres e perda da capacidade mitocondrial espermática após o período de equilíbrio do sêmen canino na presença de crioprotetor (glicerol) (LUCIO et al., 2016a). O crioprotetor promove estresse osmótico, o qual altera o volume celular e o metabolismo bioenergético do espermatozoide, com consequente redução da capacidade mitocondrial e aumento da formação de radicais livres. Tais alterações reduzem a motilidade espermática e geram peroxidação lipídica da membrana celular (BAILEY et al., 2000).

Após a descongelação do sêmen canino, os espermatozoides apresentam queda da motilidade, baixa resistência ao estresse oxidativo, aumento da

peroxidação lipídica e danos às membranas plasmática e do acrossomo, quando comparada aos processos de refrigeração e de equilíbrio (LUCIO et al., 2016a). Também foi observado aumento de espermatozoides com baixo potencial mitocondrial, o que pode contribuir para o aumento do estresse oxidativo e redução da motilidade espermática (LUCIO et al., 2016a).

Na tentativa de minimizar os efeitos deletérios das ROS às células espermáticas durante a criopreservação, a adição de antioxidantes aos meios de refrigeração/congelação de sêmen canino em diferentes concentrações foi testada, sendo obtidos resultados variáveis (BECCAGLIA et al., 2009; NEAGU et al., 2010; MICHAEL et al., 2010; LUCIO et al., 2016b).

3. CONSIDERAÇÕES FINAIS

Estudos relacionados ao perfil proteico do plasma seminal e das células espermáticas foram conduzidos a fim de determinar proteínas biomarcadoras da fertilidade masculina, ou aquelas que possam auxiliar no diagnóstico de alterações reprodutivas em diferentes espécies. No entanto, ainda há poucos relatos científicos relacionados à espécie canina.

A alta produção de ROS durante a criopreservação do sêmen também ocorre na espécie canina, o que pode prejudicar a qualidade espermática e a fertilidade. O melhor entendimento desse processo durante as biotecnologias do sêmen nesta espécie pode auxiliar a criação de protocolos de refrigeração/congelação de sêmen mais eficientes, que resultem em menos danos celulares devido à peroxidação lipídica.

Dentre as espécies domésticas, os cães apresentam uma grande variedade de raças e de padrões raciais, sem qualquer seleção ou melhoramento genético voltado à reprodução animal. Estas características estão intimamente relacionadas à conformação do trato reprodutivo e à composição do ejaculado, o que dificulta ainda mais o entendimento da fisiologia reprodutiva e justifica a realização de mais estudos em cães. Além disso, o cão é um excelente modelo biológico para a compreensão de processos reprodutivos de muitas outras espécies, como os

humanos e de animais selvagens. Além disso, a crescente utilização das biotecnologias reprodutivas em cães, de alto valor zootécnico, tem ressaltado a importância em se estudar esta espécie, na tentativa de incrementar os índices de fertilidade dentro de uma determinada raça.

4. REFERÊNCIAS*

AGARWAL, A.; HAMADA, A.; ESTEVES, S. C. Insight into oxidative stress in varicocele-associated male infertility: part 1. *Nature Reviews Urology*, v.9, p.678-690, 2012.

ANGRIMANI, D. S. R.; LOSANO, J. D. A.; LUCIO, C. F.; VEIGA, G. A. L.; PEREDA, M. C.; NICHI, M.; VANUCCHI, C. I. Role of residual cytoplasm on oxidative status during sperm maturation in dogs. *Animal Reproduction Science*, v.151, p.256-261, 2014a.

ANGRIMANI, D. S. R.; LUCIO, C. F.; VEIGA, G. A. L.; SILVA, L. C. G.; REGAZZI, F. M.; NICHI, M.; VANUCCHI, C. I. Sperm maturation in dogs: sperm profile and enzymatic antioxidant status in ejaculated and epididymal spermatozoa. *Andrologia*, v.46, p.814-819, 2014b.

AITKEN, R. J. Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Molecular Reproduction and Development*, v.84, p.1039-1052, 2017.

AITKEN, J. B.; NAUMOVSKI, N.; CURRY, B.; GRUPEN, C. G.; GIBB, Z.; AITKEN, R. J. Characterization of an L-amino acid oxidase in equine spermatozoa. *Biology of Reproduction*, v.92, n.5, p.125, 2015.

*ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. *NBR 6023*: informação e documentação – Referências – Elaboração. Rio de Janeiro, 2002. 24p.

AITKEN, R. J.; RYAN, A. L.; BAKER, M. A.; MCLAUGHLIN, E. A. Redox activity associated with the maturation and capacitation of mammalian spermatozoa. *Free Radical Biology and Medicine*, v.36, n.8, p.994-1010, 2004.

AITKEN, R.; PATERSON, M.; FISHER, H.; BUCKINGHAM, D.; VAN DUIN, M. Redox regulation of tyrosine phosphorylation in human spermatozoa and tis role in the control of human sperm function. *Journal of Cell Science*, v.108, p.2017-2025, 1995.

ALLEVA, R.; SCARAMUCCI, A.; MANTERO, F.; BOMPADRE, S.; LEONI, L.; LITARRU, G. The protective role of ubiquinol-10 against formation of lipid hydroperoxides in human seminal fluid. *Molecular Aspects of Medicine*, v.18, p.221-228, 1997.

ALONGE., S.; MELANDRI, M.; LEOCI, R.; LACALANDRA, G. M.; AIUDI, G. Canine prostate specific esterase (CPSE) as an useful biomarker in preventive screening programme of canine prostate: CPSE threshold value assessment and its correlation with ultrasonographic prostatic abnormalities in asymptomatic dogs. *Reproduction in Domestic Animals*, v.53, p.359-364, 2018.

AMARAL, A.; CASTILHO, J.; RAMALHO-SANTOS, J.; OLIVA, R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. *Human Reproduction Update*, v.20, n.1, p.40-62, 2014.

AMARAL, A.; LOURENÇO, B.; MARQUES, M.; SANTOS, J. R. Mitochondria functionality and sperm quality. *The Journal for the Society for Reproduction and Fertility*, p.1-34, 2013.

AONUMA, S.; OKABE, M.; KAWAGUCHI, M.; KISHI, Y. Zinc effects on mouse spermatozoa and *in vitro* fertilization. *Journal of Reproduction and Fertility*, v.63, p.463–466, 1981.

AQUINO-CORTEZ, A.; DA SILVA, L. D. M.; ARAÚJO, A. A.; DE MENEZES, E. S. B.; MOURA, A. A. A. N. Proteins of the canine seminal plasma. *Ciência Rural*, v.46, n.5, p.901-908, 2016.

AQUINO-CORTEZ, A.; PINHEIRO, B. Q.; LIMA, D. B. C.; SILVA, H. V. R.; MOTA-FILHO, A. C.; MARTINS, J. A. M.; RODRIGUEZ-VILLAMIL, P.; MOURA, A. A.; SILVA, L. D. M. Proteomic characterization of canine seminal plasma. *Theriogenology*, v.95, p.178-186, 2017.

ARVIDSON, G.; RONQUIST, G.; WIKANDER, G.; ÖJTEG, A. C. Human prostasome membranes exhibit very high cholesterol/ phospholipids ratios yielding high molecular ordering. *Biochimica et Biophysica Acta*, v.984, p.167–173, 1989.

ASHRAFZADEH, A.; NATHAN, S.; KARSANI, S. A. Comparative analysis of Mafriwal (Bos taurus x Bos indicus) and Kedah Kelantan (Bos indicus) sperm proteome identifies sperm proteins protentially responsible for higher fertility in a tropical climate. *International Journal of Molecular Science*, v.14, p.15860-15877, 2013.

ASLAM, M. K. M.; KUMARESAN, A.; RAJAK, S. K.; TAJMUL, M.; DATTA, T. K.; MOHANTY, T. K.; SRINIVASAN, A.; YADAV, S. Comparative proteomic analysis of taurine, indicine, and crossbred (Bos taurus x Bos indicus) bull spermatozoa for identification of proteins related to sperm malfunctions and subfertility in crossbred bulls. *Journal of Andrology*, v.27, n.2, p.201-11, 2006.

ASLAM, M. K. M.; KUMARESAN, A.; RAJAK, S. K.; TAIMUL, M.; DATTA, T. K.; MOHANTY, T. K.; SRINIVASAN, A.; YADAV, S. Comparative proteomic analysis of Taurine, Indicine, and crossbred (Bos taurus x Bos indicus) bull spermatozoa for identification of proteins related to sperm malfunctions and subfertility in crossbred bulls. *Theriogenology*, v.84, p.624-633, 2015.

ASSUMPÇÃO, T. I.; FONTES, W.; SOUSA, M. V.; RICARTI, C. A. O. Proteome analysis of nelore bull (*Bos taurus indicus*) seminal plasma. *Protein & Peptide*

Letters, v.12, p.813-817, 2005.

AX, R. L.; HAWKINS, H. E.; DENISE, S. K.; HOLM, T. R.; ZHANG, H. M. New developments in managing the bull. In: FIELDS; M. J.; SAND, R. S.; YELICH, J. V. *Factors Affecting Calf Crop: Biotechnology of Reproduction*. CRC Press LLC, Boca Raton, FL, USA. 2002, p.287-295.

AYAZ, A.; AGARWAL, A.; SHARMA, R.; KOTHANDARAMN, N.; CAKAR, Z.; SIKKA, S. Proteomic analysis of sperm proteins in infertile men with high levels of reactive oxygen species. *Andrologia*, v.50, p.1-10, 2018.

BAILEY, J. L.; BILODEAU, J. F.; CORNIER, N. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *Journal of Andrology*, v.21, p.1-7, 2000.

BAKER, M. A.; NAUMOVSKI, N.; HETHERINGTON, L.; WEINBERG, A.; VELKOV, T.; AITKEN, J. Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics*, v.13, p.61-74, 2013.

BARROS, T. B.; GUIMARÃES, D. B.; ARAUJO, L. R. S.; CANTANHÊDE, L. F.; FILHO, I. B. Q.; MOURA, A. A. A.; TONIOLLI, R. O estudo proteômico e o seu potencial de avaliação da fertilidade em machos de diferentes espécies domésticas. *Revista Brasileira de Reprodução Animal*, v.37, n.3, p.266-271, 2013.

BARTHELEMY, C.; KHALFOUN, B.; GUILLAUMIN, J. M.; LECOMTE, P.; BARDOS, P. Seminal fluid transferrin as an index of gonadal function in men. *Journal of Reproduction and Fertility*, v.82, p.113-118, 1988.

BARTLETT, J. D. Studies on dog semen II. Biochemical characteristics. *Journal of Reproduction and Fertility*, v.3, p.190-205, 1962.

BAUCHÉ, F.; FOUCHARD, M. H; JEGOU, B. Antioxidant system in rat testicular cells. *FEBS Lett*, v.349, p.392-396, 1994.

BECCAGLIA, M.; ANASTASI, P.; CHIGIONI, S.; LUVONI, G. C. Tris-Lecitin extender supplemented with antioxidant catalase for chilling of canine semen. *Reproduction in Domestic Animals*, v.44, n.2, p.345-349, 2009.

BELL, F. W.; KLAUSNER, J. S.; HAYDEN, D. W.; LUND, E. M.; LIEBENSTEIN, B. B.; FEENEY, D. A.; JOHNSTON, S. D.; SHIVERS, J. L.; EWING, C. M.; ISAACS, W. B. Evaluation of serum and seminal plasma markers in the diagnosis of canine prostatic disorders. *Journal of Veterinary Internal Medicine*, v.9, p.149–153, 1985.

BILODEAU, J. F.; CHATTERJEE, S.; SIRARD, M. A.; GAGNON, C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Molecular Reproduction and Development*, v.55, p.282-288, 2000.

BIRBEN, E., SAHINER, U. M., SACKESEN, C., ERZURUM, S., KALAYCI, O. Oxidative stress and antioxidant defense. *World Allergy Organization Journal*, v.5, p.9-19, 2012.

BISHT, S.; FAIQ, M.; TOLAHUNASE, M.; DADA, R. Oxidative stress and male infertility. *Nature Reviews of Urology*, v.14, p.470-485, 2017.

BJORNDHAL, L.; KVIST, U. Influence of seminal vesicular fluid on the zinc content of human spermatozoa chromatin. *International Journal of Andrology*, v.13, p.232–237, 1990.

BOVERIS, A.; CHANCE, B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochemical Journal*, v.134, p.707-716, 1973.

BRANDON, C. I.; HEUSNER, G. L.; CAUDLE, A. B.; FAYRER-HOSKEN, R. A. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. *Theriogenology*, v.52, p.863-873, 1999.

BRITO-NERY, L. T.; VAN TILBURG, M. F.; MENEZES, E. B.; MACHADO, V. P.; MOURA, A. A. A.; OLIVEIRA, E. C. S. Proteínas do plasma seminal canino: revisão. *Revista Brasileira de Reprodução Animal*, v.38, n.2, p.110-115. 2013.

BUCAK, M. N.; ATESSAHIN, A.; YUCE, A. Effect of antioxidants and oxidative stress parameters on ram semen after the freeze-thawing process. *Small Ruminant Research*, v.75, p.128-134, 2008.

BUCHMAN-SHAKED, O.; KRAIEM, Z.; GONEN, Y.; GOLDMAN, S. Presence of matrix metalloproteinases and tissue inhibitor of matrix metalloproteinase in human sperm. *Journal of Andrology*, v.23, p.702– 708, 2002.

CABALLERO, J.; FRENETTE, G.; SULLIVAN, R. Post testicular sperm maturational changes in the Bull: important role of the epididymosomes and prostasomes. *Veterinary Medicine International***,** p.1-13, 2011.

CANCEL, A. M.; CHAMPAN, D. A.; KILLIAN, G. J. Osteopontin localization in the Holstein bull reproductive tract. *Biology of Reproduction*, v.60, p.454-460, 1999.

CASSANI, P.; BECONI, M. T.; O'FLAHERTY, C. Relationship between total superoxide dismutase activity with lipid peroxidation, dynamics and morphological parameters in canine semen. *Animal Reproduction Science*, v.86, p.163-173, 2005.

CHAPDELAINE, P.; DUBÉ, J. Y.; FRENETTE, G.; TREMBLAY, R. R. Identification of arginine esterase as the major androgen-dependent protein secreted by dog prostate and preliminary molecular characterization in seminal plasma. *Journal of Andrology*, v.5, p.206-210, 1984.

CHEN, H.; CHOW, P. H.; CHENG, S. K.; CHEUNG, A. L.; CHENG, L. Y. Male genital tract antioxidant enzymes: their source, function in the female, and ability to preserve sperm DNA integrity in the golden hamster. *Journal of Andrology*, v.24, p.704-711, 2003.

CHIRINEA, V. H; SICHERLE, C. C.; LOPES, M. D. Congelamento de sêmen e sua eficiência na inseminação artificial de cães. *Revista Brasileira de Reprodução Animal*, v.37, n.2, p.164-168, 2013.

CORAZZA, M.; GUIDI, G.; ROMAGNOLI, S.; TOGNETTI, R.; BUONACCORSI; A. Serum total prostatic and non-prostatic acid phosphatase in healthy dogs and in dogs with prostatic diseases. *Journal of Small Animal Practice*, v.35, p.307-310, 1994.

DAR, M. R.; SINGH, M.; SHARMA, R.; THAKUR, S.; SHEIKH, A. A.; BHAT, A. B. Bovine fertility as regulated by sperm binding proteins: A review. *Asian Journal of Animal Veterinary Advances*, v.13, p.6-13, 2018.

DASKALOVA, D.; TSVETKOV, T.; LAZOV, K.; GRADINARSKA, D.; HRISTOVA, M.; IVANOVA, M. Canine seminal plasma – functions and interaction with capacitation. *Journal of Bioscience and Biotechonology*, p.19-24, 2017.

DAVALIEVA, K.; KIPRIJANOVSKA, S.; NOVESKI, P.; PLASESKI, T.; KOCEVSKA, B.; BROUSSARD, C.; PLASESKA-KARANFILSKA, D. Proteomic analysis of seminal plasma in men with different spermatogenic impairment. *Andrologia*, v.44, n.4, p.256-264, 2012.

DE LAMIRANDE, E.; GAGNON, C. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *International Journal of Andrology*, v.16, p. 21-25, 1993.

DE LAMIRANDE, E.; GAGNON, E. Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radical Biology & Medicine*, v.14, p.157-166, 1993.

DE LAMIRANDE, E.; JIANG. H.; ZINI, A.; KODAMA, H.; GAGNON, C. Reactive oxygen species and sperm physiology. *Reviews of Reproduction*, v.2, p.48-54, 1997.

DE LAMIRANDE E.; TSAI, C.; HARAKATA, A.; GAGNON, C. Involvement of reactive oxygen species in human sperm acrosome reaction induced by A23187 lysophosphatidylcholine and biological fluid ultrafiltrates. *Journal of Andrology*, v.19, p.585-594, 1998.

DENHARDT, D. T.; GUO, X. Osteopontin: a protein with diverse functions. *The FASEB Journal*, v.7, p.1475–1482, 1993.

DUBÉ, J. Y. Prostatic kallikreins: biochemistry and physiology. *Comparative Biochemistry and Physiology*, v.107, p.13-20, 1994.

DEPUYDT, C. E.; BOSMANS, E.; ZALATA, A.; SCHOONJANS, F.; COMHAIRE, F. H. The relation between reactive oxygen species and cytokines in andrological patients with or without male accessory gland infection. *Journal of Andrology*, v.17, p.699-707, 1996.

DESAI, N. R.; KESARÍ, K. K.; AGARWAL, A. Pathophysiological of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. *Reproductive Biology and Endocrionology*, v.7, p.2-9, 2009.

DINESH, V.; SHAMSI, M.; DADA, R. Supraphysiological free radical levels and their pathogenesis in male infertility. *Reproductive System and Sexual Disorders*, v.1, n.4, p.1-15, 2012.

EVANGELISTA, L. S. M.; FILHO M. A. C. S.; DE SOUZA, J. A. T. Inseminação Artificial Intravaginal em cadela da raça Dogo Argentino utilizando sêmen refrigerado: Relato de caso. *Publicações em Medicina Veterinária e Zootecnia*, v.10, n.3, p.244-247, 2016.

FAWCETT, D. W. Review article: The mammalian spermatozoon. *Developmental Biology*, v.44, p.394-436, 1975.

FISCHER, K. A.; VAN LEYEN, K.; LOVERCAMP, K. W.; MANANDHAR, G.; SUTOVSKY, M.; FENG, D.; SAFRANSKJ, T.; SUTOVSKY, P. 15-Lipoxygenase is a component of the mammalian sperm cytoplasmic droplet. *Reproduction*, v.130, p.213–222, 2005.

FRENETTE, G.; DUBÉ, J. Y.; TREMBLAY, R. R. Origin of Alkaline Phosphatase of Canine Seminal Plasma. *Archives of andrology*, v.16, p.235-41, 1986.

GADELHA, C. R. F.; VICENTE, W. R. R.; RIBEIRO, A. P. C.; APPARICIO, M.; COVIZZI, G. J.; CAMPOS, A. C. N. Prostatic acid phosphatase in serum and semen of dogs. *Archivos de Medicina Veterinaria*, v.45, p.321-325, 2013.

GARG, P.; VIJAY-KUMAR, M.; WANG, L.; GEWIRTZ, A. T.; MERLIN, D.; SITARAMAN, S. V. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, v.296, p.G175– G184, 2009.

GILBERT, R.; SUAREZ, S. S. Canine seminal plasma: Transport medium or signaling agent? *The Veterinary Journal*, v.195, p.131-132, 2013.

GILBOA, E.; ELKANA, Y.; RIGBI, M. Purification and properties of human acrosin. *European Journal of Biochemistry*, v.39, p.85–92, 1973.

GILMONT, R. R.; SENGER, P. L.; SYLVESTER, S. R.; GRISWOLD, M. D. Seminal transferrin and spermatogenic capability in the bull. *Biology of Reproduction*, v.43, p.151-157, 1990.

HADEN, N. P.; HICKOX, J. R.; WHISNANT, C. S.; HARDY, D. M. Systematic characterization of sperm-specific membrane proteins in swine. *Biology of Reproduction*, v.63, p.1839–1847, 2000.

HALIWELL, B. The antioxidant paradox. *Lancet*, v.355, p.1179-1180, 2000.

HATAMOTO, L. K.; SOBRINHO, B. C. A.; NICHI, M.; BARNABE, V. H.; BARNABE, R. C.; CORTADA, C. N. M. Effects of dexamethasone treatment (to mimic stress) and vitamin E oral supplementation on the spermiogram and on seminal plasma spontaneous lipid peroxidation and antioxidant enzyme activities in dogs. *Theriogenology*, v.66, p.1610-1644, 2006.

HENKEL, R. R. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian Journal of Andrology*, v.13, p.43-52, 2011.

HOLMES, S. D.; BUCCI, L. R.; LIPSHULTZ, L. I.; SMITH, R. G. Transferrin binds specifically to pachytene spermatocytes. *Endocrinology*, v.113, p.1916-1918, 1983.

HOLMES, S. D.; LIPSHULTZ, L. I.; SMITH, R. G. Regulation of transferrin secretion by human Sertoli cells cultures in the presence or absence of human peritubular cells. *The Journal of Clinical Endocrinology & Metabolism*, v.59, p.1058-1062, 1984.

HORI, T.; HAGIUDA, K.; KAWAKAMI, E.; TSUTSUI, T. Unilateral intrauterine insemination with prostatic fluid-sensitized frozen caudal epididymal sperm in beagle dogs. *Theriogenology*, v.63, p.1573-1583, 2004.

HUD, N. V.; ALLEN, M. J.; DOWNING, K. H.; LEE, J.; BALHORN, R. Identification of the elemental packing unit of DNA in mammalian sperm cells by atomic force microscopy. *Biochemical and Biophysical Research Communications*, v.193, p.1347-1354, 1993.

HULBOY, D. L.; RUDOLPH, L. A.; MATRISIAN, L. M. Matrix metalloproteinases as mediators of reproductive function. *Molecular Human Reproduction*, v.3, p.27-45, 1997.

IGUER-OUADA, M.; VERSTEGEN, J. P. Evaluation of the Hamilton Thorne computer based automated system for dog semen analysis. *Theriogenology*, v.55, p.733-749, 2001.

ISAACS, W. B.; COFFEY, D. S. The predominant protein of canine seminal plasma is an enzyme. *The Journal of Biological Chemistry*, v.259, n.18, p.11520-11526, 1984.

JONES, R.; MANN, T. Toxicity of exogenous fatty acid peroxides towards spermatozoa. *Journal of Reproduction and Fertility*, v.50, p.255–260, 1977.

JUNIEWICZ, P. E.; BARBOLT, T. A.; EGY, M. A.; FRENETTE, G.; DUBE, J. Y.; TREMBLAY, R. R. Effects of androgen and antiandrogen treatment on canine prostatic arginine esterase. *Prostate*, v.17, p.101-111, 1990.

KIKUCHI, M.; MIZOROKI, S.; KUBO, T.; OHIWA, Y.; KUBOTA, M.; YAMADA, N.; ORINO, K.; OHNAMI, Y.; WATANABE, K. Seminal plasma lactoferrin but not transferrin reflects gonadal function in dogs. *Journal of Veterinary Medical Science*, v.65, p.679-684, 2003.

KILLIAN, G. J.; CHAPMAN, D. A.; ROGOWSKI, L. A. Fertility-associated proteins in Holstein bull seminal plasma. *Biology of Reproduction*, v.49, p.1202-1207, 1993.

KOBAYASHI, T.; MIYAZAKI, T.; NATORI, M.; NOZAWA, S. Protective role of superoxide dismutase in human sperm motility: superoxide dismutase activity and lipid peroxide in human seminal plasma and spermatozoa. *Human Reproduction*, v.6, p. 987-991, 1991.

KOPPERS, A. J.; GARG, M. L.; AITKEN, R. J. Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radical Biology & Medicine*, v.48, p.112–119, 2010.

KRIEGER, J. N.; REIN, M. F. Canine prostatic secretions kill *Trichomonas vaginalis*. *Infection and immunity*, v.37, n.1, p.77-81, 1982.

KUMAR, V.; HASSAN, M. D. I.; KASHAV, T.; SINGH, T. P.; YADAV, S. Heparin-binding proteins of human seminal plasma: purification and characterization. *Molecular Reproduction and Development*, v.75, p.1767-1774, 2008.

KUTZLER, M. Semen collection in the dog. *Theriogenology*, v.64, p.747-754, 2005.

LEVY, X.; MIMOUNI, P. Hyperplasie benigne de la prostate: Actualites. *Le Point veterinaire*, v.293, p.39–43, 2009.

LEWIS, S. E.; STERLING E. S.; YOUNG, I. S.; THOMPSON, W. Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertility and Sterility*, v.67, p.142-147, 1997.

LINDSEY, M. L.; ESCOBAR, G. P.; DOBRUCKI, L. W.; GOSHORN, D. K.; BOUGES, S.; MINGOIA, J. T.; MCCLISTER JR., D. M.; SU, H.; GANNON, J.; MACGILLIVRAY, C.; LEE, R. T.; SINUSAS, A. J.; SPINALE, F. G. Matrix metalloproteinase-9 gene deletion facilitates angiogenesis after myocardial infarction. *American Journal of Physiology-Heart and Circulatory Physiology*, v.290, p.H232–H239, 2006.

LOBASCIO, A. M.; DE FELICI, M.; ANIBALDI, M.; GRECO, P.; MINASI, M. G.; GRECO, E. Involvement of seminal leukocytes, reactive oxygen species, and sperm mitochondrial of seminal leukocytes, reactive oxygen species, and sperm mitochondrial membrane potential in the DNA damage of the human spermatozoa. *Andrology*, v.3, p.165-170, 2015.

LUCHTEFELD, M.; DREXLER, H.; SCHIEFFER, B. 5-Lipoxygenase is involved in the angiotensin II-induced NAD(P)H-oxidase activation. *Biochemical and Biophysical Research Communications*, v.308, p.668-672, 2003.

LUCIO, C. F.; SILVA, L. C. G.; REGAZZI, F. M.; ANGRIMANI, D. S. R.; NICHI, M. ASSUMPÇÃO, M. E. O.; VANNUCCHI, C. I. Effect of reduced glutathione (GSH) in

canine sperm cryopreservation: In vitro and in vivo evaluation. *Cryobiology*, v.72, p.135-140, 2016a.

LUCIO, C. F.; REGAZZI, F. M.; SILVA, L. C.; ANGRIMANI, D. S.; NICHI, M.; VANNUCCHI CI. Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. *Theriogenology*, v.85, p.1568-75, 2016b.

MANDA, K.; UENO, M.; MORITAKE, T.; ANZAI, K. A-lipoic acid attenuates X-irradiation-induced oxidative stress in mice. *Cell Biology Toxicology*, v. 23, p.129-137, 2007.

MANN T.; LUTWAK-MANN C. Biochemistry of Seminal Plasma and Male Accessory Fluids; Application to Andrological Problems. In: *Male Reproductive Function and Semen***.** Springer, London, 1981. p.269-336.

MAKKER, K.; AGARWAL, A.; SHARMA, R. Oxidative stress & male infertility. *Indian Journal of Medical Research*, v.129, p.357-367, 2009.

METAYER, S.; DACHEUX, F.; DACHEUX, J. L.; GATTI, J. L. Comparison, characterization, and identification of proteases and protease inhibitors in epididymal fluids of domestic mammals. Matrix metalloproteinases are major fluid gelatinases. *Biology of Reproduction*, v.66, p.1219–1229, 2002.

MCENTEE, M.; ISAACS, W.; SMITH, C. Adenocarcinoma of the canine prostate: immunhistochemical examination for secretory antigens. *The Prostate*, v.11, n.2, p.163-170, 1987.

MICHAEL, A.; ALEXOPOULOS, C.; PONTIKI, E.; HADJIPAPAVLOU-LITINA, D. J.; SARATSIS, P.; BOSCOS, C. Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, v.68, p.204-212, 2007.

MICHAEL, A. J.; ALEXOPOULOS, C.; PONTIKI, E. A.; HADJIPAVLOU-LITINA, D. J.; SARATSIS, P.; VERVERIDIS, H. N.; BOSCOS, C. M. Effect of N-acetyl-L-cysteine supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Reproduction in Domestic Animals*, v.45, p.201-207, 2010.

MIESCHER, F. Das Protamin, eine neue organische Basis aus den Samenfäden des Rheinlachses. *Berichte der Deutschen Chemischen Gesellschaft*, v.7, p.376, 1874.

MILARDI, D.; GRANDE, G.; VINCENZONI, F.; MESSANA, I.; PONTECORVI, A.; DE MARINIS, L.; CASTAGNOLA, M.; MARANA, R. Proteomic approach in the identification of fertility pattern in seminal plasma of fertile men. *Fertility and Sterility*, v.97, p.67-73, 2012.

MILLER, D. J.; WINER, M. A.; AX, R. L. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. *Biology of Reproduction*, v.42, p.899-915, 1990.

MOGIELNICKA-BRZOZOWSKA, M.; KORDAN, W. Isolation and characterization of zinc-binding proteins of canine seminal plasma. *Polish Journal of Veterinary Sciences*, v.15, p.493-498, 2011.

MOGIELNICKA-BRZOZOWSKA, M.; KOWALSKA, N.; FRASER, L.; KORDAN, W. Proteomic characterization of zinc-binding proteins of canine seminal plasma. *Reproduction in Domestic Animals*, v.50, p.1017-21, 2015.

MOHRI, H. Amino-acid composition of ‘‘Tubulin’’ constituting microtubules of sperm flagella. *Nature*, v.217, p.1053-1054, 1968.

MOORE, A. I.; SQUIRES, E. L.; GRAHAM, J. K. Effect of seminal plasma on the cryopreservation of equine spermatozoa. *Theriogenology*, v.63, p.2372-2381, 2005.

MORIELLI, T.; O'FLAHERTY, C. Oxidative stress impairs function and increases redox protein modification in human spermatozoa. *Reproduction*, v.149, p.113-123, 2015.

MOURA, A. A.; HASAN, K.; DAVID, A. C.; GARY, J. K. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *Journal of Andrology*, v.27, n.2, p.201-211, 2005.

NEAGU, V. R.; GARCIA, B. M.; RODRIGUEZ, A. M.; FERRUSOLA, C. O.; BOLANOS, J. M.; FERNANDEZ, L. G.; TAPIA, J. A.; PENA, F. J. Determination of glutation peroxidase and superoxide dismutase activities in canine seminal plasma and tis realtion with sperm quality and lipid peroxidation post thaw. *Theriogenology*, v.75, p.10-16, 2011.

OCHSENDORF, F. R. Infections in the male genital tract and reactive oxygen species. *Human Reproduction Update*, v.5, p.399-420, 1999.

OLIVA, R.; DIXON, G. H. Vertebrate protamine genes and the histone-to-protamine replacement reaction. *Progress in Nucleic Acid Research and Molecular Biology*, v.40, p.25–94, 1991.

OLIVA, R.; MARTÍNEZ-HEREDIA, J.; ESTANYOL, J. M. Proteomics in the Study of the Sperm Cell Composition, Differentiation and Function, Systems. *Biology in Reproductive Medicine*, v.54, n.1, p.23-36, 2008.

OLIVEIRA, P. F.; TOMAS, G. D.; DIAS, T. R.; MARTINS, A. D.; RATO, L.; ALVES, M. G. SILVA, B. M. White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. *Reproductive Biomedicine Online*, v.31, p.544-556, 2015.

PAASCH, U.; GRUNEWALD, S.; AGARWAL, A.; GLANDERA, H. J. Activation pattern of caspases in human spermatozoa. *Fertility and Sterility*, v.81, p.802-809, 2004.

PENÃ, F. J.; NUÑEZ-MARTÍNEZ, I.; MORÁN, J. M. Semen Technologies in dog breeding: an update. *Reproduction in Domestic Animals*, v.41, n.2, p.21-29, 2006.

PLANTE, M.; DE LAMIRANDE, E.; GAGNON, C. Reactive oxygen species released by activated neuthrophils, but not by deficient spermatozoa, are sufficient to affect normal sperm motility. *Fertility and Sterility*, v.62, p.387-393, 1994.

REGO, J. P.; CRISP, J. M.; MOURA, A. A.; NOUWENS, A. S.; LI, Y.; VENUS, B.; CORBET, N. J.; CORBET, D. H.; BURNS, B. M.; BOE-HANSEN, G. B.; MCGOWAN, M. R. Seminal plasma proteome of electroejaculated *Bos indicus* bulls. *Animal Reproduction Science*, v.148, p.1-17, 2014.

RIPATHI, U. K.; ASLAM, M. K. M.; PANDEY, S.; NAYAK, S.; CHHILLAR, S.; SRINIVASAN, A.; MOHANTY, T. K.; KADAM, P. H.; CHAUHAN, M. S.; YADAV, S.; KUMARESAN, A. Differential proteomic profile of spermatogenic and Sertoli cells from peri-pubertal testes of three different bovine breeds. *Frontier in Cell and Developmental Biology*, v.2, p.1-9, 2014.

RIVLIN, J.; MENDEL, J.; RUBINSTEIN, S.; ETKOVITZ, N.; BREITBART, H. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biology of Reproduction*, v.70, p.518–522, 2004.

RODRIGUES, B. A.; DONÁ, A. V.; RODRIGUES, J. L. Influência da secreção prostática autóloga sobre a viabilidade de espermatozóides caninos no pós-descongelamento. *Brazilian Journal of Veterinary Research and Animal Science*, v.36, p.316-23, 1999.

RODRÍGUEZ-MARTÍNEZ, H.; KVIST, U.; ERNERUDH, J.; SANZ, L.; CALVETE, J. J. Seminal Plasma Proteins: What Role Do They Play? *American Journal of Reproductive Immunology*, v.66, n.1, p.11-22, 2011.

RONQUIST, G.; BRODY, I. The prostasomes: its secretion and function in man. *Biochimica et Biophysica Acta*, v.822, p.203-218, 1985.

ROTA, A.; STROM, B.; LINDE-FORSBERG, C. Effects of seminal plasma and three extenders on canine semen stored at 4°C. *Theriogenology*, v.44, p.885-900, 1995.

SAENGSOI, W.; WEI-YAU, S.; SHYU, C-L.; WUB, J-T; WARINRAK, C.; LEE, W-M; CHEGN, F-P. Detection of matrix metalloproteinase (MMP)-2 and MMP-9 in canine seminal plasma. *Animal Reproduction Science*, v.127, p.114-119, 2011.

SAEZ, F.; FRENETTE, G.; SULLIVAN, R. Epididymosomes and prostasomes: their roles in post-testicular maturation of the sperm cells. *Journal of Andrology*, v.24, p.149–154, 2003.

SALAMONSEN, L. A.; WOLLEY, D. E. Matrix metalloproteinases in normal menstruation. *Human of Reproduction*, v.2, p.124-133, 1996.

SALEH, R. A.; AGARWAL, A.; KANDIRALI, E.; SHARMA, R. K.; THOMAS, A. J.; NADA, E. A; EVENSON, D. P.; ALVAREZ, J. G. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertility and Sterility*, v.78, p.215-224, 2002.

SAWYER, D. E.; MERCER, B. G.; WIKLENDT, A. M.; AITKEN, R. J. Analysis of gene-specific DNA damage and single-strand DNA breaks induced by pro-oxidant treatment of human spermatozoa in vitro. *Mutation Research*, v.529, p.21–34, 2003.

SHAMAN, J. A.; YAMAUCHI, Y.; WARD, W. S. Function of the Sperm Nuclear Matrix. *Archives of Andrology*, v.53, n.3, p.135-140, 2007.

SIES, H. Strategies of antioxidant defense. *European Journal of Biochemistry*, v. 215, p. 213-219, 1993.

SIRIVAIDYAPONG, S.; URSEM, P.; BEVERS, M. M.; COLENBRANDER, B. Effect of prostatic fluid on motility, viability and acrosome integrity of chilled and frozen-thawed dog spermatozoa. *Journal of Reproduction and Fertility Supplement*, v.57, p.383-386, 2001.

SMITH, T. B.; DUN, M. D.; SMITH, N. D.; CURRY, B. J.; CONNAUGHTON, H. S.; AITKEN, R. J. The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *Journal of Cell Science*, v.126, p.1488-1497, 2013.

SOUZA, F. F.; TONIOLLO, G. H. Marcadores de tecido prostático no cão. *Revista de Educação Continuada CRMV-SP*, v.4, f.3, p.63-70, 2001.

SOUZA, F. F.; MARTINS, M. I. M.; FERNANDES, C. E. S.; RIBOLLA, P. E. M.; LOPES, M. D. Heparin-binding proteins of canine seminal plasma. *Theriognelogy*, v.66, p.1606-1609, 2006a.

SOUZA, F. F.; MARTINS, M. I. M.; LOPES, M. D. Vasectomy effect on canine seminal plasma biochemical components and their correlation with seminal parameters. *Theriogenology*, v.66, p.1621-25, 2006b.

SOUZA, F. F. Proteínas do sêmen do cão são importantes ou não na fertilização? *Revista Brasileira de Reprodução Animal*, v.13, n.1, p.108-114, 2007a.

SOUZA, F. F.; BARRETO, C. S.; LOPES, M. D. Characteristics of seminal plasma proteins and their correlation with canine semen analysis. *Theriogenology*, v.68, p.100-106, 2007b.

SOUZA, F. F.; CHIRINEA, V. H.; MARTINS, M. I. M.; LOPES, M. D. Osteopontin in seminal plasma and sperm membrane of dogs. *Reproduction in Domestic Animals*, v.44, n.2, p.283-286, 2009.

STERNLICHT, M. D.; WERB, Z. How matrix metalloproteinases regulate cell behavior. *Annual Review of Cell and Developmental Biology*, v.17, p.463–516, 2001.

STRZEZEK, R.; JANOWSKY, T. Enzymatic markers of canine prostate. *Medycyna Weterynaryina*, v.59, p.6-9, 2003.

STRZEZEK, R.; KOZIOROWSKA-GILUN, M.; KOWALOWKA, M.; STRZEZEK, J. Characteristics of antioxidant system in dog semen. *Polish Journal of Veterinary Science*, v.12, p.55-60, 2009.

STRZEZEK, R.; SZEMPLINSKA, K.; FILIPOWICZ, K.; KORDAN, W. Semen characteristics and selected biochemical markers of canine seminal plasma in various seasons of the year. *Polish Journal of Veterinary Sciences*, v.18., n.1, p.13-18, 2015.

SYLVESTER, S. R.; GRISWOLD, M. D. The testicular iron shuttle: a “nurse” function of the Sertoli cells. *Journal of Andrology*, v.15, p.381-385, 1994.

TAVILANI, H.; GOODARZI, M. T.; DOOSTI, M.; VAISI-RAYGANI, A.; HASSANZADEH, T.; SALIMI, S. ET AL. Relationship between seminal antioxidant enzymes and the phospholipid and fatty acid composition of spermatozoa. *Reproductive Biomedicine Online*, v.16, p.649-656, 2008.

THIPPESWAMY, V. B.; LAYEK, S. S.; KUMARESAN, A.; MOHANTY, T. K.; GUPTA, A. K.; CHAKRAVARTY, A. K. MANIMARAN, A.; PRASAD, S. Effects of pedigree and exotic genetic inheritance on semen production traits of dairy bulls. *Asian Pacific Journal of Reproduction*, v.3, p.13-17, 2014.

TILIGADA, E. Chemotherapy: induction of stress responses. *Endocrine-Related Cancer*, v.13, p.115-124, 2006.

TOSIC, J; WALTON, A. Metabolism of spermatozoa. The formation and elimination of hydrogen peroxide by spermatozoa and effects on motility and survival. *Biochemical Journal*, v.47, p.199-212, 1950.

TREMELLEN, K. Oxidative stress and male infertility – a clinical perspective. *Human Reproduction Update*, v.14, p.243-58, 2008.

TRIPATHI, U. K.; MUHAMMAD ASLAM M. K.; PANDEY, S.; NAYAK, S.; CHHILLAR, S.; SRINIVASAN, A.; MOHANTY, T. K.; KADAM, P. H.; CHAUHAN, M. S.; YADAV, S.; KUMARESAN, A. Differential proteomic profile of spermatogenic and Sertoli cells from peripubertal testes of three different bovine breeds. *Frontier in Cell and Developmental Biology*, v.2, p.2-9, 2014.

UCHOA, D. C. *Água de coco em pó (ACP106c) como diluente para conservação de sêmen e inseminação artificial na espécie canina***.** Tese (Doutorado) Renorbio - Universidade Estadual do Ceará, Fortaleza. 2011. 207p.

UPRETI, G. C.; JENSEN, K.; MUNDAY, R.; DUGANZICH, D. M.; VISHWANATH, R.; SMITH, J. F. Studies on aromatic amino acid oxidase activity in ram spermatozoa: Role of pyruvate as an antioxidant. *Animal Reproduction Science*, v.51, p.275–287, 1998.

VARILOVÁ, T., SEMÉNKOVA, H., HORAK, P., MADERA, M., PACAKOVA, V., TICHA, M., STULIK, K. Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins. *Journal of Separation Science*, v.29, n.8, p.1110-1115, 2006.

VIEIRA, N. M. G.; LOSANO, J. D. A.; ANGRIMANI, D. S. R.; KAWAI, G. K. V.; BICUDO, L. C.; RUI, B. R.; DA SILVA, B. C. S.; ASSUMPÇÃO, M. E. O. D.; NICHI, M. Induced sperm oxidative stress in dogs: Susceptibility against different reactive oxygen species and protective role of seminal plasma. *Theriogenology*, v.108, p.39-45, 2018.

WAGNER, H.; CHENG, J. W.; KO, E. Y. Role of reactive oxygen species in male infertility: An updated review of literature**.** *Arab Journal of Urology*, v.16, p.35-43, 2018.

WARINRAK, C.; WU, J-T.; HSU, W-L.; LIAO, J-W.; CHANG, S-C.; CHENG, F-P. Expression of matrix metalloproteinases (MMP-2, MMP-9) and their inhibitors

(TIMP-1, TIMP-2) in canine testis, epididymis and semen. *Reproduction in Domestic Animals*, v.50, p.48-57, 2015.

WATSON, P. F. The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, v.60-61, p.481-492, 2000.

WEBER, D; MILKOVIC, L.; BENNETT, S. J.; GRIFFITHS, H. R.; ZARKOVIC, N.; GRUNE, T. Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies. *Redox Biology*, v. 1, p. 226-233, 2013.

WITTAYARAT, M.; ITO A.; KIMURA, T.; NAMULA, Z.; LUU, V. V.; DO, L. T. K.; SATO, Y.; TANIGUCHI, M.; OTOI, T. Effects of green tea polyphenol on the quality of canine semen after long-term storage at 5 ºC. *Reproductive Biology*, v.13, p.251-254, 2013.

WOLF, K., KAYACELEBI, H., URHAUSEN, C., PIECHOTTA, M., MISCHKE, R., KRAMER, S.; EINSPANIEER, A.; GÜENZEL-APEL, A. Testicular steroids, prolactin, relaxin and prostate gland markers in peripheral blood and seminal plasma of normal dogs and dogs with prostatic hyperplasia. *Reproduction in Domestic Animals*, v.47, n.6, p.243–246, 2012.

WRIGHT, C.; MILNE, S.; LEESON, H. Sperm DNA damage caused by oxidative stress: modifiable clinical, lifestyle and nutritional factors in male infertility. *Reproductive Biomedicine Online*, v.28, p.684-703, 2014.

YAMASHIRO, H.; NARITA, K.; SUGIMURA, S.; SUGAWARA, A.; HOSHINO, Y.; SAKURAI, M.; YOKOO, M.; KONNO, T.; YOSHIDA, M.; SATO, E. Influence of the prostatic fluid from the first and third fractions of the ejaculates on the cryosurvival of poodle dog sperm. *American Journal of Animal Veterinary Science*, v.4, p.14-20, 2009.

YEUNG, C.; COOPER, T.; DE GEYTER, M.; DE GEYTER C.; ROLF, C.; KAMISCHKE, A.; NIESCHLAG, E. Studies on the origin of redox enzymes in seminal plasma and their relationship with results of in-vitro fertilization. *Molecular Human Reproduction*, v.4, p.835-839, 1998.

YIN, H.Z., VOGEL, M.M., SCHNEIDER, M., ERCOLE, C., ZHANG, G., SINHA, A.A., WILSON, M.J. Gelatinolytic proteinase activities in human seminal plasma. *Journal of Reproduction and Fertility*, v.88, p.491–501, 1990.

ZAGO, M. P.; OTEIZA, P. I.; The antioxidant properties of zinc: interactions with iron and antioxidants. *Free Radical Biology and Medicine*, v.31, p.266-274, 2001.

ZELLI, R.; BELLEZZA, I.; RAMBOTTI, M. G.; MINELLI, A.; POLISCA, A. Ultrastructural and Enzymatic Activity of Membranous Vesicles Isolated from Canine Seminal Plasma. *Reproduction in Domestic Animals*, v.48, p.252–257, 2013.

ZHANG W.; XIAO, S.; AHN, D. U. Protein oxidation: Basic principles and implications for meat quality. *Critical Reviews in Food Science and Nutrition*, v.53, p.1191-1201, 2013.

HIPÓTESES

1) A raça influencia a composição proteica do plasma seminal e dos espermatozoides de cães.

2) A retirada do plasma seminal é benéfica para a refrigeração do sêmen de cães.

3) O plasma seminal altera a produção de espécies reativas ao oxigênio e de enzimas antioxidantes durante a refrigeração do sêmen.

OBJETIVOS

Objetivo Geral

1) Verificar se a raça canina interfere na composição proteica do plasma seminal e das células espermáticas.

2) Avaliar a viabilidade espermática de cães no sêmen refrigerado com ou sem plasma seminal.

3) Correlacionar as proteínas encontradas no plasma seminal e nas células espermáticas com a refrigeração do sêmen.

Objetivos Específicos

- Identificar o perfil proteico do plasma seminal e das células espermáticas de cães de diferentes raças estudadas;
- Identificar marcadores proteicos relacionados com as características espermáticas em cães;
- Quantificar no plasma seminal a produção de espécies reativas ao oxigênio e de enzimas antioxidantes durante a refrigeração do sêmen canino;
- Avaliar a viabilidade espermática de cães no sêmen refrigerado com ou sem plasma seminal.

CAPÍTULO 2

Artigo redigido de acordo com as normas da revista *Biology of Reproduction*, com exceção da numeração das páginas e da disposição das figuras e dados suplementares.

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Insights into the influence of canine breed on proteomic profile of the spermatozoa and seminal plasma

Running title: Proteomic of semen from different purebred dogs

Summary Sentence: Some canine breeds exhibit different proteomic profile of seminal plasma and spermatozoa proteins.

Key words: fertility, proteomics, mass spectrometry, semen, dog

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Abstract

It is known that cattle of different breeds exhibit different protein pattern in the ejaculate that may influence fertility. Is it the same with canine species? This study verified the protein expression of seminal plasma and spermatozoa of four different purebred dogs (Golden Retriever, Great Dane, Bernese Mountain Dog and Maremmano-Abruzzese Sheepdog). The sperm-rich fraction and a portion of prostatic fluid were collected by digital manipulation. Semen samples were evaluated for motility (subjective and by computer-assisted sperm analysis); vitality and morphology (eosin/nigrosine stain). Mitochondrial potential, plasma and acrosomal membranes of spermatozoa were evaluated by flow cytometry. The spermatozoa and seminal plasma were separated by centrifugation and prepared individually for proteomic analysis by Q-Tof mass spectrometer. We described all spermatozoa and seminal plasma proteins found and their respective genic ontology. According to cluster formation of spermatozoa proteins, high correlation was observed among Golden Retriever and *Maremmano-Abruzzese Sheepdog*, and among Great Dane and Bernese Mountain Dog. Bernese Mountain Dog, Great Dane and Maremmano-Abruzzese Sheepdog showed high correlation in cluster formation of seminal plasma proteins. These correlations may be possibly related to genic proximity among evaluated breeds. Further studies focused on semen proteins of different

purebred dogs need to be performed to improve biotechnologies of reproduction in this species.

Introduction

In the late 1800's clubs were performed to propagate specific types of dogs and competitions were developed to reward the creation of perfect specimens of breed based on physical criteria [1]. Ever since many new breeds were developed, being currently recognized over 340 breeds [www.akc.org].

Purebred domestic dogs were created through backcrossing and inbreeding to fix desired traits, which is still a routinely practice in canine reproduction in order to fix desirable phenotypic or behavioral characteristics, although it may influence semen quality and impair biotechnologies of reproduction results [2-4].

The semen quality is highly correlated with the fertilizing capacity of the spermatozoa, which is influenced by several factors including integrity of sperm plasma and acrosome membranes, motility, morphology, mitochondrial potential and the presence of proteins in the ejaculate [5-7]. Some proteins have functions related to sperm quality and fertility [8-12] and may be potential biomarkers candidates for fertility (i.e. heparin binding proteins) and reproductive diseases (i.e. canine prostate-specific esterase and acid alkaline phosphatase). However, further studies on the composition of canine seminal plasma and its specific biological function are necessaries [13].

Few studies were conducted about proteomic of canine semen and almost them was done using seminal pool of different canine breeds, mixed breed or not described breeds, disregarding the difference that might exist among them [9,10,12,14-18]. In cattle, differences on protein profile of semen that are important for reproduction were observed

among *B. taurus, Bos indicus* and crossbred bulls (*B. taurus* x *B. indicus*) [7,19]. However, this difference has not yet been demonstrated in canine species.

To better understand and contribute to improve the biotechnologies of reproduction in dogs, humans and some endangered wild-species in which dogs are an experimental model, this study aimed to describe and evaluate the difference of four canine breeds (Golden Retriever, Great Dane, Bernese Mountain Dog and Maremmano-Abruzzese Sheepdog) on proteomic of seminal plasma and spermatozoa.

Materials and Methods

Ethical aspects

This study was approved by the Ethical Committee for the Use of Animals in Research of the FMVZ-UNESP with the permit number: 0007/2017 – CEUA.

Reagents

All reagents used were of high purity and purchased from Sigma Aldrich Sigma-Aldrich (St. Louis, MO, USA), Waters Corp. (Barueri, São Paulo, Brazil), GE Healthcare Life Sciences (São Paulo, São Paulo, Brazil), Merck S.A. (São Paulo, São Paulo, Brazil) and Thermo Fisher Scientific (São Paulo, São Paulo, Brazil), otherwise will be stated.

Semen collection and sample preparation

Ejaculates of 13 healthy purebred dogs with ages between 1 and 5 years, from different kennels were used in this study. Animals were divided according to breed, Golden Retriever (n = 3), Bernese Mountain Dog (n = 4), Great Dane (n = 3), and *Maremmano-Abruzzese Sheepdog* (n = 3). Entire second fraction and a portion of the third semen fraction were collected into a silicone funnel attached to a graduated plastic tube by manual stimulation of the penis in the presence of a teaser bitch.

In the kennel a 10 µL of semen were subjectively evaluated on pre-warmed glass slide (37º C) covered with a glass coverslip under light microscope (100X magnification) for motility (0-100%) and vigor (0-5). Sperm morphology and vitality were evaluated with a semen smear (5 µL) stained with eosin/nigrosine (5 µL) (BotuVital®, Botupharma, Botucatu, Brazil) and observed 200 spermatozoa under a light microscope at 1,000X magnification, in which the spermatozoa stained in red were considered dead, and those not stained as live. Sperm morphology was classified in major and minor defects according to Bloom et al. [12].

An aliquot of 50 x 10^6 spermatozoa from each ejaculate was diluted in 1 mL of TRIS-egg yolk-glucose extender [198 mM TRIS, 67 mM citric acid, 45 mM glucose, 20% yolk egg, 0.2 mg/mL amikacin sulphate and 1% Orvus WA Paste® (Procter & Gamble, Cincinnati, Ohio, USA), pH 6,8 and osmolarity 300 mOsmol/L] was chilled at 5º C in a foam box (Botuflex®, Botupharma, Botucatu, São Paulo, Brazil) and transported to the laboratory for computer-assisted sperm analysis of total and progressive motility parameters (CASA, Hamilthon-Thorne Computer-Aided Semen Analyzer, 10 Ivos version, HTR-IVOS 10 Analyzer, Hamilthon Thorne Research, Beverly, MA, USA) according to setup from Center of Study in Animal Reproduction (CERAN, FMVZ, UNESP, Botucatu, São Paulo, Brazil). For this, 10 µL of semen sample was placed into a pre-warmed chamber (Makler, chamber depth 10.0 µm, stage position 14.3 mm, Hamilton Thorne Research, Beverly, MA, USA) at 37º C. A minimum of 2,000 sperm cells were analyzed.

Flow cytometry assay

Flow cytometry evaluation of plasma and acrosome sperm membranes integrity and mitochondrial potential were assessed (Fortessa LSR BD, Becton Dickinson, Mountain View, CA, USA; equipped with 488nm, 100mW blue lasers; 640nm, 40mW

red and 405nm violet). For this study, the excitation used was 488nm and the emission filters used were 695/40nm (MitoSOXTM Red and propidium iodide), 530/30nm (SYTOX Green FITC-PSA and DHR). For excitation at 640nm, the emission filter was 660/20nm (MitoSOXTM Red) and for excitation at 405nm, the emission filter was 450/50nm (Hoechst 33342). At least 10,000 cells per sample were analyzed and the data were evaluated by software from the same manufacturer (BD FACSDiva ™ software v 6.1, Becton Dickinson, Mountain View, CA, USA).

For plasma and acrosome membranes integrity evaluation the association of Hoechst 33342, propidium iodide and FITC-PSA (*Pisum sativum agglutinin* conjugated to fluorescein isothiocyanate) were used according to Freitas-Dell'aqua et al. [21]. Thus, in a sample of 200 µL of semen diluted in TALP-PVA at the concentration of 5 x 10^6 spermatozoa/mL were added 7 µM of Hoechst, 1.5 µM of propidium iodide and 2187 ng/mL of FITC-PSA. Sample was incubated for 5 min at 37º C, protected from light. At the end of this period, the sample was evaluated by flow cytometry. Only spermatozoa with integrity plasma and acrosome membranes were considered as normal.

For evaluation of mitochondrial potential an association of Hoechst 33342, SYTOX Green (cell marking with plasma membrane damage, catalog nº S34860, Thermo Scientific, São Paulo, São Paulo, Brazil), MitoStatus Red (cell marking with mitochondrial potential, catalog nº 564697, BD Biosciences, São Paulo, São Paulo, Brazil) and MitoSOXTM Red (cell marking with generation of superoxide anion in the mitochondrial matrix, M36008, Thermo Scientific, São Paulo, São Paulo, Brazil) were used according to Freitas-Dell'Aqua et al. [22]. A semen sample of 200 µL was added TALP-PVA in a final concentration of 5 x 10^6 spermatozoa/mL, and 7 µM Hoechst, 0.05 µM SYTOX Green, 20 µM MitoStatus Red and 2 µM MitoSOXTM Red, and incubated

111 at 37º C for 15 min. Spermatozoa were classified into two groups, normal cells and cells
112 with high mitochondrial potential measured according to fluorescence intensity.

113 **Protein analysis**

114 After semen collection and analysis, the seminal plasma was separated from sperm
115 cells by centrifugation at 800g for 10 min and seminal plasma (supernatant) was frozen
116 at -20º C until protein analysis. Spermatozoa (pellet) was washed three times with a buffer
117 (50 mmol TRIS pH 7.2) containing protease inhibitors (0.8 mmol EDTA, 1.0 µg/mL
118 aprotinin, 1.0 µg/mL leupeptin e 35.0 µg/mL phenylmethylsulfonil fluoride - PMSF) and
119 maintained at -20ºC until protein extraction.

120 Seminal plasma (n = 16) was frozen at ice bath, recentrifuged at 10,000g for 30
121 min at 4º C. A protein solubilization buffer (150 mM NaCl 1% Triton X-100, 1% sodium
122 deoxycholate, 0.1% SDS) [23], was added to spermatozoa samples (n =13, except Belgian
123 Malinois dogs) at a final concentration of 200 x 10^6 spermatozoa/mL.

124 Spermatozoa were sonicated according to Baker et al. [24] and modified by Souza
125 et al. [10]. A 3.0 mm probe was used and 20% amplitude for 30 s, in ice bath, performed
126 in 10 series, with 1 min arrest between series. After sonication, the samples were
127 centrifuged at 10,000 g at 4º C for 30 min.

128 Total proteins concentration was determined in duplicate by A280 method
129 spectrophotometer (NanoDrop A280, Thermo Scientific NanoDrop One Microvolume
130 UV-VIS Spectrophotometers, Waltham, Massachusetts, EUA).

131 In-solution digestion of seminal plasma proteins was performed according to
132 Codognoto et al. [25].

133 SDS-PAGE was used to remove extraction buffer components from spermatozoa
134 samples which may compromise the nanochromatography column. A total of 50 µg
135 protein was used in 12% separation gel at a vertical mini-gel (Hoefer MiniVE Vertical

Electrophoresis System, GE HealthCare, São Paulo, SP, Brasil). The run was stopped as the sample reached the separation gel, in which a unique band was formed. Then, the gel was fixed in a solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid for 60 min and stained with colloidal coomassie blue [26-28]. Gel was maintained under distilled water until background whitening and then stained protein band was cut off. Bands were distained with 50% (v/v) methanol, 2.5% (v/v) acetic acid in ultra-pure water for 1 h, at room temperature. Solution was removed and added again. This procedure was repeated for more three times. Distain solution was removed, and acetonitrile 100% (v/v) was added for 5 min. Acetonitrile was removed, and this procedure was repeated once. Remaining acetonitrile was evaporated in a vacuum concentrator for 2 to 3 min. Then, 10 mM DTT in 100 mM ammonium bicarbonate were added and incubate for 30 min at room temperature. A fast spin was performed to remove DTT solution and 50 mM IAA in 100 mM ammonium bicarbonate was added and protected from light for 30 min. A fast spin was performed to remove IAA solution.

Gel fragments were washed with 100 mM ammonium bicarbonate for 10 min and the solution was removed. Gel dehydration was done with acetonitrile 100% (v/v), incubated for 5 min at room temperature and removed. The gels were rehydrated with 100 mM ammonium bicarbonate for 10 min. A fast spin was done to remove this solution and the gels were dehydrated again with acetonitrile as previously described for two more times. Remaining solution was evaporated in a vacuum concentrator for 2 to 3 min and 30 to 50 µL of trypsin solution (20 µL trypsin in 1,000 µL of cold 50 mM ammonium bicarbonate, at a final concentration of 20 ng/µL of trypsin) was added and the gels were rehydrated in an ice bath for 30 min. A fast spin was performed to remove the exceed trypsin solution and 5 to 20 µL of 50 mM ammonium bicarbonate were added until cover the gels, at 37º C, overnight. Then, 10 µL of 5% formic acid (v/v) in ultra-pure water was

added and incubated at room temperature for 10 min, make a fast spin and save the supernatant in another tube. A 12 µL of 5% formic acid (v/v) in 5% acetonitrile (v/v) were added until cover the gels, incubated for 10 min at room temperature. A fast spin was done, and the supernatant was deposited at the same tube contained the previously supernatant. This last step was repeated. The sample was dried until obtain approximately 1 µL and storage at -20º C for mass spectrometry.

For mass spectrometry, the samples were thawed, diluted in 0.1% formic acid in the proportion of 0.7 µg protein/µL, homogenized in a tube shaker and centrifuged at 1,100g for 5 min. Next, 20 µL of the supernatant was deposited in specific tubes for analysis in the mass spectrometer (Clear glass 12 x 32 mm screw neck total recovery vial with lid, Waters Corporation, Milford, MA, USA).

For protein analysis by mass spectrometry, 4.5 µL aliquot resulting from peptide digestion was separated by a C18 (100 µm x 100 mm) RP-nano UPLC column (Waters nanoACQUITY UPLC, Waters Corporation, Milford, MA, USA) coupled to the Q-Tof mass spectrometer (Micromass® Q-Tof PREMIER Mass Spectrometer, Waters Corporation, Milford, MA, USA) with a nanoelectrospray source at a flow rate of 0.600 µL/min. The samples were evaluated in duplicate. A gradient of 2 to 90% acetonitrile in 0.1% formic acid was maintained for 45 min. The voltage of the nanoelectrospray was maintained at 3.5 kV, cone voltage of 30 V and source temperature of 100 µC. The instrument was operated in top three mode in which a mass spectrum (MS) is acquired followed by MS/MS of the three most intense peaks detected. After MS/MS fragmentation, the ion was maintained in the exclusion list for 60 s. For endogenous cleavage peptides analysis, a real exclusion time was used.

Spectra were acquired using the MassLynx™ software v.4.1 (Waters Corporation, Milford, MA, USA) and the raw data files were converted to a peak list format (.mgf,

186 mascot generic format) without adding the scans and searched against
187 UniprotSProt_012015 (http://www.uniprot.org/) *Mammalia* taxonomy database, using
188 the Mascot tool 2.3.02 *version* and Mascot Distiller MDRO 2.4.0.0 *version* (Matrix
189 Science Inc, Boston, MA, USA). Relative quantification of each protein in the mixture
190 was determined by exponentially modified protein abundance index (emPAI), obtained
191 from Mascot Distiller software [29].

192 Search parameters included trypsin as protease, with a maximum of 1 cleavage
193 lost; carbamidomethylation of cysteine as fixed modification and methionine oxidation
194 as a variable modification. Tolerance of 0.1 Da for both precursor (MS) and fragment
195 (MS/MS) of ions and monoisotopic molecular mass was used.

Data analysis

196 Variables were submitted to normality test of Shapiro Wilk. Total subjective
197 motility, vigor; computer-assisted sperm analysis of total and progressive motility;
198 normal sperm, minor and major defects, sperm vitality, plasma and acrosome membranes
199 integrity, mitochondrial potential and total protein from spermatozoa and seminal plasma
200 were evaluated by one-way ANOVA. All pairwise multiple comparison procedures were
201 performed using Tukey test. Data were analyzed using the statistical software
202 SigmaPlot® 11.0. Results were reported as mean ± standard error and statistically
203 significant level of $P \leq 0.05$ was considered.

204 MetaboAnalyst 3.0 software [30] was used to perform proteomic statistical
205 analysis. Initially, data were normalized by the sum of protein emPAI of each canine
206 breed. This value was divided by the total protein sum, and the result was used for the
207 statistical analysis. Multivariate analysis was performed by the same software, using
208 principal component analysis (PCA) and the partial least squares-discriminant analysis

(PLS-DA). PCA describes the sample variations between groups (canine breeds), summarizing data into much fewer variables described as scores, which are weighted average of the original variables. PLS-DA indicates the protein relevance in the characterization of the groups, indicating the importance of a classification and calculates the variables in the projection score (VIP score). Important proteins were considered as VIP score was $\alpha \geq 1.0$ [31]. Group division was performed by a hierarchical clustering, using data provided by the user. Each sample begins as a separate cluster and the algorithm proceeds to combine them until all samples belong to one cluster [30].

After proteins identification, Venn diagram of spermatozoa and seminal plasma proteins was made online at http://bioinformatics.psb.ugent.be/webtools/Venn/. The annotation of the gene ontology of the proteins was obtained in the UniprotKB (www.uniprot.org) [32] using molecular function, biological process and cellular component categories. Figures on gene ontology were assembled in the *Panther version* 10 (http://www.pantherdb.org) [33].

Results

No statistical difference was observed for percentage of subjective motility (89.38 ± 0.63), vigor (4.60 ± 0.15), computer-assisted sperm analysis of progressive (70.26 ± 1.10) and total motility (85.69 ± 1.11), normal sperm (85.95 ± 1.94), minor (7.77 ± 1.28) and major defects (6.90 ± 0.77); vitality by eosin/nigrosine stain (98.04 ± 0.99); flow cytometry parameters of plasma and acrosome membrane integrity (80.49 ± 1.74), and mitochondrial potential (80.21 ± 2.01) among breeds.

There was no difference among breeds for total protein (mean ± standard error) concentration from spermatozoa (2.86 ± 0.37 g/dL; 2.41 ± 0.02 g/dL; 2.54 ± 0.13 g/dL; 2.91 ± 0.28 g/dL) and seminal plasma (3.19 ± 1.29 g/dL, 3.37 ± 0.43 g/dL, 3.70 ± 0.53

g/dL, 5.67 ± 1.66 g/dL, 2.11 ± 0.21 g/dL) for Golden Retriever, Great Dane, Bernese Mountain Dog and Maremmano-Abruzzese Sheepdog, respectively.

A total of 162 proteins were identified by mass spectrometry, 47 proteins from spermatozoa (Suppl. 1), 109 from seminal plasma (Suppl. 2) and 6 were common to spermatozoa and seminal plasma (Suppl. 3) (Fig. 1C). Among breeds, 7 (Fig. 1A) and 18 (Fig. 1B) proteins were common for spermatozoa and seminal plasma, respectively. Individual proteins for each specific breed were found in spermatozoa and seminal plasma; Golden Retriever showed 17 and 14; Great Dane 7 and 21; Bernese Mountain Dog 10 and 13; and Maremmano-Abruzzese Sheepdog 2 and 16, respectively.

PCs sum (axis X = PC1 and axis Y = PC2) was 64.3% for spermatozoa (Fig. 2A) and 51.1% for seminal plasma (Fig. 2B) proteins, both above 50%. According to PLS-DA, the results were classified by a VIP score ($\alpha > 1.0$) in which five proteins (serum albumin, two isoforms of epididymal secretory protein E1, polyhomeotic-like protein 3 and keratin type II cytoskeletal 1) were important to separate the breeds according to spermatozoa proteins (Fig. 3A).

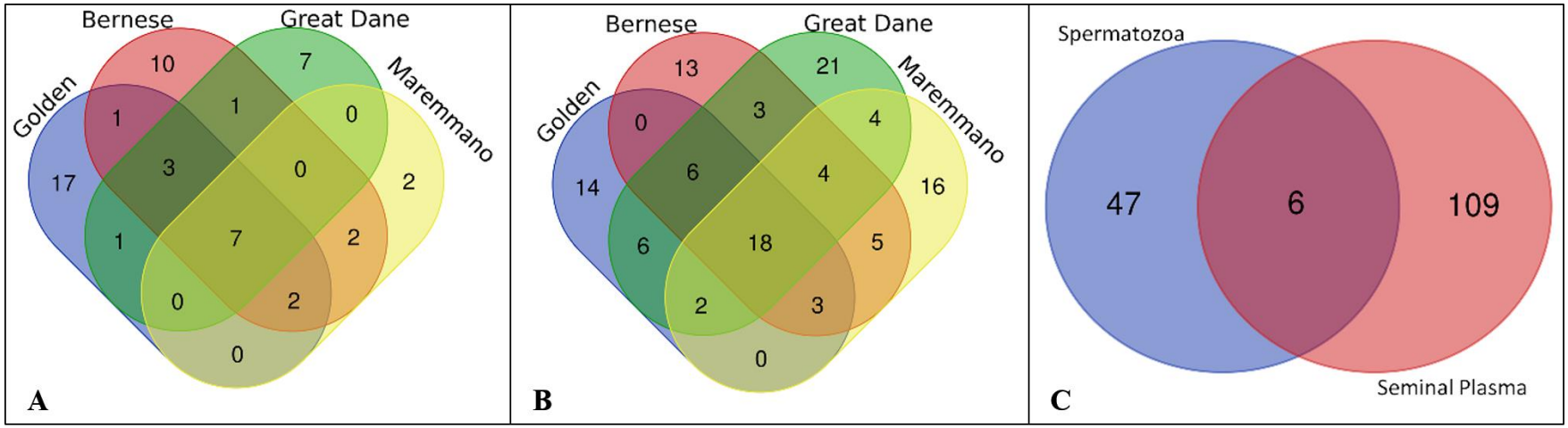
Ten proteins (ras-related protein Rab-2A, tubulin alpha-3 chain, tubulin alpha-3E chain, keratin type II cytoskeletal 7, tubulin beta-4B chain, phospholipid hydroperoxide glutathione peroxidase mitochondrial, keratin type II cytoskeletal 1, acrosin binding protein, outer dense fiber protein 2 and voltage-dependent anion-selective channel protein 2) were considered relevant in seminal plasma according to PLS-DA ($\alpha > 1.0$) (Fig. 3B).

Considering cluster formation, two large groups (Bernese Mountain Dog and Great Dane; Golden Retriever and Maremmano-Abruzzese Sheepdog) were formed according to spermatozoa proteins (Fig. 4); and one large group composed by Bernese Mountain Dog, Great Dane and Maremmano-Abruzzese Sheepdog and a single group composed by Golden Retriever were formed by according to seminal plasma proteins

(Fig. 5).

Differences has been found on gene ontology of the proteins found in spermatozoa (Fig. 6) for all breeds analyzed together in relation by individual breeds. Binding was the main molecular function of all breeds analyzed together and individually by breed. According to breed, the main biological process was cellular process (23%), localization (23%) and metabolic process (23%) for Golden Retriever; cellular process (23%) for Bernese Mountain Dog; and metabolic process (26% for both) for Great Dane and Maremmano-Abruzzese Sheepdog. The main cellular component was organelle (43%) for Golden Retriever; protein-containing complex (29% and 25%, respectively) for Bernese Mountain Dog and Maremmano-Abruzzese Sheepdog; and organelle (33%) and protein-containing process (33%) for Great Dane. The main protein class was hydrolase (19%), nucleic acid binding (19%) and transfer/carrier protein (19%) for Golden Retriever; transfer/carrier protein (22%) for Bernese Mountain Dog; nucleic acid binding (21%) for Great Dane; and transcription factor (33%) for Maremmano-Abruzzese Sheepdog.

Gene ontology of seminal plasma proteins (Fig. 7) of all breed analyzed together was the same gene ontology analyzed by breed, except for Golden Retriever (28%), Bernese Mountain Dog (30%) and Maremmano-Abruzzese Sheepdog (33%) that showed oxidoreductase as the main protein class; while Great Dane showed hydrolase (16%) as the main protein class.



277

278 **Figure 1** Venn diagram of proteins observed in spermatozoa among breeds (A), seminal plasma among breeds (B) and total spermatozoa and

279 seminal plasma proteins (C). Diagram made online at: http://bioinformatics.psb.ugent.be/webtools/Venn/

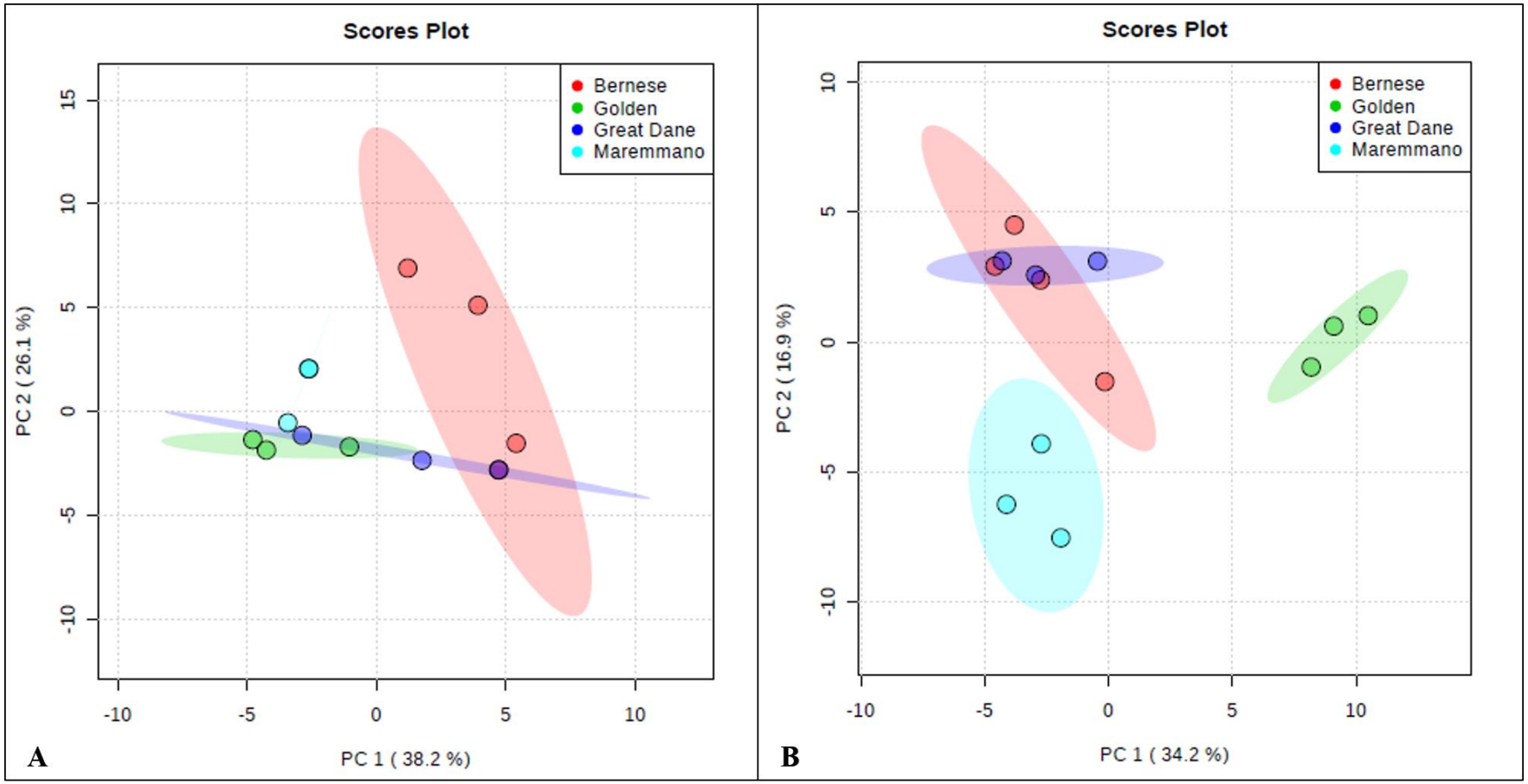


Figure 2. Principal component analysis (PCA) showing the scores plot for spermatozoa (A) and seminal plasma proteins (B).

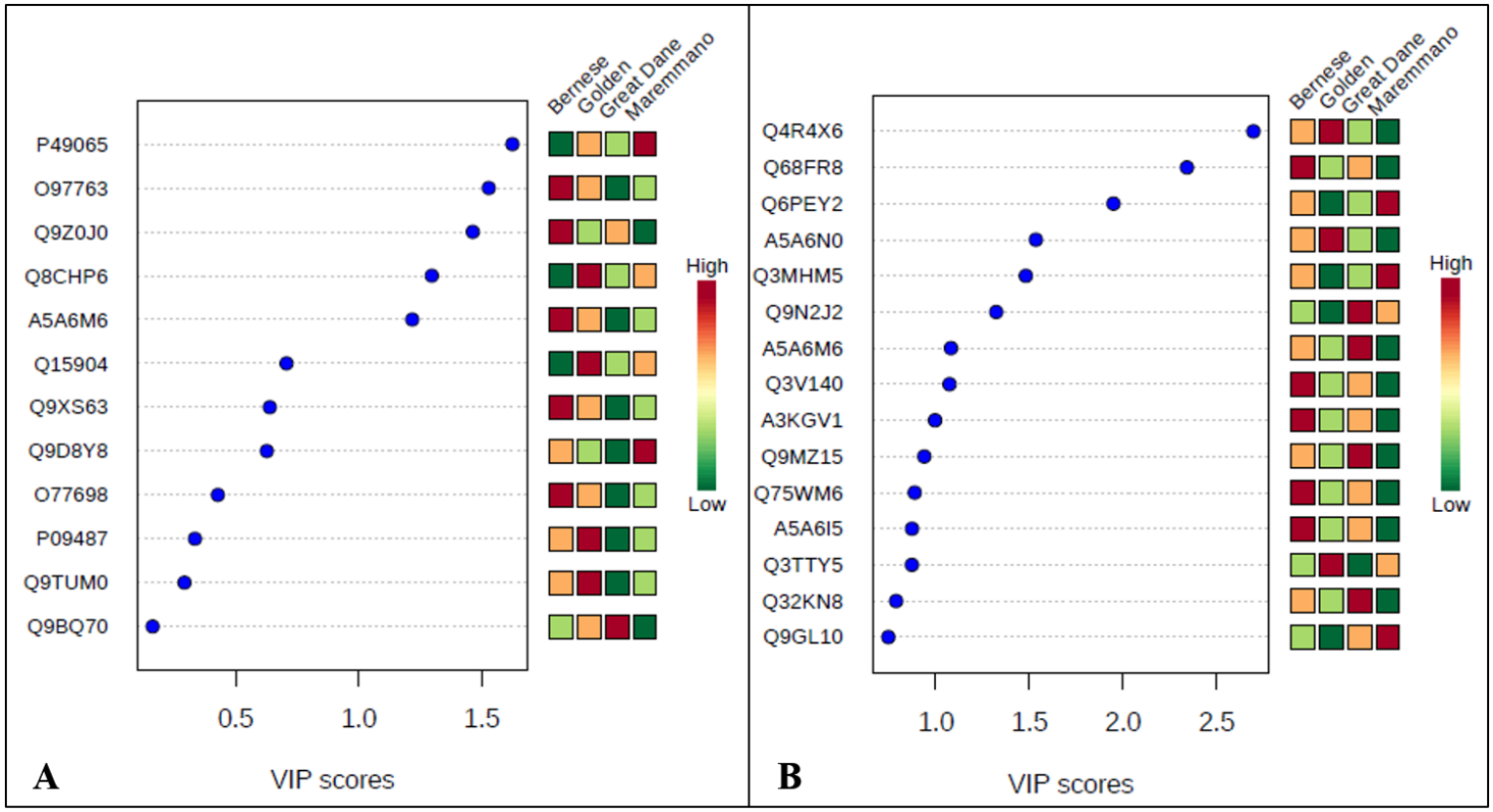


Figure 3. Variable importance projection score (VIP score) classified by PLS-DA for spermatozoa (A) and seminal plasma (B) proteins (represented by proteins identity). The intensity of protein expression was represented by colored boxes on the right side of the figures for each breed. Dark red color means high protein expression, whereas dark green color means low expression. Proteins showing VIP score (α) > 1.0 are relevant.

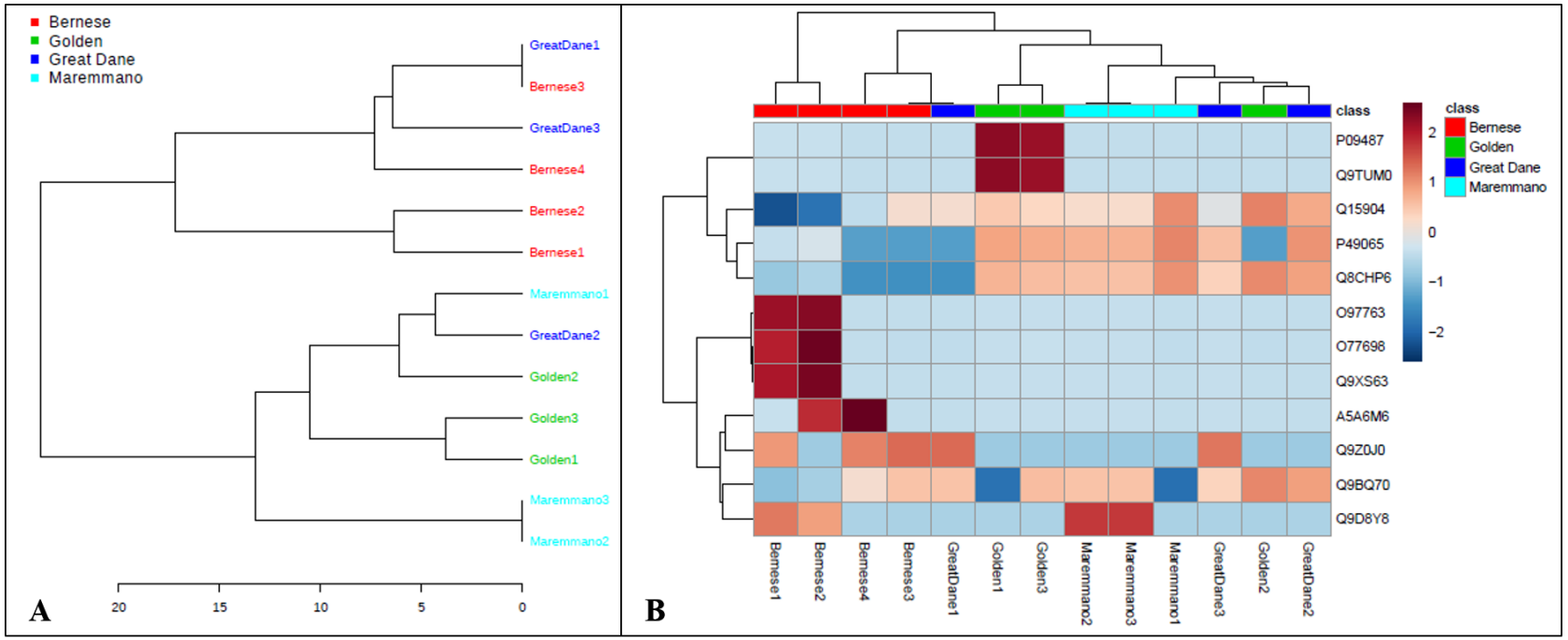


Figure 4. Dendogram (A) and heatmap (B) of spermatozoa proteins (represented by identification number) of each breed. Note high correlation between Bernese Mountain Dog (red) and Great Dane (dark blue); and Golden Retriever (green) and Maremmano-Abruzzese Sheepdog (light blue).

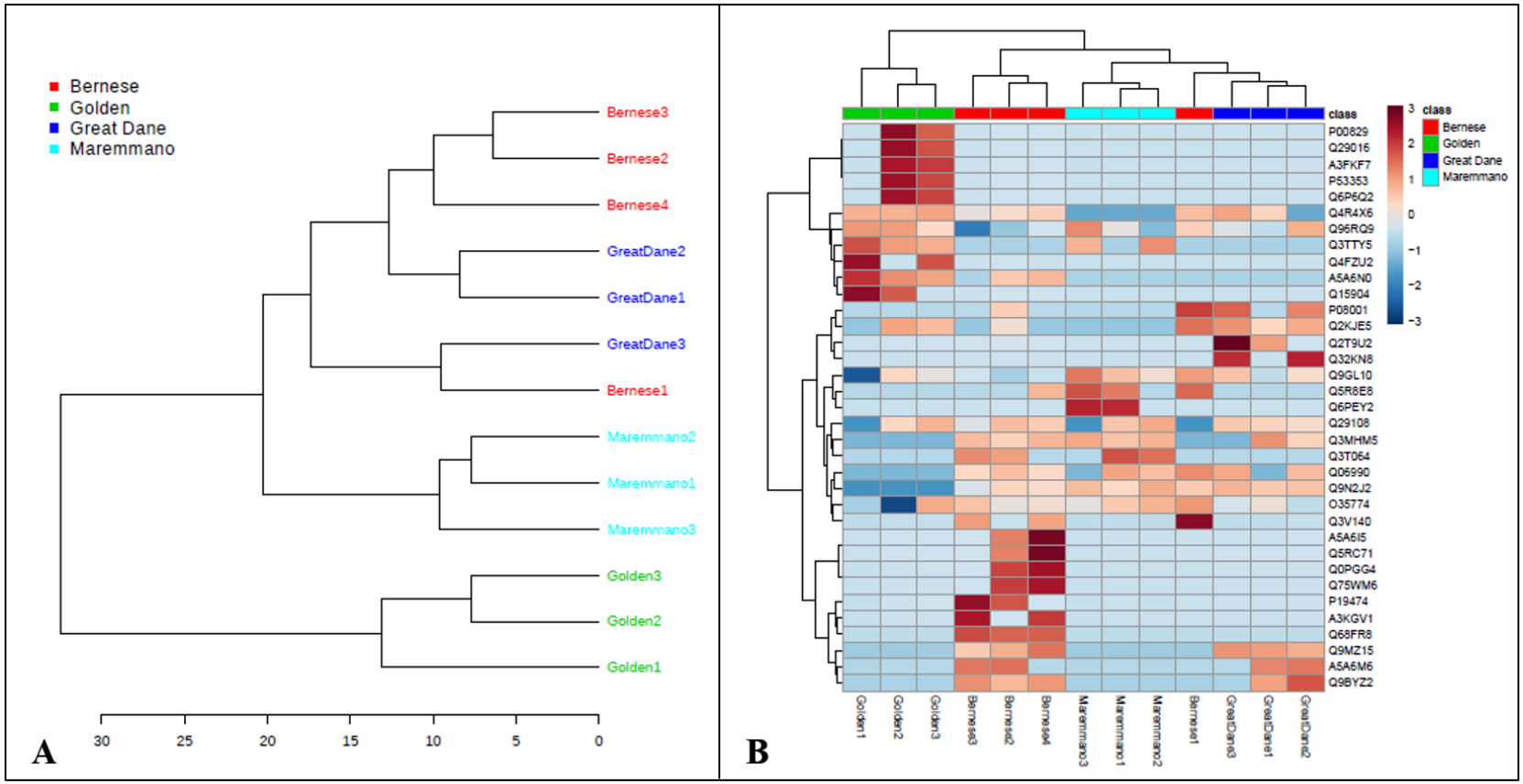


Figure 5. Dendogram (A) and heatmap (B) of seminal plasma proteins (represented by proteins identity) of each breed. Note high correlation between Great Dane (dark blue) and Maremmano-Abruzzese Sheepdog (purple), Bernese Mountain Dog (red) and Belgian Malinois (light blue), and a single group composed by Golden Retriever (green) and only one Belgian Malinois dog (light blue).

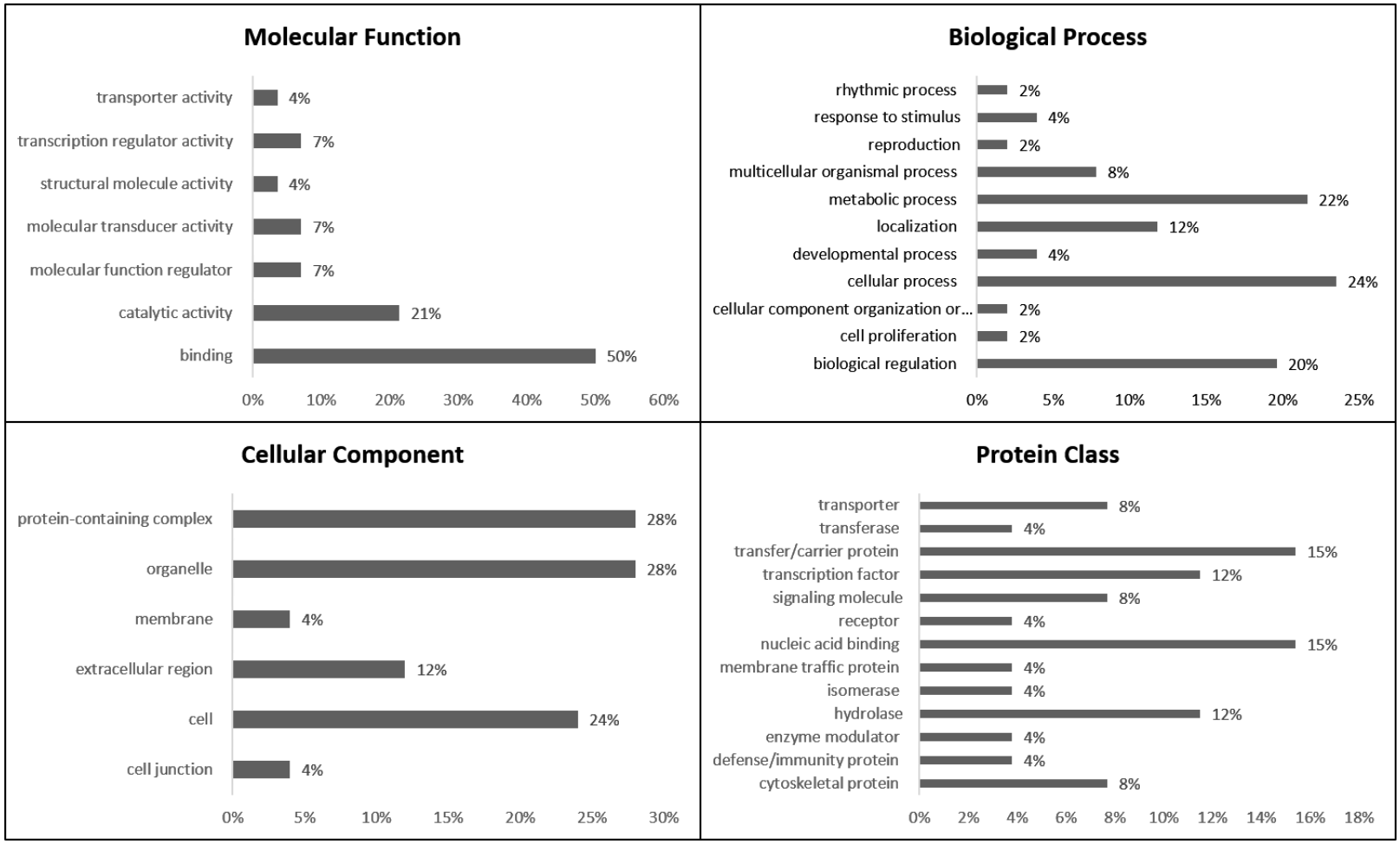


Figure 6. Gene ontology of spermatozoa proteins from all canine breeds, considering molecular function, cellular component, biological process and protein class.

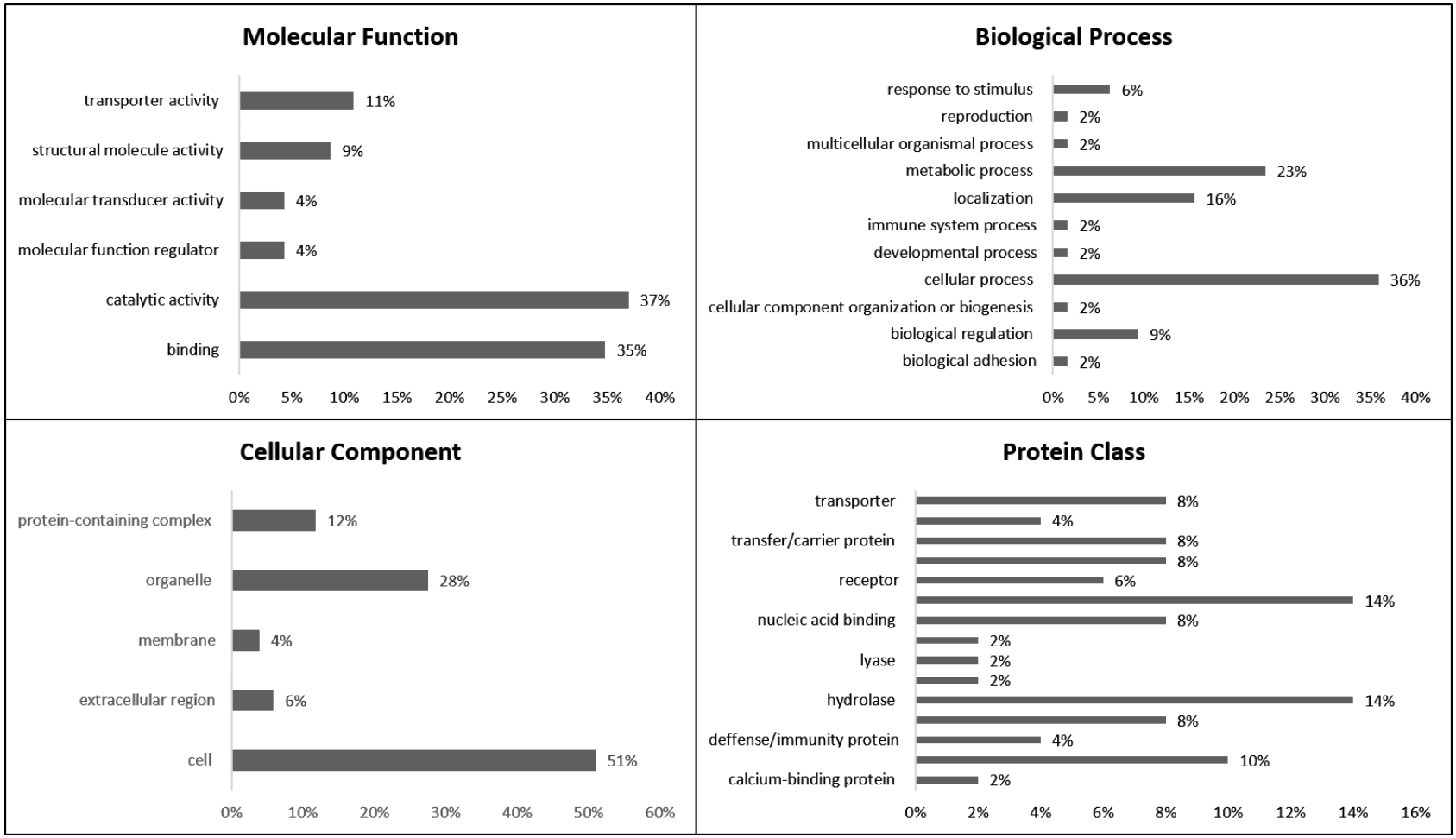


Figure 7. Gene ontology of seminal plasma proteins from all canine breeds, considering molecular function, cellular component, biological process and protein class.

Discussion

Sperm parameters obtained in the present study by CASA and flow cytometry are considered to indicate good sperm quality in accordance with previous studies with canine semen [34-36]. Although *in vitro* evaluation of sperm parameters does not guarantee the ability of the sperm to fertilize an oocyte, are useful indicators of deleterious effects on cells [36]. Therefore, the proteins found in our experiment were obtained of dogs with good seminal parameters.

Studies on isolated proteins of canine seminal plasma were performed, being identified acid alkaline phosphatase [37], arginine esterase [38], lactoferrin [8], heparin binding proteins [10,14], canine prostate-specific esterase [17], metalloproteinases [12], antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) [39-41] and zinc binding proteins [16]. These findings contributed significantly to the better understand of canine seminal plasma influence on sperm function and fertility. Sperm proteins has also been studied in cattle [7,19], human [42] and horse [43], but there is no study focused on dogs, otherwise concerning isolated proteins.

Total concentration of soluble protein from spermatozoa and seminal plasma was similar among breeds, but lower concentration of soluble protein was obtained in spermatozoa than the seminal plasma. Our finds corroborate with Aquino-Cortez et al. [18] which analyzed the sperm-rich fraction (1.76 g/dL ± 0.20) and prostatic fluid (1.56 g/dL ± 0.27) of canine semen individually. In the present study the total concentration of soluble proteins of sperm-rich fraction and the prostatic fluid were analyzed together, which justifies almost twice the value found by Aquino-Cortez et al. [18].

Based on the recent reviewed literature, this is the first research to show differences on proteomic profile of seminal plasma and spermatozoa in the ejaculate of

different canine breeds. Few common proteins were expressed among breeds in seminal plasma (18/122) and spermatozoa (7/47), showing that there are differences in the expression of these proteins according to breeds and possibly individual variation. PCA sum was above 50% for spermatozoa and seminal plasma proteins, which confirms the accuracy of the division between groups. Results of cluster analysis of spermatozoa and seminal plasma proteins demonstrate that was not possible to group dogs by single breeds, except for Golden Retriever for seminal plasma proteins, however two groups were formed by high correlation between breeds (Bernese Mountain Dog and Great Dane; Golden Retriever and Maremmano-Abruzzese Sheepdog) for sperm proteins. For seminal plasma proteins one large group was composed by Bernese Mountain Dog, Great Dane and Maremmano-Abruzzese Sheepdog; and one small group was composed by only for Golden Retriever.

Parker [1] described a genomic analysis of canine breeds and demonstrated by clustering the relationship between dog breeds based on SNP markers. In this clustering was evident the high genomic proximity of Bernese Mountain Dog and Great Dane, and these two breeds are also correlated with Golden Retriever. Unfortunately, the Maremmano-Abruzzese Sheepdog breed was not included in this clustering, so we cannot affirm the genomic proximity among this breed and the others. This may explain, at least in part, the correlation observed on clustering formation among two or three breeds for spermatozoa and seminal plasma proteins in our study. These results show that despite differences on seminal plasma and spermatozoa proteomic profile among canine breeds, correlations with some breeds are still present, and this may be related to genomic proximity.

Gene ontology of seminal plasma proteins was the same for all breeds, except for protein class. In contrast, only the molecular function remained the same for all breeds

for spermatozoa protein. Pattern of protein expression was different among breeds, even among that with high correlation between them in clustering formation of seminal plasma and spermatozoa proteins. Maremmano-Abruzzese Sheepdog showed seven underexpressed proteins of the ten relevant seminal plasma proteins and Bernese Mountain Dog showed three overexpressed proteins of the five most relevant spermatozoa proteins. Unfortunately, we could not explain why the pattern of protein expression was different even among breeds with high correlation in clustering formation. Once there was no difference on semen quality of dogs, this is cannot be considered as a cause of altered pattern of protein expression.

All domestic dogs have a common ancestor, but the purebred domestic dogs were created through backcrossing and inbreeding to fix desired traits, resulting in a much-reduced level of heterozygosity compared to wild or non-breed dogs [2,3]. They are kept relatively pure, but outcrossing has been known to occur, preventing complete homozygosity. In addition, the same breed in different countries may be subjected to slightly different selective pressures maintaining some level of heterozygosity across the breed [1]. Heterogeneity that can occur in purebred reinforce that the protein expression in the ejaculate may be different between dogs within a same breed. In cattle, differences in proteomic of seminal plasma [44] and spermatozoa [7,19] were observed in *B. indicus*, *B. taurus* and crossbred bulls, evidencing the influence of the breed on fertility in this species.

Five proteins were considered relevant for proteomic of spermatozoa, serum albumin, two isoforms of epididymal secretory protein E1, polyhomeotic-like protein 3 and keratin type II cytoskeletal 1.

Serum albumin changes sperm cholesterol and phospholipids carrying during sperm capacitation and fertilization, and plasma membrane destabilization occurs through

374 an action on the membrane lipid phase by albumin [45,46]. Destabilization of membrane
375 is associated with increase permeability of calcium, which chelates anionic phospholipid
376 molecules in adjacent membrane lipid bilayers [47] to promote fusion between the plasma
377 membranes and outer acrosome membrane [45]. Bovine serum albumin produces
378 multifactorial benefits on sperm functions as antioxidant capacity, increase post-thaw
379 motility, protects against cold shock, maintains DNA integrity after freezing-thawing,
380 stimulate capacitation and acrosome reaction [48,49]. Addition of bovine serum albumin
381 in extenders for ram sperm cryopreservation [49], bull [48] and buffalo [50] improved
382 seminal quality post-thawing. In boar semen this protein resulted in higher post-thaw
383 motility, low oxidative stress, integrity of plasmalemma and acrosome after 11 days
384 storage at 18º C [51]. These data show the importance of serum albumin for sperm quality
385 even during cryopreservation.

386 While spermatozoa pass through the epididymis some proteins are secreted from
387 the epididymal epithelium to sustain the sperm maturation for acquisition of motility and
388 fertility [52]. Both isoforms of epididymal secretory protein E1 are one of these proteins
389 that are produced by epididymis and bind to sperm plasma membrane [53]. An amino
390 acid sequence identity has been shown between epididymal secretory protein E1 and a
391 protein purified from the surface of epididymal sperm of rats [53]. This protein has
392 already been described in canine seminal plasma, although its function was not been
393 reported [18].

394 Polyhomeotic-like protein 3 is a component of the polycomb group multiprotein
395 PRC1-like complex that is present in all cells [54]. This complex assembles at specific
396 DNA sequences, called polycomb response elements, in *Hox* genes and other target genes
397 to repress transcription through enzymatic modification of histones and chromatin
398 compaction [54,55]. This is the first time that this protein is described in sperm cell.

Within the mammalian spermatozoa there is a substantial proportion of keratin present in the heads and tails related to DNA protection and zinc binding on sperm tail [56-58]. Zinc is present in relatively high concentrations within keratins and is necessary for normal keratogenesis [59] and for spermatogenesis in mammals [60]. We found keratin type II cytoskeletal 1 in spermatozoa and seminal plasma proteins. Keratin type II cytoskeletal 7 is another protein of keratin family that we detected only in seminal plasma. According to the literature reviewed until the moment, this is the first time that this protein is described in semen. We hypothesized that its function may be similar to those keratin proteins found in spermatozoa.

Canine seminal plasma contains secretions derived from the testis, epididymis and prostate composed by a variety of carbohydrates, ions, minerals, lipids and proteins [14,16,18,61]. According to proteomic of seminal plasma, ten proteins were considered relevant.

Ras-related protein Rab-2A is located in the acrosome membrane of spermatozoa and is associated with capacitation, acrosome formation, regulates vesicular transport and membrane fusion [62,63]. This protein participates in the binding of capacitated spermatozoa to the zona pellucida by facilitating the passage of zona-recognition molecules from the interior of the acrosome to the sperm surface through the formation and transport of acrosome vesicles [24,64]. Moreover, this protein was negatively correlated with litter size in boar semen [65].

Multiple isoforms of tubulin, tubulin alpha-3 chain, tubulin alpha-3E chain and tubulin beta-4B chain, were identified in canine seminal plasma. These cytoskeletal proteins are the main structural components of microtubules and are involved with the flagella movement and may present α or β isotypes that can display high molecular heterogeneity [66,67]. Tubulins are found in cell membranes [68] and regulate

intracellular reorganization in germ cells during spermiogenesis, which is essential for fertility [69]. This protein was also found on sperm membrane of Morada Nova rams [70].

Acrosin-binding protein is a phosphoprotein calcium-dependent that is phosphorylated in the acrosome and is related to sperm capacitation in boar [71]. This protein has already been described in pig and guinea pig [72,73], human [74] and mouse [Tardif et al., 2012]. Vilagran et al. [71] related that higher amounts of this protein in semen confer higher acrosome resistance to freeze-thawing procedures, retaining acrosin maturation during semen cryopreservation, preventing early sperm capacitation.

Outer dense fiber protein 2 is localized in the outer dense fibers of sperm flagella and in the fibrous sheath [76,77] and are involved with sperm hyperactivation and motility [63,78].

Phospholipid hydroperoxide glutathione peroxidase mitochondrial protein suppresses the effects of mitochondrial reactive oxidant species that are produced by oxidative phosphorylation, thus prevents apoptotic cell death [79]. Abundance of this protein in spermatozoa suggests that this unique antioxidant enzyme might be involved in the development of spermatozoa [80]. Besides, this protein is one of the major selenoproteins in spermatids, being important for both production and motility of spermatozoa [80].

Voltage-dependent anion-selective channel protein 2 is present in the mitochondrial outer membrane, outer dense fiber of flagella or in the sperm acrosome region [81,82]. This protein regulates the transport of various ions and metabolites including the transmembrane influx of calcium [83]. Calcium is necessary for the acquisition of sperm fertilization capacity in the female reproductive tract after ejaculation [84]. According to Kwon et al. [85], blocking voltage-dependent anion-selective channel protein 2 in spermatozoa caused detrimental effects on capacitation,

449 acrosome reaction, fertilization and embryo development. These authors suggest that this
450 protein may be used as marker of male infertility and as target of male contraceptive.

451 Sperm proteins in canine seminal plasma were also described by Aquino-Cortez
452 et al. [18]. These authors attributed this fact to the result of spermatozoa rupture and
453 protein leakage during freezing of samples without cryoprotectant and to the interaction
454 between sperm cells and seminal plasma during maturation in the epididymis and after
455 ejaculation. In human, 70% of proteins identified in seminal plasma are also part of
456 spermatozoa proteome, which indicates a mechanism of protein transport between
457 spermatozoa and seminal plasma [86].

458 Except for polyhomeotic-like protein 3 and keratin type II cytoskeletal 7, which
459 is the first time that these proteins are described in semen, all the proteins found in
460 spermatozoa and seminal plasma of dogs in this research has already been described in
461 semen of another species such as mouse [46,53,58,78,87], human [80,85,88,89], boar
462 [65,71], bull [66,81,82] and ram [70]. Some of the proteins identified in this current study
463 are being described for the first time in semen of dogs.

464 The profile of seminal plasma and spermatozoa proteins from the ejaculate of four
465 canine breeds were described. Correlations among Golden Retriever and Maremmano-
466 Abruzzese Sheepdog, Great Dane and Bernese Mountain Dog in proteomic of
467 spermatozoa was found; while Maremmano-*Abruzzese Sheepdog*, Great Dane and
468 Bernese Mountain Dog were correlated in seminal plasma profile. Further studies focused
469 on semen proteins of different purebred dogs need to be performed to improve
470 biotechnologies of reproduction in this species.

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References

[1] Parker HG. Genomic analyses of modern dog breeds. Mamm Genome 2012;23(1-2):19-27.

[2] Vonholdt BM, Pollinger JP, Lohmueller KE, Han E, Parker HG, Quignon P, Degenhardt JD, Boyko AR, Earl DA, Auton A, Reynolds A, Bryc K, et al. Genome-wide SNP and haplotype analyses reveal arich history underlying dog domestication. Nature 2010;464:898–902.

[3] Boyko AR, Quignon P, Lin L, Schoenebeck JJ, Degenhardt JD, Lohmueller KE, Zhao K, Brisbin A, Parker HG, VonHoldt BM, Cargill M, Auton A, et al. A simple genetic architecture underlies morphological variation in dogs. PLoS Biol 2010;8(8):e1000451.

[4] Mascarenhas RM, Antonio RPT. Influence of breed and individual variation in the quality of frozen canine semen. J Vet Sci Technol 2018;9(1):1-5.

[5] De Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. Rev Reprod 1997;2:48-54.

[6] Peña FJ, Núñez-Martínez I, Morán JM. Semen Technologies in dog breeding: an update. Reprod Dom Anim 2006;41(2):21-9.

[7] Ashrafzadeh A, Nathan S, Karsani SA. Comparative analysis of Mafriwal (Bos taurus x Bos indicus) and Kedah Kelantan (Bos indicus) sperm proteome identifies sperm proteins potentially responsible for higher fertility in a tropical climate. Int J Mol Sci 2013;14:15860-15877.

[8] Kikuchi M, Mizoroki S, Kubo T, Ohiwa Y, Kubota M, Yamada N, Orino K, Ohnami Y, Watanabe K. Seminal plasma lactoferrin but not transferrin reflects gonadal function in dogs. J Vet Med Sci 2003;65:679-684.

[9] Souza FF, Barreto CS, Lopes MD. Characteristics of seminal plasma proteins and their correlation with canine semen analysis. Theriogenology 2007;68:100-6.

[10] Souza FF, Chirinéa VH, Martins MIM, Lopes MD. Osteopontin in seminal plasma and sperm membrane of dogs. Reprod Dom Anim 2009;44(2):283-286.

[11] Mogielnicka-Brzozowska M, Kordan W. Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive process in mammals. Pol J Vet Sci 2011;14(3):489-499.

[12] Saengsoi S, Wei-Yau S, Shyu CL, Wu JT, Warinrak C, Lee WM, Cheng FP. Detection of matrix metalloproteinase (MMP)-2 and MMP-9 in canine seminal plasma. Anim Reprod Sci 2011;127:114-119.

[13] Aquino-Cortez A, Da Silva LDM, Araújo AA, De Menezes ESB, Moura AAAN. Proteins of the canine seminal plasma. Ciência Rural 2016;46(5):901-908.

[14] Souza FF, Martins MIM, Fernandes CES, Ribolla PEM, Lopes MD. Heparin-binding proteins of canine seminal plasma. Theriogenology 2006;66:1606-1609.

[15] Warinrak C, Wu J-T, Hsu W-L, Lia J-W, Chang S-C, Cheng F-P. Expression of matrix metalloproteinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in canine testis, epididymis and semen. Reprod Dom Anim 2015;50:48-57.

[16] Mogielnicka-Brzozowska M, Kowalska N, Fraser L, Kordan W. Proteomic characterization of zinc-binding proteins of canine seminal plasma. Reprod Dom Anim 2015;50:1017-1021.

[17] Alonge, S, Melandri M, Leoci R, Lacalandra GM, Aiudi G. Canine prostate specific esterase (CPSE) as an useful biomarker in preventive screening programme of canine prostate: CPSE threshold value assessment and its correlation with ultrasonographic prostatic abnormalities in asymptomatic dogs. Reprod Dom Anim 2018;53:359-364.

[18] Aquino-Cortez A, Pinheiro BQ, Lima DBC, Siva HVR, Mota-Filho AC, Martins JAM, Rodriguez-Villamil P, Moura AA, Silva LDM. Proteomic characterization of canine seminal plasma. Theriogenology 2017;95:178-186.

[19] Aslam MKM, Kumaresan A, Rajak SK, Tajmul J, Datta TK, Mohanty TK, Srinivasan A, Yadav S. Comparative proteomic analysis of taurine, indicine, and crossbred (Bos taurus x Bos indicus) bull spermatozoa for identification of proteins related to sperm malfunctions and subfertility in crossbred bulls. Theriogenology 2015;84:624-633.

[20] Bloom E. A systematic search for abnormalities in testis-epididymis in pedigree bulls in Denmark. Studies on pathological conditions in the testis, epididymis, and accessory sex glands in the bull. Copenhagen: Royal Veterinary and Agricultural University, 1972:1-36.

[21] Freitas-Dell'Aqua CP, Guasti PN, Monteiro GA, Maziero RRD, Dell'Aqua JJA, Papa FO. Flow cytometric analysis of fertile and subfertile frozen stallion spermatozoa. Anim Reprod 2012;9:941.

[22] Freitas-Dell´aqua CP, Sancler-Silva YFR, Silva-Júnior ER, Dell´Aqua JJA, Papa FO. Determination of intracellular and mitochondrial superoxide generation and high mitochondrial membrane potential in equine sperm using flow cytometry. J Equine Vet Sci 2016;43:S69-S70.

568 [23] Peach M, Marsh N, Macphee DJ. Protein solubilization: attend to the choice of lysis
569 buffer. In: Kurien BT, Scofield RH (eds), Protein electrophoresis, methods and protocols,
570 New York: Humana Press;2012:37-47.
571
572 [24] Baker MA, Reeves G, Hetherington L, Aitken JR. Analysis of proteomic changes
573 associated with sperm capacitation through the combined use of IPG-strip
574 prefractionation followed by RP chromatography LC-MS/MS analysis. Proteomics
575 2010;10:482-495.
576
577 [25] Codognoto VM, Yamada PH, Schmith RA, de Ruediger FR, Scott C, de Faria
578 Lainetti P, Brochine S, de Paula Freitas-Dell'Aqua C, de Souza FF, Oba E. Functional
579 insights into the role of seminal plasma proteins on sperm motility of buffalo. Anim
580 Reprod Sci 2018;195:251-58.
581
582 [26] Neuhoff V, Stamm R, Eibl H. Clear background and highly sensitive protein staining
583 with Coomassie Blue dyes in polyacrylamide gels: A systematic analysis. Electrophoresis
584 1985;6(9):427-448.
585
586 [27] Neuhoff V, Arold N, Taube D, Erhardt W. Improved staining of proteins in
587 polyacrylamide gels including isoelectric focusing gels with clear background at
588 nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis
589 1988;9(6):255-262.
590

591 [28] Luo C, Yang X, Fu Q, Sun M, Ouyang Q, Chen Y, Ji H. Picoliter-volume aqueous
592 droplets in oil: Electrochemical detection and yeast cell electroporation. Electrophoresis
593 2006;27:1977-1983.
594
595 [29] Ishihama Y, Oda Y, Tabata T, Sato T, Nagasu T, Rappsilber J, Mann M.
596 Exponentially modified protein abundance index (emPAI) for estimation of absolute
597 protein amount in proteomics by the number of sequenced peptides per protein. Mol Cell
598 Proteomics 2005;4:1265-72.
599
600 [30] Xia J, Wishart DS. Using MetaboAnalyst 3.0 for comprehensive metabolomics data
601 analysis. Curr. Protoc. Bioinformatics 2006;55:1-91.
602
603 [31] Checa A, Bedia C, Jaumot J. Lipidomic data analysis: Tutorial, practical guidelines
604 and applications. Anal Chim Acta 2015;885:1-16.
605
606 [32] Boutet E, Lieberherr D, Tognolli M, Schneider M, Parit B, Bridge AJ, Poux S,
607 Bougueleret L, Xenarios I. UniProtKB/Swiss-Prot, the manually annotated section of
608 the Uniprot Knowledgebase: how to use the entry view. Methods Mol Biol 2016;
609 1374:23-54.
610
611 [33] Huaiyu M, Poudel S, Muruganujan A, Casagrande JT, Thomas PD. PANTHER
612 version 10: expanded protein families and functions, and analysis tools. Nucleic Acids
613 Res 2016;44:336–342.
614

615 [34] Iguer-ouada M, Verstegen JP. Long-term preservation of chilled canine semen:
616 effect of commercial and laboratory prepared extenders. Theriogenology 2001;55:671-
617 684.
618
619 [35] Cheuquemán C, Bravo P, Treunlén F, Giojalas LC, Villegas J, Sánchez R, Risopatrón
620 J. Sperm membrane functionality in the dog assessed by flow cytometry. Reprod Dom
621 Anim 2012;47(1):39-43.
622
623 [36] Kasimanickam VR, Kasimanickam RK, Memon MA, Rogers HA. Effect of
624 extenders on sperm mitochondrial membrane, plasma membrane and sperm kinetics
625 during liquid storage of canine semen at 5º C. Anim Reprod Sci 2012;136:139-45.
626
627 [37] Zelli R, Bellezza I, Rambotti MG, Minelli A, Polisca A. Characteristics of seminal
628 plasma proteins and their correlation with canine semen analysis. Theriogenology
629 2007;68:100-106.
630
631 [38] Dubé JY. Prostatic kallikreins: biochemistry and physiology. Comp Biochem
632 Physiol 1994;107:13-20.
633
634 [39] Cassani P, Beconi MT, O'Flaherty C. Relationship between total superoxide
635 dismutase activity with lipid peroxidation, dynamics and morphological parameters in
636 canine semen. Anim Reprod Sci 2005;86:163-173.
637
638 [40] Strzezek R, Koziorowska-Gilun M, Kowalówka M, Strzezék J. Characteristics of
639 antioxidant system in dog semen. Pol J Vet Sci 2009;12:(1)55-60.

640 [41] Angrimani DSR, Lucio CF, Veiga GAL, Silva LCG, Regazzi FM, Nichi M,
641 Vannucchi CI. Sperm maturation in dogs: sperm profile and enzymatic antioxidant status
642 in ejaculated and epididymal spermatozoa. Andrologia 2014;46:814-819.
643
644 [42] Johnston DS, Wooters J, Kopf GS, Qiu Y, Roberts KP. Analysis of the human sperm
645 proteome. Ann NY Acad Sci 2005;1061:190–202.
646
647 [43] Swegen A, Curry BJ, Zamira G, Lambourne SR, Smith ND, Aitken RJ. Investigation
648 of the stallion sperm proteome by mass spectrometry. Reproduction 2014;1-23.
649
650 [44] Assumpção TI, Fontes W, Sousa MV, Ricarti CAO. Proteome analysis of nelore bull
651 (Bos taurus indicus) seminal plasma. Protein & Peptide Letters 2005;12:813-817, 2005.
652
653 [45] Davis BK, Hungund BJ. Effect of modified membrane vesicles from seminal plasma
654 on the fertilizing capacity of rabbit spermatozoa. Biochem Biophys Res Commun 1976;
655 69(4):1004-10.
656
657 [46] Davis BK, Byrne R, Hungund B. Studies on the mechanism of capacitation II.
658 Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin
659 during capacitation in vitro. Biochim Biophys Acta 1979;558:257-266.
660
661 [47] Papahadjopoulos D, Vail WJ, Jacobson K, Poste G. Cochleate lipid cylinders:
662 formation byfusionn of unilamellar lipid vesicles. Biochim Biophys Acta
663 1975;394(3):483-91.
664

[48] Ulysal O, Bucak MN. Effect of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen. Acta Vet Brno 2007;76:383-390.

[49] Alçay S, Toker MB, Gökçe E, Önder NT, Üstüner B, Nur Z. Long term incubation resilience of post-thaw ram semen diluted with lecithin-based extender supplemented with bovine serum albumin. Kafkas Univ Vet Fak Derg 2019;25(3):291-97.

[50] Rahman ZU, Anwar M, Andrabi SMH, Mehmood A, Ali L, Ahmad H. Effect of bovine serum albumin in extender on post-thaw quality and in vivo fertility of buffalo bull semen. Buffalo Bulletin 2015;34(4):417-21.

[51] Lee S-H, Park C-K. Antioxidative effects of magnetized extender containing bovine serum albumin on sperm oxidative stress during long-term liquid preservation of boar semen. Biochem Biophy Res Commun 2015;464(2):467-72.

[52] Okamura N, Kiuchi S, Tamba M, Kashima T, Hiramoto S, Baba T, et al. A porcine homolog of the major secretory protein of human epididymis, HE1, specifically binds cholesterol. Biochim Biophys Acta Mol Cell Biol Lipids 1999;1438:377-87.

[53] Xu W, Ensrud KM, Hamilton DW. The 26 kD Protein Recognized on Rat Cauda Epididymal Sperm by Monoclonal Antibody 4E9 Has Internal Peptide Sequence That Is Identical to the Secreted Form of Epididymal Protein E1. Mol Reprod Dev 1997;46:377382.

690 [54] Schwartz YB, Pirrotta V. Polycomb complexes and epigenetic states. Curr. Opin Cell
691 Biol 2008;20:266–273.
692
693 [55] Simon JA, Kingston RE. Occupying chromatin: Polycomb mechanisms for getting
694 to genomic targets, stopping transcriptional traffic, and staying put. Mol. Cell
695 2013;49:808–824.
696
697 [56] Zittle CA, O'Dell RA. Chemical Studies of Bull Spermatozoa. J Biol Chem
698 1941;140:899-907.
699
700 [57] Henricks DM, Mayer DT. Isolation and characterization of a basic keratin-like
701 protein from mammalian spspermatozoa. Exp Cell Res 1965;40:402-412.
702
703 [58] Calvin HI, Bleau G. Zinc-Thiol complexes in keratin-like structures of rat
704 spermatozoa. Exp Cell Res 1974;86:280-284.
705
706 [59] Underwood EJ. Trace Elements in Human and Animal Nutrition. 3rd ed. New York:
707 Academic Press;1971.
708
709 [60] Mann T. The Biochemistry of Semen and of the Male Reproductive Tract. 2nd ed.
710 London: Methuen; 1964:210-211.
711
712 [61] Bartlett DJ. Studies on dog semen II. Biochemical characteristics. J Reprod Fertil
713 1962;3:190-205.
714

715 [62] Mountjoy JR, Xu W, McLeod D, Hyndman D, Oko R. RAB2A: a major
716 subacrosomal protein of bovine spermatozoa implicated in acrosomal biogenesis. Biol
717 Reprod 2008;79:223–232.
718
719 [63] Kwon WS, Rahman MS, Lee, J-S, Kim J, Yoon S-J, Park Y-J, You Y-A, Hwang S,
720 Pang M-G. A comprehensive proteomic approach to identifying capacitation related
721 proteins in boar spermatozoa. BMC Genomics 2014;15:897.
722
723 [64] Tulsiani DR, Zeng HT, Abou-Haila A. Biology of sperm capacitation: evidence for
724 multiple signalling pathways. Soc Reprod Fert Supp 2007;63:257–272.
725
726 [65] Kwon W-S, Rahman MS, Lee J-S, Yoon S-J, Park Y-J, Pang M-G. Discovery of
727 predictive biomarkers for litter size in boar spermatozoa. Mol Cell Proteomics 2015;
728 14(5):1230-40.
729
730 [66] Rtidiger M, Plessmann U, Riidiger A-H, Weber K. Fl tubulin of bull sperm is
731 polyglycylated. FEBS Letters 1995;364:147-151.
732
733 [67] Gagnon C, White D, Cosson J, Huitorel P, Eddé B, Desbruyères E, Paturle-
734 Lafanechère L, Multigner L, Job D, Cibert C. The polyglutamylated lateral chain of alpha-
735 tubulin plays a key role in flagellar motility. J Cell Sci 1996;109:1545-1553.
736
737 [68] Wolff J. Plasma membrane tubulin. Biochim Biophys Acta 2009;1788:1415–33.
738

739 [69] Kierszenbaum AL, Rivkin E, Tres LL. Cytoskeletal track selection during cargo
740 transport in spermatids is relevant to male fertility. Spermatogenesis 2011;1:221–30.
741
742 [70] Van Tilburg MF, Rodrigues MAM, Moreira RA, Moreno FB, Monteiro-Moreira
743 ACO, Cândido MJD, Moura AA. Membrane-associated proteins of ejaculated sperm
744 from Morada Nova rams. Theriogenology 2013;79(9):147-61.
745
746 [71] Vilagran I, Castillo J, Bonet S, Sancho S, Yeste M, Estanyol JM, Oliva R. Acrosin-
747 binding protein (ACRBP) and triosephosphate isomerase (TPI) are good markers to
748 predict boar sperm freezing capacity. Theriogenology 2013;80:443-550.
749
750 [72] Polakoski K, Parrish RK. Boar proacrosin, purification and preliminary activation
751 studies of proacrosin isolated from ejaculated boar sperm. J Biol Chem 1977;252:1888-
752 94.
753
754 [73] Baba T, Niida Y, Michikawa Y, Kashiwabara S, Kodaira K, Takenaka M, et al. An
755 acrosomal protein, sp32, in mammalian sperm is a binding protein specific for two
756 proacrosins and an acrosin intermediate. J Biol Chem 1994;269:10133–40.
757
758 [74] Whitehurst AW, Xie Y, Purinston SC, Cappell KM, Swanik JT, Larson B, et al.
759 Tumor antigen acrosin binding protein normalizes mitotic spindle function to promote
760 cancer cell proliferation. Cancer Res 2010;70:7652–61.
761

762 [75] Tardif S, Guvonnet B, Cormier N, Cornwall GA. Alteration in the processing of the
763 ACRBP/sp32 and sperm head/acrosome malformations in proprotein convertase 4
764 (PCSK4) null mice. Mol Hum Reprod 2012;18:296–307.
765
766 [76] Shao X, Tarnasky HA, Lee JP, et al. Spag4, a novel sperm protein, binds outer dense-
767 fiber protein Odf1 and localizes to microtubules of manchette and axoneme. Dev Biol
768 1999;211:109-23.
769
770 [77] Fujita A, Nakamura K-I, Kato T, Watanabe N, Ishizaki T, Kimura K, Mizoguchi A,
771 Narumiya S. Ropporin, a sperm-specific binding protein of rhophilin, that is localized in
772 the fibrous sheath of sperm flagella. J Cell Sci 2000;113:103-112.
773
774 [78] Mariappa D, Aladakatti RH, Dasari SK, Sreekumar A, Wolkowicz M, Van der Hoorn
775 F, Seshagiri PB. Inhibition of tyrosine phosphorylation of sperm flagellar proteins, outer
776 dense fiber protein-2 and tektin-2, is associated with impaired motility during capacitation
777 of hamster spermatozoa. Mol Reprod Dev 2010;77:182-93.
778
779 [79] Arai M, Imai H, Koumura T, Yoshida M, Emoto K, Umeda M, Chiba N, Nakagawa
780 Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role
781 in preventing oxidative injury to cells. J Biol Chem 1999;274:4924–4933.
782
783 [80] Imai H, Suzuki K, Ishizaka K, Ichinose S, Oshima H, Okayasu I, Emoto K, Umeda
784 M, Nakagawa Y. Failure of the expression of phospholipid hydroperoxide glutathione
785 peroxidase in the spermatozoa of human infertile males. Biol Reprod 2001;64:674-683.
786

787 [81] Hinsch KD, De Pinto V, Aires VA, Schneider X, Messina A, Hinsch E. Voltage
788 dependent anion-selective channels VDAC2 and VDAC3 are abundant proteins in bovine
789 outer dense fibers, a cytoskeletal component of the sperm flagellum. J Biol Chem
790 2004;279:15281–8.
791
792 [82] Triphan X, Menzel VA, Petrunkina AM, Cassar_a MC, Wemheuer W, Hinsch KD,
793 Hinsch E. Localisation and function of voltage-dependent anion channels (VDAC) in
794 bovine spermatozoa. Pflugers Arch 2008;455:677–86.
795
796 [83] Gincel D, Zaid H, Shoshan-Barmatz V. Calcium binding and translocation by the
797 voltage-dependent anion channel: a possible regulatory mechanism in mitochondrial
798 function. Biochem J 2001;15:147–55.
799
800 [84] Chang MC. Fertilizing capacity of spermatozoa deposited into the fallopian tubes.
801 Nature 1951;168:697–8.
802
803 [85] Kwon WS, Park YJ, Mohamed ESA, Pang MG. Voltage-dependent anion channels
804 are a key factor of male fertility. Fertil Steril 2013;99(2):354-61.
805
806 [86] Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R,
807 Gibb Z, Crossett B, Tsikis G, Labas V, Harichaux G, Grupen CG, De Graaf SP. Proteomic
808 characterization and cross species comparison of mammalian seminal plasma. J Proteom
809 2013;91:13-22.
810

[87] Bunch DO, Welch JE, Magyar PL, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S protein distribution during mouse spermatogenesis. Biol Reprod 1998;58:834-841.

[88] Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci 2004,101:16501-16506.

[89] Parte PP, Rao P, Redij S, Lobo V, D'Souza SJ, Gajbhiye R, Kulkarni V. Sperm phosphoproteome profiling by ultra performance liquid chromatography followed by data independent analysis (LC-MSE) reveals altered proteomic signatures in asthenozoospermia. J Proteomics 2012;75:5861-5871.

824 **Supplement 1.** Proteins found in seminal plasma of evaluated dogs and their respective genic ontology.

Protein	Gene	ID	Organism	Molecular function	Biological process	Cellular component
5-methylcytosine rRNA methyltransferase NSUN4	NSUN4	Q96CB9	*Homo sapiens*	methyltransferase activity; rRNA (cytosine-C5-)-methyltransferase activity; small ribosomal subunit rRNA binding	mature ribosome assembly; positive regulation of mitochondrial translation; rRNA methylation	mitochondrion
60 kDa heat shock protein, mitochondrial	HSPD1	Q5NVM5	*Pongo abelii*	ATP binding; isomerase activity	protein refolding; response to cold	mitochondrion; cytoplasm; protein-containing complex
Aconitate hydratase, mitochondrial	Aco2	Q9ER34	*Rattus norvegicus*	3 iron, 4 sulfur cluster binding; 4 iron, 4 sulfur cluster binding; aconitate hydratase activity; iron ion binding	citrate metabolic process; isocitrate metabolic process; liver development; response to isolation stress; tricarboxylic acid cycle	cytosol; mitochondrion
Acrosin	ACR	Q9GL10	*Ovis aries*	amidase activity; drug binding; fucose binding; mannose binding; serine-type endopeptidase activity	acrosome reaction; activation of adenylate cyclase activity; single fertilization	acrosomal vesicle
Acrosin	Acr	P23578	*Mus musculus*	amidase activity; drug binding; fucose binding; mannose binding; peptidase	acrosome matrix dispersal; acrosome reaction; activation of adenylate cyclase	acrosomal matrix; acrosomal vesicle; golgi-associated vesicle; protein-containing complex

				activity; serine-type endopeptidase activity	activity; binding of sperm to zona pellucida; penetration of zona pellucida; protein catabolic process; response to steroid hormone; single fertilization	
Acrosin	ACR	P08001	*Sus scrofa*	amidase activity; drug binding; fucose binding; mannose binding; serine-type endopeptidase activity	acrosome reaction; activation of adenylate cyclase activity; single fertilization	acrosomal matrix; protein-containing complex
Acrosin-binding protein	ACRBP	Q29016	*Sus scrofa*	-	acrosome assembly; sperm capacitation	extracellular region or secreted; acrosomal membrane; acrosomal vesicle
Acrosin-binding protein	ACRBP	Q60485	*Cavia porcellus*	-	acrosome assembly	extracellular region or secreted; acrosomal membrane; acrosomal vesicle
Acrosin-binding protein	ACRBP	Q8NEB7	*Homo sapiens*	-	acrosome assembly; fertilization	extracellular region or secreted; nucleus; acrosomal membrane; acrosomal vesicle
Acrosin-binding protein	ACRBP	Q3V140	*Mus musculus*	peptidase activator activity	acrosome assembly; fertilization;	extracellular region or secreted; acrosomal

					spermatid development	membrane; acrosomal vesicle
Acrosin-binding protein	Acrbp	Q6AY33	*Rattus norvegicus*	-	acrosome assembly	extracellular region or secreted; acrosomal membrane; acrosomal vesicle
Acrosomal protein SP-10	ACRV1	Q06990	*Papio hamadryas*	-	-	acrosomal vesicle
Actin, cytoplasmic 1	ACTB	Q0PGG4	*Bos mutus grunniens*	ATP binding	-	cytoskeleton; nucleus; plasma membrane; dense body; focal adhesion; protein-containing complex
Actin, cytoplasmic 1	ACTB	Q4L0Y2	*Spermophilus citellus*	ATP binding	-	cytoskeleton; nucleus; plasma membrane; dense body; focal adhesion; protein-containing complex
A-kinase anchor protein 4	Akap4	O35774	*Rattus norvegicus*	protein kinase A binding	cell projection organization; flagellated sperm motility; protein localization	cytoplasm; motile cilium; sperm fibrous sheath; sperm principal piece
Alpha-1-antiproteinase	*N/A*	P12725	*Ovis aries*	serine-type endopeptidase inhibitor activity	-	extracellular region or secreted; golgi apparatus; plasma membrane; vesicle
Alpha-2-HS-glycoprotein	AHSG	P12763	*Bos taurus*	cysteine-type endopeptidase inhibitor activity	acute-phase response; negative regulation of bone mineralization; negative regulation of endopeptidase	extracellular region or secreted; golgi apparatus; plasma membrane; vesicle

					activity; ossification; positive regulation of phagocytosis; regulation of inflammatory response	
Alpha-enolase	Eno1	P04764	*Rattus norvegicus*	enzyme binding; heat shock protein binding; magnesium ion binding; phosphopyruvate hydratase activity; protein heterodimerization activity; protein homodimerization activity	canonical glycolysis; cellular response to hypoxia; ERK1 and ERK2 cascade; glycolytic process; protein heterotetramerization	cytosol; plasma membrane; cell cortex region; cell surface; cytoplasm; growth cone; membrane raft; neuron projection
Apolipoprotein A-I	APOA1	P15497	*Bos taurus*	amyloid-beta binding; apolipoprotein A-I receptor binding; chemorepellent activity; cholesterol binding; cholesterol transporter activity; enzyme binding; heat shock protein binding; high-density lipoprotein particle binding;	adrenal gland development; blood vessel endothelial cell migration; cholesterol biosynthetic process; cholesterol efflux; cholesterol homeostasis; cholesterol import; endothelial cell proliferation; glucocorticoid	extracellular region or secreted; endocytic vesicle

		high-density lipoprotein particle receptor binding; identical protein binding; phosphatidylcholine-sterol O-acyltransferase activator activity; phospholipid binding	metabolic process; G protein-coupled receptor signaling pathway; high-density lipoprotein particle assembly; high-density lipoprotein particle remodeling; integrin-mediated signaling pathway; lipid storage; lipoprotein biosynthetic process; lipoprotein metabolic process; negative regulation of cell adhesion molecule production; negative regulation of cytokine secretion involved in immune response; negative regulation of heterotypic cell-cell adhesion; negative regulation of hydrolase activity; negative regulation	

		of inflammatory response; negative regulation of interleukin-1 beta secretion; negative regulation of tumor necrosis factor-mediated signaling pathway; negative regulation of very-low-density lipoprotein particle remodeling; peptidyl-methionine modification; phosphatidylcholine biosynthetic process; phospholipid efflux; phospholipid homeostasis; positive regulation of cholesterol efflux; positive regulation of cholesterol esterification; positive regulation of hydrolase activity; positive regulation of lipid biosynthetic process; positive	

		regulation of phagocytosis; positive regulation of phospholipid efflux; positive regulation of Rho protein signal transduction; positive regulation of stress fiber assembly; positive regulation of substrate adhesion-dependent cell spreading; protein oxidation; protein stabilization; regulation of Cdc42 protein signal transduction; regulation of cholesterol transport; regulation of intestinal cholesterol absorption; regulation of protein phosphorylation; reverse cholesterol transport; triglyceride catabolic process; triglyceride	

					homeostasis; very-low-density lipoprotein particle remodeling; vitamin transport	
Apolipoprotein A-I	APOA1	P0DMB0	*Tursiops truncatus*	lipid binding	cholesterol metabolic process; lipid transport; lipoprotein metabolic process; positive regulation of cholesterol efflux; positive regulation of phagocytosis; positive regulation of phospholipid efflux; protein stabilization	extracellular region or secreted
ATP synthase subunit alpha, mitochondrial	ATP5F1A	A5A6H5	*Pan troglodytes*	ADP binding; ATP binding; proton-transporting ATP synthase activity; rotational mechanism	ATP synthesis coupled proton transport	mitochondrion; plasma membrane
ATP synthase subunit beta, mitochondrial	ATP5F1B	Q0QEP2	*Mesocricetus auratus*	ATP binding; proton-transporting ATP synthase activity; rotational mechanism	ATP synthesis coupled proton transport	mitochondrion; proton-transporting ATP synthase complex; catalytic core F(1)

ATP synthase subunit beta, mitochondrial	ATP5F1B	P00829	*Bos taurus*	angiostatin binding; ATPase activity; ATP binding; MHC class I protein binding; proton-transporting ATPase activity, rotational mechanism; proton-transporting ATP synthase activity, rotational mechanism	angiogenesis; cellular response to interleukin-7; lipid metabolic process; mitochondrial ATP synthesis coupled proton transport; negative regulation of cell adhesion involved in substrate-bound cell migration; positive regulation of blood vessel endothelial cell migration; regulation of intracellular pH	mitochondrion; plasma membrane; cell surface; proton-transporting ATP synthase complex; catalytic core F(1)
Centrosomal protein of 44 kDa	CEP44	Q08DB0	*Bos taurus*	-	-	cytoskeleton; cytoplasm; midbody
Complement C3	C3	Q2UVX4	*Bos taurus*	C5L2 anaphylatoxin chemotactic receptor binding; endopeptidase inhibitor activity	amyloid-beta clearance; cell surface receptor signaling pathway involved in cell-cell signaling; complement activation; complement activation, alternative pathway;	extracellular region or secreted; cell surface; protein-containing complex

		complement activation, classical pathway; complement-dependent cytotoxicity; complement-mediated synapse pruning; fatty acid metabolic process; inflammatory response; neuron remodeling; positive regulation of activation of membrane attack complex; positive regulation of angiogenesis; positive regulation of apoptotic cell clearance; positive regulation of glucose transmembrane transport; positive regulation of G protein-coupled receptor signaling pathway; positive regulation of lipid

					storage; positive regulation of phagocytosis, engulfment; positive regulation of protein phosphorylation; positive regulation of receptor-mediated endocytosis; positive regulation of type IIa hypersensitivity; positive regulation of vascular endothelial growth factor production; regulation of triglyceride biosynthetic process; response to bacterium; vertebrate eye-specific patterning	
Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	COX4I1	P00423	*Bos taurus*	cytochrome-c oxidase activity	mitochondrial electron transport; cytochrome c to oxygen	mitochondrion; nucleus; integral component of membrane; respiratory chain complex IV
Dynein light chain 1, cytoplasmic	DYNLL1	P61273	*Macaca fascicularis*	motor activity	apoptotic process; microtubule-based process; negative	cytoskeleton; mitochondrion ; nucleus; kinetochore

						regulation of phosphorylation	
E3 ubiquitin-protein ligase TRIM21	TRIM21	P19474	*Homo sapiens*	DNA binding; identical protein binding; RNA binding; ubiquitin-protein transferase activity; zinc ion binding	cell cycle; innate immune response; interferon-gamma-mediated signaling pathway; negative regulation of NF-kappaB transcription factor activity; negative regulation of protein deubiquitination; negative regulation of viral release from host cell; negative regulation of viral transcription; positive regulation of autophagy; positive regulation of cell cycle; positive regulation of DNA-binding transcription factor activity; positive regulation of viral entry into host cell; protein autoubiquitination; protein	cytosol; nucleus; autophagosome; cytoplasm; cytoplasmic vesicle; p-body; ribonucleoprotein complex

					destabilization; protein monoubiquitination; protein polyubiquitination; protein trimerization; protein ubiquitination; regulation of type I interferon production; response to interferon-gamma	
Fibrinogen beta chain	FGB	P02676	*Bos taurus*	signaling receptor binding	adaptive immune response; innate immune response; platelet activation; protein polymerization	extracellular region or secreted
Fibrinogen gamma-B chain	FGG	P12799	*Bos taurus*	metal ion binding; signaling receptor binding	platelet activation; protein polymerization	extracellular region or secreted
Fructose-bisphosphate aldolase A	ALDOA	A5A6I5	*Pan troglodytes*	fructose-bisphosphate aldolase activity	fructose 1,6-bisphosphate metabolic process; glycolytic process; protein homotetramerization	cytosol; I band; M band
Glutathione S-transferase Mu 1	GSTM1	Q9N0V4	*Bos taurus*	glutathione transferase activity	glutathione metabolic process;	cytosol

					xenobiotic catabolic process	
Glutathione S-transferase Mu 1	Gstm1	P10649	*Mus musculus*	glutathione binding; glutathione transferase activity; identical protein binding; nickel cation binding; protein heterodimerization activity; protein homodimerization activity; protein kinase binding; steroid binding	cellular response to drug; glutathione metabolic process; xenobiotic catabolic process	cytosol; extracellular region or secreted; myelin sheath; protein-containing complex
Glutathione S-transferase Mu 2	Gstm2	P15626	*Mus musculus*	enzyme binding; glutathione binding; glutathione transferase activity; protein heterodimerization activity; protein homodimerization activity	cellular detoxification of nitrogen compound; glutathione metabolic process; nitrobenzene metabolic process; xenobiotic catabolic process	cytosol; extracellular region or secreted; plasma membrane; cytoplasm; protein-containing complex
Glutathione S-transferase Mu 2	GSTM2	Q5R8E8	*Pongo abelii*	glutathione transferase activity	glutathione metabolic process	cytoplasm
Glutathione S-transferase Mu 3	GSTM3	Q9BEA9	*Macaca fuscata fuscata*	glutathione transferase activity	glutathione metabolic process	cytoplasm

Glutathione S-transferase Mu 5	Gstm5	Q9Z1B2	*Rattus norvegicus*	glutathione transferase activity; protein homodimerization activity	glutathione metabolic process; xenobiotic catabolic process	cytosol
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	A3FKF7	*Mustela putorius furo*	glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity; microtubule binding; NAD binding; NADP binding; peptidyl-cysteine S-nitrosylase activity	glucose metabolic process; glycolytic process; microtubule cytoskeleton organization; neuron apoptotic process; peptidyl-cysteine S-trans-nitrosylation; protein stabilization; regulation of translation	cytoskeleton; cytosol; nucleus; cytoplasm; GAIT complex
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAPDHS	Q2KJE5	*Bos taurus*	glyceraldehyde-3-phosphate dehydrogenase (NAD+) (phosphorylating) activity; NAD binding; NADP binding	glucose metabolic process; glycolytic process	cytosol
Hexokinase-1	HK1	Q5RC71	*Pongo abelii*	ATP binding; glucose binding; hexokinase activity	cellular glucose homeostasis; glycolytic process; hexose metabolic process	cell

Histone H2B type 1-K	HIST1H2BK	Q2M2T1	*Bos taurus*	DNA binding; protein heterodimerization activity	nucleosome assembly	nucleus; nucleosome
Histone H3.3C-like	*N/A*	Q3SZB8	*Bos taurus*	nucleosomal DNA binding; protein heterodimerization activity	-	nucleus; nucleosome
Histone H4	QtsA-19327	Q4R362	*Macaca fascicularis*	DNA binding; protein heterodimerization activity	DNA-templated transcription, initiation	nucleus; nucleosome
Inositol polyphosphate 5-phosphatase OCRL-1	OCRL	Q01968	*Homo sapiens*	GTPase activator activity; inositol phosphate phosphatase activity; phosphatidylinositol -4,5-bisphosphate 5-phosphatase activity; Rac GTPase binding	cilium assembly; inositol phosphate dephosphorylation; inositol phosphate metabolic process; in utero embryonic development; lipid metabolic process; membrane organization; phosphatidylinositol biosynthetic process; phosphatidylinositol dephosphorylation; regulation of GTPase activity; regulation of small GTPase mediated signal	cytosol; endosome; golgi apparatus; nucleus; plasma membrane; clathrin-coated pit; clathrin-coated vesicle; membrane; phagocytic vesicle membrane; photoreceptor outer segment

					transduction; signal transduction	
Izumo sperm-egg fusion protein 4	Izumo4	D3Z690	*Mus musculus*	-	-	extracellular region or secreted
Keratin, type I cytoskeletal 10	KRT10	P06394	*Bos taurus*	protein heterodimerization activity; structural molecule activity	positive regulation of epidermis development; protein heterotetramerizatio n	cytoskeleton
Keratin, type I cytoskeletal 10	Krt10	P02535	*Mus musculus*	cytoskeletal protein binding; protein heterodimerization activity; structural molecule activity	cellular response to calcium ion; epidermis development; epithelial cell differentiation; intermediate filament organization; keratinocyte development; positive regulation of epidermis development; protein heterotetramerizatio n	cytoskeleton; cell surface
Keratin, type I cytoskeletal 10	Krt10	Q6IFW6	*Rattus norvegicus*	protein heterodimerization activity; structural	keratinocyte differentiation; peptide cross-	cytoskeleton; plasma membrane; cytoplasm

					constituent of epidermis	linking; positive regulation of epidermis development; protein heterotetramerizatio n	
Keratin, type cytoskeletal 13	**I**	Krt13	Q6IFV4	*Rattus norvegicus*	structural molecule activity	cellular response to retinoic acid; response to radiation; tongue morphogenesis	cytoskeleton
Keratin, type cytoskeletal 15	**I**	KRT15	O77727	*Ovis aries*	structural molecule activity	-	cytoskeleton
Keratin, type cytoskeletal 15	**I**	Krt15	Q6IFV3	*Rattus norvegicus*	scaffold protein binding; structural constituent of cytoskeleton	intermediate filament-based process	cytoskeleton
Keratin, type cytoskeletal 17	**I**	KRT17	A5A6M0	*Pan troglodytes*	structural molecule activity	hair follicle morphogenesis	cytoskeleton; cytoplasm
Keratin, type cytoskeletal 19	**I**	KRT19	Q5R8S9	*Pongo abelii*	structural molecule activity	-	cytoskeleton
Keratin, type cytoskeletal 20	**I**	Krt20	Q9D312	*Mus musculus*	structural molecule activity	apoptotic process; cellular response to starvation; intermediate filament organization; regulation of protein secretion	cytoskeleton; cytosol; cytoplasm

Keratin, type II cytoskeletal 1	Krt1	P04104	*Mus musculus*	carbohydrate binding; protein heterodimerization activity; structural constituent of epidermis	complement activation, lectin pathway; establishment of skin barrier; negative regulation of inflammatory response; peptide cross-linking; protein heterotetramerizatio n	cytoskeleton; extracellular region or secreted; plasma membrane; membrane	
Keratin, type II cytoskeletal 1	KRT1	A5A6M6	*Pan troglodytes*	protein heterodimerization activity	protein heterotetramerizatio n	cytoskeleton; plasma membrane	
Keratin, type II cytoskeletal 1b	Krt77	Q6IG01	*Rattus norvegicus*	-	-	cytoskeleton	
Keratin, type II cytoskeletal epidermal 2	Krt2	Q3TTY5	*Mus musculus*	cytoskeletal protein binding; structural constituent of epidermis; structural molecule activity	epidermis development; intermediate filament organization; keratinization; keratinocyte activation; keratinocyte development; keratinocyte migration; keratinocyte	cytoskeleton; plasma membrane	

					proliferation; peptide cross-linking; positive regulation of epidermis development	
Keratin, type II cytoskeletal 2 oral	Krt76	Q3UV17	*Mus musculus*	-	epidermis development; pigmentation; sebaceous gland development	cytoskeleton
Keratin, type II cytoskeletal 5	KRT5	Q5XQN5	*Bos taurus*	-	epithelial cell differentiation; negative regulation of epithelial cell proliferation	cytoskeleton
Keratin, type II cytoskeletal 5	KRT5	A5A6M8	*Pan troglodytes*	-	-	cytoskeleton
Keratin, type II cytoskeletal 5	Krt5	Q6P6Q2	*Rattus norvegicus*	-	-	cytoskeleton
Keratin, type II cytoskeletal 6A	Krt6a	Q4FZU2	*Rattus norvegicus*	-	morphogenesis of an epithelium; wound healing	cytoskeleton
Keratin, type II cytoskeletal 7	KRT7	A5A6N0	*Pan troglodytes*	-	-	cytoskeleton
Lactotransferrin	LTF	O77698	*Bubalus bubalis*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; bone morphogenesis; innate immune	extracellular region or secreted; specific granule

		response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation;	

					positive regulation of osteoblast differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
Lactotransferrin	LTF	Q9TUM0	*Camelus dromedarius*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; bone morphogenesis; innate immune response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development;	extracellular region or secreted; specific granule

					negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of osteoblast differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
L-amino-acid oxidase	IL4I1	Q96RQ9	*Homo sapiens*	L-amino-acid oxidase activity;	cellular amino acid catabolic process; L-	extracellular region or secreted; lisossome

				oxidoreductase activity	phenylalanine catabolic process	
L-lactate dehydrogenase A-like 6B	LDHAL6B	Q9BYZ2	*Homo sapiens*	L-lactate dehydrogenase activity	carbohydrate metabolic process; pyruvate metabolic process	mitochondrion; nucleus
L-lactate dehydrogenase C chain	LDHC	Q9TSX5	*Sus scrofa*	L-lactate dehydrogenase activity	carbohydrate metabolic process; carboxylic acid metabolic process	cytoplasm
L-lactate dehydrogenase C chain	LDHC	Q29563	*Vulpes vulpes*	L-lactate dehydrogenase activity	carbohydrate metabolic process; carboxylic acid metabolic process	cytoplasm
Lysozyme C, milk isozyme	*N/A*	Q6B411	*Bos taurus*	lysozyme activity	cytolysis; defense response to Gram-negative bacterium; defense response to Gram-positive bacterium; metabolic process	-
Lysozyme C-1	*N/A*	P12067	*Sus scrofa*	lysozyme activity	cytolysis; defense response to Gram-negative bacterium; defense response to Gram-positive bacterium; digestion; metabolic process	extracellular region or secreted

Transport and Golgi organization protein 1 homolog	Mia3	Q8BI84	*Mus musculus*	cargo receptor activity	cell migration involved in sprouting angiogenesis; cellular response to oxidised low-density lipoprotein particle stimulus; chondrocyte development; collagen fibril organization; COPII-coated vesicle cargo loading; endoplasmic reticulum organization; endoplasmic reticulum to Golgi vesicle-mediated transport; exocytosis; lipoprotein transport; negative regulation of cell adhesion; negative regulation of cell migration; negative regulation of leukocyte cell-cell adhesion; negative regulation of	endoplasmic reticulum; golgi apparatus; integral component of membrane; intracellular membrane-bounded organelle

					lymphocyte migration; positive regulation of bone mineralization; positive regulation of leukocyte migration; protein localization to endoplasmic reticulum exit site; protein secretion; protein transport; vesicle cargo loading; wound healing	
Myeloperoxidase	Mpo	P11247	*Mus musculus*	heme binding; heparin binding; metal ion binding; peroxidase activity	aging; defense response to bacterium; defense response to fungus; hydrogen peroxide catabolic process; hypochlorous acid biosynthetic process; low-density lipoprotein particle remodeling; negative regulation of growth of symbiont in host; oxidation-reduction process; removal of superoxide radicals;	extracellular region or secreted; lysosome; mitochondrion; nucleus; cytoplasm; intracellular membrane-bounded organelle; secretory granule

					respiratory burst involved in defense response; response to food; response to gold nanoparticle; response to lipopolysaccharide; response to mechanical stimulus; response to yeast	
Olfactory receptor 4D10	OR4D10	Q8NGI6	*Homo sapiens*	G protein-coupled receptor activity;olfactory receptor activity	G protein-coupled receptor signaling pathway	plasma membrane; integral component of membrane
Outer dense fiber protein 2	ODF2	Q2T9U2	*Bos taurus*	-	cell differentiation; multicellular organism development; spermatogenesis	cytoskeleton; sperm flagellum
Outer dense fiber protein 2	ODF2	Q5BJF6	*Homo sapiens*	Rab GTPase binding; structural molecule activity	centriole-centriole cohesion; ciliary basal body-plasma membrane docking; cilium organization; G2/M transition of mitotic cell cycle; multicellular organism development; protein localization;	cytoskeleton; cytosol; nucleus; centriolar subdistal appendage; ciliary transition fiber; cilium; sperm flagellum

					regulation of cilium assembly; regulation of G2/M transition of mitotic cell cycle; spermatid development	
Outer dense fiber protein 2	Odf2	A3KGV1	*Mus musculus*	Rab GTPase binding;structural constituent of cytoskeleton	centriole-centriole cohesion; cilium organization; multicellular organism development; protein localization; regulation of cilium assembly; spermatid development	cytoskeleton; centriolar subdistal appendage; ciliary transition fiber; cilium; sperm flagellum
Outer dense fiber protein 2	Odf2	Q6AYX5	*Rattus norvegicus*	-	centriole-centriole cohesion; cilium organization; multicellular organism development; protein localization; spermatid development	cytoskeleton; centriolar subdistal appendage; ciliary transition fiber
Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A	INPP5J	Q15735	*Homo sapiens*	inositol-1,3,4,5-tetrakisphosphate 5-phosphatase activity; inositol-1,4,5-trisphosphate 5-	inositol phosphate dephosphorylation; inositol phosphate metabolic process; negative regulation	cytosol; plasma membrane; cytoplasm; dendritic shaft; growth cone; ruffle

				phosphatase activity; inositol-polyphosphate 5-phosphatase activity; phosphatidylinositol -3,4,5-trisphosphate 5-phosphatase activity; phosphatidylinositol -4,5-bisphosphate 5-phosphatase activity; SH3 domain binding	of microtubule polymerization; negative regulation of neuron projection development; negative regulation of peptidyl-serine phosphorylation; phosphatidylinositol biosynthetic process; phosphatidylinositol dephosphorylation	
Phospholipid hydroperoxide glutathione peroxidase	GPX4	Q9N2J2	*Bos taurus*	glutathione peroxidase activity; identical protein binding; peroxidase activity; phospholipid-hydroperoxide glutathione peroxidase activity	chromatin organization; multicellular organism development; negative regulation of ferroptosis; protein polymerization; response to oxidative stress; spermatogenesis	cytosol; mitochondrion; nucleus; protein-containing complex
Protein MENT	MENT	Q9BUN1	*Homo sapiens*	-	regulation of cell population proliferation	extracellular region or secreted
Pyruvate dehydrogenase E1	PDHB	P11966	*Bos taurus*	pyruvate dehydrogenase (acetyl-transferring)	acetyl-CoA biosynthetic process from pyruvate;	mitochondrion; pyruvate dehydrogenase complex

component subunit beta, mitocondrial				activity; pyruvate dehydrogenase activity	glucose metabolic process; tricarboxylic acid cycle	
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	Q5RE79	*Pongo abelii*	pyruvate dehydrogenase (acetyl-transferring) activity; pyruvate dehydrogenase (NAD+) activity; pyruvate dehydrogenase activity	acetyl-CoA biosynthetic process from pyruvate; glucose metabolic process; tricarboxylic acid cycle	mitochondrion; nucleus; pyruvate dehydrogenase complex
Pyruvate kinase PKM	PKM	Q5NVN0	*Pongo abelii*	ATP binding; kinase activity; magnesium ion binding; potassium ion binding; pyruvate kinase activity	positive regulation of sprouting angiogenesis	nucleus; cytoplasm
RalBP1-associated Eps domain-containing protein 2	Reps2	Q80XA6	*Mus musculus*	calcium ion binding	-	cytoplasm
Ras-related protein Rab-2A	RAB2A	Q4R4X6	*Macaca fascicularis*	GDP binding; GTPase activity; GTP binding	Golgi organization; protein transport; vesicle-mediated transport	endoplasmic reticulum; golgi apparatus, endoplasmic reticulum-golgi intermediate compartment membrane
RNA-binding protein MEX3A	MEX3A	A1L020	*Homo sapiens*	metal ion binding; RNA binding	-	cytosol; nucleus, p-body

Ropporin-1	ROPN1	Q3T064	*Bos taurus*	-	cilium organization; flagellated sperm motility; protein localization to cilium; regulation of protein phosphorylation; sperm capacitation	motile cilium
Saccharopine dehydrogenase-like oxidoreductase	SCCPDH	Q3T067	*Bos taurus*	oxidoreductase activity	glycolipid biosynthetic process	mitochondrion; plasma membrane; lipid droplet
Saccharopine dehydrogenase-like oxidoreductase	SCCPDH	Q5R5C9	*Pongo abelii*	oxidoreductase activity	-	-
Serotransferrin	TF	Q29443	*Bos taurus*	ferric iron binding; ferric iron transmembrane transporter activity	cellular iron ion homeostasis	extracellular region or secreted; cell
Serpin A3-2	SERPINA3-2	A2I7M9	*Bos taurus*	serine-type endopeptidase inhibitor activity	negative regulation of endopeptidase activity	extracellular region or secreted; chromaffin granule; cytoplasmic vesicle
Serum albumin	ALB	P02768	*Homo sapiens*	antioxidant activity; chaperone binding; copper ion binding; DNA binding; drug binding; enterobactin binding; exogenous protein binding; fatty acid binding;	cellular protein metabolic process; cellular response to starvation; high-density lipoprotein particle remodeling; maintenance of mitochondrion location; negative	endoplasmic reticulum; extracellular region or secreted; golgi apparatus; nucleus; platelet alpha granule lumen; protein-containing complex

				identical protein binding; pyridoxal phosphate binding; toxic substance binding	regulation of apoptotic process; negative regulation of programmed cell death; negative regulation of protein oligomerization; platelet degranulation; post-translational protein modification; receptor-mediated endocytosis; retina homeostasis	
Serum albumin	ALB	P14639	*Ovis aries*	enterobactin binding; lipid binding; metal ion binding	-	extracellular region or secreted
Solute carrier family 2, facilitated glucose transporter member 3	SLC2A3	P47843	*Ovis aries*	dehydroascorbic acid transmembrane transporter activity; D-glucose transmembrane transporter activity; glucose binding; glucose transmembrane transporter activity	glucose transmembrane transport	plasma membrane; acrosomal membrane; cell projection; integral component of membrane; perikaryon

Solute carrier family 2, facilitated glucose transporter member 3	SLC2A3	Q5R608	*Pongo abelii*	glucose binding; glucose transmembrane transporter activity	glucose transmembrane transport	plasma membrane; cell projection; integral component of membrane; perikaryon
Sperm acrosomal protein FSA-ACR.1	*N/A*	P53353	*Vulpes vulpes*	-	-	acrosomal vesicle
Sperm acrosome membrane-associated protein 1	Spaca1	Q9DA48	*Mus musculus*	-	acrosome assembly; spermatogenesis	acrosomal membrane; inner acrosomal membrane; integral component of membrane
Superoxide dismutase [Mn], mitochondrial	SOD2	Q8HXP0	*Callithrix jacchus*	manganese ion binding; superoxide dismutase activity	age-dependent response to reactive oxygen species; regulation of transcription by RNA polymerase II	mitochondrial
Superoxide dismutase [Mn], mitochondrial	SOD2	Q5FB30	*Macaca nemestrina*	manganese ion binding; superoxide dismutase activity	-	mitochondrion
Testis-specific H1 histone	H1FNT	Q75WM6	*Homo sapiens*	ATP binding; double-stranded DNA binding; nucleosomal DNA binding	chromosome condensation; multicellular organism development; negative regulation of chromatin silencing; negative regulation of DNA recombination; nucleosome	nucleus

					positioning; regulation of transcription, DNA-templated; spermatid nucleus elongation; spermatogenesis; sperm chromatin condensation	
Transmembrane protein 190	TMEM190	E1BJD3	*Bos taurus*	-	hematopoietic progenitor cell differentiation	inner acrosomal membrane; integral component of membrane
Tubulin alpha-3 chain	TUBA3	Q32KN8	*Bos taurus*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle	cytoskeleton, cytoplasm
Tubulin alpha-3 chain	Tuba3a	Q68FR8	*Rattus norvegicus*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle	cytoskeleton; cytoplasm
Tubulin alpha-3E chain	TUBA3E	Q6PEY2	*Homo sapiens*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle	cytoskeleton, nucleus, cytoplasm
Tubulin alpha-8 chain	TUBA8	Q2HJB8	*Bos taurus*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton	cytoskeleton, cytoplasm

						organization; mitotic cell cycle	
Tubulin chain	**beta-1**	Tubb1	A2AQ07	*Mus musculus*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle; spindle assembly	cytoskeleton, cytoplasm
Tubulin chain	**beta-4B**	TUBB4B	Q3MHM5	*Bos taurus*	GTPase activity; GTP binding; structural constituent of cytoskeleton	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle	cytoskeleton, cytoplasm
Tubulin chain	**beta-5**	TUBB5	Q2KJD0	*Bos taurus*	GTPase activity; GTP binding; MHC class I protein binding; structural constituent of cytoskeleton; ubiquitin protein ligase binding	microtubule-based process; microtubule cytoskeleton organization; mitotic cell cycle; regulation of synapse organization; spindle assembly	cytoskeleton; nucleus; cell body; cytoplasm; cytoplasmic ribonucleoprotein granule
Voltage-dependent anion-selective channel protein 2		VDAC2	Q9MZ15	*Sus scrofa*	nucleotide binding; porin activity; voltage-gated anion channel activity	-	mitochondrion; pore complex
Voltage-dependent anion-selective channel protein 3		VDAC3	Q9TT13	*Oryctolagus cuniculus*	nucleotide binding; porin activity; voltage-gated anion channel activity	behavioral fear response; learning; neuron-neuron synaptic transmission;	mitochondrion; pore complex

					regulation of cilium assembly	
Voltage-dependent anion-selective channel protein 3	VDAC3	Q5R7V4	*Pongo abelii*	nucleotide binding; porin activity; voltage-gated anion channel activity	regulation of cilium assembly	mitochondrion; pore complex
V-type proton ATPase subunit S1	ATP6AP1	Q15904	*Homo sapiens*	ATP binding; proton-transporting ATPase activity, rotational mechanism; proton-transporting ATP synthase activity, rotational mechanism; Rab GTPase binding; transporter activity	ATP hydrolysis coupled proton transport; cellular iron ion homeostasis; cellular response to increased oxygen levels; establishment of organelle localization; insulin receptor signaling pathway; ion transmembrane transport; pH reduction; positive regulation of bone resorption; positive regulation of ERK1 and ERK2 cascade; positive regulation of exocytosis; positive regulation of osteoblast differentiation; positive regulation of	endoplasmic reticulum; endosome; extracellular region or secreted; plasma membrane; endoplasmic reticulum-Golgi intermediate compartment membrane; integral component of membrane; proton-transporting two-sector ATPase complex; proton-transporting V-type ATPase, V1 domain

				osteoclast development; proton transmembrane transport; regulation of cellular pH; transferrin transport		
Zona pellucida-binding protein 1	ZPBP	Q29108	*Sus scrofa*	binding of sperm to zona pellucia	extracellular region or secreted; acrosomal membrane	

825

826 **Supplement 2.** Proteins found in spermatozoa of all dogs evaluated and their respective genic ontology.

Protein	Gene	ID	Organism	Molecular function	Biological process	Cellular component
Alkaline phosphatase, tissue-nonspecific isozyme	ALPL	P09487	*Bos taurus*	alkaline phosphatase activity; metal ion binding	biomineral tissue development	extracellular region or secreted; plasma membrane; anchored component of membrane
Apolipoprotein A-I	APOA1	P0DJG0	*Pan troglodytes*	cholesterol binding; cholesterol transporter activity; high-density lipoprotein particle receptor binding; phosphatidylcholine-sterol O-acyltransferase activator	cholesterol biosynthetic process; cholesterol efflux; cholesterol homeostasis; high-density lipoprotein particle assembly; lipoprotein metabolic	extracellular region or secreted

				activity; phospholipid binding	process; phospholipid efflux; positive regulation of cholesterol efflux; positive regulation of cholesterol esterification; positive regulation of lipid biosynthetic process; positive regulation of phagocytosis; positive regulation of phospholipid efflux; protein stabilization; regulation of cholesterol transport; reverse cholesterol transport; triglyceride catabolic process; very-low-density lipoprotein particle remodeling	
Apolipoprotein A-II	APOA2	P0DJG2	*Gorilla gorilla gorilla*	apolipoprotein receptor binding; cholesterol binding; high-density lipoprotein particle binding; high-density lipoprotein particle receptor binding; lipase inhibitor activity; phosphatidylcholine binding	cholesterol homeostasis; cholesterol metabolic process; cholesterol transport; high-density lipoprotein particle assembly; high-density lipoprotein particle remodeling;	extracellular region or secreted

					lipoprotein metabolic process; low-density lipoprotein particle remodeling; negative regulation of lipase activity; peptidyl-methionine modification; positive regulation of phagocytosis; protein oxidation; protein stabilization; triglyceride-rich lipoprotein particle remodeling	
Apolipoprotein C-III	APOC3	P02656	*Homo sapiens*	cholesterol binding; enzyme regulator activity; high-density lipoprotein particle receptor binding; lipase inhibitor activity; phospholipid binding	cholesterol efflux; cholesterol homeostasis; chylomicron assembly; chylomicron remnant clearance; chylomicron remodeling; G protein-coupled receptor signaling pathway; high-density lipoprotein particle remodeling; lipoprotein metabolic process; negative regulation of cholesterol import;	endosome; extracellular region or secreted

		negative regulation of fatty acid biosynthetic process; negative regulation of high-density lipoprotein particle clearance; negative regulation of lipid catabolic process; negative regulation of lipid metabolic process; negative regulation of lipoprotein lipase activity; negative regulation of low-density lipoprotein particle clearance; negative regulation of receptor-mediated endocytosis; negative regulation of triglyceride catabolic process; negative regulation of very-low-density lipoprotein particle clearance; negative regulation of very-low-density lipoprotein particle remodeling; phospholipid efflux;	

					regulation of Cdc42 protein signal transduction; retinoid metabolic process; reverse cholesterol transport; triglyceride catabolic process; triglyceride homeostasis; triglyceride metabolic process; very-low-density lipoprotein particle assembly	
CDK5 regulatory subunit-associated protein 1	Cdk5rap1	Q9JLH6	*Rattus norvegicus*	4 iron, 4 sulfur cluster binding; metal ion binding; N6-isopentenyladenosine methylthiotransferase activity; protein-containing complex binding; protein kinase regulator activity	brain development; cell population proliferation; mitochondrial tRNA modification; negative regulation of cyclin-dependent protein serine/threonine kinase activity; positive regulation of mitochondrial translation; positive regulation of translational fidelity; regulation of cyclin-dependent protein serine/threonine kinase activity;	mitochondrion

						regulation of neuron differentiation; regulation of protein kinase activity	
Checkpoint protein HUS1	Hus1	Q8BQY8	*Mus musculus*	-		cellular response to DNA damage stimulus; cellular response to ionizing radiation; DNA damage checkpoint; double-strand break repair via homologous recombination; embryo development ending in birth or egg hatching; intra-S DNA damage checkpoint; meiotic DNA integrity checkpoint; mitotic cell cycle checkpoint; mitotic DNA replication checkpoint; negative regulation of DNA replication; nucleotide-excision repair; protein phosphorylation; regulation of protein phosphorylation; response to UV;	nucleus; cytoplasm; site of double-strand break

					telomere maintenance	
Chromogranin-A	CHGA	Q9XS63	*Equus caballus*	-	adenylate cyclase-activating adrenergic receptor signaling pathway involved in cardiac muscle relaxation; defense response to Gram-negative bacterium; defense response to Gram-positive bacterium; endocrine process; mast cell activation; mast cell chemotaxis; mast cell cytokine production; mast cell degranulation; negative regulation of catecholamine secretion; negative regulation of hormone secretion; negative regulation of insulin secretion; positive regulation of dense core granule biogenesis	extracellular region or secreted; lysosome; chromaffin granule; secretory granule; transport vesicle membrane
Clusterin	CLU	Q29482	*Equus caballus*	amyloid-beta binding; low-density lipoprotein particle receptor binding; misfolded protein binding; protein-	cell morphogenesis; central nervous system myelin maintenance;	cytoskeleton; cytosol; endoplasmic reticulum;

			containing complex binding; tau protein binding; ubiquitin protein ligase binding; unfolded protein binding	chaperone-mediated protein complex assembly; chaperone-mediated protein folding; immune complex clearance; intrinsic apoptotic signaling pathway; microglial cell activation; microglial cell proliferation; negative regulation of amyloid-beta formation; negative regulation of amyloid fibril formation; negative regulation of cellular response to thapsigargin; negative regulation of cellular response to tunicamycin; negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage; negative regulation of protein homooligomerization; negative regulation of response to endoplasmic reticulum stress;	extracellular region or secreted; mitochondrion; nucleus; apical dendrite; cell periphery; cell surface; chromaffin granule; cytoplasm; intracellular membrane-bounded organelle; neurofibrillary tangle; perinuclear region of cytoplasm; synapse

		positive regulation of amyloid-beta formation; positive regulation of apoptotic process; positive regulation of intrinsic apoptotic signaling pathway; positive regulation of neurofibrillary tangle assembly; positive regulation of neuron death; positive regulation of NF-kappaB transcription factor activity; positive regulation of nitric oxide biosynthetic process; positive regulation of proteasomal ubiquitin-dependent protein catabolic process; positive regulation of protein homooligomerization; positive regulation of receptor-mediated endocytosis; positive regulation of tau-protein kinase activity; positive regulation of tumor	

					necrosis factor production; positive regulation of ubiquitin-dependent protein catabolic process; protein import; protein stabilization; regulation of amyloid-beta clearance; regulation of cell population proliferation; regulation of neuronal signal transduction; response to misfolded protein; response to virus	
Clusterin	CLU	Q9XSC5	*Oryctolagus cuniculus*	misfolded protein binding; ubiquitin protein ligase binding; unfolded protein binding	chaperone-mediated protein folding; immune complex clearance; intrinsic apoptotic signaling pathway; negative regulation of amyloid fibril formation; negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage; negative regulation of protein	cytosol; endoplasmic reticulum; extracellular region or secreted; mitochondrion; nucleus; chromaffin granule; cytoplasm; intracellular membrane-

					homooligomerization; positive regulation of apoptotic process; positive regulation of intrinsic apoptotic signaling pathway; positive regulation of NF-kappaB transcription factor activity; positive regulation of proteasomal ubiquitin-dependent protein catabolic process; positive regulation of receptor-mediated endocytosis; positive regulation of ubiquitin-dependent protein catabolic process; protein stabilization; regulation of cell population proliferation; response to misfolded protein	bounded organelle; perinuclear region of cytoplasm
Clusterin	Clu	P05371	*Rattus norvegicus*	misfolded protein binding; protein N-terminus binding; ubiquitin protein ligase binding; unfolded protein binding	aging; cell morphogenesis; cellular response to growth factor stimulus; central	cytosol; endoplasmic reticulum; extracellular region or

		nervous system myelin maintenance; chaperone-mediated protein complex assembly; chaperone-mediated protein folding; endocrine pancreas development; estrous cycle; immune complex clearance; intrinsic apoptotic signaling pathway; microglial cell activation; microglial cell proliferation; negative regulation of amyloid-beta formation; negative regulation of amyloid fibril formation; negative regulation of apoptotic process; negative regulation of intrinsic apoptotic signaling pathway in response to DNA damage; negative regulation of protein homooligomerization; neuron projection morphogenesis; positive regulation of	secreted; mitochondrion; nucleus; aggresome; apical dendrite; chromaffin granule; cytoplasm; growth cone; neurofibrillary tangle; neuron projection; perinuclear region of cytoplasm

			amyloid-beta formation; positive regulation of apoptotic process; positive regulation of cell differentiation; positive regulation of cell population proliferation; positive regulation of gene expression; positive regulation of intrinsic apoptotic signaling pathway; positive regulation of neurofibrillary tangle assembly; positive regulation of neuron death; positive regulation of NF-kappaB transcription factor activity; positive regulation of nitric oxide biosynthetic process; positive regulation of proteasomal ubiquitin-dependent protein catabolic process; positive regulation of receptor-mediated endocytosis; positive	

						regulation of tau-protein kinase activity; positive regulation of tumor necrosis factor production; positive regulation of ubiquitin-dependent protein catabolic process; protein import; protein stabilization; regulation of amyloid-beta clearance; regulation of cell population proliferation; regulation of neuronal signal transduction; regulation of neuron death; response to light stimulus; response to misfolded protein; response to potassium ion; response to wounding; spermatogenesis	
Cortactin-binding protein 2	CTTNBP2	Q2QLA2	*Equus caballus*	-	-	-	cell cortex; dendritic spine

Double-headed protease inhibitor, submandibular gland	*N/A*	P08479	*Vulpes vulpes*	serine-type endopeptidase inhibitor activity	-	extracellular region or secreted
NPC intracellular cholesterol transporter 2	NPC2	P61916	*Homo sapiens*	cholesterol binding; cholesterol transporter activity; enzyme binding; sterol binding	cholesterol efflux; cholesterol homeostasis; cholesterol metabolic process; cholesterol transport; glycolipid transport; intracellular cholesterol transport; intracellular sterol transport; low-density lipoprotein particle clearance; neutrophil degranulation; phospholipid transport; regulation of isoprenoid metabolic process; response to virus; sterol transport	endoplasmic reticulum; extracellular region or secreted; lysosome
NPC intracellular cholesterol transporter 2	Npc2	Q9Z0J0	*Mus musculus*	cholesterol binding; cholesterol transporter activity; enzyme binding; sterol binding	cholesterol efflux; cholesterol homeostasis; cholesterol metabolic process; cholesterol transport; intracellular cholesterol transport; intracellular sterol	endoplasmic reticulum; extracellular region or secreted; lysosome

					transport; sterol transport	
NPC intracellular cholesterol transporter 2	NPC2	O97763	*Sus scrofa*	cholesterol binding; cholesterol transporter activity; sterol binding	cholesterol efflux; cholesterol metabolic process; cholesterol transport; intracellular cholesterol transport; sterol transport	endoplasmic reticulum; extracellular region or secreted; lysosome
Fer-1-like protein 5	FER1L5	A0AVI2	*Homo sapiens*	-	myoblast fusion	plasma membrane; integral component of membrane
Fibrinogen alpha chain	FGA	P02671	*Homo sapiens*	extracellular matrix structural constituent; metal ion binding; signaling receptor binding; structural molecule activity	adaptive immune response; blood coagulation; blood coagulation, common pathway; blood coagulation, fibrin clot formation; cell-matrix adhesion; cellular protein-containing complex assembly; cellular protein metabolic process; extracellular matrix organization; fibrinolysis; induction of bacterial agglutination; innate immune response; negative regulation of	endoplasmic reticulum; extracellular region or secreted; plasma membrane; cell cortex; cell surface; platelet alpha granule; platelet alpha granule lumen; synapse

		blood coagulation, common pathway; negative regulation of endothelial cell apoptotic process; negative regulation of extrinsic apoptotic signaling pathway via death domain receptors; plasminogen activation; platelet aggregation; platelet degranulation; positive regulation of ERK1 and ERK2 cascade; positive regulation of exocytosis; positive regulation of heterotypic cell-cell adhesion; positive regulation of peptide hormone secretion; positive regulation of protein secretion; positive regulation of substrate adhesion-dependent cell spreading; positive regulation of vasoconstriction; post-translational	

					protein modification; protein-containing complex assembly; protein polymerization; response to calcium ion; toll-like receptor signaling pathway	
Glyoxalase domain-containing protein 5	GLOD5	A6NK44	*Homo sapiens*	-	-	-
Granulocyte-macrophage colony-stimulating factor receptor subunit alpha	Csf2ra	Q00941	*Mus musculus*	cytokine binding; cytokine receptor activity; granulocyte colony-stimulating factor binding; granulocyte colony-stimulating factor receptor activity	cellular response to granulocyte macrophage colony-stimulating factor stimulus	plasma membrane; integral component of membrane; receptor complex
High affinity cGMP-specific 3',5'-cyclic phosphodiesterase 9A	Pde9a	Q8QZV1	*Rattus norvegicus*	3',5'-cyclic-GMP phosphodiesterase activity; metal ion binding	cGMP catabolic process; cGMP-mediated signaling; cGMP metabolic process; positive regulation of cardiac muscle hypertrophy	endoplasmic reticulum; golgi apparatus; plasma membrane; perikaryon; perinuclear region of cytoplasm
Histone H2B type 1-K	HIST1H2BK	Q2M2T1	*Bos taurus*	DNA binding; protein heterodimerization activity	nucleosome assembly	nucleus; nucleosome
Inhibitor of growth protein 5	Ing5	Q9D8Y8	*Mus musculus*	chromatin binding; metal ion binding; methylated histone binding	DNA replication; histone H3 acetylation; negative	nucleus

					regulation of cell population proliferation; negative regulation of growth; positive regulation of apoptotic process; positive regulation of apoptotic signaling pathway; positive regulation of transcription, DNA-templated; protein acetylation	
Keratin, type I cytoskeletal 19	KRT19	P51856	*Potorous tridactylus*	-	-	cytoskeleton
Keratin, type I cytoskeletal 9	KRT9	P35527	*Homo sapiens*	structural constituent of cytoskeleton	cornification; epidermis development; intermediate filament organization; keratinization; skin development; spermatogenesis	cytoskeleton; cytosol; extracellular region or secreted; nucleus; membrane
Keratin, type II cytoskeletal 1	KRT1	A5A6M6	*Pan troglodytes*	protein heterodimerization activity	protein heterotetramerization	cytoskeleton; plasma membrane
Keratin, type II cytoskeletal 73	Krt73	Q6IG03	*Rattus norvegicus*	-	-	cytoskeleton
Lactotransferrin	LTF	O77698	*Bubalus bubalis*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response;	extracellular region or

	bone morphogenesis; innate immune response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of osteoblast	secreted; specific granule

					differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
Lactotransferrin	LTF	Q9TUM0	*Camelus dromedarius*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; bone morphogenesis; innate immune response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily	extracellular region or secreted; specific granule

					member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of osteoblast differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
Lactotransferrin	Ltf	P08071	*Mus musculus*	cysteine-type endopeptidase inhibitor activity; heparin binding; iron ion binding; lipopolysaccharide binding; peptidase activity; protein serine/threonine kinase activator activity; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; antimicrobial humoral immune response mediated by antimicrobial peptide; bone morphogenesis; defense response to Gram-negative bacterium; innate	extracellular region or secreted; cell surface; cytoplasm; other organism cell membrane; protein-containing complex; secretory

	immune response in mucosa; ion transport; iron ion homeostasis; killing of cells of other organism; membrane disruption in other organism; negative regulation by host of viral process; negative regulation of apoptotic process; negative regulation of ATPase activity; negative regulation of cysteine-type endopeptidase activity; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11	granule; specific granule

		production; negative regulation of viral genome replication; negative regulation of viral process; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of I-kappaB kinase/NF-kappaB signaling; positive regulation of NF-kappaB transcription factor activity; positive regulation of osteoblast differentiation; positive regulation of osteoblast proliferation; positive regulation of protein serine/threonine kinase activity; positive regulation of toll-like receptor 4 signaling pathway; regulation of	

					cytokine production; regulation of tumor necrosis factor production	
Mucin-19	MUC19	Q7Z5P9	*Homo sapiens*	-	O-glycan processing; stimulatory C-type lectin receptor signaling pathway	extracellular region or secreted; golgi apparatus; plasma membrane
Nephrocystin-4	NPHP4	O75161	*Homo sapiens*	structural molecule activity	actin cytoskeleton organization; cell-cell adhesion; ciliary basal body-plasma membrane docking; flagellated sperm motility; hippo signaling; negative regulation of canonical Wnt signaling pathway; photoreceptor cell maintenance; photoreceptor cell outer segment organization; positive regulation of bicellular tight junction assembly; protein localization to ciliary transition zone; retina	cytoskeleton; cytosol; nucleus; bicellular tight junction; cell-cell junction; ciliary base; ciliary transition zone; non-motile cilium; photoreceptor connecting cilium; ribbon synapse

					development in camera-type eye; signal transduction; visual behavior	
Origin recognition complex subunit 1	ORC1	Q9JI69	*Cricetulus griseus*	ATP binding; chromatin binding; DNA binding; metal ion binding	DNA replication	nucleus
Peptidyl-prolyl cis-trans isomerase B	Ppib	P24368	*Rattus norvegicus*	cyclosporin A binding; peptidyl-prolyl cis-trans isomerase activity; RNA polymerase binding; unfolded protein binding	bone development; chaperone-mediated protein folding; nuclear transport; positive regulation by host of viral genome replication; positive regulation of multicellular organism growth; protein peptidyl-prolyl isomerization; protein refolding; protein stabilization	endoplasmic reticulum; nucleus; melanosome; perinuclear region or cytoplasm; protein-containing complex
Period circadian protein homolog 1	Per1	Q8CHI5	*Rattus norvegicus*	chromatin DNA binding; transcription corepressor binding; transcription factor binding; transcription regulatory region sequence-specific DNA binding	circadian regulation of gene expression; circadian regulation of translation; circadian rhythm; entrainment of circadian clock by photoperiod; histone H3 deacetylation; negative regulation of glucocorticoid receptor signaling	nucleus; cytoplasm

			pathway; negative regulation of I-kappaB kinase/NF-kappaB signaling; negative regulation of JNK cascade; negative regulation of transcription, DNA-templated; negative regulation of transcription by RNA polymerase II; positive regulation of transcription by RNA polymerase II; posttranscriptional regulation of gene expression; regulation of circadian rhythm; regulation of cytokine production involved in inflammatory response; regulation of hair cycle; regulation of p38MAPK cascade; regulation of sodium ion transport; response to light stimulus	

Polyhomeotic-like protein 3	Phc3	Q8CHP6	*Mus musculus*	DNA binding; zinc ion binding	multicellular organism development	nucleus
Prostaglandin-H2 D-isomerase	PTGDS	O97921	*Equus caballus*	prostaglandin-D synthase activity; retinoid binding; small molecule binding	negative regulation of male germ cell proliferation; prostaglandin biosynthetic process; regulation of circadian sleep/wake cycle, sleep	endoplasmic reticulum; extracellular region or secreted; golgi apparatus; nucleus; perinuclear region or cytoplasm
Prostaglandin-H2 D-isomerase	PTGDS	Q29562	*Ursus arctos*	prostaglandin-D synthase activity; retinoid binding; small molecule binding	prostaglandin biosynthetic process; regulation of circadian sleep/wake cycle, sleep	endoplasmic reticulum; extracellular region; extracellular space; golgi apparatus; nuclear membrane; perinuclear region of cytoplasm
Protein Spindly	Spdl1	Q3KR99	*Rattus norvegicus*	enzyme binding; kinetochore binding	cell division; establishment of mitotic spindle orientation; mitotic metaphase plate congression; mitotic	cytoskeleton; nucleus; condensed chromosome outer

					spindle assembly checkpoint; protein localization to kinetochore	kinetochore; cytoplasm
Protein Wnt-7a	WNT7A	Q1KYK4	*Aotus trivirgatus*	signaling receptor binding	multicellular organism development; Wnt signaling pathway	extracellular region or secreted
Serum albumin	ALB	P02769	*Bos taurus*	DNA binding; drug binding; enterobactin binding; fatty acid binding; metal ion binding; pyridoxal phosphate binding; toxic substance binding	cellular response to starvation; maintenance of mitochondrion location; negative regulation of apoptotic process	extracellular region or secreted; protein-containing complex
Serum albumin	ALB	Q5XLE4	*Equus asinus*	DNA binding; drug binding; enterobactin binding; fatty acid binding; metal ion binding; pyridoxal phosphate binding; toxic substance binding	cellular response to starvation; maintenance of mitochondrion location; negative regulation of apoptotic process	extracellular region or secreted; protein-containing complex
Serum albumin	ALB	P49064	*Felis catus*	DNA binding; drug binding; enterobactin binding; fatty acid binding; metal ion binding; pyridoxal phosphate binding; toxic substance binding	cellular response to starvation; maintenance of mitochondrion location; negative regulation of apoptotic process	extracellular region or secreted; protein-containing complex
Serum albumin	ALB	A6YF56	*Mesocricetus auratus*	enterobactin binding; lipid binding; metal ion binding	-	extracellular region or secreted

Serum albumin	ALB	P49065	*Oryctolagus cuniculus*	DNA binding; drug binding; enterobactin binding; fatty acid binding; metal ion binding; pyridoxal phosphate binding; toxic substance binding	cellular response to starvation; maintenance of mitochondrion location; negative regulation of apoptotic process	extracellular region or secreted; protein-containing complex
Serum albumin	ALB	P14639	*Ovis aries*	enterobactin binding; lipid binding; metal ion binding	-	extracellular region or secreted
Transcription elongation factor SPT5	SUPT5H	Q5R405	*Pongo abelii*	-	regulation of DNA-templated transcription, elongation; regulation of transcription by RNA polymerase II	nucleus
Transcription factor 25	TCF25	Q9BQ70	*Homo sapiens*	DNA binding; DNA-binding transcription factor activity	heart development; negative regulation of transcription by RNA polymerase II	nucleus
Transcriptional regulator ATRX	ATRX	Q7YQM3	*Pongo pygmaeus*	ATP binding; chromatin binding; DNA binding; helicase activity; histone binding; metal ion binding	cellular response to hydroxyurea; chromatin remodeling; DNA damage response, signal transduction by p53 class mediator; DNA repair; DNA replication-independent nucleosome	nucleus; telomeric heterochromatin

						assembly; negative regulation of telomeric RNA transcription from RNA pol II promoter; nucleosome assembly; positive regulation of nuclear cell cycle DNA replication; positive regulation of telomere maintenance; positive regulation of transcription by RNA polymerase II; regulation of histone H3-K9 trimethylation; replication fork processing	
Transformation/transcription domain-associated protein	TRRAP	Q9Y4A5	*Homo sapiens*	transcription activity	coregulator	beta-catenin-TCF complex assembly; DNA repair; histone acetylation; histone deubiquitination; histone H2A acetylation; histone H4 acetylation; protein deubiquitination; regulation of	golgi apparatus; nucleus

					transcription, DNA-templated	
TRIO and F-actin-binding protein	TRIOBP	Q9H2D6	*Homo sapiens*	actin filament binding; GTP-Rho binding; myosin II binding; ubiquitin protein ligase binding	actin modification; auditory receptor cell stereocilium organization; barbed-end actin filament capping; cell cycle; cell division; positive regulation of substrate adhesion-dependent cell spreading; sensory perception of sound	cytoskeleton; nucleus; cytoplasm; focal adhesion; midbody
tRNA wybutosine-synthesizing protein 4	Lcmt2	Q8BYR1	*Mus musculus*	protein C-terminal carboxyl O-methyltransferase activity; tRNA methyltransferase activity	C-terminal protein methylation; tRNA methylation; wybutosine biosynthetic process	-
V-type proton ATPase subunit S1	ATP6AP1	Q15904	*Homo sapiens*	ATP binding; proton-transporting ATPase activity, rotational mechanism; proton-transporting ATP synthase activity, rotational mechanism; Rab GTPase binding; transporter activity	ATP hydrolysis coupled proton transport; cellular iron ion homeostasis; cellular response to increased oxygen levels; establishment of organelle localization; insulin receptor signaling pathway; ion transmembrane transport; pH reduction; positive	endoplasmic reticulum; endosome; extracellular region or secreted; plasma membrane; endoplasmic reticulum-golgi intermediate compartment membrane;

					regulation of bone resorption; positive regulation of ERK1 and ERK2 cascade; positive regulation of exocytosis; positive regulation of osteoblast differentiation; positive regulation of osteoclast development; proton transmembrane transport; regulation of cellular pH; transferrin transport	integral component of membrane; proton-transporting two-sector ATPase complex; proton-transporting V-type ATPase, V1 domain
Xylosyltransferase 1	Xylt1	Q9EPI1	*Rattus norvegicus*	acetylglucosaminyltransferase activity; metal ion binding; protein xylosyltransferase activity	chondroitin sulfate biosynthetic process; chondroitin sulfate proteoglycan biosynthetic process; embryonic skeletal system development; heparan sulfate proteoglycan biosynthetic process; negative regulation of axon regeneration; ossification involved in bone maturation; proteoglycan biosynthetic process;	extracellular region or secreted; golgi apparatus

					response to interleukin-1	

827

828 **Supplement 3.** Common proteins found in seminal plasma and spermatozoa of evaluated dogs and their respective genic ontology.

Protein	Gene	ID	Organism	Molecular function	Biological process	Cellular component
Histone H2B type 1-K	HIST1H2BK	Q2M2T1	*Bos taurus*	DNA binding; protein heterodimerization activity	nucleosome assembly	nucleus; nucleosome
Keratin, type II cytoskeletal 1	KRT1	A5A6M6	*Pan troglodytes*	protein heterodimerization activity	protein heterotetramerization	cytoskeleton; plasma membrane
Lactotransferrin	LTF	O77698	*Bubalus bubalis*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; bone morphogenesis; innate immune response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast	extracellular region or secreted; specific granule

					development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of osteoblast differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
Lactotransferrin	LTF	Q9TUM0	*Camelus dromedarius*	iron ion binding; serine-type endopeptidase activity	antibacterial humoral response; antifungal humoral response; bone morphogenesis;	extracellular region or secreted; specific granule

		innate immune response in mucosa; ion transport; iron ion homeostasis; negative regulation of apoptotic process; negative regulation of lipopolysaccharide-mediated signaling pathway; negative regulation of osteoclast development; negative regulation of single-species biofilm formation in or on host organism; negative regulation of tumor necrosis factor (ligand) superfamily member 11 production; ossification; positive regulation of bone mineralization involved in bone maturation; positive regulation of chondrocyte proliferation; positive regulation of osteoblast	

					differentiation; positive regulation of osteoblast proliferation; regulation of cytokine production; regulation of tumor necrosis factor production	
Serum albumin	ALB	P14639	*Ovis aries*	enterobactin binding; lipid binding; metal ion binding	-	extracellular region or secreted
V-type proton ATPase subunit S1	ATP6AP1	Q15904	*Homo sapiens*	ATP binding; proton-transporting ATPase activity, rotational mechanism; proton-transporting ATP synthase activity, rotational mechanism; Rab GTPase binding; transporter activity	ATP hydrolysis coupled proton transport; cellular iron ion homeostasis; cellular response to increased oxygen levels; establishment of organelle localization; insulin receptor signaling pathway; ion transmembrane transport; pH reduction; positive regulation of bone resorption; positive regulation of ERK1 and ERK2 cascade; positive regulation of exocytosis; positive	endoplasmic reticulum; endosome; extracellular region or secreted; plasma membrane; endoplasmic reticulum-golgi intermediate compartment membrane; integral component of membrane; proton-transporting two-sector ATPase complex; proton-transporting V-

		regulation of osteoblast differentiation; positive regulation of osteoclast development; proton transmembrane transport; regulation of cellular pH; transferrin transport	type ATPase, V1 domain

829

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Seminal plasma positively influences the quality of canine semen stored at 5ºC

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Abstract

Studies regarding the main role of seminal plasma in the canine species, mainly during semen refrigeration, was little mentioned and its function remains controversial. Thus, this study aimed to evaluate the prostatic fluid influence on oxidative stress and sperm quality on canine semen stored at 5 ºC for seven days. Three pools of eight healthy dogs ejaculate were collected by digital manipulation of the penis. The first three ejaculate emissions were discarded and a portion of the first and third fractions, and the entire sperm-rich fraction were collected. The morphology, computer-assisted sperm motility (CASA), integrity of spermatozoa (eosin/nigrosine stain), integrity of plasma and acrosomal membranes, superoxide and hydrogen peroxide production, mitochondrial potential, lipid peroxidation, oxygen reactive species production (induced and spontaneous thiobarbituric acid assay) and antioxidant enzymes production (ELISA) were evaluated every 48 and 72 hours (M0, M48, M96 and M168) until 168 hours (seven days) in chilled extended semen of dogs at 5º C with or without seminal plasma. The obtained data were analyzed by two-way ANOVA and multivariate analysis by Bonferroni test. Cooled samples with seminal plasma showed higher total motility (TM), progressive motility (PM), velocity average pathway (VAP), velocity in straight line (VSL), rapid spermatozoa, movement index (IND MOV), integrity of plasma and acrosomal membranes (MPAI), mitochondrial potential, lower lateral head amplitude (ALH) and lower medium and slow spermatozoa. According to time of refrigeration, PM in M0 > M168 (P =0.041); VSL in M0 > M168 (P = 0.036) and M48 > M168 (P = 0.011); ALH in M0 < M96 and M168 (P = 0.009, P = 0.020, respectively); STR in M0 > M48, M96 and M168 (P = 0.045, P = 0.008, P = 0.002, respectively); LIN in M0 > M96 and M168 (P = 0.002, P < 0.001, respectively); WOB in M0 > M96 and M168 (P = 0.007, P = 0.002, respectively); and IND MOV in M0 and M48 > M96 (P < 0.001, P = 0.008,

respectively), and M0, M48 and M96 > M168 (P < 0. 001, P < 0.001, P = 0.020, respectively). The seminal plasma exerts positive influence on sperm motility parameters, viability and mitochondrial function during dog semen extended and stored at 5 ºC for seven days.

Keywords: chilled semen, oxidative stress, dog, spermatozoa.

1. Introduction

Chilled dog semen has allowed a relatively inexpensive, simple and successful method to the increasing demand of import and export semen with the development of pure dog breeding [1]. Chilled semen has been often used in relation to frozen-thawed semen due the easy handling and higher pregnancy and whelping rates with the same methods of insemination in dogs [2,3]. It may be sent for longer distances or kept for more than two days before insemination [4,5], or the semen may be cryopreserved for long-term storage [6].

During dog semen collection for cooling, it is recommended to obtain only the second fraction (sperm-rich fraction) of the ejaculate [1], because the harmful effects that prostatic fluid exerts upon spermatozoa during preserving dog semen at low temperatures [7]. However, the inability of the professional to perform the digital manipulation of the penis associated with the movement of the animal may impair the precise separation of the three fractions during semen collection, and a portion of the seminal plasma may be cooled with the sperm cells [1,7].

The canine seminal plasma has many components, among them proteins and antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase), that act to protect the spermatozoa during fertilization [8,9]. However, studies regarding the main

role of seminal plasma in the canine species, mainly during semen refrigeration, was little mentioned and its function remains controversial [9-11].

When the spermatozoa are submitted to low temperatures a lot of reactive oxygen species (ROS) production occurs [12,13]. ROS are physiologically produced by sperm cells during mitochondrial oxidative phosphorylation for synthesis of adenosine triphosphate (ATP) [14]. The production of cytoplasmic glucose-6-phosphate dehydrogenase is another spermatic physiological source of ROS [15]. To occur fertilization and adequate sperm function, ROS and antioxidants may have a balance [16].

Spermatozoa have limited content of antioxidant enzymes due to its low cytoplasm content and high quantity of polyunsaturated fatty acids on its plasma membrane. The imbalance between ROS and antioxidants production are more susceptible to occur leading to oxidative stress [17]. The oxidative stress negatively influences sperm motility, morphology, DNA integrity, carbohydrates and proteins functionality, impairing fertilization [18]. Thus, this study aimed to evaluate the prostatic fluid influence on oxidative stress and sperm quality on canine semen stored at 5 ºC for seven days.

2. Material and Methods

2.1. Ethical aspects

This study was approved by the Ethical Committee for the Use of Animals in Research of the FMVZ-UNESP with the permit number: 0007/2017 – CEUA

2.2. Reagents

Unless otherwise stated, all reagents used were of high purity and purchased from Sigma Aldrich Sigma-Aldrich (St. Louis, MO, USA), Cayman Chemical (Ann Arbor, MI, EUA), Merck S.A. (São Paulo, São Paulo, Brazil).

2.3. Semen collection and immediate analysis

The ejaculate of eight healthy dogs aged between one and five years, from different kennels at São Paulo State, Brazil, were used for this study. In order to obtain higher volume and sperm cell concentration, the dog's ejaculates were pooled (n = 3) prior to evaluation. The first three ejaculate emissions were discarded and a portion of the first and third fractions, and the entire sperm-rich fraction were collected into a silicone funnel attached to a graduated plastic tube by manual stimulation of the penis in the presence of a teaser bitch when possible [19].

The semen was immediately subjectively analyzed at the kennel for volume (mL), total motility (0 to 100%), vigor (0 to 5 score), concentration (x 10^6/mL), sperm morphology and vitality (eosin/nigrosine) under light microscopy. Total motility and vigor were assessed by placing a drop of 10 µL of semen on a pre-warmed glass slide (38ºC) applying a coverslip and visualized under a light microscope at 400x magnification. The concentration was accessed by a Neubauer chamber after semen dilution of 1:100 in formol-saline.

A 5 µL of semen and 5 µL of Eosin/Nigrosin stain (BotuVital®, Botupharma, Botucatu, Brazil) were placed on a pre-warmed glass slide, and the smear was air-dried and evaluated under a light microscope (1,000X magnification) with immersion oil. A total of 200 sperm cells were counted. Spermatozoa colored in red were considered damaged (membrane lesion), while no colored spermatozoa were considered intact (membrane integrity). Sperm morphology was classified according to Bloom [20].

After immediate evaluation the pooled semen samples (n = 3) were divided into another two samples (n = 6). One sample was centrifuged at 800g / 15 min [21] to remove seminal plasma and added TRIS-egg-yolk extender [198 mM TRIS, 67 mM citric acid, 45 mM glucose, 20% yolk egg, 0.2 mg/mL amicacin sulphate and 1% Orvus (Orvus WA

Paste®, Procter & Gamble, Cincinnati, Ohio, USA), pH 6,8 and osmolarity 300 mOsmol/L] until a final concentration of 50 x 10^6 sperm/mL. The other sample was diluted with the same extender in the presence of the seminal plasma until a final concentration of 50 x 10^6 sperm/mL (minimum dilution of 1:3). The both samples were chilled at 5ºC in a styrofoam box for semen transportation (BotuFlex, BotuPharma®, Botucatu, Brazil) and taken to the laboratory for additional analysis. The mean time of semen transportation to the laboratory was three hours.

2.4. Laboratorial analysis

All samples were evaluated upon arrival (M0) and at 48 (M48), 96 (M96) and 168 (M168) hours of refrigeration for morphology, membrane integrity (eosin-nigrosine stain), computer-assisted sperm analysis, flow cytometry parameters, oxidative stress (induced and spontaneous thiobarbituric acid reactive substances tests) and antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase). The samples remained at 5ºC for seven days in a temperature-controlled cooler.

2.4.1. Sperm kinematic evaluation

At the laboratory, sperm kinetic parameters were analyzed by computer-assisted sperm analysis (CASA, Hamilthon-Thorne Computer-Aided Semen Analyzer, 10 Ivos version, HTR-IVOS 10 Analyzer, Hamilthon Thorne Research, Beverly, MA, USA) according to setup from Center of Study in Animal Reproduction (CERAN, FMVZ, UNESP, Botucatu, São Paulo, Brazil) and included image capture (frames per sec: 60 Hz), image capture (No. of frames: 30), cell detection (min contrast: 30), cell detection (min cell size: 30 pixels), defaults (cell size: 5 pixels), defaults (cell intensity: 40), progressive cells (VAP: 70.0 μ/s), progressive cells (STR: 80.0%), slow cells (static, VAP cut-off: 30 μ/s), slow cells (static, VSL cut-off: 20 μ/s), illumination/intensity (3,600), illumination/photometer (125), video source (dark field: 60 Hz), static intensity gates

(min and max: 0.48 and 1.45), static elongation gates (min and max: 0 and 97), chamber type (Makler, chamber depth 10.0 μm, stage position 14.3 mm, Hamilton Thorne Research, Beverly, MA, USA), temperature (38º C) and automatic field selection. Briefly, a 10 μL of semen sample was placed into a Makler chamber at 37 ºC and analyzed for motility parameters of total motility (TM, %), progressive motility (PM., 153 %), average path velocity (VAP, μm/s), straight line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, mm), head displacement frequency = beat cross frequency (BCF, Hz), straightness (STR, %) which indicates the linearity of the mean trajectory and is defined as (VSL/VAP) x 100, linearity (LIN, %) which is the relation between the linear displacement of the sum of the displacements during the measurement time and which is defined as (VSL/VCL) x 100. In addition, according to the low cut VAP (LVV) and medium cut VAP (MVV), the total sperm population was divided into four categories: fast (VAP>MVV), medium (LVV<VAP<MVV), slow (VAP<LVV) and static (proportion of cells without movement). With VAP and VCL values, the wobble (WOB, %) was calculated by (VAP/VCL) x 100. The parameters found in CASA were used to calculate the velocity index and sperm movement index, according to Agarwal et al. [22], modified by Nuñez-Martínez et al. [23]: spermatic velocity index (IND VEL) = (VCL x 0.87) + (VSL x 0.76) + (VAP x 0.90) + (ALH x 0.92); and spermatic movement index (IND MOV) = (VSL x 0.59) + (VAP x 0.37) + (LIN x 0.95) + (STR x 0.89) + (WOB x 0.83).

2.4.2. Assessment of sperm membranes integrity, mitochondrial potential, superoxide production, hydrogen peroxide production and lipid peroxidation

The spermatozoa were also evaluated by flow cytometry for integrity of the plasma and acrosomal sperm membranes, mitochondrial potential, superoxide production and hydrogen peroxide production. The equipment used for all flow cytometry analysis

was Fortessa LSR BD (Becton Dickinson, Mountain View, CA, USA); equipped with 488nm, 100mW blue lasers; 640nm, 40mW red and 405nm violet. For this study, the excitation used was 488nm; and the emission filters used were 695/40nm (MitoSOXTM Red and propidium iodide), 530/30nm (SYTOX Green FITC-PSA and DHR). For excitation at 640nm, the emission filter was 660/20nm (MitoSOXTM Red) and for excitation at 405nm, the emission filter was 450/50nm (Hoechst 33342). At least 10,000 cells per sample were analyzed and the data were evaluated by a program from the same manufacturer (BD FACSDiva ™ software v 6.1, Becton Dickinson, Mountain View, CA, USA).

For plasma and acrosomal membranes integrity evaluation the association of Hoechst 33342, propidium iodide and FITC-PSA (*Pisum sativum agglutinin* conjugated to fluorescein isothiocyanate) were used according to Freitas-Dell'aqua et al. [24]. Thus, in a sample of 200 μL of semen diluted in TALP-PVA at the concentration of 5 x 10^6 spermatozoa/mL were added 7 μM of Hoechst, 1.5 μM of propidium iodide and 2187 ng of FITC-PSA. The sample was incubated for 5 min at 37º C, protected from light. At the end of this period, the sample was evaluated by flow cytometry. Spermatozoa were classified into four groups, damaged plasma membrane and normal acrosomal membrane (MPLAI); both damaged plasma and acrosomal membranes (MPAL); integrity plasma and acrosomal membranes (MPAI); and integrity plasma membrane and damaged acrosomal membrane (MPIAL).

For evaluation of mitochondrial potential and superoxide (O_2) production in the mitochondrial matrix was used the association of Hoechst 33342, SYTOX Green (cell marking with plasma membrane damage, catalog nº S34860, Thermo Scientific, São Paulo, São Paulo, Brazil), MitoStatus Red (cell marking with mitochondrial potential, catalog nº 564697, BD Biosciences, São Paulo, São Paulo, Brazil) and MitoSOXTM Red

(cell marking with generation of superoxide anion in the mitochondrial matrix, M36008, Thermo Scientific, São Paulo, São Paulo, Brazil) according to Freitas-Dell'Aqua et al. [25]. A semen sample of 200 μL was added TALP-PVA in a final concentration of 5 x 10^6 spermatozoa/mL, and 7 μM Hoechst, 0.05 μM SYTOX Green, 20 μM MitoStatus Red and 2 μM MitoSOXTM Red, and incubated at 37º C for 15 min.

Dihydrorhodamine 123 (catalog nº D23806, Thermo Scientific, São Paulo, São Paulo, Brazil) was used to evaluate the intracellular production of hydrogen peroxide (H_2O_2). A solution of 1.5 μM of propidium iodide (diluted in TALP-PVA) and 1 μM of dihydrorhodamine 123 (diluted in DMSO) were added to 500 μL of the diluted semen in 207 TALP-PVA. The sample was incubated at 37º C for 20 minutes protected from light and analyzed. Spermatozoa were classified into four groups, normal cells; cells with high O_2 concentration; cells with high mitochondrial potential; cells with high H_2O_2 production. The concentration of mitochondrial potential, O_2 and H_2O_2 production were measured according to fluorescence intensity.

Lipid peroxidation assessments were carried out with the fluorescent probe C11-BODYPY (D-3861; Molecular Probes, Carlsbad, CA, USA), is a lipophilic fluorophore sensitive to react with oxygen and peroxynitrite and once oxidized converts its fluorescence from red to green. Each aliquot of semen (2 million sperm/mL TALP-PVA extended in 489.5 μL) was added C11BODIPY581/591 (0.5 μL, solution 1 mg / mL), propidium iodide (5 μl, solution 50 μg/mL), Hoescht 33,342 (5 μL, solution 100 μg/mL) and then the mixture was incubated for at 37 °C for 30 min. After incubation, the samples were washed two times at 300 g for 5 min and the pellet resuspended in 500 μL of TALP-PVA and then analyzed by flow cytometry [26].

2.4.3. Oxidative stress

The spontaneous measurement of thiobarbituric acid reactive substances (TBARS) was done by assessing a product of lipid peroxidation called malondialdehyde (MDA), as first described by Ohkawa et al. [27]. The concentration of MDA indirectly measures the amount of sperm oxidative stress when two molecules of thiobarbituric acid reacts with one molecule of MDA at high temperatures and low pH. The resulting pink color of this reaction is quantified with a spectrophotometer at 532 nm wavelength.

For this, 500 µl of all seminal samples were centrifuged at 800g / 10 min / 4ºC to recover the supernatant. To precipitate proteins, 600 µL of 10% trichloroacetic acid solution (v:v) were mixed with 300 µl of the precentrifuged supernatant and centrifuged at 20,0817g / 15 min / 5ºC. After centrifugation, 700 µl of thiobarbituric acid 1% in 0.05 N sodium hydroxide were added to 700 µl of the supernatant (1:1), placed in cryotubes and boiled in a water bath at 100 ºC for 15 min and subsequently cooled in an ice bath at 0º C to stop the chemical reaction. The samples were centrifuged at 20,817 g/ 5ºC / 15 min and the TBARS were quantified using a spectrophotometer at a wavelength of 532 nm and the results were compared with a standard curve previous prepared with a standard solution of MDA. The results are expressed as nanograms of TBARS/mL.

Induced TBARS test according to Nichi et al. [28] and Losano et al. [29] was performed to analyze the sperm susceptibility to oxidative stress. For this, 800 µL containing 40 x 10^6 spermatozoa were centrifuged at 800 g/ 10 min / 4ºC. The supernatant was discarded, and the cells were resuspended with phosphate-buffered saline (PBS) (pH 7.0, dilution 1:2) and recentrifuged as previous describe to remove the remains of extender. The cells were resuspended with PBS at a final concentration of 40 x 10^6 sperm/ 0.4mL. Lipid peroxidation was induced by adding ferrous sulphate (100 µL; 4 mM) and sodium ascorbate (100 µL, 20 mM) to 0.4 mL of sperm suspension. This solution was

incubated for 2 hours at 37ºC [30]. Then, 1 mL of a solution (v:v) of trichloroacetic acid 10 % was added to 0.5 mL of the incubation mixture (dilution 1:2) and centrifuged at 10,000g / 30 min / 15ºC to precipitate protein. Following this, 500 µL of the supernatant was mixed in 500 µL of 1% (v:v) TBA 1 % diluted in 0.05 N sodium hydroxide in a glass tube and placed into a boiling water bath (100 ºC) for 20 min, and immediately cooled in an ice bath (0º C) to stop chemical reaction. The TBARS were quantified ate a spectrophotometer at a wavelength of 532 nm (Ultrospec 3300 Pro, Amersham Biosciences). The results were compared with a standard curve previously prepared with a standard solution of MDA, and the lipid-peroxidation index was described as nanograms of TBARS/10^6 sperm.

2.4.4. Antioxidant enzymes activity

The activity of catalase, glutathione peroxidase and superoxide dismutase were assessed by Catalase Assay Kit (Cayman Chemical, Ann Arbor, Michigan, EUA, n. 707002), Glutathione Peroxidase Assay Kit (Cayman Chemical, Ann Arbor, Michigan, EUA, nº 703102) and Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, Michigan, EUA, nº 706002), according to manufacturer's specification.

2.5. Data analysis

All variables were submitted to normality test of Shapiro Wilk. The semen variables from subjective analyses, computer-assisted sperm analysis, flow cytometry parameters, spontaneous and induced TBARS evaluated by two-way ANOVA. All pairwise multiple comparison procedures were performed using Bonferroni test.

Outliers data obtained from spontaneous and induced TBARS results were eliminated according to Andrade and Ogliari [31]. Values one and a half times lower than the first quartile (Q1 – 1,5 DI), or one and a half times higher than the third quartile (Q3 + 1,5 DI) were considered outliers. The Q1 is the first quartile, Q3 the third quartile and

DI the interquartile distance (DI = Q3 – Q1). Values beyond these limits, in a normal distribution, are 0.7% of the results [31].

Data were analyzed using the statistical software Sigma Plot® 11.0. The results were reported as mean ± standard error and statistically significant level of $P \leq 0.05$ was considered.

3. Results

All the results are summarized at table 1. The sperm kinematics parameters of MT, PM, VAP, rapid and IND MOV, MPAI, hydrogen peroxide production and mitochondrial potential were significantly higher during canine semen refrigeration with seminal plasma ($P = 0.012$, $P = 0.002$, $P = 0.005$, $P < 0.001$, $P < 0.001$, $P = 0.008$, respectively); while medium and slow spermatozoa (CASA parameters) were significantly low ($P = 0.038$, $P = 0.002$, $P = 0.007$, respectively). No statistical difference was observed for normal spermatozoa, minor and major defects, membrane integrity, superoxide production, lipid peroxidation, induced and spontaneous TBARS. Unfortunately, it was not possible to quantify the production of catalase, superoxide dismutase and glutathione peroxidase in semen samples according to protocol described by Cayman kits manufactures for plasma. The values obtained were out of the standard curve range and we did not have an additional kit and enough samples to perform new tests.

According to time of refrigeration, PM in M0 > M168 ($P = 0.041$); VSL in M0 > M168 ($P = 0.036$) and M48 > M168 ($P = 0.011$); ALH in M0 < M96 and M168 ($P = 0.009$, $P = 0.020$, respectively); STR in M0 > M48, M96 and M168 ($P = 0.045$, $P = 0.008$, $P = 0.002$, respectively); LIN in M0 > M96 and M168 ($P = 0.002$, $P < 0.001$, respectively); WOB in M0 > M96 and M168 ($P = 0.007$, $P = 0.002$, respectively); and IND MOV in

M0 and M48 > M96 ($P < 0.001$, $P = 0.008$, respectively), and M0, M48 and M96 > M168 ($P < 0.001$, $P < 0.001$, $P = 0.020$, respectively).

Table 1. Results expressed by mean ± standard error of computer analysis of sperm kinematic parameters, percentage of normal sperm, major defects, minor defects, integrity of plasma membrane (eosin-nigrosine stain), intact plasma and acrosomal membranes, mitochondrial potential, superoxide production, hydrogen peroxide production, lipid peroxidation, concentration of thiobarbituric acid reactive substances (spontaneous and induced TBARS), and antioxidants concentration (catalase, superoxide dismutase and glutathione peroxidase) in chilled semen of dogs in the presence or absence of seminal plasma, upon arrival at the laboratory (M0), 48 (M48), 96 (M96) and 168 (M168) hours of refrigeration.

Sperm parameters	M0		M48		M96		M168	
	+SP	-SP	+SP	-SP	+SP	-SP	+SP	-SP
TM (%)	88.7 ± 1.2*	77.7 ± 5.2	90.3 ± 1.8*	81.3 ± 3.7	84.0 ± 2.5*	74.0 ± 6.5	79.0 ± 4.7*	68.7 ± 9.4
PM (%)	73.7 ± 0.7^{*a}	63.3 ± 3.8^{a}	70.7 ± 1.2^{*ab}	64.7 ± 2.6^{ab}	67.7 ± 1.5^{*ab}	55.0 ± 5.0^{ab}	62.3 ± 4.7^{*b}	50.7 ± 6.9^{b}
VAP (µm/s)	128.4 ± 2.1*	114.9 ± 2.3	133.6 ± 5.9*	123.7 ± 6.6	133.0 ± 9.0*	112.9 ± 5.9	116.8 ± 5.5*	109.9 ± 3.0
VSL (µm/s)	113.0 ± 2.3^{*a}	102.1 ± 0.8^{a}	113.8 ± 3.6^{*ab}	105.8 ± 3.9^{ab}	112.9 ± 6.6^{*abc}	94.5 ± 4.1^{abc}	99.5 ± 4.8^{*c}	90.6 ± 2.5^{c}
VCL (µm/s)	161.6 ± 3.7	146.7 ± 5.7	183.3 ± 16.6	168.1 ± 16.5	192.1 ± 22.5	161.3 ± 12.0	166.6 ± 6.5	163.5 ± 3.1
ALH (µm)	5.6 ± 0.2^{*a}	5.6 ± 0.2^{a}	6.8 ± 0.5^{*ab}	6.7 ± 0.6^{ab}	7.7 ± 0.7^{*b}	6.9 ± 0.5^{b}	7.0 ± 0.1^{*b}	7.3 ± 0.2^{b}
BCF (Hz)	18.7 ± 1.3	18.2 ±0.4	20.2 ± 1.3	18.5 ± 1.2	19.8 ± 1.6	20.1 ± 0.4	19.4 ± 0.3	20.6 ± 0.4
STR (%)	87.0 ± 0.6^{a}	87.7 ± 1.7^{a}	83.7 ± 1.3^{b}	84.7 ± 1.3^{b}	83.7 ± 1.2^{b}	83.0 ± 0.6^{b}	84.0 ± 0.0^{b}	81.3 ± 0.3^{b}
LIN (%)	70.7 ± 0.3^{a}	70.3 ± 2.4^{a}	63.7 ± 3.0^{ab}	65.3 ± 3.3^{ab}	61.0 ± 2.6^{b}	60.7 ± 1.8^{b}	61.7 ± 0.9^{b}	57.7 ± 0.3^{b}
Rapid (%)	85.3 ± 1.5*	71.7 ± 5.2	86.0 ± 2.0*	76.0 ± 4.0	80.0 ± 2.0*	66.7 ± 6.8	73.3 ± 5.8*	61.7 ± 8.8
Medium (%)	3.3 ± 0.3*	6.3 ± 0.3	4.3 ± 0.7*	5.3 ± 0.7	3.7 ± 0.3*	7.0 ± 1.0	5.3 ± 1.3*	6.7 ± 1.2
Slow (%)	11.3 ± 1.2*	21.7 ± 5.0	9.7 ± 1.8*	18.3 ± 3.8	15.7 ± 2.3*	25.7 ± 6.2	20.3 ± 4.5*	27.0 ± 5.0
Static (%)	0.3 ± 0.3	0.7 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.7 ± 0.3	0.3 ± 0.3	4.3 ± 4.3
WOB (%)	79.5 ± 1.1^{a}	78.4 ± 1.5^{a}	73.5 ± 3.2^{ab}	74.2 ± 3.2^{ab}	70.0 ± 3.2^{b}	70.3 ± 2.2^{b}	70.1 ± 0.9^{b}	67.2 ± 0.7^{b}
IND VEL	339.8 ± 8.0	322.0 ± 15.4	342.6 ± 9.6	416.8 ± 32.3	361.9 ± 16.4	349.1 ± 47.9	347.3 ± 11.4	327.7 ± 13.9
IND MOV	324.7 ± 2.3^{*a}	312.7 ± 3.9^{a}	312.5 ± 2.3^{*a}	307.3 ± 2.5^{a}	306.3 ± 1.3^{*b}	287.4 ± 3.2^{b}	293.4 ± 6.1^{*c}	277.1 ± 3.0^{c}

Normal spermatozoa (%)	87.8 ± 1.8	88.7 ± 0.4	90.0 ± 3.1	81.7 ± 3.7	82.5 ± 5.6	86.2 ± 4.2	84.7 ± 1.9	87.7 ± 1.9
Major defects (%)	5.2 ± 0.2	3.7 ± 0.8	3.2 ± 0.3	6.7 ± 1.2	5.0 ± 2.0	3.2 ± 1.2	4.3 ± 0.7	4.2 ± 0.6
Minor defects (%)	7.0 ± 1.8	8.7 ± 1.0	6.8 ± 2.9	12.2 ± 2.6	12.5 ± 3.9	10.7 ± 3.4	11.0 ± 2.6	8.2 ± 1.3
Membrane integrity (E/N) (%)	97.8 ± 1.2	96.5 ± 2.3	98.7 ± 0.9	93.7 ± 2.0	98.7 ± 0.7	93.7 ± 3.0	96.0 ± 2.0	99.7 ± 0.3
MPAI (%)	74.2 ± 1.9*	57.6 ± 6.6	74.1 ± 1.4*	62.9 ± 4.2	75.5 ± 0.3*	61.3 ± 4.2	68.0 ± 5.7*	51.4 ± 8.0
Mitochondrial potential (%)	75.3 ± 1.4*	62.6 ± 7.6	75.1 ± 1.1*	62.7 ± 3.9	74.5 ± 0.3*	62.2 ± 4.6	64.3 ± 6.1*	56.0 ± 9.7
Superoxide production (%)	23.1 ± 3.7	27.8 ± 4.2	24.6 ± 3.8	36.5 ± 4.7	24.6 ± 2.7	34.9 ± 4.4	38.6 ± 7.6	41.9 ± 12.1
H_2O_2 production (%)	56.0 ± 22.6*	38.4 ± 17.2	84.4 ± 2.7*	44.1 ± 6.2	75.8 ± 11.1*	48.2 ± 24.3	83.7 ± 12.9*	70.7 ± 13.1
Lipid peroxidation (%)	14.6 ± 4.4	12.6 ± 4.7	12.8 ± 7.9	15.0 ± 8.8	29.4 ± 9.9	33.6 ± 9.1	28.9 ± 11.3	24.2 ± 10.1
Spontaneous TBARS (ng/mL)	573.6 ± 74.9	380.5± 216.3	503.1 ± 258.1	660.9 ± 254.8	487.3 ± 219.1	444.7 ± 294.2	494.7 ± 234.4	624.8 ± 482.6
Induced TBARS (ng/10^6sperm)	848.4 ±169.1	962.7 ± 411.2	663.3 ± 175.0	761.8 ± 290.6	746.0 ± 198.0	704.2 ± 298.8	802.9 ± 148.5	927.2 ± 402.9

* Represents difference between +SP and -SP at $P \leq 0.05$.

[a,b,c] Superscripts represents difference between moments (M0, M48, M96, M168) of samples evaluation at $P \leq 0.05$.

Abbreviations: +SP, with seminal plasma; -SP, without seminal plasma; TM, total motility; PM, progressive motility; VAP, velocity average pathway; VSL, velocity in straight line; VCL, curvilinear velocity; ALH, amplitude lateral head; BCF, beat cross-frequency; STR, straightness; LIN, linearity; WOB, wobble; IND VEL, spermatic velocity index; IND MOV, spermatic movement index; E/N, eosin-nigrosine stain; MPAI, intact of plasma and acrosomal membranes; H_2O_2, hydrogen peroxide; TBARS, thiobarbituric acid reactive substances.

4. Discussion

The exact role of the seminal plasma in the canine species is not well understood [9-11], however is known that prostatic fluid is detrimental for long term-conservation of semen, so its recommended that it must be removed by centrifugation prior cooling [1].

In the current study, significant higher TM, PM, VAP, rapid spermatozoa, IND MOV, MPAI, peroxide hydrogen production and mitochondrial potential was observed when the seminal plasma was chilled with extended dog semen, at a minimum dilution of 1:3 (semen / extender) and final concentration of 50 x 10^6 spermatozoa/mL. During cryopreservation the sperm mitochondria and plasma membrane are the most demanded organelles, likely responsible for the majority loss in motility and fertility [32]. Sperm motility and integrity are one of the most important parameters used to predict spermatozoa fertilizing capacity, which are also regulated by ATP production *via* oxidative phosphorylation in sperm mitochondria [7,32,33]. In this case, in addition to the protective effect exerted by the diluent, seminal plasma is also important to sperm motility and integrity during cooling at 5 ºC.

The peroxide hydrogen is considered a spermicide with toxic effects to the spermatozoa, promoting decreased respiration and motility [34]. Although the samples chilled with seminal plasma exhibited high peroxide hydrogen production; the membranes integrity, mitochondrial potential and sperm motility were not affected. Barron et al. [35] reported that low concentrations of peroxide hydrogen had stimulating effect on sperm respiration, otherwise Tosic and Walton [34] did not find this correlation.

The seminal plasma of dogs has a lot of components such as antioxidants (catalase, glutathione peroxidase, superoxide dismutase) [8], lipidic membranous vesicles (prostasomes) [36] and a variety of proteins [9,36-41] which protect the spermatozoa and

support the capacitation, hyperactivation, acrosomal reaction and the fecundation processes [42-45].

The presence of antioxidant enzymes protects the sperm cells against oxidative stress reacting with ROS [46]. Angrimani et al. [8] detected the presence of glutathione peroxidase, catalase and superoxide dismutase in seminal plasma of dogs. Once was not possible to measure the presence of these antioxidant in this current study; we cannot affirm that these enzymes contributed to maintain good sperm parameters in samples cooled with seminal plasma.

Some of seminal plasma proteins found in canine semen like matrix metalloproteinase 2 [36], phospholipid hydroperoxide glutathione peroxidase [47], ropporin-1, outer dense fiber protein 2 [48,49], glyceraldehyde 3-phosphate dehydrogenase testis-specific [50,51,52], tubulin alpha 3 – E chain, tubulin alpha 3 chain and tubulin alpha beta-4B chain [53,54] are correlated with sperm motility. Thus, the presence of these seminal plasma proteins may contribute to the maintenance of sperm motility during canine semen cooled at 5º C.

According to time of refrigeration, the sperm kinematic parameters of PM, VLS, STR, LIN WOB and IND MOV were decreasing while ALH increased despite the presence of seminal plasma. The removal of seminal plasma from spermatozoa by centrifugation may damage spermatozoa and influence their fertility ability [55]. Rijsselaere et al. [7] observed that centrifuging dog sperm at 720 g for 5 min it the best strategy to maintain functional parameters of the spermatozoa and acceptable loss of sperm cells even after three days of storage at 4º C. The centrifugation protocol used in this current study was previous described by Cunha and Lopes [21] when canine semen was centrifuged in the presence of autologous seminal plasma or with extenders at 800 g for 15 minutes. These authors did not find difference in sperm motility, vigor and membrane integrity after centrifugation. Although

motility parameters were reducing during time in the present study, membranes integrity, mitochondrial potential, sperm morphology, lipid peroxidation and ROS production (induces and spontaneous TBARS) did not change.

As observed by Moore et al. [56] for stallion, in dogs the that total removal of seminal plasma may be not beneficial to maintenance fertilizing capacity of spermatozoa. The maintenance of a portion of autologous seminal plasma after centrifugation may be interesting to maintain sperm parameters of chill extended dog semen at 5º C. Nothling et al. [57] observed that the addition of autologous seminal plasma to post-thaw canine semen increased fertility rate after intravaginal insemination. Despite we did not perform artificial insemination, the chilled extended dog semen with seminal plasma may exert better fertility results after insemination than without seminal plasma.

The seminal plasma exerts positive influence on sperm motility parameters, viability and mitochondrial function during dog semen extended and stored at 5 ºC for seven days. These results may be attributed to seminal plasma components such as some proteins.

5. Conflicts of interest

The authors have not declared any conflicts of interest.

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8. References

[1] Peña FJ, Núñez-martínez I, Morán JM. Semen Technologies in dog breeding: an update. Reprod Dom Anim 2006;41:21-29.

[2] Farstad W. Bitch fertility after natural mating and after artificial insemination with fresh of frozen semen. J Small Anim Pract 19984;25:561-565.

[3] Linde-Forsberg C. Biology of reproduction and modern reproductive technology. In: Ruvinsky A, Sampson J (eds). The genetics of the dog. New York: CABI Publishing; 2001. p.402-23.

[4] Province CA, Amann RP, Pickett BW, Squires EL. Extenders for preservation of canine and equine spermatozoa at 5 C. Theriogenology 1984;22:409–415.

[5] Ponglowhapan S, Essen-Gustavsson B, Linde-Forsberg C. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. Theriogenology 2004;62:1498–517.

[6] Thomassen R, Farstad W. Artificial insemination in canids: A useful tool in breeding and conservation. Theriogenology 2009;71:190–199.

[7] Rijsselaere T, Van Soom A, Maes D, De Kruif A. Effect of technical settings on canine semen motility parameters measured by the Hamilton-Thorne analyzer. Theriogenology 2003;60:1553-1568.

[8] Angrimani DSR, Lucio CF, Veiga GAL, Silva LCG, Regazzi FM, Nichi M, Vannucchi CI. Sperm maturation in dogs: sperm profile and enzymatic antioxidant status in ejaculated and epididymal spermatozoa. Andrologia 2014;46:814-819.

[9] Aquino-Cortez A., Pinheiro BQ, Lima DBC, et al. Proteomic characterization of canine seminal plasma. Theriogenology 2017;95:178-186.

[10] Gilbert R, Suarez SS. Canine seminal plasma: Transport medium or signaling agent? Vet J 2013;195:131-132.

[11] Brito-Nery LT, Van Tilburg MF, Menezes EB, Machado VP, Moura AAA, Oliveira ECS. Protein patterns of canine seminal plasma. Rev Bras Reprod Anim 2013;38:110-115.

[12] Lucio Cf, Regazzi FM, Silva LCG, Angrimani DSR, Nichi M, Vannucchi CI. Oxidative stress at different stages of two-step semen cryopreservation procedures in dogs. Theriogenology 2016;85:1568-75.

[13] Vieira NMG, Losano JDA, Angrimani DSR, Kawai GKV, Bicudo LC, Rui BR, Da Silva BDCS, Assumpção MEOD, Nichi M. Induced sperm oxidative stress in dogs: Susceptibility against different reactive oxygen species and protective role of seminal plasma. Theriogenology 2018;108:39-45.

[14] Boveris A., Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J 1973;134:707–716.

[15] Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. J Androl 1996;17:276–87.

[16] Wagner H, Cheng JW, Ko EY. Role of reactive oxygen species in male infertility: An updated review of literature. Arab J Urol 2018;16:35–43.

[17] Aitken RJ, De Iuliis, GN. On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod 2010;16:3–13.

[18] Aitken RJ, Smith TB, Jobling MS, et al. Oxidative stress and male reproductive health. Asian J Androl 2014;16:31-38.

[19] Kutzler M. Semen collection in the dog. Theriogenology 2005;64:747-754.

[20] Bloom E. A sistematic search for abnormalities in testis-epidydimid in pedigree bulls

in Denmark. Copenhagen: Royal Veterinary and Agricultural University, 1972:1-36.

[21] Cunha ICN, Lopes MD. Effect of centrifugation on quality of canine semen. Arq. Bras. Med. Vet. Zootec 2009;61:104-109.

[22] Agarwal A, Sharma RK, Nelson DR. New semen quality scores developed by principal component analysis of semen characteristics. J Anrol 2003;24:343-352.

[23] Nuñez-Martínez I, Moran JW, Peña FJA. Three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod Dom Anim 2006;41:408-415.

[24] Freitas-Dell'Aqua CP, Guasti PN, Monteiro GA, Maziero RRD, Dell'Aqua JJA, Papa FO. Flow cytometric analysis of fertile and subfertile frozen stallion spermatozoa. Anim Reprod 2012;9:941.

[25] Freitas-Dell´aqua CP, Sancler-Silva YFR, Silva-Júnior ER, Dell´Aqua JJA, Papa FO. Determination of intracellular and mitochondrial superoxide generation and high mitochondrial membrane potential in equine sperm using flow cytometry. J Equine Vet Sci 2016; 43:S69-S70.

[26] Guasti PN, Freitas-Dell'aqua CP, Maziero RRD, Monteiro GA, Hartwig FP, Lisboa FP, Papa PM, Papa FO. Lipid peroxidation and generation of hydrogen peroxide from subfertile stallion spermatozoa during storage at refrigeration temperature. Reprod Fertil Dev 2012;25:20-20.

[27] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:551-358.

[28] Nichi M, Rijsselaere T, Losano J, Angrimani, D, Kawai G, Goovaerts I, Van Soom A, Barnage VH, De Clercg J, Bols P. Evaluation of epididymis storage temperature and cryopreservation conditions for improved mitochondrial membrane potential, membrane integrity, sperm motility and in vitro fertilization in bovine epididymal sperm. Reprod Dom Anim 2017;52:257-263.

[29] Losano JDA, Angrimani DSR, Redondo R, Brito MM, Rui BR, Kawai GKV, Mendes, CM, Assumpção MEOD, Nichi M. Deleterious effect of high carnosine concentrations in extenders during sperm cryopreservation in dogs. J Vet Androl 2017;2:2542-3045.

[30] Gomez E., Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function. Int J Androl 1998;21:81-94.

[31] Andrade DF, Ogliari PJ. Statistics for agricultural and biological sciences with notions of experimentation [in Portuguese]. 1ª ed. Florianópolis: UFSC; 2007.

[32] Gonzalez-Fernandez L, Morrell JM, Pena FJ, Macias-Garcia B. Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. Theriogenology 2012;78:415–422.

[33] Moraes CR, Meyers S. The sperm mitochondrion: Organelle of many functions. Anim Reprod Sci 2018;194:71-80.

[34] Tosic J, Walton A. Metabolism of spermatozoa. The formation and elimination of hydrogen peroxide by spermatozoa and effects on motility and survival. Bioch J 1950;47:199-212.

[35] Barron ESG, Flood V., Gasvoda B. Biol Bu. Woodse Hole 1949;97:51.

[36] Mogielnicka-Brzozowska M., Kowalska N., Fraser L., Kordan W. Proteomic characterization of zinc-binding proteins of canine seminal plasma. Reprod Dom Anim 2015;50:1017-1021.

[37] Bell FW, Klausner JS, Hayden DW, Lund EM, Liebenstein BB, Feeney DA, Johnston SD, Shivers JL, Ewing CM, Isaacs WB. Evaluation of serum and seminal plasma markers in the diagnosis of canine prostatic disorders. J Vet Int Med 1985;9:149–153.

[38] Kikuchi M., Mizoroki S, Kubo T, Ohiwa Y, Kubota M, Yamada N, Orino K, Ohnami Y, Watanabe K. Seminal plasma lactoferrin but not transferrin reflects gonadal function in dogs. J Vet Med Sci 2003;65:679-684.

[39] Saengsoi S, Wei-Yau S, Shyu CL, Wu JT, Warinrak C, Lee WM, Cheng FP. Detection of matrix metalloproteinase (MMP)-2 and MMP-9 in canine seminal plasma. Anim Reprod Sci 2011;127:114-119.

[40] Zelli R, Bellezza I, Rambotti MG, Minelli A, Polisca A. Ultrastructural and enzymatic activity of membranous vesicles isolated from canine seminal plasma. Reprod Dom Anim 2013;48:252–257.

[41] Strzezek R, Szemplinska K, Filipowicz K, Kordan W. Semen characteristics and selected biochemical markers of canine seminal plasma in various seasons of the year. Polish J Vet Sci 2015;18:13-18.

[42] Varilová T, Seménkova H, Horak P, Madera M, Pacáková V, Tichá M, Stulík K. Affinity liquid chromatography and capillary electrophoresis of seminal plasma proteins. J Sep Sci 2006;29:1110-1115.

[43] Mogielnicka-Brzozowska M, Fraser L, Czarzasta J, Kordan W. Isolation and characterization of zinc-binding proteins of canine seminal plasma. Polish J Vet Sci 2011;15:493-498.

[44] Davalieva K, Kiprijanovska S, Noveski P, Plaseski T, Kocevska B, Broussard C, Plaseska-Karanfilska D. Proteomic analysis of seminal plasma in men with different spermatogenic impairment. Andrologia 2012;44:256-264.

[45] Milardi D, Grande G, Vincenzoni F, Messana I, Pontecorvi A, De Marinis L, Castagnola M, Marana R. Proteomic approach in the identification of fertility pattern in seminal plasma of fertile men. Fertil Steril 2012;97:67-73.

[46] Sies H. Strategies of antioxidant defense. Europ J Biochem 1993;215:213-219.

[47] Imai H, Suzuki K, Ishizaka K, Ichinose S, Oshima H, Okavasu I, Emoto K, Umeda M, Nakagawa Y. Failure of the expression of phospholipid hydroperoxide glutathione peroxidase in the spermatozoa of human infertile males. Biol Reprod 2001;64:674-683.

[48] Mariappa D, Aladakatti RH, Dasari SK, Sreekumar A, Wolkowicz M, Van Der Hoorn F, Seshagiri PB. Inhibition of tyrosine phosphorylation of sperm flagellar proteins, outer dense fiber protein-2 and tektin-2, is associated with impaired motility during capacitation of hamster spermatozoa. Mol Reprod Dev 2010;**77**:182-93.

[49] Kwon WS, Rahman MS, Lee JS, Kim J, Yoon SJ, Park YJ, You YA, Hwang S, Pang MG. A comprehensive proteomic approach to identifying capacitation related proteins in boar spermatozoa. BMC Genomics 2014;15:897.

[50] Bunch DO, Welch JE, Magyar PL, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S protein distribution during mouse spermatogenesis. Biol Reprod 1998;58:834-841.

[51] Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci 2004;101:16501-16506.

[52] Kuravsky ML, Aleshin VV, Frishman D, et al. Testis-specific glyceraldehyde-3-phosphate dehydrogenase: origin and evolution. BMC Evol Biol 2011;11:160.

[53] Rtidiger M, Plessmann U, Riidiger A-H, Weber K. Fl tubulin of bull sperm is polyglycylated. FEBS Letters 1995;364:147-151.

[54] Gagnon C, White D, Cosson J, Huitorel P, Eddé B, Desbruyères E, Paturle-Lafanechère L, Multigner L, Job D, Cibert C. The polyglutamylated lateral chain of alpha-tubulin plays a key role in flagellar motility. J Cell Sci 1996;109:1545-1553.

[55] Sharma RK, Vemulapalli S, Kohn S, Agarwal A. Effect of centrifuge speed, refrigeration medium, and sperm washing medium on cryopreserved sperm quality after thawing. Arch Androl 1997;39:37-38.

[56] Moore A, Squires EL, Graham JK. Effect of plasma on the cryopreservation of equine spermatozoa. Theriogenology 2005;63(9):2372-81.

[57] Nothling JO, Shuttleworth R, De Haas K. Homologous prostatic fluid added to frozen-thawed dog spermatozoa prior to intravaginal insemination of bitches resulted in better fertility than albumin-free TALP. Theriogenology 2005;63:1469-1480.

CAPÍTULO 3

CONSIDERAÇÕES FINAIS

A partir dos trabalhos científicos apresentados, foi possível observar uma variação racial na composição das proteínas do plasma seminal e das células espermáticas caninas. No entanto, algumas raças estudadas apresentaram correlação quanto à composição proteica do sêmen, e esta correlação pode estar possivelmente relacionada à proximidade genética entre as raças descritas no presente estudo.

O sêmen canino refrigerado a 5ºC na presença do plasma seminal apresentou melhores resultados em relação ao refrigerado na ausência do plasma. Possivelmente a presença de proteínas presentes no plasma seminal deve ser um dos fatores que contribuem com a maior viabilidade do sêmen a baixas temperaturas.

Mais estudos com relação às proteínas do sêmen de diferentes raças caninas, bem como sobre as principais funções desempenhadas pelo plasma seminal canino durante à criopreservação do sêmen, devem ser realizados com o intuito de aprimorar os resultados obtidos com as biotecnologias reprodutivas aplicadas a esta espécie e àquelas em vias de extinção.