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The Impacts of Inbreeding and Hybridization on Sperm Quality in Felids and Canids.

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The Impacts of Inbreeding and Hybridization on Sperm Quality in Felids and Canids.

A dissertation submitted in partial satisfaction
of the requirements for the degree Doctor of Philosophy
in Biology

by
Audra Alexandra Huffmeyer
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ABSTRACT OF THE DISSERTATION

The Impacts of Inbreeding and Hybridization on Sperm Quality in Felids and Canids.

by

Audra Alexandra Huffmeyer

Doctor of Philosophy in Biology

University of California, Los Angeles, 2021

Professor Robert Wayne, Chair

Normal sperm form and function is essential to fertilization of oocytes and species reproduction. Large carnivores, particularly large cats, experience reduced sperm quality at higher rates than other mammals due to genetic isolation caused by habitat loss and urbanization. Reduction in home ranges for large carnivores is the primary consequence of habitat loss and urbanization that leads to inbreeding and genetic isolation.

In this dissertation, we explore the impacts of inbreeding and hybridization on sperm quality in two felid species, the domestic cat and mountain lion (*Felis domesticus* and *Puma concolor*) and two canid species, wolf and coyote, (*Canis lupus* and *Canis latrans*). To assess the extent to which inbreeding, and hybridization reduces sperm quality in felid and canid species, we focused our investigation on three systems. (1) We completed a comparative study using a

single cell RNA-Seq approach to identify differentially expressed genes between morphologically normal and abnormal sperm from domestic cats with normospermia (< 60% abnormal sperm production per ejaculate) and teratospermia (> 60% abnormal sperm production per ejaculate) to elucidate genes and pathways associated with abnormal sperm function. We found normal sperm from cats with teratospermia have a gene expression profile similar to abnormal sperm from males with teratospermia. There was also downregulation of cGMP pathways and kinase phosphorylation pathways. (2) We surveyed male mountain lions for abnormalities associated with inbreeding depression during live captures, after mortality events, and from images collected from camera traps between December 2019 and December 2020 in California (Santa Monica Mountains, Santa Susanna Mountains, Santa Ana Mountains, and Eastern Peninsula). Specifically, we looked for evidence of teratospermia, cryptorchidism, and distal tail kinks. For teratospermia, we extracted testes from five males (postmortem). Epididymal sperm evaluations revealed all males were teratospermic. Across all samples, on average, 93% of observed spermatozoa were abnormal. Further, we found 4 individuals exhibited distal tail kinks, one unilaterally cryptorchid male and one male with testes that differed greatly in size - likely reflecting asynchronous migration of the testes during puberty. Finally, (3) we assessed gene expression patterns between the coyote (*Canis latrans*) and gray wolf (*C. lupus*) whose potential to hybridize may underlie the origins of the red wolf (*Canis rufus*) and Eastern wolf (*Canis lycaon*). Here we examined ejaculates from gray wolves and F1 and F2 hybrids between a western coyote and a western gray wolf and employed a comparative RNA-Seq approach to assess expression differences between ejaculates from gray wolf males and their wolf-coyote hybrid offspring. Our RNAseq analyses identified 9 transcripts between wolves and F1 and F2 offspring that were differentially expressed (DE). However, we found 425

DE transcripts between F1-F2 offspring suggesting the potential for reproductive incompatibility between hybrid generations.

The dissertation of Audra Alexandra Huffmeyer is approved.

Budhan Pukazhenthi

Thomas Smith

Karen Sears

Victoria Sork

Robert Wayne, Chair

University of California, Los Angeles

2021

Dedicated to the mountain lions of Southern California that provided invaluable samples for Chapter 2.

TABLE OF CONTENTS

Acknowledgements

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Chapter 1: Differential gene expression patterns in spermatozoa with normal morphology in semen from domestic cats with teratospermia and normospermia

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A. Huffmeyer assisted with the sample collection, completed the lab work, bioinformatic analyses, and writing of the manuscript. B. Pukazhenthi assisted with the sample collection and writing of the manuscript. R Wayne assisted in the support, data analysis and writing of the manuscript. This research was supported by the National Science Foundation's Graduate Research Fellowship [Grant No. DGE-1650604].

Chapter 2: First reproductive patterns of inbreeding depression in Southern California male mountain lions (*Puma concolor*).

Currently in press, second round of reviews for *Theriogenology*

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AAH collected samples, completed the epididymal semen analyses, and wrote the manuscript. JAS collected field data and edited the manuscript. WTV collect field data and edited the manuscript. SPDR collected field data and edited the manuscript. RKW provided funding and edited the manuscript. We would like to thank Dr. Buddha Pukazhenthi, Dr. Pierre Comizzoli, and Dr. Melody Roelke for their advice on sampling. We would like to thank Dr. Karen Sears for permissions to use her microscope for the morphological assessments. We thank Denise Kamradt for producing the Figure 1 map. For the SMM study, we thank the many NPS biologists, technicians, and interns who assisted with fieldwork. For the SAM and ePR studies, we thank field technicians Jamie Bourdon and Rich Codington, as well as all the other field staff who have assisted in capture activities over the course of the long-term studies. Lastly, we acknowledge the following funding agencies: The National Science Foundation's Graduate Research Fellowship [Grant No. DGE-1650604], the National Geographic Explorer Grant, the California

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Chapter 3: Gene expression patterns in semen from wolf-coyote hybrids.

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CSA collected the ejaculate samples. AAH extracted the RNA, generated cDNA libraries, and completed the bioinformatic analyses. AAH and RKW completed the first drafts of the manuscript. All authors edited and contributed to revisions on the final manuscript. The authors thank L. David Mech, Karen Baumen, and Peggy Callahan at the Wildlife Science Center for allowing us to use the samples and providing us with the meta data that allowed us to complete this project. This material is based upon work supported by the National Science Foundation's Graduate Research Fellowship under Grant No. DGE-1650604.

Curriculum Vitae

Audra A. Huffmeyer

EDUCATION

Master of Science, University of Michigan- Ann Arbor. Major: Ecology and Evolutionary Biology, Graduation: May 2015

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TEACHING EXPERIENCE

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- Associate Teaching Assistant, Life Sciences, University of California, Los Angeles, LS 7B: Genetics, Ecology, and Evolution (Winter 2017, Winter 2918).
- Co-Facilitator, PEERS, Life Sciences, University of California, Los Angeles, (September 2016- April 2017)
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AWARDS

- National Geographic Early Career Award, Monitoring the effects of small population size and inbreeding on seminal quality in mountain lions. Fall 2017- Present.
- National Science Foundation GRIP Award, Proteomic analyses of teratospermic and normospermic samples. Fall 2017.
- National Science Foundation GRIP Award, Differential gene expression in abnormal sperm cells. Fall 2016.
- Competitive Edge Award, ULCA, Summer 2015
- National Science Foundation Graduate Research Fellowship Award, Spring 2015.
- Minority Student Award, Smithsonian Institution, Awarded twice: Summer 2015 & Summer 2014.
- National Science Foundation Travel Grant to Emerging Researchers National Conference in STEM, February 2012 & February 2014.

PUBLICATIONS

Huffmeyer, Audra A., Budhan S. Pukazhenthi, and Robert K. Wayne. "Differential gene expression patterns in spermatozoa from normospermic and teratospermic domestic cats." Animal Reproduction Science (2021): 106698.

SELECTED TALKS

Survey of Conservation Genomics. University of California, Santa Cruz. Virtual. March 2021.

How to mentor and stand up for women of Color in STEM Workshop. NE Consortium WOCShop. Virtual. December 2020.

How to mentor and stand up for women of Color in Marine Biology Workshop. The 6th International Marine Conservation Congress. Virtual, August 2020

She is an Entrepreneur Panelist, Bruin Women in Business & Startup UCLA, March 2020.

Social Entrepreneur 101 Panelist, NET IMPACT- UCLA, Feb 2020.

Single Cell RNA-Seq analysis of abnormal and normal sperm cell in the domestic cat. Global Biodiversity Genomics Conference. Washington DC, February 2017.

RNA benchwork at RNA-seq Workshop. Asilomar, California September 2015.

SERVICE

STEM Ambassador, UCLA Graduate Admissions (Summer 2016- Present)
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Chapter 1: Differential gene expression patterns in spermatozoa with normal morphology in semen from domestic cats with teratospermia and normospermia

ABSTRACT

Large percentages of abnormal sperm, termed teratospermia, are associated with poor fertility in cats, many of which are threatened with extinction from their natural habitats. Even normal appearing spermatozoa from felids with teratospermia may have a compromised capacity for motility and fertilization indicating there are factors affecting the fertilization capacity of all sperm regardless of morphology. There was a comparative study conducted using the RNA-Seq approach to identify differentially expressed genes between morphologically normal and abnormal sperm from domestic cats with normospermia and teratospermia to elucidate genes and pathways associated with abnormal sperm function. Normal sperm from cats with teratospermia have a gene expression profile similar to abnormal sperm from males with teratospermia. There was also downregulation of cGMP pathways, which may be associated with a lesser sperm motility in ejaculates from males with teratospermia. Kinase phosphorylation pathways also were downregulated in normal spermatozoa from ejaculates of males with teratospermia. Results indicate that analysis of sperm gene expression provides a more precise assessment of sperm function in semen of cats with teratospermia and facilitates identification of molecular abnormalities that may lead to compromised fertilization capacity.

Keywords: Felidae; Gene expression; Inbreeding; Spermatozoa: Teratospermia.

1. Introduction

Ejaculates with more than 60% malformed sperm have a phenotype termed teratospermia and generally there is a lesser fertilizing capacity when these ejaculates are used for artificial insemination (Pukazhenthi et al., 2001, 2006). Teratospermia is prevalent in many mammals, such as humans, mice, rats, dogs, and cats (Pukazhenthi et al., 2006; Platts et al., 2007 Ferenčaković et al., 2017). In felids, 28 of the 37 extant species have teratospermia, a value far greater than other surveyed families (Pukazhenthi et al., 2006). The most severe cases occur in wild felids and include species with lesser heterozygosity due to greater population densities in regional areas and inbreeding, such as the cheetah (*Acinonyx jubatus*) and Florida panther (*Puma concolor coryi*; Johnson et al., 2010). There is a close association between lesser heterozygosity and impaired sperm quality (Fitzpatrick and Evan, 2009). Little is known about the genes that contribute to abnormal sperm production in cats. Reproduction success in inbred species is generally less due to genome wide effects, such as lesser heterozygosity, increased deleterious alleles, and small population size (Keller and Waller, 2002; Robinson et al., 2019). The large prevalence of abnormal sperm in ejaculates in species where inbreeding has occurred adds to this precarious circumstance. The most notable example is the Florida panther, where all males captured between 1985 and 1990 were cryptorchid and approximately 94% of their ejaculates were comprised of abnormal sperm. As a result, reproduction was markedly impaired in this population (Roelke et al., 1993).

Distinct phenotypic differences are evident in sperm from cat donor males with normospermia and those with teratospermia. Specifically, there are distinct differences in sperm concentration, sperm motility, and percentage of morphologically normal sperm (Terrell et al., 2010). Teratospermic donor males produce more sperm per ejaculate, perhaps as compensatory

mechanism for producing larger numbers of normal appearing sperm (Pukazhenthi et al., 1998b). Circulating testosterone concentrations in the ejaculate of donor domestic cats with teratospermia are markedly less than in those with normospermia (Howard et al., 1990). Furthermore, there are also distinct differences in sperm sub-populations during maturation in the epididymis of domestic cats with normospermia and teratospermia (Gutiérrez-Reinoso and García-Herreros, 2016).

Microscopic assessments are the most common method for diagnosing teratospermia. Even morphologically normal spermatozoa from cats with teratospermia have less motility and a compromised capacity for fertilization (Pukazhenthi et al., 2006). These findings result in concerns in non-domesticated felids that have teratospermia because of the small numbers of morphologically normal spermatozoa in their ejaculate. If morphologically normal spermatozoa from teratospermic ejaculates function abnormally, assumptions about the importance of phenotypic indicators of fertility may be flawed. Results after conducting sperm chromatin structure assays indicate there are effects of teratospermia in the nucleus of spermatozoa in domestic cats (Penfold et al., 2003). Recent advances in conducting single cell transcriptomic procedures provide for more sensitive and accurate assessments of cell function. Specifically, analysis of differences in gene expression between normal and abnormal spermatozoa from semen donor males with normospermia and teratospermia could provide new insights into molecular mechanism affecting fertilization capacity of spermatozoa.

In the present study, there was use of a single cell RNA-Seq analyses to compare normal spermatozoa from domestic cats with normospermia with four morphotypes (normal, coiled tail, bent-midpiece and cytoplasmic droplet) to those with teratospermia. The goal in conducting this study was to assess differences in gene expression among all four sperm morphotypes using

Differential Expression (DE) analyses. The hypothesis was that gene expression profiles in sperm with the different morphotypes is distinct based on results from previous studies with humans and infertile strains of mice, such as oligospermia (small sperm count), azoospermia (ejaculates lacking sperm), teratospermia (>60% abnormal sperm), and asthenospermia (markedly less sperm motility). These conditions are marked by distinct mRNA and small noncoding RNA (miRNA and piRNA) profiles affecting mis-expression and downregulation of important genes for sperm function (Platts et al., 2007; Montjean et al., 2012; Tian et al., 2018). Furthermore, because male infertility is associated with sperm morphology, to a marked extent, it was hypothesized that sperm morphotypes (Figure 1) from domestic cats also have distinct mRNA and small noncoding RNA profiles that may facilitate prediction of reproductive failure.

Domestic cats serve as important animals for studying human diseases and disorders in other felid species (Schraeder et al., 1993, Willet et al., 1997, Fox et al., 2000). Domestic cats share more than 200 homologous heritable genetic defects with humans (O'Brien et al., 2002) and there is a large amount of synteny between the domestic cat and the human genome (O'Brien et al., 2002) as well as similar sex chromosome organization (Murphy et al., 1999). Domestic cats and humans are also similar because both species are prone to teratospermia (Ali and Grimes, 1989). In human males, deleterious, heritable variants contribute to malformed spermatozoa that are present in ejaculates of men with teratospermia (Bronson et al., 2017). In the present research, we compare inbred and outbred domestic cats to identify candidate genes associated with teratospermia. If differences in gene expression between sperm morphotypes from inbred and outbred domestic cats can be detected, then reduced fertility in non-domestic species can likely be analyzed using this approach and newly developed genomic techniques. Consequently, results from studies with domestic cats may provide for a greater understanding of

molecular mechanisms involved in reproduction and testis function both in wild feline species and humans. The present study is the first in which there is evaluation of gene expression to enhance understanding of abnormal felid spermatozoa.

2. Materials and methods

2.1. Sperm collection

All samples used in this study were derived from previously banked samples collected by researchers at the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA with there being institutional permits for sample collections (Pukazhenthi et al., 1998b, 2002; Terrell et al., 2010). Donors of semen were part of a long-term research colony of outbred and inbred cats maintained at SCBI. Inbred cats were either purchased from commercial cattery or were intentionally inbred for other projects. Ejaculates were collected using electroejaculation procedures and then cryopreserved (normospermia, $n = 5$ cats; teratospermia, $n = 5$ cats; one ejaculate per cat) as described in Pukazhenthi et al. (1988b) and supplemental methods.

2.2. Sperm thawing

There is routine cryopreservation (bank) of domestic cat sperm at ~50 million per ml in the laboratory where the present study was conducted. Cryopreserved samples (straws, two straws per cat) were removed from liquid nitrogen and thawed individually, first in air for 10 s, then in a 37 ˚C water bath for 30 s. Straws were cut and the contents emptied into a sterile Eppendorf microcentrifuge tube. Sperm suspensions were then diluted 1:2 with fresh Ham's F10 (Hepes modification), centrifuged (300 x *g*; 8 min) and supernatant was discarded. Resulting sperm pellets were re-suspended in 0.05 mL of fresh Ham's F10 and assessed for sperm motility. For this study,

two ejaculates from five semen donor males that were normospermia and five that were teratospermia were thawed (50 million per ml, 12.5 million per straw; Table 1). Washed spermatozoa were mixed with 7% polyvinylpyrollidone solution (Sage *In Vitro* Fertilization, Inc., Turmbull, CY, USA) to immobilize the sperm.

2.3. Isolating spermatozoa using micromanipulation

Individual spermatozoa were isolated using aspiration procedures while utilizing an inverted microscope equipped with micromanipulators. A sperm injection pipette (MIC-50-0; ORIGIO Inc., Charlottesville, VA, USA) was used to aspirate spermatozoa (Comizzoli et al., 2006). Replicates (n = 6-9) included ten spermatozoa of each morphotype: normal, coiled tail, and bent midpiece with a cytoplasmic droplet (Figure 1, Table 1). The three sperm morphotypes are the most common in inbred domestic cats and were relatively easy to isolate (Howard et al., 1993). The presence of cytoplasmic droplets may indicate the sperm are immature (Howard et al., 1993). The bent-midpiece or bent tail of spermatozoa with a cytoplasmic droplet attached is one of the most common morphotypes in males with teratospermia and thus was selected for analysis in the present study to increase statistical power. Isolated spermatozoa were centrifuged (8,000 x *g*; 10 min) in micro-centrifuge tubes to pellet sperm and then flash frozen in liquid nitrogen using standard protocols (Comizzoli et al., 2002).

2.4. Library preparation, sequencing, and mapping

We used the QIAseq FX Single Cell RNA Library kit to extract total RNA and synthesize cDNA libraries (Trevino et al., 2017). The quantity and quality of the library preparations were evaluated using a Bioanalyzer (Agilent Technologies). The libraries were sequenced at Fulgent

Genetics in Los Angeles California on an Illumina HiSeq 4000 in one lane. The raw reads were trimmed with Trim Galore! 0.3.1 to remove adaptors and filter poor quality sequences. The resulting trimmed reads were aligned to the most recent domestic cat genome (*Felis catus* 9.0) using STAR (Dobin et al., 2013). Using the mapped reads, genes were identified and gene matrix was developed for downstream RNA-Seq analyses with HTSeq (Anders and Huber, 2010). HTSEQ yielded 26,686 transcripts. The HTSEQ gene matrix was uploaded to R.

2.5. Data processing, normalization, and differential expression (DE) analyses

Prior to normalization and differential expression analyses, poor-quality reads, lesser coverage reads (<10) were filtered, and poor-quality cells/libraries were entirely removed using an alpha cut off 0.1. In R, DESeq2 was used (Love et al., 2017) to complete five differential expression (DE) analyses (Table 2). For each DE analysis, gene counts and sperm morphotype information were uploaded to a CounTdataSet object. DESeq2 estimated size factors were estimated for each single cell library using the "median ratio method" or Equation 5 in Anders and Huber (2010) and overdispersion for each gene to account for library differences before completing the DE analysis. Extraction and analyses of genes with an FDR adjusted *P*-value < 0.05 were used for downstream analyses (Supplemental Information for R script). A plotPCA function in DESeq2 was used to generate principal component analyses.

2.6. Data visualization

The DE genes were queried using BioMart (Cunningham et al., 2019) for gene designation, gene description, and RNA type to determine if the transcript was protein coding or non-protein coding. The PANTHER (version 14.1, Mi et al., 2019) program was used to

determine the functional classification of the DE genes and g: Profiler (Reimand et al., 2016) was used to complete an over-representation analyses (ORA) of the upregulated and downregulated genes. For the ORA analyses, the reference genome was used as the background data set for the GO analyses and there was application of a Benjamini-Hochberg FDR correction (Benjamini and Hochberg 1995). The ORA included queries for Gene Ontology and KEGG Reactome pathways. The ggplot2 in R was used to create volcano plots and the program Pheatmaps (Kolde et al., 2015) to generate a heatmap of the top 20 expressed genes. With Pheatmap, normalized gene expression data was used to visualize clustering of cell libraries as well as clustering of the 20 genes expressed to the greatest extent.

3.0. Results

3.1. Differential gene expression analyses – normospermia (*normal, N_n) compared with teratospermia (normal, T_n)*

Results from the DESeq2 analyses between morphologically normal-appearing sperm from cats that were N_n and T_n indicated there were 16 DE mRNA transcripts with an FDR adjusted *P*-value < 0.05 (Supplemental Information Table 1). The results from the BioMart analyses indicated there were 14 of the transcripts that were protein coding, one long non-coding RNA (lincRNA, ENSFCAG00000043684), and one pseudogene (ENSFCAG00000015640). Of the 16 mRNA transcripts, nine were in greater abundance and seven were lesser abundance in morphologically normal-appearing sperm from cats with normospermia than those with teratospermia. The pseudogenes and lincRNA transcript were present in greater abundance. Of the nine transcripts present in greater abundance, seven were protein coding. All mRNA

transcripts that were in relatively greater abundance in morphologically normal-appearing sperm from cats with normospermia than those with teratospermia were protein coding.

The results from the PANTHER *GO* functional analyses led to classification of 16 DE transcripts into six functional categories: binding (GO: 0005488), catalytic activity (GO: 0003824), molecular function regulator (GO: 0098772), molecular transducer activity (GO: 0060089), transcription regulator activity (GO: 01400110), and transporter activity (GO: 0005215, Figure 2, A1). Results from conducting the separate GO functional classification analyses of the DE transcripts present in greater abundance and DE transcripts present in lesser abundance indicated there was transcription regulator activity only associated with DE transcripts present in greater abundance. Molecular transducer activity was only associated with DE transcripts present in lesser abundance (Figure 2, A2 and A3). Both up- and down-regulated genes were associated with binding, catalytic, molecular function regulator, and transporter activity.

3.2. Differential mRNA transcript abundances – Normospermia (normal, N_n) compared with teratospermia (tightly coiled tail, T_c)

Results in comparisons of gene expression in morphologically normal sperm to those from donor males with normospermia (N_n) in spermatozoa with a tightly coiled tail defect from a semen donor male with teratospermia (T_c) indicated there were five DE mRNA transcripts with an FDR adjusted $P < 0.05$ (Supplemental Information Table 3 and Figure 2, C1). All DE mRNA transcripts were for protein coding genes. Two of the DE mRNA transcripts were in relatively greater abundance, and the other three transcripts were in relatively lesser abundance in sperm with tightly coiled tails from males with teratospermia as compared with those with

normospermia (Figures 2, C2 and C3). The results from conducting the GO analyses led to classifications of five DE transcripts into two functional groups: binding (GO: 0005488) and catalytic activity (GO:0003824). The two mRNA transcripts in relatively greater abundance in sperm with tightly coiled tails from males with teratospermia as compared with those with normospermia were associated with the binding functional group and the three DE transcripts present in lesser abundance were associated with binding and catalytic functional groups.

3.3. Differential abundances of mRNA transcripts – Normospermia (normal, N_n) compared with teratospermia (bent midpiece with a cytoplasmic droplet, T_d)

Results from the DESeq2 analyses led to identification of 45 DE mRNA transcripts between normal spermatozoa from normospermic (N_n) donors and spermatozoa from teratospermic donors with a bent midpiece and a cytoplasmic droplet (T_d; FDR adjusted *P*-value < 0.05; Supplemental Information Table 2 and Figure 4). Ten of the mRNA transcripts were in greater abundance and 35 transcripts were in lesser abundances in males with normospermia and spermatozoa from males with teratospermia with a bent midpiece and a cytoplasmic droplet. The mRNA transcripts in greater abundance included those for one pseudogene, one small nuclear RNA, and those for eight protein coding genes (Table 3). The mRNA transcripts in lesser abundance comprise those for 34 protein coding genes and one lincRNA transcript (Supplemental Information Table 2).

The results from the PANTHER *GO* functional analyses led to classification of the 45 DE transcripts into five functional groups: binding (GO: 0005488), catalytic activity (GO: 0003824), molecular transducer activity (GO: 0060089), transcription regulator activity (GO: 0140110), and transporter activity (GO: 0005215, Figure 2-B1). The up-regulated DE transcripts represent

three functional categories: binding, catalytic activity, and transcription regulator activity (Figure 2-B2). The DE transcripts in lesser relative abundance in males with normospermia and spermatozoa from males with teratospermia with a bent midpiece and a cytoplasmic droplet represented four functional categories: binding, catalytic activity, molecular transducer activity, and transporter activity (Figure 2-B3).

3.4. Relative abundance of the top 20 mRNA transcripts

The results from conducting the Pheatmap analyses indicated that normal spermatozoa from semen samples of donor males with teratospermia (T_n) do not cluster separately from abnormal sperm morphotypes from males with teratospermia (T_c or T_d; Figure 3, Panels A and B).

3.5. Over representation analyses

An over-representation analyses (ORA) was performed on the DE genes from N_n-T_n, N_n-T_c, and N_n-T_d to determine if the DE genes present is larger abundance and the DE genes present in lesser abundance were associated with GO terms over enriched compared to the reference domestic cat genome (Supplement Information Tables 5-9). Importantly, the results from the three ORA analyses led to identification of GO terms associated with kinase phosphorylation. Specifically, the following GO terms are associated with kinase phosphorylation: GO:004690: cyclic nucleopeptide-dependent protein kinase activity (Supplemental Information Table 7 and Table 9), GO:008239: cGMP-dependent protein kinase activity (Supplemental Information Table 7), GO:004690 cyclic nucleotide-dependent protein kinase activity (Supplemental Information

Table 9), GO:0030551 cGMP binding, GO:0030551 cyclic nucleotide binding (Supplemental Information Table 9).

4. Discussion

In the present study, differential gene expression, based on abundances of mRNA transcripts between sperm with normal and abnormal morphotypes from semen of normospermic and teratospermic domestic cat males was investigated. Results indicate gene expression of normal spermatozoa from domestic male cats with normospermia differs from spermatozoa in semen collected from males with teratospermia. The results also indicate normal appearing sperm from males with teratospermia have aberrant gene expression profiles comparable to abnormal sperm from these males. These findings lead to the conclusion that normal sperm from semen of male domestic cats with teratospermia may be functionally compromised. The observed differences in gene expression profiles may contribute to lesser sperm motility and subsequently to a lesser capacity for ovum penetration (Pukazhenthi et al., 2000).

The results from the DESeq2 analyses indicated there was down-regulation of genes and pathways associated with the kinase phosphorylation in males with teratospermia. Kinase phosphorylation is responsible for capacitation and fertilization in felid species. Results from previous studies of kinase inhibitor indicate these pathways are disrupted in sperm from teratospermic males (Pukazhenthi et al., 1998). In the present study, mechanisms by which kinase phosphorylation may be disrupted in sperm from males with teratospermia were identified. Specifically, kinase phosphorylation was impaired as a consequence of down regulation of *PRKG, PRKG2, PRKACB*, *PIK3C2B*, *SPHKAP,* ADRB3, *MADD* genes, as well as genes associated with cyclic nucleopeptide-dependent protein kinase activity (GO:004690), and

cGMP-dependent protein kinase activity (GO:008239). The change in functions that occur due to kinase phosphorylation are not yet fully understood in spermatozoa especially with regard to how the kinase phosphorylation induces sperm capacitation and facilitation of fertilization processes (Ickowicz et al., 2012). Results from protein kinase inhibition studies in mammals, including domestic cats and wild felids, supports the general importance of this pathway in sperm capacitation and phosphorylation (Carrera et al., 1996; Pukazhenthi et al., 1998b; Leyton et al., 2012).

Down-regulated DE genes associated with kinase phosphorylation include *PRKG*, *PRKG2*, and *PRKACB* (protein kinase cGMP-dependent 1, protein kinase cGMP-dependent 2, and protein kinase cAMP-activated catalytic subunit beta) belong to the same family of proteins that bind to and inhibit tyrosine kinase receptors (Hoffman et al., 2009). The *PRKG* and *PRKG2* genes are major downstream effectors but not much is known about the physiological changes that occur as a result of kinase phosphorylation in sperm. The cGMP-dependent protein kinases are encoded by two genes, *PRGK* and *PRGK2,* both of which are down-regulated in this study (N_n-T_d, Table 4, and N_n-T_c). Therefore, there is likely a downstream effect on the cGMP-dependent protein kinase activity related to capacitation and fertilization. Results from a study in which there was knock-out of the *PRKACA* and *PRKACB* genes in mice indicated these genes may be associated with sperm motility because mice with the knocked-out subunits had normal spermatogenesis but sperm from these mice had lesser forward sperm motility (Skålhegg et al., 2002). Consequently, any dysregulation or mutation of the *PRKACB* gene may contribute to reduced motility of sperm. The *SPHKAP* gene is an A-kinase anchor protein that may contribute to actions of cAMP and sphingosine in signaling pathways (Lacana et al., 2002). The A-kinase anchoring proteins comprise a large group of proteins important for binding to the regulator

subunit of protein kinase A, a component of the kinase phosphorylation pathway (Edwards and Scott, 200). Specifically, A-kinase anchoring proteins are important in regulation of flagella and cilia function. A whole genome sequencing effort for the cheetah led to identification of fixed deleterious variants in the *AKAP4* gene as the probable cause for abnormal sperm production and teratospermia (Dobrynin et al., 2015).

In human sperm, the mean concentrations of cAMP are generally ~100x greater than the mean concentration of cGMP and cAMP in the body and is the main regulatory factor for kinase phosphorylation (Willipinski-Stapelfeldt et al., 2004). In the present study, however, there was no downregulation of cAMP pathways, down regulation of only one A-kinase anchor protein (*SPHKAP)* in sperm from males with teratospermia. However, there was down regulation of genes and pathways associated with cGMP, including cyclic nucleopeptide-dependent protein kinase activity (GO:004690); cGMP-dependent protein kinase activity (GO:008239); cyclic nucleotide-dependent protein kinase activity (GO:004690); cGMP binding (GO:0030551); and cyclic nucleotide binding (GO:0030551.). Activation of the cGMP pathways result in mediation of sperm motility as a result of activation of cyclic nucleotide-gated (CNG) calcium channels that have been detected in the sperm flagellum (cGMP, Weisner et al., 1998; Cisneros-Mejorado and Herara, 2012). An influx of calcium across the CNG calcium channels is essential for sperm flagellar movement and capacitation. Hence, downregulation of cGMP pathways may lead to compromising of sperm motility and capacitation in males with teratospermia. This is an important finding because spermatozoa from felids with teratospermia have a lesser motility and capacitation *in vitro* likely due to the downregulation of cGMP pathways.

In addition to functional enrichment of the DE genes and pathways, many of the identified DE genes are concentrated in regions of the A1 and B1 chromosomes. This suggests

that these genes may be located in a Long Run of Homozygosity (ROH, contiguous lengths of homozygous genotypes) that are common in genomes of inbred animals (Robinson et al., 2016; Robinson et al.,; 2019) and may be associated with enrichment of deleterious gene variations. There is a strong correlation between decreased heterozygosity and teratospermia (increased abnormal sperm production; Fitzpatrick and Evans, 2014) but the molecular mechanisms underlying this remain poorly understood. Increased inbreeding, however, results in loss of heterozygosity which leads to deleterious gene variants that can be expressed in the sperm (Beichman et al., 2019, Kyriazis et al., 2019). A study in cattle with relatively reduced sperm quality is associated with inbreeding and genes in ROHs affecting spermatogenesis which may have contributed to reduced sperm quality (Ferenčaković et al., 2017). Similarly, in the present study there were 18 DE genes located on the A1 and B1 chromosome. A few of these are directly associated with spermatogenesis and kinase phosphorylation (spermatogenesis associated 13, *FYB1, PRKG2, ADRB3, and CXCL13*). Prior to the present study, there was not a clear understanding of how lesser heterozygosity and reduced sperm quality were associated. Considering the results from the present study, it is suggested that in future studies should explore genes in regions of ROHs to examine the effects of inbreeding on sperm structure and function.

An essential qualification to these conclusions is that findings in the present study are based on an aggregation of ten spermatozoa of the same morphotype per library to meet the minimum RNA input requirements for the QIAseq FX Single Cell RNA Library kit. Each library, therefore, represents the total gene expression profile of ten cells. Probing of normal sperm cells from males with teratospermia may provide evidence that there is a fraction of these sperm that have a gene expression profile similar to a normal sperm cell from a male with

normospermia. Additionally, cryopreservation may lead to alterations in gene expression profiles and, therefore, could have affected the results from the present study (references??). Furthermore, in future studies gene expression analyses of confirmed normal appearing spermatozoa from males with teratospermic samples which exhibit reduced motility and fertilization capacity should be explored. In the present study, we document for the first time that gene expression profiles of sperm differ by morphotype, and that those of similar phenotype but from normal and teratospermic ejaculates differ in gene expression patterns. Future work should include larger sample sizes, profiling additional sperm morphologies, and assessing gene expression of sperm in other felid species to extend our results.

5. Conclusions

In conclusion, microscope evaluations of sperm morphology to predict male infertility phenotypes do not account for gene expression abnormalities. In the present study, normal appearing sperm from domestic cats with teratospermia have compromised gene expression profiles comparable to abnormal sperm which indicates normal sperm from males with teratospermia may be functionally compromised. Secondly, with the approach in the present study, several A-kinase anchoring proteins genes were identified as candidate genes associated with teratospermia and downregulation of kinase phosphorylation pathways that may be useful in fertility diagnosis. Furthermore, we find downregulation of cGMP pathways may be associated with lesser sperm motility and may lead to alteration in the fertilization capacity of spermatozoa from males with teratospermia. An important conservation implication of our results is that felids with teratospermia may have an even smaller proportion of morphologically normal sperm. For example, the clouded leopard (*Neofelis nebulosa*) is an endangered species with 84% of the sperm

in ejaculates being abnormal (Pukazhenthi et al., 2006). If the findings from the present study are applicable to other felid species with teratospermia, the majority of the normal appearing sperm may have molecular abnormalities leading to compromised reproduction. Conceivably, a gene expression approach may lead to more accurate predictions of fertility, and more effective breeding designs, than analysis of sperm morphology alone.

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Author Contribution Statement

A Huffmeyer assisted with the sample collection, completed the lab work, bioinformatic analyses, and writing of the manuscript. B Pukazhenthi assisted with the sample collection and writing of the manuscript. R Wayne assisted in the support, data analysis and writing of the manuscript.

Competing interest's statement

The authors declare no competing interests regarding the publication of this article.

References

Ali, J. I., & Grimes, E. M. 1989. Sperm morphology: unstained and stained smears in fertile and infertile men. Archv Andry, 22, 191-195. https://doi.org/10.3109/01485018908986771

Anders, S., Pyl, P.T., Huber, W., 2015. HTSeq-A Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169. https://doi.org/10.1093/bioinformatics/btu638

Beichman, A.C., Koepfli, K.P., Li, G., Murphy, W., Dobrynin, P., Kliver, S., Tinker, M.T., Murray, M.J., Johnson, J., Lindblad-Toh, K. and Karlsson, E.K., 2019. Aquatic adaptation and depleted diversity: a deep dive into the genomes of the sea otter and giant otter. *Mol Bio Evol*. *36*, 2631-2655. https://doi.org/10.1093/molbev/msz101

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x

Bronson, R, Mikhailik, A., Schwedes, A., DGnatenko, D., HaTchwell, E., 2017. Detection of candidate nectin gene mutations in infertile men with severe teratospermia. J. Ass. Rep. 34, 1295-1302.

Carrera, A., Moos, J., Ning, X.P., Gerton, G.L., Tesarik, J., Kopf, G.S., Moss, S.B., 1996. Regulation of protein tyrosine phosphorylation in human sperm by a calcium/calmodulin-dependent mechanism: Identification of A Kinase Anchor Proteins as major substrates for tyrosine phosphorylation. Dev. Biol. 180, 284–296. https://doi.org/10.1006/dbio.1996.0301

Cisneros-Mejorado, A., Sánchez Herrera, D.P., 2012. CGMP and cyclic nucleotide-gated channels participate in mouse sperm capacitation. FEBS Lett. 586, 149–153. https://doi.org/10.1016/j.febslet.2011.12.006

Comizzoli, P., Wildt, D.E., Pukazhenthi, B.S., 2006. Poor centrosomal function of cat testicular spermatozoa impairs embryo development in vitro after intracytoplasmic sperm injection. Biol. Reprod. 75, 252–260. https://doi.org/10.1095/biolreprod.106.051342

Crosier, A.E., Pukazhenthi, B.S., Henghali, J.N., Howard, J., Dickman, A.J., Marker, L., Wildt, D.E., 2006. Cryopreservation of spermatozoa from wild-born Namibian cheetahs (Acinonyx jubatus) and influence of glycerol on cryosurvival. Cryobiology 52, 169–181. https://doi.org/10.1016/j.cryobiol.2005.10.011

Cunningham, F., Achuthan, P., Akanni, W., Allen, J., Amode, M. R., Armean, I. M., Cummins, C., 2019. Ensembl 2019. *Nuc Ac Res*. *47*, 745-751. https://doi.org/10.1093/nar/gky1113

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., Gingeras, T.R., 2013. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635

Dobrynin, P., Liu, S., Tamazian, G., Xiong, Z., Yurchenko, A.A., Krasheninnikova, K., Kliver, S., Schmidt-Küntzel, A., Koepfli, K.P., Johnson, W., Kuderna, L.F.K., García-Pérez, R., Manuel, M. de, Godinez, R., Komissarov, A., Makunin, A., Brukhin, V., Qiu, W., Zhou, L., Li, F., Yi, J., Driscoll, C., Antunes, A., Oleksyk, T.K., Eizirik, E., Perelman, P., Roelke, M., Wildt, D., Diekhans, M., Marques-Bonet, T., Marker, L., Bhak, J., Wang, J., Zhang, G., O'Brien, S.J., 2015. Genomic legacy of the African cheetah, Acinonyx jubatus. Genome Biol. 16. https://doi.org/10.1186/s13059-015-0837-4

Ferenčaković, M., Sölkner, J., Kapš, M., science, I.C.-J. of dairy, 2017, undefined, n.d. Genome-wide mapping and estimation of inbreeding depression of semen quality traits in a cattle population. Elsevier. 6, 472104730. https://doi.org/10.3168/jds.2016-12164Get

Fitzpatrick, J.L., Evans, J.P., 2014. Postcopulatory inbreeding avoidance in guppies. J. Evol. Biol. 27, 2585–2594. https://doi.org/10.1111/jeb.12545

Fox, P.R., Maron, B.J., Basso, C., Liu, S.K., Thiene, G., 2000. Spontaneously occurring arrhythmogenic right ventricular cardiomyopathy in the domestic cat: A new animal model similar to the human disease. Circulation 102, 1863–1870. https://doi.org/10.1161/01.CIR.102.15.1863

Gutiérrez-Reinoso, M. A., & García-Herreros, M., 2016. Normozoospermic versus teratozoospermic domestic cats: differential testicular volume, sperm morphometry, and subpopulation structure during epididymal maturation. Asn J Andro. *18*, 871-878. https://doi.org/10.4103/1008-682X.187583

Hofmann, F., Bernhard, D., Lukowski, R., Weinmeister, P., 2009. CGMP regulated protein Kinases (cGK). Handb. Exp. Pharmacol. https://doi.org/10.1007/978-3-540-68964-5_8

Howard, J., Bush, M., Wildt, D.E., 1991. Teratospermia in domestic cats compromises penetration of zona□free hamster ova and cat zonae pellucidae. J. Androl. 12, 36–45. https://doi.org/10.1002/j.1939-4640.1991.tb00211.x

Ickowicz, D., Finkelstein, M., Breitbart, H., 2012. Mechanism of sperm capacitation and the acrosome reaction: Role of protein kinases. Asian J. Androl. 14, 816-821. https://doi.org/10.1038/aja.2012.81

Keller, L.F., Waller, D.M., 2002. Inbreeding effects in wild populations. Trends Ecol. Evol. 17, 230-241. https://doi.org/10.1016/S0169-5347(02)02489-8

Kolde, R., MR Kolde, 2015. Package "pheatmap." R Packag. 1.

Kyriazis, C.C., Wayne, R.K. and Lohmueller, K.E., 2020. Strongly deleterious mutations are a primary determinant of extinction risk due to inbreeding depression. *Evol Let (pre-print).* https://doi.org/10.1101/678524

Lacana, E., Maceyka, M., Milstien, S., Spiegel, S., 2002. Cloning and characterization of a protein kinase A anchoring protein (AKAP)-related protein that interacts with and regulates sphingosine kinase 1 activity. J. Biol. Chem. 277, 32947–32953. https://doi.org/10.1074/jbc.M202841200

Leyton, L., LeGuen, P., Bunch, D., Saling, P.M., 1992. Regulation of mouse gamete interaction by a sperm tyrosine kinase. Proc. Natl. Acad. Sci. U. S. A. 89, 11692–11695. https://doi.org/10.1073/pnas.89.24.11692

Long, J.A., Wildt, D.E., Wolfe, B.A., Critser, J.K., DeRossi, R. V., Howard, J., 1996. Sperm capacitation and the acrosome reaction are compromised in teratospermic domestic cats. Biol. Reprod. 54, 638–646. https://doi.org/10.1095/biolreprod54.3.638

Love, M., Anders, S., Huber, W., 2017. Analyzing RNA-seq data with DESeq2. Bioconductor 2, 1–63.

Mi, H., Muruganujan, A., Huang, X., Ebert, D., Mills, C., Guo, X., Thomas, P.D., 2019. Protocol update for large-scale genome and gene function analysis with the PANTHER classification system (v.14.0). Nat. Protoc. 14, 703–721. https://doi.org/10.1038/s41596-019-0128-8

Montjean, D., De La Grange, P., Gentien, D., Rapinat, A., Belloc, S., Cohen-Bacrie, P., Menezo, Y., Benkhalifa, M., 2012. Sperm transcriptome profiling in oligozoospermia. J. Assist. Reprod. Genet. 29, 3–10. https://doi.org/10.1007/s10815-011-9644-3

O'Brien, S. J., Menotti-Raymond, M., Murphy, W. J., Yuhki, N., 2002. The feline genome project. *Anl Rev Gen*. *36*, 657-686. https://doi.org/10.1146/annurev.genet.36.060602.145553

Penfold, L., Jost, L., Evenson. D., Wildt, D. 2003. Normospermic versus teratospermic domestic cat sperm chromatin integrity evaluated by flow cytometry and intracytoplasmic sperm injection. *Biol Reprod.* 69, 1730-1735. https://doi.org/10.1095/biolreprod.103.016089

Platts, A.E., Dix, D.J., Chemes, H.E., Thompson, K.E., Goodrich, R., Rockett, J.C., Rawe, V.Y., Quintana, S., Diamond, M.P., Strader, L.F., Krawetz, S.A., 2007. Success and failure in human spermatogenesis as revealed by teratozoospermic RNAs. Hum. Mol. Genet. 16, 763–773. https://doi.org/10.1093/hmg/ddm012

Pukazhenthi, B.S., Wildt, D.E., Howard, J.G., 2001. The phenomenon and significance of teratospermia in felids. J. Reprod. Fertil. Suppl., 57, 423-433.

Pukazhenthi, B.S., Long, J.A., Wildt, D.E., Ottinger, M.A., Armstrong, D.L., Howard, J., 1998. Regulation of sperm function by protein tyrosine phosphorylation in diverse wild felid species. J. Androl. 19, 675–685. https://doi.org/10.1002/j.1939-4640.1998.tb02077.x

Pukazhenthi, B.S., Neubauer, K., Jewgenow, K., Howard, J.G., Wildt, D.E., 2006. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology 66, 112–121. https://doi.org/10.1016/j.theriogenology.2006.03.020

Pukazhenthi, B.S., Wildt, D.E., Ottinger, M.A., Howard, J., 1998. Inhibition of domestic cat spermatozoa acrosome reaction and zona pellucida penetration by tyrosine kinase inhibitors. Mol. Reprod. Dev. 49, 48–57. https://doi.org/10.1002/(SICI)1098-2795(199801)49:1<48::AID-MRD6>3.0.CO;2-O

Pukazhenthi, B., Spindler, R., Wildt, D., Bush, L.M., Howard, J.G., 2002. Osmotic properties of spermatozoa from felids producing different proportions of pleiomorphisms: Influence of adding and removing cryoprotectant. Cryobiology 44, 288–300. https://doi.org/10.1016/S0011-2240(02)00035-4

Reimand, J., Arak, T., Adler, P., Kolberg, L., Reisberg, S., Peterson, H., Vilo, J., 2016. g:Profiler-a web server for functional interpretation of gene lists (2016 update). Nucleic Acids Res. 44, W83–W89. https://doi.org/10.1093/nar/gkw199

Robinson, J.A., Ortega-Del Vecchyo, D., Fan, Z., Kim, B.Y., Vonholdt, B.M., Marsden, C.D., Lohmueller, K.E., Wayne, R.K., 2016. Genomic flatlining in the endangered island fox. Curr. Biol. 26, 1183–1189. https://doi.org/10.1016/j.cub.2016.02.062

Robinson, J.A., Räikkönen, J., Vucetich, L.M., Vucetich, J.A., Peterson, R.O., Lohmueller, K.E., Wayne1, R.K., 2019. Genomic signatures of extensive inbreeding in Isle Royale wolves, a population on the threshold of extinction. Sci. Adv. 5, 757–786. https://doi.org/10.1126/sciadv.aau0757

Roelke, M.E., Martenson, J.S., O'Brien, S.J., 1993. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr. Biol. 3, 340-350. https://doi.org/10.1016/0960-9822(93)90197-V

Schraeder, P.L., Lathers, C.M., 1983. Cardiac neural discharge and epileptogenic activity in the cat: An animal model for unexplained death. Life Sci. 32, 1371–1382. https://doi.org/10.1016/0024-3205(83)90813-5

Schuh, S.M., Hille, B., Babcock, D.F., 2007. Adenosine and catecholamine agonists speed the flagellar beat of mammalian sperm by a non-receptor-mediated mechanism. Biol. Reprod. 77, 960–969. https://doi.org/10.1095/biolreprod.107.062562

Skålhegg, B.S., Huang, Y., Su, T., Idzerda, R.L., Stanley McKnight, G., Burton, K.A., 2002. Mutation of the Cα subunit of PKA leads to growth retardation and sperm dysfunction. Mol. Endocrinol. 16, 630–639. https://doi.org/10.1210/me.16.3.630

Terrell, K. A., Wildt, D. E., Anthony, N. M., Bavister, B. D., Leibo, S. P., Penfold, L. M., Crosier, A. E., 2010. Evidence for compromised metabolic function and limited glucose uptake in spermatozoa from the teratospermic domestic cat (Felis catus) and cheetah (Acinonyx jubatus). *Bio Reprod*. *83*, 833-841. https://doi.org/10.1095/biolreprod.110.085639

Tian, H., Lv, M., Li, Z., Peng, D., Tan, Y., Wang, H., Li, Q., Li, F., Liang, W., 2018. Semen-specific miRNAs: Suitable for the distinction of infertile semen in the body fluid identification? Forensic Sci. Int. Genet. 33, 161–167. https://doi.org/10.1016/j.fsigen.2017.12.010

Trevino, S.G., Nkhoma, S.C., Nair, S., Daniel, B.J., Moncada, K., Khoswe, S., Banda, R.L., Nosten, F., Cheeseman, I.H., 2017. High-resolution single-cell sequencing of malaria parasites. Genome Biol. Evol. 9, 3373–3383. https://doi.org/10.1093/gbe/evx256

Wildt, D.E., Bush, M., Howard, J.G., O'Brien, S.J., Meltzer, D., Van Dyk, A., Ebedes, H., Brand, D.J., 1983. Unique seminal quality in the South African cheetah and a comparative evaluation in the domestic cat. Biol. Reprod. 29, 1019–1025. https://doi.org/10.1095/biolreprod29.4.1019

Wildt, D.E., 1994. Endangered species spermatozoa: Diversity, research, and conservation, in: Function of Somatic Cells in the Testis. Springer New York, pp. 1–24. https://doi.org/10.1007/978-1-4612-2638-3_1

Willett, B.J., Flynn, J.N., Hosie, M.J., 1997. FIV infection of the domestic cat: An animal model for AIDS. Immunol. Today. 18, 182-189 https://doi.org/10.1016/S0167-5699(97)84665-8

Willipinski-Stapelfeldt, B., Lübberstedt, J., Stelter, S., Vogt, K., Mukhopadhyay, A.K., Müller, D., 2004. Comparative analysis between cyclic GMP and cyclic AMP signaling in human sperm. Mol. Hum. Reprod. 10, 543–552. https://doi.org/10.1093/molehr/gah065

Table 1

Description of sperm phenotypes (n = 4), donor types (n = 2), number of libraries (n = 29), and number of cats (n = 10).

Morphotype	Donor	Number of libraries	Number of cats
Normal	Normospermia	6	5
Normal	Teratospermia	9	5
Coiled tail	Teratospermia	7	4
Bent-midpiece with a cytoplasmic droplet	Teratospermia	7	3

Table 2

Description of experimental design for DE analyses (n = 5); Of the five analyses, results from three allowed for determination of DE genes used for *GO* analyses.

Differential Expression Analyses	initials
Normopsermia (normal) X Teratospermia (normal)*	N_n-T_n
Normospermia (normal) X Teratospermia (coiled tail) *	N_n-T_c
Normospermia (normal) X Teratospermia (Bent midpiece with a cytoplasmic droplet) *	N_n-T_d
Teratospermia (normal) X Teratospermia (coiled tail)	T_n-T_c
Teratospermia (normal) X Teratospermia (bent midpiece with a cytoplasmic droplet)	T_n-T_d

*Results from these comparative analyses indicated these were differentially expressed genes.

Table 3

Summary of seminal traits of samples (adapted from Pukazhenthi et al., 2000)

	Normospermia	Teratospermia
Ejaculate volume	0.2 ± 0.2	0.2 ± 0.1
Sperm concentration (X10^6/ml)	172 ± 13.2	196 ± 15.3
Sperm motility (%)	87.5 ± 3.2	83.6 ± 5.1
Morphologically normal sperm (%)	75.2 ± 3.2	36.1 ± 3.5
Head defects (%)	0.2 ± 0.1	1.6 ± 0.2
Midpiece defects (%)	11.3 ± 3.2	28.6 ± 2.4
Flagellum defects (%)	13.3 ± 2.1	33.7 ± 3.1

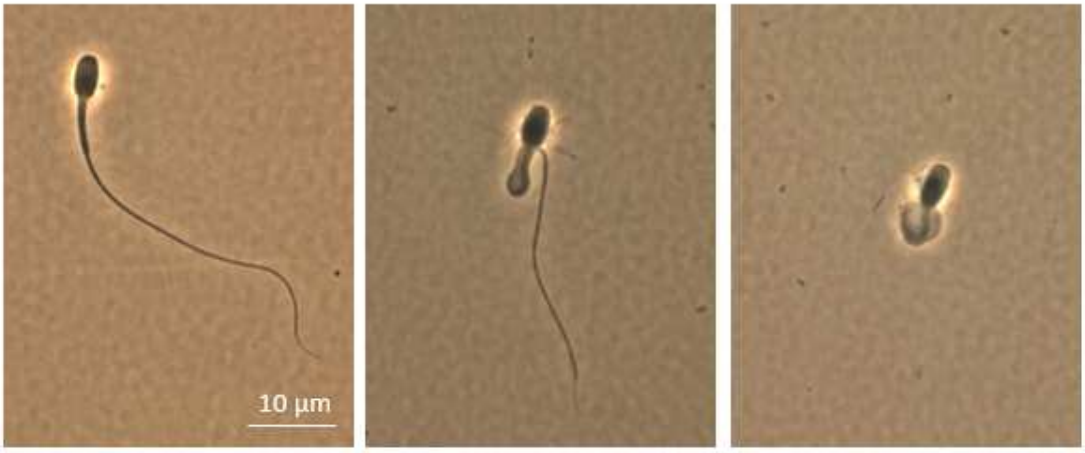

Fig. 1. Representative phase-contrast photomicrographs (1,000 x magnification) of domestic cat spermatozoa; A) normal, B) bent-midpiece with cytoplasmic droplet, and C) tightly coiled tail.

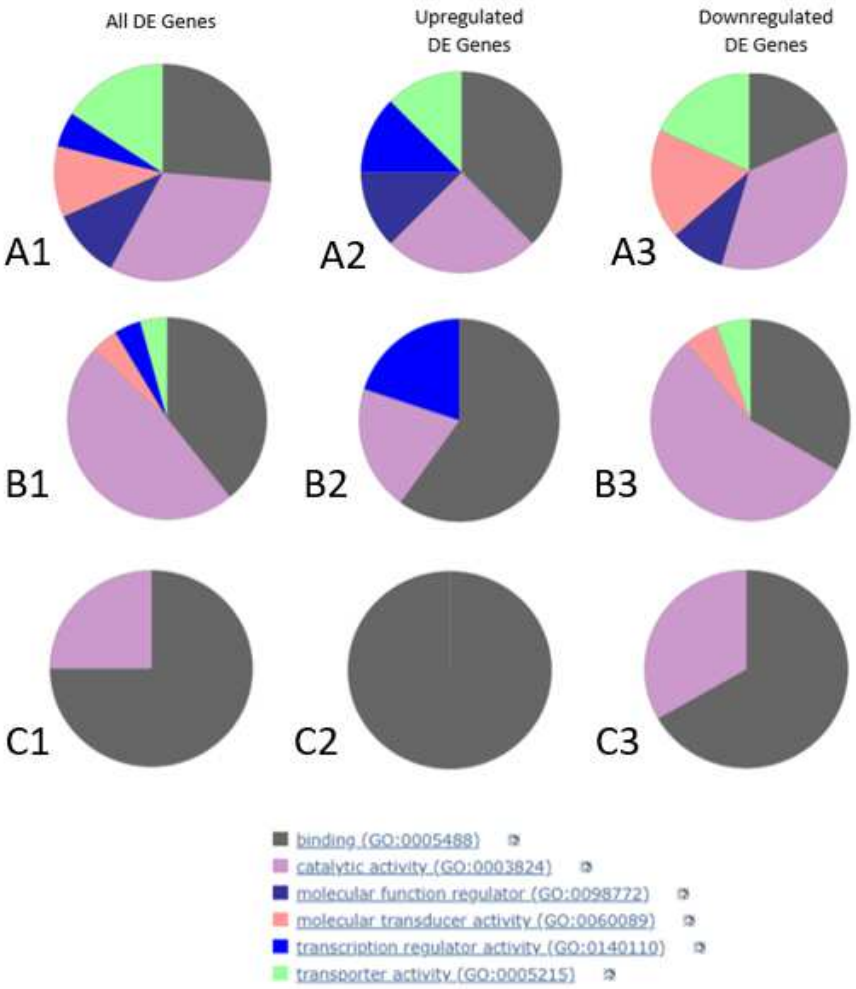

Fig. 2. Pie chart of PANTHER *GO* functional analyses: (a). N_n-T_n; (b) N_n-T_d; (c) N_n-T_c.

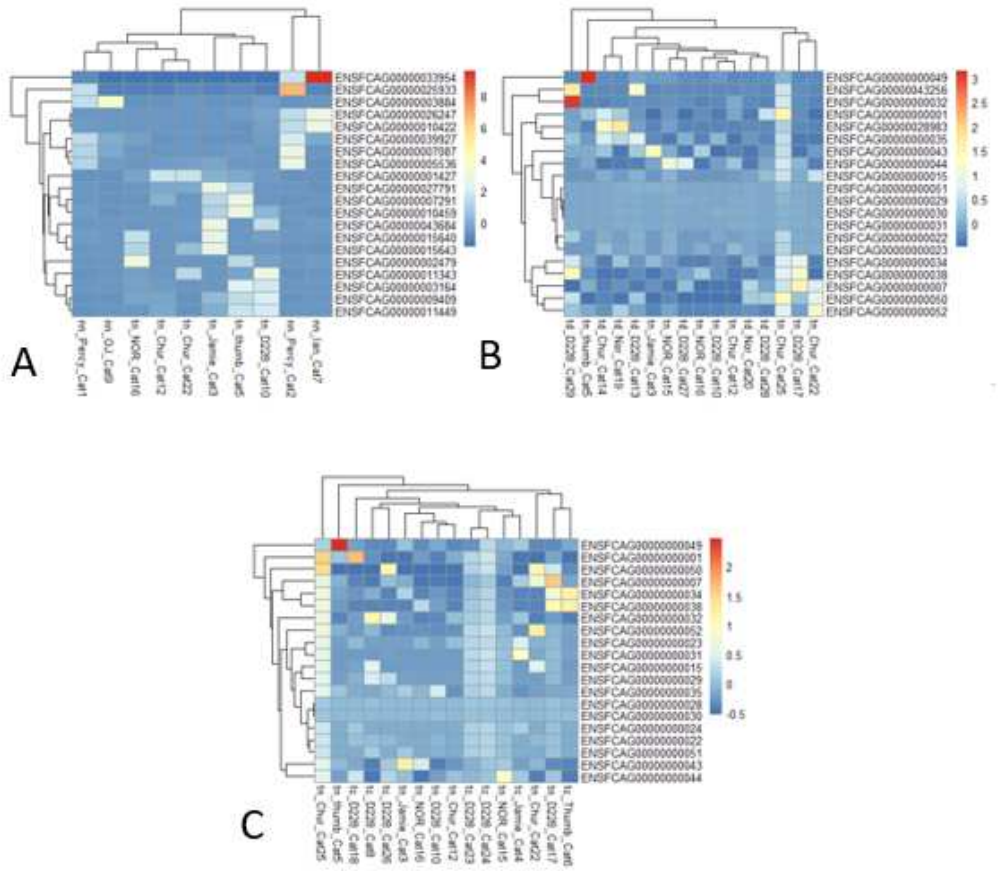

Figure 3. Heatmap of top 20 expressed genes: Top 20 transcripts expressed between (a) N_n-T_n; (b) N_n-T_d; (c) N_n-T_c; Normal spermatozoa of semen from domestic cats with teratospermia have a dissimilar heat profile compared with normal spermatozoa from domestic cats with normospermia and a more similar heatmap profile to spermatozoa with a bent midpiece and a cytoplasmic droplet and tightly coiled tail abnormalities.

Supplemental Information

Methods

Sperm Collection (continued)

Briefly, following anesthesia with Telazol® (tiletamine and zolazepam; 4.5 mg/kg; Zoetis, Parsippany, NJ, USA) a well lubricated Teflon rectal probe (1 cm diameter) with three longitudinal electrodes and an electro-stimulator (P.T. Electronics, Boring, OR, USA) was used to administer 80 stimuli (at a low voltage of 2 – 5 volts) for a 20 to 30 min interval. Each stimulation session was comprised of a slow increase in voltage until there was the desired voltage with there being maintenance of this voltage for 2 to 3 sec and then a decrease to the time of cessation of stimulations. The overall stimulation regimen was comprised of three series (each included 20-30 stimulations) with cessation for 5 min between series: Series I – 2V (ten stimulations), 3V (ten stimulations), and 4V (ten stimulations); Series II – 3V (ten stimulations), 4V (ten stimulations), and 5V (ten stimulations); Series III – 4V (ten stimulations) and 5V (ten stimulations) (Wildt et al., 1984).

Sperm cryopreservation

Ejaculates were assessed for volume, sperm total motility, sperm forward progression, and sperm concentration (Long et al., 1996, Pukazhenthi et al., 1998b). All samples were assessed for sperm morphology using a phase-contrast microscope (1,000 x magnification) to determine if the donors were classified as being normospermia or teratospermia specimens. After the sperm analyses, the samples were cryopreserved by placing the samples in liquid nitrogen vapor. For cryopreservation, ejaculates were diluted 1:1 (v/v) in Ham's F10 (Hepes modification) containing 5% fetal bovine serum, penicillin-streptomycin, pyruvate, and glutamine (Pukazhenthi et al., 1998b). Diluted samples were centrifuged (300 x *g*; 8 min), supernatant removed, and sperm pellets were re-suspended in Test Yolk Buffer (Refrigeration medium; Irvine Scientific, Santa Ana, CA). An equal volume of Test Yolk Buffer Freezing medium (containing 8% glycerol) was transferred to a

previously unused microcentrifuge tube. Both tubes were then placed in a tube float in a glass beaker containing 300 mL of water that was at room temperature. The beaker was placed in a walk-in cold room for about 3 hours, or until the samples were 5˚C. After the samples were at 5 ˚C, the TYB 8% glycerol was added step-wise at 15-minute intervals. The first and second procedures resulted in addition of one-fourth the volume of TYB 8% glycerol and the third process resulted in addition of the remaining volume of TYB 8% glycerol. At 10 minutes after the final addition of TYB 8% glycerol, the sperm samples were packaged in pre-cooled in 0.25 ml cut plastic straws, heat sealed and frozen over liquid nitrogen vapor using a manual two-stage procedure. Specifically, straws were placed 8.3 cm above liquid nitrogen for 1 minute, then 2.5 cm above liquid nitrogen for 1 minute and then plunged into the liquid nitrogen for storage (Crosier et al., 2006).

Results

Results from DESeq2 analysis between morphologically normal sperm from domestic cats with teratospermia and spermatozoa with a cytoplasmic droplet indicated one DE mRNA transcript in greater abundance with an FDR adjusted $P < 0.05$ (Table 5 and Fig 8). Results from BioMart analyses indicated the transcript as a pseudogene. Results from the DESeq2 analyses of normal appearing sperm and sperm with a coiled tail from a domestic cat with teratospermia did not indicate there were any DE genes.

Supplemental Tables and Figures

Table S1

DE transcripts and genes for N_n-T_n; There was extraction and analyses of DE genes with an FDR adjusted *P*-value < 0.05 for downstream analyses; Of the 16 transcripts only 12 mapped to protein coding genes.

Transcript IDs	baseMean	log2 Fold Change	padj	RNA type	Gene name
ENSFCAG00000033954	60.63444	-9.57632	0.03599	protein coding	CXCL13
ENSFCAG00000007087	61.76416	-9.10419	0.02824	protein coding	SLC30A8
ENSFCAG00000003884	98.90854	-9.08270	0.03960	protein coding	NAA25
ENSFCAG00000025933	2104.51414	-9.04613	0.01241	protein coding	
ENSFCAG00000010422	143.39888	-7.81029	0.02824	protein coding	ADRB3
ENSFCAG00000026247	1609.54353	-7.24836	0.01845	protein coding	FAP
ENSFCAG00000005536	180.56337	-6.62388	0.03599	protein coding	DENND5B
ENSFCAG00000015643	346.05527	7.88725	0.02170	protein coding	AXIN1
ENSFCAG00000009409	75.11920	8.00163	0.03599	protein coding	CABIN1
ENSFCAG00000011343	181.03700	8.04288	0.02824	protein coding	IGSF3
ENSFCAG00000027791	285.44088	8.29812	0.01845	protein coding	JADE1
ENSFCAG00000015640	292.53445	8.83809	0.01845	pseudogene	
ENSFCAG00000001427	76.62819	9.25884	0.01421	protein coding	
ENSFCAG00000002479	31.47621	22.15520	0.00000	protein coding	RAB3A
ENSFCAG00000043684	32.75056	22.20350	0.00000	lincRNA	
ENSFCAG00000003164	83.25036	23.51113	0.00000	protein coding	SLCO3A1

Table S2

DE Genes N_n-T_d; There was extraction and analyses of DE genes with an FDR adjusted *P*-value < 0.05 for downstream analyses.

Transcript ID	baseMean	log2 Fold Change	padj	RNA Type	Gene
ENSFCAG00000043810	69.42319734	-12.10276581	0.000624005	protein_coding	RIMKLA
ENSFCAG00000010962	42.07039541	-10.70337903	0.002311475	protein_coding	ZP4
ENSFCAG00000035314	131.2069167	-10.51842603	0.006682653	protein_coding	RAD1
ENSFCAG00000028340	198.5472585	-10.48876229	0.003786582	protein_coding	SPHKAP
ENSFCAG00000010422	144.1848243	-9.997050516	0.002311475	protein_coding	ADRB3
ENSFCAG00000010783	48.35115965	-9.405223895	0.006682653	protein_coding	NOC4L
ENSFCAG00000007814	276.2402743	-9.248040399	0.000951629	protein_coding	PRKG2
ENSFCAG00000043889	54.79034959	-8.859795794	0.032905045	protein_coding	CCDC198
ENSFCAG00000026247	1554.45707	-8.782345275	0.000951629	protein_coding	FAP
ENSFCAG00000033996	42.42321196	-8.717166509	0.014496102	protein_coding	SYT4
ENSFCAG00000025987	86.75618936	-8.514684014	0.024937292	protein_coding	TWISTNB
ENSFCAG00000043835	54.38487599	-8.458609761	0.036263916	lincRNA	
ENSFCAG00000022636	30.69275396	-8.342203775	0.048133139	protein_coding	APLF
ENSFCAG00000022167	31.48216905	-8.298899985	0.027024195	protein_coding	HEXA
ENSFCAG00000033149	54.47154329	-8.277938201	0.008998317	protein_coding	GTF2A1
ENSFCAG00000008209	95.09375934	-8.149241633	0.010983494	protein_coding	DSCAM
ENSFCAG00000026363	69.2187214	-8.144122473	0.01301008	protein_coding	FNIP1
ENSFCAG00000022865	103.1581687	-8.059556649	0.006682653	protein_coding	ATF7IP
ENSFCAG00000007915	167.0077517	-7.790621132	0.014877588	protein_coding	
ENSFCAG00000008084	15.89241794	-7.582942474	0.042190307	protein_coding	FYB1
ENSFCAG00000044413	31.36076059	-7.168812382	0.032905045	protein_coding	SP8
ENSFCAG00000003236	54.62574459	-7.146888516	0.026889705	protein_coding	IPO11
ENSFCAG00000023040	16.37405215	-6.952274255	0.036263916	protein_coding	AP1AR
ENSFCAG00000009957	22.25523261	-6.922677901	0.024937292	protein_coding	MOXD1
ENSFCAG00000031652	19.34439518	-6.885681534	0.033459348	protein_coding	NAF1

ENSFCAG00000042883	22.59267877	-6.854376518	0.048133139	protein_coding	C5orf51
ENSFCAG00000011732	13.09301823	-6.787348813	0.036263916	protein_coding	TMED8
ENSFCAG00000003321	25.48594815	-6.786346199	0.024937292	protein_coding	SV2C
ENSFCAG00000026268	232.4136087	-6.766761433	0.02985065	protein_coding	INPP4B
ENSFCAG00000028255	66.1567576	-6.405355968	0.041738226	protein_coding	PHLDB2
ENSFCAG00000000211	53.24583395	-6.403212834	0.036263916	protein_coding	USP25
ENSFCAG00000008554	25.87440635	-6.397981896	0.032905045	protein_coding	HGF
ENSFCAG00000014472	49.11467449	-6.12317851	0.038680179	protein_coding	USP53
ENSFCAG00000031885	104.1644114	-6.079222195	0.02985065	protein_coding	ARMC4
ENSFCAG00000027890	20.02597328	-5.933556843	0.042190307	protein_coding	PRKACB
ENSFCAG00000000924	75.62889525	6.20161221	0.018236201	protein_coding	CIT
ENSFCAG00000014850	36.07890222	6.378582879	0.036263916	protein_coding	PIK3C2B
ENSFCAG00000015277	41.40650377	7.12105386	0.038680179	protein_coding	PHC1
ENSFCAG00000002529	187.5514908	7.572500383	0.014414383	protein_coding	MADD
ENSFCAG00000004530	53.26221318	9.000181924	0.01301008	protein_coding	PBX2
ENSFCAG00000016209	16.05626454	22.31155782	2.35E-11	protein_coding	SNRPA
ENSFCAG00000037580	26.62520616	22.42150011	2.99E-12	snRNA	RF00026
ENSFCAG00000043256	23.50656565	23.53104544	1.69E-12	pseudogene	
ENSFCAG00000015142	46.51856985	24.02472109	9.69E-14	protein_coding	NINJ1
ENSFCAG00000028983	70.88602289	25.07103912	8.13E-20	protein_coding	PRR5L

Table S3

DE genes N_n x T_c (n = 5); There was extraction and analyses of genes with an FDR adjusted P-value < 0.05 for downstream analyses.

	baseMean	log2FoldChange	padj	RNA type	Gene
ENSFCAG00000005514	37.33039386	-26.02640485	1.23E-17	protein_coding	PRPF4
ENSFCAG00000026247	1257.653481	-9.64816342	0.003858643	protein_coding	FAP
ENSFCAG00000007814	231.3668965	-8.740551666	0.015836924	protein_coding	PRKG2
ENSFCAG00000008801	32.69886012	22.96736148	4.96E-12	protein_coding	MOCOS
ENSFCAG00000015453	20.5268066	23.31875917	1.11E-15	protein_coding	BICDL1

Table S4

DE genes T_n T_d (n = 1); Results from this analysis indicated there was one DE gene.

Gene ID	baseMean	log2FoldChange	padj	RNA type	Description
ENSFCAG00000043256	25.7156312	23.94626217	1.08E-12	pseudogene	transmembrane protein 158 [Source:NCBI gene;Acc:111558414

Table S5

N_n-T_n downregulated g:Profiler overrepresentation analysis.

GO.ID	Description	p.Val	FDR	Genes
GO:0000298	endopolyphosphatase activity	0.011667	0.011667	ENSFCAG00000025933
GO:0046983	protein dimerization activity	0.011667	0.011667	ENSFCAG00000033954, ENSFCAG00000007087, ENSFCAG00000010422, ENSFCAG00000026247
GO:0034432	bis(5'-adenosyl)-pentaphosphatase activity	0.011667	0.011667	ENSFCAG00000025933
GO:0034431	bis(5'-adenosyl)-hexaphosphatase activity	0.011667	0.011667	ENSFCAG00000025933
GO:0004939	beta-adrenergic receptor activity	0.011667	0.011667	ENSFCAG00000010422
GO:0031735	CCR10 chemokine receptor binding	0.011667	0.011667	ENSFCAG00000033954
GO:0031724	CXCR5 chemokine receptor binding	0.011667	0.011667	ENSFCAG00000033954
GO:0031699	beta-3 adrenergic receptor binding	0.011667	0.011667	ENSFCAG00000010422
GO:0015052	beta3-adrenergic receptor activity	0.011667	0.011667	ENSFCAG00000010422
GO:0008486	diphosphoinositol-polyphosphate diphosphatase activity	0.011667	0.011667	ENSFCAG00000025933
GO:0051380	norepinephrine binding	0.011963	0.011963	ENSFCAG00000010422
GO:0048248	CXCR3 chemokine receptor binding	0.011963	0.011963	ENSFCAG00000033954
GO:0042803	protein homodimerization activity	0.011963	0.011963	ENSFCAG00000007087, ENSFCAG00000010422, ENSFCAG00000026247
GO:0001664	G protein-coupled receptor binding	0.014721	0.014721	ENSFCAG00000033954, ENSFCAG00000010422
GO:0050072	m7G(5')pppN diphosphatase activity	0.015547	0.015547	ENSFCAG00000025933
GO:0051379	epinephrine binding	0.017001	0.017001	ENSFCAG00000010422
GO:0008239	dipeptidyl-peptidase activity	0.018283	0.018283	ENSFCAG00000026247
GO:0045236	CXCR chemokine receptor binding	0.019423	0.019423	ENSFCAG00000033954
GO:0031690	adrenergic receptor binding	0.021176	0.021176	ENSFCAG00000010422
GO:0004935	adrenergic receptor activity	0.021176	0.021176	ENSFCAG00000010422
GO:0004596	peptide alpha-N-acetyltransferase activity	0.021176	0.021176	ENSFCAG00000003884

GO:0004551	nucleotide diphosphatase activity	0.021176	0.021176	ENSFCAG00000025933
GO:1901338	catecholamine binding	0.023623	0.023623	ENSFCAG00000010422
GO:0017134	fibroblast growth factor binding	0.027922	0.027922	ENSFCAG00000033954
GO:0005385	zinc ion transmembrane transporter activity	0.027922	0.027922	ENSFCAG00000007087
GO:0005102	signaling receptor binding	0.02854	0.02854	ENSFCAG00000033954, ENSFCAG00000010422, ENSFCAG00000026247
GO:0048020	CCR chemokine receptor binding	0.029069	0.029069	ENSFCAG00000033954
GO:0072509	divalent inorganic cation transmembrane transporter activity	0.029069	0.029069	ENSFCAG00000007087
GO:0004177	aminopeptidase activity	0.041081	0.041081	ENSFCAG00000026247
GO:0008009	chemokine activity	0.041081	0.041081	ENSFCAG00000033954
GO:0042802	identical protein binding	0.041081	0.041081	ENSFCAG00000007087, ENSFCAG00000010422, ENSFCAG00000026247
GO:0046915	transition metal ion transmembrane transporter activity	0.041081	0.041081	ENSFCAG00000007087
GO:0042379	chemokine receptor binding	0.046814	0.046814	ENSFCAG00000033954
GO:1901911	adenosine 5'-(hexahydrogen pentaphosphate) catabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:0043542	endothelial cell migration	0.03474	0.03474	ENSFCAG00000033954, ENSFCAG00000026247
GO:0060242	contact inhibition	0.03474	0.03474	ENSFCAG00000026247
GO:0060244	negative regulation of cell proliferation involved in contact inhibition	0.03474	0.03474	ENSFCAG00000026247
GO:0061088	regulation of sequestering of zinc ion	0.03474	0.03474	ENSFCAG00000007087
GO:0071543	diphosphoinositol polyphosphate metabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:0097325	melanocyte proliferation	0.03474	0.03474	ENSFCAG00000026247
GO:1901906	diadenosine pentaphosphate metabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:1901909	diadenosine hexaphosphate catabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:1901908	diadenosine hexaphosphate metabolic process	0.03474	0.03474	ENSFCAG00000025933

GO:0035768	endothelial cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:1901910	adenosine 5'-(hexahydrogen pentaphosphate) metabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:1904848	negative regulation of cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:1904847	regulation of cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:2000544	regulation of endothelial cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:1902362	melanocyte apoptotic process	0.03474	0.03474	ENSFCAG00000026247
GO:1901907	diadenosine pentaphosphate catabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:0035766	cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:2001027	negative regulation of endothelial cell chemotaxis	0.03474	0.03474	ENSFCAG00000033954
GO:0010710	regulation of collagen catabolic process	0.03474	0.03474	ENSFCAG00000026247
GO:0015961	diadenosine polyphosphate catabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:0015959	diadenosine polyphosphate metabolic process	0.03474	0.03474	ENSFCAG00000025933
GO:0010716	negative regulation of extracellular matrix disassembly	0.03474	0.03474	ENSFCAG00000026247
GO:0002025	norepinephrine-epinephrine-mediated vasodilation involved in regulation of systemic arterial blood pressure	0.03474	0.03474	ENSFCAG00000010422
GO:2000545	negative regulation of endothelial cell chemotaxis to fibroblast growth factor	0.03474	0.03474	ENSFCAG00000033954
GO:0032119	sequestering of zinc ion	0.040078	0.040078	ENSFCAG00000007087
GO:0001993	regulation of systemic arterial blood pressure by norepinephrine-epinephrine	0.041511	0.041511	ENSFCAG00000010422
GO:0090132	epithelium migration	0.041511	0.041511	ENSFCAG00000033954, ENSFCAG00000026247
GO:0010631	epithelial cell migration	0.041511	0.041511	ENSFCAG00000033954, ENSFCAG00000026247
GO:0017196	N-terminal peptidyl-methionine acetylation	0.041511	0.041511	ENSFCAG00000003884
GO:0035754	B cell chemotaxis	0.041511	0.041511	ENSFCAG00000033954

GO:0002024	diet induced thermogenesis	0.042088	0.042088	ENSFCAG00000010422
GO:0090130	tissue migration	0.042088	0.042088	ENSFCAG00000033954, ENSFCAG00000026247
GO:1900119	positive regulation of execution phase of apoptosis	0.043395	0.043395	ENSFCAG00000026247
GO:0071850	mitotic cell cycle arrest	0.043395	0.043395	ENSFCAG00000026247
GO:0010715	regulation of extracellular matrix disassembly	0.043395	0.043395	ENSFCAG00000026247
GO:0018206	peptidyl-methionine modification	0.044389	0.044389	ENSFCAG00000003884
GO:0003085	negative regulation of systemic arterial blood pressure	0.044389	0.044389	ENSFCAG00000010422
GO:0010819	regulation of T cell chemotaxis	0.044389	0.044389	ENSFCAG00000033954
GO:0010820	positive regulation of T cell chemotaxis	0.044389	0.044389	ENSFCAG00000033954
GO:0040015	negative regulation of multicellular organism growth	0.044389	0.044389	ENSFCAG00000010422
GO:1903054	negative regulation of extracellular matrix organization	0.044389	0.044389	ENSFCAG00000026247
GO:0031649	heat generation	0.044389	0.044389	ENSFCAG00000010422

Table 6

N_n X T_d upregulated.

GO.ID	Description	p.Val	FDR	Genes
KEGG:00562	Inositol phosphate metabolism	0.04671914	0.04671914	ENSFCAG00000014850
KEGG:03040	Spliceosome	0.04671914	0.04671914	ENSFCAG00000016209
KEGG:04070	Phosphatidylinositol signaling system	0.04671914	0.04671914	ENSFCAG00000014850

Table 7

N_n X T_d down regulated.

GO.ID	Description	p.Val	FDR	Genes
GO:0004690	cyclic nucleotide-dependent protein kinase activity	0.009777669	0.009777669	ENSFCAG00000007814, ENSFCAG00000027890
KEGG:04923	Regulation of lipolysis in adipocytes	0.011210606	0.011210606	ENSFCAG00000010422, ENSFCAG00000007814, ENSFCAG00000027890
KEGG:04924	Renin secretion	0.011210606	0.011210606	ENSFCAG00000010422, ENSFCAG00000007814, ENSFCAG00000027890
KEGG:04970	Salivary secretion	0.011210606	0.011210606	ENSFCAG00000010422, ENSFCAG00000007814, ENSFCAG00000027890

Table 8

N_n X T_c upregulated.

GO.ID	Description	p.Val	FDR	Genes
GO:0008265	Mo-molybdopterin cofactor sulfurase activity	0.002385	0.002385	ENSFCAG00000008801
GO:0102867	molybdenum cofactor sulfurtransferase activity	0.002385	0.002385	ENSFCAG00000008801
GO:0030151	molybdenum ion binding	0.006359	0.006359	ENSFCAG00000008801
GO:0016783	sulfurtransferase activity	0.008583	0.008583	ENSFCAG00000008801
GO:0034452	dynactin binding	0.008583	0.008583	ENSFCAG00000015453
GO:0016782	transferase activity, transferring sulfur-containing groups	0.03332	0.03332	ENSFCAG00000008801
GO:0030170	pyridoxal phosphate binding	0.03332	0.03332	ENSFCAG00000008801
GO:0070279	vitamin B6 binding	0.03332	0.03332	ENSFCAG00000008801
GO:0006777	Mo-molybdopterin cofactor biosynthetic process	0.009725	0.009725	ENSFCAG00000008801
GO:0019720	Mo-molybdopterin cofactor metabolic process	0.009725	0.009725	ENSFCAG00000008801
GO:0043545	molybdopterin cofactor metabolic process	0.009725	0.009725	ENSFCAG00000008801
GO:0051189	prosthetic group metabolic process	0.009725	0.009725	ENSFCAG00000008801
GO:0055107	Golgi to secretory granule transport	0.009725	0.009725	ENSFCAG00000015453
GO:0047496	vesicle transport along microtubule	0.047188	0.047188	ENSFCAG00000015453
GO:0099518	vesicle cytoskeletal trafficking	0.047188	0.047188	ENSFCAG00000015453
KEGG:00790	Folate biosynthesis	0.003176	0.003176	ENSFCAG00000008801

Table 9

N_n X T_c downregulated.

GO.ID	Description	p.Val	FDR	Genes
GO:0030621	U4 snRNA binding	0.020737	0.020737	ENSFCAG00000005514
GO:0004690	cyclic nucleotide-dependent protein kinase activity	0.020737	0.020737	ENSFCAG00000007814
GO:0017070	U6 snRNA binding	0.020737	0.020737	ENSFCAG00000005514
GO:0004692	cGMP-dependent protein kinase activity	0.020737	0.020737	ENSFCAG00000007814
GO:0008239	dipeptidyl-peptidase activity	0.020737	0.020737	ENSFCAG00000026247
GO:0030553	cGMP binding	0.025914	0.025914	ENSFCAG00000007814
GO:0030551	cyclic nucleotide binding	0.047435	0.047435	ENSFCAG00000007814
GO:0017069	snRNA binding	0.047435	0.047435	ENSFCAG00000005514
GO:0004177	aminopeptidase activity	0.047435	0.047435	ENSFCAG00000026247
GO:0060244	negative regulation of cell proliferation involved in contact inhibition	0.015978	0.015978	ENSFCAG00000026247
GO:1902362	melanocyte apoptotic process	0.015978	0.015978	ENSFCAG00000026247
GO:0097325	melanocyte proliferation	0.015978	0.015978	ENSFCAG00000026247
GO:0010716	negative regulation of extracellular matrix disassembly	0.017974	0.017974	ENSFCAG00000026247
GO:0010710	regulation of collagen catabolic process	%9171	0.019171	ENSFCAG00000026247
GO:0060242	contact inhibition	0.019968	0.019968	ENSFCAG00000026247
GO:0010715	regulation of extracellular matrix disassembly	0.023953	0.023953	ENSFCAG00000026247
GO:0071850	mitotic cell cycle arrest	0.023953	0.023953	ENSFCAG00000026247
GO:1903054	negative regulation of extracellular matrix organization	0.023953	0.023953	ENSFCAG00000026247
GO:1900119	positive regulation of execution phase of apoptosis	0.023953	0.023953	ENSFCAG00000026247
GO:0030574	collagen catabolic process	0.030478	0.030478	ENSFCAG00000026247
GO:0010712	regulation of collagen metabolic process	0.035903	0.035903	ENSFCAG00000026247
GO:1900117	regulation of execution phase of apoptosis	0.035903	0.035903	ENSFCAG00000026247
GO:0022617	extracellular matrix disassembly	0.035903	0.035903	ENSFCAG00000026247
GO:0071158	positive regulation of cell cycle arrest	0.04485	0.04485	ENSFCAG00000026247
GO:1903053	regulation of extracellular matrix organization	0.04485	0.04485	ENSFCAG00000026247
GO:0071001	U4/U6 snRNP	0.018838	0.018838	ENSFCAG00000005514

GO:0071438	invadopodium membrane	0.018838	0.018838	ENSFCAG00000026247
GO:0071437	invadopodium	0.025113	0.025113	ENSFCAG00000026247
GO:0071005	U2-type precatalytic spliceosome	0.027603	0.027603	ENSFCAG00000005514
GO:0097526	spliceosomal tri-snRNP complex	0.027603	0.027603	ENSFCAG00000005514
GO:0046540	U4/U6 x U5 tri-snRNP complex	0.027603	0.027603	ENSFCAG00000005514
GO:0045178	basal part of cell	0.027603	0.027603	ENSFCAG00000026247
GO:0015030	Cajal body	0.027603	0.027603	ENSFCAG00000005514
GO:0071011	precatalytic spliceosome	0.027603	0.027603	ENSFCAG00000005514
GO:0097525	spliceosomal snRNP complex	0.040732	0.040732	ENSFCAG00000005514
GO:0030532	small nuclear ribonucleoprotein complex	0.040732	0.040732	ENSFCAG00000005514
GO:0005684	U2-type spliceosomal complex	0.040732	0.040732	ENSFCAG00000005514
GO:0120114	Sm-like protein family complex	0.043368	0.043368	ENSFCAG00000005514
KEGG:04730	Long-term depression	0.047287	0.047287	ENSFCAG00000007814
KEGG:04923	Regulation of lipolysis in adipocytes	0.047287	0.047287	ENSFCAG00000007814
KEGG:04713	Circadian entrainment	0.047287	0.047287	ENSFCAG00000007814
KEGG:04540	Gap junction	0.047287	0.047287	ENSFCAG00000007814
KEGG:04970	Salivary secretion	0.047287	0.047287	ENSFCAG00000007814
KEGG:04924	Renin secretion	0.047287	0.047287	ENSFCAG00000007814
KEGG:04611	Platelet activation	0.048179	0.048179	ENSFCAG00000007814
KEGG:03040	Spliceosome	0.048179	0.048179	ENSFCAG00000005514

Table 10

Location of differentially expressed genes on the domestic cat genome.

Gene description	Chr	Gene start (bp)	Gene end (bp)
spermatogenesis associated 13 [Source:NCBI gene;Acc:101095787]	A1	4515576	4600154
folliculin interacting protein 1 [Source:NCBI gene;Acc:101093329]	A1	110830760	110977674
importin 11 [Source:NCBI gene;Acc:105260725]	A1	132248091	132451250
synaptic vesicle glycoprotein 2C [Source:NCBI gene;Acc:101100360]	A1	142732512	142898300
chromosome A1 C5orf51 homolog [Source:NCBI gene;Acc:101094157]	A1	208037685	208062236
FYN binding protein 1 [Source:NCBI gene;Acc:101095954]	A1	210219342	210377725
RAD1 checkpoint DNA exonuclease [Source:NCBI gene;Acc:101080512]	A1	214063739	214071177
transmembrane protein 158 [Source:NCBI gene;Acc:111558414]	A2	15034661	15035573
hepatocyte growth factor [Source:NCBI gene;Acc:493705]	A2	87720026	87800181
TWIST neighbor [Source:NCBI gene;Acc:101086962]	A2	113916104	113947421
Sp8 transcription factor [Source:NCBI gene;Acc:111559511]	A2	114895658	114901237
aprataxin and PNKP like factor [Source:NCBI gene;Acc:101092931]	A3	88194563	88279200
adrenoceptor beta 3 [Source:NCBI gene;Acc:493930]	B1	46802626	46805557
nuclear assembly factor 1 ribonucleoprotein [Source:NCBI gene;Acc:101100126]	B1	67185521	67245748
inositol polyphosphate-4-phosphatase type II B [Source:NCBI gene;Acc:101089005]	B1	86207486	86611338
ubiquitin specific peptidase 53 [Source:NCBI gene;Acc:101095738]	B1	106860449	106928068
adaptor related protein complex 1 associated regulatory protein [Source:NCBI gene;Acc:101085120]	B1	113342934	113385266
protein kinase cGMP-dependent 2 [Source:NCBI gene;Acc:101091618]	B1	141312197	141417926
adrenoceptor beta 3 [Source:NCBI gene;Acc:493930]	B1	46802626	46805557
jade family PHD finger 1 [Source:NCBI gene;Acc:101081978]	B1	98102028	98161579
C-X-C motif chemokine ligand 13 [Source:NCBI gene;Acc:101096888]	B1	144697473	144704765
PBX homeobox 2 [Source:NCBI gene;Acc:101097316]	B2	33084788	33090142
monooxygenase DBH like 1 [Source:NCBI gene;Acc:101100815]	B2	122225351	122318812
hexosaminidase subunit alpha [Source:NCBI gene;Acc:101091627]	B3	35588614	35615645
coiled-coil domain containing 198 [Source:NCBI gene;Acc:101095265]	B3	106793324	106821024
transmembrane p24 trafficking protein family member 8 [Source:NCBI gene;Acc:101085552]	B3	124429594	124458026
general transcription factor IIA subunit 1 [Source:NCBI gene;Acc:101089945]	B3	127911680	127956134
armadillo repeat containing 4 [Source:NCBI gene;Acc:101088848]	B4	25778809	25960501
polyhomeotic homolog 1 [Source:NCBI gene;Acc:101097991]	B4	43481601	43501964
activating transcription factor 7 interacting protein [Source:NCBI gene;Acc:101094607]	B4	50185628	50296139

Chapter 2: First reproductive patterns of inbreeding depression in Southern California male mountain lions (*Puma concolor*).

Abstract

Long-term studies of mountain lions (*Puma concolor*) in Southern California have documented persistent small population sizes and the lowest genetic variation of any mountain lion population, except for the Federally endangered mountain lion subspecies, the Florida panther (*Puma concolor coryi)*. Overwhelming molecular evidence indicates inbreeding depression in these populations, physical evidence is lacking. The primary goal of this study was to assess male mountain lions, in Southern California, for teratospermia (>60% abnormal sperm production), one of the first signs of inbreeding depression in mountain lions that is associated with decreased reproduction and population decline. December 2019 to December 2020, we surveyed male mountain lions during live captures, after mortality events, and in images collected from camera traps in Santa Monica Mountains, Santa Susanna Mountains, Santa Ana Mountains, and the Eastern Peninsula for the following known physical abnormalities associated with inbreeding depression in mountain lions: teratospermia, cryptorchidism, and distal tail kinks. To assess for teratospermia, we extracted testes from five males (P56, P76, P78, M267, and M252) postmortem to assess morphology. Epididymal sperm evaluations revealed all males were teratospermic. Across all samples, on average, 93% of observed spermatozoa were abnormal. Further we examined 43 mountain lions (males and females) for distal tail kinks and observed 4 individuals affected (P81 and three males in camera trap images). We also examined 21 male mountain lions for cryptorchidism and observed one unilaterally cryptorchid male (P81) and one male with testes that differed greatly in size likely reflecting asynchronous migration of

the testes during puberty. In conclusion, our studies identified nine individuals (out of 43 mountain lions) exhibiting physical signs of inbreeding depression. These reproductive and physical signs of inbreeding depression in Southern California mountain lions increase the urgency of conservation efforts in the region.

Key Words: Cryptorchidism; Inbreeding; Distal tail kink; Puma concolor; Teratospermia

1. Introduction

Southern California contains several geographically distinct mountain lion (*Puma concolor*) populations. The most well-studied populations are found in the Santa Ana Mountains (SAM) [1] [2}, Eastern Peninsular (ePR)], Santa Susanna Mountains (SSM), and Santa Monica Mountains (SMM) [3]. All four mountain lion populations are small and isolated by freeways and urbanization from other populations in adjacent less-developed areas [3] [4]. However, regions nonetheless sustain viable populations of mountain lions. This fragmented urban landscape provides a model for persistence of large carnivores in other metropolitan areas worldwide.

Inbreeding depression likely will threaten the persistence of mountain lions in Southern California populations given their geographic and genetic isolation due to human expansion and urbanization that causes biodiversity loss [5] [6] [7]. Moreover, genetic studies of the SMM and SAM populations have confirmed high levels of inbreeding [3] [4], approaching that of the Florida mountain lion [8]. In the Florida population, inbreeding led to physiological and reproductive problems including extensive sperm defects [9] and high levels of cryptorchidism, a congenital condition in which one or both testes fail to descend into the scrotal sac [10]. The Florida population was only restored to viability in the 1990s by a genetic rescue initiative

involving the introduction of 8 female mountain lions from Texas [8]. Prior to the genetic rescue, studies of Florida panther sperm found that ejaculates were comprised of mostly abnormal sperm (misshapen heads, kinked and coiled tails) and exhibited a phenotype termed teratospermia occurring when >60% of sperm are abnormal [11]. Abnormal sperm and non-motile sperm are compromised in their ability to fertilize ova [12]. In addition to teratospermia, a high percentage of male Florida panthers prior to genetic rescue were unilaterally or bilaterally cryptorchid [8] [12]. Failure of the testes to descend, cryptorchidism, results in reduced testosterone production, reduced sperm production, failure of reproduction, and population decline [8]. Teratospermia is found in twenty-eight species of Felidae [13]. Genetic factors, such as the loss in heterozygosity due to inbreeding are linked to teratospermia [14], with more severe conditions associated with endangered species that have encountered a genetic bottleneck and increased homozygosity [14]. The exact genetic underpinnings of teratospermia and cryptorchdism are unknown. But there is a strong correlation between low heterozygosity and a high percentage of abnormal sperm production [14]. This correlation has been found in the cheetah (*Acinonyx jubatus),* the Florida panther, the Indian lion (*Panthera leo persica*), and the Ngorongoro Crater lion (*Panthera leo)* [14]. Consequently, teratospermia, which may be common in isolated and inbred large cat populations, reduces fertility [15]. We hypothesize that mountain lions in Southern California suffer from reduced sperm quality due to inbreeding associated with population isolation. The primary goal of this project was to opportunistically sample mountain lions in the SMM and SAM, as well as adjacent populations (SSM and ePR), to assess sperm quality, and to document other signs of inbreeding depression such as cryptorchidism and distal tail kinks. Consequently, we collected testes from male mountain lions and extracted epididymal sperm for microscopic

evaluation. Further, we assessed the status of testicular descent at physical exams and postmortem, and we checked for kinks in the distal vertebrae of the tail.

2. Materials and Methods

2.1. Study area

Santa Monica Mountains National Recreation Area (SMMNRA; 34°05'N, 118°46'W) is located in the Greater Los Angeles area in Los Angeles and Ventura Counties, California. The Santa Monica Mountains (SMM), part of SMMNRA south of the 101 Freeway, is a 600 km^2 area that includes federal, state, and privately-owned lands. The SMM are bordered by the Pacific Ocean to the south, freeways, and urbanization to the north and east, and agricultural and developed areas to the west. We also studied mountain lions in areas north of the SMM in the Simi Hills and Santa Susana Mountains (SSM, Figure 1).

The Santa Ana Mountains (Figure 1, SAM, 33-34°N, 117-118°W) is a mountain range between Los Angeles and San Diego, approximately 2,070 km^2 in area, with approximately 1,533 km^2 of that area considered to be mountain lion habitat (Benson et al., 2019). The SAM includes conserved lands controlled by federal, state, county, and nonprofit entities, as well as private land. The SAM are surrounded by large urban and suburban environments as well as native habitats, with freeways to the east, specifically Interstate 15, and north, and bounded on the west by the Pacific Ocean.

The eastern Peninsular Ranges (ePR) are a much larger area of habitat than SAM and include several distinct but contiguous mountain ranges, including the Palomar, San Jacinto, Volcan, Cuyumaca, and Santa Rosa Mountains. The wildlife habitat there is owned/managed by a combination of federal, state, county, city, and nonprofit entities, as well as private individuals,

and includes several small to medium-sized cities as well as rural and exurban development. The ePR is bounded on the north by Interstate 10, to the east by the Salton Sea and desert, to the south by the U.S. – Mexico border, and to the west by Interstate 15.

2.2. Capture and Monitoring

2.2.1 National Park Service Study in SMM and SSM

Mountain lions were captured using foot cable-restraints (also known as Aldrich foot-snares), baited cage-traps, or by treeing them with trained hounds. We immobilized mountain lions with ketamine hydrochloride combined with medetomidine hydrochloride administered intramuscularly. This immobilization method does not allow for electroejaculation procedures which is why we sampled epididymal sperm from individual postmortems. Captured animals were monitored for the duration of the time they were immobilized, and we took standard morphological measurements, determined sex and weight for each animal, and estimated age based on body size and tooth wear [3]. Adults and subadults were fitted with a global positioning system (GPS) radio-collar (Vectronic Aerospace, GPS Plus & Vertex Plus model, Berlin, Germany) equipped with a VHF beacon. We programmed collars to collect locations every two hours starting in the evening (1700) and ending in the morning (0500) and collect a single location during the day (1300). We tracked survival using remotely accessed GPS telemetry data and generally responded to GPS mortality alerts within 24 hours of detection to collect the carcass for necropsy. Capture and handling procedures were permitted through a scientific collecting permit with the California Department of Fish and Wildlife (SCP #5636) and the National Park Service Institutional Animal Care and Use Committee.

2.2.2 UC Davis study for ePR and SAM

The UC Davis team utilized cage traps. The field team collected road-killed deer and stored them frozen for later use at bait sites. When mountain lions use of an area was detected by tracks or trail camera, road-killed deer were placed at bait sites and checked daily. If feeding by a mountain lion at a bait site was detected, cage traps were set and monitored continuously via radio-transmitters affixed to the trap doors. Cameras that could transmit images from the site via cell systems were also used at trap sites if possible. Traps were checked within half an hour after detection of signals indicating a cage door had closed and / or receipt of pictures indicating a cougar had entered a trap. This assured that no animal was left within a trap unattended for lengthy periods of time, minimizing the potential for self-inflicted injury within the trap.

Animals were anesthetized with either a tiletamine-zolazepam or medetomidine-ketamine combination by jab stick syringe, blow dart, or dart rifle (in the case of hound captures). Capture methods were approved by the UC Davis Institutional Animal Use and Care Committee (Permit #17233), and the California Department of Fish and Wildlife (Scientific Collecting Permit #9875). A wildlife veterinarian or trained biologists directed all cougar captures and oversaw administration and monitoring of all anesthetic drugs, conducted all physical exams and tissue sampling, and fitted GPS-collars. Each animal captured was physically examined, including assessment of the testes for presence and size, and measured, weighed, ear tagged, and tattooed with their individual study ID number in one ear (unless anesthetic considerations dictated not completing one or more of these items). Blood samples and an ear punch were taken for disease and genetic evaluation, and nasal, pharyngeal, and rectal swabs were taken to assess for disease presence. Fecal samples were taken for assessment of intestinal parasites if possible, and a whisker was taken from most animals.

We applied Very High Frequency ("VHF"; MOD500 Telonics,Mesa, AZ), and/or Global Positioning System ("GPS"; Simplex P-1D, Televilt, Lindesberg, Sweden; TGW 3580, Telonics, Mesa, AZ; GPS4400S, GPS3300S, and GlobalstarTrack S and M, Lotek, Ontario, Canada) radio collars on pumas if their body weight exceeded 22.7 kg. GPS locations were collected at varying time intervals from every 5 minutes to every 6 hours depending on specific study objectives. For some young males collars were not placed if a 6-month drop-off unit was not available for the collar. Captured mountain lions were released at the site of capture after recovery from anesthesia and monitored regularly. Mortalities were investigated and necropsies performed, when possible, based on carcass condition.

2.3. Field surveys for cryptorchidism and distal tail kinks

The UC Davis Wildlife Health Center has conducted studies of the SAM, and ePR, and the National Park Service in the SMM and SSM mountain lion populations for nearly two decades [2] [3], and the capture and post-mortem protocols (see Supplemental Materials) include skin surface palpation and measurement of the testes to identify any cryptorchid males and assessment of the tail for abnormalities including distal kinks. The SMM/SSM study also used motion-triggered remote cameras to identify mountain lions with distal tail kinks from January-December 2020. A total of 14 camera traps were installed throughout the entire SMM and one in the SSM to target uncollared mountain lions and opportunistically check for tail abnormalities.

2.4. Collection of testes

From December 2019 to-December 2020, fourteen mountain lions that were hit by cars or succumbed to wounds due to male-on-male combat were recovered. No individuals were culled as part of this research program. Of the fourteen males, we sampled five male mountain lions in

Southern California after mortality events: P56, P76, P78, M267, and M252. P56 and P78 were from the SMM; P76 was from the SSM; M267 was from the SAM; and M252 was from ePR (Fig. 1) whole carcass, or testes were preserved within 24 hours from death. The testes were excised with scalpels either in the field or by the California Animal Health and Food Safety Lab. Postmortem spermatozoa can survive in the epididymis and reproductive tract intact for a few days, and once excised or if the body is preserved (in a refrigerator) epididymal spermatozoa can survive at 2 degrees C for as long as a week [16]. The testes were transported to the Wayne laboratory in phosphate buffered saline (PBS) on ice at 4°C [16]. In the laboratory, the testicles were measured with calipers, weighed on a scale, and washed with PBS, and the vas deferens and epididymis were extracted and analyzed by the first author, Audra Huffmeyer.

2.5. Visualization of sperm

The excised vas deferens and epididymis were minced with a scalpel to release the spermatozoa in a petri dish containing a 250 μL tris-citric acid solution [17] [18]. Aliquots of the sperm solution were then used for semen analyses. The semen sample was assessed for sperm morphology with a Leica DMC 4500 microscope. Ten μL of the sperm solution were placed on a hemocytometer and the sample was observed under a light microscope (magnification X 1000). This procedure was repeated nine times per individual mountain lion to obtain more than one hundred spermatozoa per individual, when possible. Only sperm morphology was assessed. Since we extracted epididymal sperm, additional information was not possible with sperm harvested in this study. We did not analyze sperm concentration or kinematic parameters.

2.5. Analysis of variance of semen characteristics

We compared the semen analysis data from this study with Barone et al., [19] and did a one-way analysis of variance (ANOVA) to test for significant differences among mountain lion populations in the percentage of structurally normal sperm. Following the ANOVA, we used a Fisher least-significant-difference procedure to compare individual means with a Bonferroni correction to determine the alpha levels for significance [19]. The first analysis included all lion populations from our study and Barone et al., [19]: Southern California (n=5), Florida (n=16), Colorado (n=7), Texas (n=9), Latin America (n=15), and a captive population (n=30). The second analyses included all populations except the Florida population to determine if Southern California lions are unique among less inbred populations in the percentage of structurally normal sperm.

3. Results

3.1. Captures and monitoring

3.1.1 SMM and SSM

During the study period, we examined 12 individuals (6 males and 6 females) from SMM and SSM. There were 8 live capture events (4 males: P78, P79, P81, P78 and 4 females: P80, P65, P19, P77) where one individual, P78, was captured twice. There were 6 mortality events (4 males: P56, P76, P-P, P78 and 2 females: P67, P-Q) where the bodies of the animals were taken to California Animal Health & Food Safety Laboratory Lab in San Bernadino for full necropsy, including P78. We collected testes from 3 of the 4 deceased males. We examined all 6 males, alive and postmortem, for cryptorchidism, and we examined all 12 individuals for distal tail kinks.

3.1.2 SAM and ePR

During the study period, we examined 31 individuals in SAM and ePR. There were 10 live captures (5 males: M250, M251, M252, and M254 and 5 females) and 21 mortality events (10 males and 11 females). We collected testes from 2 of the 10 deceased males. We examined all males 15 males for cryptorchidism, and we examined 31 individuals for distal tail kinks.

3.2. Seminal assessments

In total we assessed epididymal sperm from 5 male mountain lions: P56, P76, P78, M267, and M252. Testes from P56 were 44 mm long and 26 mm wide (Supplemental Table 1), and one hundred and forty-eight sperm were obtained from P56 (Table 1). Testes for P76 were 40 mm long and 25 mm wide (Supplemental Table 1), and we obtained 128 epididymal spermatozoa (Table 1). For P78, the left testis was smaller than the right: the left testis measured 25 cm long and 22 cm wide; and the right testis measured 42 cm long and 23 cm wide. We extracted 143 spermatozoa from P78. Testes from M267 were 40 mm long and 26 mm wide (Supplemental Table 1), and 183 epididymal spermatozoa were obtained (Table 1). Testes from M252 were 25 mm long and 18 mm wide (Supplemental Table 1), and we obtained 25 spermatozoa (Table 1, Fig. 2).

Across the five individuals, abnormal sperm accounted for an average of 93% (range 90% - 97%; Table 1) of the sample which was dominated by primary abnormalities that result from errors during spermatogenesis (mean 76%, range 65% - 92%; Table 1). The Southern California samples were unique in showing significantly reduced levels of structurally normal sperm compared to other populations, in the ANOVA, with and without the Florida populations (ANOVA, $F = 27.376$, $d.f. = 65$, $P < 0.00001$) (Table 2; Supplemental Figs. 1 and 2, Barone et al., 1994).

3.3. Cryptorchidism and distal tail kinks

In SMM, SSM, SAM, and ePR we examined 21 males, during live captures and postmortem, for cryptorchidism. We observed one cryptorchid male (P81) during a live capture event and one male with testes that differed greatly in size and weight likely reflecting asynchronous migration of the testes during puberty after a mortality event (P78, Fig. 3, Supplemental Table 1B). Across all populations in Southern California, we examined 41 mountain lions for distal tail kinks. We observed four affected individuals: P81 during a live capture (Fig. 3) and three other individuals in camera trap photos (Fig 4).

Male P81 was captured on March 04, 2020, in the SMM and was documented to have a distal tail kink (Fig. 4) and unilateral cryptorchidism. P81 was collared and released after the live capture. Through examining remote camera photos beginning in March 2020, we have identified three other individuals with distal tail kinks, one in the SSM, and two in the SMM (Fig. 4). One photo was obtained at the same site where P81 was captured, and another was in the eastern Santa Monica Mountains across the 405, a major freeway (Fig. 1). Based on the dates and locations where they were obtained, as well as physical differences in the tails, we are confident that these are different individuals.

4. Discussion

Long-term modeling that predicted population decline and reduced levels of genetic variation suggests that the SMM and SAM mountain lion populations are threatened by extinction [5] [6]. Consequently, in April 2020, mountain lions in Southern California received temporary threatened species status (https://fgc.ca.gov/CESA#ml). Inbreeding depression may further threaten the populations, and our study provides the first physical evidence that mountain lions in

Southern California are experiencing inbreeding depression as reduced fitness because of lower survival, mating, or reproduction [20]. Here we present nine individuals exhibiting physical signs of inbreeding depression in Southern California. Specifically, a high percentage of abnormal sperm (> 90%) is associated with reduced fertility in model species [12]. As we find greater than this value in all 5 sperm samples our results suggest reductions in fertility may be widespread and have the potential to impact the population growth rate, heightening the threat of extinction.

All five male mountain lions sampled were teratospermic. Epididymal sperm morphological assessments indicate an abundance of abnormal sperm compared to other felid species and to other mountain lion populations in the USA (Table 2, Supplemental Figs. 1 and 2) [19]. Primary sperm abnormalities accounted for the majority of the observations in epididymal sperm samples. Primary phenotypes, such as macrocephaly, microcephaly, abnormal acrosome, and tightly coiled tail are generally the result of developmental dysfunction during spermatogenesis, whereas secondary abnormalities are likely due to extraction procedures [21]. Specifically, sperm with tail defects have difficulties swimming through the vaginal canal to the oocyte [12]. Sperm with head defects and missing acrosomes may not be equipped with the enzymes necessary to break through the zona pellucida and membrane of the oocyte [12].

The only known felid population to have more abnormal sperm than the mountain lions in Southern California was the Florida mountain lion prior to genetic rescue [13]. However, Pukazhenthi et al. compared fresh semen samples from several felid species collected via electroejaculation [13], and the spermatozoa observed in this study were extracted from the epididymis and vas deferens. Although we did not use sperm collected from electroejaculation, epididymal sperm is commonly used to diagnose male infertility phenotypes, including teratospermia [22]. We acknowledge the small sample size in our study, and historically,

mountain lions in North America have had poorer sperm quality than those in Latin America. This difference is likely due to the bottleneck that occurred when the species migrated from South America to North America about 10,000 years ago (Supplemental Fig. 1 & 2) [19] [23].

The Florida mountain lion population is also the only documented wild felid population in the USA to exhibit a high incidence of cryptorchidism and distal tail kinks [8] [19]. Florida mountain lions experienced a severe population decline due to habitat loss and fragmentation. We identified three mountain lions in the Santa Monica Mountains and one in the Santa Susana Mountains with a distal tail kink. These two traits may be recessive deleterious variants that are linked and co-inherited. The five mountain lions sampled for teratospermia did not exhibit cryptorchidism, but of the six total males that we examined in 2020, one was cryptorchid (P81) and one had potentially asynchronous testes migration (P78, an etiology associated with cryptorchidism). Previously, we had not identified any cryptorchid males or distal tail kinks in the SMM since 2002, and only one distal tail kink had been identified in the SAM since 2001 [13, also shown in Fig. 1]. Moreover, there was extensive use of remote cameras to detect mountain lions throughout the SAM and the SSM prior to 2020, without evidence of distal tail kinks. So, it may be that inbreeding depression has developed or accelerated more recently, particularly in the SMM and surrounding region. In a statewide analysis, the SAM and the SMM region (referred to as Central Coast South in [24]) both had very low genetic diversity and small effective population sizes compared to other populations around the state [24].

Inbreeding depression is measurable at the molecular level. There is a strong correlation between decreased heterozygosity and teratospermia, but the molecular mechanisms underlying this relationship remain poorly understood [14]. However, increased inbreeding results in the loss of heterozygosity which exposes deleterious variants that can be expressed in the sperm [25]

[26]. This observation suggests that the genes that give rise to teratospermia, cryptorchidism, and distal tail kinds may be co-inherited in a long run of homozygosity (ROH), something that is common in inbred genomes [27] [28] and may be rich in deleterious variation [27]. The genetic consequences of habitat fragmentation in large carnivores are well documented, and these genetic impacts likely decrease sperm quality through inbreeding. Moreover, genome-wide studies of mountain lions in the SMM region, including ones that originated north of the 101 Freeway as well as south, revealed low diversity regions in their genomes that are not shared between the two populations [7]. This finding suggests that efforts to increase population connectivity in both the SAM and SMM would allow complementation of population specific ROHs that harbor deleterious homozygous variants in genes or promoter regions [26], thereby reducing the chances of unfavorable phenotypes.

Male fertility assessments may be a useful variable to identify potential founder individuals that would serve to rescue genetically impoverished populations such as the SMM and SAM mountain lions We suggest that the use of male fertility assessments can assist with identifying source populations for genetic rescue. Secondly, we recommend analysis of spermatozoa at the gene expression level because even the normal appearing sperm from teratospermic ejaculates may have aberrant gene expression levels and difficulties fertilizing ova [27]. Lastly, genetic rescue was extremely beneficial for Florida panthers, but population viability modeling indicates that it will be needed repeatedly in the future [29]. In California, because larger and genetically more diverse populations exist nearby, maintaining effective linkages between these populations could provide a long-term solution that may negate the need for assisted genetic rescue. We propose sustaining and restoring habitat corridors between

populations of mountain lions in Southern California to increase genetic diversity and mitigate physical signs of inbreeding depression.

Sperm quality assessments in large, wild carnivore species are rare. Here, we show that mortality events in an at-risk population provide useful information on epididymal sperm morphology. Reduced sperm quality, the reduction of fertility, specifically normal sperm morphology, in an already threatened population of mountain lions, along with the first appearance of cryptorchidism and the wider distribution of distal tail kinks, suggests that the mountain lions in these Southern California populations are more seriously threatened than previously thought based on genetic diversity information alone. Male mountain lion fertility in Southern California should be further assessed by methods outlined in this paper and electrostimulation or urethral catheterization of live individuals, if possible [30], to better document the geographic extent of inbreeding depression.

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References

[1] Beier P, Choate D, Barrett RH. American Society of Mammalogists Movement Patterns of Mountain Lions during Different Behaviors Published by: American Society of Mammalogists L: https://www.jstor.org/stable/1382599.

[2] Vickers TW, Sanchez JN, Johnson CK, Morrison SA, Botta R, Smith T, et al., Survival and Mortality of Pumas (Puma concolor) in a Fragmented, Urbanizing Landscape. PLoS One 2015;10:e0131490. https://doi.org/10.1371/journal.pone.0131490.

[3] Riley SPD, Serieys LEK, Pollinger JP, Sikich JA, Dalbeck L, Wayne RK, et al., Individual behaviors dominate the dynamics of an urban mountain lion population isolated by roads. Curr Biol 2014;24:1989–94. https://doi.org/10.1016/j.cub.2014.07.029.

[4] Ernest HB, Vickers TW, Morrison SA, Buchalski MR, Boyce WM. Fractured Genetic Connectivity Threatens a Southern California Puma (Puma concolor) Population. PLoS One 2014;9:e107985. https://doi.org/10.1371/journal.pone.0107985.

[5] Benson JF, Sikich JA, Riley SPD. Individual and Population Level Resource Selection Patterns of Mountain Lions Preying on Mule Deer along an Urban-Wildland Gradient. PLoS One 2016;11: e0158006. https://doi.org/10.1371/journal.pone.0158006.

[6] Benson JF, Mahoney PJ, Vickers TW, Sikich JA, Beier P, Riley SPD, et al., Extinction vortex dynamics of top predators isolated by urbanization. Ecol Appl 2019;29: e01868. https://doi.org/10.1002/eap.1868.

[7] Saremi NF, Supple MA, Byrne A, Cahill JA, Coutinho LL, Dalén L, et al., Puma genomes from North and South America provide insights into the genomic consequences of inbreeding. Nat Commun 2019; 10:1–10. https://doi.org/10.1038/s41467-019-12741-1.

[8] Johnson WE, Onorato DP, Roelke ME, Land ED, Cunningham M, Belden RC, et al., Genetic restoration of the Florida panther. Science (80-) 2010; 329:1641–5. https://doi.org/10.1126/science.1192891.

[9] Hedrick PW, Fredrickson R. Genetic rescue guidelines with examples from Mexican wolves and Florida panthers. Conserv Genet 2010; 11:615–26. https://doi.org/10.1007/s10592-009-9999-5.

[10] Onorato D, Belden C, Cunninghan M, Land D, McBride R, Roelke M. Long-term research on the Florida panther (Puma concolor coryi): historical findings and future obstacles to population persistence. In: Macdonald D, Loveridge A, editors. Biol. Conserv. Wild Felids, Oxford, UK: Oxford University Preoss; 2010, p. 453–69.

[11] Roelke M, Martenson J, Biology SO. The consequences of demographic reduction and genetic depletion in the endangered Florida panther. Curr Biol 1993; 3:340–50.

[12] Pukazhenthi BS, Wildt DE, Ottinger MA, Howard J. Inhibition of Domestic Cat Spermatozoa Acrosome Reaction and Zona Pellucida Penetration by Tyrosine Kinase Inhibitors 1998;49:48–57. https://doi.org/10.1002/(SICI)1098-2795(199801)49:1

[13] Pukazhenthi BS, Neubauer K, Jewgenow K, Howard JG, Wildt DE. The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology 2006; 66:112–21. https://doi.org/10.1016/j.theriogenology.2006.03.020.

[14] Fitzpatrick JL, Evans JP. Conservation biology Reduced heterozygosity impairs sperm quality in endangered mammals. Biol Lett 2009; 5:320–3. https://doi.org/10.1098/rsbl.2008.0734.

[15] Pukazhenthi BS, Wildt DE, Howard JG. The phenomenon and significance of teratospermia in felids. J Reprod Fertil Suppl 2001; 57:423–33.

[16] Maksudov GY, Shishova N V, Katkov II. In the cycle of life: Cryopreservation of post-mortem sperm as a valuable source in restoration of rare and endangered species All Russian institution for Environmenthal protection. 2009.

[17] Lima DBC, Silva TFP da, Aquino-Cortez A, Leiva-Revilla J, Silva LDM da. Vitrification of testicular tissue from prepubertal cats in cryotubes using different cryoprotectant associations. Theriogenology 2018; 110:110–5. https://doi.org/10.1016/j.theriogenology.2017.12.037.

[18] Duselis AR, Vrana PB. Harvesting sperm and artificial insemination of mice. J Vis Exp 2007:184. https://doi.org/10.3791/184.

[19] Barone MA, Roelke ME, Howard J, Brown JL, Anderson AE, Wildt DE. Reproductive Characteristics of Male Florida Panthers: Comparative Studies from Florida, Texas, Colorado, Latin America, and North American Zoos. J Mammal 1994;75:150–62. https://doi.org/10.2307/1382247.

[20] Hedrick, P. W. Garcia-Dorado A. Understanding Inbreeding Depression, Purging, and Genetic Rescue. Trends Ecol Evol 2016;31:940–952.

[21] Wildt DE, Bush M, Howard JG, O'Brien SJ, Meltzer D, Van Dyk A, et al., Unique Seminal Quality in the South African Cheetah and a Comparative Evaluation in the Domestic Cat. Biol Reprod 1983;29:1019–25. https://doi.org/10.1095/biolreprod29.4.1019.

[22] Gutiérrez-Reinoso M, García-Herreros M. Normozoospermic versus teratozoospermic domestic cats: Differential testicular volume, sperm morphometry, and subpopulation structure during epididymal maturation. Asian J Androl 2016;18:871–8. https://doi.org/10.4103/1008-682X.187583.

[23] O'Brien Stephen J., Johnson Warren. The Evolution of CATS. Sci Am 2007:68–75.

[24] Gustafson KD, Gagne RB, Vickers TW, Riley SPD, Wilmers CC, Bleich VC, et al., Genetic source–sink dynamics among naturally structured and anthropogenically fragmented puma populations. Conserv Genet 2019;20:215–27. https://doi.org/10.1007/s10592-018-1125-0.

[25] Beichman AC, Koepfli KP, Li G, Murphy W, Dobrynin P, Kliver S, et al., Aquatic Adaptation and Depleted Diversity: A Deep Dive into the Genomes of the Sea Otter and Giant Otter. Mol Biol Evol 2019;36:2631–55. https://doi.org/10.1093/molbev/msz101.

[26] Kyriazis CC, Wayne RK, Lohmueller KE. Strongly deleterious mutations are a primary determinant of extinction risk due to inbreeding depression. Evol Lett 2021;5:33–47. https://doi.org/10.1002/evl3.209.

[27] Robinson JA, Ortega-Del Vecchyo D, Fan Z, Kim BY, Vonholdt BM, Marsden CD, et al., Genomic Flatlining in the Endangered Island Fox. Curr Biol 2016;26:1183–9. https://doi.org/10.1016/j.cub.2016.02.062.

[28] Huffmeyer AA, Pukazhenthi BS, Wayne RK. Differential gene expression patterns in spermatozoa from teratospermic and normospermic domestic cats. Anim Reprod Sci 2021;226:106698. https://doi.org/10.1016/j.anireprosci.2021.106698.

[29] Kerk M, Onorato DP, Hostetler JA, Bolker BM, Oli MK. Dynamics, Persistence, and Genetic Management of the Endangered Florida Panther Population. Wildl Monogr 2019;203:3–35. https://doi.org/10.1002/wmon.1041.

[30] Swanson W, Bateman H, Vansandt L. Urethral catheterization and sperm vitrification for simplified semen banking in felids. Reprod Domest Anim 2017;52:255–60. https://doi.org/10.1111/rda.12863.

Tables and Figures

Table 1. Morphological characteristics of epididymal spermatozoa from testes extracted from male mountain lions in southern California, 2020.

Morphology	P56	P76	P78	M267	M252
Normal	15	13	4	13	1
	Primary abnormalities				
Macrocephalic	1	1	2	2	0
Microcephalic	6	2	4	1	16
Bicephalic	5	0	1		0
Abnormal acrosome	8	5	6	2	1
Abnormal Midpiece	2	0	5	0	0
No Midpiece	2	0	0	0	0
Tightly coiled tail	80	75	95	131	6
Biflagellate	1	0	0	0	0
	Secondary abnormalities				
Bent midpiece w/droplet	20	26	16	24	0
Bent midpiece w/o droplet	3	0	0	0	0
Bent tail w/droplet	0	0	10	4	1
Bent tail w/o droplet	0	0	0	0	0
Proximal droplet	0	1	0	3	0
Distal droplet	5	5	0	1	0
Bent neck	0	0	0	2	0
Total primary	105	83	113	136	23
Total secondary	28	32	26	34	1
Total	148	128	143	183	25
% normal sperm	10%	10%	3%	7%	4%
% abnormal sperm	90%	90%	97%	93%	96%

Table 2. ANOVA statistical analyses of structurally normal sperm from southern California and the Barone et al. (1994) dataset.

	Population							
	S. California (*n* = 5)	Florida (*n* = 16)	Texas (*n* = 9)	Colorado (*n* = 7)	Latin America (*n* = 15)	Captive (*n* = 30)	*P* (with Florida)	P (without Florida)
Structurally normal sperm (%)	7.0 ± 3	6.5 ± 0.7	14.0 ± 3.5	16.3 ± 2.1	39.4 ± 2.9	16.5 ± 1.9	<0.00001	<0.00001

Figure 1: Location of mountain lions with distal tail kinks (kinked tails), cryptorchidism, and teratospermia in southern California. The distal tail kink (kinked tail) in the Santa Ana Mountains was first reported in Ernest et al., 2014.

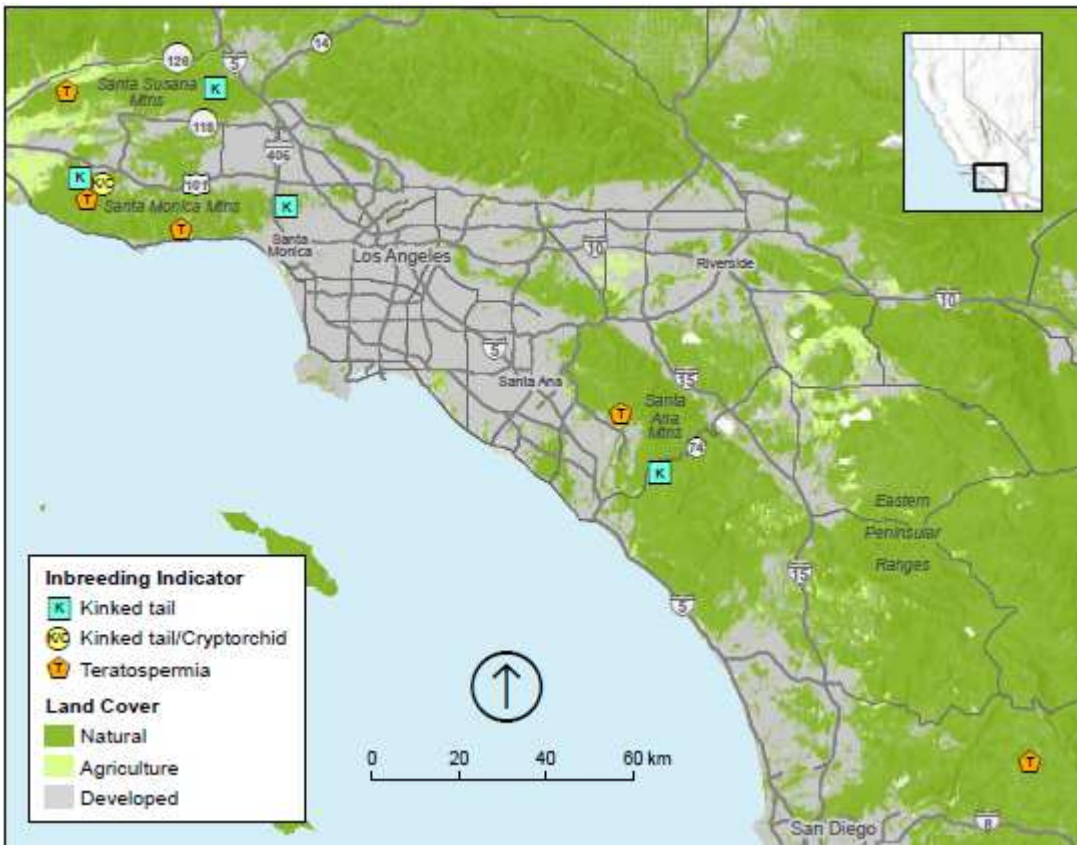


Figure 2 A-C 500X magnification. A. Image of spermatozoa with tightly coiled tail from P56 sample. B. Images of various sperm abnormalities from M267. C. Microcephalic sperm from M252.

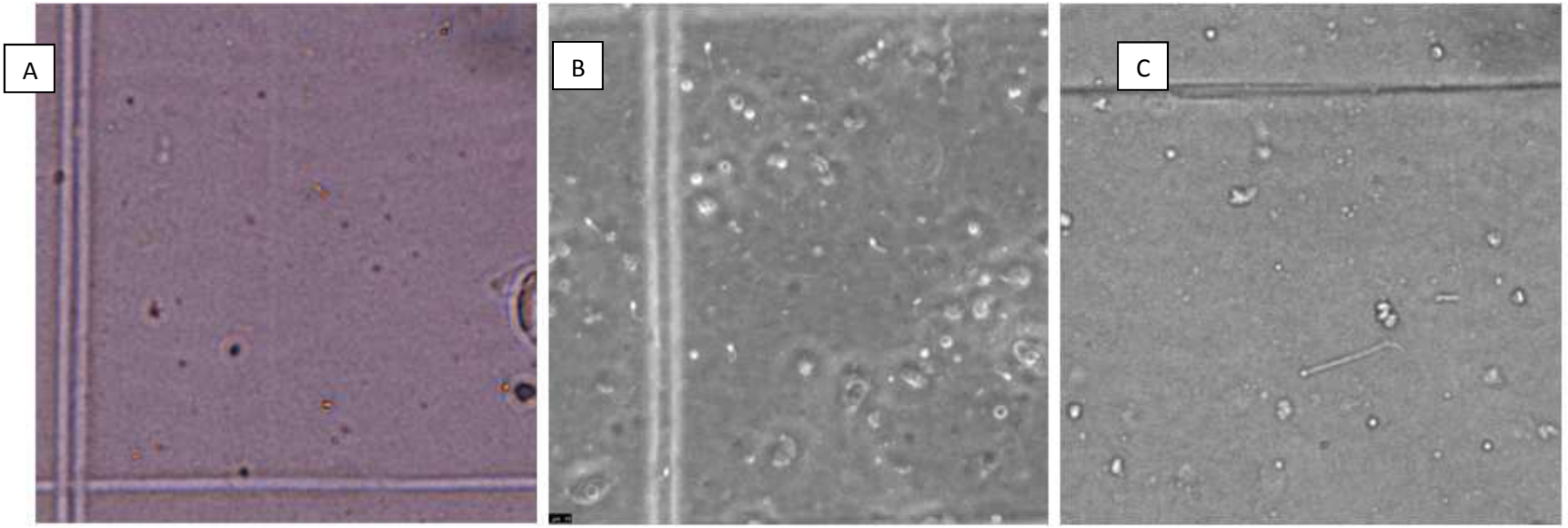


Figure 3 A-E. Image of testes from P56 (A), P76 (B), P78 (C), M267 (D), M 252 (E). Scale for A-B is noted in the image and grid size for C-E is 20mm

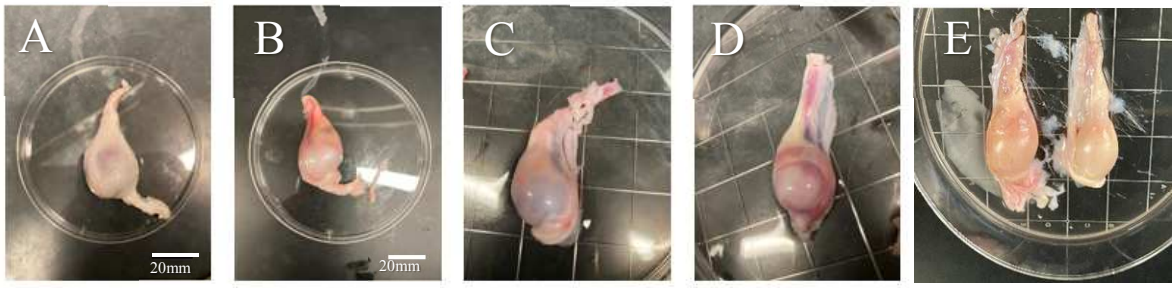
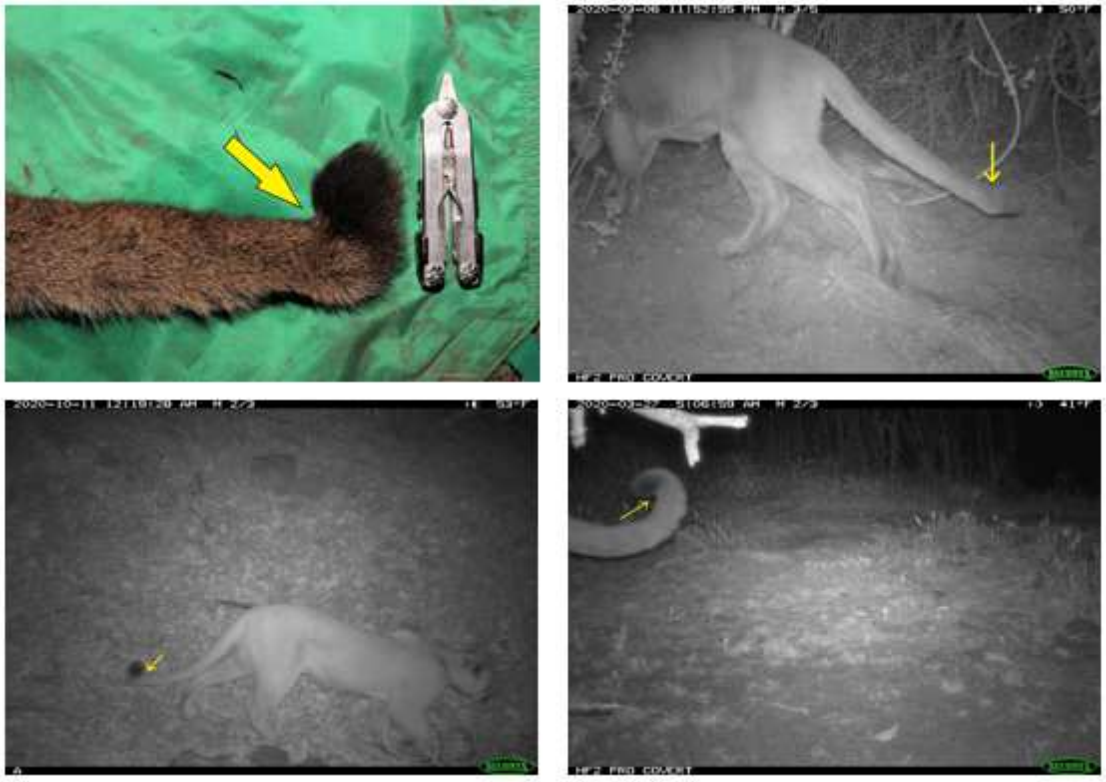


Figure 4. Images of distal tail kinks. (A). Image of P81's distal tail kink. (B). Image of distal tail kink observed on uncollared mountain lion in Western SSM. (C). Image of distal tail kink on uncollared mountain lion in SSM. (D). Image of distal tail kink observed on an uncollared mountain lion in the Eastern SSM.



Supplemental Data

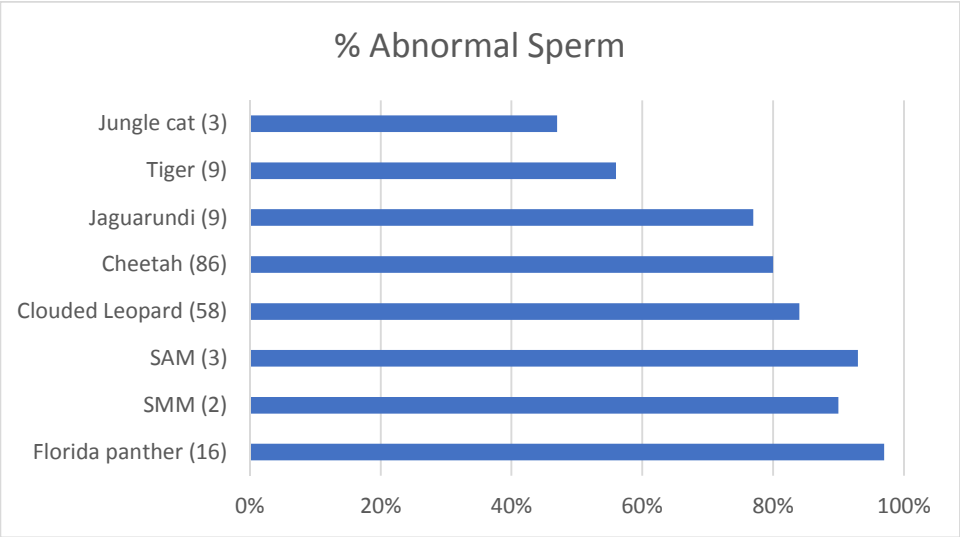
Table S1A. Location and measurements for harvested testes (both testes of similar size).

Individual	Location	Age (years)	Testes length (mm)	Testes width (mm)	Testes weight (g)
P56	SMM	5	44	26	9.7
P76	Simi	2	40	25	9.3
M267	SAM	3	40	26	9.2
M252	ePR	1.5	25	18	9

Table S1B. Location and measurements for harvested testes (testes are different sizes).

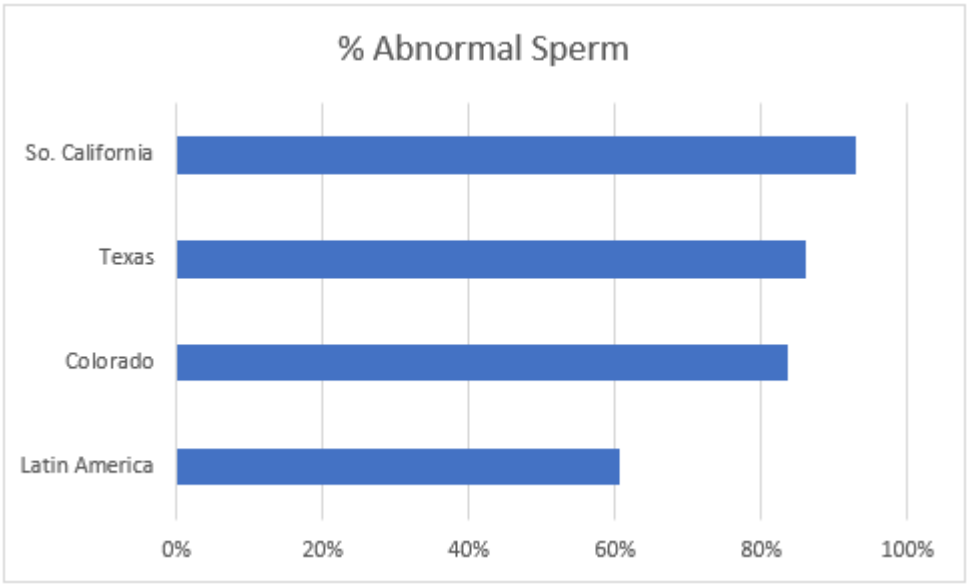
Individual	Location	Age (years)	L testes length (mm)	L testes width (mm)	L testes weight (g)	R testes length (mm)	R testes width (mm)	R testes weight (g)
P78	SMM	3	25	22	6.8	42	23	9.2

Figure S1. Percent abnormal sperm production in felids (Adapted et al. from Pukazhenthi et al., 2006, SAM= Santa Ana Mountains and eastern Peninsular Ranges, SMM= Santa Monica Mountains).



Numbers in parenthesis represent number of individuals sampled.

Figure S2. Percent abnormal sperm production in mountain lions (Adapted et al. from Barone et al., 1994). Southern California (n=5), Colorado (n=7), Texas (n=9), Latin America (n=15).



Chapter 3: Gene expression patterns in semen from wolf-coyote hybrids.

Abstract

We assessed gene expression patterns of the coyote (*Canis latrans*) and gray wolf (*C. lupus*) whose potential to hybridize may underlie the origins of the red wolf (*Canis rufus*) and Eastern wolf (*Canis lycaon*). We examined ejaculates from gray wolves and F1 and F2 hybrids between a western coyote and a western gray wolf and employed a comparative RNA-Seq approach to assess expression differences between ejaculates from gray wolf males and their wolf-coyote hybrid offspring. Our RNAseq analyses identified 9 transcripts between wolves and F1 and F2 offspring that were differentially expressed (DE). However, we found 425 DE transcripts between F1-F2 offspring suggesting the potential for reproductive incompatibility between hybrid generations. This study highlights the use of gene expression analysis to further study possible reproductive isolating mechanisms between closely related non model species.

Keywords: Canidae, hybridization, reproductive genomics, RNA-Seq.

Introduction

The genetic origins of the red wolf (*Canis rufus*) of the American Southeast and eastern wolves in the Great Lakes region are a topic of considerable debate (Mech et al., 2014). Interspecific hybridization between female coyotes (*C. latrans*) and male gray wolves (*C. lupus*) is hypothesized for the origin the red wolf and wolf-like canids in the Great Lakes Region (Roy *et al.,* 1996; Murry *et al.,* 2015). An alternate hypothesis posits hybridization between gray wolves, an eastern wolf species (*C. lycaon*) and coyotes contribute to the genetic structure of wolf-like canids in the Great Lakes region (Wilson et al., 2009). A prime disagreement among researchers is whether male wolves and female coyotes can produce healthy offspring (Mech et

al., 2014). To determine if hybridization was possible, artificial insemination (AI) in captivity of a female western coyote with western gray wolf semen was preformed (Mech *et al.,* 2014). One of the three artificial insemination events resulted in a successful pregnancy and produced six healthy, viable hybrid offspring (Mech et al., 2014). The other two artificial insemination attempts failed; one female coyote ate her pups; one female coyote resorbed a fetus and produced a dead fetus via c-section (Mech et al.)., 2014). These unsuccessful pregnancies may be evidence of reproductive compatibility that limits wild mating of western gray wolves and coyotes. Finally, the adult offspring (F1s) from the successful artificial insemination event were housed in the same pen and unintentionally produced another generation of hybrids through an incestuous mating (F2s).

Admixture between distinct species can result in hybrid dysgenesis and unfitness which may result from a variety of causes such as intergenomic conflict (Fitzpatrick 2008). Notably, admixture between distinct species can result in hybrid breakdown in the F2 generation which decreases incompatibility between genes, resulting in decreased fertility or reproductive failure of hybrids (Oka et al., 2004). However, evidence of hybrid breakdown is limited in mammal hybrids, and little is known about how hybrid breakdown impacts sperm function, a basic element of fertility in mammals (Turelli *et al.,* 2001; Maheshwari and Barbash 2011). Microscopic assessments of semen (vigor, motility, volume, and sperm count) are traditionally used to probe how interspecific hybridization affects fertility. However, hybrid breakdown and other reproductive isolating mechanisms may cause sperm dysfunction at the molecular level and only recently have molecular techniques been developed to assess misexpression in sperm via RNA-Seq of single sperm cells or semen samples (Sharma *et al.,* 2015; Hermann *et al.,* 2018; Huffmeyer et al., 2021). Additionally, reproductive isolation is difficult to study in large non

model mammals with long generation times. With sperm samples from gray wolves and their F1 and F2 hybrids, we had the opportunity to assess gene expression in wolf-coyote crosses.

Semen was collected and assessed as a part of the artificial insemination study. Semen quality of the F1s and F2s varied, some suffered from lowered sperm motility compared to the wolves (Mech *et al.,* 2017), but the lowered sperm motility was well within the normal values for domestic dogs. Lowered sperm motility can be associated with genetic, behavioral, and environmental factors (Pacheco *et al.,* 2011). A molecular study in the same captive system from blood samples suggested that epigenetic regulation of deleterious genes may mitigate the effect of mismatched parental genotypes (vonHoldt *et al.,* 2017). To access differences in gene expression patterns in the reproductive tissues, we extracted RNA from semen samples collected from 2 wolves and 4 hybrid offspring and employed RNA-Seq to assess differential expression between male wolf and their wolf-coyote hybrid offspring. We assessed the differences in gene expression of protein coding genes and non-protein coding genes using EdgeR and linear modeling. We only analyzed protein coding RNA transcripts. We hypothesize that there is a difference in genes expression patterns between wolves and their hybrids including possible dysregulation of genes important for gametic function that may contribute to the failed pregnancies and observed reduced sperm motility in the wolf-coyote hybrids (vonHoldt *et al.,* 2016; Brekke 2016).

Methods

Sample Collection

Samples were collected at the Wildlife Science Center in Stacy, MN via electroejaculation (Table 1). The semen samples were collected from the wolves and hybrids under general

anesthesia (6.25 mg/kg IM ketamine hydrochloride: Ketaset, Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO, USA, and xylazine: Rompun, Bayer Corp., Shawnee Mission, KS, USA) as described in Mech et al. (2014). The samples were collected for semen evaluations and cryopreservation. The results of the semen analyses were published in Mech et al., (2014; 2017). Samples were shipped from the St. Louis Zoo in liquid nitrogen and then stored in a -80 C freezer until initiation of the extraction procedure. We used the cryopreserved samples for the RNA-Seq analyses.

RNA Extraction and Library Preparations

Although the laboratory analyses were randomized with regard to the order of extraction and all RNA extractions and library preparations were completed in the same batch, we requested sperm samples after they were collected and thus could not randomize the original sperm collection. Nonetheless, sires and offspring sperm were collected near the same date but separated by one year (January 2014 and February 2015, respectively).

The RNA extractions from the ejaculated were completed in one batch for 8 samples: 4 male wolves, 2 F1s and 2F2s. Semen contains both sperm and other extracellular vesicles (EVs) and therefore, some RNA may not be from sperm. However, each ejaculate was washed 2-3 time with PBS and then pelleted which should remove most extra-cellular material. Total RNA was isolated from the samples with TRIZol reagent (Invitrogen) according to manufacturer's protocols. The RNA was washed using buffers and spin columns from the RNeasy mini elute kit (Qiagen). The quality and quantity of the RNA extractions were assessed with a NanoDrop 1000 (Thermo Fisher Scientific) and Bioanalyzer (Agilent Technologies). The NanoDrop 1000 indicated the samples were free from proteins and organic compound contamination and a

Bioanalyzer (Agilent Technologies) confirmed we extracted RNAs free of 18S and 28S rRNA as predicted for sperm (Ostermeier *et al.,* 2002).

We used the extracted RNA to generate cDNA libraries with the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen; Moll *et al.,* 2014) to capture ployadenylated RNAs. The library preparations were completed in one batch for the 8 samples. The quantity and quality of the library preparations were evaluated with a Bioanalyzer. The eight libraries were sequenced in one lane on a HiSeq 4000 (150 bp PE, Illumina) at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley.

Trimming, Alignment, and Reads Counts

The fastq.gz sequencing files were uploaded to Bluebee's genomics analysis platform (https://www.bluebee.com). We employed the Integrated QuantSeq 2.2.3 data analysis pipeline on the Bluebee Platform for trimming, mapping, and generating read counts. The Bluebee Platform used bbduk v35.92 (Bushnell 2017) to remove adapter sequences, poly-A tails, and low-quality reads. Given the close relationship between dogs, wolves and coyotes (Chavez et al., 2019), the trimmed reads were aligned and mapped to the well annotated dog genome (CanFam3.1) with STAR v2.5.2.a (Dobin et al., 2012) The mapped reads were indexed with samtools index v.13 (Li et al., 2009) and read counts were generated with HTSeq (Anders *et al.,* 2015). Noncoding RNAs and mitochondrial RNAs were removed before downstream analyses.

Relatedness metric

We calculated relatedness from the information and pedigree in Mech et al. (2014; 2017). The sires originated from the same den in British Columbia and were either siblings or half siblings. We did not include wolf 487, the sire of CW2 and CW4 to balance sample size across treatments and reduce sample relatedness. Specifically, wolves 480, 494, 499, and 500 are uncles

to CW2 and CW4. The F1s CW2 and CW4 are brothers, hence they share 50% of their DNA. However, CW2 sired CW1-F2 and CW2-F2 with his sister CW1, so they share 0.75% of their DNA with other individuals in the study.

Differential Expression (DE) Analyses

We performed DE analyses to identify genes differentially expressed in male wolves compared to their F1 and F2 hybrids (Table 2) using edgeR (Robinson *et al.,* 2010) and Limma (Ritchie *et al.,* 2015). Because two of the libraries were not of high quality, we downsampled the number of male wolves from four to two, removing the low-quality samples to maintain even quality and the same number of samples across the hybrid generations. Prior to normalization we used edgeR to filter low quality reads and low coverage reads (<10). Raw counts were transformed to CPM using the cpm function in edgeR. Subsequently, lowly expressed genes were removed (cpm<10 were removed). The remaining reads were normalized using the trimmed mean of M-values normalization method (TMM method, Robinson and Oshlack, 2010) in edgeR. TMM normalization is most appropriate for small samples sizes such as those in this study (Robinson and Oshlack, 2010).

We created a design matrix for each sample. The design matrix included information regarding species (gray wolf or hybrid -f1 or f2), age, sampling date, and relatedness (Table 1). In Limma, using the lmFit function, we normalized the reads and design matrix to fit the data with linear models to determine which genes are expressed at different levels between comparison of the three generations. We completed a gene ontology (GO) analysis of the top upregulated and downregulated genes using *WebGestalt* (Wang *et al.,* 2013). We set the

reference genome, the dog (CanFam3.1), as the background data set for the GO analyses and applied a Benjamini-Hochberg FDR correction (Benjamini and Hochberg 1995).

Data Visualization

DE genes were queried using BioMart (Cunningham et al., 2019) for gene designation, gene description. The PANTHER (version 14.1, Mi et al., 2019) program was used to determine the functional classification of the DE genes. The plotPCA, was used to create PCAs. The ggplot2 function in R was used to create volcano plots and the program Pheatmaps (Kolde and Kolde, 2015) to generate a heatmap of the top 3000 expressed genes. With Pheatmap, we used the normalized gene expression data to visualize clustering of the libraries as well as clustering of the top 3000 expressed genes.

Identifying mapping bias

To identify instances of mapping bias using the CanFam3.1 reference for the gray wolf, and hybrid wolf-coyote samples, we queried the DE gene sequences from CanFam3.1 using BioMart. Then using blastn, we queried the gray wolf, domestic dog, the red fox (as an outgroup, *Vulpes vulpes*), and an unpublished low-quality coyote genome in our collection. We aligned the sequences and completed a pairwise analysis and calculated percent identity to determine how similar the coding sequences were to the reference genome (the dog genome, CanFam3.1).

Results

Differential Expression Analyses

We tested for an effect of age, sampling date, and relatedness and did not observe an effect. However, there was an observed effect on generation.

Wolves vs F1 hybrids

The DE analyses identified 2 upregulated and 12 downregulated protein coding transcripts in wolves and F1 hybrids (Fig. 1, Supplemental Fig. 1). Of the 14 transcripts, 9 were associated with genes: IL21, LNP1, DPP10, ADH4, CRYM, GYG2, SMIM33, KDR, and LPCAT4 (Table 2). The *GO* analyses of the DE genes were associated with two GO IDs: binding (GO:0005488) and catalytic activity (GO: 0003824).

Wolves vs F2 hybrids

The DE analyses identified 9 downregulated transcripts. Of the 9 transcripts only two were associated with genes: LMBRIL and NCF2 (Table 3, Supplemental Fig. 2). The transcripts were associated with four GO IDs: binding (GO: 0005488, 33% of transcripts), catalytic activity (GO: 0003824, 33% of transcripts), molecular function regulator (GO: 0098772, 17% of transcripts), and molecular transducer activity (GO: 0060089, 17% of transcripts).

F1s vs F2 hybrids

The DE analyses identified 425 DE transcripts for F1s vs F2s comparisons of gene expression. Of the 425 transcripts 30 were upregulated and 395 were downregulated (Supplemental Table 1, Supplemental Fig. 3). One hundred and ninety-seven of the transcripts mapped to genes and seven GO IDs: binding (GO:0005488), catalytic activity (GO:0003824), molecular function regulator (GO:0098772), molecular transducer activity (GO:0060089), structural molecule activity (GO:0005198), transcription regulator activity (GO:0140110), transporter activity (GO:0005215). The downregulated transcripts are associated with 168 genes and are associated with the following GO terms binding, catalytic activity, molecular function regulator, molecular transducer activity, structural molecule activity, transcription regulator activity, and transporter

activity. The upregulated transcript map to 29 genes and are associated with the following GO terms: binding, catalytic activity, molecular transducer activity, transcription regulator activity, and transporter activity. Clearly, the F1s vs F2 hybrids DE analysis shows a dramatic increase in DE genes, suggesting dysregulation in the F2 generation.

Mapping bias

We found a 0.00067% and 0.00073% difference in sequences from the reference dog genome compared to the gray wolf and coyote genes, respectively, suggesting minimal bias (see Supplemental Figure 4). Similarly, we did not detect any substantial differences (> 1.00%) between the three species across the 112 (of 208) genes that were DE (Supplemental Figure S4).

Discussion

Although previous genomic analyses suggest crosses between gray wolves and western coyotes are common (vonHoldt et al., 2016), crosses between gray wolves and western coyotes were never directly studied in captivity until recently. All DE analyses (Wolf-F1, Wolf-F2, and F1-F2) identified significant DE genes and transcripts suggesting an effect of hybridization on gene expression at the gametic level. Since there is one generation of inbreeding, we tested for relatedness and did not observe an effect, however, given the small samples size, we cannot be certain that inbreeding/relatedness does not impact our results. The wolf-F1 and wolf-F2 analyses identified nine DE genes and transcripts whereas the F1-F2 analyses identified 425 transcripts that mapped to 197 known genes. This substantial increase in the number of DE transcripts suggests that a second generation of admixture further differentiates wolf-coyote hybrids. One potential reproductive barrier in an admixed population is hybrid breakdown where reproductive failures appear in the F2 and subsequent generations (Li et al. 1996). Hybrid breakdown can readily be studied in model organisms such as some plants (Li et al. 1996), mice

(Oka et al., 2003), and nematodes (Dey et al., 2008) due to their short generation times and suitability for crossing under controlled conditions. However, hybrid breakdown is difficult to study in wild large carnivores because of the logistical problems in raising sufficient numbers in captivity under controlled conditions and their long generation times. Researchers had suggested that male wolves could not successfully mate with female coyotes (Mech et al. 2011). However, their ability to mate successfully was proven by a controlled cross and distinct differences in sperm quality were observed between pure wolves, F1s, and F2s (Mech et al. 2014). The difference in sperm quality suggest there maybe underlying genetic consequences on subsequent generations of wolf and coyote crosses and the potential for hybrid breakdown.

The wolf-F1 DE analysis identified only 14 genes as differentially expressed relative to pure male wolves. One gene, kinase insert domain receptor (KRD), has a known association with sperm function and fertility. Specifically, decreased gene expression of KRD is associated with reduced fertility in male humans caused by cryptorchidism, astenozoospermia, and oligospermia (Alvarez-Palomo et al., 2019). KRD is downregulated in hybrid F1s. This finding suggests the potential for reduced reproductive fitness in F1s compared to pure gray wolves. The other DE genes were unrelated to each other or sperm quality and function. The wolf-F2 DE analyses identified 2 DE genes, NCF2 and LMBR1L which do not have known associations with sperm function or fertility. The F1-F2 DE analyses yielded 197 DE genes. However, there is no functional difference in GO terms between upregulated and downregulated genes. The sharp increase in the number of DE genes identified in F1-F2 analyses indicate there are more genetic differences or incompatibilities between F1-F2 than wolf-F1s and wolf-F2s. Consequently, our findings imply that male hybrids in later generations may not be as reproductively fertile as earlier generations of hybrids or pure wolves.

Analysis of epigenetic regulation and gene expression should be important tools to study hybrid breakdown and reproductive isolation. Consistent with vonHoldt et. al. (2016), we found only a few genes that appear to be differentially expressed in gray wolves as compared with their F1 and F2 hybrids (< 15 genes). These findings suggest that in general, epigenetic differences between wolves and their hybrids are minimal. However, our results also show much stronger expression differences between the F1 and the F2 generations. In the wild, wolf-coyote hybrids zones are geographically limited and only found in Minnesota, Ontario, and Quebec despite the co-occurrence of the two species elsewhere (Lehman *et al.,* 1991). In this region, there is biased introgression of coyote mitochondrial DNA into gray wolf populations suggesting that successful mating generally occurs between male gray wolves and female coyotes. Mate choice was suggested as an explanation for these results because male coyotes are smaller than male wolves so female wolves prefer to mate with larger male wolves (Hinton et al. 2018). However, our results indicate there may be expression differences that affect fertility in the F2 generation and this reduced fertility may conceivably involve a sex bias.

Our results highlight the importance of using gene expression analysis of sperm to explore reproductive function in hybrid zones of large mammals with long generation times. However, our conclusions are limited by a small sample size and relatedness between samples. Nonetheless, even with a small sample size, our targeted approach could identify known genes associated with reduced sperm motility in wolf-coyote hybrids and a strong effect on DE between the F1 and F2 generations. Moreover, even small samples sizes in RNA-Seq studies of sperm can be potentially informative with regard to guiding further research (Huffmeyer et al., 2021). Clearly, however, our results suggest that molecular evidence of DE across generations does not preclude hybridization in the wild. There are other mechanisms, including heterosis, and

high rates of dispersal that may maintain large hybrid zones despite some infertility (Barton and Gale 1993; Charlesworth et al. 2003). Using natural models to explore the effects of hybridization on sperm quality will expand our understanding of hybrid dysfunction and the mechanisms involved in reproductive isolation. Our results also highlight the efficacy of using a genome of a closely related species when a reference genome and GFF files are not available.

Author Contributions

CSA collected the ejaculate samples. AAH extracted the RNA, generated cDNA libraries, and completed the bioinformatic analyses. AAH and RKW completed the first drafts of the manuscript. All authors edited and contributed to revisions on the final manuscript.

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Data Accessibility Statement

By the time this article is published the raw sequencing data and gene count data will be made available on NCBI's Gene Expression Omnibus.References

References

Anders, S., P. T. Pyl, and W. Huber. (2014). HTSeq - A Python framework to work with high-throughput sequencing data. *Bioinformatics*. DOI: 10.1101/002824.

Anderson, S., M. D. Bruijn, A. Coulson, I. Eperon, F. Sanger, and I. Young. (1982). Complete sequence of bovine mitochondrial DNA conserved features of the mammalian mitochondrial genome. *Journal of Molecular Biology* 156:683–717.

Barton, N.H. and Gale, K.S. (1993). Genetic analysis of hybrid zones. *Hybrid zones and the evolutionary process*13-45.

Benjamini, Y., and Y. Hochberg. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society: Series B (Methodological)* 57:289–300.

Benson, J. F., B. R. Patterson, and T. J. Wheeldon. (2012). Spatial genetic and morphologic structure of wolves and coyotes in relation to environmental heterogeneity in a canis hybrid zone. *Molecular Ecology* 21:5934–5954.

Bourc'His, D., and O. Voinnet. (2010). A Small-RNA Perspective on Gametogenesis, Fertilization, and Early Zygotic Development. *Science* 330:617–622.

Brekke, T. D. (2016). Extreme hybrid growth, genomic imprinting, the large X effect, and the drivers of speciation in mammals. University of Montana, Missoula, Montana.

Bushnell, B. (2017). BBTools software package. *URL http://sourceforge. net/projects/bbmap*

Charlesworth, B., Charlesworth, D. and Barton, N.H. (2003). The effects of genetic and geographic structure on neutral variation. *Annual Review of Ecology, Evolution, and Systematics* 34: 99-125.

Chekulaeva, M., and W. Filipowicz. (2009). Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Current Opinion in Cell Biology* 21:452–460.

Chen, Q., M. Yan, Z. Cao, X. Li, Y. Zhang, J. Shi, G.-H. Feng, H. Peng, X. Zhang, Y. Zhang, J. Qian, E. Duan, Q. Zhai, and Q. Zhou. (2015). Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science* 351:397–400.

Conine, C. C., F. Sun, L. Song, J. A. Rivera-Pérez, and O. J. Rando. (2018). Small RNAs Gained during Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. *Developmental Cell* 46.

Darr, C. R., L. E. Moraes, R. E. Connon, C. C. Love, S. Teague, D. Varner, and S. A. Meyers. (2017). The relationship between mitochondrial DNA copy number and stallion sperm function. *Theriogenology* 94: 94-99.

Dobin, A., C. A. Davis, F. Schlesinger, J. Drenkow, C. Zaleski, S. Jha, P. Batut, M. Chaisson, and T. R. Gingeras. (2012). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–21.

Eggan, K., H. Akutsu, J. Loring, L. Jackson-Grusby, M. Klemm, W. M. Rideout, R. Yanagimachi, and R. Jaenisch. (2001). Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proceedings of the National Academy of Sciences* 98:6209–6214.

Ellison, C. K., and R. S. Burton. (2010). Cytonuclear conflict in interpopulation hybrids: the role of RNA polymerase in mtDNA transcription and replication. *Journal of Evolutionary Biology* 23: 528-538.

Esteller, M. 2011. Non-coding RNAs in human disease. Nature Reviews Genetics 12:861–874.

Fitzpatrick, B. M. (2008). Hybrid Dysfunction: Population Genetic and Quantitative Genetic Perspectives. *The American Naturalist* 171:491–498.

Gapp, K., A. Jawaid, P. Sarkies, J. Bohacek, P. Pelczar, J. Prados, L. Farinelli, E. Miska, and I. M. Mansuy. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature Neuroscience* 17:667–669.

Guo, H., Y. Gong, B. He, and R. Zhao. (2017). Relationships between mitochondrial DNA content, mitochondrial activity, and boar sperm motility. *Theriogenology* 87:276–283.

Gopalakrishnan, Shyam, Mikkel-Holger S. Sinding, Jazmín Ramos-Madrigal, Jonas Niemann, Jose A. Samaniego Castruita, Filipe G. Vieira, Christian Carøe et al., (2018). Interspecific gene flow shaped the evolution of the genus Canis. *Current Biology* 28: 3441-3449.

Haider, S., B. Ballester, D. Smedley, J. Zhang, P. Rice, and A. Kasprzyk. (2009). BioMart Central Portal—unified access to biological data. *Nucleic Acids Research* 37.

He, L., and G. J. Hannon. (2004). MicroRNAs: small RNAs with a big role in gene regulation. *Nature Reviews Genetics* 5:522–531.

Hermann, B. P., K. Cheng, A. Singh, L. R.-D. L. Cruz, K. N. Mutoji, I.-C. Chen, H. Gildersleeve, J. D. Lehle, M. Mayo, B. Westernströer, N. C. Law, M. J. Oatley, E. K. Velte, B. A. Niedenberger, D. Fritze, S. Silber, C. B. Geyer, J. M. Oatley, and J. R. Mccarrey.

(2018). The Mammalian Spermatogenesis Single-Cell Transcriptome, from Spermatogonial Stem Cells to Spermatids. *Cell Reports* 25.

Heuvel, L. V. D., and J. Smeitink. (2001). The oxidative phosphorylation (OXPHOS) system: nuclear genes and human genetic diseases. *BioEssays* 23:518–525.

Ho, H., and S. Suarez. (2001). Hyperactivation of mammalian spermatozoa: function and regulation. *Reproduction* 519–526.

Hutchison, C. A., J. E. Newbold, S. S. Potter, and M. H. Edgell. (1974). Maternal inheritance of mammalian mitochondrial DNA. *Nature* 251:536–538.

Josefsson, C., B. Dilkes, and L. Comai. (2006). Parent-Dependent Loss of Gene Silencing during Interspecies Hybridization. *Current Biology* 16:1322–1328.

Kao, S.-H., H.-T. Chao, H.-W. Liu, T.-L. Liao, and Y.-H. Wei. (2004). Sperm mitochondrial DNA depletion in men with asthenospermia. *Fertility and Sterility* 82:66–73.

Koblmüller, S., Nord, M., Wayne, R. K., & Leonard, J. A. (2009). Origin and status of the Great Lakes wolf. *Molecular ecology*, *18*: 2313-2326.

Krol, J., I. Loedige, and W. Filipowicz. (2010). The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics* 11:597–610.

Lane, N. (2011). Mitonuclear match: Optimizing fitness and fertility over generations drives ageing within generations. *BioEssays* 33:860–869.

Larsson, N.-G., J. Wang, H. Wilhelmsson, A. Oldfors, P. Rustin, M. Lewandoski, G. S. Barsh, and D. A. Clayton. (1998). Mitochondrial transcription factor A is necessary for mtDNA maintance and embryogenesis in mice. *Nature Genetics* 18:231–236.

Lehman, N., A. Eisenhawer, K. Hansen, L. D. Mech, R. O. Peterson, P. J. P. Gogan, and R. K. Wayne. (1991). Introgression of Coyote Mitochondrial DNA Into Sympatric North American Gray Wolf Populations. *Evolution* 45:104.

Li, Heng, Bob Handsaker, Alec Wysoker, Tim Fennell, Jue Ruan, Nils Homer, Gabor Marth, Goncalo Abecasis, and Richard Durbin. (2009). "The sequence alignment/map format and SAMtools." *Bioinformatics* 25:2078-2079.

Lishko, P., M. Miller, and S. Mansell. (2016). The Role of Sperm Ion Channels in Reproduction. *Ion Channels in Health and Disease* 223–238.

Love, M. I., W. Huber, and S. Anders. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology* DOI: 10.1101/002832.

Lu, J., G. Getz, E. A. Miska, E. Alvarez-Saavedra, J. Lamb, D. Peck, A. Sweet-Cordero, B. L. Ebert, R. H. Mak, A. A. Ferrando, J. R. Downing, T. Jacks, H. R. Horvitz, and T. R. Golub. (2005). MicroRNA expression profiles classify human cancers. *Nature* 435:834–838.

Maheshwari, S., and D. A. Barbash. (2011). The Genetics of Hybrid Incompatibilities. *Annual Review of Genetics* 45:331–355.

Mech, L. D., B. W. Christensen, C. S. Asa, M. Callahan, and J. K. Young. (2014). Production of Hybrids between Western Gray Wolves and Western Coyotes. *PLoS ONE* 9.

Mech, L. D., C. S. Asa, M. Callahan, B. W. Christensen, F. Smith, and J. K. Young. (2017). Studies of wolf x coyote hybridization via artificial insemination. *Plos One* 12.

Miki, Kiyoshi (2007). Energy metabolism and sperm function. *Society of Reproduction and Fertility supplement* 65: 309-325.

Moll, P., M. Ante, A. Seitz, and T. Reda. (2014). QuantSeq 3′ mRNA sequencing for RNA quantification. *Nature Methods* 11:i-iii.

Mosher, R. A., C. W. Melnyk, K. A. Kelly, R. M. Dunn, D. J. Studholme, and D. C. Baulcombe. (2009). Uniparental expression of PolIV-dependent siRNAs in developing endosperm of Arabidopsis. *Nature* 460:283–286.

Murray, D. L., G. Bastille-Rousseau, J. R. Adams, and L. P. Waits. (2015). The Challenges of Red Wolf Conservation and the Fate of an Endangered Species Recovery Program. *Conservation Letters* 8:338–344.

Obernosterer, G. (2006). Post-transcriptional regulation of microRNA expression. *RNA* 12:1161–1167.

Oconnell, M., N. Mcclure, and S. Lewis. (2002). The effects of cryopreservation on sperm morphology, motility and mitochondrial function. *Human Reproduction* 17:704–709.

Ostermeier, G. C., D. J. Dix, D. Miller, P. Khatri, and S. A. Krawetz. (2002). Spermatozoal RNA profiles of normal fertile men. *The Lancet* 360:772–777.

Pacheco, S. E., E. A. Houseman, B. C. Christensen, C. J. Marsit, K. T. Kelsey, M. Sigman, and K. Boekelheide. (2011). Integrative DNA Methylation and Gene Expression Analyses

Identify DNA Packaging and Epigenetic Regulatory Genes Associated with Low Motility Sperm. *PLoS ONE* 6.

Piomboni, P., R. Focarelli, A. Stendardi, A. Ferramosca, and V. Zara. (2011). The role of mitochondria in energy production for human sperm motility. *International Journal of Andrology* 35:109–124.

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth. 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Research 43.

Robinson, M. D., & Oshlack, A. (2010). A scaling normalization method for differential expression analysis of RNA-seq data. *Genome biology*, *11*:3.

Robinson, M. D., D. J. Mccarthy, and G. K. Smyth. (2009). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140.

Roy, M. S., Geffen, E., Smith, D., & Wayne, R. K. (1996). Molecular genetics of pre□1940 red wolves. *Conservation Biology*, *10*(5), 1413-1424.

Rutledge, L. Y., Garroway, C. J., Loveless, K. M., & Patterson, B. R. (2010). Genetic differentiation of eastern wolves in Algonquin Park despite bridging gene flow between coyotes and grey wolves. *Heredity*, *105*:520.

Ryan, M. E., J. R. Johnson, and B. M. Fitzpatrick. (2009). Invasive hybrid tiger salamander genotypes impact native amphibians. *Proceedings of the National Academy of Sciences* 106:11166–11171.

Sackton, T. B., R. A. Haney, and D. M. Rand. (2003). Cytonuclear Coadaptation In Drosophila: Disruption Of Cytochrome C Oxidase Activity In Backcross Genotypes. *Evolution* 57:2315.

Schagdarsurengin, U., and K. Steger. (2016). Epigenetics in male reproduction: effect of paternal diet on sperm quality and offspring health. *Nature Reviews Urology* 13:584–595.

Scott, R. J., M. Spielman, J. Bailey, and H. G. Dickinson. (1998). Parent-of-origin effects on seed development in Arabidopsis thaliana. *Development* 125:3329–3341.

Sharma, U., C. C. Conine, J. M. Shea, A. Boskovic, A. G. Derr, X. Y. Bing, C. Belleannee, A. Kucukural, R. W. Serra, F. Sun, L. Song, B. R. Carone, E. P. Ricci, X. Z. Li, L. Fauquier, M. J. Moore, R. Sullivan, C. C. Mello, M. Garber, and O. J. Rando. (2015). Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science* 351:391–396.

Singh, A. P., and S. Rajender. (2015). CatSper channel, sperm function and male fertility. *Reproductive BioMedicine Online* 30:28–38.

Stanford, K. I., M. Rasmussen, L. A. Baer, A. C. Lehnig, L. A. Rowland, J. D. White, K. So, A. L. D. Sousa-Coelho, M. F. Hirshman, M.-E. Patti, O. J. Rando, and L. J. Goodyear. (2018). Paternal Exercise Improves Glucose Metabolism in Adult Offspring. *Diabetes* 67:2530–2540.

Thomson, J. M. (2006). Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes & Development* 20:2202–2207.

Turelli, M., N. H. Barton, and J. A. Coyne. 2001. Theory and speciation. Trends in Ecology & Evolution 16:330–343.

Vonholdt, B. M., J. A. Cahill, Z. Fan, I. Gronau, J. Robinson, J. P. Pollinger, B. Shapiro, J. Wall, and R. K. Wayne. (2016). Whole-genome sequence analysis shows that two endemic species of North American wolf are admixtures of the coyote and gray wolf. *Science Advances* 2.

Vonholdt, B., E. Heppenheimer, V. Petrenko, P. Croonquist, and L. Y. Rutledge. (2017). Ancestry-Specific Methylation Patterns in Admixed Offspring from an Experimental Coyote and Gray Wolf Cross. *Journal of Heredity* 108:341–348.

Wang, J., D. Duncan, Z. Shi, and B. Zhang. (2013). WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Research* 41.

Watkins, A. J., I. Dias, H. Tsuro, D. Allen, R. D. Emes, J. Moreton, R. Wilson, R. J. M. Ingram, and K. D. Sinclair. (2018). Paternal diet programs offspring health through sperm- and seminal plasma-specific pathways in mice. *Proceedings of the National Academy of Sciences* 115:10064–10069.

Xie, L., X. Zhu, Y. Hu, T. Li, Y. Gao, Y. Shi, and S. Tang. (2008). Mitochondrial DNA Oxidative Damage Triggering Mitochondrial Dysfunction and Apoptosis in High Glucose-Induced HRECs. *Investigative Opthalmology & Visual Science* 49:4203.

Table 1. Sampling information.

Sample Name	Name	Species	Age (months)*	Relatedness**
494	Tristan	Wolf	22	0.25-0.50
480	Napolean	Wolf	22	0.25-0.50
CW1-F2	Xavier	Hybrid-F2	22	0.75
CW2-F2	Logan	Hybrid-F2	22	0.75
CW2	Brian	Hybrid-F1	22	0.75
CW4	Kepplar	Hybrid-1 F1	22	0.50

*Age refers to age at sampling. The wolves and hybrid F1s were sampled February 28, 2015. The hybrid F2s were sampled February 16, 2016.

**The relatedness metric refers to the percentage each individual is related to other individuals in the study. See the Supplemental Materials for how this value was calculated.

Table 2. Differentially expressed genes between Wolves X F1s

Ensembl ID	Gene Name	BaseMean	log2FoldChange
ENSCAFG00000040225	-	126.98	-23.99
ENSCAFG00000004004	IL21	121.19	-23.93
ENSCAFG00000029353	LNP1	120.30	-23.92
ENSCAFG00000004970	DPP10	110.50	-23.81
ENSCAFG00000026370	-	100.25	-23.67
ENSCAFG00000031667	ADH4	95.79	-23.61
ENSCAFG00000017807	CRYM	94.015	-23.59
ENSCAFG00000011113	GYG2	90.01	-23.53
ENSCAFG00000031017	SMIM33	83.77	-23.43
ENSCAFG00000039569	-	83.77	-23.43
ENSCAFG00000035207	-	81.54	-23.39
ENSCAFG00000002079	KDR	78.87	-23.35
ENSCAFG00000020907	-	82.78	23.17
ENSCAFG00000007814	LPCAT4	100.12	23.43

Table 3 Differentially expressed genes between wolves x F2s

Ensembl ID	Gene Name	baseMean	log2FoldChange
ENSCAFG00000008679	LMBR1L	127.89	-25.203
ENSCAFG00000036473	-	109.05	-24.98
ENSCAFG00000038487	-	101.90	-24.89
ENSCAFG00000038719	-	102.92	-24.90
ENSCAFG00000013314	NCF2	78.50	-24.54
ENSCAFG00000023416	-	83.32	-24.62
ENSCAFG00000032345	-	86.99	-24.67
ENSCAFG00000024629	-	78.42	-24.54
ENSCAFG00000036532	-	75.97	-24.49

Figure 1. Heat map of top 25 expressed DE genes between wolves and F2s.

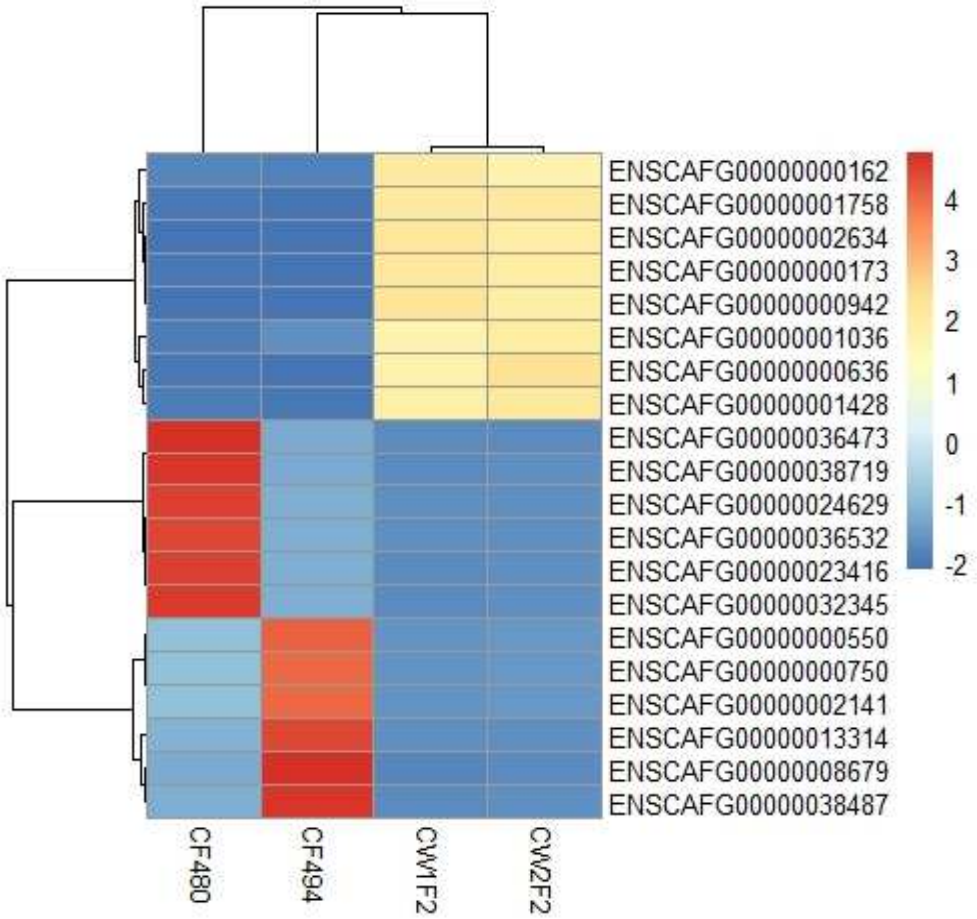
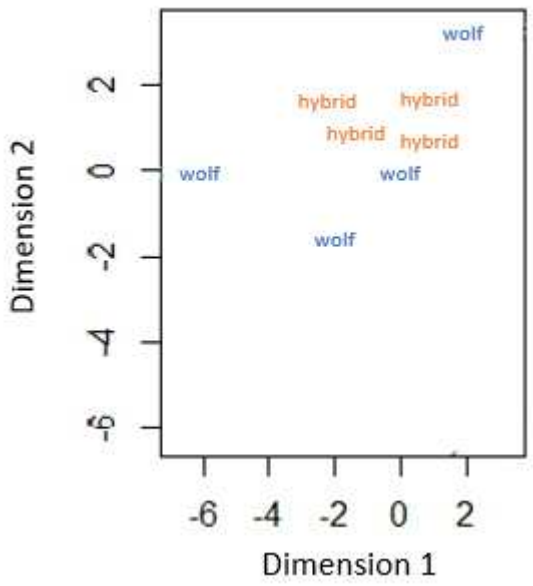


Figure 2. PCA comparing wolves to hybrids.



Supplemental Material

Figure S1. Volcano Plot: Wolves and F1s

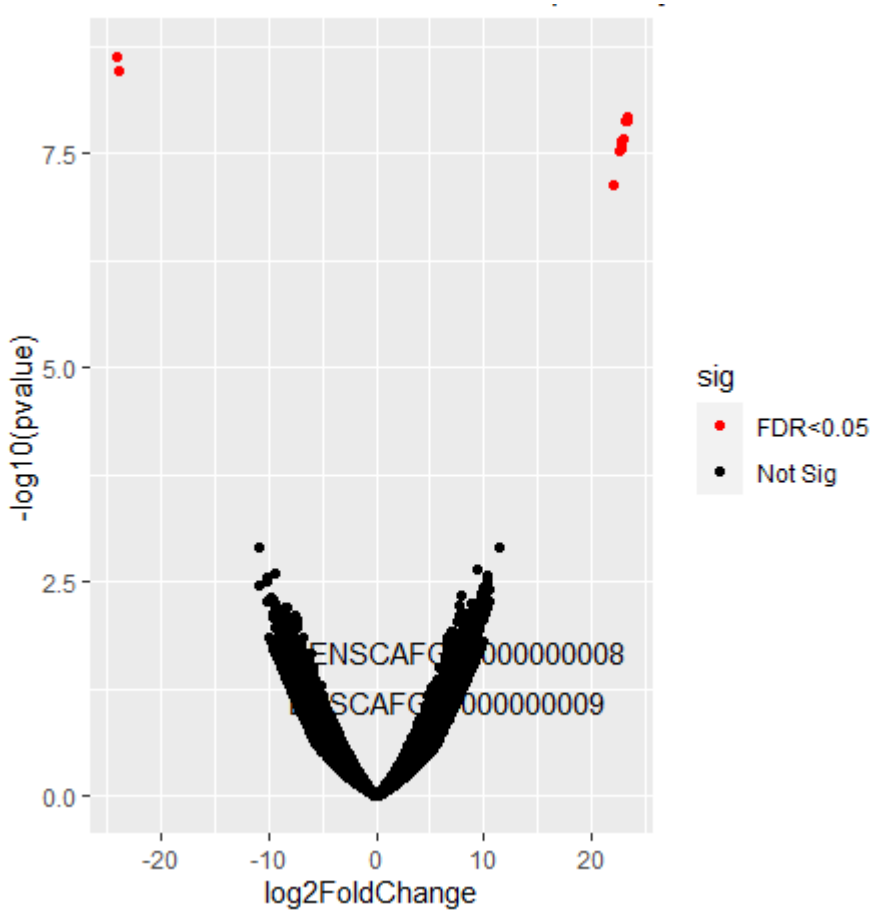


Figure S2. Volcano Plot: Wolves and F2s

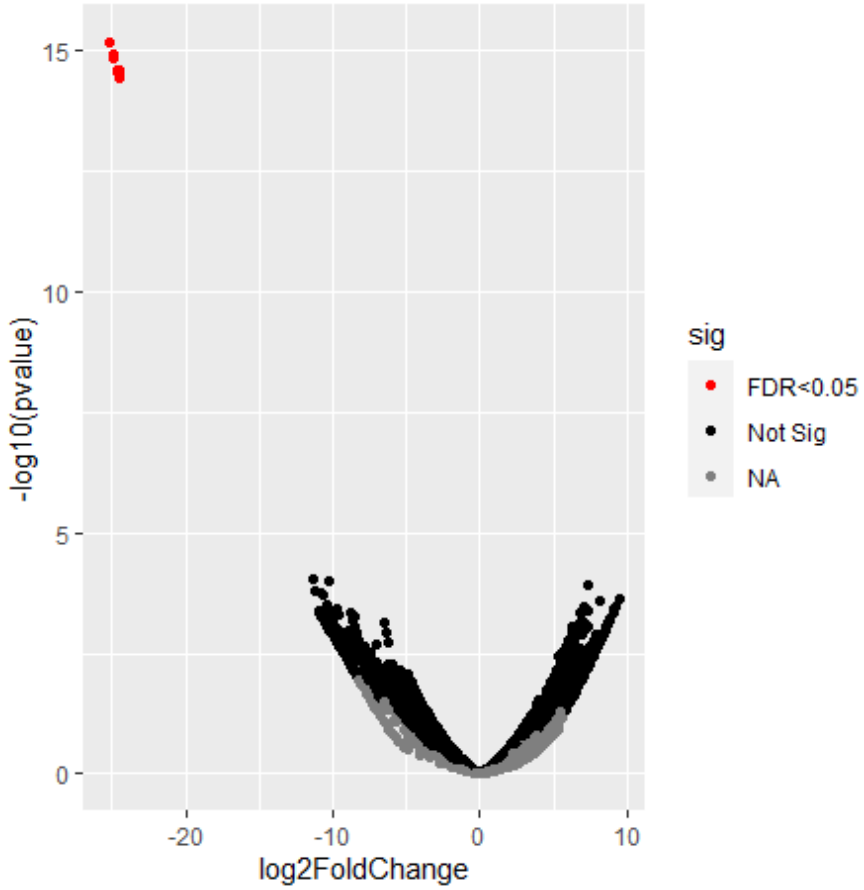


Figure S3. Volcano Plot: F1s and F2s

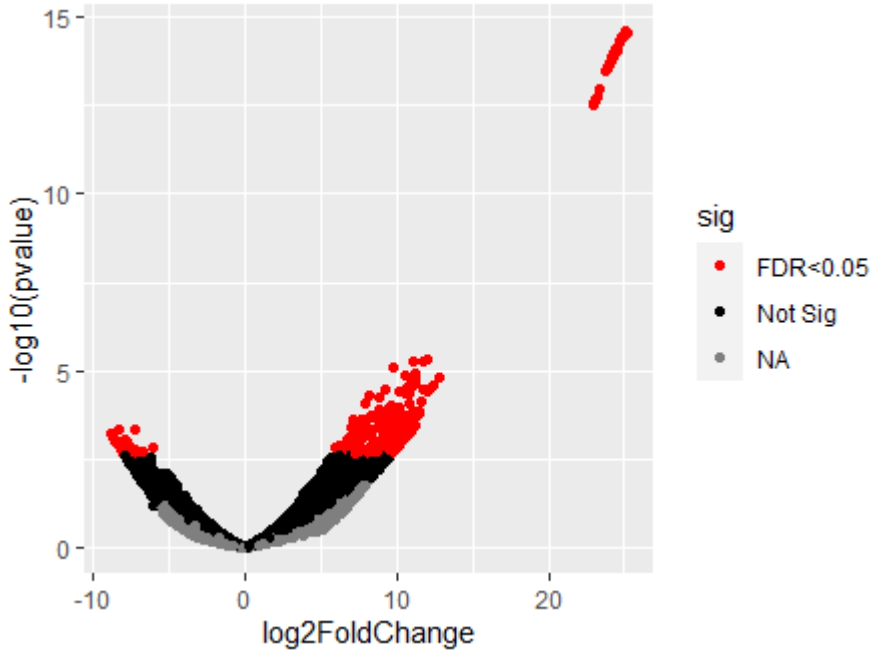


Figure S4. Percent Identity to the CanFam3.0 Reference Genome.

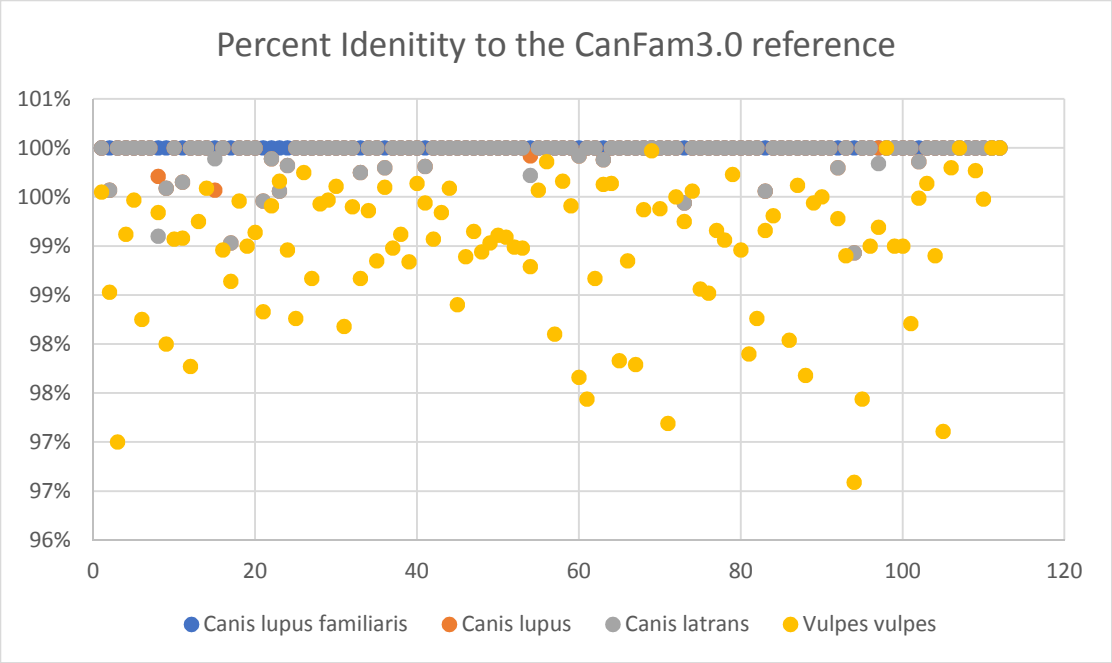

Percent Idenitity to the CanFam3.0 reference
101%
100%
100%
99%
99%
98%
98%
97%
97%
96%
0
20
40
60
80
100
120
Canis lupus familiaris
Canis lupus
Canis latrans
Vulpes vulpes

Table S1. DE transcripts and genes F1s and

Gene name	Gene stable ID
COL23A1	ENSCAFG00000000246
MYRFL	ENSCAFG00000000439
PNLDC1	ENSCAFG00000000740
IRF1	ENSCAFG00000000851
TRIB1	ENSCAFG00000001077
TGFBI	ENSCAFG00000001091
	ENSCAFG00000001341
CARD10	ENSCAFG00000001502
LRRC19	ENSCAFG00000001712
MAMDC2	ENSCAFG00000001890
	ENSCAFG00000001971
IL18R1	ENSCAFG00000002138
RHAG	ENSCAFG00000002141
SYK	ENSCAFG00000002171
GNE	ENSCAFG00000002304
COL15A1	ENSCAFG00000002525
SLC17A5	ENSCAFG00000002658
	ENSCAFG00000002855
KLK13	ENSCAFG00000002883
GJB7	ENSCAFG00000003051
PLEK	ENSCAFG00000003260
KIDINS220	ENSCAFG00000003323
EPHA7	ENSCAFG00000003332
CSF3R	ENSCAFG00000003376
ADD2	ENSCAFG00000003407
INHBA	ENSCAFG00000003550
MAP3K8	ENSCAFG00000003980
C5AR1	ENSCAFG00000004175
SSTR2	ENSCAFG00000004498
CKM	ENSCAFG00000004507
GLB1	ENSCAFG00000004652
ATP2B2	ENSCAFG00000005076
SPOPL	ENSCAFG00000005188
	ENSCAFG00000005321
EHD3	ENSCAFG00000005348
GRM7	ENSCAFG00000005583
RASGRP3	ENSCAFG00000005870
TMEM132C	ENSCAFG00000006849
GRAMD1A	ENSCAFG00000007157
	ENSCAFG00000007385

HHEX	ENSCAFG00000007502
	ENSCAFG00000008026
ADGRV1	ENSCAFG00000008218
NUP160	ENSCAFG00000008299
MYL9	ENSCAFG00000008540
SAMHD1	ENSCAFG00000008659
RXFP1	ENSCAFG00000008672
DISP2	ENSCAFG00000009083
TOX2	ENSCAFG00000009354
TSPAN18	ENSCAFG00000009416
HPGDS	ENSCAFG00000010032
SLC17A6	ENSCAFG00000010093
TSPAN32	ENSCAFG00000010112
NFKB2	ENSCAFG00000010155
	ENSCAFG00000010596
RBM20	ENSCAFG00000010785
RIN3	ENSCAFG00000010958
MRPS35	ENSCAFG00000010963
GJA8	ENSCAFG00000011071
	ENSCAFG00000011098
PRKX	ENSCAFG00000011187
SNAI1	ENSCAFG00000011499
SEZ6L	ENSCAFG00000011694
CRYBA4	ENSCAFG00000011809
ARV1	ENSCAFG00000011897
HJURP	ENSCAFG00000011899
DTX3L	ENSCAFG00000011948
GBX2	ENSCAFG00000012148
UGT8	ENSCAFG00000012173
HMX2	ENSCAFG00000012699
CD3E	ENSCAFG00000012802
CSPG5	ENSCAFG00000013156
MGMT	ENSCAFG00000013263
NFKBIA	ENSCAFG00000013418
ZBTB16	ENSCAFG00000013538
	ENSCAFG00000013899
PTPN6	ENSCAFG00000014463
PTGER2	ENSCAFG00000014701
DTWD1	ENSCAFG00000014926
FMO3	ENSCAFG00000014992
SLC35F4	ENSCAFG00000015255
ASMTL	ENSCAFG00000015291
	ENSCAFG00000016084

SERPINE2	ENSCAFG00000016228
	ENSCAFG00000016319
KCNIP4	ENSCAFG00000016537
ITGAL	ENSCAFG00000016603
PANK3	ENSCAFG00000017049
FABP6	ENSCAFG00000017275
SERPINA1	ENSCAFG00000017646
TRPV2	ENSCAFG00000018012
GUCY2F	ENSCAFG00000018034
CAMK2A	ENSCAFG00000018163
NPC1	ENSCAFG00000018183
LONRF3	ENSCAFG00000018363
JAG2	ENSCAFG00000018401
	ENSCAFG00000018434
	ENSCAFG00000018468
KCNJ12	ENSCAFG00000018532
ADGRE1	ENSCAFG00000018571
GHR	ENSCAFG00000018579
UNC119	ENSCAFG00000018713
CDH6	ENSCAFG00000018951
CDH10	ENSCAFG00000018996
CDH12	ENSCAFG00000019028
	ENSCAFG00000019092
ZNF185	ENSCAFG00000019176
ISG15	ENSCAFG00000019348
PRSS57	ENSCAFG00000019702
CSF1	ENSCAFG00000019798
BCAR3	ENSCAFG00000020133
U6	ENSCAFG00000020928
U2	ENSCAFG00000021122
	ENSCAFG00000021573
U6	ENSCAFG00000021594
	ENSCAFG00000021724
U3	ENSCAFG00000022065
	ENSCAFG00000022712
SULT1C3	ENSCAFG00000023398
CYP4F22	ENSCAFG00000023401
	ENSCAFG00000023589
	ENSCAFG00000023691
ARSH	ENSCAFG00000024515
	ENSCAFG00000024629
	ENSCAFG00000025750
	ENSCAFG00000025897

SNORA62	ENSCAFG00000026036
	ENSCAFG00000027609
U6	ENSCAFG00000027817
U6	ENSCAFG00000027903
U6	ENSCAFG00000028111
	ENSCAFG00000028575
CAVIN1	ENSCAFG00000028879
UCHL3	ENSCAFG00000029050
GATA4	ENSCAFG00000029087
	ENSCAFG00000029124
	ENSCAFG00000029288
	ENSCAFG00000029454
	ENSCAFG00000029864
	ENSCAFG00000030361
PDZRN3	ENSCAFG00000030541
CA10	ENSCAFG00000030895
U6	ENSCAFG00000030954
E2F3	ENSCAFG00000031036
U6	ENSCAFG00000031070
	ENSCAFG00000031244
C30H15orf48	ENSCAFG00000031501
NUDT2	ENSCAFG00000031608
	ENSCAFG00000031694
ZNF200	ENSCAFG00000031888
OLFM1	ENSCAFG00000032227
	ENSCAFG00000032341
DDIT4	ENSCAFG00000032469
COTL1	ENSCAFG00000032529
FGF10	ENSCAFG00000032695
MXD1	ENSCAFG00000032746
	ENSCAFG00000033370
	ENSCAFG00000033413
	ENSCAFG00000034971
	ENSCAFG00000035885
	ENSCAFG00000036143
	ENSCAFG00000036805
	ENSCAFG00000037762
	ENSCAFG00000038198
	ENSCAFG00000038312
	ENSCAFG00000038356
	ENSCAFG00000039391
	ENSCAFG00000039434
	ENSCAFG00000039476

ENSCAFG00000039509
ENSCAFG00000040286
ENSCAFG00000040611

Table S2. Percent identity comparison for *Canis lupus*, *Canis latrans*, and *Vulpes vulpes* (outgroup) to the reference genome CanFam3.0.

	IL21	LNP1	DPP10	ADH4	CRYM	GYG2	SMIM33	KDR	LPCAT4	NCF2	COL23A1	MYRFL	PNLDC1	IRF1
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	99.57%	100%	100%	100%	100%	100%	99.71%	99.59%	100%	99.65%	100%	100%	100%
Canis latrans	100%	99.57%	100%	100%	100%	100%	100%	99.10%	99.59%	100%	99.65%	100%	100%	100%
Vulpes vulpes	99.55%	98.53%	97%	99.12%	99.47%	98.25%		99.34%	98%	99.07%	99.08%	97.77%	99.25%	99.59%
	TRIB1	TGFBI	CARD10	LRRC19	MAMDC2	IL18R1	RHAG	GRM7	GNE	COL15A1	SLC17A5	KLK13	PLEK	SLC17A6
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	99.57%	100%	99.03%	100%	100%	100%	99.46%	99.89%	99.56%	99.82%	100%	100%	100%	100%
Canis latrans	99.89%	100%	99.03%	100%	100%	100%	99.46%	99.89%	99.56%	99.82%	100%	100%	100%	100%
Vulpes vulpes		98.96%	98.64%	99.46%	99%	99.14%	98.33%	99.41%	99.66%	98.96%	98.26%	99.75%	98.67%	99.43%
	KIDINS220	HPGDS	CSF3R	ADD2	TOX2	RXFP1	MYL9	SSTR2	CKM	GLB1	ATP2B2	SPOPL	EHD3	SAMHD1
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100.00%	100%	100%	100%	100%
Canis lupus	100%	100%	100%	100%	99.75%	100%	100%	99.80%	100%	100%	100%	100%	99.81%	100%
Canis latrans	100%	100%	100%	100%	99.75%	100%	100%	99.80%	100%	100%	100%	100%	99.81%	100%
Vulpes vulpes	99.47%	99.61%	98.18%	99.40%	98.67%	99.36%	98.85%	99.60%	98.98%	99.12%	98.84%	99.64%	99.44%	99.07%
	RASGRP3	TMEM132C	GRAMD1A	HHEX	ADGRV1	NUP160	TSPAN32	NFKB2	RBM20	RIN3	MRPS35	GJA8	PRKX	SNAI1
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	100.00%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.92%	100%	100%
Canis latrans	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.72%	100%	100%
Vulpes vulpes	99.34%	99.59%	98.40%	98.89%	99.15%	98.94%	99.03%	99.11%	99.09%	98.99%	98.98%	98.79%	99.57%	99.86%
	SEZ6L	CRYBA4	ARV1	HJURP	DTX3L	GBX2	UGT8	HMX2	CD3E	CSPG5	MGMT	NFKBIA	ZBTB16	PTPN6
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	100%	100%	99.92%	100%	100%	99.88%	100%	100%	100%	100%	100%	100%	100%
Canis latrans	100%	100%	100%	99.92%	100%	100%	99.88%	100%	100%	100%	100%	100%	100%	100%
Vulpes vulpes	98.10%	99.66%	99.41%	97.66%	97.44%	98.67%	99.63%	99.64%	97.83%	98.85%	97.79%	99.37%	99.97%	99.38%
	PTGER2	DTWD1	FMO3	SLC35F4	ASMTL	SERPINE2	KCNIP4	ITGAL	PANK3	FABP6	SERPINA1	TRPV2	CAMK2A	NPC1
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	100%	99.44%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.56%	100%
Canis latrans	100%	100%	99.44%	100%	100%	100%	100%	100%	100%	100%	100%	100%	99.56%	100%
Vulpes vulpes	97.19%	99.50%	99.25%	99.56%	98.56%	98.52%	99.16%	99.06%	99.73%	98.96%	97.90%	98.26%	99.16%	99.31%
	MXD1	JAG2	KCNJ12	ADGRE1	GHR	UNC119	CDH6	CDH10	CDH12	FGF10	ISG15	PRSS57	CSF1	COTL1
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	100%	100%	100%	100%	100%	100%	99.80%	100%	98.93%	100%	100%	100%	100%
Canis latrans	100%	100%	100%	100%	100%	100%	100%	99.80%	100%	98.93%	100%	100%	99.84%	100%
Vulpes vulpes		98.04%	99.62%	97.68%	99.44%	99.50%		99.28%	98.90%	96.59%	97.44%	99%	99.19%	100%
	BCAR3	SULT1C3	CYP4F22	CAVIN1	UCHL3	GATA4	PDZRN3	CA10	E2F3	C30H15orf48	NUDT2	ZNF200	OLFM1	DDIT4
Canis lupus familiaris	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis lupus	100%	100%	100%	99.86%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Canis latrans	100%	100%	100%	99.86%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Vulpes vulpes	99%	99%	98.21%	99.49%	99.64%	98.90%	97.11%	99.80%	100%		99.77%	99.48%	100%	100%