AN ABSTRACT OF THE THESIS OF

Rachel M. Hegedus for the degree of Honors Baccalaureate of Science in Animal Sciences presented on February 28, 2014. Title: Comparison of Canine Spermatozoa Motility, Morphology, and RNA Integrity Using Three Different Cell Purification Solutions.

Abstract approved:

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Transcriptome gene expression studies of sperm RNA have been utilized in a variety of different species to investigate causes of male infertility. Previous research investigators have optimized cell separation and RNA isolation techniques for each species of interest. To date, no study has been completed for dogs. The objective of this thesis research was to investigate the efficacy of various cell separation techniques in separating sperm cells from somatic cells in the ejaculate to yield a sample of total pure sperm RNA that could be used for a future downstream application. Comparisons were made between the conventional swim-up method and two commercial density gradient centrifugation (DGC) solutions (Bovipure™ and Equipure™, Nidacon International, Mölndal, Sweden) Prior to and just following cell separation, total motility, normal morphology, and sperm count were determined for each method. Following cell separation, total RNA was isolated from each sample and RNA quantity and quality was determined via spectrophotometry and reverse transcriptase polymerase chain reaction. The presence of somatic cell RNA was used to determine purity of the cell separation method. The DGC methods were superior in separating sperm with higher percent normal morphology and higher total motility than the swim-up method. Additionally, the DGC methods were superior at producing a more pure RNA sample than samples not treated with any

separation technique (control). This research shows that the DGC methods should be used to separate canine sperm cells prior to RNA isolation for sperm-specific transcriptome applications.

Keywords: cell separation, density gradient centrifugation, infertility, sperm, swim-up

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# Comparison of Canine Spermatozoa Motility, Morphology, and RNA Integrity Using Three Different Cell Purification Solutions

by

Rachel M. Hegedus

A PROJECT

Submitted to

Oregon State University

University Honors College

In partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Animal Sciences (Honors Scholar)

Presented February 28, 2014
Commencement June 2014

Honors Baccalaureate of Science in Animal Sciences project of Rachel Morgen Hegedus

presented on February 28, 2014.

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Rachel Morgen Hegedus, Author

ACKNOWLEDGEMENTS

This project would not have been possible without the incredible mentorship and leadership from my thesis advisor, Dr. Kutzler. I want to thank her for her assistance and encouragement throughout the entire process. Additionally, I want to thank my committee members, Dr. Estill and Dr. Menino for their knowledge, expertise and guidance. The graduate students in the Theriogenology lab, Caitlin Donovan, Libby Fellows, and Justine Gullaba, were great supporters and gave me such good advice for my thesis and my future. I especially thank Libby for teaching me the process of RNA isolation that started this whole project.

I had a lot of help throughout this research project: Ashley Doherty and Ellie Bohrer performed cell concentration analysis on all semen samples, Stephanie Bollmann and Dr. Massimo Bionaz assisted in the primer design process, Caprice Rosato and the Center for Genome Research and Biocomputing allowed me access to their thermocycler for my RT-PCR protocols and Dr. Alfred Menino provided his laboratory and guidance for the agarose gel and UV visualization process of RT-PCR products. Finally, Caitlin Donovan wrote the code for the program in SAS and used it to evaluate the data. Their help and guidance were instrumental in completing this project.

Finally, I thank my family and friends for supporting me throughout the research process and never letting me give up.

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# DEDICATION

This thesis is dedicated to my grandfather, with whom I share an unwavering passion for animals.

***Comparison of Canine Spermatozoa Motility, Morphology, and RNA Integrity Using Three Different Cell Purification Solutions***

# CHAPTER ONE

# INTRODUCTION AND LITERATURE REVIEW

## 1.1 NORMAL SPERM PHYSIOLOGY AND FUNCTION

### 1.1.1 Male Gamete Formation

The development and maturation of sperm cells (spermatogenesis) is a process that occurs in several stages; the stage from the spermatogonium to the spermatid is called *spermatocytogenesis* and the stage from the differentiation of the spermatid to the mature spermatozoon is called *spermiogenesis*. Spermatocytogenesis consists of various mitotic and meiotic divisions that take place as spermatogonia divide from diploid stem cells to haploid germ cells. The process ensures that a population of spermatogonia is preserved for future rounds of spermatogenesis (Johnston et al. 2001). The meiotic phase of the cycle consists of a division from primary spermatocytes to secondary spermatocytes (Meiosis I) and secondary spermatocytes to spermatids (Meiosis II).

The spermiogenic process consists of the differentiation of the spermatid into a spermatozoon. This process is divided into four phases which transform the spermatid into the morphologic characteristics of mature spermatozoa. The *Golgi phase* is marked

by the development of the acrosome. The acrosome contains enzymes (acrosin, hyaluronidase, esterases, acid hydrolases) essential for fertilization and penetration of the oocyte zona pellucida. The flagellum begins to form during the Golgi phase via the proximal and distal centrioles. The *cap phase* is characterized by the acrosome elongating over the spermatid nucleus. The *acrosomal phase* continues with the acrosome stretching over the nucleus and the elongation of the nucleus itself, producing the characteristic shape of the sperm. The flagellum continues to develop during the acrosomal phase and the manchette microtubules also begin to form. Finally, the *maturation phase* consists of the formation of the postnuclear cap from the manchette and consolidation of mitochondria into the midpiece of the flagella to form the streamlined shape of the cell.

In addition to the changes of the cell shape, important changes within the cell are occurring. One of the most relevant is the replacement of the histones in condensed DNA by protamines. Protamines are nuclear proteins that have sulfhydryl groups (which allows for disulfide bonding) and are extremely compact (which allows for nuclear condensation) (Zini et al. 2001). With the intense condensation of DNA, translation of transcripts into mRNA and proteins is halted until fertilization occurs.

It is important to note that spermatozoa are not motile at the time of release into the seminiferous tubule lumen. As spermatozoa travel through the epididymis, motility is achieved by extrusion of the cytoplasmic droplet that was formed during spermiogenesis. Additional disulfide cross-linking occurs during the condensation of DNA by protamines while the cell travels through the epididymis (Zini et al. 2001).

### 1.1.2 Role of Spermatozoa in Fertilization

Before a sperm is capable of fertilizing an oocyte, it must undergo an additional series of cellular activities. The first is *capacitation*, a process whereby the seminal plasma proteins that coated the sperm during ejaculation are lost due to interactions with the female reproductive tract. This exposes key molecules that bind to the zona pellucida of the ovulated oocyte.

Fertilization occurs in several steps following capacitation of sperm. First, the spermatozoa gain hyperactive motility once capacitation is complete and the cells have reached the uterine tube. The sperm cell then binds to the zona pellucida of the oocyte using binding proteins expressed on the sperm cell membrane. Zona protein 3 on the zona pellucida specifically acts to bind these proteins on the sperm plasma membrane. This is the primary zona binding region. Further binding occurs during the acrosome reaction.

The acrosome reaction is important for the spermatozoa to penetrate the zona pellucida and for modification of the equatorial segment of the sperm plasma membrane to allow it to fuse with the oocyte plasma membrane. Vesiculation occurs when the sperm plasma membrane fuses at multiple sites with the outer acrosomal membrane and enzymes that were carried in the acrosome are released into small vesicles. The enzymes (acrosin and proacrosin) facilitate sperm penetration of the zona pellucida.

Fusion of the equatorial segment of the sperm plasma membrane and occyte plasma membrane is facilitated by a fusion protein located on the oocyte membrane. The oocyte cytoplasm engulfs the spermatozoon, the nuclear membrane of the spermatozoon is broken down, and decondensation of the male DNA occurs by reduction of the various disulfide cross-links formed by protamines. While in the oocyte cytoplasm but before

fusion of male and female genetic material, the male DNA is referred to as the male pronuclei. Fusion of the male and female pronuclei occurs to complete fertilization and embryogenesis begins.

## 1.2 GENETIC CAUSES OF MALE INFERTILITY

There are a variety of genetic anomalies, deletions, and mutations that are known and speculated to cause male infertility. Nearly 30% of male infertility cases in humans are due to genetic factors (Li and Zhou 2012).

Several genes encoding proteins required for spermatogenesis have been studied as potential locations where infertility-causing discrepancies could occur. The proto-oncogene-cp-kit (KIT) and the KIT-ligand/stem cell factor gene (KITLG) are both involved in the differentiation of primordial germ cells into spermatogonia (Leeb et al. 2005; Leeb 2007). Mutation in these genes in mice cause sterility (Leeb et al. 2005; Leeb 2007; Manova et al. 1990; Dolci et al. 1991; Godin et al. 1991; Kissel et al. 2000). An additional form of the KIT gene, produced by alternative splicing, may be involved in oocyte activation post fertilization (Leeb et al. 2005; Leeb 2007; Sette et al. 1997).

In non-human primate species, microdeletions on the Y-chromosome in the regions of $AZB_b$ and $AZF_a$ negatively affect spermatogenesis (Leeb et al. 2005; Leeb 2007; Ferlin et al. 2006). However, the effects of these mutations on spermatogenesis are not known in the dog.

With the complete sequencing of the whole dog genome in 2002, gene comparisons between dogs and other species became possible (Leeb 2007). By examining the previously identified genes that affect fertility in these other species, the

genes that potentially need to be investigated in dogs can be targeted. Further examination of the dog genome must be done to identify species specific proteins that may play crucial roles in the spermatogenic process because there are hundreds of genes that control this mechanism. Despite this, caution must be taken when comparing reproductive related genes across species because they have been shown not to be highly conserved (Leeb 2007). Target genes responsible for spermatogenesis include the androgen receptor, the synaptonemal complex protein 3, and the FSH receptor (Ferlin et al. 2006; Leeb et al. 2005).

Genes responsible for encoding proteins during spermiogenesis that are expressed sperm-specifically include the protamine family of genes which have crucial roles in DNA condensation (Leeb 2007; Li and Zhou 2012; Ostermeier et al. 2004; Monjean et al. 2012; Boerke et al. 2007). Genes encoding for proteins necessary for sperm maturation within the seminal plasma in the epididymis include cysteine-rich secretory proteins, alpha 1 actinin, and gamma 2 actin (Leeb et al. 2005; Leeb 2007; Cohen et al. 2000; Wimmers et al. 2005).

## 1.3 SPERMATOZOA RNA

Interest in the presence of spermatozoa RNA is a relatively new area in molecular and genetic research. Much controversy has arisen out of this research over how the transcripts survive the incredible condensation of chromatin during the sperm maturation process and what roles they play in the various functions of spermatozoa. The majority of research regarding these questions has been completed using human spermatozoa with studies using various animals being more recent. Although the spermatozoa RNA in the

male dog has yet to be significantly studied, looking at the results and conclusions of research regarding other mammalian species can give a good idea of what particular transcripts are promising markers for infertility.

### 1.3.1 Spermatozoa RNA Origins and Roles

Historically, the sperm cell was thought to be transcriptionally and translationally inactive following condensation of the nuclear DNA during spermiogenesis. However, in the mid 20$^{th}$ century, several reports using radioactive probes demonstrated that these probes could be integrated into spermatozoa RNA and protein (Miller and Ostermeier 2006a; Bhargava 1957; Abraham and Bhargava 1963). Miller and Ostermeier (2006a) confirmed the presence of RNA transcripts but did not conclusively find spermatozoa to be active in transcription and translation. Since then, various RNA transcripts have been identified, most importantly being mRNA (transcripts derived from DNA and used to translate DNA into proteins) (Li and Zhou 2012; Ostermeier et al. 2002; Kramer and Krawetz 1997; Miller et al. 1994; Miller et al. 1999; Richter et al. 1999). A key feature contributing to the "dormant" transcriptional state of the spermatozoa is the obvious absence of 18S and 28S rRNA (required in the structure of cytoplasmic ribosome for translation of mRNA to protein) (Lalancette et al. 2009; Krawetz 2005; Ostermeier et al. 2002; Hamatani 2011). However, the presence of 55S mitochondrial ribosomes may indicate that some transcription and translation can occur in the cell (Hamatani 2011; Gur and Breitbart 2006).

The earliest hypothesis of the role of spermatozoa RNA stemmed from the key process of nuclear condensation during the process of spermiogenesis. The replacement

of histones for protamines allows for intense compaction of the genetic material, allowing the streamlined morphology of the sperm cell. This condensation leads to the inability of the cell to perform transcriptional activities because access to the genes encoding the cellular machinery is inhibited. As a result, the spermatozoa RNA that has been confirmed to be present in the cell was initially thought to be remnants of the genes expressed during spermatogenesis and the sperm maturation process (Platts et al. 2007; Lalancette et al. 2009; Hamatani 2011; Li and Zhou 2012). These transcripts were thought to have no function in any activity in which the cell may participate (Boerke et al. 2007). Studies investigating transcripts involved in these processes have helped to support this hypothesis but recent research has suggested that reverse transcriptases are active in mature sperm and can initiate translation of exogenous mRNA transcripts or complete protein translation from nuclear encoded genes via mitochondrial type ribosomes (Giordano et al. 2000; Gur and Brietbart 2006; Li and Zhou 2012).

Additional research has provided convincing evidence for a role of spermatozoa RNA in early embryo development (Ostermeier et al. 2004). There are six spermatozoa mRNA transcripts involved in the development of the zygote (Ostermeier et al. 2004; Li and Zhou 2012). Though the exact mechanisms through which the mRNA works is unknown, resulting proteins could be involved in embryogenesis, morphogenesis, and implantation (Boerke et al. 2007). Additionally, there is strong evidence that some of the mRNAs persist until the activation of the embryonic genome (Boerke et al. 2007).

Another function of spermatozoa RNA is to deliver exogenous DNA and mRNA to the oocyte (Miller et al. 1999; Li and Zhou 2012; Boerke et al. 2007; Chan et al. 2000; Sciamanna et al. 2003; Giordano et al. 2000). Furthermore, there is evidence that these

exogenous mRNA transcripts can be reverse transcribed into cDNA and then translated into various proteins, indication that RNA may have a crucial role in early embryonic development (Sciamanna et al. 2003; Li and Zhou 2012; Giordano et al. 2000). It has been hypothesized that both these endogenous and exogenous RNA transcripts (primarily small RNAs like miRNAs, endo-siRNAs, and piRNAs) delivered by the sperm cell can exert epigenetic effects on the developing embryo (Boerke et al. 2007; Miller and Ostermeier 2006b; Miller et al. 2005; Das et al. 2013; Dadoune 2009; Cuzin and Rassoulzadegan 2010; Daxinger and Whitelaw 2012; Puri et al. 2010). More research must be done to uncover the specific mechanisms and implications of this potential mRNA translation activity in the mature spermatozoa.

### 1.3.2 Transcriptome Analysis of Spermatozoa

Recent research has developed transcriptome expression profiles of spermatozoa RNA due to the interest surrounding its potential functions in infertility. Through technologies such as real time polymerase chain reaction and microarray, differential expression levels of transcripts can be elucidated between samples. Identification of differences in the expression of these RNAs from infertile versus fertile males will give more insight into the particular sperm RNA transcripts that may have an effect on infertility in the male.

The research involving both human and animal spermatozoa RNA transcriptome analysis has presented a variety of transcripts that have been summarized in Table 1.3.2. There have been no transcriptome analysis studies for the dog.

Studies in the bull suggest that the majority of up-regulated transcripts in high fertility bulls are critical for fertilization in that these genes are responsible for cellular transport, structural molecules, cell shape and development, and receptor activity (Feugang et al. 2010). A recent study investigating the spermatozoa RNA transcriptome of the stallion showed that most genes were involved in cell structure, G-protein coupled receptors, membrane transport, ion channels, and mitochondrial ribosomal functions (Das et al. 2013). These cell functions are important for capacitation, fertilization events, and the acrosome reaction (Das et al. 2013; Moore et al. 1993; Etkovitz et al. 2009; Teves et al. 2009). The conservation of these highly expressed genes can be seen through comparison of transcriptome analysis studies across species. Identification and characterization of these transcripts can be useful indicators of markers for genetic infertility.

Table 1.3.2 Transcripts Identified through Comparative Transcriptome Analysis

| Species | Gene Symbol | Role and Relation to Male Infertility | Reference |
|---|---|---|---|
| Human | PRM1 | Chromatin condensation, motility<br>Reduced in infertile men<br>Relation to azoospermia | Carreau et al. 2007; Steger et al. 2003; Jodar et al. 2012 |
| Human | PRM2 | Chromatin condensation, motility | Li and Zhou 2012; Ostermeier et al. 2004 Montjean et al. 2012; Boerke et al. 2007; |
| Human | clusterin | Oocyte-penetrating capacity<br>Zygote development | Li and Zhou 2012; Boerke et al. 2007; Ostermeier et al. 2004 |
| Human | PLC-ζ | Induction of $Ca^{2+}$ oscillations<br>Fertilization<br>Activation of oocyte | Sone et al. 2005; Saunders et al. 2002; Yoda et al. 2004; Kouchi et al. 2004; Yoon et al. 2008; Krawetz 2005; Li and Zhou 2012; Platts et al. 2007; Boerke et al. 2007; Hamatani 2011 |
| Human | c-myc | Capacitation<br>Acrosome reaction<br>Fertilization | Li and Zhou 2012; Lambard et al. 2004; Miller et al. 2005; Kumar et al. 1993; Carreau et al. 2007 |
| Human | eNOS and nNOS | Capacitation<br>Motility – motility inhibited by high concentrations | Li and Zhou 2012; Rosselli et al. 1995; Miller et al. 2005; Lambard et al. 2004; Carreu et al. 2007 |
| Human | P450 aromatase | Motility<br>Mutation causes low sperm count, reduced | Carreau et al. 2007; Li and Zhou 2012; Jedrzejczak et al. |

| | | | |
|---|---|---|---|
| | | motility | 2006; Aquila et al. 2002; Rochira et al. 2005; Miller et al. 2005 |
| Human | TSSK6 | Motility | Li and Zhou 2012; Bissonnette et al. 2009 |
| Human | ADAM5P | Motility | Li and Zhou 2012; Bissonnette et al. 2009 |
| Human | GP130 | Motility<br>Reduced expression in asthenozoospermic men | Cai et al. 2006; Li and Zhou 2012 |
| Human | VASA | Germ cell development | Li and Zhou 2012; Guo et al. 2007 |
| Human | HSPA2 | Sperm maturity, fertility and function | Li and Zhou 2012 |
| Human | BDNF | Spermatogenesis regulation<br>Reduced in oligoasthenozoospermic men | Zheng et al. 2011; Li and Zhou 2012 |
| Human | TrKA | Receptor for nerve growth factors important in development<br>Reduced in oligoasthenozoospermic men | Li et al. 2010; Li and Zhou 2012 |
| Human | AKAP4 | Regulation of G-protein coupled receptors<br>Important protein of sperm flagellum sheath<br>Motility | Miki et al. 2002; Appert-Collin et al. 2006; Li and Zhou 2012; Boerke et al. 2007 |
| Human | IGR-receptor | Regulated by spermatozoa miRNA<br>Involved in embryonic growth | Krawetz 2005; Li and Zhou 2012 |
| Human | DKK2 | miRNA that inhibits the wingless signaling pathway<br>Involved in morphogenetic patterning | Li and Zhou 2012; Mao and Niehrs 2003 |
| Human | KRAB transcription family | Early embryonic maintenance<br>Methylation of genomic regions, imprinted genes | Lalancette et al. 2009; Bissonnette et al. 2009; Wiznerowicz et al. 2007 |

| | | | |
|---|---|---|---|
| Human | GA17 | Sperm-oocyte interaction | Boerke et al. 2007; Miller and Ostermeier 2006b |
| Human | COX5B | Subunit of terminal mitochondrial respiratory transport enzyme<br>Function unknown | Boerke et al. 2007; Miller and Ostermeier 2006b |
| Human | TFAM | Mitochondrial transcription factor<br>Function unknown | Boerke et al. 2007; Miller and Ostermeier 2006b |
| Human | FOXG1B | Early embryo patterning<br>Present only in fertilized embryos | Krawetz 2005; Boerke et al. 2007 |
| Human | WNT5A | Cellular differentiation, morphogenetic patterning<br>Present only in fertilized embryos | Krawetz 2005; Boerke et al. 2007 |
| Human | STAT-4 | Activates transcription<br>Cell cycle control | Boerke et al. 2007 |
| Human | ACRV1 | Acrosomal protein<br>Reduced in teratozoospermic men | Hamatani 2011 |
| Human | SPAM1 | Acrosomal protein<br>Reduced in teratozoospermic men | Hamatani 2011 |
| Human | ODF1-4 | Non tubulin parts of sperm flagella<br>Reduced in teratozoospermic men<br>May be involved in motility | Hamatani 2011 |
| Human | TPX-1 | Motility<br>Reduced in asthenozoospermic patients | Wang et al. 2004; Jodar et al. 2012 |
| Human | LDHC | Motility<br>Reduce in asthenozoospermic patients | Wang et al. 2004; Jodar et al. 2012 |
| Human | HILS | Involved in nuclear condensation<br>Reduced in asthenozoospermic patients | Jedrzejczak et al. 2007; Jodar et al. 2012 |
| Human | TNP1 | Transition protein during replacement of histones with protamines<br>Reduced in asthenozoospermic patients | Kempisty et al. 2007; Jodar et al. 2012; Jedrzejczak et al. 2007 |

| | | | |
|---|---|---|---|
| Human | TNP2 | Transition protein during replacement of histones with protamines<br>Reduced in asthenozoospermic patients | Kempisty et al. 2007; Jodar et al. 2012; Jedrzejczak et al. 2007 |
| Human | ANXA2 | Calcium binding protein<br>Motility initiation, regulation<br>Reduced in asthenozoospermic men | Jodar et al. 2012 |
| Human | BRD2 | Transcriptional regulator<br>Spermatogenesis<br>Reduced in asthenozoospermic men | Jodar et al. 2012 |
| Human | OAZ3 | Involved in motility<br>Reduced in asthenozoospermic men | Jodar et al. 2012; Tokuhiro et al. 2009 |
| Human | SPZ1 | Transcription factor<br>Spermatogenesis<br>Down regulated in infertile men | Montjean et al. 2012; Horowitz et al. 2005 |
| Human | CREM | Spermatogenesis<br>Down regulated in infertile men | Montjean et al. 2012; Liu et al. 2004; Blendy et al. 1996; Horowitz et al. 2005; Liu et al. 2010 |
| Human | MEA1 | Spermatogenesis<br>Down regulated in infertile men | Montjean et al. 2012; Ohinata et al. 2002 |
| Human | SPATA4 | Spermatogenesis<br>Down regulated in infertile men | Montjean et al. 2012 |
| Human | JMJDIA | Histone modification<br>Spermatogenesis<br>Down regulated in infertile men | Liu et al. 2010; Montjean et al. 2012 |
| Bull | CSN2 | Ion transport for fertilization | Feugang et al. 2010 |
| Bull | Protamine 1 | DNA packaging, stabilization<br>Motility | Feugang et al. 2010; D'Occhio et al. 2007; Galeraud-Denis et al. 2007; O'Brien and Zini 2005 |

| | | | |
|---|---|---|---|
| Bull | ZMYND11 | Transcriptional repressor<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | SUMO1 | Ubiquitin modifier, nuclear transport, transcriptional regulation<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | SEP15 | Translation termination<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | MTFR1 | Mitochondrial fission regulator, protection from oxidative stress?<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | GALK1 | Galactose metabolism<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | ID2 | Inhibition of transcription factors, negative regulation of cell differentiation<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | ZEP14 | Function unknown<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | MADCAM1 | Cell adhesion molecule, immune response<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | MTF2 | Transcription factor<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Bull | PAFAH1B1 | Platelet activating factor<br>Abundant in high fertility males | Fuegang et al. 2010 |
| Stallion | MMP1 | Spermatogenesis | Das et al. 2013; Saengsoi et al. 2011 |
| Stallion | MMP3 | Spermatogenesis | Das et al. 2013; Saengsoi et al. 2011 |
| Stallion | TNP2 | Transition protein during replacement of histones with protamines<br>Chromatin structure | Das et al. 2013; Zhao et al. 2001 |

| | | | |
|---|---|---|---|
| Stallion | PRM1 | Chromatin structure | Das et al. 2013; Bench et al. 1996 |
| Stallion | PKM2 | Pyruvate metabolism, energy production<br>High in high fertility bovine males | Das et al. 2013; Peddinti et al. 2008 |
| Stallion | GRP94 | Sperm maturation | Das et al. 2013; Kameshwari et al. 2010 |
| Stallion | COL2A1 | Testes development | Das et al. 2013 |
| Stallion | FBXO9 | Sperm differentiation | Das et al. 2013; Paillisson et al. 2005 |
| Stallion | CASP1 | Protease activated for apoptosis<br>Spermatogenesis | Das et al. 2013; Bader et al. 2010; Cavalcanti et al. 2011 |
| Stallion | CRISP2 | Capacitation<br>Sperm-egg fusion | Das et al. 2013; Arangasamy et al. 2011 |
| Stallion | CRISP3 | Protection from degradation | Das et al. 2013; Arangasamy et al. 2011 |
| Stallion | NEMF | Sperm-egg interactions | Das et al. 2013 |
| Stallion | CTNNBIP1 | Transcription regulation<br>Spermatogenesis | Das et al. 2013; Boyer et al. 2012 |
| Stallion | LCP1 | Sperm maturation | Das et al. 2013; Yamazaki et al. 2006 |
| Stallion | DNTTIP2 | Chromatin remodling | Das et al. 2013; Fujita et al. 2003 |
| Stallion | FGD3 | May function in motility | Das et al. 2013; Huber et al. 2008 |
| Stallion | LYRM1 | Mitochondrial membrane polarization<br>Function unknown | Das et al. 2013; Cao et al. 2010 |
| Stallion | PDIA4 | Spermatogensis<br>Sperm maturation<br>Sperm-oocyte fusion | Das et al. 2013; Dun et al. 2012 |

## 1.4 SPERMATOZOA RNA ISOLATION TECHNIQUES

Several factors must be considered when isolating RNA from sperm. The ejaculate sample may contain many cell types other than sperm and a cell separation technique must be utilized in order to separate sperm cells from somatic cells (Goodrich et al. 2013; Lewis 2007). Because of the subtle differences between the sperm cell membranes of different species, the development of an optimal protocol for RNA isolation from sperm cells is necessary for each individual species (Das et al. 2013; Varner and Johnson 2007; Krawetz 2005; Gilbert et al. 2007). Sperm cells contain comparatively low amounts of RNA compared to somatic cells (Goodrich et al. 2013; Goodrich et al. 2007; Das et al. 2010; Krawetz 2005; Gilbert et al. 2007). Finally, the rRNA in sperm cells is cleaved during the process of spermatogenesis to stop translational activity. Therefore, quality assessment using tools such as a Bioanalyzer cannot be utilized because the sperm cell lacks rRNA markers (Goodrich et al. 2013; Johnson et al. 2011; Goodrich et al. 2007).

### 1.4.1 Cell Purification Methods for Separation of Sperm Cells in the Ejaculate

Cell separation techniques have been developed in order to purify the sperm from ejaculate samples for successful freezing and reproductive application. The seminal plasma in the ejaculate must be removed prior to cryopreservation or use for various reproductive technologies because it has characteristics that impair the initiation of capacitation and fertilization (Allamaneni et al. 2005; Mortimer 2000; Yanagimachi 1994; Rogers et al. 1983). One important consideration is that repeated centrifugations of

ejaculate samples results in the production reactive oxygen radical species (ROS) by these contaminating cells which causes damage to the sperm membrane and DNA, reducing the fertilizing ability of the sperm (Younglai et al. 2001; Twigg et al. 1998b; Moohan and Lindsay 1995; Aitken and Clarkson 1988; Alvarez et al. 1987). Centrifugation techniques have been developed in order to reduce the possibility of ROS forming. Additionally, separation of semen samples initiates capacitation of sperm, which destabilizes sperm membranes, leading to the acrosome reaction and allowing sperm to acquire fertility (Samardzijia et al. 2006; Centola et al. 1998; Van Soom and de Kruif 1996; Yanagimachi 1994). In regards to semen separation for genomic analysis, it is critical that all the somatic cells are removed from the sample so that the RNA isolated is exclusively from sperm cells and that the integrity of the nucleic acids is preserved. However, the initiation of capacitation could be beneficial for downstream RNA isolation, which requires the destabilization of the sperm membrane and lysing of the cell. Although a definitive conclusion on a superior method has not been discerned, comparisons among methods in their efficacy on fresh and frozen semen from various species have been reported (Ricci et al. 2009; Younglai et al. 2001; Moohan and Lindsay 1995; Allamaneni et al. 2005; Mehmood et al. 2009; Sakkas et al. 2000; Natali 2011).

The swim-up method of separating cells in an ejaculate samples is one of the oldest and most common techniques used (Jameel 2008). First described by Mahadevan and Baker (1984), the technique pellets the sperm in the ejaculate and overlays the sample with a nutrient medium. During a period of incubation, the motile morphologically normal spermatozoa swim-up in to the overlaying nutrient medium (Jameel 2008). It is important to note that the success of this method is based upon the

number of layers in the pellet; if many layers of cells exist in the sperm pellet, the cells at the bottom will never get the chance to reach the nutrient medium (Jameel 2008). Swim-up has been hypothesized to be a superior method of separation if a sample with normal sperm count, motility, and morphology is being processed (Natali 2011). When patients present with sperm with various abnormalities such as oligozoospermia, a method other than swim-up is advised because of the low yield (although high quality) of sperm from the swim-up method (Jameel 2008; Burr et al. 1996; Natali 2011).

The other major method of sperm separation that has been developed is density gradient centrifugation. This method is preferred to yield motile and morphologically normal sperm (Natali 2011; Sakkas et al. 2000; Allamaneni et al. 2005). Many commercial products are available which consist of colloidal silica suspension in either an isotonic salt solution or HEPES-buffered human tubal fluid (Phillips et al. 2012). Various dilutions are made to form a discontinuous gradient over which an aliquot of sperm is laid. After a period of centrifugation, the motile, morphologically normal sperm cells are found at the bottom of the tube while abnormal cells can be removed with the supernatant (Natali 2011).

PureSperm® (Nidacon International, Mölndal, Sweden) is a commercially available density gradient that has been widely studied in the human, dog, bull, brown bear, and marmoset (Phillips et al. 2012; Dorado et al. 2011; Maxwell et al. 2007; Nicolas et al. 2012; Hernandez-Lopez et al. 2005). Percoll (Pharmacia, Uppsala, Sweden) is another density gradient used to separate sperm. In human applications, the latter was shown to have potential effects on cleavage rates, embryo development, and endotoxic

effects on sperm membranes and has since been recommended against (Samardzijia et al. 2006; Phillips et al. 2012; Strehler et al. 1998; Chen and Bongso 1999; Mendes et al. 2003; Mortimer 2000). However, it is still widely used in many non-human applications (Phillips et al. 2012).

When comparing the two density gradient centrifugation products for canine semen samples, PureSperm® was reported to be superior to Percoll (Phillips et al. 2012). BoviPure™, another product by Nidacon International, was specifically designed according to the size and density of bull sperm. BoviPure™ was shown to be better than Percoll in cleavage rate and embryo production when bull sperm was processed with both products (Smardzija et al. 2006).

Some studies conclude that density gradient centrifugation is superior to the swim-up method (Allamaneni et al. 2005) while others conclude that both methods are effective in separating viable sperm from the rest of the ejaculate (Mehmood et al. 2009; Moohan and Lindsay 1995). Of interest to this thesis are the studies comparing these methods in separating sperm with nuclear anomalies from sperm with normal DNA. Sakkas and coworkers (2000) found that the swim-up method was not as efficient as density gradient centrifugation in separating sperm with a low percentage of nuclear anomalies while the density gradient centrifugation significantly reduced the percentage of sperm with nuclear anomalies. The density gradient centrifugation methods also reduced the percentage of sperm with DNA damage (Sakkas et al. 2000). However, Younglai et al. (2001) found that the swim-up method as well as the density gradient centrifugation methods resulted in low rates of DNA damage. Another study found that DNA damage was significantly reduced using the swim-up method but not the density

gradient centrifugation method (Zini et al. 2000). Overall, a definitive conclusion on the superiority of either method has not been reached when looking at various sperm characteristic evaluations.

### 1.4.2 Available RNA Extraction Methods

Several sperm RNA isolation methods have been reported. Goodrich and colleagues (2007) demonstrated that the RNAeasy Isolation kit (Qiagen) was an adequate system to isolate spermatozoa RNA from human sperm. This kit utilizes a guanidine-based lysis buffer with β-mercaptoethanol for extraction. The TRIzol® reagent (Ambion®, Carlsbad, CA) and accompanying protocol utilizes a guanidine-phenol-chloroform extraction. This method has been used to isolate stallion and bull spermatozoa RNA (Das et al. 2013; Feugang et al. 2010). Additional modifications to the manufacturer's protocol for TRIzol® have been made, including heating the reagent to 65ºC (Gilbert et al. 2007; Bissonnette et al. 2009; Ostermeier et al. 2005).

Bissonnette and coworkers (2009) compared the RNAeasy kit (Qiagen), the RNA isolation kit (Gentra System), the standard TRIzol® procedure (Ambion®) and the heated TRIzol® on bovine spermatozoa. The first two kits utilize a spin column, which serves to bind the membrane of the cells in order to separate the RNA. These were unsuccessful because of the low amounts of RNA in spermatozoa and the high volume of cells which clogged the spin column (Bissonnette et al. 2009). The heated TRIzol® method yielded a higher concentration of RNA than the TRIzol® method alone (Bissonnette et al. 2009). Interestingly, the heated TRIzol® protocol also yielded broader gene diversity across high and low motility samples in down-stream transcriptome

analysis but negatively affected RNA integrity (Bissonnette et al. 2009). It is important to emphasize that care must be taken when heating these reagents as they are extremely volatile and caustic, with the potential to cause severe chemical burns both internally and externally to the operator.

### 1.4.3 Evaluation of Spermatozoa RNA Yield and Purity

Because RNA is isolated from sperm cells most commonly to be later utilized in a transcriptome study, it is critical that the sample be devoid of any contaminating DNA or RNA from somatic cells that may have been in the samples. Following extraction of RNA from the sperm sample, a DNAse treatment to remove any contaminating DNA is standard. The Ambion® product, TURBO DNA-*free*®, is a common kit used to remove this DNA (Das et al. 2010; Goodrich et al. 2013). After DNAse treatment, various measures of RNA purity and yield are available to determine the integrity of the sample.

An initial analysis of extracted RNA can be done using a spectrophotometer. Values that can be determined from this method are the concentration of RNA in the sample and the $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios. Nucleic acid concentration is readily measured by looking at absorbance at a 260nm wavelength (IMPLEN). The $A_{260}/A_{280}$ ratio indicates protein contamination in an isolated RNA or DNA sample. The normal values for a pure sample range from 1.8 to 2. The $A_{260}/A_{230}$ ratio indicates DNA contamination in an RNA sample. If DNA contamination is present, the value should be less than 2.0. If there is no DNA contamination, the value should be greater than 2.0 (IMPLEN).

Somatic cell RNA quality is determined using a Bioanalyzer, which gives an accurate depiction of RNA concentration and integrity (Agilient Technologies, Inc.). For a typical RNA sample isolated from somatic cells, the real time feedback graph shows two distinct peaks, indicating the presence of 18S rRNA and 28S rRNA. From the curve in the graph, a RNA integrity number (RIN) can be assigned to the sample; a RIN 8 or above is considered a pure RNA sample. However, Cappallo-Obermann and colleagues (2011) found that cell purification technique and the use of a DNAse treatment could affect the rRNA content and purity of the sample.

Although the Bioanalayzer system for determining quality is a useful tool for RNA from somatic cells, sperm cells present a challenge because of the degradation of rRNA during the sperm maturation process (Johnson et al. 2011). As explained in Section 1.1.1 of this thesis, the sperm cell is transcriptionally silent, due in part to the expulsion of the excess cytoplasm (which contains translational machinery) during spermiogenesis. Therefore, there is an absence of intact 18S and 28S rRNAs on eletrophoretic analyses (Johnson et al. 2011). Interestingly, analysis of total sperm RNA revealed that about 80% of the total RNA in mature spermatozoa was portions of 18S and 28S rRNAs, not intact transcripts (Johnson et al. 2011). Johnson et al. (2011) additionally found that 28S rRNA in spermatozoa have preferential sites of cleavage where the transcripts are cut during spermiogenesis. From these findings, it is speculated that rRNAs are cleaved to stop translation activity in addition to the expulsion of the cytoplasm which obviously is not successful on its own at extruding all rRNAs from the sperm cell (Johnson et al. 2011).

Because an electrophoretic analysis (e.g. the Bioanalyzer) has not been developed to give an accurate depiction of sperm cell purity, the final step in determining RNA

quality in sperm is using reverse-transcriptase polymerase chain reaction (RT-PCR). Various studies which have isolated RNA from mature spermatozoa used RT-PCR as a quality control mechanism in the RNA isolation protocol (Goodrich et al. 2013; Goodrich et al. 2007; Das et al. 2013; Gilbert et al. 2007; Bissonnette et al. 2009; Feugang et al. 2010). In RT-PCR, two primers should be used; one primer that is a specific transcript present in sperm and the other that is a specific transcript only found in somatic cells. RNA samples are then reverse transcribed into cDNA using each of these primers. A sample in which the somatic cell specific transcript is amplified indicates that it contains somatic cells and is contaminated. Samples containing only sperm should amplify only the sperm cell specific transcript.

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# CHAPTER TWO

# MATERIALS AND METHODS

## 2.1 COLLECTION OF EJACULATE AND SPERM EVALUTION

### 2.1.1 Ejaculate Collection

Semen collection was performed as previously described by Kutzler (2005). Materials needed for collection included a 15mL conical tube labeled with the dog's name and date (15mL Presterilised Centrifuge Tubes, VWR International, West Chester, PA, Cat. No. 89004-368), a plastic semen collection cone fitted over the conical tube (Canine Disposable Collection Cones, Next Generation® Exodus Breeders Corporation, York, PA, Cat. No. 931), gloves for the handler (Powder-free Purple Nitrile Gloves, VWR International, West Chester, PA, Cat. No. 40101-344), and non-spermicidal lubricant (Non-Spermicidal Sterile Lubricating Jelly, First Priority, Inc., Elgin, IL, Cat. No. LU040PC). Privately owned intact male dogs were used in the study. Semen collections were performed at the private homes of the dog's owners, with some of the males utilizing an ovariectomized teaser bitch.

Semen collection was performed by digital manipulation of the penis. The collection cone was placed over the penis so all of the ejaculate would be collected into the attached conical tube. All three fractions of the ejaculate were collected together. After semen collection, lubricant was applied to the penis to avoid hairs from the prepuce

or abdomen from sticking to the penis during detumescence. Semen was transported immediately to the laboratory for evaluation and processing.

### 2.1.2 Sperm Evaluation

Semen samples were evaluated for motility, morphology, and concentration. For the motility evaluation, a small drop of semen was placed on a warmed glass microscope slide and covered with a cover slip. Using bright field microscopy at 40X magnification, the percent total sperm motility (moving) and percent progressive sperm motility (moving forward in a progressive manner) of each sample were recorded. A percentage no more accurate than 5% was assigned for both total and progressive motility.

For the sperm morphology evaluation, a small drop of semen was placed on one end of a labeled glass microscope slide. A small drop of eosin-nigrosin stain (Society for Theriogenology Morphology Stain, Montgomery, AL) was then placed next to the drop of semen and the two drops were mixed gently. A second microscope slide was held at a 45º to the slide containing the sample and pushed across it to create a stained semen smear on the slide. These slides were allowed to air dry and then were stored. The percent normal morphology was performed using bright field microscopy at 100X magnification under oil immersion. Two hundred sperm from each slide were evaluated and abnormalities were recorded. In addition, because eosin and nigrosin is a viability stain, the percent live sperm was also determined for each ejaculate.

For the sperm concentration evaluation, 20µL of ejaculate was diluted into a BMP Leukochek 1.98mL dilution reservoir using a capillary pipette supplied by the manufacturers (Biomedical Polymers Inc., Gardner, MA, Cat. No. BMP-LUKCHK-50).

Following dilution, sperm concentration was determined using a hemocytometer slide with bright field light microscopy. The number of sperm in the large center grid on each side of the hemocytometer were counted and averaged to determine the sperm concentration in the ejaculate in million sperm per mL. Ashley Doherty and Ellie Bohrer performed all of the sperm concentrations.

Each ejaculate was then divided into three equal aliquots. In each ejaculate, one aliquot was not treated with any cell separation technique. The untreated aliquot served as a control within each ejaculate to which the cell separation techniques could be compared. As soon as the sperm evaluation was completed, control aliquots were centrifuged at 670xg for 10 minutes at room temperature in a swing out rotor to pellet the sperm cells. The supernatant was then aspirated off and discarded and the pellet was transferred to a labeled, RNAse-free/DNAse-free microcentrifuge tube for immediate RNA isolation.

## 2.2 SPERM CELL SEPARATION

Three techniques were evaluated in this study for their ability to effectively separate sperm cells from non-sperm cells (somatic cells) within the ejaculate. Each technique employed different procedures which are outlined here.

### 2.2.1 The Swim-Up Method

The swim-up method was performed as previously described by Jameel (2008). The semen aliquot was centrifuged in a 15mL conical tube at 400xg for 15 minutes at room temperature in a swing out rotor centrifuge. The supernatant was discarded and

each pellet was resuspended in 2.5mL of Ham's F-10 Nutrient Mix (Gibco®, Grand Island, NY, Cat. No. 11550-043). The sample was centrifuged again at 400xg for 15 minutes and the supernatant was discarded. The pellet was then carefully overlaid with 1mL of Ham's F-10 and the conical tube was transferred to an incubator (37ºC and 5% $CO_2$) at a 45º incline. After a one hour incubation, all of the supernatant was transferred into a labeled RNAse-free/DNAse-free microcentrifuge tube. Sperm motility, morphology, and concentration were repeated prior to RNA isolation.

### 2.2.2 The Equipure™ Method

The procedures used to process aliquots with Equipure™ (Nidacon International, Mölndal, Sweden) utilize a one layer density centrifugation technique. Equipure™ has an osmolality of 300-310 mOsm and a pH of 7.3 to 7.8 (Nidacon Interantional Equipure™ MSDS). Using a sterile needle and syringe, 3mL of Equipure™ was added to a 15mL conical tube. 2mL of the aliquot of semen was carefully layered over the top of the Equipure™ making sure not to disrupt the interface between the two solutions. The tube was centrifuged at 300xg for 25 minutes at room temperature in a swing out rotor. The supernatant was aspirated off the pellet in a circular motion and the pellet was transferred into a labeled RNAse-free/DNAse-free microcentrifuge tube. Sperm motility, morphology, and concentration was repeated prior to RNA isolation.

### 2.2.3 The Bovipure™ Method

Bovipure™ (Nidacon International, Mölndal, Sweden) is a density centrifugation technique that utilizes two gradient layers. Bovipure™ has an osmolality of 290-300

mOsm and a pH of 7.5 to 8.5 (Nidacon International, Bovipure™ MSDS). BoviDilute™ has an osmolality of 300-310 mOsm and a pH of 7.3 to 7.8 (Nidacon International, BoviDilute™ MSDS). The top layer of the gradient was prepared by combining 2 parts Bovipure™ with 3 parts BoviDilute™ (Nidacon International, Mölndal, Sweden). The bottom layer of the gradient was prepared by combining 4 parts Bovipure™ with 1 part BoviDilute™. Both layers were prepared prior to processing the semen and stored at 4ºC.

Using a sterile needle and syringe, 2mL of the Bovipure™ bottom layer was added into a 15mL conical tube and 2mL of Bovipure™ top layer was carefully layered over the top making sure not to disrupt the interface between the two layers. Semen (2mL) was layered over the top layer without disrupting the interface. The conical tube was centrifuged at 300xg for 25 minutes at room temperature in a swing out rotor. The supernatant was aspirated off the pellet in a circular motion and the pellet was transferred into a labeled RNAse-free/DNAse-free microcentrifuge tube. Sperm motility, morphology, and concentration were repeated prior to RNA isolation.

## 2.3 RNA ISOLATION

### 2.3.1 RNAse Elimination

Because of the fragile nature of RNA and the abundance of RNAses in the environment, care was taken to prepare the work area before RNA isolation. Bench paper was laid down on all work surfaces and labeled as an "RNAse-free/DNAse-free zone." This paper was replaced every week. Before ejaculate samples were collected, the pipettors, pipette tip racks, test tube racks, reagent bottles, labeling markers, and other

work equipment were wiped down with a Kim-wipe sprayed with RNAseZap® (Ambion®, Carlsbad, CA, Cat. No. AM9780) to eliminate RNAses.

### 2.3.2 RNA Isolation

RNA isolations were performed using TRIzol® Reagent (Ambion®, Carlsbad, CA, Cat No. 15596-026) according to the manufacturer's instructions. All steps were performed under a fume hood because of the caustic nature of TRIzol®. To prepare for the first steps in the RNA isolation protocol, the refrigerated microcentrifuge was turned on and set to a temperature of 4ºC. TRIzol® reagent (1mL) was added to each 0.25mL sample in a microcentrifuge tube. Cells were lysed by repeatedly pipetting up and down with a small pipette tip for 2-3 minutes. The lysed samples were incubated at room temperature in the TRIzol® reagent for 15 minutes. Chloroform (0.2mL) was added to each microcentrifuge tube after the 15 minute incubation and then shaken vigorously by hand for 15 seconds. The samples were incubated again at room temperature for 3 minutes. The samples were centrifuged at 12,000xg for 15 minutes at 4ºC. During this process, the sample shifts in different phases with the bottom (pink) organic layer containing the phenol, chloroform, and protein, the middle (white) interphase layer containing the DNA, and the top (clear) aqueous layer containing the RNA.

The aqueous phase (400µL) was transferred into a new, labeled RNAse-free microcentrifuge tube. 0.5mL of 100% isopropanol was added and the sample was incubated for 10 minutes at room temperature. The samples were centrifuged at 12,000xg for 10 minutes at 4ºC. According to the protocol, the RNA following centrifugation was expected to appear as a gel-like pellet but no visible pellet was seen in any sample. The

supernatant was pipetted off and the pellet was washed with 1mL of 70% ethanol. The sample was vortexed for a few seconds and then centrifuged at 7500xg for 5 minutes at 4ºC. The supernatant was pipetted off and the pellet was air dried for 5 to 10 minutes. It was important not to let the pellet dry completely as this would lead to loss of RNA stability. However, if the RNA was still partially dissolved in the ethanol, it would result in lower purity. After drying, the RNA pellet was re-suspended in 50µL of RNAse-free/DNAse-free water (Invitrogen™, Carlsbad, CA, Cat. No. 10977-015) by passing the solution up and down a couple times through the pipette tip. These samples were incubated in a water bath at 57ºC for 10 to 15 minutes.

### 2.3.3 DNAse Treatment

DNAse treatment with the TURBO DNA-free™ kit (Invitrogen™, Carlsbad, CA, Cat. No. AM1907) was performed according to the manufacturer's instructions to eliminate any contaminating DNA in the RNA samples that may have been introduced by pipetting errors during the phase separation in the RNA isolation protocol. For each 50µL RNA sample, 5µL of 10X TURBO DNAse buffer and 1µL of TURBO DNAse was added and the solution was mixed gently. The samples were incubated at 37ºC for 25 minutes. After incubation, 5µL of re-suspended DNAse Inactivation Reagent was added and the solution was mixed well. The samples were incubated at room temperature for 5 minutes, occasionally (2-3 times) mixing the contents by flicking the tube. The samples were then centrifuged at 10,000xg for 1.5 minutes at room temperature and the supernatant was transferred into a new, labeled RNAse-free tube.

### 2.3.4 Determining RNA Concentration and Purity

After the DNAse treatment, the RNA concentration (ng/µL) and purity ($A_{260}/A_{280}$, $A_{260}/A_{230}$) were determined using a spectrophotometer (NanoPhotometer Version 2.1, IMPLEN, Munich, Germany). The cover to the spectrophotometer was removed and the black stage was pulled from its spot in the machine. The spectrophotometer was turned on and allowed to start and calibrate. After this was complete, the black stage was put back into its slot and wiped down with a Kim-wipe. The steps to choose the program for RNA were as follows:

1) "Label Guard Applications" on the main menu

2) "RNA" on the next menu

3) "Lid Factor 10" was made sure to be highlighted and "OK" was chosen

RNAse-free/DNAse-free water (2µL) was placed on the viewing circle of the black stage, being careful not to touch the glass surface. The lid labeled "Factor 10" was then placed on the black stage over the sample and the button for "Blank" was pressed. The lid was removed and the water was wiped off the stage with a Kim-wipe. Then, 2µL of each sample was placed on the viewing circle of the stage, the "Factor 10" lid replaced, and the "Sample" button pushed. The RNA concentration and absorbance values (for determining purity) were displayed on the screen and recorded. The lid was then removed and the stage was wiped down with a Kim-wipe between samples. After determining RNA concentration and purity, the samples were stored at -80ºC until needed for RT-PCR.

## 2.4 DETERMINING THE RNA QUALITY

To determine the quality of the RNA samples, a reverse-transcriptase polymerase chain reaction (RT-PCR) experiment was performed using two genes, one specific to sperm cells (protamine-2) and one found in somatic cells but not in sperm cells (protein tyrosine phosphatase receptor type C - PTPRC). Any amplification of PTPRC transcript in the RNA sample would indicate somatic cell contamination in the sperm sample.

### 2.4.1 Primer Design

The sequences for protamine-2 and PTPRC genes were identified on the NCBI gene website (http://www.ncbi.nlm.nih.gov/gene/). Introns and exons for each gene identified and intron-spanning primers were designed using Primer3 online software (http://bioinfo.ut.ee/primer3-0.4.0/). Primers had a "GC" content of less than 60%, had no hairpins or primer-dimer potential, and were between 18 and 20 base pairs in length. Details on the primers used can be found in Table 2.4.1. All primers were synthesized by Sigma-Aldrich (St. Louis, MO). Upon arrival, primers were re-suspended to a 100µM stock solution and then 10µL of the stock solution was added to 90µL of RNAse-free/DNAse-free sterile water in new, labeled microcentrifuge tubes to create 10µM aliquots of each primer. Primers were stored at -20ºC until use for RT-PCR.

Table 2.4.1 Intron spanning primer sequences for canine Protamine-2 (PRM-2) and PTPRC transcripts

| Gene | Primer Sequence | RNA/cDNA amplicon (bp) | Genomic DNA amplicon (bp) | Annealing temperature (ºC) |
|---|---|---|---|---|
| PRM-2 | *Forward:* AGCGAACATCCACAGCAT *Reverse:* TGTATCTCCTCCTCCTGACC | 252 | 343 | 59ºC |
| PTPRC | *Forward:* AGCGCAGAAACAGAAGAAGT *Reverse:* ACAGACACACACACCCAAAG | 621 | 2116 | 59ºC |

### 2.4.2 Reverse Transcriptase Polymerase Chain Reaction

In preparation for RT-PCR, a set up sheet was constructed outlining each RNA sample's concentration, $A_{260}/A_{280}$ ratio, the amount of sample added to the reaction tube for 100ng total RNA, and the amount of RNAse-free/DNAse-free sterile water added to the reaction tube to reach a final reaction volume of 50µL (See Appendix C). RT-PCR reactions were performed in strips of eight RNAse-free/DNAse-free tubes. A number was assigned to each sample indicating the date on which RT-PCR was done, the strip it was in, the tube it was in, and the identity of the primer used. Reaction tubes were labeled with the strip tube number on the cap and the sample information on the side (date of RNA isolation, dog's first initial, and cell separation method).

RT-PCR reactions were performed using reagents from the Superscript® One-Step RT-PCR with Platinum® *Taq* kit (Invitrogen™, Carlsbad, CA, Cat. No. 10928-042) and according to the manufacturer's instructions. Reaction reagents were added to each tube on ice in the following order:

1) Calculated amount of RNAse-free/DNAse-free sterile water for that sample

2) 25µL 2X Reaction Mix

3) 2.5µL each forward and reverse primer

4) Calculated amount of RNA sample for 100ng

5) 1µL RT/Platinum *Taq* Mix

Samples were transported on ice to the Center for Genome Research and Biocomputing (CGRB) on Oregon State University's campus to be run in the Bio-RAD, DNA Engine Peltier Thermal Cycler (Hercules, CA). The thermocycler was programmed

with cycling parameters named “RACHEL” under the folder “<MAIN>”. The cycling parameters were as follows:

Step 1: 55ºC for 30 minutes

Step 2: 94ºC for 2 minutes

Step 3: 94ºC for 15 seconds

Step 4: 59ºC for 30 seconds

Step 5: 68ºC for 1 minute

Step 6: 34 more cycles starting at Step 3

Step 7: 72ºC for 5 minutes

Step 8: 4ºC until end

After RT-PCR, the reaction tubes were stored at -20ºC until gel electrophoresis.

### 2.4.3 Visualizing RT-PCR Products

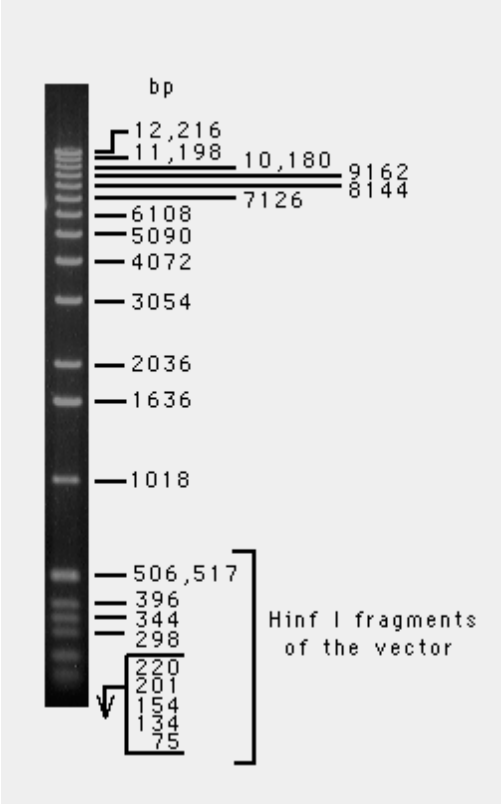
These steps were performed in Dr. Alfred Menino’s reproductive physiology laboratory (Withycombe 135, Corvallis, OR). cDNA products from the RT-PCR were visualized on 2% agarose gels. For each gel, 1.6g of agarose (Sigma-Aldrich, St. Louis, MO, Cat. No. A9539-100G) was measured into a 250mL flask containing 78.4mL of distilled water and 1.6mL of 50X TAE (prepared in Menino lab, Appendix D). A plastic stir bar was added and the flask was heated in the microwave until the mixture was boiling briefly. The mixture was checked periodically to make sure that it was not boiling over and that all the agarose was dissolved. The flask was then removed from the microwave and place on a stir plate. The flask was allowed to cool to about 60ºC (hot to touch but not burning). A gel cast was assembled and well comb was adjusted to make

sure it was properly positioned in the case (not too low). The cooled mixture was then carefully poured into the gel cast, making sure no bubbles were introduced. Once the gel was set, the well comb and rubber sides to the gel cast were carefully removed. The gel was placed in the electrophoresis machine, making sure about 2 to 3mm of TAE Buffer (prepared in Menino lab, see Appendix D) covered the top of the gel.

Samples for electrophoresis were prepared with 2µL of gel loading buffer and 10µL of cDNA (RT-PCR product). DNA ladder samples were prepared with 2µL of 1 Kb DNA Ladder (Invitrogen™, Carlsbad, CA, Cat. No. 15615-016), 2µL of gel loading buffer and 8µL of sterile water. The total volume of each sample (12µL) was then pipetted into a pre-assigned lane in the gel. Gel lane assignments can be seen in Appendix E. The gel was then run at 100V for about an hour until the lowest blue band was about 1 inch from the bottom of the gel.

The gel stain solution was prepared by adding 30µL of SYBR® Green nucleic acid gel stain (Invitrogen™, Carlsbad, CA, Cat. No. S-7563) to 300mL of TAE Buffer in a Tupperware® container covered with paper to eliminate light from quenching the dye solution. A stir bar was used to mix the solution. Once the gel was removed from the electrophoresis machine, it was placed in the Tupperware® container with gel stain solution and a stir bar. The Tupperware® container was then placed on a stir plate and the gel was allowed to stain for up to 30 minutes. The gel was then removed from the gel stain solution and put into a distilled water bath on an oscillator to rinse for 5 minutes. The gel was visualized using the GelLogic 212 Pro machine (Carestream Health, Woodbridge, CT) and images were digitally saved.

Figure 2.4.3 1 Kb DNA Ladder (Invitrogen™, Carlsbad, CA, Cat. No. 15615-016)



## 2.5 DATA ANALYSIS

Data for each individual sample from semen evaluation, the spectrophotometer, and RT-PCR visualization was organized in spreadsheets using Microsoft Office's Excel program (Redmond, WA).

The coefficient of variation was calculated for the control samples of each dog for total motility, morphology, and sperm count semen evaluations using the Excel program. The standard deviation of the control samples was divided by the average and multiplied by 100% to give a percentage value for the coefficient of variation.

Statistical analyses for the semen evaluation, the spectrophotometer, and the RT-PCR visualization results were performed by Caitlin Donovan using the PROC MIXED platform in SAS (V. 9.3, SAS Institute Inc., Cary, NC). To evaluate sample purity for RT-PCR analysis, samples with only sperm-specific transcripts ("pure") were scored as 100% and samples containing somatic cell transcript were scored as 0%. A cell separation method that produced only "pure" samples was considered 100% effective. The experiment unit (individual dog), replication, and separation technique were fixed effects for the scoring. Normality was verified by the Shapiro-Wilk test. Data for all analyses were expressed as the least squares mean ± standard error of the mean (LSM±SD) and significance for all results was defined as $p<0.05$.

## 2.6 REFERENCES

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# CHAPTER THREE

# RESULTS

## 3.1 SEMEN EVALUATION

### 3.1.1 Motility

Total and forward, progressive motility results are summarized in Table 3.1. The total motility decreased significantly following all of the cell separation methods. Additionally, both density gradient centrifugation methods had significantly higher total motility compared to the swim-up method. However, there was no difference between the Equipure™ and Bovipure™ methods in total motility (p=0.68).Because of the viscous nature of the density gradient centrifugation solutions, forward, progressive motility results were unreliable and therefore not used in the comparison of the cell separation methods.

### 3.1.2 Morphology

Percent normal morphology results are summarized in Table 3.1. The percent normal morphology did not differ following either density gradient centrifugation solution compared to samples before the cell separation method was applied, the control samples (Bovipure™ p=0.16 and Equipure™ p=0.71). However, percent normal morphology was significantly lower following the swim-up method compared to either of the two density gradient centrifugation methods or the control samples.

### 3.1.3 Sperm Count

Total sperm count from before separation (control) and after each cell separation method are summarized in Table 3.1. The sperm count decreased significantly following all of the cell separation methods. However, there was no difference between any of the cell separation methods; Bovipure™ vs. swim-up ($p=0.69$); Equipure™ vs. swim-up ($p=0.97$); Equipure™ vs. Bovipure™ ($p=0.59$).

### 3.1.4 Coefficient of Variation for Control Samples

The coefficient of variation ranged from 2.4% to 13.5% for the control samples of the total motility evaluation which were within the acceptable range of 0-30% (Brown, 1998). For the morphology evaluation, control samples had a coefficient of variation ranging from 2.1% to 16.7%. The coefficients of variation for control sperm count samples ranged from 25.8% to110.7% and are likely to be the result due to the fractions of the canine's ejaculate. A failure to completely collect the second fraction would result in a lower sperm count for that sample.

Table 3.1 Semen evaluation results before (control) and after each method of cell separation. Results are reported in least squares means±standard error of the means.

| | **Control** | **Equipure™** | **Bovipure™** | **Swim-Up** |
|---|---|---|---|---|
| Total motility (%) | 89±4 | 52±5 | 55±4 | 12±9 |
| *Forward, progressive motility (%) | 80±0.13 | 36±0.33 | 21±0.2 | 1±0.01 |
| Normal morphology (%) | 78±2 | 79±3 | 83±2 | 37±5 |
| Sperm count ($x10^6$) | 369±44 | 117±50 | 82±42 | 120±87 |

*Represents results only from before the separation method was applied to samples

## 3.2 SPECTROPHOTOMETER

### 3.2.1 RNA Concentration

The RNA concentration from all experiments is summarized in Table 3.2. The RNA concentration was not different in any of the treatments compared to the untreated, control samples; swim-up ($p=0.37$); Equipure™ ($p=0.2$); Bovipure™ ($p=0.48$). There was no difference in the RNA concentrations between the samples separated by the swim-up method and by the Equipure™ method ($p=0.09$) and between the samples separated by the Equipure™ gradient and the Bovipure™ gradient ($p=0.6$).

### 3.2.2 $A_{260}/A_{280}$ Ratio

The $A_{260}/A_{280}$ ratio results are summarized in Table 3.2. There was no difference between the ratios of the untreated, control samples and the swim-up ($p=0.98$) samples, the Equipure™ ($p=0.15$) samples, or the Bovipure™ ($p=0.62$) samples. Additionally, there was no difference between the Bovipure™ ($p=0.79$) and Equipure™ (0.35) techniques when compared to the swim-up method. Finally, there was no difference between the two density gradient centrifugation methods ($p=0.39$).

### 3.2.3 $A_{260}/A_{230}$ Ratio

The $A_{260}/A_{230}$ ratio results are summarized in Table 3.2. There was no difference between the ratios of the untreated, control samples and the swim-up ($p=0.17$) samples, the Equipure™ ($p=0.15$) samples, or the Bovipure™ ($p=0.41$) samples. Additionally, there was no significance between the Bovipure™ and the swim-up method ($p=0.1$).

However, Equipure$^{TM}$ had a higher $A_{260}/A_{230}$ ratio than the swim-up method ($p=0.029$), indicating that there was higher DNA contamination in the swim-up samples.

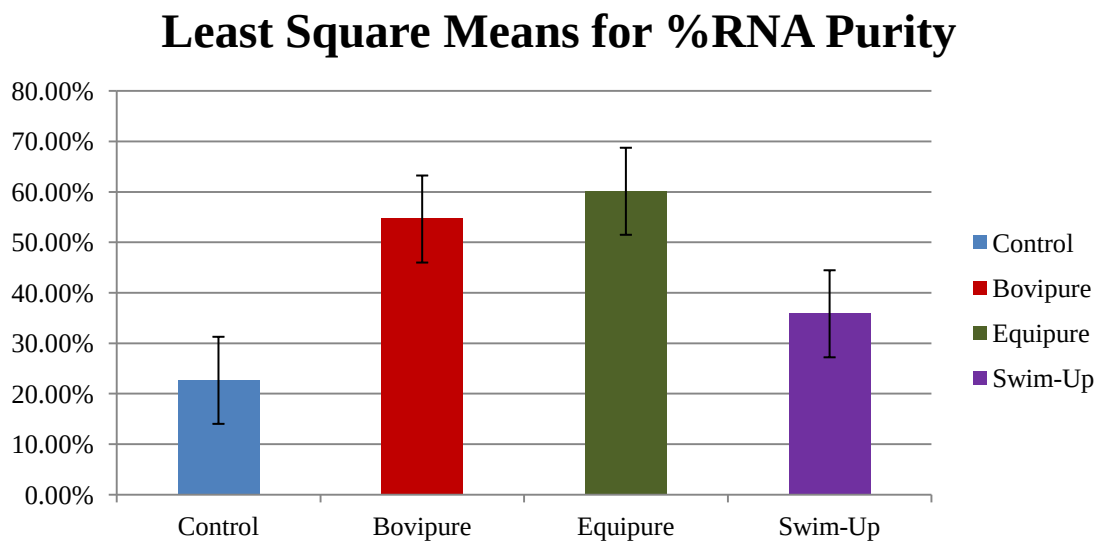
Table 3.2 Spectrophotometer results from each cell separation method. Results are reported in least squares means±standard error of the means.

| | **Control** | **Equipure™** | **Bovipure™** | **Swim-Up** |
|---|---|---|---|---|
| RNA concentration (ng/μL) | 60.51±19.30 | 94.76±25.73 | 78.90±19.22 | 24.76±43.83 |
| $A_{260}/A_{280}$ ratio | 1.06±0.036 | 1.13±0.047 | 1.09±0.036 | 1.06±0.081 |
| $A_{260}/A_{230}$ ratio | 0.20±0.018 | 0.24±0.026 | 0.22±0.018 | 0.15±0.04 |

## 3.3 RT-PCR RESULTS

The RT-PCR results are summarized in Figure 3.3. Sperm samples separated by Bovipure™ ($p=0.033$) or Equipure™ ($p=0.015$) had fewer contaminating somatic cells compared to untreated, control samples. The samples separated by the swim-up method were not more pure than the untreated, control samples ($p=0.538$). However, when comparing the three cell separation methods, the results did not differ significantly.

Figure 3.3 RT-PCR results for each cell separation method including samples receiving no cell separation method (controls). Results are expressed as least square means±standard error of the means. 0% represents a sperm sample contaminated with somatic cells and a value of 100% represents a sperm samples with no somatic cell contamination.



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# CHAPTER FOUR

# DISCUSSION

## 4.1 SEMEN EVALUATION

There have been numerous studies comparing the effects of cell separation techniques on sperm characteristics in humans, dogs, bulls, and buffalo (Phillips 2012; Samardzija et al. 2006; Sakkas et al. 2000; Ricci et al. 2009; Mehmood et al. 2009; Allamaneni et al. 2005; Moohan and Lindsay 1995). Several studies have investigated the effects of the density gradient centrifugation method and the swim-up method on motility, survival, sperm count, acrosome integrity, viability, and chromatin anomalies in these various species (Sakkas et al. 2000; Ricci et al. 2009; Mehmood et al. 2009; Allamaneni et al. 2005; Moohan and Lindsay 1995). However, a definitive conclusion on which cell separation technique is better has not been determined (Natali 2011). Comparison between the swim-up method and the density gradient method in evaluating canine sperm RNA purification has not been done previously. Additionally, this study was interested in the effect on sperm characteristics (total motility, normal morphology, and sperm count) of the swim-up and density gradient centrifugation techniques. Irrespective of cell separation technique employed, our results indicate that there is a significant decrease in total sperm motility following cell separation, although the density gradient methods resulted in significantly higher total motility than the swim-up method. Additionally, percent normal morphology of the samples recovered by the density gradient methods was superior to that of the swim-up method. Since there were no

significant differences between the Equipure™ and the Bovipure™, we could recommend the Equipure™ in a clinical setting because it is a single layer gradient and requires fewer steps to use the product for cell separation.

## 4.2 RNA PURITY

The current study is the first to compare the effects of sperm separation techniques on the expression of sperm-specific transcripts in canine RNA. Our study shows that the Equipure™ and Bovipure™ methods were superior in producing pure samples (uncontaminated by somatic cell RNA) when compared to samples not treated with any cell separation technique. These results validate other studies' use of density gradient centrifugation techniques in sperm separation prior to RNA isolation (Goodrich et al. 2013; Bissonnette et al. 2009; Goodrich et al. 2007; Das et al. 2010; Johnson et al. 2011; Feugang et al. 2010).

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# CHAPTER FIVE

# CONCLUSIONS AND FUTURE DIRECTIONS

## 5.1 CONCLUSIONS

In the present study, we have found that the density gradient centrifugation technique is superior to the swim-up method for separation of sperm with high total motility and normal morphology. Additionally, sperm RNA isolated from samples treated with the density gradient centrifugation solutions exhibited higher purity than samples not treated with any cell separation technique. These finding indicate that density gradient centrifugation of semen is an important step to achieving high quality, pure RNA samples from viable sperm for use in downstream application.

## 5.2 FUTURE DIRECTIONS

The cell separation and RNA isolation techniques described in this study should be utilized in future research to evaluate the role of spermatozoa RNA in genetic causes of infertility. Sperm transcriptome analysis in the human, bull and stallion have identified a variety of potential RNA transcripts that may be genetic markers to investigate when looking at genetic infertility in the male dog. For example, the protamine-1 transcript is involved in chromatin packing and condensation and speculated to be important for conferring motility to the sperm in the human, bull and stallion (Carreau et al. 2007; Steger et al. 2003; Jodar et al. 2012; Feugang et al. 2010; D'Occhio et al. 2007; Galeraud-Denis et al. 2007; O'Brien and Zini 2005; Das et al. 2013; Bench et al 1996). The

conservation of this gene across species and its similar role in conferring motility and fertility to sperm indicate that protamine-1 would be an important gene of interest in future canine infertility transcriptome analyses. Additional genes identified in the present study (Table 1.3.2) may also be targeted for future research into genetic causes of male dog infertility.

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# APPENDICES

APPENDIX A: SEMEN COLLECTION AND EVALUATION SHEET

Libido/ease of collection: Poor / Fair / Good / Excellent
Teaser bitch present: Yes / No
Stage of cycle: _____

| | Color | Volume (mL) | Concentration (sperm/mL) | Total Sperm/ejaculate |
|---|---|---|---|---|
| Fraction 1 | | | | |
| Fraction 2 | | | | |
| Fraction 3 | | | | |

Total Motile Spermatozoa:_____%
Progressively Motile Spermatozoa:_____%
Speed/velocity of motility (0-5):_____

Morphology: Stain(s) utilized:_____ Number counted: 200
%Live:_____
%Normal:_____
%Head defects:_____
%Midpiece defects:_____
%Tail defects:_____
%other defects:_____

**COMMENTS:**

**DOG INFORMATION**
Name:_____
Date of Collection:_____
Breed:_____
Color:_____
Date of Birth:_____
Owner name:_____
Phone number:_____

Table A.1 Semen evaluation results. “PMNM” represents the percentage of sperm that are progressively motile with normal morphology. “NA” indicates that the information is not available.

| Sample | Separation Method | Total Motility | Progressive Motility | Live | Normal | Total Sperm/ejaculate ($x10^6$) | PMNM |
|---|---|---|---|---|---|---|---|
| 1-15 Vegas | Control | 95% | 80% | NA | 63% | 9.975 | 50% |
| 2-5 Vegas | Control | >99% | 95% | 90% | 90% | 337.5 | 85% |
| 2-12 Vegas | Control | 95% | 85% | 94% | 90% | 456.5 | 77% |
| 4-17 Vegas | Control | 85% | 75% | 89% | 84% | 94.0 | 63% |
| 5-8 Vegas | Control | >95% | 90% | 98% | 85% | 598.0 | 77% |
| 5-15 Vegas | Control | 95% | 90% | 90% | 72% | 356.25 | 65% |
| 1-15 Vegas | Swim-Up | 5% | 1% | NA | 48% | 0.75 | <1% |
| 2-5 Vegas | Swim-Up | 10% | 1% | 46% | 49% | 0.75 | <1% |
| 2-12 Vegas | Swim-Up | 10% | 1% | 54% | 27% | 0.5 | <1% |
| 1-15 Vegas | Equipure™ | 50% | 75% | NA | 54% | 28.0 | 40% |
| 2-5 Vegas | Equipure™ | 35% | 10% | 87% | 88% | 73.5 | 9% |
| 2-12 Vegas | Equipure™ | 90% | 70% | 92% | 78% | 23.5 | 54% |
| 4-17 Vegas | Bovipure™ | 35% | 5% | 83% | 88% | 36.5 | 4% |
| 5-8 Vegas | Bovipure™ | 75% | 40% | 75% | 40% | 142.0 | 16% |
| 5-15 Vegas | Bovipure™ | 90% | 40% | 83% | 92% | 39.75 | 37% |
| 1-22 James | Control | 99% | 85% | 85% | 78% | 33.75 | 66% |
| 2-5 James | Control | 95% | 90% | 88% | 86% | 187.0 | 77% |
| 2-12 James | Control | >99% | 80% | 92% | 74% | 21.25 | 59% |
| 4-17 James | Control | 70% | 35% | 92% | 68% | 100.89 | 24% |
| 5-8 James | Control | 95% | 85% | 99% | 68% | 129.0 | 57% |
| 5-15 James | Control | 85% | 80% | 99% | 55% | 461.5 | 44% |
| 1-22 James | Swim-Up | 60% | 1% | 48% | 63% | 1.0 | <1% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2-5 James | Swim-Up | 5% | 1% | 42% | 32% | 1.0 | <1% |
| 2-12 James | Swim-Up | 5% | 1% | 49% | 29% | 1.25 | <1% |
| 1-22 James | Equipure™ | 50% | 10% | 87% | 90% | 141.25 | 9% |
| 2-5 James | Equipure™ | 50% | 5% | 91% | 88% | 100.25 | 4% |
| 2-12 James | Equipure™ | 30% | 1% | 96% | 66% | 61.0 | <1% |
| 4-17 James | Bovipure™ | 55% | 5% | 65% | 70% | 29.0 | 4% |
| 5-8 James | Bovipure™ | 45% | 5% | 91% | 78% | 57.0 | 4% |
| 5-15 James | Bovipure™ | 70% | 5% | 96% | 69% | 170.5 | 3% |
| 3-26 Rio | Control | 90% | 80% | 79% | 63% | 33.75 | 50% |
| 4-2 Rio | Control | >99% | 85% | 90% | 87% | 70.13 | 74% |
| 4-23 Rio | Control | 905 | 75% | 90% | 84% | 102.0 | 63% |
| 3-26 Rio | Equipure™ | 15% | 5% | 26% | 48% | 21.5 | 2% |
| 4-2 Rio | Equipure™ | 40% | 10% | 79% | 77% | 135.75 | 8% |
| 4-23 Rio | Equipure™ | 40% | 10% | 89% | 91% | 64.0 | 9% |
| 3-26 Rio | Bovipure™ | 30% | 1% | 90% | 93% | 25.75 | <1% |
| 4-2 Rio | Bovipure™ | 20% | 15% | 88% | 87% | 20.5 | 13% |
| 4-23 Rio | Bovipure™ | 65% | 15% | 94% | 92% | 42.0 | 14% |
| 3-26 Mauna | Control | 95% | 75% | 93% | 71% | 74.25 | 53% |
| 4-2 Mauna | Control | 95% | 80% | 95% | 78% | 865.25 | 62% |
| 4-23 Mauna | Control | 90% | 70% | 94% | 78% | 208.0 | 54% |
| 3-26 Mauna | Equipure™ | 20% | 1% | 20% | 39% | 28.75 | <1% |
| 4-2 Mauna | Equipure™ | 25% | 5% | 93% | 84% | 32.25 | 4% |
| 4-23 Mauna | Equipure™ | 55% | 20% | 87% | 93% | 20.75 | 19% |
| 3-26 Mauna | Bovipure™ | 50% | 1% | 91% | 81% | 17.0 | <1% |
| 4-2 Mauna | Bovipure™ | 30% | 10% | 88% | 86% | 22.75 | 9% |
| 4-23 Mauna | Bovipure™ | 75% | 5% | 88% | 89% | 58.75 | 4% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4-2 Yonkers | Control | >99% | 97% | 94% | 91% | 1102.0 | 88% |
| 4-16 Yonkers | Control | >95% | 90% | 95% | 94% | 774.0 | 84% |
| 4-23 Yonkers | Control | 95% | 90% | 95% | 90% | 593.25 | 81% |
| 4-2 Yonkers | Equipure™ | 50% | 25% | 80% | 99% | 292.25 | 25% |
| 4-16 Yonkers | Equipure™ | 95% | 85% | 74% | 92% | 230.0 | 78% |
| 4-23 Yonkers | Equipure™ | 95% | 70% | 91% | 95% | 66.25 | 67% |
| 4-2 Yonkers | Bovipure™ | 75% | 55% | 61% | 78% | 62.5 | 43% |
| 4-16 Yonkers | Bovipure™ | 90% | 40% | 63% | 97% | 290.0 | 39% |
| 4-23 Yonkers | Bovipure™ | 60% | 20% | 91% | 95% | 71.0 | 19% |
| 4-2 Baloo | Control | 85% | 80% | 97% | 75% | 304.5 | 60% |
| 4-16 Baloo | Control | 90% | 65% | 63% | 65% | 364.0 | 42% |
| 4-23 Baloo | Control | 95% | 85% | 96% | 82% | 500.5 | 70% |
| 4-2 Baloo | Equipure™ | 15% | 1% | 78% | 93% | 87.0 | <1% |
| 4-16 Baloo | Equipure™ | 95% | 70% | 85% | 80% | 65.0 | 56% |
| 4-23 Baloo | Equipure™ | 90% | 65% | 82% | 95% | 77.0 | 62% |
| 4-2 Baloo | Bovipure™ | 35% | 1% | 82% | 81% | 20.0 | <1% |
| 4-16 Baloo | Bovipure™ | 90% | 55% | 76% | 82% | 104.0 | 45% |
| 4-23 Baloo | Bovipure™ | 65% | 30% | 90% | 84% | 44.75 | 25% |
| 1-17 Garrett | Control | 99% | 80% | 93% | 64% | 48.0 | 51% |
| 2-14 Garrett | Control | >99% | 95% | 83% | 64% | 29.6 | 61% |
| 2-19 Garrett | Control | 95% | 90% | 100% | 91% | 85.25 | 81% |
| 4-16 Garret | Control | 75% | 50% | 90% | 85% | 141.0 | 42% |
| 4-24 Garrett | Control | 80% | 70% | 68% | 74% | 372.0 | 52% |
| 5-8 Garrett | Control | 75% | 70% | 99% | 76% | 202.5 | 53% |
| 1-17 Garrett | Swim-Up | 40% | 5% | 66% | 32% | 0.25 | 2% |
| 2-14 Garrett | Swim-Up | 5% | 1% | 60% | 17% | 0.5 | <1% |
| 2-19 Garrett | Swim-Up | 5% | 1% | 73% | 34% | 0.5 | <1% |
| 1-17 Garrett | Equipure™ | 97% | 90% | 94% | 68% | 5.25 | 61% |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 2-14 Garrett | Equipure™ | 75% | 40% | 96% | 88% | 8.5 | 35% |
| 2-19 Garrett | Equipure™ | 0% | 1% | 95% | 90% | 18.0 | <1% |
| 4-16 Garret | Bovipure™ | 20% | 5% | 77% | 84% | 285.0 | 4% |
| 4-24 Garrett | Bovipure™ | 65% | 10% | 68% | 74% | 75.75 | 7% |
| 5-8 Garrett | Bovipure™ | 25% | 5% | 99% | 86% | 21.25 | 4% |
| 4-16 Tsavo | Control | >95% | 85% | 98% | 94% | 522.75 | 80% |
| 4-24 Tsavo | Control | 90% | 85% | 83% | 87% | 687.5 | 74% |
| 5-8 Tsavo | Control | 90% | 85% | 98% | 90% | 136.0 | 76% |
| 4-16 Tsavo | Equipure™ | 85% | 75% | 91% | 92% | 34.0 | 69% |
| 4-24 Tsavo | Equipure™ | 95% | 85% | 90% | 91% | 45.5 | 77% |
| 5-8 Tsavo | Equipure™ | 65% | 10% | 97% | 94% | 41.75 | 9% |
| 4-16 Tsavo | Bovipure™ | 65% | 40% | 88% | 94% | 11.75 | 38% |
| 4-24 Tsavo | Bovipure™ | 55% | 15% | 92% | 93% | 34.75 | 14% |
| 5-8 Tsavo | Bovipure™ | 70% | 15% | 95% | 92% | 20.25 | 14% |

Table A.2 Dog identification, age, breed, and indication if a teaser bitch was required for collection.

| **Dog** | **ID Number** | **Age** | **Breed** | **Teaser Required?** |
|---|---|---|---|---|
| Vegas | 1 | 5 years | Brittany | Yes |
| James | 2 | 2 years | Brittany | No |
| Garrett | 3 | 5.5 years | German Shorthair Pointer | No |
| Rio | 4 | 7 years | Brittany | Yes |
| Mauna | 5 | 2 years | Brittany | Yes |
| Yonkers | 6 | 14 months | Bernese Mountain Dog | Yes |
| Baloo | 7 | 22 months | Bernese Mountain Dog | Yes |
| Tsavo | 8 | 21 months | German Shorthair Pointer | Yes |

# APPENDIX B: COMPLETE SPECTROPHOTOMETER RESULTS

Table B.1 RNA Concentration, $A_{260}/A_{280}$ ratio, and $A_{260}/A_{230}$ ratio for each sample as determined by spectrophotometry

| Dog | Treatment | RNA Concentration (ng/µl) | $A_{260}/A_{280}$ | $A_{260}/A_{230}$ |
|---|---|---|---|---|
| 1-15 Vegas | Control | 83.6 | 1.229 | 0.135 |
| 1-17 Garrett | Control | 47.2 | 1.168 | 0.26 |
| 1-22 James | Control | 40.8 | 1.159 | 0.224 |
| 2-5 Vegas | Control | 74.4 | 1.12 | 0.268 |
| 2-5 James | Control | 66.4 | 1.099 | 0.222 |
| 2-12 Vegas | Control | 174 | 1.211 | 0.25 |
| 2-12 James | Control | 136 | 1.122 | 0.266 |
| 2-14 Garrett | Control | 155 | 1.155 | 0.281 |
| 2-19 Garrett | Control | 168 | 1.176 | 0.259 |
| 3-26 Rio | Control | 218 | 1.143 | 0.312 |
| 3-26 Mauna | Control | 314 | 1.217 | 0.37 |
| 4-2 Rio | Control | 92.4 | 1.191 | 0.173 |
| 4-2 Mauna | Control | 41.2 | 1.157 | 0.234 |
| 4-2 Yonkers | Control | 60.8 | 1.216 | 0.193 |
| 4-2 Baloo | Control | 115 | 1.226 | 0.238 |
| 4-16 Yonkers | Control | 72.8 | 1.04 | 0.203 |
| 4-16 Baloo | Control | 165 | 1.087 | 0.25 |
| 4-16 Tsavo | Control | 114 | 1.032 | 0.24 |
| 4-16 Garrett | Control | 39.6 | 0.971 | 0.174 |
| 4-17 Vegas | Control | 54 | 0.925 | 0.175 |
| 4-17 James | Control | 33.2 | 0.838 | 0.233 |
| 4-23 Yonkers | Control | 132 | 1.092 | 0.253 |
| 4-23 Baloo | Control | 50 | 1.05 | 0.252 |
| 4-23 Rio | Control | 0 | 0 | 0 |
| 4-23 Mauna | Control | 54 | 1.134 | 0.184 |
| 4-24 Tsavo | Control | 77.2 | 1.116 | 0.204 |
| 4-24 Garrett | Control | 70.8 | 1.099 | 0.234 |
| 5-8 Tsavo | Control | 129 | 1.142 | 0.242 |
| 5-8 Garrett | Control | 47.6 | 1.133 | 0.166 |
| 5-8 Vegas | Control | 46.4 | 1.196 | 0.272 |
| 5-8 James | Control | 30.8 | 1.167 | 0.241 |
| 5-15 Vegas | Control | 49.2 | 1.183 | 0.173 |
| 5-15 James | Control | 42.4 | 1.165 | 0.268 |

| | | | | |
|---|---|---|---|---|
| 1-15 Vegas | Swim-Up | 127 | 1.196 | 0.261 |
| 1-17 Garrett | Swim-Up | 60.8 | 1.078 | 0.245 |
| 1-22 James | Swim-Up | 90.8 | 1.188 | 0.266 |
| 2-5 Vegas | Swim-Up | 86.8 | 1.119 | 0.246 |
| 2-5 James | Swim-Up | 50 | 1.116 | 0.206 |
| 2-12 Vegas | Swim-Up | 105 | 1.159 | 0.003 |
| 2-12 James | Swim-Up | 108 | 1.163 | 0.171 |
| 2-14 Garrett | Swim-Up | 109 | 1.147 | 0.228 |
| 2-19 Garrett | Swim-Up | 158 | 1.142 | 0.276 |
| 1-15 Vegas | Equipure™ | 802 | 1.454 | 0.838 |
| 1-17 Garret | Equipure™ | 28.4 | 1.164 | 0.202 |
| 1-22 James | Equipure™ | 122 | 1.151 | 0.295 |
| 2-5 Vegas | Equipure™ | 106 | 1.138 | 0.225 |
| 2-5 James | Equipure™ | 69.6 | 1.094 | 0.284 |
| 2-12 Vegas | Equipure™ | 171 | 1.212 | 0.242 |
| 2-12 James | Equipure™ | 87.6 | 1.21 | 0.192 |
| 2-14 Garrett | Equipure™ | 132 | 1.171 | 0.257 |
| 2-19 Garrett | Equipure™ | 275 | 1.199 | 0.332 |
| 3-26 Rio | Equipure™ | 305 | 1.183 | 0.366 |
| 3-26 Mauna | Equipure™ | 388 | 1.228 | 0.447 |
| 4-2 Rio | Equipure™ | 63.2 | 1.17 | 0.173 |
| 4-2 Mauna | Equipure™ | 80.4 | 1.149 | 0.259 |
| 4-2 Yonkers | Equipure™ | 70.8 | 1.238 | 0.274 |
| 4-2 Baloo | Equipure™ | 94.4 | 1.21 | 0.188 |
| 4-16 Yonkers | Equipure™ | 65.2 | 1.045 | 0.209 |
| 4-16 Baloo | Equipure™ | 73.2 | 1.058 | 0.247 |
| 4-16 Tsavo | Equipure™ | 97.2 | 1.061 | 0.208 |
| 4-23 Yonkers | Equipure™ | 139 | 1.164 | 0.27 |
| 4-23 Baloo | Equipure™ | 112 | 1.111 | 0.247 |
| 4-23 Rio | Equipure™ | 22.8 | 1.018 | 0.125 |
| 4-23 Mauna | Equipure™ | 48.8 | 1.119 | 0.191 |
| 4-24 Tsavo | Equipure™ | 39.2 | 1.054 | 0.271 |
| 5-8 Tsavo | Equipure™ | 36.8 | 1.373 | 0.113 |
| 3-26 Rio | Bovipure™ | 344 | 1.193 | 0.411 |
| 3-26 Mauna | Bovipure™ | 240 | 1.174 | 0.327 |
| 4-2 Rio | Bovipure™ | 61.2 | 1.159 | 0.172 |
| 4-2 Mauna | Bovipure™ | 83.2 | 1.162 | 0.254 |
| 4-2 Yonkers | Bovipure™ | 165 | 1.245 | 0.284 |

| | | | | | |
|---|---|---|---|---|---|
| 4-2 Baloo | Bovipure™ | 58.4 | 1.217 | 0.214 | 266PT, 366PR |
| 4-16 Yonkers | Bovipure™ | 209 | 1.127 | 0.264 | 258PT, 358PR |
| 4-16 Baloo | Bovipure™ | 68.8 | 1.049 | 0.22 | 271PT, 371PR |
| 4-16 Tsavo | Bovipure™ | 64.8 | 1.012 | 0.167 | 285PT, 385PR |
| 4-16 Garrett | Bovipure™ | 49.6 | 1.008 | 0.136 | 276PT, 376PR |
| 4-17 Vegas | Bovipure™ | 40 | 0.909 | 0.274 | 216PT, 316PR |
| 4-17 James | Bovipure™ | 36.8 | 0.929 | 0.274 | 224PT, 324PR |
| 4-23 Yonkers | Bovipure™ | 74 | 1.171 | 0.219 | 263PT, 363PR |
| 4-23 Baloo | Bovipure™ | 90 | 1.087 | 0.28 | 274PT, 374PR |
| 4-23 Rio | Bovipure™ | 48.8 | 1.184 | 0.187 | 241PT, 341PR |
| 4-23 Mauna | Bovipure™ | 64.8 | 0.149 | 0.217 | 252PT, 352PR |
| 4-24 Tsavo | Bovipure™ | 46.8 | 1.083 | 0.225 | 288PT, 388PR |
| 4-24 Garrett | Bovipure™ | 112 | 1.098 | 0.25 | 278PT, 378PR |
| 5-8 Tsavo | Bovipure™ | 70.8 | 1.273 | 0.159 | 293PT, 393PR |
| 5-8 Garrett | Bovipure™ | 32.8 | 1.323 | 0.102 | 282PT, 382PR |
| 5-8 Vegas | Bovipure™ | 68.4 | 1.188 | 0.227 | 218PT, 318PR |
| 5-8 James | Bovipure™ | 57.6 | 1.18 | 0.223 | 226PT, 326PR |
| 5-15 Vegas | Bovipure™ | 48.8 | 1.13 | 0.195 | 222PT, 322PR |
| 5-15 James | Bovipure™ | 46.8 | 1.206 | 0.27 | 228PT, 328PR |

APPENDIX C: SET UP SHEET FOR RT-PCR SAMPLES

RNA Sample Information:

| NAME | $A_{260}$ | $A_{260/280}$ | Concentration (ng/µl) - in 50µl RNAse/DNAse Free Water |
|---|---|---|---|
| | | | |
| | | | |
| | | | |

Reverse Transcriptase - PCR Samples

| Sample Number | RNA source | Forward Primer | Reverse Primer | Amount of RNA Sample to Add | Amount $H_2O$ added |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

Add to each tube in this order (on ice):
1. Enough **RNAse/DNAse free water** to reach 50 µl reaction volume (see above)
2. 25µl **2x Reaction Mix**
3. 2.5µl **forward primer** from stock 10µM solution
4. 2.5µl **reverse primer** from stock 10µM solution
5. 100 ng **template RNA** - µl varies by sample (**see above)**
6. 1µl **RT/Platinum *Taq* Mix**

# APPENDIX D: 50X TAE PREPARATION AND TAE BUFFER PREPARATION

## *50X TAE Preparation*

50X TAE preparation took place in Dr. Alfred Menino's reproductive physiology laboratory on Oregon State University's campus (Withycombe 135, Corvallis, OR). 250mL of the reagent was prepared. 60.5g of TRIS base (Avantor Performance Materials, Center Valley, PA, Cat. No. 4109-01) was dissolved in 150mL of $ddH_2O$ using a stir bar. 14.3mL of glacial acetic acid (EMD Millipore, Darmstadt, Germany, Cat. No. AX0073-9) was added to the TRIS base mixture. Finally, 25mL of 0.5M EDTA at pH 8.0 was added to the mixture. The final volume was adjusted to 250mL using $ddH_2O$ and the mixture was labeled with the date and stored at room temperature.

## *TAE Buffer Preparation*

TAE Buffer was used for electrophoresis of the agarose gels and for preparation of the nucleic acid stain solution. 1000mL of TAE buffer was prepared when needed. 20mL of 50X TAE buffer was combined with 980mL of $ddH_2O$ in a 1L glass bottle to prepare the TAE buffer. The solution was labeled with the date and stored at room temperature.

APPENDIX E: AGAROSE GEL RESULTS

Figure E.1 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16 – 1000bp DNA ladder; 2, 3 – no template negative controls; 4 – PTPRC positive control; 5 – Protamine-2 positive control.

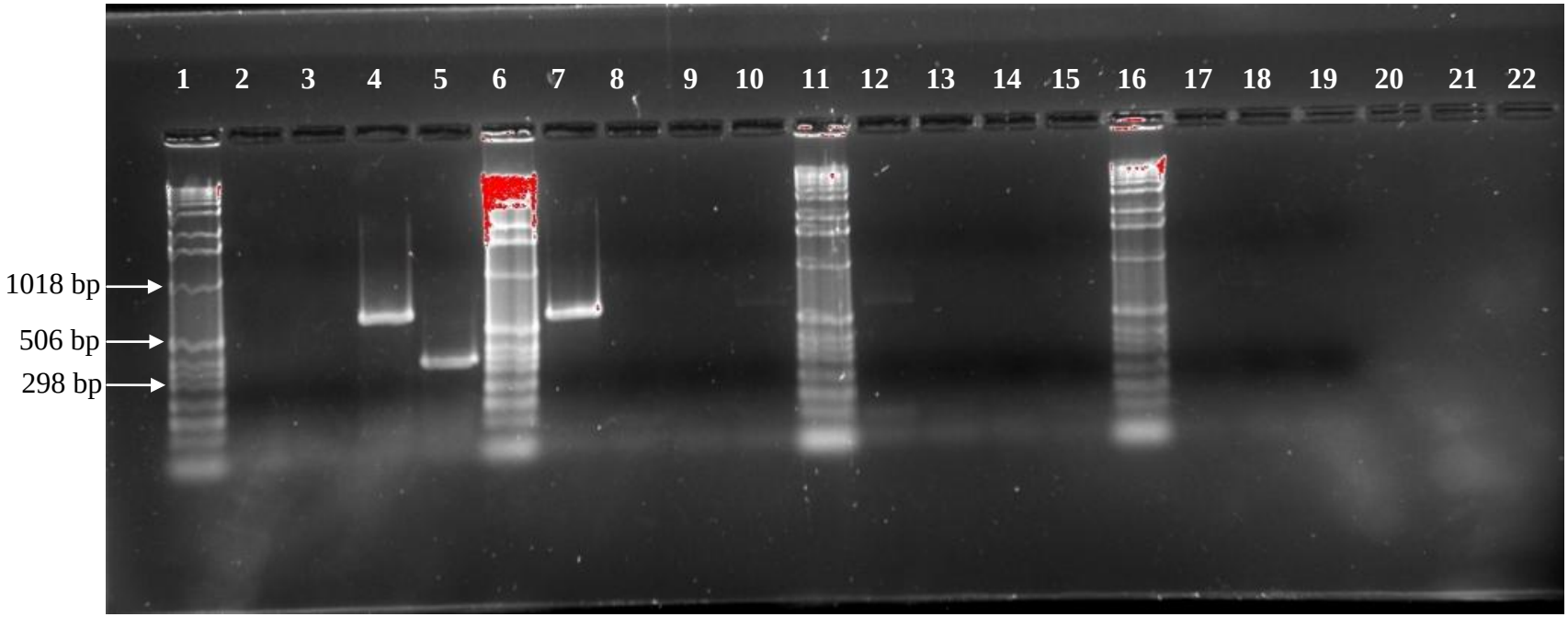



Table E.1 Lane identification for Figure E.1 C=control, E=Equipure™, S=swim-up

| **Lane** | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 3 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Date** | 2/19 | 2/19 | 2/19 | 1/15 | 1/15 | 1/15 | 2/5 | 2/5 | 2/5 | 2/12 | 2/12 |
| **Method** | C | E | S | C | E | S | C | E | S | C | E |

Figure E.2 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16 – 1000bp DNA ladder

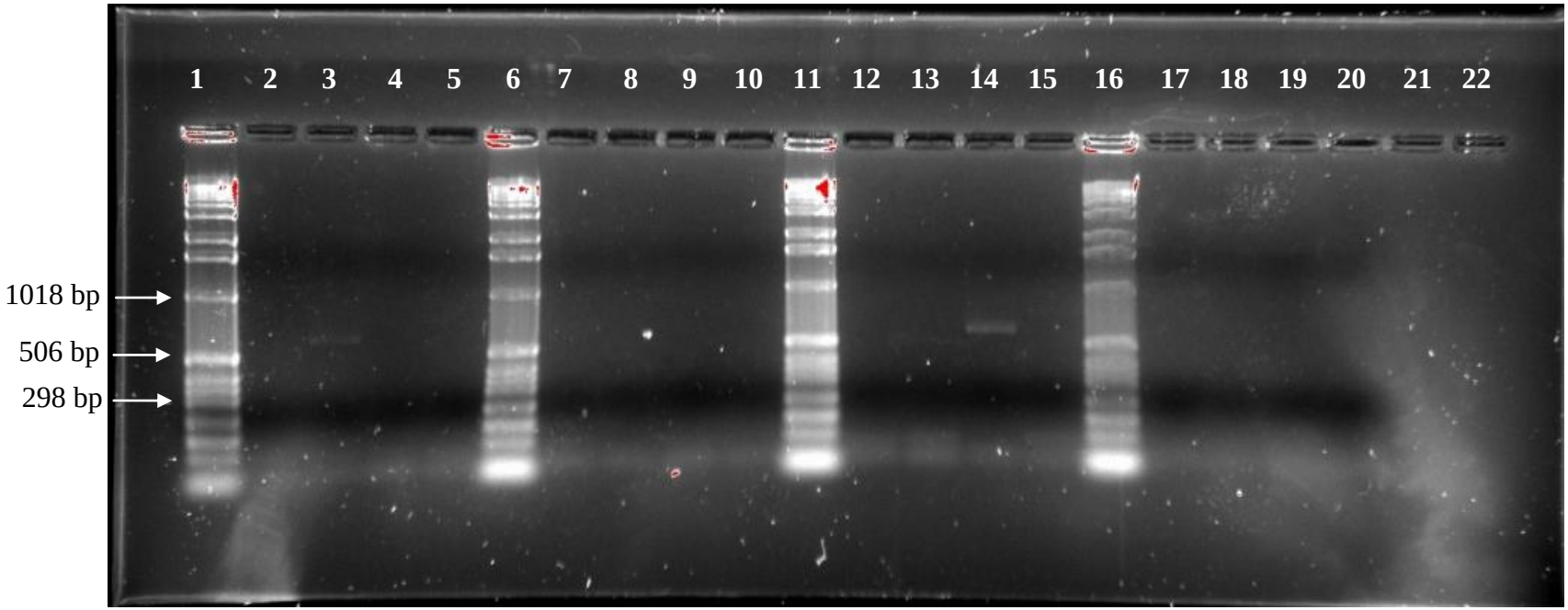



Table E.2 Lane identification for Figure E.2 C=control, E=Equipure™, S=swim-up

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Date** | 2/12 | 1/22 | 1/22 | 1/22 | 2/5 | 2/5 | 2/5 | 2/12 | 2/12 | 2/12 | 1/17 | 1/17 | 1/17 | 2/14 | 2/14 | 2/14 |
| **Method** | S | C | E | S | C | E | S | C | E | S | C | E | S | C | E | S |

Figure E.3 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16 – 1000bp DNA ladder; 2, 3 – no template negative controls; 4 – PTPRC positive control; 5 – Protamine-2 positive control

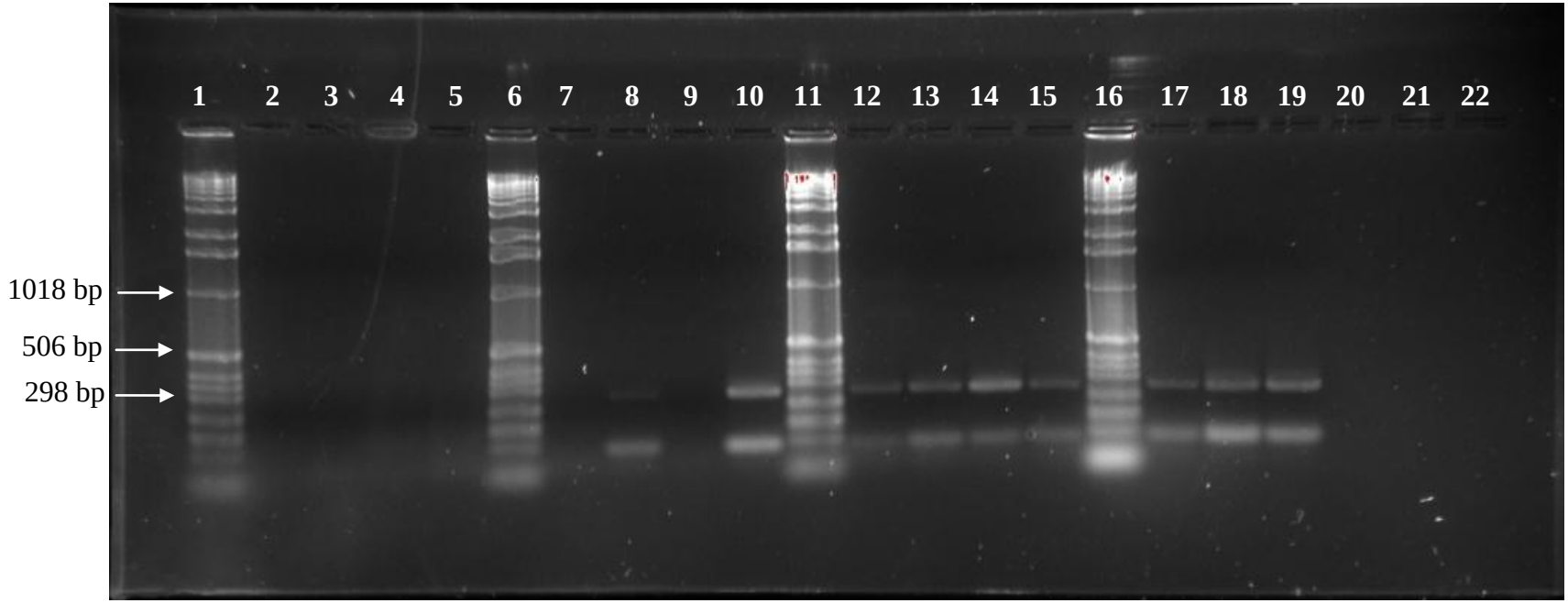



Table E.3 Lane identification for Figure E.3 C=control, E=Equipure™, S=swim-up

| **Lane** | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 3 | 3 | 3 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Date** | 2/19 | 2/19 | 2/19 | 1/15 | 1/15 | 1/15 | 2/5 | 2/5 | 2/5 | 2/12 | 2/12 |
| **Method** | C | E | S | C | E | S | C | E | S | C | E |

Figure E.4 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16 – 1000bp DNA ladder

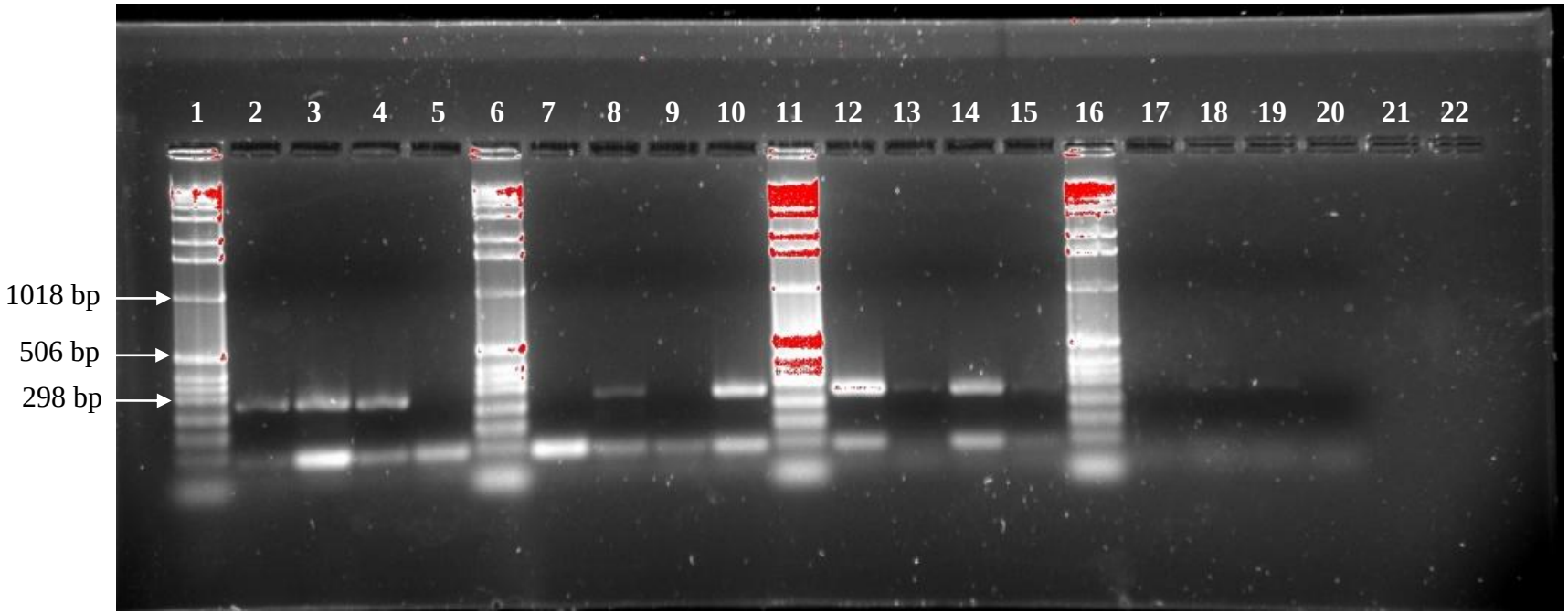



Table E.4 Lane identification for Figure E.4 C=control, E=Equipure™, S=swim-up

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 3 | 3 | 3 |
| **Date** | 2/12 | 1/22 | 1/22 | 1/22 | 2/5 | 2/5 | 2/5 | 2/12 | 2/12 | 2/12 | 1/17 | 1/17 | 1/17 | 2/14 | 2/14 | 2/14 |
| **Method** | S | C | E | S | C | E | S | C | E | S | C | E | S | C | E | S |

Figure E.5 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder; 2, 3 – no template negative controls; 4 – PTPRC positive control; 5 – Protamine-2 positive control.

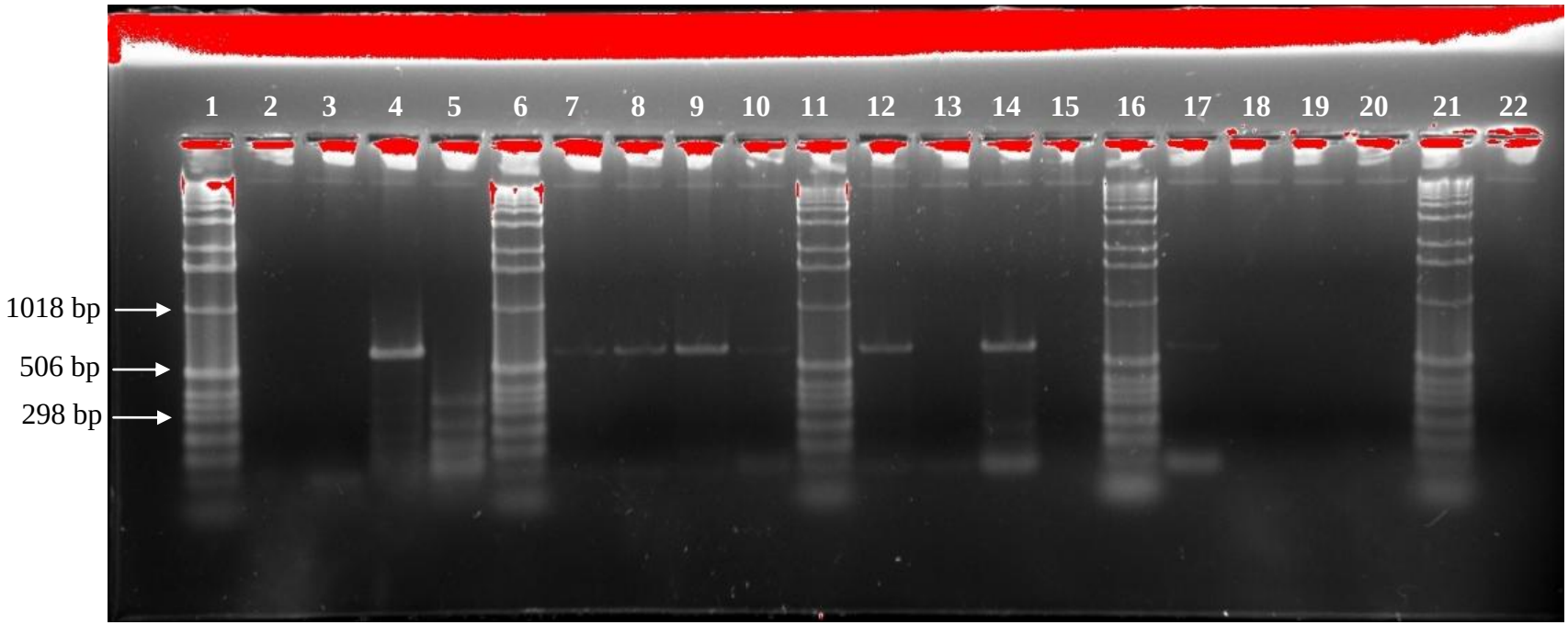



Table E.5 Lane identification for Figure E.5 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 8 |
| **Date** | 4/17 | 4/17 | 5/8 | 5/8 | 5/15 | 5/15 | 4/17 | 4/17 | 5/8 | 5/8 | 5/15 | 5/15 | 5/8 |
| **Method** | C | B | C | B | C | B | C | B | C | B | C | B | C |

Figure E.6 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

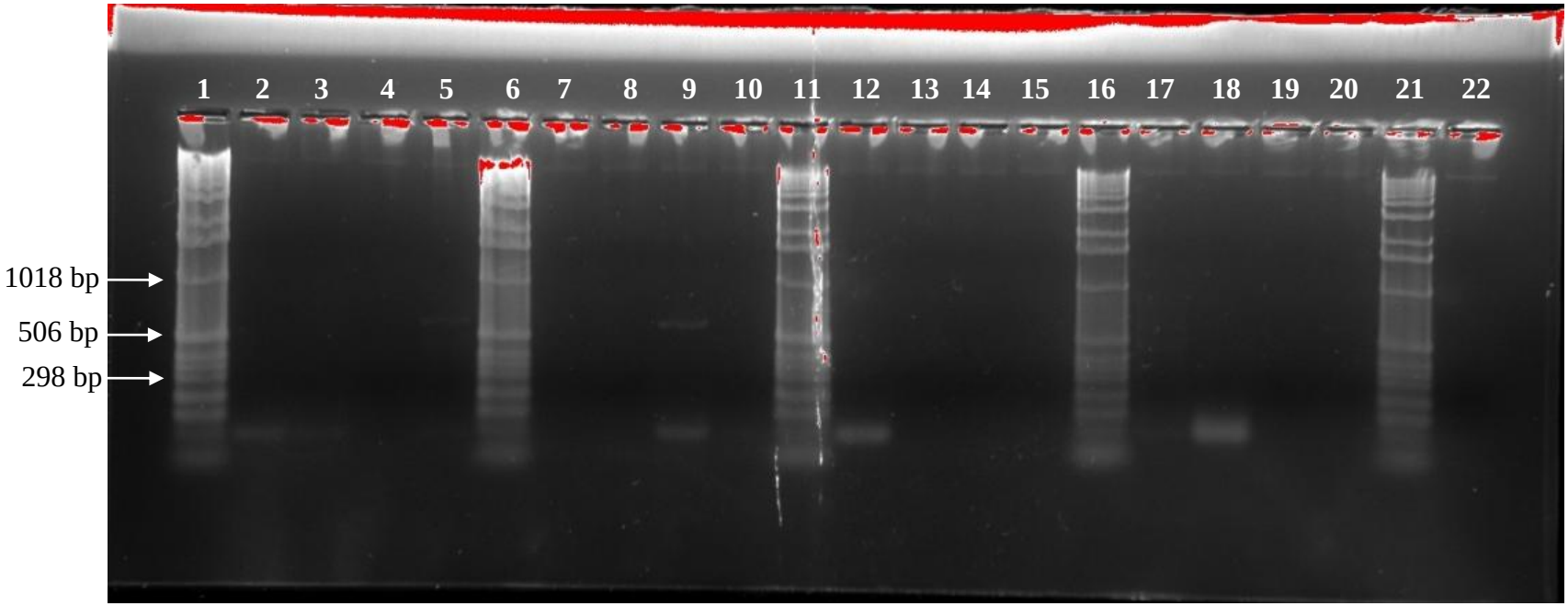



Table E.6 Lane identification for Figure E.6 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 8 |
| **Date** | 3/26 | 3/26 | 3/26 | 4/2 | 4/2 | 4/2 | 4/23 | 4/23 | 4/23 | 3/26 | 3/26 | 3/26 | 4/2 | 4/2 | 4/2 | 4/23 | 5/8 |
| **Method** | C | E | B | C | E | B | C | E | B | C | E | B | C | E | B | C | E |

Figure E.7 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

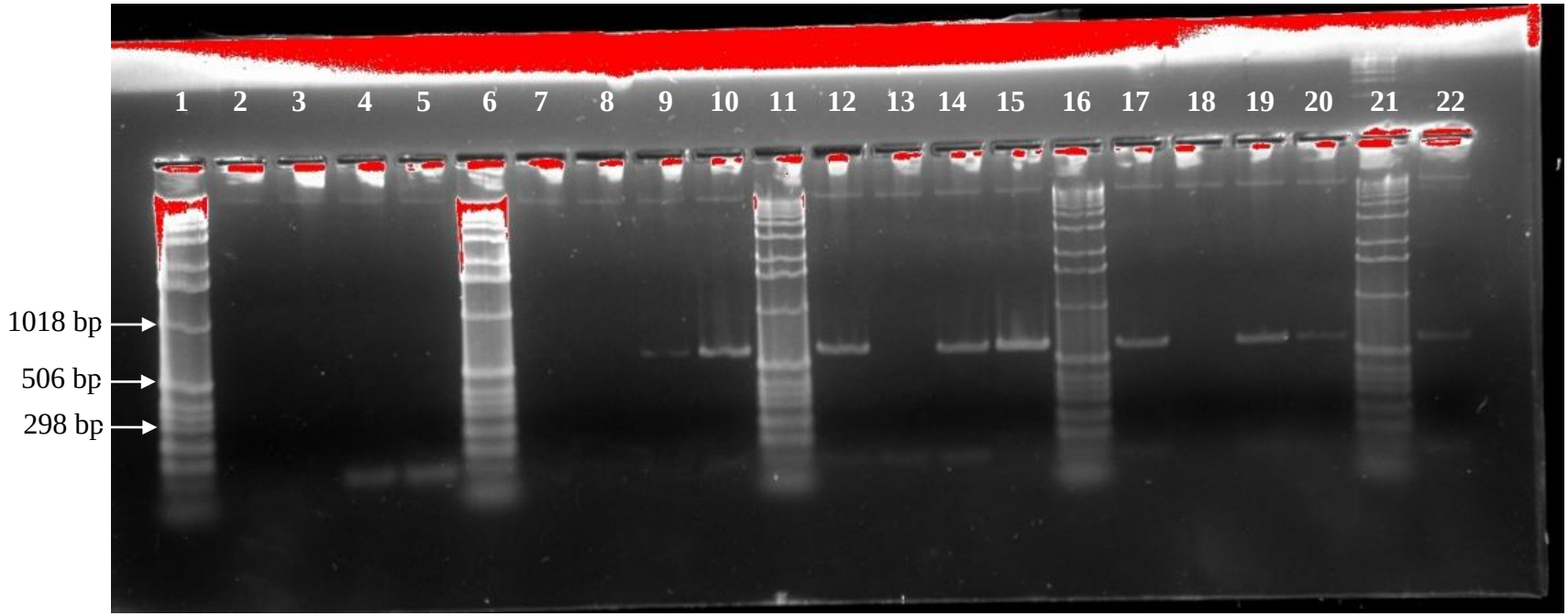



Table E.7 Lane identification for Figure E.7 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 5 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 7 | 7 | 8 |
| **Date** | 4/23 | 4/23 | 4/2 | 4/2 | 4/2 | 4/16 | 4/16 | 4/16 | 4/23 | 4/23 | 4/23 | 4/2 | 4/2 | 4/2 | 4/16 | 4/16 | 5/8 |
| **Method** | E | B | C | E | B | C | E | B | C | E | B | C | E | B | C | E | B |

Figure E.8 Agarose gel images showing samples amplified with PTPRC. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

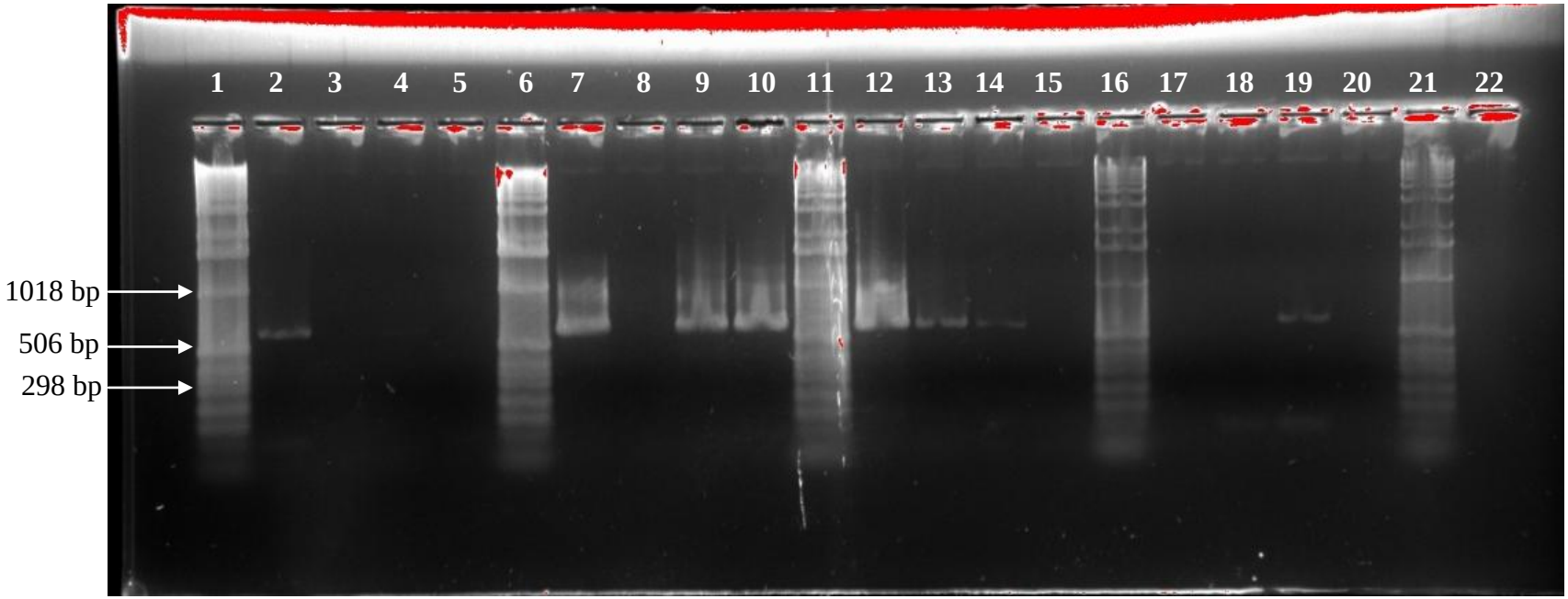



Table E.8 Lane identification for Figure E.8 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 7 | 7 | 7 | 7 | 3 | 3 | 3 | 3 | 3 | 3 | 8 | 8 | 8 | 8 | 8 | 8 |
| **Date** | 4/16 | 4/23 | 4/23 | 4/23 | 416 | 4/16 | 4/24 | 4/24 | 5/8 | 5/8 | 4/16 | 4/16 | 416 | 4/24 | 4/24 | 4/24 |
| **Method** | B | C | C | B | C | B | C | B | C | B | C | E | B | C | E | B |

Figure E.9 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder; 2, 3 – no template negative controls; 4 – PTPRC positive control; 5 – Protamine-2 positive control.

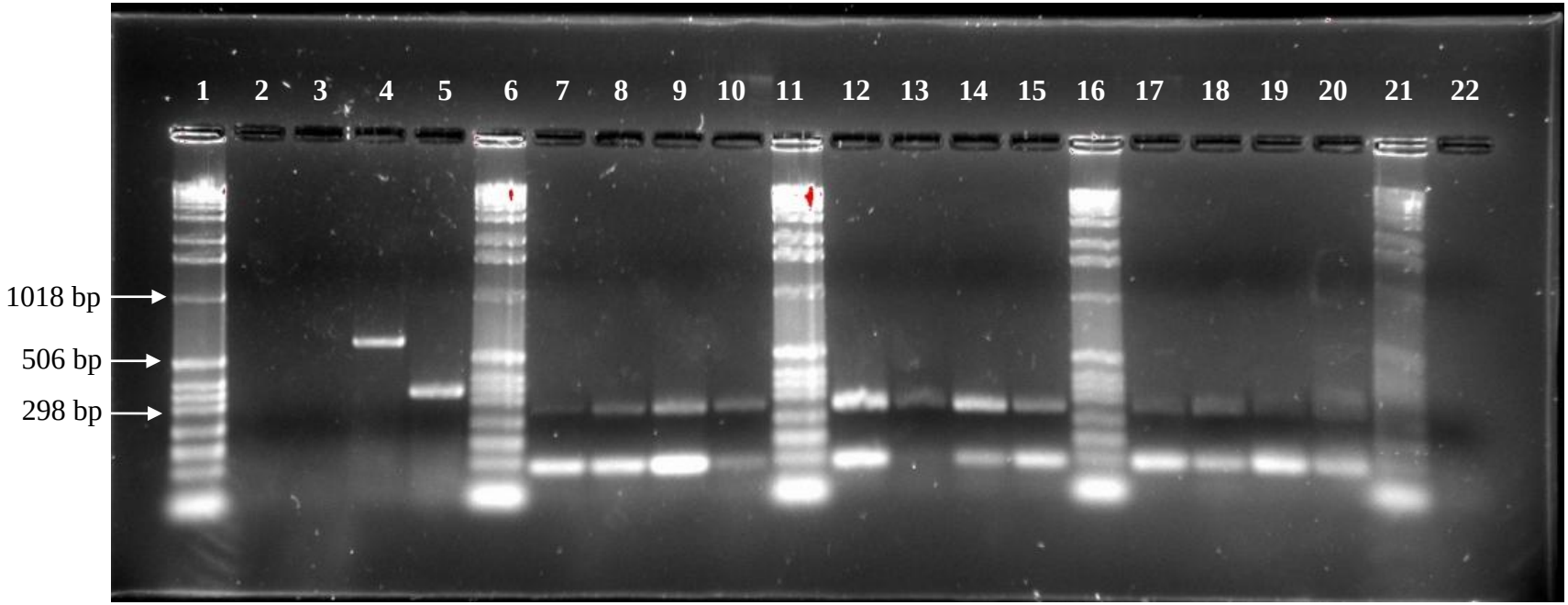



Table E.9 Lane identification for Figure E.9 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 1 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 8 |
| **Date** | 4/17 | 4/17 | 5/8 | 5/8 | 5/15 | 5/15 | 4/17 | 4/17 | 5/8 | 5/8 | 5/15 | 5/15 | 5/8 |
| **Method** | C | B | C | B | C | B | C | B | C | B | C | B | C |

Figure E.10 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

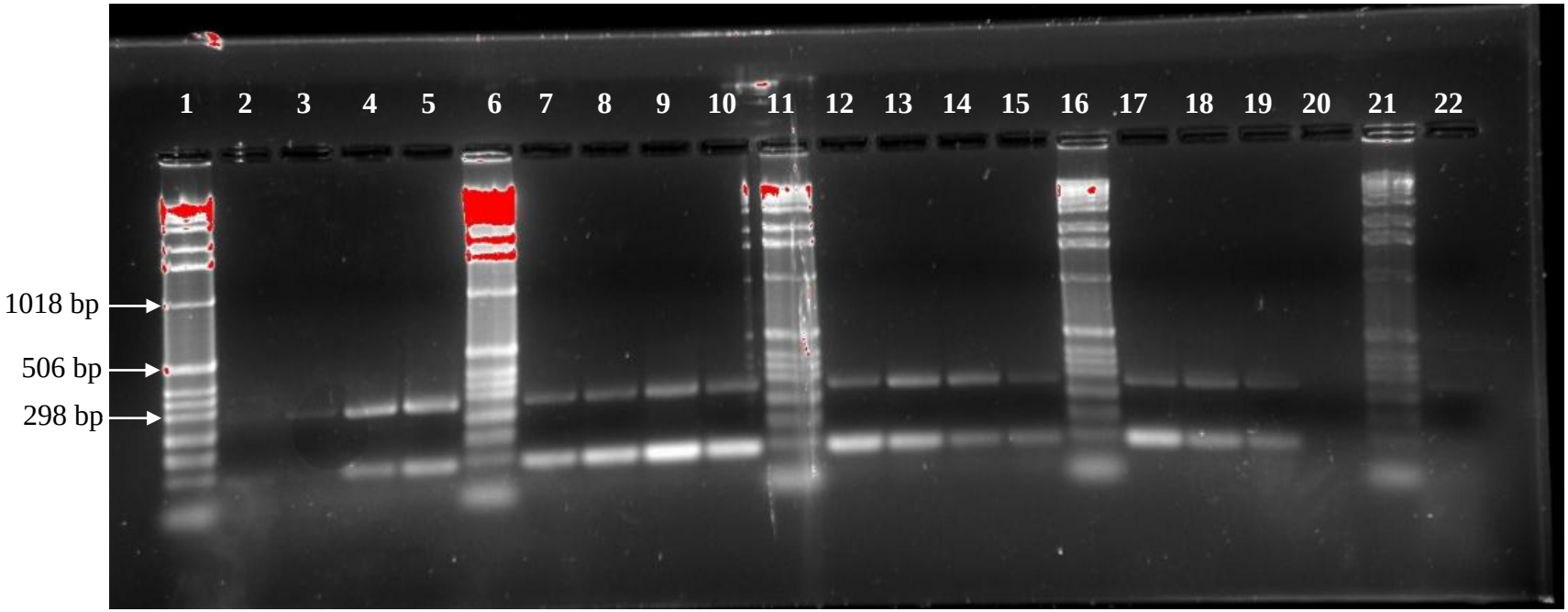



Table E.10 Lane identification for Figure E.10 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 8 |
| **Date** | 3/26 | 3/26 | 3/26 | 4/2 | 4/2 | 4/2 | 4/23 | 4/23 | 4/23 | 3/26 | 3/26 | 3/26 | 4/2 | 4/2 | 4/2 | 4/23 | 5/8 |
| **Method** | C | E | B | C | E | B | C | E | B | C | E | B | C | E | B | C | E |

Figure E.11 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

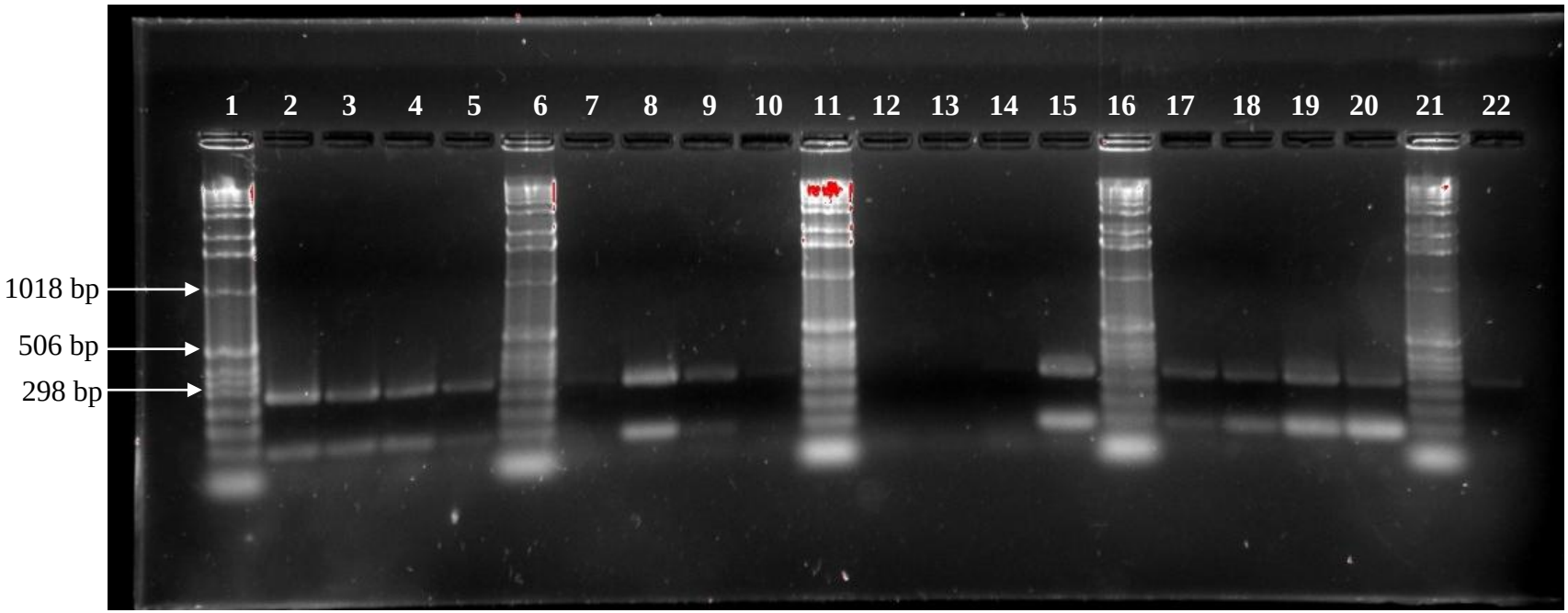



Table E.11 Lane identification for Figure E.11 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 5 | 5 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 7 | 7 | 7 | 7 | 7 | 8 |
| **Date** | 4/23 | 4/23 | 4/2 | 4/2 | 4/2 | 4/16 | 4/16 | 4/16 | 4/23 | 4/23 | 4/23 | 4/2 | 4/2 | 4/2 | 4/16 | 4/16 | 5/8 |
| **Method** | E | B | C | E | B | C | E | B | C | E | B | C | E | B | C | E | B |

Figure E.12 Agarose gel images showing samples amplified with Protamine-2. Lanes: 1, 6, 11, 16, 21 – 1000bp DNA ladder

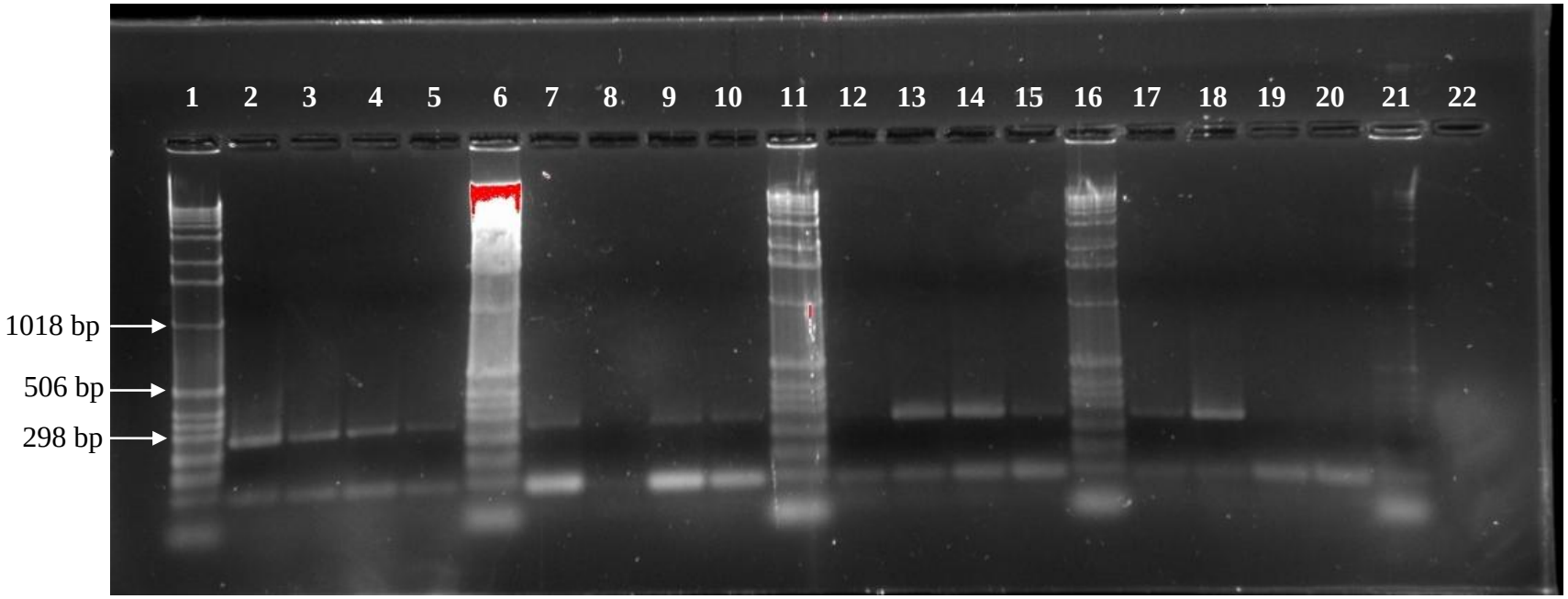



Table E.12 Lane identification for Figure E.12 C=control, E=Equipure™, B=Bovipure™

| **Lane** | 2 | 3 | 4 | 5 | 7 | 8 | 9 | 10 | 12 | 13 | 14 | 15 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Dog ID** | 7 | 7 | 7 | 7 | 3 | 3 | 3 | 3 | 3 | 3 | 8 | 8 | 8 | 8 | 8 | 8 |
| **Date** | 4/16 | 4/23 | 4/23 | 4/23 | 416 | 4/16 | 4/24 | 4/24 | 5/8 | 5/8 | 4/16 | 4/16 | 416 | 4/24 | 4/24 | 4/24 |
| **Method** | B | C | C | B | C | B | C | B | C | B | C | E | B | C | E | B |

# APPENDIX F: Abstracts, Presentations, and Posters

## Appendix F.1 Summary

My initial reason for embarking on this journey of my thesis was to fulfill graduation requirements for my Honors undergraduate degree. Along the way, I have been unexpectedly blessed with opportunities to present my work at professional conferences and for casual audiences. Not only have these experiences given me confidence in my public speaking abilities but they have also exposed me to the exciting and ever-changing world of research.

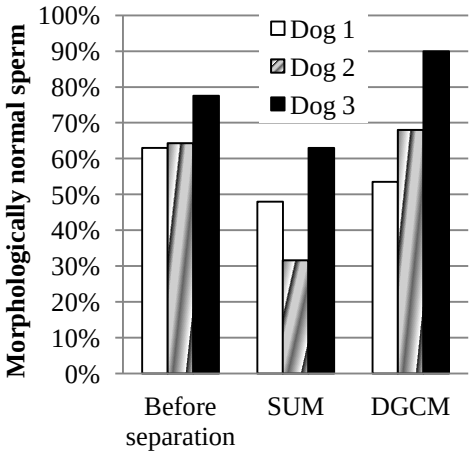
Appendix F.2 Abstract accepted for presentation at the Society for Theriogenology 2013 Conference in Louisville, KY.

**Evaluation of Canine Sperm Morphology Using Two Techniques for Sperm Separation**

Rachel Hegedus, Michelle Kutzler
Department of Animal and Rangeland Sciences, Oregon State University, Corvallis, OR 97331;

**Introduction** Veterinarians often encounter subfertile male dogs whose poor quality semen is intended for artificial insemination. Several methods for the elimination of abnormal sperm are available. Density gradient centrifugation (DGCM) and the swim-up method (SUM) have both been shown to be effective in separating sperm but their efficacy in recovery of morphologically normal canine sperm has not been compared. The objective of this study was to compare sperm morphology following these two methods of sperm separation. With the success and widespread use of commercially-available DGCM for horses [1], we hypothesized that this method would yield a higher percentage of morphologically normal sperm than the swim-up method.

**Methods** Semen was manually collected from three dogs who had sired a litter within a year from semen collection. Samples were divided into three aliquots of equal volume. Sperm morphology was assessed immediately prior to sperm separation using an eosin-nigrosin stain and by counting 200 sperm under oil immersion. For the DGCM, Equipure™ (Nidacon International, Mölndal, Sweden) was overlaid with the semen sample and centrifuged for 30 min at 100 x *g* as previously described for horses [1]. For the SUM, the semen sample was centrifuged twice for 15 min at 215 x *g* in Ham's F-10 and then incubated at 37˚C in 5% $CO_2$ at a 45˚ angle for 60 min as previously described for humans [2]. Sperm morphology was again assessed following sperm separation. Using a paired Student's t test (Microsoft Office Excel 2007, Redmond, WA) the percent of morphologically normal sperm before and after each of the separation methods was conmpared. Significance was defined as $p<0.05$.




**Results** There was no significant difference in the percent of morphologically normal sperm before separation and following DGCM. However, there was a trend towards a lower percentage of morphologically normal sperm in samples separated using the SUM (see Figure below) than in fresh semen ($p<0.06$) and after DGCM ($p<0.09$).

**Discussion** Equipure™ density gradient centrifugation is easier to perform than other DGCMs reported in dogs [3] because it only requires one overlay layer. In addition, there is no need to perform any additional washing steps after using this method of separation. Ongoing studies are comparing sperm RNA purity and yield between these methods.

**References**

[1] Das PJ et al. *Theriogenology* 2010;74:1099-1106.
[2] Jameel T. *J Pak Med Assoc* 2008;58:71-74.
[3] Dorado J et al. *Theriogenology* 2011;76:381-385.

Appendix F.3 Presentation for the 2013 Society for Theriogenology Conference in Louisville, KY.

## Evaluation of Canine Sperm Morphology Using Two Techniques for Sperm Separation

Rachel Hegedus

Oregon State University
Department of Animal and Rangeland Sciences
University Honors College

## Introduction

- Infertility in male dogs can arise due to multiple factors
  - Memon *Theriogenology*. 2007;68:322-328
- Genetics has been hypothesized to be a potential cause of male dog infertility
  - Hamatani *Reproductive Biology Online* 2011;22:103-105
- Genetic evaluation of infertility in dogs requires separation of sperm from other cells in the ejaculate
  - Ensures RNA isolated is from sperm cells only

## Separation of Sperm from Ejaculate

- Swim Up
  - Separates motile morphologically normal sperm from immotile sperm
    - Jameel *J Pak Med Assoc*.2008;58:71-74
- Density gradient centrifugation
  - Nidacon International has developed products that are species specific (BoviPure™, PureSperm®)
  - For separating equine sperm: EquiPure™
    - Used for isolating total RNA
    - Did not report on changes in % normal morphology before or after their somatic cell removal technique
      - Das et al. *Theriogenology*.2010;74:1099-1106

## Objectives

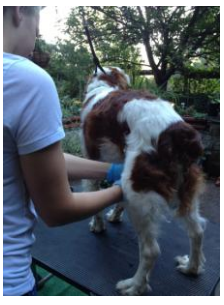
- Our goal was to evaluate methods of sperm separation techniques for the removal of somatic cells from the ejaculate and their effectiveness at removing abnormally morphological sperm from the sample
  - Experiment 1: Swim up and EquiPure™
  - Experiment 2: EquiPure™ and BoviPure™

## Hypothesis

- Experiment 1
  - Density gradient centrifugation method (EquiPure™) would yield a higher percentage of morphologically normal sperm than the swim up method
- Experiment 2
  - The percentage of morphologically normal sperm recovered by EquiPure™ would not differ from BoviPure™

## Methods

- Experiment 1: Semen was manually collected three times from three dogs
- Experiment 2: Semen was manually collected three times from eight dogs
- For both experiments, semen was divided into aliquots of equal volume

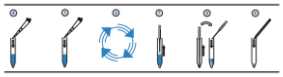


## Methods – Experiment 1

- Swim Up:
  - Sample of semen was centrifuged at 400xg for 15 minutes and supernatant was removed
  - Pellet was re-suspended in 2.5ml of Ham's F-10 and centrifuged under the same parameters
  - Pellet was overlaid with 1ml of Ham's F-10 and inclined at 45° for one hour at 37°C and 5% $CO_2$

## Methods – Experiments 1 and 2

- EquiPure™:
  - 3ml of EquiPure™ was overlaid with one aliquot of semen
  - Tube was centrifuged for 30 minutes at 300xg at room temperature
  - Supernatant was aspirated off in a circular motion

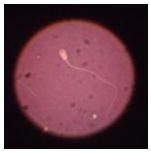


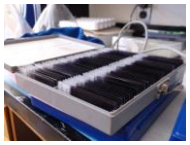
## Methods – Experiment 2

- BoviPure™:
  - Solutions for the top and bottom layer were made using BoviPure™ and BoviDilute™ prior to each separation according to the manufacturer's instructions
  - 2ml of the bottom layer was gently overlaid with 2ml of the top layer
  - The aliquot of semen was layered over this prepared gradient
  - Tube was centrifuged at 300xg for 25 minutes at room temperature
  - Supernatant was aspirated off in a circular motion

## Methods

- Sperm morphology was performed prior to semen separation and after each method using an eosin-nigrasin stain and counting 200 sperm under oil immersion

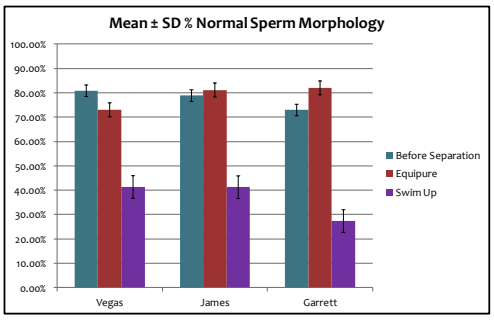




## Methods – Data Analysis

- Percent normal morphology was compared for each of the sperm separation method to the initial sample before separation
- Percent normal morphology was also compared between the two sperm separation methods in each experiment
- An unpaired, two tailed student's T-Test (Microsoft Office Excel 2007) was used
- Significance was defined as $P<0.05$

## Results – Experiment 1

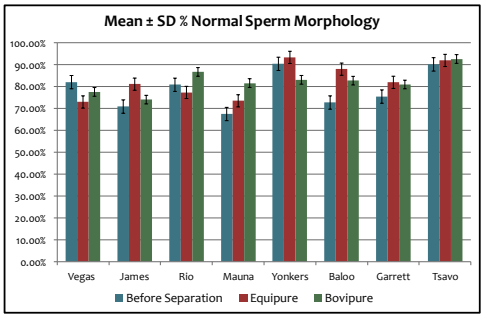


## Results – Experiment 1

- Fewer morphologically normal sperm after swim up method compared to before separation (P=0.001)
- No significant difference in percent of morphologically normal sperm before separation and after the EquiPure™ separation (P=0.765)
- Fewer morphologically normal sperm after the swim up method compared to EquiPure™ (P=0.001)

| | Before Separation | Swim Up | EquiPure™ |
|---|---|---|---|
| Mean | 77.53% | 78.72% | 36.64% |
| Standard Deviation | 2.37% | 2.87% | 4.64% |

## Results – Experiment 2




## Results – Experiment 2

- No significant difference in percent morphologically normal sperm before separation and after either the EquiPure™ (P=0.375) or BoviPure™ (P=0.328) density gradient centrifugation method
- No significant difference in percent morphologically normal sperm between the EquiPure™ and BoviPure™ methods (P=0.976)

| | Before Separation | EquiPure™ | BoviPure™ |
|---|---|---|---|
| Mean | 78.78% | 82.56% | 82.46% |
| Standard Deviation | 3.04% | 2.80% | 1.98% |

## Discussion

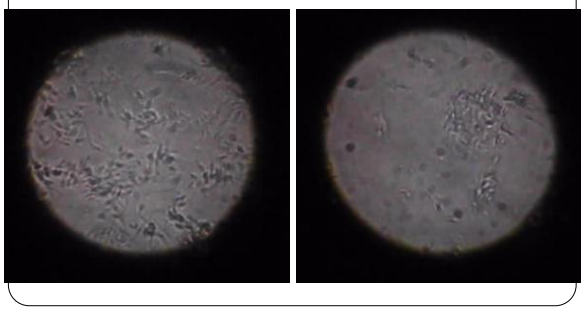
- We conclude that the density gradient centrifugation method is superior to the swim up method for the recovery of morphologically normal sperm
- For canine sperm, either the EquiPure™ or BoviPure™ product could be effective at separating morphologically normal sperm from abnormal sperm

## Discussion

- However, further evaluation of both centrifugation methods shows that it does not yield a high percentage of progressively motile sperm
  - Due in part because the viscosity of the media, the sperm stick together in clumps that move slowly because the tails are still beating

## Motility

EquiPure™ BoviPure™



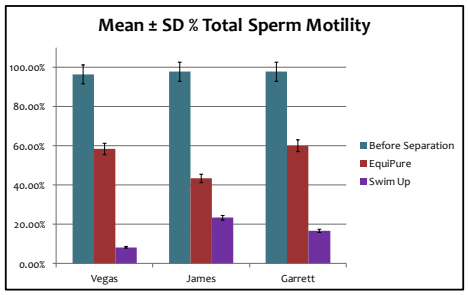
## Ongoing Studies

- Ongoing studies using the sperm separated using these techniques (swim up, EquiPure™, BoviPure™) are being done to:
  - Compare sperm RNA purity after isolation
  - Compare sperm RNA yield after isolation
- Initial data analysis suggests there is no significant difference in the RNA concentration isolated after separation with any method (swim up, EquiPure™, or BoviPure™)
- Reverse transcriptase PCR analysis is currently underway to determine the presence or absence of somatic cell specific genes in the isolated RNA

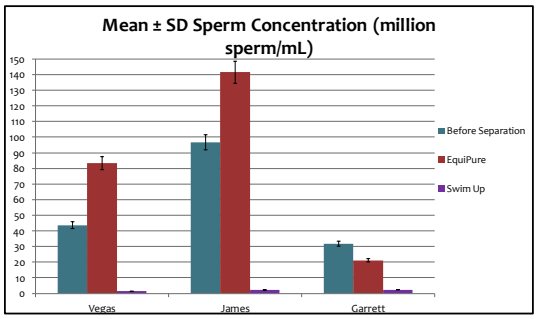
## Acknowledgements


- Special thanks to:
  - Dr. Michelle Kutzler, DVM, PhD, DACT
  - Dr. Charles Estill VMD, PhD, DACT
  - Reni Stewart, Howard Meyer, Lisa Loeffler, Linda Lyster, and Sherry Schoorl for participating with their dogs
  - Oregon State University Honors College
  - Oregon State University Department of Animal and Rangeland Sciences


## Experiment 1 Motility




## Experiment One Concentration




## Experiment 1 Summary

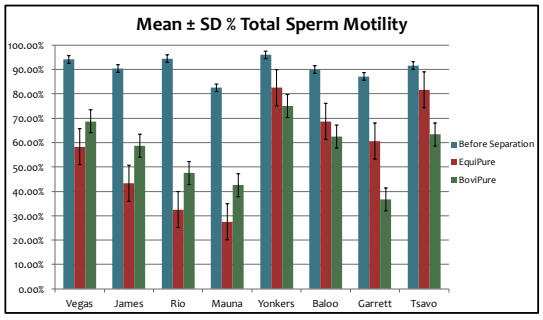
| % Total Sperm Motility | | | |
|---|---|---|---|
| | Before Separation | Swim Up | EquiPure™ |
| Mean | 97.22% | 16.11% | 53.89% |
| Standard Error | 0.44% | 4.34% | 5.30% |

•Significant difference between total motility before separation and after both methods (swim up, EquiPure™) of sperm separation

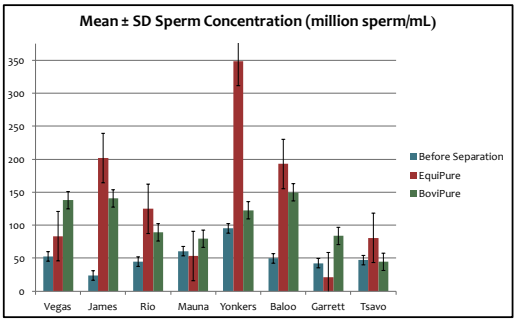
| Sperm Concentration (million sperm/mL) | | | |
|---|---|---|---|
| | Before Separation | Swim Up | EquiPure™ |
| Mean | 57.389 | 1.944 | 82.005 |
| Standard Error | 19.934 | 0.309 | 34.758 |

•Significant difference between sperm concentration before separation and after the swim up method of sperm separation

## Experiment 2 Motility



## Experiment 2 Concentration




## Experiment 2 Summary

| % Total Sperm Motility | | | |
|---|---|---|---|
| | Before Separation | EquiPure™ | BoviPure™ |
| Mean | 90.80% | 56.91% | 56.88% |
| Standard Error | 1.56% | 7.40% | 4.72% |

- Significant difference between total motility before separation and after both methods (EquiPure™, BoviPure™) of sperm separation
- EquiPure™ and BoviPure™ are extremely similar in the total motility of the sample after separation with these methods

| Sperm Concentration (million sperm/mL) | | | |
|---|---|---|---|
| | Before Separation | EquiPure™ | BoviPure™ |
| Mean | 52.007 | 138.297 | 105.911 |
| Standard Error | 7.201 | 37.492 | 13.129 |

- Significant difference between sperm concentration before separation and after both methods (EquiPure™, BoviPure™) of sperm separation

Appendix F.4 Presentation for the November 2013 Chintimini Kennel Club Meeting in Corvallis, OR.

# Comparison of Canine Sperm Motility, Morphology, and RNA Quality using 2 Techniques for Sperm Separation

Rachel Hegedus

Oregon State University
Department of Animal and Rangeland Sciences
University Honors College

## Introduction

- Infertility in male dogs can arise due to multiple factors
- Genetics has been hypothesized to be a potential cause of male dog infertility
  - 30% of infertility cases in human males are predicted to be caused by genetics
- Interest in identifying these genetic factors has been area of recent research interest across species

## Genetic Infertility Studies

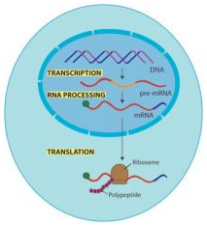
- Two characteristics of sperm help determine infertility
  - Spermatogenesis
  - Fertilization events
- Various processes in each activity of sperm are dependent on various expression of proteins
  - Differential expression of these proteins in sperm can be used to determine infertility
- Spermatozoal RNA is the tool to evaluate genetic infertility

## Brief Introduction to RNA

DNA → mRNA → protein

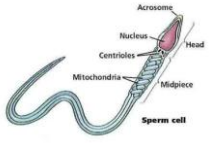
•mRNA represents only the portion of the DNA that is being expressed in the cell

•mRNA is used to translate the message of DNA into proteins that carry out specific functions in the cell




## Why Spermatozoal RNA?

- The sperm cell is transcriptionally and translationally dormant – no active protein expression!
  - How does this happen?




During spermatogenesis, the sperm cell adopts its characteristic shape

The DNA has to undergo intense condensation to fit inside the nucleus
-Uses protamines

Remnant RNA from spermatogenesis represent the genes that were expressed during this process

## Why Spermatozoal RNA?

- Spermatozoal RNA has functions in fertilization and early embryonic development
  - mRNA is delivered to the oocyte
  - Functions still need to be researched but could include:
    - Embryogenesis
    - Activation of the embryonic genome
    - Implantation

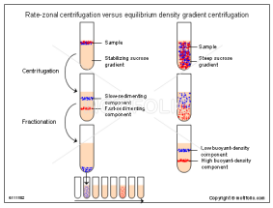
## Transcriptome Analysis

- Use sperm RNA to compare expression levels of the various transcripts between fertile and infertile males
  - Identify genes of interest that may influence fertility
- Has been done in the human, mouse, bull, and stallion

Spermatozoal RNA needs to be extracted from the cell in order to perform transcriptome analysis!!

## Separation of Sperm from Ejaculate

- Swim Up
  - Separates motile morphologically normal sperm from immotile sperm
- Density gradient centrifugation
  - Nidacon International has developed products that are species specific
    - BoviPure™,
    - PureSperm®
    - EquiPure™




Nidacon

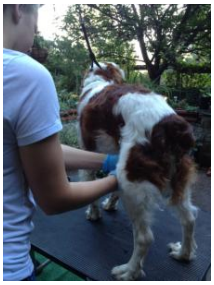
## Objectives

- Our goal was to evaluate methods of sperm separation techniques for the removal of somatic cells from the ejaculate for RNA isolation and their effectiveness at removing abnormally morphological sperm from the sample
  - Experiment 1: Swim up and EquiPure™
  - Experiment 2: EquiPure™ and BoviPure™

- Evaluations on: motility, morphology, concentration, RNA quantity, RNA quality

## Methods

- Experiment 1: Semen was manually collected three times from three dogs
- Experiment 2: Semen was manually collected three times from eight dogs
- For both experiments, semen was divided into aliquots of equal volume

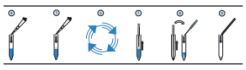


## Methods – Experiment 1

- Swim Up:
  - Sample of semen was centrifuged at 400xg for 15 minutes and supernatant was removed
  - Pellet was re-suspended in 2.5ml of Ham's F-10 and centrifuged under the same parameters
  - Pellet was overlaid with 1ml of Ham's F-10 and inclined at 45° for one hour at 37°C and 5% $CO_2$

## Methods – Experiments 1 and 2

- EquiPure™:
  - 3ml of EquiPure™ was overlaid with one aliquot of semen
  - Tube was centrifuged for 30 minutes at 300xg at room temperature
  - Supernatant was aspirated off in a circular motion

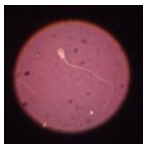


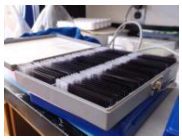
## Methods – Experiment 2

- BoviPure™:
  - Solutions for the top and bottom layer were made using BoviPure™ and BoviDilute™ prior to each separation according to the manufacturer's instructions
  - 2ml of the bottom layer was gently overlaid with 2ml of the top layer
  - The aliquot of semen was layered over this prepared gradient
  - Tube was centrifuged at 300xg for 25 minutes at room temperature
  - Supernatant was aspirated off in a circular motion

## Methods

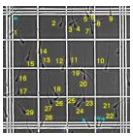
- Sperm morphology was performed prior to semen separation and after each method using an eosin-nigrasin stain and counting 200 sperm under oil immersion

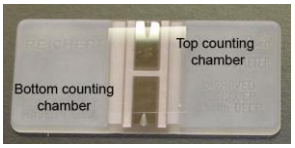




## Methods

- Motility was evaluated under a microscope prior to cell separation and after each method
- Concentration was evaluated prior to cell separation and after each method using the standard technique of a hemocytometer






## Methods

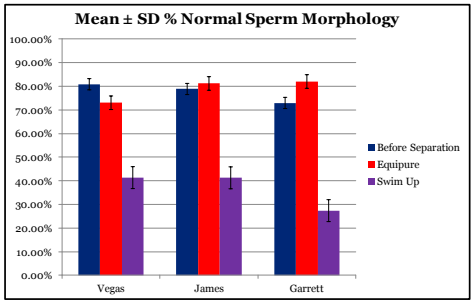
- RNA Isolation was performed using a phenol-chloroform extraction method with TRIzol
- RNA quantity and quality was evaluated after extraction using a spectrophotometer
  - Concentration of RNA in ng/µl
  - $A_{260}/A_{280}$ ratio
- RT-PCR was performed with gene specific transcripts to determine the presence or absence of contaminating somatic cell RNA
  - PTPRC – somatic cell specific
  - Protamine 2 – sperm cell specific

## Methods – Data Analysis

- Percent normal morphology was compared for each of the sperm separation method to the initial sample before separation
- Percent normal morphology was also compared between the two sperm separation methods in each experiment
- The same comparisons were made for the motility and concentration data as well
- An unpaired, two tailed student's T-Test (Microsoft Office Excel 2007) was used
- Significance was defined as $P<0.05$

Analysis of the data on cell separation techniques on RNA quantity and quality is ongoing!

## Results – Experiment 1

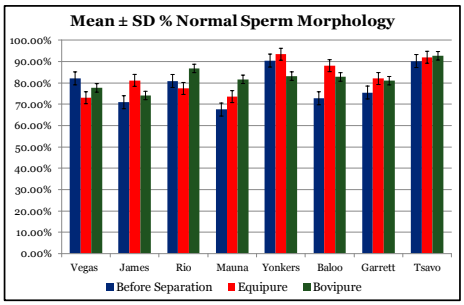


## Results – Experiment 1

- Fewer morphologically normal sperm after swim up method compared to before separation (P=0.001)
- No significant difference in percent of morphologically normal sperm before separation and after the EquiPure™ separation (P=0.765)
- Fewer morphologically normal sperm after the swim up method compared to EquiPure™ (P=0.001)

| | Before Separation | Swim Up | EquiPure™ |
|---|---|---|---|
| Mean | 77.53% | 78.72% | 36.64% |
| Standard Deviation | 2.37% | 2.87% | 4.64% |

## Results – Experiment 2




## Results – Experiment 2

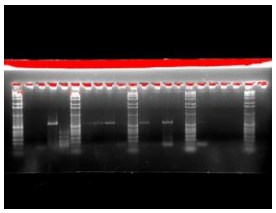
- No significant difference in percent morphologically normal sperm before separation and after either the EquiPure™ (P=0.375) or BoviPure™ (P=0.328) density gradient centrifugation method
- No significant difference in percent morphologically normal sperm between the EquiPure™ and BoviPure™ methods (P=0.976)

| | Before Separation | EquiPure™ | BoviPure™ |
|---|---|---|---|
| Mean | 78.78% | 82.56% | 82.46% |
| Standard Deviation | 3.04% | 2.80% | 1.98% |

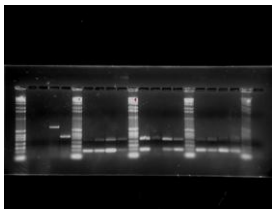
## Other Results

- Ongoing studies using the sperm separated using these techniques (swim up, EquiPure™, BoviPure™) are being done to:
  - Compare sperm RNA purity after isolation
  - Compare sperm RNA yield after isolation
- Initial data analysis suggests there is no significant difference in the RNA concentration isolated after separation with any method (swim up, EquiPure™, or BoviPure™)
- Reverse transcriptase PCR analysis is currently underway to determine the presence or absence of somatic cell specific genes in the isolated RNA

## Example of RT-PCR Results



Amplification of PTPRC transcripts



Amplification of Protamine transcripts

## Discussion

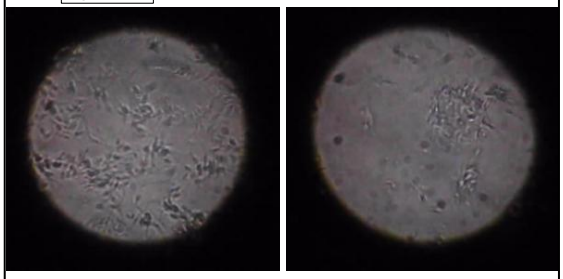
- We conclude that the density gradient centrifugation method is superior to the swim up method for the recovery of morphologically normal sperm
- For canine sperm, either the EquiPure™ or BoviPure™ product could be effective at separating morphologically normal sperm from abnormal sperm

## Discussion

- Motility is a important factor in fertility
- However, further evaluation of both centrifugation methods shows that it does not yield a high percentage of progressively motile sperm
  - Due in part because the viscosity of the media, the sperm stick together in clumps that move slowly because the tails are still beating
- Results also showed that there was a significant difference between total motility before separation and after both methods (swim up and density gradient centrifugation)

## Motility

EquiPure™ BoviPure™



## Future Studies

•Although initial data suggests that RNA quantity may not differ between separation techniques, RNA quality is crucial to further transcriptome analysis studies

•Goal is that comparisons of spermatozoal RNA expression profiles between idiopathic infertile male dogs will be compared to normal fertile males

- •Potential to also compare between dogs exhibiting low motility (asthenozoospermia) or poor morphology (teratozoospermia) to normal fertile spermatozoal RNA profiles

## Acknowledgements


- Special thanks to:
  - Dr. Michelle Kutzler, DVM, PhD, DACT
  - Dr. Charles Estill VMD, PhD, DACT
  - Dr. Alfred Menino
  - Reni Stewart, Howard Meyer, Lisa Loeffler, Linda Lyster, and Sherry Schoorl for participating with their dogs
  - Oregon State University Honors College
  - Oregon State University Department of Animal and Rangeland Sciences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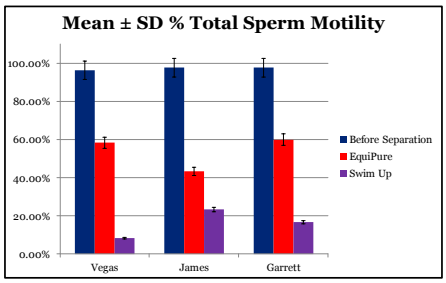
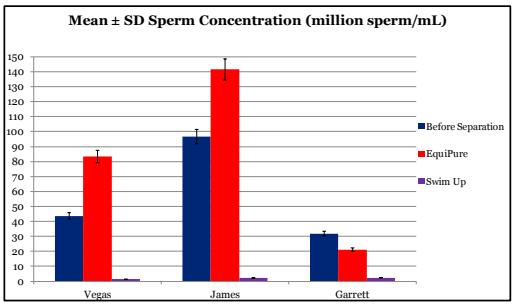
## Questions??

## Experiment 1 Motility



## Experiment One Concentration




## Experiment 1 Summary

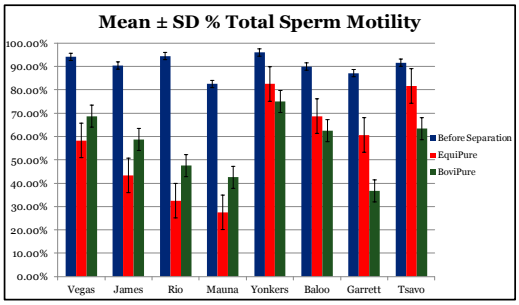
| % Total Sperm Motility | | | |
|---|---|---|---|
| | Before Separation | Swim Up | EquiPure™ |
| Mean | 97.22% | 16.11% | 53.89% |
| Standard Error | 0.44% | 4.34% | 5.30% |

•Significant difference between total motility before separation and after both methods (swim up, EquiPure™) of sperm separation

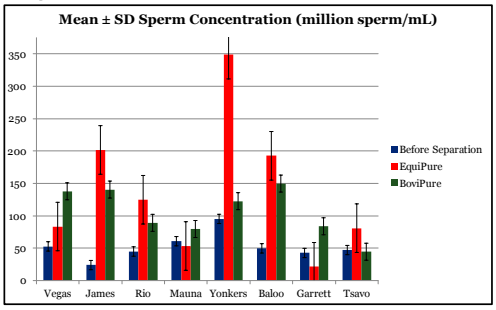
| Sperm Concentration (million sperm/mL) | | | |
|---|---|---|---|
| | Before Separation | Swim Up | EquiPure™ |
| Mean | 57.389 | 1.944 | 82.005 |
| Standard Error | 19.934 | 0.309 | 34.758 |

•Significant difference between sperm concentration before separation and after the swim up method of sperm separation

## Experiment 2 Motility




## Experiment 2 Concentration




## Experiment 2 Summary

| % Total Sperm Motility | | | |
|---|---|---|---|
| | Before Separation | EquiPure™ | BoviPure™ |
| Mean | 90.80% | 56.91% | 56.88% |
| Standard Error | 1.56% | 7.40% | 4.72% |

•Significant difference between total motility before separation and after both methods (EquiPure™, BoviPure™) of sperm separation
•EquiPure™ and BoviPure™ are extremely similar in the total motility of the sample after separation with these methods

| Sperm Concentration (million sperm/mL) | | | |
|---|---|---|---|
| | Before Separation | EquiPure™ | BoviPure™ |
| Mean | 52.007 | 138.297 | 105.911 |
| Standard Error | 7.201 | 37.492 | 13.129 |

•Significant difference between sperm concentration before separation and after both methods (EquiPure™, BoviPure™) of sperm separation

Appendix F.5 Abstract submitted for presentation at the Society for Theriogenology 2014 Conference in Portland, OR.

**Comparison of canine spermatozoa RNA concentrations and purity using two density gradient centrifugation solutions**

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[b]Dept of Animal Science, University of California, Davis, CA

**Introduction** Sperm must be separated from other cells in the ejaculate prior to RNA isolation in order to provide a pure sample for analysis of genetic causes of infertility. Several methods for elimination of contaminating somatic cells have been described including swim-up and density gradient centrifugation (DGC). In a previous study, we found the swim-up method yielded fewer morphologically normal sperm than DGC.[1] The objective of this study was to compare RNA concentration and purity following separation of dog sperm by DGC products commercialized for horses and cattle. Because of the two layer density gradient, we hypothesized that the cattle product would be more effective at removing somatic cells, yielding a purer sperm RNA sample.

**Methods** Semen was manually collected up to six replicates from eight dogs and divided into three aliquots of equal volume. Two DGC products were used according to the manufacturer's instructions (Equipure™ and Bovipure™, Nidacon International, Molndal, Sweden). No cell separation technique was applied to the control samples. Total RNA was isolated using Trizol® reagent (Ambion®, Carlsbad, CA) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically with a NanoPhotometer® (IMPLEN, Munich, Germany). Primers specific for canine sperm gene protamine-2 (PRM2) were designed using Primer3 software as well as information from the NCBI gene bank and synthesized by Sigma-Aldrich (St. Louis, MO). Gene specific transcripts were reverse-transcribed from the total RNA using Superscript® One-Step RT-PCR with Platinum® *Taq* kit (Invitrogen™, Carlsbad, CA) according to the manufacturer's instructions. Results were visualized on 2% agarose gels stained with SYBR® Green nucleic acid gel stain (Invitrogen™, Carlsbad, CA) utilizing the GelLogic 212 Pro Imaging System (Carestream Health, Woodbridge, CT). RNA concentrations and purity were compared between separation methods using a PROC MIXED platform in SAS (V.9.3, SAS Institute Inc, Cary, NC). Data were reported as least squares mean±SEM.

**Results** RNA concentration did not differ significantly between separation methods. However, both separation methods were significantly greater in amplifying the PRM2 transcript (purity) compared to the control. Results are summarized in Table 1.

| Table 1. *****p<0.05 compared to control | Control | Equipure™ | Bovipure™ |
|---|---|---|---|
| RNA concentration (ng/µl) | 60.50±19.30 | 94.76±25.73 | 78.90±19.22 |
| Purity (100%=pure, 0%=contaminated) | 23%±10% | 60%±13%* | 55%±10%* |

**Discussion** The two DGC products may have yielded similar results because size of the dog sperm head (5µmX7µm) is intermediate to the stallion (3µmX6µm) and bull (5µmX9.5µm).[2]

**Keywords** Dog, ejaculate, primers, protamine-2, sperm separation

**References** [1]Hegedus R, Kutzler M. Evaluation of canine sperm morphology using two techniques for sperm separation. 2013 Annual Meeting Society for Theriogenology (abstract).
[2]Roberts SJ. Veterinary Obstetrics and Genital Diseases Theriogenology. 3rd ed. Devon, UK: David and Charles, Inc.; 1986.